UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS BIOMÉDICAS
INSTITUTO DE FISIOLOGÍA CELULAR

MUERTE NEURONAL INDUCIDA POR LA INHIBICIÓN GLUCOLÍTICA: POSIBLE PAPEL DEL INCREMENTO DE CALCIO INTRACELULAR Y ESTRÉS OXIDATIVO

T E S I S

QUE PARA OBTENER EL GRADO ACADÉMICO DE

DOCTORA EN CIENCIAS

P R E S E N T A

M EN C. KARLA HERNÁNDEZ FONSECA

DIRECTORA DE TESIS:
DRA. MA DE LOURDES MASSIEU TRIGO

COMITE TUTORAL:
DR. ARTURO HERNÁNDEZ CRUZ
DR. MAURICIO DÍAZ MUÑOZ

MÉXICO, D.F. ABRIL, 2008

EL PRESENTE TRABAJO SE LLEVÓ A CABO BAJO LA DIRECCIÓN DE LA DRA. LOURDES MASSIEU TRIGO EN EL DEPARTAMENTO DE NEUROCIENCIAS DEL INSTITUTO DE FISIOLOGÍA CELULAR DE LA UNIVERSIDAD NACIONAL AUTONÓMA DE MÉXICO.

ESTE TRABAJO SE REALIZÓ CON EL APOYO DEL CONSEJO NACIONAL DE CIENCIA Y TECNOLOGÍA (CONACYT, PROYECTO 48645-Q), ASI COMO POR LA BECA DE DOCTORADO OTORGADA POR EL CONACYT (167146) Y LA DIRECCIÓN GENERAL DE ESTUDIOS DE POSGRADO (DGEP).

A LA BIOL. TERESA MONTIEL MONTES POR SU COLABORACION EN EL TRABAJO EXPERIMENTAL

A MIS PAPITOS, POR SU INFINITO AMOR, POR SU APOYO INCONDICIONAL A CADA UNO DE MIS PROYECTOS, PERO ESPECIALMENTE POR ENSEÑARME A SER QUIEN SOY.

A JORGE, POR HACER MI VIDA MÁS QUE PERFECTA. TE AMO!!!!

A MIS HERMANOS,

JUAN, LUIS, VÍCTOR Y LESLY.

A JIBARITO.

AGRADECIMIENTOS

A la Dra. Lourdes Massieu Trigo, gracias por tu constante enseñanza y motivación, pero sobre todo por guiarme en este sinuoso camino del doctorado.

A mi Comité Tutoral, Dra. Lourdes Massieu Trigo, Dr. Mauricio Díaz Muñoz y al Dr. Arturo Hernández Cruz, por el tiempo que me dedicaron a lo largo del doctorado.

A los integrantes del jurado, la Dra. María Eugenia Gonsebatt Bonaparte, la Dra. Clorinda Arias Álvarez, la Dra. Mahara Valverde Ramírez, la Dra. Lourdes Massieu Trigo, el Dr. Ignacio Camacho Arroyo, el Dr. Mauricio Díaz Muñoz, el Dr. Rolando Hernández Muñoz, quienes aportaron ideas y sugerencias a este trabajo.

Al Dr. Julio Morán Andrade, a la Dra. Herminia Pasantes Morales y al Dr. José Pedraza Chaverri por su cooperación en la realización de algunas técnicas experimentales necesarias para la publicación de los resultados presentados en este trabajo.

A Tere Montiel, gracias por todas tus enseñanzas y ayuda, pero especialmente por esos consejos y apapachos tan necesarios en momentos difíciles.

A Perla y Mari, gracias por todos los buenos momentos que hemos pasado juntas. NO TE MUERAS DIEGO!!!!!!!!!!

A mis compañeros de laboratorio: Mari, Perla, Octavio, Alberto, Ana, Blanca y Cris, por compartir esta pasión por la investigación, así como, por todos esos momentos de alegría en el laboratorio. Y especialmente a Ceci por todos los martes de journal.

A todos los amigos del instituto: Benito, Oscar, Fernando Peña, Rogelio, Lemus, Victor, Yuridia, Luis (ñoño Tapia), Juan Carlos y Octavio (Manitas).

ÍNDICE

I. RESUMEN

La muerte neuronal mediada por la activación de los receptores a glutamato de tipo N-metil-D-aspartato (NMDA), se ha asociado a diferentes condiciones patológicas como la isquemia, la hipoglucemia y algunas enfermedades neurodegenerativas. Este proceso de muerte se conoce como excitotoxicidad y está relacionado con el aumento en los niveles de glutamato extracelular, y en la concentración de calcio (Ca^{2+}) interno por la entrada de este catión a través del receptor NMDA. Entre los procesos activados por Ca^{2+} está la producción de especies reactivas de oxígeno (EROS), que desencadena otro proceso conocido como estrés oxidativo. Se sabe que la toxicidad del glutamato se facilita durante la falla energética, condición asociada a la isquemia y a la hipoglucemia. Aunque el substrato energético principal en el cerebro es la glucosa, en algunas condiciones, como la inanición, el ayuno prolongado, la hipoglucemia y durante el periodo de amamantamiento, la concentración sanguínea de los cuerpos cetónicos, acetoacetato (AcAc) y β-hidroxibutirato (β-HB) aumenta, y pueden ser utilizados en el cerebro como substratos energéticos alternativos a la glucosa.

En el presente estudio investigamos si la inhibición glucolítica inducida por el yodoacetato (IOA), un inhibidor de la enzima glucolítica, gliceraldehído 3-fosfato deshidrogenasa, induce muerte neuronal a través de un mecanismo excitotóxico, relacionado con la producción de EROS y con el incremento en la concentración de Ca^{2+} intracelular, en cultivos de neuronas de hipocampo de rata. La exposición de las neuronas a 50 μM de IOA induce una disminución lenta y progresiva en los niveles de ATP y muerte neuronal parcial, mientras que la exposición a 100 μM de IOA induce una disminución rápida y aguda en los niveles de ATP, así como una muerte neuronal masiva. Así mismo, observamos que la producción de EROS es dosis y tiempo dependiente. Tanto la muerte, como la producción de EROS inducidas por inhibición glucolítica moderada son prevenidas eficientemente por un bloqueador de los receptores a glutamato de tipo NMDA, la ausencia de calcio externo, el atrapador de Ca^{2+} intracelular,

Bapta-AM, por antioxidantes (principalmente vitamina E) y por cuerpos cetónicos; mientras que en el caso de la condición aguda ambos procesos son prevenidos eficientemente por la vitamina E, cuerpos cetónicos y el Bapta-AM; pero no por el medio libre de Ca^{2+} ni por el bloqueador de los receptores NMDA. El tratamiento con AcAc y D-β-HB previene la reducción de los niveles de ATP, pero no así el L- β-HB, aunque disminuye la producción de EROS y la muerte neuronal, atribuyéndosele este efecto a una acción antioxidante. La exposición a IOA induce el aumento en la concentración de Ca^{2+} intracelular de una manera dependiente de la dosis.

Los resultados sugieren que el daño neuronal inducido por la inhibición glucolítica puede implicar diferentes mecanismos, dependiendo de la intensidad del déficit energético. Durante la inhibición glucolítica moderada un componente excitotóxico está claramente implicado, mientras que durante la falla energética severa, el estrés oxidativo tiene un papel preponderante. En ambas condiciones el proceso de muerte está asociado con un aumento en la concentración de calcio intracelular. En el caso de la falla energética moderada, esta estaría mediado por los receptores NMDA, mientras que durante la falla energética severa, la alteración de los sistemas de regulación de la homeostasis del calcio intracelular podría tener un papel preponderante. Por otra parte, los resultados sugieren que la reducción de la producción de EROS por los cuerpos cetónicos está mediada por su efecto metabólico través del mantenimiento del metabolismo mitocondrial. Aunque, con base en los resultados obtenidos podemos concluir que el efecto protector de dichos compuestos, principalmente del β-HB, está relacionado con su acción atrapadora de radicales libres, principalmente de $^{\bullet}OH$.

II. ABSTRACT

Neuronal death induced by activation of the N-methyl-D-aspartic acid (NMDA) glutamate receptor subtype has been associated with different pathological conditions such as, ischemia, hypoglycemia and some neurodegenerative diseases. This process of cell death is known as excitotoxic and is related to the increase in glutamate extracellular levels and augmented intracellular calcium (Ca^{2+}), due to the influx of this ion through NMDA receptor. Increased production of reactive oxygen species (ROS) is one of the processes activated by Ca^{2+} inducing oxidative stress. It is well known that glutamate toxicity is highly facilitated during energy failure. Glucose is the main energy substrate in brain, however in some conditions such as prolonged fasting, starvation, hypoglycemia, and during the suckling period, the blood concentration of ketone bodies, acetoacetate (AcAC) and β-hydroxybutyrate (β-HB) increases and they can be used as alternative energy substrates by brain.

In the present study we have investigated whether glycolysis inhibition induced by iodoacetate (IOA), an inhibitor of the glycolytic enzyme, Glyceraldehyde 3-phosphate dehydrogenase, induces neuronal death through an excitotoxic mechanism, related to ROS production and increased intracellular Ca^{2+} concentration, in hippocampal cultured neurons. Exposure of 50 μM of IOA to neurons induces a slow and progressive decrease in ATP levels and partial neuronal death; while exposure to 100 μM IOA induces a fast and acute reduction of ATP levels, as well as a massive neuronal death. In addition, we observed that ROS production is dose and time dependent. Both neuronal death and ROS production induced by moderate glycolysis inhibition, are efficiently prevented by blockade of NMDA receptors, the absence of extracellular Ca^{2+}, the Ca^{2+} chelator Bapta-AM, antioxidants (mainly vitamin E), and the ketone bodies; While in the case of the acute condition both processes are efficiently prevented by vitamin E, ketone bodies and Bapta-AM; but not by Ca^{2+}-free medium, nor by blockade of NMDA receptors. Treatment with AcAc and D-β-HB, but not with L-β-HB prevented the reduction of ATP levels, while the three compounds reduced

ROS production and the neuronal death. Exposure to IOA induces the increase in the intracellular Ca^{2+} concentration, which is dose dependent.

Results suggest that neuronal damage induced by glycolysis inhibition involves different mechanisms depending on the intensity of the energy deficit. During moderate glycolysis inhibition an excitotoxic component is involved, while during acute inhibition oxidative stress might play a major role. In both conditions neuronal death is associated with increased intracellular Ca^{2+}. In the case of moderate energy failure this is mainly mediated by the NMDA receptor, while during severe energy failure, alterations in the systems regulating the intracellular Ca^{2+} levels might have a mayor role. Results suggest that reduction in ROS production by ketone bodies is mediated by its metabolic effect through the supplementation of mitochondrial metabolism. However, the present results lead us to conclude that the protective effect of these compounds, mainly of β-HB, is related to its free radical scavenger activity, mainly $^{\bullet}OH$.

III. ORGANIZACIÓN DE LA TESIS

La presente tesis está organizada en: Introducción, Antecedentes, Hipótesis, Objetivos, Resultados, Discusión, Conclusiones y Apéndice. En la sección de metodología, solamente se incluye un diagrama de flujo de los métodos, ya que los mismos están descritos detalladamente en los artículos incluidos en los resultados.

La revisión sobre los distintos sistemas de regulación del calcio intracelular y su papel en la en la muerte excitotóxica y el estrés oxidante, se incluye en la revisión (artículo 1):

Hernández-Fonseca K., Massieu L., Díaz-Muñoz M. 2007. ***Interplay between intracellular calcium handling and reactive oxygen species. Role of mitochondria and endoplasmic reticulum***, publicada como capítulo en el libro "The Neurochemistry of Neuronal Death", editado por L. Massieu, C. Arias y J. Morán, Ed. Research Singpost, Kerala, India.

En los Antecedentes se hace referencia a un trabajo previo directamente relacionado con esta tesis', que formó parte de mi tesis de maestría y que se incluye como consulta en el apéndice. (artículo 4):

Hernández-Fonseca K., Massieu L. 2005. **"Disruption of endoplasmic reticulum calcium stores is involved in neuronal death induced by glycolysis inhibition in cultured hippocampal neurons"**, J. Neurosci. Res. 2005, 82: 196-205.

En los Resultados se incluyen dos artículos (artículos 2 y 3):

Hernández-Fonseca K., Massieu L. 2008. **"Calcium-dependent production of reactive oxygen species is involved in neuronal damage induced during glycolysis inhibition in cultured hippocampal neurons"**. J. Neurosci. Res., DOI: 10.1002/jnr.21634

Haces M.L., Hernández-Fonseca K., Medina-Campos O.N., Montiel T., Pedraza-Chaverri J., Massieu L. 2008. "**Antioxidant capacity contributes to protection of ketone bodies against oxidative damage induced during hypoglycemic conditions".** Exp. Neurol. DOI:10.1016/expneurol.2007.17.029.

Sólo los resultados de los experimentos *in vitro* presentados en este artículo forman parte de esta tesis.

En la Discusión, se resumen y se integra la discusión conjunta de ambos artículos.

IV. ABREVIATURAS

AAE	Aminoácidos excitadores
AcAc	Acetoacetato
Acetil-CoA	Acetil coenzima A
EROS	Especies Reactivas de Oxígeno
$FADH_2$	Flavin adenin dinucleotido
FMN	Favin mononucelótido
GAPDH	Gliceraldehído 3-fosfato deshidrogenasa
GPx	Glutatión peroxidasa
GSH	Glutatión
H^+	Protón
IOA	Yodoacetato (por sus siglas en inglés)
NADH	Nicotinamida adenin dinucleótido reducido
PFK-1	Fosfofructocinasa -1
SNC	Sistema nervioso central
β-HB	β-hidroxibutirato

V. INTRODUCCIÓN

Metabolismo energético en el cerebro

El cerebro humano es un órgano muy caro en cuanto a la energía que requiere para su funcionamiento debido a que aunque sólo representa el 2% del peso corporal requiere del 40-60% del ATP total producido (Hyder et al., 2006; Shulman et al., 2004). La energía que requiere el cerebro para mantener su funcionamiento puede equipararse a la que necesita un músculo de la pierna de un humano que corre un maratón o la energía requerida por el riñón para llevar a cabo su trabajo osmótico (Attwell y Laughling, 2001). Aproximadamente el 75% de la energía consumida por el cerebro se utiliza en señalización celular, mientras que el 25% restante sirve para mantener actividades cerebrales esenciales, como: síntesis y degradación de proteínas, recambio de nucleótidos y fosfolípidos, transporte axonal y transporte mitocondrial de protones (H^+) (Attwell y Laughling, 2001). Se ha propuesto que la alta demanda energética del cerebro, se debe a que alrededor del 95% de las sinápsis son glutamatérgicas y GABAérgicas, y el balance entre la excitación y la inhibición requiere de un gran consumo de energía (Patel et al., 2005; Waldvogel et al., 2000).

El cerebro requiere de un aporte continuo de oxígeno y glucosa para sustentar sus necesidades energéticas basales. El Sistema Nervioso Central (SNC) presenta una capacidad muy limitada de utilizar reservas alternativas de energía. Aunque en algunas condiciones, como el ayuno prolongado, puede metabolizar cuerpos cetónicos (acetoacetato y β-hidroxibutirato), el cerebro no es capaz de metabolizar las grasas. Adicionalmente, el cerebro posee una reserva muy limitada de glucógeno (0.1 g/100 g de tejido fresco, comparado con 1 g/100 g en músculo y 6-10 g/100 g en hígado), que en condiciones de ausencia de glucosa puede sostener por algunos minutos el funcionamiento cerebral.

A nivel cerebral, la glucosa alimenta diferentes vías metabólicas. Es el principal sustrato para la producción de ATP y NADH, por su oxidación a través de la glucólisis y del ciclo del ácido cítrico (ciclo de Krebs) (Fig. 1). Interviene en

la biosíntesis de aminoácidos y neurotransmisores por vía anaplerótica del ciclo de Krebs; interviene en la síntesis de glucógeno; y mediante la vía del ciclo de las pentosas produce ribosa 5-fosfato y NADPH, necesarios para la síntesis de nucleótidos y lípidos, respectivamente (Tabernero et al., 1996).

En la mayoría de las células de mamífero, el transporte de glucosa y de otras hexosas ocurre a través de un mecanismo saturable de difusión facilitada que depende principalmente de la participación de proteínas de transporte de la familia SLC2, que está formada por 13 proteínas transportadoras, GLUT1-GLUT12 y por el transportador de mio-inositol (HMIT) (Uldry y Thorens, 2004). Con excepción del GLUT1, el cual se expresa ubicuamente, las otras proteínas transportadoras de esta familia presentan un patrón de expresión tejido y célula especifico. Dada la heterogeneidad celular en el cerebro, no es sorprendente que se haya reportado que la mayoría, sino todas las proteínas de esta familia de transportadores, estén presentes en el cerebro de mamífero (Duelli y Kuschinsky, 2001; Dwyer et al., 2002; Maher et al., 1994; Vannucci et al., 1997a). Sin embargo, la mayoría de estos transportadores son ineficientes en el transporte de glucosa ya que presentan una baja afinidad (GLUT5, GLUT6, GLUT11 y HMIT), lo cual está compensado por la gran cantidad de transportadores en la membrana de las neuronas. Algunos, tienen una localización y concentración limitada en el SNC (GLUT2 y GLUT4), mientras que para otros su localización y capacidad de transporte no ha sido determinada (GLUT8 y GLUT10). En el cerebro de mamíferos el transporte de glucosa predominantemente lo realizan los transportadores GLUT1 y GLUT3 (McEwen y Reagan, 2004; Vannucci et al., 1997b).

El transportador de glucosa GLUT1, fue el primero en clonarse, y sus características cinéticas están exhaustivamente estudiadas (Mueckler et al., 1985). Debido al patrón de glicosilación de GLUT1, se han detectado dos variantes de peso molecular (45 y 55 kDa) en el SNC de mamífero, lo cual no representa diferencias en estructura proteica o características cinéticas (Birnbaum et al., 1986). La forma de mayor peso molecular (55 kDa) es expresada en eritrocitos y en células endoteliales que forman la barrera

hematoencefálica (Simpson et al., 2007). La forma de 45 kDa de GLUT1 se localiza en células gliales, así como en las membranas basolateral y apical de los plexos coroideos. En condiciones fisiológicas hay una expresión muy limitada de GLUT1 de 45 kDa en neuronas, aunque su expresión puede incrementarse como respuesta a un estrés ambiental o en condiciones de cultivo (Gerhart et al., 1994; Lee y Bondy, 1993). El número de transportadores GLUT1 en las membranas de las células endoteliales de la barrera hematoencefálica es mucho mayor que en los astrocitos (400 pmol/mg proteína y 5.8-7.3 pmol/mg proteína respectivamente) (Simpson et al., 2001; Vannucci et al., 1997b).

El transportador GLUT3, originalmente se clonó en una línea celular de músculo esquelético de feto humano (Nagamatsu et al., 1992). En el cerebro se ha localizado casi exclusivamente en neuronas (Gerhart et al., 1992; Maher et al., 1992; Nagamatsu et al., 1993), en una concentración de 9.5 pmol/mg proteína (Maher et al., 1996). Durante la maduración cerebral, el incremento en la expresión de GLUT3 precede a la expresión de la forma de 45 kDa de GLUT1 en células gliales y coincide con la maduración neuronal, sinaptogénesis, actividad funcional e incremento en la tasa de utilización de glucosa (Vannucci, 1994).

La concentración de glucosa en la sangre es del orden de 5-8 mM y la del cerebro de 1.5-2.5 mM en humanos y roedores adultos (Erecinska et al., 2004). La glucosa se captura del plasma al cerebro por el GLUT1 de 55 kDa presente en las células endoteliales que tiene una km = 3 mM. La baja afinidad del GLUT1 para la glucosa intracelular permite incrementar la concentración de este azúcar en el lumen de las células endoteliales y mantener un adecuado suplemento a los astrocitos (Simpson et al., 2007).

Los astrocitos capturan la glucosa por medio del GLUT1 de 45 kDa (km= 8 mM), mientras que en las neuronas la captura de este azúcar es a través del transportador GLUT3 (km = 2.8 mM). Esto explica en parte porque la capacidad de transporte de glucosa en membranas aisladas de neuronas es 9 veces mayor que en las membranas de astrocitos (Maher et al., 1996). Esto permite que las neuronas acumulen la glucosa a una concentración de 1.2 mM, comparado con

0.9 mM de los astrocitos (Simpson et al., 2007). Esta característica es particularmente importante, debido a que aproximadamente el 75% de la energía que consume el SNC se relaciona con la transmisión sináptica (Attwell y Laughlin, 2001).

Glucólisis

La respiración celular es el proceso por medio del cual las células obtienen energía (ATP) a partir de la oxidación de las moléculas de combustible por el oxígeno, y se lleva a cabo en tres etapas. En la primera etapa, las moléculas de hidratos de carbono (principalmente glucosa, pero alternativamente ácidos grasos y algunos aminoácidos) son oxidados hasta producir fragmentos de dos átomos de carbono en forma de grupos acetilo de acetil coenzima A (acetil-CoA). Esta primera etapa es conocida como glucólisis y se lleva a cabo en ausencia de oxígeno (O_2). En la segunda etapa, los grupos acetilo son incorporados al ciclo de Krebs, los cuales son enzimáticamente oxidados a dióxido de carbono (CO_2); la energía liberada es conservada en acarreadores de electrones reducidos (NADH y $FADH_2$). En la tercera etapa de la respiración, esas coenzimas reducidas son de nuevo oxidadas, liberando protones (H^+) y electrones. Los electrones son transferidos al oxígeno (el aceptor final de electrones) vía cadena transportadora de electrones, conocida como cadena respiratoria. En el curso de la transferencia de electrones, la gran cantidad de energía liberada es conservada en forma de ATP, por un proceso llamado fosforilación oxidativa.

Hace millones de años se originaron los primeros organismos en una atmósfera anaerobia, por tanto, la glucólisis se considera como la vía metabólica más primitiva. Está presente en todas las formas de vida actuales. De hecho la glucólisis es la única vía metabólica en los animales que produce ATP en ausencia de oxígeno. La glucólisis (del griego glycos = dulce y lysis ruptura), también denominada ruta de Embden-Meyerhof fue inicialmente descubierta por Eduard Buchner en 1897 al estudiar la fermentación en levaduras, pero la

elucidación de la vía completa en levaduras (por Otto Warburg y Hans von Euler-Chelpin) y en músculo (por Gustav Embdeb y Otto Meyerhof) fue hasta 1930.

La glucólisis es el origen del metabolismo energético y consta de una secuencia de 10 reacciones enzimáticas, en las que la glucosa es oxidada, produciendo 2 moléculas de piruvato, 4 ATP y 2 NADH (Fig. 1). Las dos enzimas limitantes en la glucólisis son la hexocinasa y la fosfofructocinasa-1 (PFK-1, por sus siglas en inglés). Bajo condiciones normales la tasa de oxidación de la glucosa en el cerebro, no se regula por su transporte sino por su fosforilación a glucosa 6-fosfato, reacción catalizada irreversiblemente por la hexocinasa (Km = 40 μM) (Qutub y Hunt, 2005). La hexocinasa se inhibe por su producto, glucosa 6-fosfato, ATP y por su unión a membranas mitocondriales (Wilson, 2003). Hay cuatro subtipos de hexocinasa (I–IV o A-D) en mamífero, que varían en localización subcelular, cinética, afinidad por diferentes sustratos y función (Bustamante y Petersen, 1980). La concentración de hexocinasa en el cerebro se correlaciona con la utilización de glucosa local (Wilson, 1980, 2003). Adicionalmente se ha demostrado que su actividad pero no su concentración, parece ser el paso limitante en el metabolismo de la glucosa cerebral. En condiciones de demanda energética se incrementa la actividad de la hexocinasa para iniciar la glucólisis.

La PFK-1 es la enzima reguladora más importante de la glucólisis, cataliza la reacción de fructosa-6-fosfato a fructuosa 1-6-bifosfato. Es una enzima alostérica conformada por 4 subunidades, se inhibe por ATP, Mg^{2+} y citrato, y es activada por K^{+}, PO_4^{3-}, 5'-AMP, ADP y fructosa 2,6-bifosfato (Dunaway, 1983). En condiciones basales, el ATP y citrato en el cerebro inhiben la actividad de la PFK-1. Existen diferentes subtipos de subunidades codificados a partir de un mismo gen, que se han encontrado en conejos, ratas y humanos (Mhascar y Dunaway, 1995). El subtipo C es el que se encuentra ampliamente expresado en el cerebro. La habilidad del tejido para utilizar la glucosa depende de la expresión y la composición de subunidades que conforman a la cinasa (Dunaway, 1983). A diferencia del subtipo M presente en el músculo, el subtipo

C presenta una baja afinidad por la fructuosa 6-fosfato y menor inhibición por ATP (Kasten y Dunaway, 1993).

Las moléculas de NADH producidas en la glucólisis se translocan a la mitocondria por medio de la lanzadera de malato-aspartato. Esta última, es la ruta más importante para el transporte de equivalentes reductores en el SNC (Palmeiri et al., 2001). La actividad de este sistema es mayor en neuronas que en astrocitos debido a que se encuentra estrechamente relacionado con la síntesis de neurotransmisores (Palaiologos et al., 1998).

Metabolismo oxidativo

El producto final de la glucólisis en condiciones aerobias, el piruvato es transportado a la mitocondria por medio de un sistema de proteínas acarreadoras. Una vez en la mitocondria el piruvato se descarboxila formando acetil-CoA mediante la acción catalítica del complejo enzimático piruvato deshidrogenasa (PDH), localizado en la matriz mitocondrial. El PDH está conformado por tres enzimas (piruvato deshidrogenasa, dihidrolipoil transacetilasa y dihidrolipoil deshidrogenasa) presentes en multicopia y requiere de 5 cofactores (NAD^+, tiamina pirofosfato, co-enzima A, FAD^+ y ácido lipóico) (Reed, 2001). Su actividad es regulada por fosforilación, Ca^{2+}, Mg^{2+} y ATP.

El metabolismo oxidativo de la glucosa se lleva a cabo en la mitocondria en condiciones aerobias y está dividido en dos vías muy importantes: el ciclo de Krebs y la cadena transportadora de electrones. El ciclo Krebs recibe su nombre en honor a su descubridor Sir Hans Krebs, quien propuso los elementos clave de esta vía en 1937. Este ciclo consiste en una serie de 8 reacciones enzimáticas sucesivas, en las que se generan 1 GTP, 3 NADH y 1 $FADH_2$, a modo de conservar la energía liberada en tales reacciones (Fig. 1). La enzima piruvato deshidrogenasa y algunas de las enzimas que participan en el ciclo de Krebs, como la citrato sintasa, la isocitrato deshidrogenasa y la α-cetoglutarato deshidrogenasa son inhibidas por altas concentraciones de ATP y NADH y son activadas por Ca^{2+}.

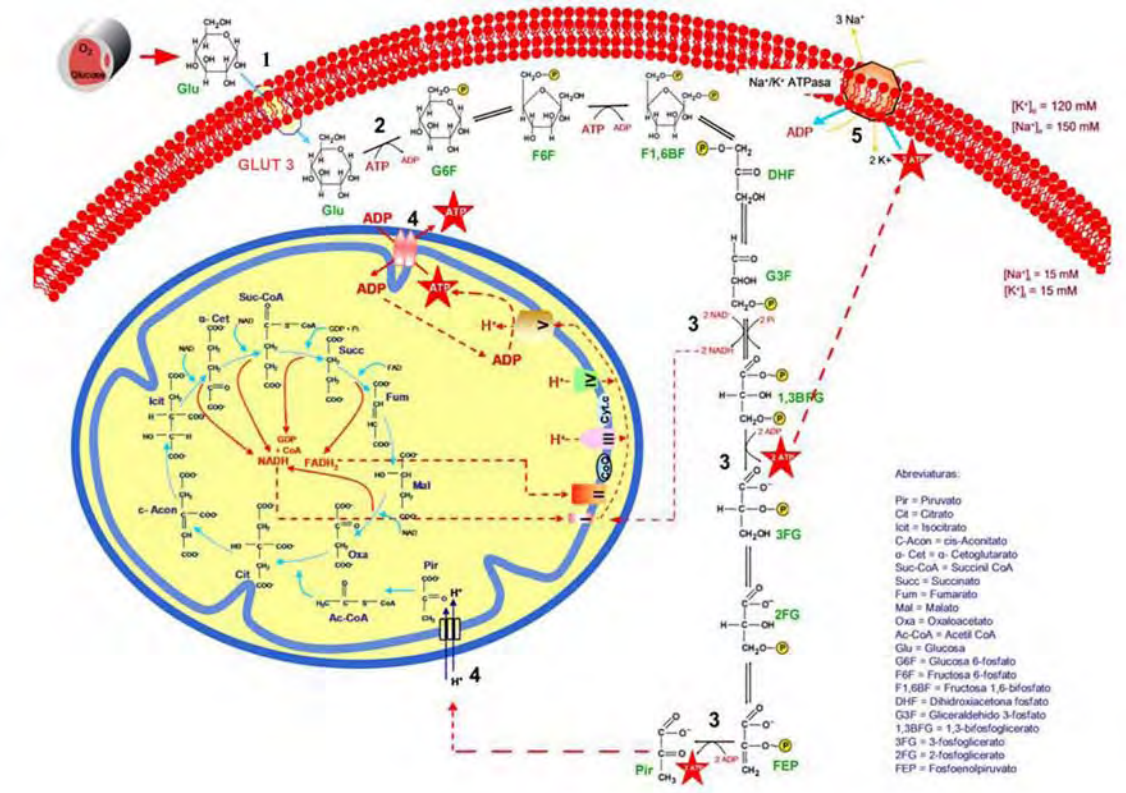


Figura 1. Metabolismo energético cerebral. La glucosa proveniente de los vasos sanguíneos es capturada por los transportadores localizados en la membrana de neuronas y astrocitos (GLUT3 y GLUT5, respectivamente) (1). La primera fase de la oxidación de la glucosa se lleva a cabo a través de la vía glucolítica (2). El metabolismo glucolítico (etapa anaeróbica) constituye una serie de 10 reacciones sucesivas en que la ganancia energética final radica en la formación de 2 NADH y 2 ATP (3). El piruvato (producto final de la vía glucolítica) es transportado a la mitocondria, en donde es oxidado a 30 ATP, 1 GTP, 3 NADH, 1 FADH2 a través de la fosforilación oxidativa (etapa aeróbica) (4). Se ha propuesto que el ATP proveniente del metabolismo glucolítico está involucrado en la función de la ATPasa Na^+/K^+ localizada en la membrana celular (5). De esta forma el desbalance en el equilibrio electroquímico generado por la activación de receptores y canales durante la neurotransmisión glutamatérgica esta relacionado con la función del metabolismo glucolítico. Cambios en la neurotransmisión glutamatérgica en el hipocampo de la rata durante la inhibición metabólica. Relevancia para el daño neuronal asociado a algunas enfermedades neurológicas. 2007.

La segunda etapa del metabolismo oxidativo comienza con la transferencia de electrones donados por los NADH y $FADH_2$ producidos en la glucólisis y el ciclo de Krebs. La re-oxidación de estas moléculas se lleva a cabo en la cadena respiratoria mitocondrial, que consiste de una serie secuencial de activación de acarreadores de electrones, de los cuales, la mayoría son

proteínas integrales con grupos prostéticos capaces de aceptar y donar uno o dos electrones. Estas proteínas se encuentran agrupadas en 4 complejos proteicos (I-IV) localizados en la membrana interna de la mitocondria.

El complejo I de la cadena transportadora de electrones, también denominada como NADH deshidrogenasa o NADH:ubiquinona oxidoreductasa capta dos electrones del NADH, y está conformada por 42-43 diferentes cadenas poli-peptídicas. Tiene una estructura en forma de "L", en la cual, el brazo más largo forma parte de una proteína integral de membrana y el brazo corto se extiende dentro de la matriz con una región hidrofílica que contiene un grupo Flavin Mononucleótido (FMN) y al menos seis centros hierro-azufre (Navarro y Boveris, 2007). La NADH deshidrogenasa lleva a cabo dos procesos simultáneamente: 1) la transferencia de un hidroxilo a la ubiquinona (transportador liposoluble) y 2) la translocación de cuatro H^+ a través de membrana, contribuyendo al gradiente de protones.

El complejo II o succinato deshidrogenasa, es un complejo enzimático que participa tanto en el ciclo de Krebs, como en la cadena respiratoria mitocondrial; está conformado por 5 grupos prostéticos y cuatro diferentes subunidades proteicas. Este complejo presenta en su sitio catalítico un grupo FAD y un centro hierro-azufre necesarios para la transferencia de electrones a la ubiquinona, además de grupos hemo b en el dominio membranal hidrofóbico (Cecchini, 2003). Los electrones transferidos a la ubiquinona vía complejo I y II, generan una forma reducida de la ubiquinona denominada ubiquinol (QH_2) que puede difundir libremente por la membrana.

El complejo III o complejo citocromo bc_1, está estructuralmente conformado por 9-10 polipéptidos, de los cuales, tres de ellos se asocian con centros redox. Estos centros son b_{562}, b_{566} y c_1 hemo y un grupo de [2Fe-2S] (Hatefi, 1985). Adicionalmente, dos semi-ubiquinonas se unen a dos dominios del complejo III (Crofts, 2004). Este complejo realiza la transferencia de electrones del QH_2 al citocromo c, el cual es una proteína periférica localizada en el espacio intermembranal que transfiere los electrones del complejo III al Cu_A del complejo IV. Al mismo tiempo, transloca dos protones hacia el espacio

intermembranal a través de la membrana, por los dos electrones transportados desde el ubiquinol. El complejo IV o citocromo oxidasa, es una enzima conformada por dos iones Cu (Cu_A) asociados a los centros 2Fe-2S. La subunidad I contiene 2 grupos hemo, designados como *a* y *a3*, además de otro ión Cu (Cu_B). Los grupos hemo *a3* y Cu_B forman un centro binuclear que acepta electrones del grupo hemo *a* y los transfiere al O_2, para generar 2 moléculas de H_2O a partir de los cuatro electrones transferidos por el citocromo c, consumiendo 4 H^+ de la matriz mitocondrial. Al mismo tiempo, se translocan cuatro H^+ al espacio intermembranal, por los cuatro electrones.

El flujo de electrones a través de los cuatro complejos en el que se involucran los donadores (NADH, succinato, FADH), y el aceptor (O_2), se genera mediante el potencial de oxidación. Los electrones se mueven hacia los componentes que tienen una energía de oxidación positiva. La diferencia en el potencial redox de los acarreadores de electrones definen las reacciones que son exergónicas y que proveen la energía libre requerida para el bombeo de H^+ dentro del espacio intermembranal. Los complejos I, III y IV funcionan como bombas de H^+ que actúan en serie con respecto al flujo de electrones y en paralelo con el circuito de H^+. El bombeo de H^+ se genera por la energía libre de las reacciones de oxidación acopladas, que involucra el movimiento de H^+ de la parte negativa de la mitocondria (la matriz) a la parte positiva (espacio intermembranal), generando un gradiente electroquímico, denominado como fuerza protón-motriz.

La síntesis de ATP se genera por el paso de protones a través del complejo ATP sintetasa (complejo V). La ATP sintetasa es una ATPasa de tipo F que está formada por dos subunidades: F_1, una proteína de membrana externa, y F_0, proteína integral de membrana. La F_1 se compone de 5 subunidades (α, β, γ, δ, y Є) que forman el dominio catalítico. El movimiento de las subunidades de la ATP sintetasa es vital para su funcionamiento, y se conoce que puede rotar a 50-100 veces/s. La rotación se produce en la F_0 siguiendo el flujo electroquímico de H^+ (Walker et al., 1995). Se estima que se requieren aproximadamente 3-4 H^+ para dar lugar a 1 molécula de ATP (Chance, 1977; Mitchell y Moyle, 1965). El

proceso de síntesis de ATP es termodinámicamente posible debido a que la transferencia de electrones genera energía suficiente, 34 kJ/mol por par de electrones, que se conserva en el gradiente de protones para dirigir la formación de 1 mol ATP (se requieren 32 kJ/mol ATP). Al mecanismo involucrado en el proceso de síntesis de ATP se le conoce como teoría quimiosmótica y fue sugerida por primera vez en el año 1961 por Peter Mitchell. Por su parte, al proceso en el cual se forma ATP a través de la transferencia de electrones se le denomina fosforilación oxidativa (porque el oxígeno capta los protones formando H_2O) y fue descubierta en el año 1948 por Eugene Kennedy y Albert Lehninger (Fig. 1).

La oxidación completa de una molécula de glucosa hasta CO_2 + H_2O genera de 30-32 moléculas de ATP.

Cuerpos cetónicos como sustratos alternativos a la glucosa en el cerebro

En la mayoría de los mamíferos, la acetil-CoA formada durante la oxidación de los ácidos grasos puede entrar al ciclo de Krebs o puede ser convertida en el hígado en cuerpos cetónicos, acetona, acetoacetato (AcAc) y β-hidoxibutirato (β-HB) (Owen et al., 1967; Sokoloff, 1973; Gjedde y Crone, 1975; Guzmán y Blázquez, 1975). La acetona es producida en pequeñas cantidades y exhalada. El AcAc y el β-HB son transportados en el torrente sanguíneo a otros tejidos, donde son oxidados en el ciclo de Krebs y proveen la energía requerida por tejidos como el musculo esquelético y cardíaco y la corteza renal. Como ya se mencionó anteriormente, el cerebro utiliza preferentemente a la glucosa como fuente de energía, pero en condiciones de ayuno y en animales inmaduros durante el periodo de amamantamiento puede utilizar al AcAc y β-HB.

El primer paso en la síntesis de cuerpos cetónicos es la condensación enzimática de dos moléculas de acetil-CoA, catalizada por la tiolasa formando acetoacetil-CoA. El acetoacetil-CoA se condensa con un acetil-CoA formando β-hidroxi-β-metilglutaril-CoA (HMG-CoA), el cual es cortado por la liasa HMG-CoA

generando AcAc y acetil-CoA. El AcAc producido es reversiblemente reducido a D-β-HB por la D-β-HB deshidrogenasa, una enzima mitocondrial.

En tejidos extra-hepáticos, el D-β-HB es oxidado a AcAc por la D-β-HB deshidrogenasa. El AcAc se convierte a acetoacetil-CoA por la transferencia de la CoA del succinil-CoA, un intermediario del ciclo de Krebs, en una reacción catalizada por la β-cetoacil-CoA transferasa. El acetoacetil-CoA es cortado por una tiolasa produciendo dos moléculas de acetil-CoA, las cuales entran al ciclo de Krebs (Fig. 2).

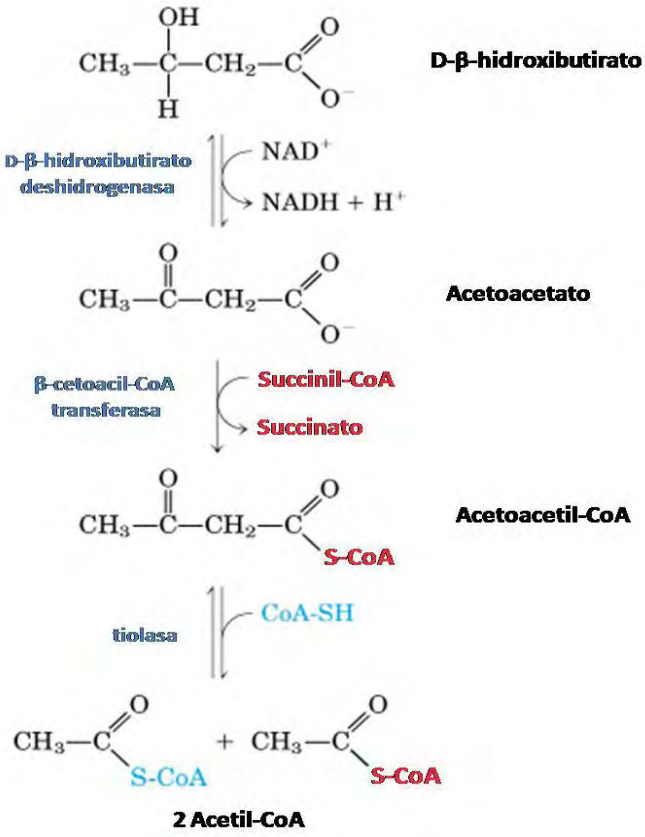


Figura 2. Formación de acetil-CoA a partir de D-β-HB y AcAc. El D-β-HB es sintetizado en el hígado pasa al torrente sanguíneo, y de allí a otros tejidos donde es convertido a acetoacetato, el cual es condensado con una coenzima A donada del succinil-CoA y posteriormente cortado por una tiolasa. El acetil-CoA formado es usado para la producción de energía.

En diversos estudios se ha observado un incremento en la captura y oxidación de los cuerpos cetónicos en estado de cetosis, resultado de dietas ricas en grasas y en algunas condiciones patológicas, como la diabetes, hipoglucemia o hipoxia (Pollay y Stevens, 1980; Kirsch y D′ Alercy, 1984; Hawkins et al., 1986; Nehlig, 1996). Por otra parte, el ayuno prolongado y las

dietas cetogénicas disminuyen la frecuencia y la severidad de las crisis epilépticas en modelos animales (Hori et al., 1997; Bough et al., 2000; Yudkoff et al., 2001), por lo que las dietas cetogénicas ser benéficas para el tratamiento de niños con epilepsia refractaria (Freeman et al., 1998). Además, trabajos pioneros demuestran un incremento en la utilización de cuerpos cetónicos durante condiciones de hipoxia (Kirsch y D′ Alercy, 1984). Recientemente, estudios in vitro han demostrado que la administración de cuerpos cetónicos restaura la actividad sináptica y preserva la integridad morfológica de células después de periodos de privación de glucosa o inhibición glucolítica en rebanadas de hipocampo (Izimi et al., 1998; Massieu et al., 2003). También se ha sugerido que estos compuestos favorecen el mantenimiento del potencial de membrana mitocondrial, disminuyen la liberación del citocromo c y previenen la activación de la caspasa 3 durante condiciones de hipoxia (Masuda et al., 2005). Además, recientemente un estudio in vitro muestra que el AcAc previene el daño oxidativo inducido por glutamato en células en cultivo (Noh et al., 2006). Por otra parte, estudios in vivo demuestran que el tratamiento con cuerpos cetónicos preserva los niveles de ATP y previenen el daño asociado a la hipoxia, la isquemia (Suzuki et al., 2001, 2002; Masuda et al., 2005) y la excitotoxicidad inducida durante la inhibición metabólica (García y Massieu, 2001; Massieu et al., 2003; Mejía-Toiber et al., 2006). Resultados similares se han encontrado en ratones tratados con la toxina mitocondrial 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP), un modelo de la enfermedad de Parkinson (Tieu et al., 2003).

Neurotransmisión glutamatérgica

El glutamato es un aminoácido no esencial en la dieta, que forma parte estructural de las proteínas, y su síntesis está involucrada en el metabolismo de la glucosa y aminoácidos. El glutamato es el neurotransmisor excitador más abundante en el sistema nervioso de mamíferos (Collingridge y Lester, 1989; Fonnum, 1984). Se encuentra a una concentración de entre 5-15 µmol/g tejido (Erecinska y Silver, 1990; Perry et al., 1987). En el espacio sináptico en

condiciones de reposo, se han reportado concentraciones de 0.6-5 μM dependiendo del método de cuantificación utilizado. Se estima que entre el 80–90% de las sinápsis en el cerebro son glutamatérgicas. Las neuronas glutamatérgicas aportan del 80%-88% del contenido total de glutamato del cerebro (Ottersen et al., 1992; Storm-Mathisen et al., 1983). El glutamato es una molécula excitadora que promueve la sobrevivencia neuronal principalmente durante el desarrollo; sin embargo, el incremento descontrolado de su concentración en el espacio extracelular lo convierte en una molécula generadora de daño neuronal y muerte. Cuando el glutamato se libera de la terminal presináptica ocurre la activación de sus receptores en milisegundos. La activación de los receptores glutamatérgicos y de cualquier receptor, es un proceso estocástico en donde el receptor puede activarse y desactivarse sucesiva y azarosamente.

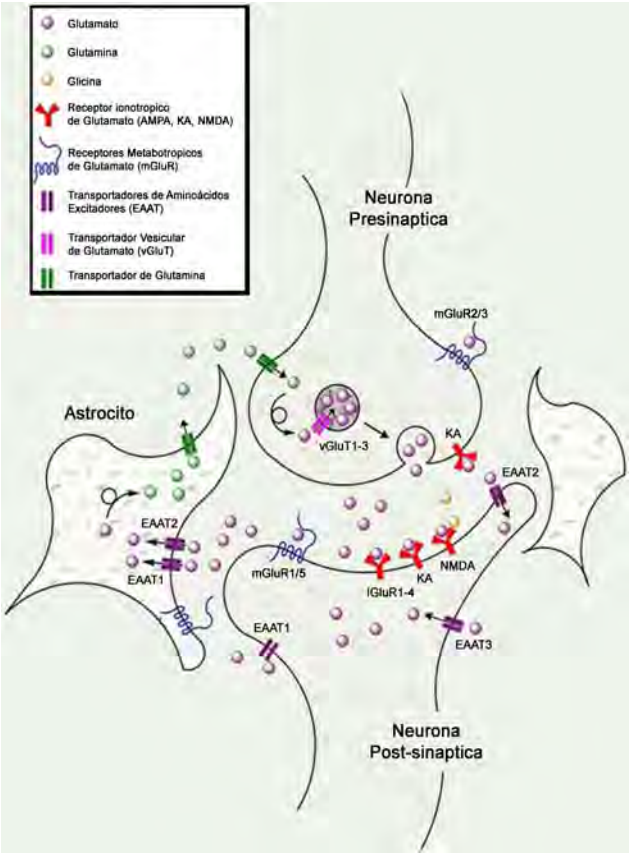


Figura 3. Esquema de la neurotransmisión glutamatérgica. El glutamato es liberado por exocitosis durante la despolarización de la pre-sinápsis. Una vez en la hendidura sináptica, el glutamato activa a sus receptores post-sinápticos ionotrópicos y metabotrópicos. Existen 3 tipos de receptores ionotrópicos: Kainato (KA), NMDA y AMPA. En el caso del receptor NMDA, el glutamato requiere de la glicina (co-agonista), para activar a este receptor. Los receptores metabotrópicos son: pre-sinápticos (mGluR2-3) y post-sinápticos (mGluR1-5). El glutamato es eliminado de la hendidura sináptica por transportadores de glutamato tanto neuronales (EAAT1-3) como gliales (EAAT1-2). Una vez en las células gliales, éste es transformado a glutamina y liberado al espacio extracelular, para ser capturado por la presinápsis y de nuevo transformado en glutamato. El glutamato es de nuevo vesiculizado y liberado en una despolarización subsecuente.

Receptores a glutamato

El glutamato interactúa con dos tipos generales de receptores, los ionotrópicos y los metabotrópicos (Fig. 3). Los receptores ionotrópicos se denominan de acuerdo a su agonista farmacológico, y pueden ser diferenciados gracias a sus propiedades farmacológicas y electrofisiológicas en tres grupos: N-metil-D-aspartato (NMDA), kainato (KA) y α-amino-3-hidroxi-5-metilisoxasolpropionato (AMPA) (Watkins y Olverman, 1987). Los receptores a AMPA y kainato comparten algunas características, por lo cual son llamados receptores no-NMDA. Los tres receptores ionotrópicos son permeables a Na^+ y K^+, mientras que el receptor NMDA y sólo algunos receptores AMPA y kainato son permeables a Ca^{2+} (Nakanishi, 1992; Seeburg y Hartner, 1993). Esta permeabilidad diferencial está dada por la combinación de subunidades que conforman el receptor. Los receptores metabotrópicos están acoplados a proteínas que unen GTP (proteínas G) y modulan la producción de mensajeros intracelulares (Fig. 3) (Ozawa et al., 1998).

Receptor NMDA

El receptor a NMDA responde a la estimulación con glutamato más tardíamente que los receptores no-NMDA y contribuye con el componente lento de la corriente excitadora postsináptica (Fig. 3). El receptor NMDA es altamente permeable a Ca^{2+} y su apertura depende del voltaje, debido a que se encuentra normalmente bloqueado por un ión Mg^{2+} que es liberado al despolarizarse la membrana, abriendo el canal. Este receptor presenta por lo menos siete sitios diferentes de unión a ligandos (Fig. 4). Estos sitios son: 1) sitio de unión al ligando endógeno (glutamato y D-aspartato); 2) sitio de unión a glicina (también es un ligando endógeno) cuya ocupación facilita la unión del glutamato con el receptor; 3) sitio de unión a poliaminas, como son la espermina y la espermidina. Estos compuestos a bajas concentraciones (μM), promueven la apertura del canal y a altas concentraciones bloquean el canal de manera dependiente de voltaje (Lynch y Guttmann, 2002); 4) sitio de unión del Mg^{2+}, que se encuentra en el poro del canal, aquí también se une el (+)-5-metil-10,11-dihidroxi-5H-

dibenzo(a,d)cicloheptano-5,10-imina maleato (MK-801), un potente antagonista farmacológico no competitivo del receptor); 5) sitio de unión a Zn^{2+}, 6) sitio modulador de unión a glutatión y 7) sitio de unión a fenciclidinas.

En la última década se ha determinado la composición de las subunidades del receptor NMDA. Este receptor es un tetrámero conformado por una subunidad NMDAR1 (NR1) en combinación con una o más subunidades NMDAR2 (NR2), y menos comúnmente una subunidad NMDAR3 (NR3-A y B) (Kemp y McKernan, 2002). Se han descrito ocho isoformas de la subunidad NR1 generadas por cortes alternativos en el mismo gen, denominadas NR1A-NR1G y una variante truncada no funcional (McBain y Mayer, 1994). La subunidad NR2, se presenta en cuatro isoformas (NR2A-NR2D), provenientes de distintos genes.

Los subtipos que forman la subunidad NR2 contienen entre 40-55% de homología en su secuencia, y un 27% con la subunidad NR1. Las subunidades NR3 presentan un 50% de homología en su secuencia, y comparten un 27% con NR1 y con NR2. Para que un receptor sea funcional requiere de la asociación de varias subunidades, siendo fundamental la presencia de al menos una NR1 y al menos una o más subunidades NR2, (NR1/NR2A, NR1/NR2B, o NR1/NR2A/NR2B) o NR1 en combinación tanto con las subunidades NR2 como NR3 (NR1/NR2A/NR3A). De hecho, el requerimiento mínimo para que un receptor sea funcional es un tetrámero compuesto de dos subunidades NR1 y dos NR2. Las subunidades NR1 y NR2A-NR2D presentan una asparagina en el sitio correspondiente a la glutamina/arginina en los receptores AMPA. Esta asparagina determina la permeabilidad al Ca^{2+} y el bloqueo del Mg^{2+} en el canal del receptor NMDA (Ozawa et al, 1998). El patrón de expresión de las subunidades NR2 y NR3 se restringe a cierto tipo de células y a un determinado periodo de desarrollo (Kew et al., 1998; Monyer et al., 1992; Watanabe et al., 1994). Durante la etapa embrionaria y de desarrollo temprano, el cerebro de los roedores y del humano, presenta principalmente la subunidad NR2B, mientras que la NR2D está presente en el diencéfalo y el cerebro medio. En los días postnatales, la expresión de la NR2A se establece en la mayoría de las regiones cerebrales, y la NR2C aparece posteriormente y de manera prominente en el

cerebelo (Cull-Candy y Leszkiewicz, 2004). En el adulto hay un decremento en la expresión de la subunidad NR2B a lo largo de la vida del individuo, lo cual se asocia con un incremento en la expresión de la subunidad NR2A (Cull-Candy y Leszkiewicz, 2004). En cambio, el patrón de expresión de la subunidad NR1, misma que es fundamental para que el receptor funcione, se mantiene desde el desarrollo embrionario y hasta el animal adulto (Ishii et al., 1993; Kutsuwada et al., 1992; Monyer et al., 1992). La subunidad NR3 también presenta un patrón de expresión espacio-temporal, ya que mientras la NR3A se expresa principalmente durante el desarrollo embrionario y llega a persistir en el adulto, la NR3B está confinada a motoneuronas somáticas del tallo y médula espinal (Nishi et al., 2001).

La proporción de las subunidades NR1/NR2 que conforman este receptor, les confiere una cinética característica de activación por glutamato. Tomando en consideración que la NR1 posee 8 diferentes isoformas de corte y empalme alternativo, y que hay 4 y 2 genes que codifican las subunidades NR2 y NR3 respectivamente, se calcula que pueden ensamblarse hasta 384 distintos tipos de receptores a NMDA. En particular, la asociación NR1/NR2A presenta una constante de activación de tiempo menor (50 ms), a la que se manifiesta con la asociación de NR1/NR2B (300 ms), esta última es muy similar a la que presenta la combinación NR1/NR2C (280 ms); siendo la combinación NR1/NR2D la que presenta la cinética más lenta (1.7 s) (Cull-Candy y Leszkiewicz, 2004). Estas características son de gran importancia en la excitabilidad neuronal.

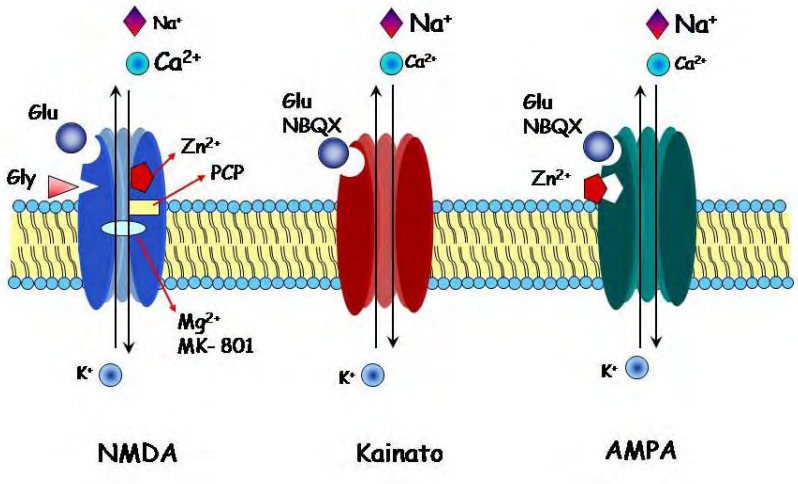


Figura 4. Representación esquemática de los diferentes tipos de receptores ionotrópicos a glutamato, y sus principales moduladores.

Receptores no-NMDA

Dentro de los receptores no-NMDA se encuentran los receptores AMPA y los receptores Kainato. Estos son complejos formados por cinco subunidades hetero-oligómericas. Ambos receptores están acoplados a canales iónicos permeables tanto a Na^+ como a K^+ (Fig. 3). La mayoría de canales asociados a receptores AMPA no son permeables a Ca^{2+}, pero algunos de estos receptores forman canales iónicos permeables a este catión (Michaelis, 1997).

Receptor AMPA

Los receptores AMPA están compuestos de una familia de cuatro subunidades (GluR1-4) codificadas por genes separados y que se ensamblan formando tetrámeros funcionales (Rosenmund et al., 1998). Las cuatro subunidades, tienen un tamaño similar (~900 aminoácidos), presentan un 68-73% de semejanza en la secuencia de aminoácidos (Ozawa et al, 1998). Los receptores AMPA, semejantes a los receptores a NMDA, tienen una composición heteromérica. Dado que los receptores AMPA permiten el paso de Na^+ como ión principal, su activación participa en la transmisión rápida de las sinápsis glutamatérgica, despolarizando a la célula e induciendo la activación de diversos canales sensibles a voltaje.

En el SNC, las propiedades funcionales del receptor AMPA están influenciadas, en gran medida, por la subunidad GluR2, ya que la presencia de está subunidad le confiere al canal una baja permeabilidad al Ca^{2+} (Bowie y Mayer, 1995). El residuo que determina la impermeabilidad al Ca^{2+} es el aminoácido arginina (R) localizado en el poro del asa, del dominio M2 de las subunidades GluR1, 3 y 4, y que corresponde a una glutamina en la subunidad GluR2 (Rosenmund et al., 1998; Seeburg y Hartner, 2003). El receptor a AMPA presenta al menos tres sitios de unión independientes en los cuales pueden actuar tanto agonistas como antagonistas (Fig. 4). El sitio de unión a glutamato es el mismo para antagonistas competitivos, como: el 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxalina (NBQX) y el 6-(1H-imidazol-1-yl)-7-nitro-2,3(1H,4H-quinoxalinediona (YM90K).

Recepto a Kainato

Los receptores a kainato están agrupados en dos familias de subunidades relacionadas, GluR5-7 y KA1-2. Todas las subunidades presentes en la estructura básica de los receptores AMPA/Kainato incluyen los cuatro segmentos transmembrales. Las subunidades GluR5-GluR7 tienen un tamaño similar (~900 aminoácidos) y una similitud del 75-80% en la secuencia de aminoácidos y 40% con las subunidades del receptor AMPA. Las subunidades KA1–KA2 son un poco más grandes que GluR5-GluR7 (~970 aminoácidos) y tienen un 70% de identidad en la secuencia de aminoácidos (Ozawa et al, 1998). Este subtipo está conformado de manera tetramérica en combinación homomérica o heteromérica. Las subunidades KA1 y KA2 se combinan en ensamble heteromérico con miembros de la familia GluR5-7 y forman receptores funcionales (Bleakman et al., 2002). Las subunidades GluR5-7 forman receptores homoméricos funcionales, pero también se combinan con KA1 y KA2 formando receptores heteroméricos con distintas propiedades farmacológicas (Alt et al., 2004). Similar a las subunidades de los receptores a NMDA, las subunidades GluR5-7 se forman por corte y empalme alternativo del mismo gen. El patrón de expresión de cada subunidad es específico para las distintas regiones cerebrales: en el hipocampo la subunidad GluR6 se encuentra localizada a nivel presináptico y postsináptico en la mayoría de las fibras musgosas de la región CA3 (Contractor et al., 2000). La subunidad GluR5 se expresa predominantemente en interneuronas de hipocampo en donde media un efecto de facilitación de la liberación de glutamato por los astrocitos. GluR5 se expresa en el lóbulo temporal, y está muy relacionada con la excitación y la epileptogénesis en la amígdala (Rogawski et al., 2003). Además de las neuronas principales de CA3, se ha identificado la presencia de receptores postsinápticos de kainato en neuronas de la amígdala lateral, en interneuronas del hipocampo, en neuronas del asta dorsal de la médula espinal, en algunas células bipolares de la retina, en el corteza cerebral y en el cerebelo (Rodríguez-Moreno, 2006).

Además de la participación de los receptores tipo kainato en la despolarización de la membrana plasmática, algunos experimentos han

propuesto que estos receptores también pueden ser de tipo metabotrópico acoplados a la activación de proteínas G y localizarse en terminales presinápticas. De esta forma, la activación de los receptores promoverá la activación de la PKC y la inhibición de la corriente de K^+ dependiente de Ca^{2+}. La consecuencia final de su activación es la repolarización de la membrana celular y la subsiguiente inhibición de la transmisión sináptica. De hecho la inhibición de la corriente asociada a la activación de los receptores a kainato incrementa drásticamente la excitabilidad de la membrana y la liberación de transmisores (Lerma, 2006).

Receptores metabotrópicos

Los receptores metabotrópicos están agrupados en al menos ocho diferentes subtipos, divididos de acuerdo a su secuencia, farmacología y al mecanismo de transducción de señales al que se encuentran acoplados. En general, poseen un largo extremo extracelular bi-lobular que representa el dominio N-terminal, el cual se ha propuesto como el sitio al que se une el glutamato de acuerdo a estudios de mutagénesis y cristalografía de rayos X. El dominio N-terminal se une mediante una región rica en cisteínas al dominio de siete segmentos transmembranales, el cual interviene en la activación de las proteínas G. El carboxilo terminal es intracelular y está involucrado en la regulación de la actividad del receptor y del tráfico del mismo, mediante su asociación con varias proteínas incluyendo la calmodulina y proteínas estructurales como las de la familia Homer y PICKI (Kew y Kemp, 2005).

Los receptores metabotrópicos se encuentran acoplados a una proteína G, que puede activar a la fosfolipasa C (PLC) o a la adenilato ciclasa y en general producen cambios funcionales y de conductancia membranal en una escala temporal más lenta que los receptores ionotrópicos (Stone y Addae, 2002). Los que están acoplados a PLC provocan la hidrólisis de fosfolípidos de inositol, produciendo segundos mensajeros como el diacilglicerol (DAG) y el inositol 1-4,5-trifosfato (IP_3). El DAG activa a la proteína cinasa C (PKC) que promueve la fosforilación de proteínas y el IP_3 moviliza Ca^{2+} de pozas

intracelulares, al activar a sus receptores en el retículo endoplásmico (Fig. 5). Algunos receptores metabotrópicos inhiben a la adenilato ciclasa (AC) y por lo tanto la formación de AMPc.

Los subtipos $mGluR_1$-$mGluR_8$ están muy relacionados en su estructura primaria. La secuencia de aminoácidos de los mGluRs muestran más del 40% de identidad y todos tienen siete regiones transmembranales. Todos los receptores mGluRs son proteínas considerablemente grandes (854-1179 aminoácidos) con una región hidrofóbica N-terminal grande de ~550 aminoácidos, una región central de ~250 aminoácidos, que como ya se mencionó, incluye los siete dominios transmembranales y una región C-terminal (Nakanishi, 1992; Hollmann y Heinemann, 1994).

Existen tres grupos de receptores metabotrópicos: grupo I que incluye a los subtipos $mGluR_1$ y $mGluR_5$ y que está acoplado positivamente a la fosfolipasa C; el grupo II que incluye a $mGluR_2$ y $mGluR_3$; y el grupo III incluyendo a $mGluR_4$, $mGluR_6$, $mGluR_7$ y $mGluR_8$, éstos dos últimos grupos inhiben la actividad de la adenilato ciclasa (Swanson et al., 2005). Los subtipos del grupo I se expresan en la mayoría de las sinápsis glutamatérgicas a nivel postsináptico. La función de este grupo está vinculada a la plasticidad sináptica, incluyendo la potenciación y la depresión a largo plazo y se relaciona con desórdenes de ansiedad. En el grupo II se encuentran los subtipos que se localizan tanto a nivel presináptico ($mGluR_2$ y $mGluR_3$) como postsináptico ($mGluR_3$). La distribución del $mGluR_2$ es abundante a nivel de hipocampo y amígdala. Funcionalmente el $mGluR_2$ está relacionado con la depresión a largo plazo. Por su parte el subtipo $mGluR_3$ se distribuye en el hipocampo y el tálamo, y se encuentra asociado a la liberación de neurotrofinas de las células gliales, y ambos subtipos también intervienen en el trastorno de ansiedad. Los subtipos del grupo III poseen la mayor diversidad en cuanto a función y localización se refiere. Los subtipos $mGluR_4$ y $mGluR_7$ se localizan tanto pre- como postsinápticamente. El subtipo $mGluR_4$ a nivel presináptico está relacionado con la plasticidad sináptica y aprendizaje. Por su parte el $Glum_7$ a nivel presináptico se localiza en las zonas activas de la sinápsis, proponiéndolo como un

autoreceptor con función inhibidora. El $mGluR_6$ es un subtipo especial ya que su expresión está confinada a las células bipolares de la retina, por lo que su función es importante en los procesos de visión. Finalmente el subtipo $Glum_8$ está principalmente localizado a nivel presináptico en las sinápsis glutamatérgicas y en algunos otros tipos. Se distribuye abundantemente en el hipocampo y la amígdala, por lo que su función está relacionada con la ruta perforante lateral.

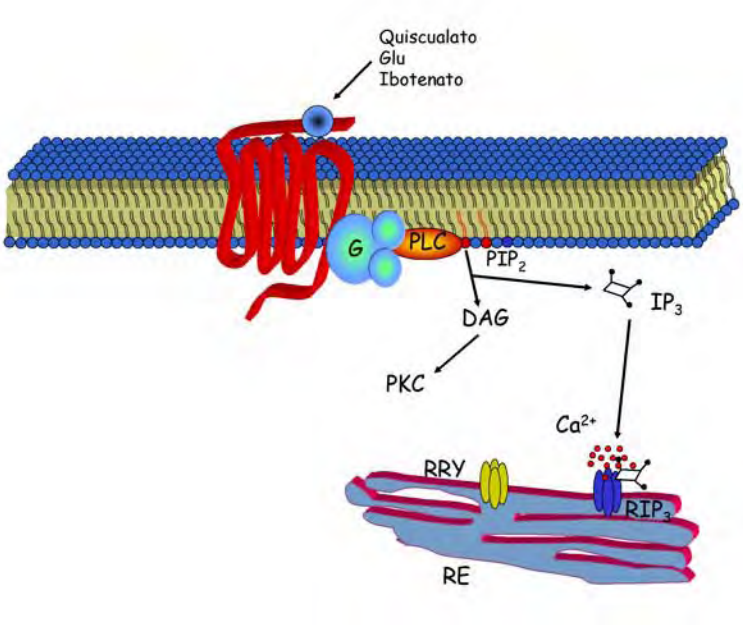


Figura 5. El receptor metabotrópico (grupo I) es activado por su ligando endógeno, el glutamato, y por agonistas farmacológicos como el quiscualato y el ibotenato. Se encuentra acoplado a la vía proteína G (G)-fosfolípasa C (PLC). La PLC cataliza la producción de diacilglicerol (DAG) que activa a una proteína cinasa C (PKC) que activa una serie de enzimas intracelulares y produce inositol-1,4,5-trifosfato (IP_3) el cual promueve la liberación de calcio de retículo endoplásmico.

Transportadores de glutamato

En el control de la neurotransmisión glutamatérgica participan diversas proteínas y tipos celulares. Entre las proteínas más importantes se encuentran los transportadores de glutamato localizados en neuronas y principalmente en las células gliales (Grewer y Rauen, 2005; Sonders et al., 2005) (Fig. 2). En la actualidad se han clonado cinco transportadores de glutamato de alta afinidad dependientes de Na^+ (Danbolt, 2001): transportador de glutamato/aspartato (GLAST) (Storck et al., 1992), transportador de glutamato tipo 1 (GLT-1) (Pines et al., 1992), acarreador de aminoácidos excitadores tipo 1 (EAAC1) (Kanai y Heideger, 1992), transportador de aminoácidos excitadores tipo 4 (EAAT4) (Fairman et al., 1995) y 5 (EAAT5) (Arriza et al., 1997).

GLAST y GLT-1 se encuentran localizados principalmente en las células gliales (Schmitt et al., 1997; Rothstein et al., 1994) y al parecer son los transportadores predominantes en el cerebro. EAAC1 ha sido localizado exclusivamente en neuronas, tanto glutamatérgicas como no-glutamatérgicas (Rothstein et al., 1994). Estudios de microscopia electrónica han revelado que este transportador solo se encuentra localizado en los somas de las neuronas y no en las terminales presinápticas (Rothstein et al., 1994). EAAT4 se encuentra exclusivamente en las dendritas de las células de Purkinje del cerebelo (Fairman et al., 1995) y EAAT5 se expresa selectivamente en las células de Müller de la retina (Arriza et al., 1997).

El funcionamiento de los transportadores de glutamato depende del gradiente electroquímico del Na^+ generado por el funcionamiento de las ATPasas Na^+/K^+ membranales. De acuerdo al modelo de Watzke y colaboradores (2001), durante la captura de glutamato intervienen varios iones, previamente a la unión del glutamato el transportador tiene unido un ión K^+. Antes de que se inicie la captura, el ión K^+ se desprende de su sitio y la captura se inicia por la unión de un ión Na^+ y posteriormente la de un ión H^+. El glutamato se une en este momento seguido de un segundo ión de Na^+. Se sabe que se requiere de la unión de tres iones Na^+ para que se lleve a cabo la captura a través de un mecanismo simporte. Sin embargo, aún no está claro si el tercer ión Na^+ se une al transportador antes o después de unirse el glutamato (Grewer y Rauen, 2005). La translocación del glutamato al interior de la célula, ya sea en una neurona o en célula glial, induce el transporte de un ion K^+ que se cree se une al transportador después de que éste ha liberado al citoplasma 3 Na^+, 1 H^+ y 1 glutamato. El transporte iónico durante la captura de glutamato es electrogénico, ya que se mueven dos cargas positivas dentro de la célula por cada ciclo completo de transporte (Grewer y Rauen, 2005). Se ha descrito la activación de una corriente entrante de Cl^- asociada a la captura de glutamato en algunos tipos de transportadores; sin embargo, a la fecha no se sabe si el canal permeable a Cl^- forma parte de la proteína del transportador o simplemente es un canal independiente (Wadiche et al., 1995).

El glutamato capturado por las células gliales es transformado a glutamina, en un proceso dependiente de ATP y Mg^{2+}, por la acción de la glutamina sintetasa (una enzima exclusiva de las células gliales) (Fig. 2). La glutamina sale de la glía a través del sistema de transporte N y regresa a las neuronas por el sistema de transporte A (Broer y Brookes, 2001). Una vez en la célula, entra a la mitocondria neuronal y es transformada a glutamato por medio de la glutaminasa activada por fosfato. De esta manera, el glutamato está nuevamente disponible para ser capturado en las vesículas y liberado mediante un estímulo despolarizante. Sin embargo, el glutamato (en astrocitos) puede ser metabolizado oxidativamente y generar α-cetoglutarato (Hertz et al., 1999; Yudkoff et al., 1993). La poza de glutamato oxidado debe de reemplazarse, lo cual ocurre a través de la actividad de la enzima piruvato carboxilasa. El ciclo mediante el cual el glutamato se transforma a glutamina en la glia, y la glutamina se transforma a glutamato en las neuronas se conoce como ciclo glutamina-glutamato y constituye una de las vías metabólicas más importantes para el reciclamiento del glutamato en la terminal presináptica de neuronas (Erecinska and Silver, 1990) (Fig. 3).

Diversos estudios han demostrado que existe una relación de 1:1 entre la oxidación de la glucosa neuronal y el ciclo glutamina-glutamato, es decir entre el consumo de glucosa y la producción de glutamina. Lo anterior, concuerda con algunos estudios *in vitro* sugiriendo que durante la captura de glutamato por la glia, el cotransporte de Na^+ estimula la glucólisis y la producción de lactato en la glia (Magistretti et al., 1999; Pellerin y Magistretti, 1994). Estas observaciones sugieren una importante relación entre la transmisión glutamátergica y el consumo de glucosa.

Excitotoxicidad

Los primeros estudios que sugirieron el papel del glutamato como neurotransmisor excitador fueron realizados por Curtis y colaboradores en 1954, en donde se observó que el glutamato y sus análogos superfundidos en células

de médula espinal de gato, provocaba un aumento en la tasa de disparo de éstas. Por otra parte en 1957, Lucas y Newhouse observaron por primera vez que la inyección intraperitoneal de glutamato en ratas producía degeneración de la capa interna en la retina. Posteriormente, Olney y colaboradores (1969) observaron que la administración de glutamato a ratones inmaduros producía neurodegeneración en algunas regiones del SNC, como el hipotálamo y otras zonas cuya barrera hematoencefálica no estaba totalmente desarrollada. Dada la correlación entre la capacidad del glutamato para despolarizar, y su potencialidad neurotóxica, Olney en 1978 acuño el término excitotoxicidad para referirse a la capacidad de los aminoácidos excitadores bajo ciertas condiciones, de destruir a las células nerviosas. Se sugirió que la muerte neuronal excitotóxica se debe al agotamiento de las pozas energéticas debido a su excitación prolongada.

En 1988, Choi demuestra que la entrada de Ca^{2+} a la célula es un factor preponderante en la muerte excitotóxica, ya que el catión activa una serie de enzimas (proteasas, endonucleasas, fosfolipasas y xantina oxidasa), y vías metabólicas que dan lugar a la desintegración de proteínas y fosfolípidos de la membrana, como la calpaina que degradará proteínas estructurales como fodrina, tubulina, y MAP2 causando alteración o rompimiento del citoesqueleto (Siman y Noszek, 1988). El metabolismo del ácido araquidónico por oxidasas genera radicales libres, que también desencadenan la oxidación de lípidos (Chan y Fishman, 1982). Lo anterior puede traer como consecuencia la generación de radicales libres, y un subsecuente daño al DNA, a proteínas y a lípidos membranales contribuyendo al deterioro y la muerte neuronal (Fig. 6)

Finalmente en los 90's, se retomó la hipótesis excitotóxica inicialmente propuesta por Olney, en donde se da énfasis a la participación del calcio y al estado energético en el proceso de muerte neuronal.

Muerte excitotóxica, isquemia e hipoglucemia.

La isquemia cerebral y la hipoglucemia severa son patologías consideradas como neurológicas agudas, ya que se manifiestan en un periodo

corto de tiempo, que comprende desde horas hasta un par de días. En éstas se ha propuesto que la muerte neuronal ocurre por un mecanismo excitotóxico.

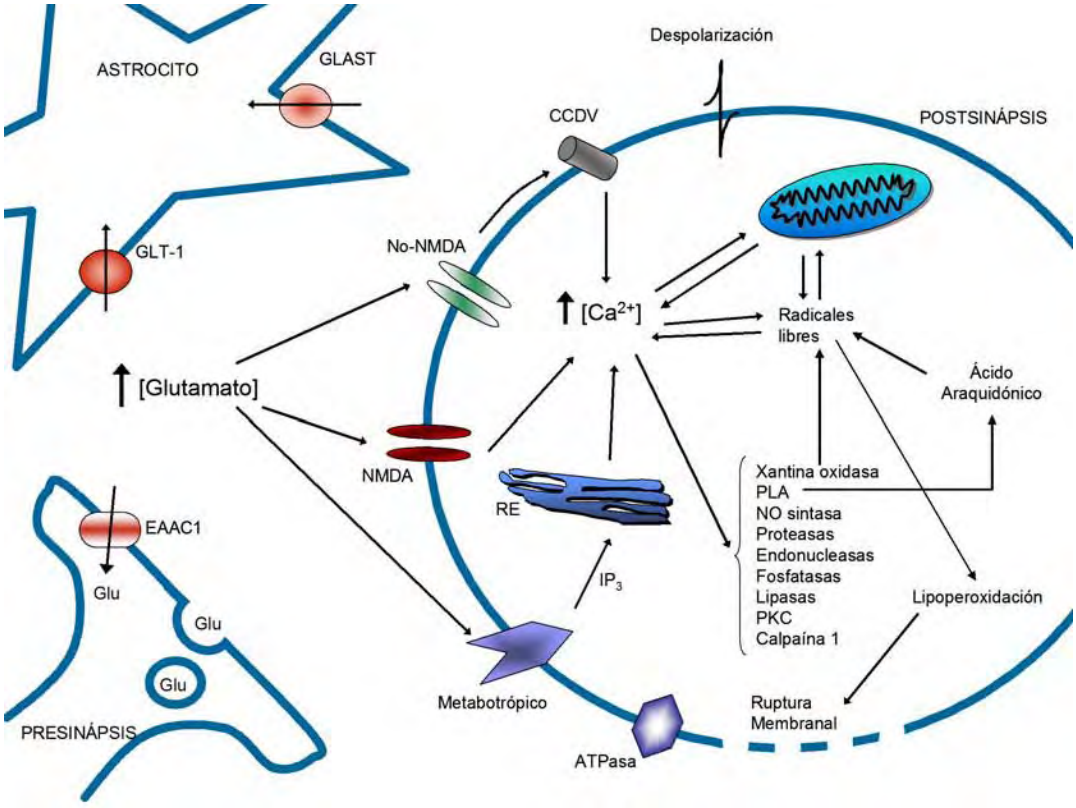


Figura 6. Posibles vías metabólicas involucradas en la muerte neuronal producida por excitotoxicidad. La activación de los receptores glutamatérgicos ionotrópicos permite una entrada masiva de calcio. El aumento en la concentración de calcio intracelular activa una serie de enzimas que contribuyen al daño neuronal. La pérdida de la integridad de la membrana plasmática da lugar a la fuga de glutamato al medio extracelular, lo que contribuye a la activación prolongada de los receptores glutamatérgicos de neuronas vecinas. A su vez, la entrada de calcio promueve la liberación de glutamato por exocitosis, creándose un sistema de retroalimentación que redunda en la sobrexcitación neuronal prolongada.

La isquemia cerebral asociada a los accidentes cerebrovasculares tiene un gran impacto debido a la severidad de sus secuelas. Los trastornos vasculares cerebrales se ubican entre las tres primeras causas de muerte en Norteamérica y en Europa Occidental, y son la causa principal de incapacidad permanente en los adultos, principalmente por el traumatismo cerebral y la

hipoxia perinatal. En México los accidentes cerebrovasculares representan la 6ª causa de muerte (INEGI, 2001). Independientemente de la causa, la isquemia cerebral se presenta cuando existe una reducción del flujo sanguíneo cerebral, provocando daño neuronal irreversible en la zona en donde se interrumpe por completo el flujo sanguíneo (foco isquémico). Sin embargo, en la zona circundante al foco isquémico, el daño neuronal se desarrolla lentamente. Esta región es denominada como zona de penumbra o zona perifocal (Massieu, 1998).

Un gran número de estudios apoyan la hipótesis de que la muerte neuronal asociada con la isquemia cerebral se debe a un proceso excitotóxico mediado por la sobre-activación de los receptores a glutamato. En experimentos de microdiálisis en diversos modelos de isquemia cerebral se ha demostrado que inmediatamente después del inicio del episodio isquémico se presenta un incremento en la concentración extracelular de los aminoácidos excitadores (AAE), glutamato y aspartato (Benveniste et al. 1984). En las regiones donde ocurre este incremento se presenta muerte neuronal días después del evento isquémico, la cual se ha denominado muerte neuronal retardada. De manera similar, durante la hipoglucemia aguda inducida por insulina, así como durante la inhibición farmacológica de la vía glucolítica en el cerebro, se produce un incremento de los niveles extracelulares de AAE (Sandberg et al., 1985; Sandberg et al., 1986; Massieu et al., 2000).

Además, diversos estudios han demostrado que los antagonistas de los receptores glutamatérgicos tanto de tipo NMDA como no-NMDA, evitan la muerte asociada a la hipoxia/isquemia y a la hipoglucemia (Simon et al. 1984; Foster et al. 1988; Wieloch et al. 1985; McCulloch et al. 1991). Asimismo, se ha encontrado que la interrupción de la inervación glutamátergica a la región isquémica o hipoglucémica, evita el daño neuronal (Benveniste et al. 1989; Wieloch et al. 1985; Sandberg et al. 1986;). Se ha propuesto que el decremento del contenido de proteína de los transportadores de glutamato (Chen et al., 2005; Kim et al., 2006; Rao et al., 2001a; Yeh et al., 2005) y el funcionamiento deficiente de los mismos (Allen et al., 2004; Mitani y Tanaka, 2003; Yeh et al.,

2005) explicaría la acumulación de AAE durante periodos de isquemia e hipoglucemia, así como durante la inhibición glucolítica. Varios grupos de investigación han propuesto que tales alteraciones preceden a la muerte neuronal (Raghavendra et al., 2000; Rao et al., 2001b; Camacho et al., 2007). Adicionalmente, se han descrito alteraciones en el patrón de expresión de algunas subunidades que forman el receptor a NMDA asociadas a eventos de isquemia, hipoglucemia e inhibición glucolítica (Gascon et al., 2005; Quintana et al., 2006; Sutcu et al., 2005; Zhang et al., 1997; Camacho et al., 2007), lo cual podría estar relacionado con la alteración en la neurotransmisión glutamatérgica y con el incremento en la susceptibilidad a la muerte neuronal mediada por la acumulación de glutamato.

Excitotoxicidad y estado energético

Como ya se mencionó anteriormente, la glucosa es el principal sustrato energético en el SNC y la demanda energética del cerebro es muy alta. Por esta razón las alteraciones en la producción de ATP pueden tener consecuencias fatales en la sobrevivencia neuronal. A pesar de que la vía glucolítica aporta solo del 4.6-7.7% del ATP total, su funcionamiento es esencial en el desarrollo óptimo de la neurotransmisión glutamatérgica (Hyder et al., 2006; Magistretti et al., 1999; Pellerin and Magistretti, 1994; Sibson et al., 1997). Durante condiciones de hipoxia/isquemia la interrupción de la irrigación sanguínea cerebral produce un estrés metabólico debido a la falta de ATP generado a través de la fosforilación a nivel de sustrato de la glucólisis, y a la fosforilación oxidativa. Cuando los requerimientos de glucosa no se satisfacen debido a la hipoglucemia o a la isquemia cerebral, se produce la muerte de las neuronas posiblemente como consecuencia de la combinación de la deficiencia energética debida, no sólo a la falta de flujo sanguíneo, sino posiblemente también a la alteración de la actividad del transporte de electrones mitocondrial (Allen et al. 1995; Zaidan y Sims, 1994; Canevari et al. 1997; Camacho et al., 2007); y al aumento en los niveles extracelulares de los aminoácidos excitadores, glutamato y aspartato (Benveniste et al. 1989). Las causas de este aumento en las concentraciones

extracelulares de estos aminoácidos no se conocen, pero se piensa que puede deberse a un funcionamiento deficiente o inverso de sus transportadores, que son dependientes de energía (Rossi et al. 2000; Allen et al., 2004; Mitani y Tanaka, 2003; Yeh et al., 2005).

En las últimas décadas se ha reconocido la importancia de la relación que existe entre los efectos tóxicos del glutamato y el estado energético de la célula (Novelli et al. 1998; Henneberry, 1989; Beal, 1993; Schinder et al. 1996). En estudios tanto *in vivo* como *in vitro* se ha demostrado que la toxicidad del glutamato y sus análogos se exacerba en presencia de inhibidores metabólicos (Novelli et al. 1998; Greene y Grenamyre, 1995; Sánchez-Carbente y Massieu, 1999; García y Massieu, 2001; Massieu et al, 2001; 2003). Estudios *in vitro* en retina de pollo han mostrado que la inhibición simultánea de la glucólisis y la cadena transportadora de electrones da lugar a la muerte neuronal, la cual puede prevenirse con antagonistas del receptor glutamatérgico de tipo NMDA (Zeevalk y Nicklas, 1990). Estudios *in vitro* han mostrado que durante condiciones de deficiencia energética, tales como la inhibición glucolítica o mitocondrial, se presentan cambios en la concentración intracelular de Na^+ y K^+, resultando en la despolarización membranal y un incremento en la concentración de Ca^{2+} intracelular (Silver et al., 1997). El colapso del gradiente iónico membranal, provoca a su vez la despolarización de la membrana y la pérdida del bloqueo del receptor NMDA por el Mg^{2+}. Esto da lugar a la sobreactivación de estos receptores aún a concentraciones fisiológicas de glutamato extracelular, dando lugar a un proceso conocido como excitotoxicidad secundaria, ya que no implica un aumento en los niveles de glutamato en el medio (Novelli et al. 1988; Zeevalk y Nicklas, 1990, 1992; Storey et al. 1992; Pang y Geddes, 1997) (Fig. 7). Estudios indican que la producción de energía a través de la vía glucolítica tiene un impacto mayor sobre el control de los gradientes iónicos que la producción de energía por la vía mitocondrial, por lo que se ha propuesto que el ATP glucolítico es el que alimenta principalmente a la ATPasa de Na^+/K^+ (Silver et al., 1997; Kahler y Reiser, 2000).

Por otra parte, algunos experimentos sugieren que el daño neuronal irreversible producido por el glutamato o sus análogos se debe a la falla mitocondrial (Ankarcrona et al. 1995; Schinder et al. 1996; Budd y Nicholls, 1996) debido a una sobre carga de calcio. Esto apoya la estrecha relación entre el estado energético celular y la muerte excitotóxica.

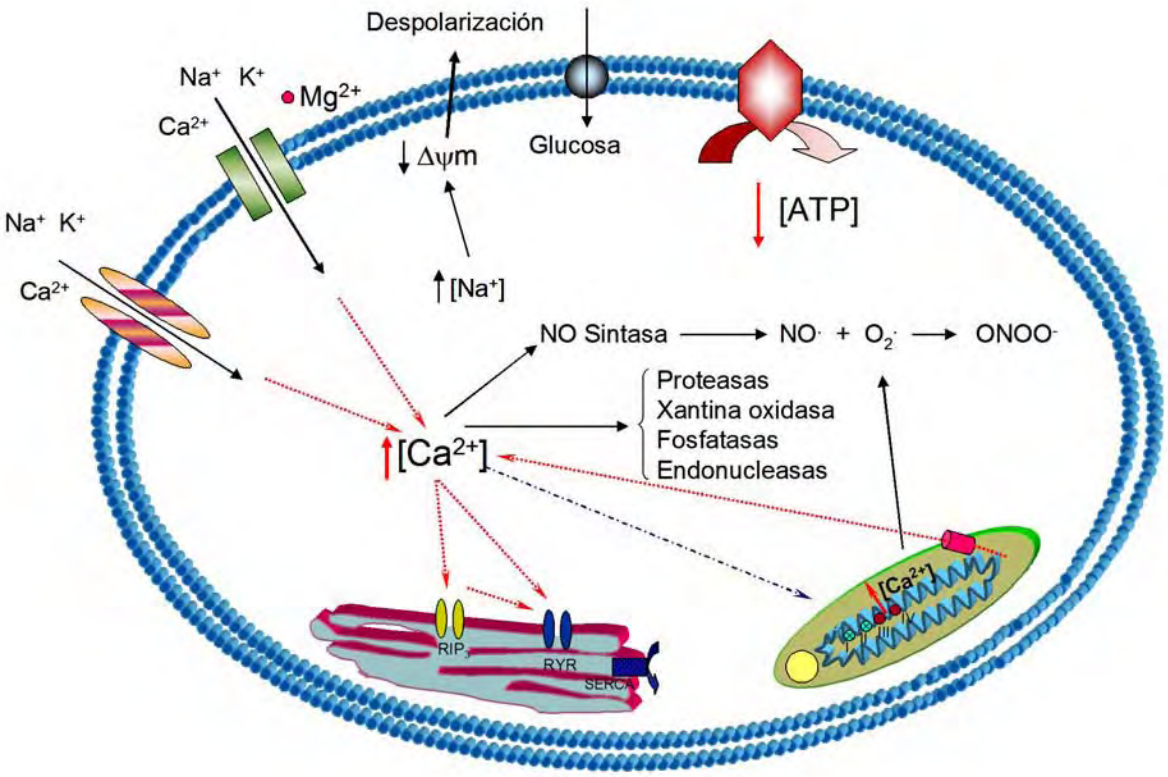


Figura 7. Mecanismos celulares involucrados en la muerte inducida por excitotoxicidad secundaria. En condiciones de deficiencia energética, la actividad de la ATPasa Na^+/K^+ es inhibida colapsando el gradiente iónico membranal, provocando a su vez la despolarización de la membrana y la pérdida del bloqueo del receptor NMDA por el Mg^{2+}. Esto permite la activación de estos receptores a concentraciones bajas de glutamato extracelular, y un incremento de calcio intracelular. Este incremento de calcio intracelular activa una serie de enzimas como proteasas, fosfatasas, endonucleasas y la oxido nítrico sintasa. El óxido nítrico ($NO^{\bullet}$) y el superóxido ($O^{\bullet}_2$) producen daño membranal por lipoperoxidación.

Homeostasis de calcio intracelular en la muerte excitotóxica

La concentración de Ca^{2+} intracelular está muy relacionada con un gran número de procesos fisiológicos. La homeostasis de Ca^{2+} intracelular es mantenida mediante diversos mecanismos membranales de extrusión de Ca^{2+}, como la ATPasa de Ca^{2+} membranal y el intercambiador Na^{+}/Ca^{2+}, y de almacenamiento, como la ATPasa del retículo endoplásmico y transportadores mitocondriales.

En las siguientes páginas se incluye una revisión que se público como capítulo del libro “The neurochemistry of Neuronal Death”, en donde se revisan los distintos sistemas encargados de regular la concentración de calcio intracelular y su relación con la producción de EROS y la muerte neuronal.

ARTICULO 1

"Interplay between intracellular calcium handling and reactive oxygen species. Role of mitochondria and endoplasmic reticulum"

(The neurochemistry of Neuronal Death, Research Signpost, 2007, Keral, India. p. 67-93)

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

The Neurochemistry of Neuronal Death, 2007: ISBN: 81-308-0086-1
Editors: Lourdes Massieu, Clorinda Arias and Julio Morán

Interplay between intracellular calcium handling and reactive oxygen species. Role of mitochondria and endoplasmic reticulum

Karla Hernández-Fonseca[1], Lourdes Massieu[1] and Mauricio Díaz-Muñoz[2]
[1]Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México, D.F. AP 70-257, CP 14610
[2]Departamento de Neurobiología Celular y Molecular, Universidad Nacional Autónoma de México, Campus Juriquilla, Qro., Querétaro, México

Abstract

The intracellular concentration of calcium is highly regulated due to its participation in a variety of physiological processes. Intracellular calcium homeostasis is maintained through the balance between calcium extruding and calcium storage mechanisms. Mitochondria and endoplasmic reticulum are the major intracellular calcium stores, while ATPases and ionic exchangers

Correspondence/Reprint request: Dr. Karla Hernández-Fonseca, Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México, D.F. 04510, AP 70-257, Mexico
E-mail: kfonseca@ifc.unam.mx

embedded in the plasma membrane constitute the main calcium extrusion systems. Calcium is a key factor in mitochondrial function since many mitochondrial enzymes are calcium-dependent. However, mitochondrial calcium overload induces mitochondrial dysfunction and the generation of free radicals. A close relationship between increased intracellular calcium, mitochondrial dysfunction and the production of reactive oxygen species is well documented, and its participation in many pathological conditions, such as hypoxia/ischemia, hypoglycemia, and excitotoxicity, has been suggested by several studies. On the other hand, recent evidence suggests an interaction between mitochondria and endoplasmic reticulum in the regulation of intracellular calcium. The role of such interaction in the cell death process has been the focus of recent studies, but its participation in the generation of reactive oxygen species (ROS) during the cell death processes is not completely understood. The aim of the present review is to discuss relevant evidence relating calcium loading in the endoplasmic reticulum, mitochondrial dysfunction, and ROS production, with neuronal death associated with several pathological conditions.

Introduction

Intracellular calcium plays an important role as a second messenger in the regulation of different cellular processes such as exocytosis, excitability, muscle contraction, metabolism, synaptic plasticity, and gene expression during development, differentiation, and proliferation [1]. The generation of calcium signals in the cell cytoplasm is regulated by the coordinated action of several systems controlling calcium entry, calcium release from intracellular stores, buffering of calcium ions by Ca-binding proteins, calcium extrusion by ionic exchangers and Ca-ATPases located at the plasma membrane, and calcium sequestration in intracellular organelles. The intracellular concentration of calcium is 10 000 fold lower than its extracellular concentration, about 100 nM and 1 mM, respectively. This concentration gradient is maintained because of the relative low membrane permeability to calcium ions, and by the action of the Ca^{2+}-ATPases (PAMCA) and the Na^{+}/Ca^{2+} exchangers from the plasma membrane [2]. The endoplasmic reticulum contributes substantially to the storage of intracellular calcium through the activity of the Ca^{2+}-ATPase from the endoplasmic reticulum (SERCA). In addition, when the cytosolic concentration of calcium rises, it is sequestered by the mitochondria through the activity of calcium uniporters [3] (Fig. 1).

Diverse physiological stimuli promote transient increases in the intracellular concentration of calcium inducing different responses. However, during pathological conditions, changes in intracellular calcium are pronounced and sustained, inducing the activation of hydrolytic enzymes and metabolic changes that initiate the degradation of the cytoskeleton and finally resulting in cell death [4].

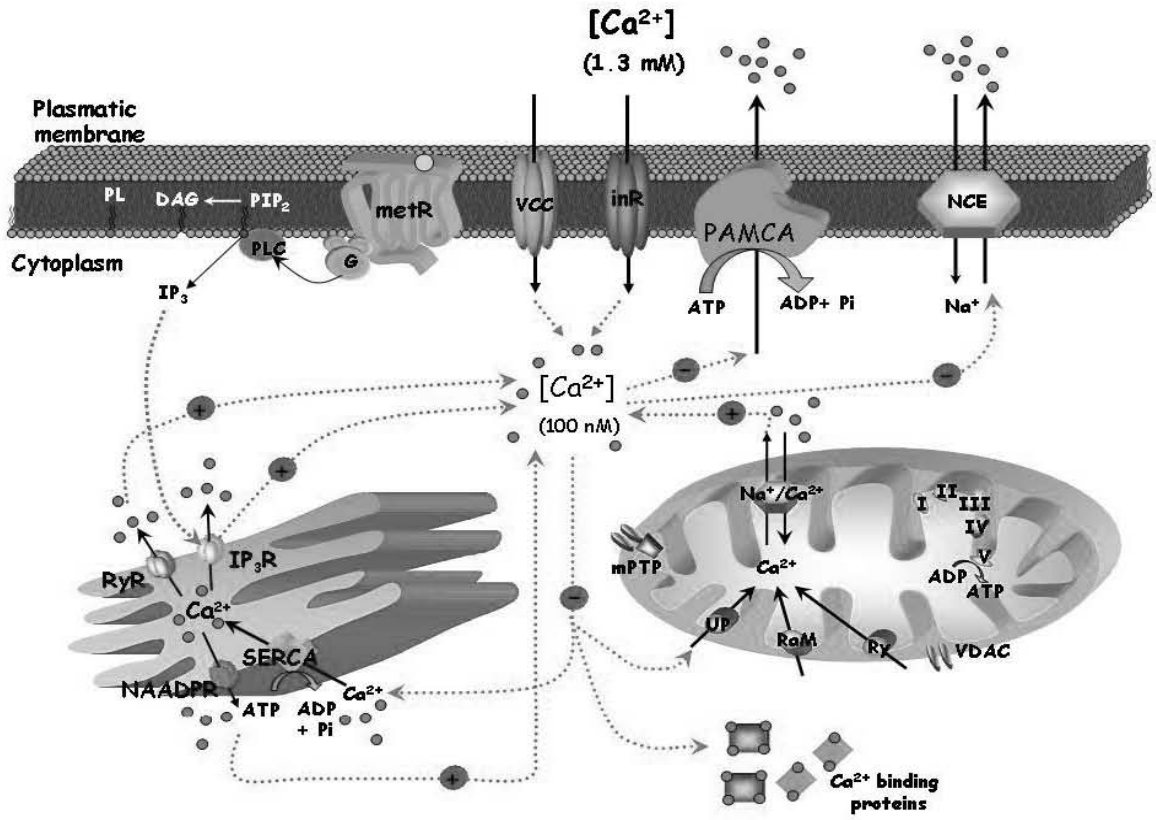


Figure 1. Mechanisms involved in the regulation of the intracellular calcium homeostasis. Calcium concentration (100 nM) is regulated by its influx through receptors (glutamate receptors) and voltage channels, and its extrusion through the membrane calcium ATPase (PAMCA) and the Na^+/Ca^{2+} exchanger (NCE). Calcium uptake systems are located in endoplasmic reticulum (ER) and mitochondria. Calcium is loaded into the ER by the calcium ATPase (SERCA) and is released through the IP_3, ryanodine (Ry in cardiac cells), and NAADP receptors. The activation of metabotropic receptors coupled to G proteins, activate phospholipase C inducing the breakdown of phosphatidylinositol 4,5-biphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). Mitochondrial calcium uptake occurs through the calcium uniporter and is released to the cytosol by the Na^+/Ca^{2+} exchanger. Calcium binding proteins in the cytosol contribute to the regulation of intracellular calcium.

Mitochondrial calcium transport

Oxidative phosphorylation in mitochondria produces 95 % of the ATP consumed by mammalian cells. In addition to ATP synthesis, mitochondria is the main site of other important metabolic reactions such as the synthesis of steroid hormones and porphyrins, the urea cycle, the oxidation of fatty acids and amino acid catabolism [5]. In many species, mitochondria constitute a complicated system of calcium uptake and release, contributing to the maintenance of the intracellular calcium homeostasis [6].

Mitochondrial calcium transport systems were described during the 70's. The outer membrane of the mitochondria is permeable to ions and molecules of 1000 Da, while the inner membrane in physiological conditions is almost impermeable. Therefore, specific transporters for ions and other molecules are present in the inner membrane, contributing to the generation of an electrochemical gradient. Mitochondrial Ca^{2+} uptake is ATP independent but it depends on the mitochondrial membrane voltage potential (ψm), which is normally around 150 – 200 mV [7]. Reports on isolated mitochondria have suggested that calcium uptake is driven by the intracellular concentration of calcium. When calcium raises up to 300 nM mitochondrial uptake takes place, although in some cells it occurs when the intracellular calcium concentration achieves up to 1 μM, [8].

The most important calcium uptake system is the calcium uniporter, which is able to transport diverse cations in the following order of selectivity: $Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+} > Mg^{2+} > Fe^{2+} > La^{3+}$ (Fig 2) [9]. Calcium influx through this channel is dependent on the electrochemical gradient. Its activation and inactivation is slow requiring about a minute to be completed. The channel is inhibited by ruthenium red and divalent cations like Sr^{2+}, Mn^{2+}, Ba^{2+}, lanthanides, and adenine nucleotides (ATP> ADP> AMP), and can be also regulated by Mg^{2+}. The uniporter is activated by cytosolic calcium, at low concentrations of calcium its activity is only marginal [10]. The uniporter can be also activated by spermine, spermidine, taurine, and its derivates [6]. After calcium uptake, the intramitochondrial concentration of calcium is re-established by the action of the Na^{+}/Ca^{2+}exchanger extruding calcium ions to the cytosol (Fig 2). Another calcium uptake system, recently identified in mitochondria, is the rapid uptake mode (RaM). This system is very efficient when mitochondria are exposed to calcium concentrations close to 400 nM, and is rapidly inhibited (0.75 sec) when the intracellular calcium concentration decreases to 100 or 200 nM. The RaM system is inhibited by ruthenium red [6] and activated by ATP and GTP [8]. On the other hand, a new isoform of the ryanodine receptor has been identified in the inner mitochondrial membrane of cardiac cells, which has been suggested to represent a new mitochondrial calcium uptake system [11].

Intramitochondrial calcium homeostasis depends on the balance between calcium uptake and calcium extrusion mechanisms. The most efficient systems for calcium extrusion are the Ca^{2+}/Na^{+} and Ca^{2+}/H^{+} exchangers [7,12]. The Ca^{2+}/Na^{+} exchange depends on Na^{+} efflux by the Na^{+}/H^{+} exchanger (Fig. 2). A second mitochondrial calcium release mechanism is constituted by a complex system composed of proteins from the inner and the outer mitochondrial membrane. This channel, known as the mitochondrial permeability transition pore (mPTP), normally opens to release calcium from the mitochondrial matrix, however sustained opening of the mPTP has been associated with calcium overload and mitochondrial dysfunction during several pathological conditions [13].

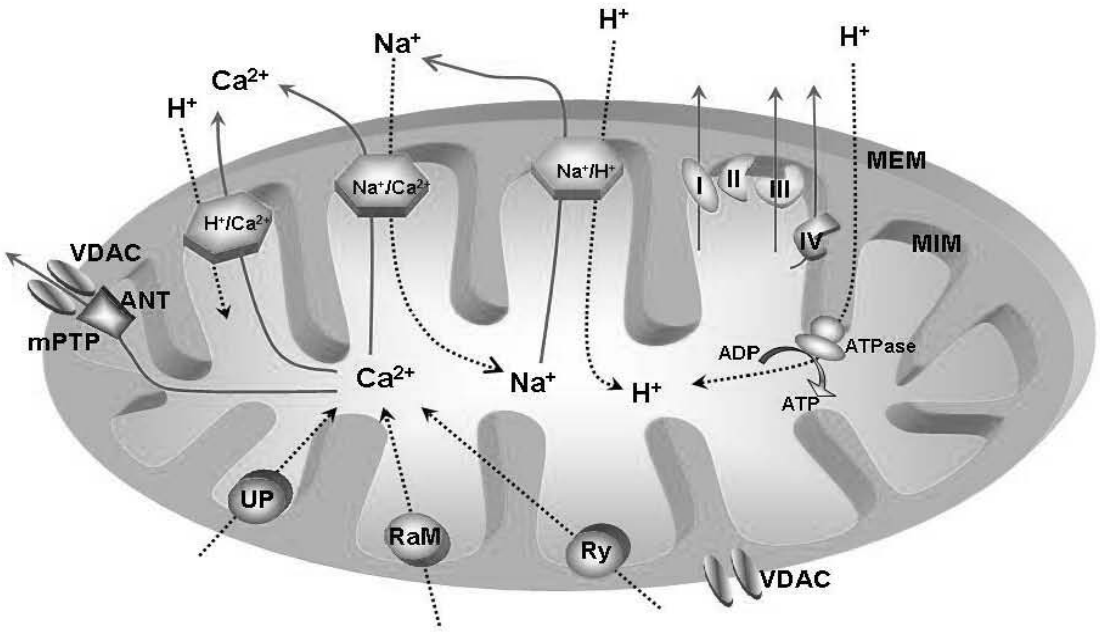


Figure 2. Mitochondrial calcium loading and extrusion systems. Calcium uptake into the mitochondria occurs through the calcium uniporter (UP), the rapid uptake system (RaM) and the mitochondrial ryanodine receptor (Ry), and extruded by the Na^+/Ca^{2+} exchanger and the permeability transition pore (mPTP). The increase in the mitochondrial Na^+ concentration is regulated by Na^+/H^+ exchanger. Protons (H^+) are extruded by the mitochondrial complexes (I, II, III, IV) generating the proton gradient necessary for the activity of the ATPase. Mitochondrial external membrane (MEM), mitochondrial internal membrane (MIM), voltage activated anion channel (VDAC), adenine nucleotide translocator (ANT).

Physiological relevance of mitochondrial calcium transport

Mitochondrial calcium uptake is physiologically relevant because it activates mitochondrial metabolism. Calcium activates mitochondrial enzymes, stimulates NADH and ATP production, and induces O_2 consumption [14,15]. Micromolar concentrations of calcium stimulate the activity of several dehydrogenases of TCA cycle, such as pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. These enzymes are also regulated by intrinsic factors such as the ADP/ATP and the NAD^+/NADH ratios, as well as pH. Others targets of calcium in mitochondria are cytochrome *c* and the ATP synthase complex [12,16], however, its regulation by calcium has not yet been elucidated. An increase in mitochondrial calcium concentration in the micromolar range might inhibit pyrophosphatase and cause mitocondrial swelling, which in turn stimulates the activation of the respiratory chain, the oxidation of fatty acids and the activity of glutaminase [17].

Mitochondrial calcium also plays a major role in the control of hormone synthesis and its release in endocrine cells. In aldosterone producing cells, mitochondrial calcium accumulation is a key step driving hormone biosynthesis within the mitochondrial matrix [18]. In cromaffin cells, a role of intramitochondrial calcium in catecholamine secretion has been suggested [19]. On the other hand, a role of mitochondria in the regulation of the space-time pattern of calcium signals has been proposed. Jouaville et al. (1995) [20] demonstrated that mitochondrial calcium uptake can modulate the shape and speed of the calcium waves induced by IP_3 in Xenopus oocytes. In addition, in mammalian cells the magnitude and speed of calcium release from endoplasmic reticulum can be modulated by the local calcium buffering in mitochondria [21]. This evidence suggests an interaction between mitochondria and endoplasmic reticulum in the regulation of the intracellular concentration of calcium and calcium signaling.

Mitochondria is the major source of free radicals in all cell types except in those specialized in free radical production such as macrophages and neutrophils. Physiologically, mitochondria generate reactive oxygen species such as the superoxide anion ($O_2^{\bullet -}$) during oxidative phosphorylation. When free radicals are excessively produced peroxidation of membrane lipids and oxidative damage to proteins and DNA, takes place damaging cells (see section of oxidative stress).

Mitochondrial damage is induced by calcium overload

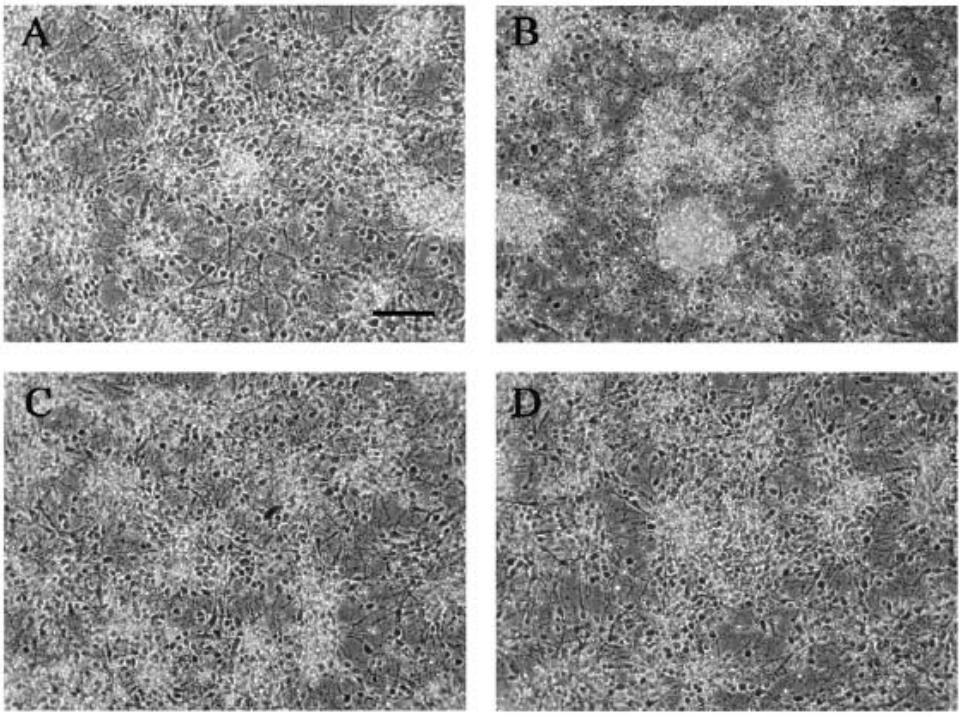
The role of intramitochondrial calcium overload in neuronal death associated with neurological disorders, such as anoxia/ischemia, is well documented. It has been observed that during the anoxic/ischemic period a substantial increase in the extracellular concentration of glutamate induces the overactivation of glutamate receptors, mainly of the N-methyl-D-aspartate (NMDA) subtype, leading to a massive influx of calcium and mitochondrial calcium overload [22, 23, 24]. The reverse operation of the Na^+/Ca^{2+} exchanger might also contribute to increased mitochondrial calcium during hypoxic/ischemic conditions [25]. As a consequence of calcium overload mitochondria depolarizes, free radical generation by this organelle is enhanced, and ATP production is impaired [24, 26, 27]. A change in mitochondrial membrane permeability and mitochondrial swelling (see below) might induce the release of cytochrome *c* and the subsequent activation of caspases triggering the apoptotic cell death cascade. On the other hand, energy failure and the activation of calcium-dependent enzymes such as calpain, xantine oxidase and nitric oxide synthase contribute to the generation of free radicals and the execution of necrotic cell death (see last section of this review). A major role of mitochondrial dysfunction in excitotoxic death is evidenced by studies showing that recovery of the mitochondrial membrane potential, the

intracellular calcium homeostasis and ATP levels prevent neuronal damage [24]. On the other hand, several studies have shown that neuronal death induced by glutamate, during conditions of metabolic inhibition, can be prevented by the supplementation of mitochondrial metabolism with energy substrates such as pyruvate and ketone bodies [28, 29, 30; Fig. 3]. Moreover, it has been shown that inhibition of mitochondrial calcium uptake during mitochondrial depolarization induced by an uncoupling agent, prevents cell death [31].

During the last decade, a predominant role of mitochondria in the initiation of both apoptotic and necrotic death has been suggested. One major component implicated in cell death, is the mPTP. The opening of the mPTP causes massive swelling, depolarization of the mitochondrial membrane, breakdown of the outer membrane, and release of intermembrane components [32]. The opening of the mPTP occurs under certain circumstances, more remarkably as a result of mitochondrial calcium overload (in the case of brain when intramitochondrial calcium exceeds a concentration of 1500 nmol/mg of protein, [33]), oxidative stress, ATP deficit, increased concentration of inorganic phosphate and mitochondrial depolarization [34]. The main consequence of the mPTP opening in mitochondrial function is the uncoupling of oxidative phosphorylation impairing ATP synthesis and stimulating the hydrolysis of glycolytically produced ATP, by the inverse operation of the ATPase. The opening of mPTP has been suggested to be critical in the triggering of necrotic neuronal death associated with glutamate toxicity and ischemia/reperfusion [32].

The mPTP is a megachannel permeant to molecules larger than 1500 kDa (glutathione, NAPDH and NADH) allowing their passage through the mitochondrial inner membrane. It can be rapidly closed by Ca^{2+} and Mg^{2+} chelators, by cyclosporin A (CsA) and by modulators of the adenine nucleotide translocator (ANT), as bongkrekic acid [34, 35]. This pore communicates mitochondrial inner and outer membranes through its association with the voltage activated anion channel (VDAC, also known as porin) in the outer membrane, with the ANT in the inner membrane, with cyclophilin D (CyP-D) in the mitochondrial matrix and possibly with other proteins [32, 35]. It has been partially reconstituted using purified VDAC and ANT loaded in liposomes [36]. CyP-D is a determinant factor in the opening of the mPTP, it translocates from the matrix to the pore during conditions of calcium overload and oxidative stress. The opening of the mPTP is inhibited by CsA and some of its analogs, such as N-methyl-valine-4-cyclosporin A (MelValCsA), but not by the immunosuppressor and calcineurin inhibitor, FK506 [37].

It has been suggested that inhibition of mPTP opening by the binding of CsA to CyP-D, is responsible for the recovery of the mitochondrial membrane potential, and the prevention of cell death induced by glutamate in cultured neurons [24, 31]. CsA also prevents neuronal death induced by hypoglycemia



E

Treatment	Cell survival (% of control)	ATP levels (pmol/μg of protein)
Control	100 ± 2.75	14.86 ± 1.24
IOA (50 μM)	54.07 ± 4.08	10.67 ± 1.34
+Pyruvate	73.93 ± 4.75*	13.74 ± 1.98
+Acetoacetate	85.43 ± 4.32*	14.52 ± 1.79*
+MK-801	88.82 ± 4.22*	ND

Figure 3. Micrographs showing the morphological changes induced by the glycolysis inhibitor, iodoacetate (IOA) (50 μM) and its protection by pyruvate and acetoacetate in hippocampal cultured neurons. Cultures exposed to IOA show many bright and refringent cells, which is indicative of death cell bodies, and less number of well-preserved dark somata (B). Cells are better preserved in the presence of pyruvate (C) and acetoacetate (D) and look basically identical to those in control cultures (A). Scale bar = 100 μM. E: Effect of the NMDA receptor antagonist, MK-801, pyruvate, and acetoacetate on survival and ATP levels during glycolysis inhibition. Survival and ATP levels were determined at 24 h and 4 h, respectively, after 30 min exposure to IOA (50 μM) in the presence or absence of MK-801 (10 μM), pyruvate (2 mM) and acetoacetate (5 mM) in cultured hippocampal neurons. Data are means ± SEM of 4 -5 independent experiments. Cell viability was assessed by the MTT reduction assay and ATP levels were determined by the luciferin-luciferase assay. Data were analyzed by one-way ANOVA followed by a Fisher's least significant differences test. *$P<0.05$ relative to IOA.

in the rat hippocampus in vivo [38], by trauma in cerebral cortex, by ischemia in the rat brain [39, 40], as well as damage induced by NMDA and the calcium ionophore A23187 in cultured neurons [41, 42]. Moreover, it has been shown that calcium depletion from the endoplasmic reticulum induced by thapsigargin, and inhibitor of the Ca^{2+} ATPase of the endoplasmic reticulum, directly induces a transitory change in mitochondrial permeability, which finally leads to necrotic death [43]. All together, these studies suggest a major role of mPTP in neuronal death associated with diverse pathological conditions and toxic agents.

In addition to the role of mitochondria in the regulation of intracellular calcium homeostasis and free radical production, it can contribute to the cell death cascade through the release of proapoptotic factors, such as cytochrome *c* and proteins like Smac/Diablo, which neutralize a series of endogenous caspase inhibitors, known as inhibitors of apoptotic proteins (IAPs) [44]. Mitochondrial calcium accumulation promotes cytochrome *c* release from the intermembrane space. Once in the cytosol cytochrome *c* binds the APAF-1 protein, ATP and pro-caspase 9, inducing the activation of caspase-9 and caspase 3, an executor caspase, promoting the death of neurons through the degradation of proteins involved in DNA repair, proteins of the cytoskeleton and DNAses, among others. The mechanism of release of cytochrome *c* is not known, but it has been suggested that it might be extruded through the mPTP [45]. An alternative more accepted hypothesis is that cytochrome *c* is released by the pores formed by pro-apoptotic proteins such as Bax and Bak [46]. Bax interacts with the VDAC forming a Bax-VDAC pore. The anti-apoptotic protein, Bcl-2, prevents the release to cytochrome *c* though the formation of heteromeric complexes with Bax preventing the formation of the Bax-VDAC complex. Bcl-2 is widely distributed in the inner mitochondrial membrane but it is also present in the endoplasmic reticulum and the Golgi complex, altering Ca^{2+} levels and preventing programmed cell death [7].

Endoplasmic reticulum as a regulator of intracellular calcium dynamics

Endoplasmic reticulum (ER) is the most important organelle to regulate the intracellular calcium dynamics. The ER is constituted by a set of cistern-forming endomembranes which are named according to the presence or absence of ribosomes as rough and smooth ER. A variety of important biochemical and physiological activities take place in this organelle: 1) Synthesis and conformational stabilization of proteins; 2) Anabolic reactions for carbohydrates and lipids, including glycogen, cholesterol, and phospholipids; 3) Processing of xenobiotics [47, 48, 49]. In addition, the ER is a dynamic compartment with a substantial capacity to store and release calcium [50]. This activity occurs by the concerted action of release channels, metabolic pumps,

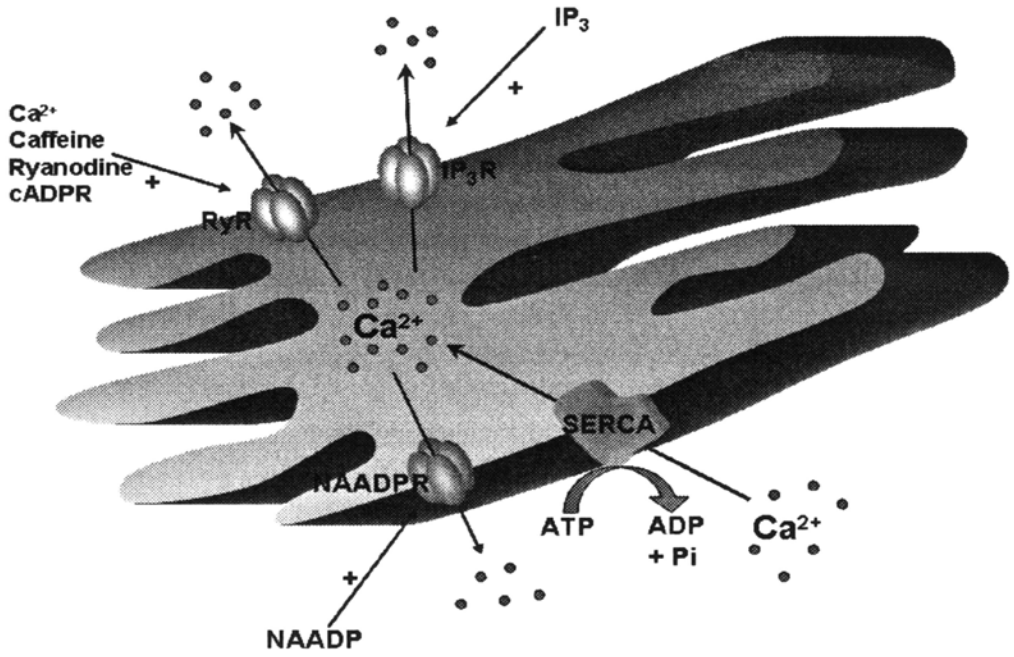


Figure 4. Mechanisms of intracellular calcium regulation in ER. The ER has different systems responsible for intracelullar calcium storage (SERCA) and release, ryanodine (RyR), IP_3 (IP_3R) receptors, and nicotinic acid-adenine dinucleotide phosphate receptor (NAADPR). RyR is activated by Ca^{2+}, caffeine, ryanodine and ADP-cyclic ribose (cADPR), while IP_3 and NAADP receptors are activated by IP_3 and NAADP, respectively.

and calcium-binding proteins. In basal conditions, a major gradient of several orders of magnitude is established between the ER lumen (≈ 0.5 mM) and the cytoplasm (≈ 100 nM) by the action of the sarco(endo)plasmic Ca^{2+} ATPase (SERCA) [51]. Calcium is released to the cytoplasm through two main channels: inositol trisphosphate and ryanodine receptors (IP_3R and RyR) [52]. The existence of a third calcium release channel which is sensitive to NAADP (nicotinic acid-adenine dinucleotide phosphate), has been also reported in sea urchin egg [53] (Fig. 4).

Sarco(endo)plasmic Ca^{2+} ATPase

Sarco(endo)plasmic Ca^{2+} ATPase is a family of P-ATPases (pumps that transport a variety of ions across the membrane) responsible for Ca^{2+} intake into the ER. An additional role has been proposed for SERCA in heat production during the thermogenesis associated with hyperthyroidism [54]. This enzyme is one of the most abundant proteins in the light sarcoendoplasmic fraction of skeletal muscle. The protein from this tissue has been crystallized, and several domains are recognized in its structure: a transmembrane region

that communicates through a stalk to the nucleotide, β-strand, central and phosphorylation domains [55]. During the catalytic cycle, an aspartate residue is phosphorylated by ATP. There are three genes in vertebrates (invertebrates show only one gene) that codify isoforms of this enzyme (~ 1000 amino acids): SERCA type 1a is expressed almost exclusively in adult fast-twitch muscle, whereas the type 1b is located in certain embryonic tissues. SERCA type 2 is the oldest and shows the most wide tissue-expression pattern. SERCA type 2a is located mainly in cardiac muscle, but also in smooth and slow-twitch muscles as well as in cerebellar purkinje cells. SERCA type 2b is found in nervous tissue equally present in neurons and different types of glial cells. There are six different forms of SERCA type 3, and all of them are located mainly in non-muscular and non-neuronal tissues [50, 51]. SERCAs are regulated basically by their substrate, the calcium cation: high calcium concentration in the cytoplasm activates SERCA activity, but in the ER lumen calcium promotes inhibition of its activity. Cardiac SERCA interacts with the anti-apoptotic protein, Bcl-2, the insulin receptor substrates IRS1/2, the EF-hand Ca^{2+}-binding protein S100A1 and acylphosphatase [51]. In addition, it is also modulated by phospholamban and sarcolipin, two peptides that decrease SERCA activity [56]. Pharmacologically, SERCA is inhibited with high selectivity by nM concentrations of thapsigargin and μM concentrations of cyclopiazonic acid [50].

Inositol trisphosphate receptor

IP_3R is an intracellular calcium release channel that plays a critical role in Ca^{2+} signaling [57], with a tetrameric arrangement and high molecular weight (≈ 300 kDa per monomer) [58]. The channel region is located at the C-terminal end, and it is characterized by six membrane-spanning helixes with the C terminus projecting into the cytoplasm. The pore domain of the channel shares some structural similarity to both voltage-gated potassium channels and other calcium channels, specially the RyR.

IP_3R activation is linked to the production of IP_3 by a different set of hormones, neurotransmitters, and ligands, all of them acting throughout specific G protein-coupled receptors or tyrosine kinases receptors in the plasma membrane. Autoradiographic studies revealed extraordinarily high densities of IP_3 binding in the cerebellum [59] with levels 100-300 times higher than those in peripheral tissues, allowing further biochemical and molecular characterization. Even though the IP_3R is mainly present in the smooth ER, it has also been found in nuclear and plasma membranes, as well in the Golgi apparatus and secretory granules. Three mammalian isoforms of the IP_3R have been identified [60], which share a common domain structure, with 60-70% sequence identity. Additional variant forms of IP_3R are derived from alternative splicing. The best characterized is the splicing of IP_3R type 1

into a longer variety which predominates in brain (SII+) and a shorter form (SII-) that is found mostly in peripheral non-neuronal tissues [61].

IP_3Rs are subjected to multiple levels of regulation. They are regulated biphasically by Ca^{2+}, showing a sharply positive cooperative increase in open probability at physiological concentrations of Ca^{2+} and inhibition at low micromolar concentrations. The calcium sensitivity varies according to the IP_3R isoform. IP_3R is phosphorylated and dephosphorylated by multiple kinases and phosphatases: 1) Cyclic-AMP-dependent protein kinase (PKA) activates Ca^{2+} release in different tissues by phosphorylating IP_3R, whereas it has been shown that protein phosphatases 1 and 2A also form a structural complex with IP_3R. 2) Activation of soluble guanylyl cyclase by nitric oxide leads to the production of cGMP and the activation of cGMP kinase 1β. This kinase phosphorylates IP_3R inhibiting its channel function. 3) Activation of G-protein-coupled signaling pathways produces diacylglycerol, which stimulates protein kinase C phosphorylation activity, activating IP_3R. 4) Activation of the B-cell receptor complex leads to the phosphorylation of BANK (B-cell scaffold protein with ankyrin repeats) by the tyrosine kinase Syk. The phosphorylation of BANK then allows the tyrosine kinase Lyn to bind to the IP_3R, allowing Lyn to phosphorylate the IP_3R and modulating channel activity. 5) calcium/calmodulin dependent protein kinase II (CAMK-II) phosphorylates IP_3R at distinct sites, and regulates calcium oscillations via IP_3R in certain systems. IP_3R modulation by CAMK-II creates a positive feedback loop that influences neurotransmitter release at the neuromuscular junction. 6) IP_3R shows the capacity to be autophosphorylated, a well known process for tyrosine kinase receptors but not for ion channel receptors [62].

ATP and other nucleotides influence IP_3R function independently of phosphorylation or energy-dependent processes. Both ATP and NADH activate IP_3R, the latter by means of an IP_3R-bound glyceraldehyde-3-phosphate dehydrogenase, which could represent a mechanism for rapid adaptation to hypoxia [63].

A number of scaffolding proteins associate with the IP_3R to promote stable localization of the IP_3R within cellular membranes, such as: 1) Ankyrin, an adapter protein which links the spectrin-based cytoskeleton to proteins in several organelles; 2) Homer, a protein induced by neural activity, which binds metabotropic glutamate receptors and IP_3R, providing regulation of IP_3-dependent signaling at excitatory synapses; 3) 4.1N Protein, a protein selectively enriched in certain neuronal populations, which, when is genetically deleted in mice, leads to neurobehavioral abnormalities. The complex between IP_3R, 4.1N and other proteins is likely to help to create the accurate ultraestructure at the postsynaptic density. In addition, binding of 4.1N to IP_3R is required for proper localization of IP_3R to the basolateral membrane in canine kidney cells; 4) Myosin, a family of proteins with a role in

cytoskeleton structure, is involved in the movement and location of diverse protein complexes. It has been demonstrated that pharmacological manipulation of the cytoskeleton can affect IP_3R-mediated Ca^{2+} signaling, and the binding of IP_3R to the heavy chain of several myosin II isoforms. 5) IP_3R itself functions as a scaffold contributing to the assemblage of several proteins at the postsynaptic density. IP_3R complexes with a variety of proteins have also been reported: G-protein coupled receptors, TRPC channels, talin, vinculin, and α-actin [64]. A number of regulatory Ca^{2+}-binding proteins directly associate with the IP_3R, such as calmodulin, calsenilin, GCAP, recoverin, hippocalcin, visinin, VIPIP, caldendrin, chromogranins, and FKBP12.

Apoptotic cell death is related to the presence of IP_3R in several cellular systems. Antisense nucleotides against IP_3R type 3 selectively prevent apoptosis in chick dorsal root ganglion cells deprived of nerve growth factor. In contrast, genetic deletion of all IP_3R subtypes from DT-40 avian B cells inhibits anti-IgM-induced apoptosis by 75%. When released from the mitochondria, the pro-apoptotic factor cytochrome *c* binds predominantly to the IP_3R, deregulating Ca^{2+} release and eventually leading to cell death [65].

A physiologically relevant interaction between IP_3R and transient receptor potential channels (TRPC) has been documented. It constitutes a large agonist-induced Ca^{2+} entry within excitable and nonexcitable cells, in regions with close proximity between plasma membrane and ER [66].

IP_3Rs can be blocked by heparin, which antagonizes the recognition between IP_3 and its receptor. However, heparin is at the same time not very specific. Recently a more selective and membrane-permeable ligand has been introduced, which very successfully inhibits IP_3Rs. This compound is called Xestospongin C and is extracted from marine sponges. It has been shown that Xestospongin C restrains IP_3-dependent calcium release in a variety of cells, and also shows an anti-apoptotic action in cultured hippocampal neurons [67, 68]. On the other hand, a compound called adenophostin has been tested successfully as a specific activator of IP_3Rs [69].

Ryanodine receptor

The ryanodine receptor (RyR) is a high-molecular weight (≈500 kDa per monomer) tetrameric protein that functions as a calcium release channel in a large variety of cells. In most of the systems, RyR is located in the sarco(endo)plasmic reticulum; however, some groups have reported that RyR is also present in the Golgi apparatus and in the nucleus [70, 71]. The existence of RyR has been described in a number of species including vertebrates (fishes, amphibians, reptiles, birds and mammals) and invertebrates (insects, crustaceans and annelids) [72, 73]. Three types of RyRs have been reported in vertebrates, situated in different chromosomal locations [74]: Type 1 is present

mainly in skeletal muscle, and it is responsible for the excitation-contraction coupling; type 2 is located primarily in cardiac muscle where it is responsible of the calcium-induced calcium release, making possible the rhythmic operation of the heart; type 3 is more ubiquitously expressed, as in the skeletal muscle of non-mammal vertebrates, in the nervous system, as well as in a great variety of tissues. In contrast, only one isoform of RyR has been documented in the genetic material of invertebrate organisms [73]. The degree of homology among different types of RyR, and between vertebrate and invertebrate isoforms is ≈ 40% [73].

The RyR shows functional and structural similarities with the IP_3R. Both receptors are high molecular weight tetramers, containing in their N-terminal section a variety of modulatory sites. The RyR modulation occurs through the action of endogenous ligands such as calcium, purines, and accessory proteins, as well as by the phosphorylating action of several kinases. The mobilization of intracellular calcium in many cells and tissues is the result of coordinated activations of both calcium release channels, the RyR and the IP_3R [75].

Through its role in intracellular calcium release, the RyR has been associated with relevant physiological events such as:

1. *Excitation-contraction coupling*. In cardiac and skeletal muscle, RyRs located in the sarcoplasmic reticulum section of the triade, are activated by the dihydropyridine receptors (DHR), which are voltage-sensitive Ca^{2+} channels. The RyR activation in each type of muscle is distinctive, since DHR directly contact the RyR in skeletal muscle, whereas in heart the interaction between the two channels is at distance. Upon activation, calcium ions released through the RyR induce conformational changes in the sarcomeric protein, troponine c, making possible muscular contraction [76].
2. *Excitation-secretion coupling*. Calcium ions needed for the secretion of molecular messengers in neurons and excitable endocrine cells, is released from internal deposits through RyR by the process of Ca^{2+}-induced Ca^{2+} release [77].
3. *Apoptosis and cellular toxicity*. Calcium released from the endoplasmic reticulum by IP_3R and RyR, has been involved in the onset and progression of cell death by apoptosis and necrosis [78]. The transfer of calcium from endoplasmic reticulum to mitochondria promotes the release of cytochrome *c* and the apoptosis inducing factor (AIF). These two factors recruit and activate caspase 9 to form the apoptosome and trigger cell death [79].
4. *Circadian rhythmicity in suprachiasmatic nuclei*. A specific circadian rhythm for the type 2 RyR has been reported in the suprachiasmatic nuclei of the rat. The peak of RyR expression is coincident with the acrophases of the metabolic and electrical activities of this circadian oscillator, and hence

the metabolic and electrical activities of this circadian oscillator, and hence a role of type 2 RyR in the keeping-time mechanism of the suprachiasmatic nuclei, has been suggested [80].

5. *Memory and learning.* Using a variety of molecular techniques, significant increase in type 2 RyR mRNA was found in the hippocampus of rats trained in an intensive water maze task. Protein levels of this calcium release channel were also demonstrated to be increased in the microsomal fractions prepared from hippocampi of the trained rats. These data suggest that type 2 RyR could be associated with structural modifications during the long-term memory storage [81].
6. *Synaptic plasticity.* Wind up and plateau potentials are examples of synaptic plasticity that have been implicated in phenomena of pain sensitization. It was shown that the activity of RyR is strictly required for the onset and maintenance of these two neuronal plastic events, suggesting the importance of intracellular calcium dynamics in the establishment of these phenomena [82].
7. *Activation of store-operated channels.* In many cells types, the emptying of intracellular Ca^{2+} stores results in the opening of store-operated Ca^{2+} channels in the plasma membrane, and the gating of a capacitative Ca^{2+} influx. RyR and IP_3R have been involved in the gating regulation of this capacitative current in regions of close proximity between plasma membrane and intracellular calcium deposits [83].

Three domains are recognized in the structure of RyRs: 1) A cytoplasmic domain which is the largest, containing the N-terminal and the regulatory sites; 2) a transmembrane domain located towards the C-terminal including the ion-permeable pore; 3) a luminal domain which interacts with calsequestrin, and is much more smaller than the cytoplasmic domain. The structure of RyR type 1, studied by electron microscopy using negative staining and metal shadowing techniques, revealed a fourfold symmetric complex having roughly the shape of a square prism (≈27x27x15 nm) with a bump projecting from the center of one face [84]. Recently, the 14 Å resolution structure of this same RyR was determined by electron cryomicroscopy and single particle reconstruction [85]. Using sequence-based fold recognition, the N-terminal region of RyR type 1 (356 amino acids) was predicted to have significant structural similarity with the IP_3-binding core region of the type 1 IP_3R.

Site-directed mutagenesis to the sequence motif GXRXGGGXGD, which is located in the C-terminal region of all RyRs and IP_3Rs, abolished [^{3}H]-ryanodine binding and reduced 97% the single channel conductance of RyR incorporated in lipid planar bilayers [86]. These data strongly suggest that this conserved region constitutes an essential part of the channel conduction pathway of RyRs. In addition, it has been shown that glutamate at position

3885 of type 3 RyR may act in a coordinated way to form the Ca^{2+} sensor in the tetrameric structure of the channel [87].

There is a growing appreciation of the central role of RyR regulation in skeletal and cardiac muscle pathologies, including malignant hyperthermia, heart disease, heart failure, and sudden cardiac death [88].

Calcium and reticular stress

It has been already mentioned that ER plays and major role in the synthesis and folding of proteins. This process is strictly calcium-dependent and highly sensitive to changes in intracellular Ca^{2+} homeostasis. Calcium ionophores depleting intracellular calcium in the lumen of ER, inhibitors of glyclosylation, oxidative stress, as well as the accumulation of misfolded proteins in ER, alter the functioning of this organelle, leading to a process known as reticular stress.

ER responds to the above-mentioned stimuli by the activation of signalling pathways such as the response to unfolded proteins (UPR), the ER-overload response and the ER associated degradation (ERAD), in order to survive reticular stress. The activation of these three pathways reduces the amount of newly synthesized proteins translocated to the lumen of the ER, reducing the load of proteins to be processed in the ER and increasing their degradation [48].

The UPR restores the functioning of the ER. Several enzymes and transcription factors participate in this response such as the double-stranded RNA protein kinase, known as PERK, the activated transcription factors 4 and 6 (ATF4, ATF6) and the inositol requiring factor 1 (IRE1). During physiological conditions, PERK, ATF6 and IRE are inhibited by the binding of the glucose-regulated chaperon protein GRP78 (Bip) (Fig. 5). During reticular stress, GRP78 dissociates from PERK, ATF6 and IRE1 and binds to misfolded proteins promoting their adecuate folding. The dissociation of GRP78 from PERK, IRE1 and ATF6 leads to their activation. ATF6 when dissociated from GRP78, is translocated to the Golgi apparatus, where its breakdown by the S1P and S2P induces its transcription factor activity [48]. Active PERK, phosphorylates the α-subunit of the eukaryotic translation initiation factor-2, (eIF2α), which inhibits protein translation. Dimerization and phosphorylation of IRE1 induces its riboendonuclease activity, splicing a 26 base pair segment of the xbp1 mRNA, inducing the translation of the $XBP1^{proc}$ protein. This protein functions as a transcription factor of genes coding for the proteins necessary for the folding and processing of proteins in ER [89] (Fig. 5).

It has been shown in diverse models of reticular stress that cell death occurs after the release of death inducing factors such as the members of the Bcl-2 family, Bcl-2, Bcl-x_L, Bax and Bik; the protein known as CHOP/GADD 153; the valosin-containing protein (VCP) and the apoptosis associated gene 2. The members of the Bcl-2 family can be pro-apoptotic or anti-apoptotic. It has

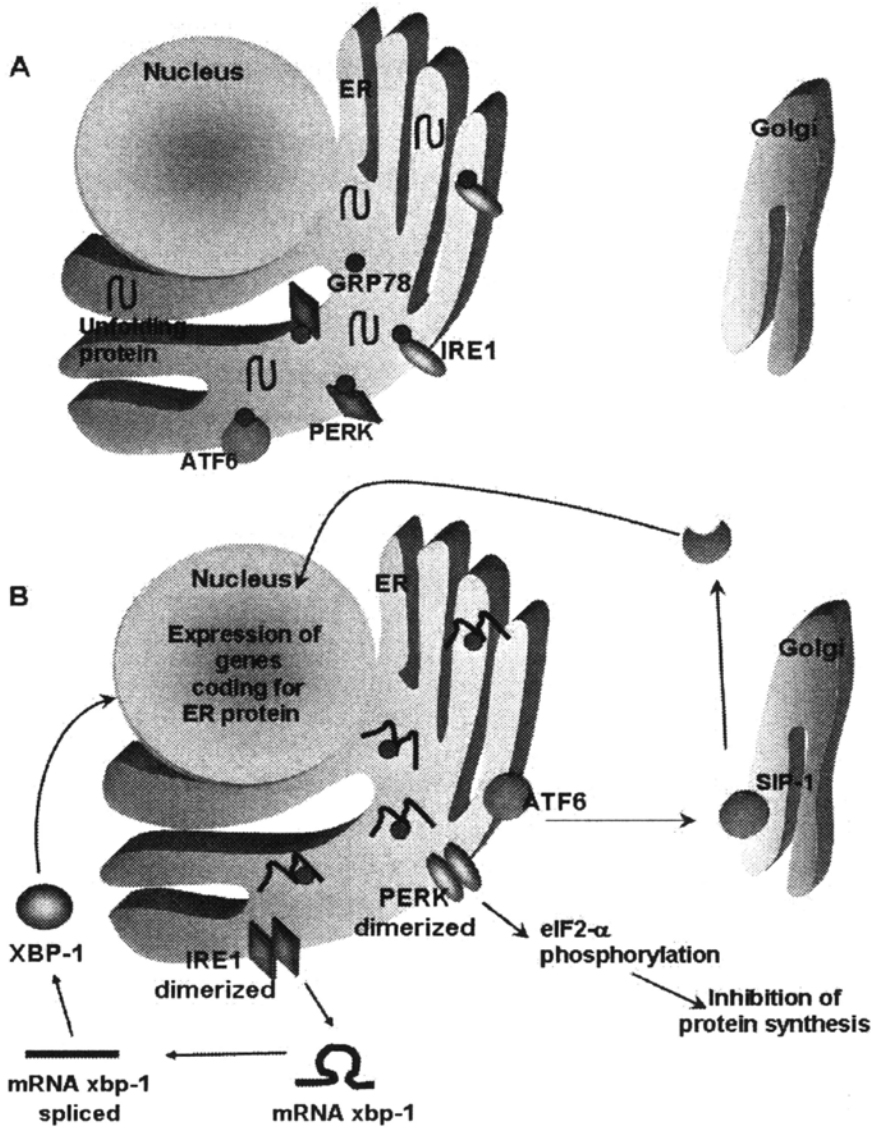


Figure 5. Biochemical pathways involved in reticular stress necessary to restore de adecuate functioning of endoplasmic reticulum (ER). The processing of proteins translocated to the ER takes place during physiological conditions. The GRP78 protein is normally bound to ATF6, PERK and IRE1 inhibiting its enzymatic activity (A). During reticular stress misfolded proteins accumulate inducing the unbinding of GRP78 from ATF6, PERK and IRE1 leading to its dimerization, phosphorylation and activation (B). The activation of PERK induces the phosphorylation of the α-subunit of eIF-2, resulting in the inhibition of protein synthesis, while active IRE1 functions as a riboendonuclease promoting the splicing of the xbp-1 mRNA and its subsequent translation to the XBP1 protein. This protein shows transcription factor activity inducing the transcription of chaperon proteins (CHOP) involved in the processing of proteins in ER lumen. ATF6 is translocated to the Golgi apparatus where it is processed by the SIP-1 protease inducing its transcription factor activity. This factor induces the expression of genes encoding proteins involved in reticular stress, such as GRP78.

been observed that the over-expression of the Bax protein induces the release of calcium from the ER, the increase in intramitochondrial Ca^{2+} loading, and the release of cytochrome *c* induced by Ca^{2+} efflux from RE [48]. When reticular stress is sustained, there is transcription of apoptotic inducing genes such as that inducing the transcription of the C/EBP (CHOP) protein, known as the growth arrest and DNA damage inducible gene 153 (GADD 153) [90].

Neuronal death resulting from ischemia/reperfusion has been associated with reticular stress, because in these conditions phosphorylation of eIF2α occurs due to the activation of PERK [91]. Moreover, the induction of the CHOP protein has been observed in the hippocampus of rats after global ischemia [92, 93]. On the other hand, altered function of ER has been related to some neurodegenerative diseases, because of the accumulation of misfolded proteins such as β-amyloid, huntingtin and α-synuclein, which have been associated with the induction of neuronal death in Alzheimer´s, Huntington´s and Parkinson´s diseases, respectively [90].

Oxidative stress

Free radicals are highly reactive molecular species containing one or more unpaired electrons in the last orbital. Due to their instability, free radicals show high affinity for the electrons present in any surrounding molecule, oxidizing their target. Among the oxygen free radicals we include, the superoxide radical ($O_2^{\bullet-}$), the hydroxyl radical ($OH^{\bullet}$), molecular oxygen (a biradical with very low reactivity) ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$). In the presence of $NO^{\bullet}$ and $O_2^{\bullet-}$ $ONOO^-$ is produced. All these free radicals are recognized as reactive oxygen species (ROS). In addition, there are other ROS that are not radicals but are also reactive, like singlet oxygen ($^1O_2$), ozone (O_3), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$), which are products of oxidative metabolism [94]. ROS are involved in several physiological and pathological processes, such as mitogenesis, the regulation of the expression of several genes [94], as well as apoptosis and damage to DNA, lipids and proteins associated with ischemia and neurodegenerative diseases [95].

Cells possess antioxidant systems for protection against the effects of excessive ROS production. These systems are enzymatic and non-enzymatic. Among the enzymatic systems we included: the manganese-dependent superoxide dismutase (SOD-Mn) located in the mitocondrial matrix, the Cu^{2+}/Zn^{2+} superoxide dismutase (SOD-Cu/Zn) located in the cytosol, superoxide reductase, catalase, glutathione reductase and other enzymes related to glutathione metabolism (GSH), such as glutathione peroxidase and the thioredoxin system. Cells also contain low molecular weight molecules, with antioxidant properties such as the vitamin A, C, and E, other compounds containing selenium, lipoid acid, and ubiquinones [96]. When the antioxidant defenses are not sufficient to neutralize excessive ROS production, or when the

antioxidant mechanisms are depleted, a condition known as oxidative stress is generated. During this condition oxidative damage to nucleic acids, lipids, and proteins occurs. The main pathways of ROS production are the xanthine/xanthine oxidase system, the cycloxygenase pathway of arachidonic acid metabolism, the electron transport chain in mitochondria, and the activated neutrophile system [94]. Mitochondria is the main intracellular source of ROS. Under physiological conditions 2-5 % of oxygen is partially reduced by the electron transport chain producing $O_2^{\bullet-}$ and subsequently H_2O_2 [8].

Oxidative stress has a critical role in a large number of pathological states, and its contribution to cell death in ischemia-reperfusion conditions and neurodegenerative diseases, has been documented. Treatment with enzymatic and non-enzymatic antioxidants prevents neuronal damage induced by glutamate and its analogs, NMDA, kainate and AMPA [97, 98, 27], as well as damage observed after hypoglycemia and ischemia [99].

Increased intracellular calcium, mitochondria, and ROS are involved in neuronal death

When oxygen and/or glucose supply is reduced to critical levels, as occurs during hypoxia/ischemia or hypoglycemia, neuronal death occurs as a result of the combination of two factors: energy failure and increased extracellular levels of the excitatory amino acids, glutamate and aspartate [22]. These amino acids are extremely toxic for neurons at high concentrations, leading to their death by an excitotoxic mechanism. Excitotoxicity is a cell death process mediated either by apoptosis or necrosis, which results from the prolonged activation of glutamate receptors [100]. The role of calcium in excitotoxic death is well documented, and the NMDA glutamate receptor channel has been suggested as the main pathway of calcium influx [23, 101]. Increased intracellular calcium will activate diverse calcium-dependent enzymes (proteases, lipases, phospholipases, DNAses, nitric oxide synthase) contributing to neuronal damage [23] (Fig. 6).

Several studies have reported that upon activation of glutamate receptors, an initial increase in the intracellular concentration of calcium is followed by a second wave of Ca^{2+}, associated with mitochondrial membrane depolarization, decreased ATP levels, ROS production, and a transitory change in the permeability of the mitochondrial membrane, leading to mitochondrial dysfunction and neuronal death [24, 26, 102]. One of the key events involved in mitochondrial dysfunction is the large increase in the intracellular concentration of calcium [103, 104], driving the activation of enzymes such as calpain, a protease involved in the conversion of xanthine dehydrogenase to xanthine oxidase, generating superoxide [105], and phospholipases, causing damage to proteins and mitochondrial lipids [106]. Excessive intracellular calcium also results in the opening of the mPTP [45]. Mitochondrial dysfunction

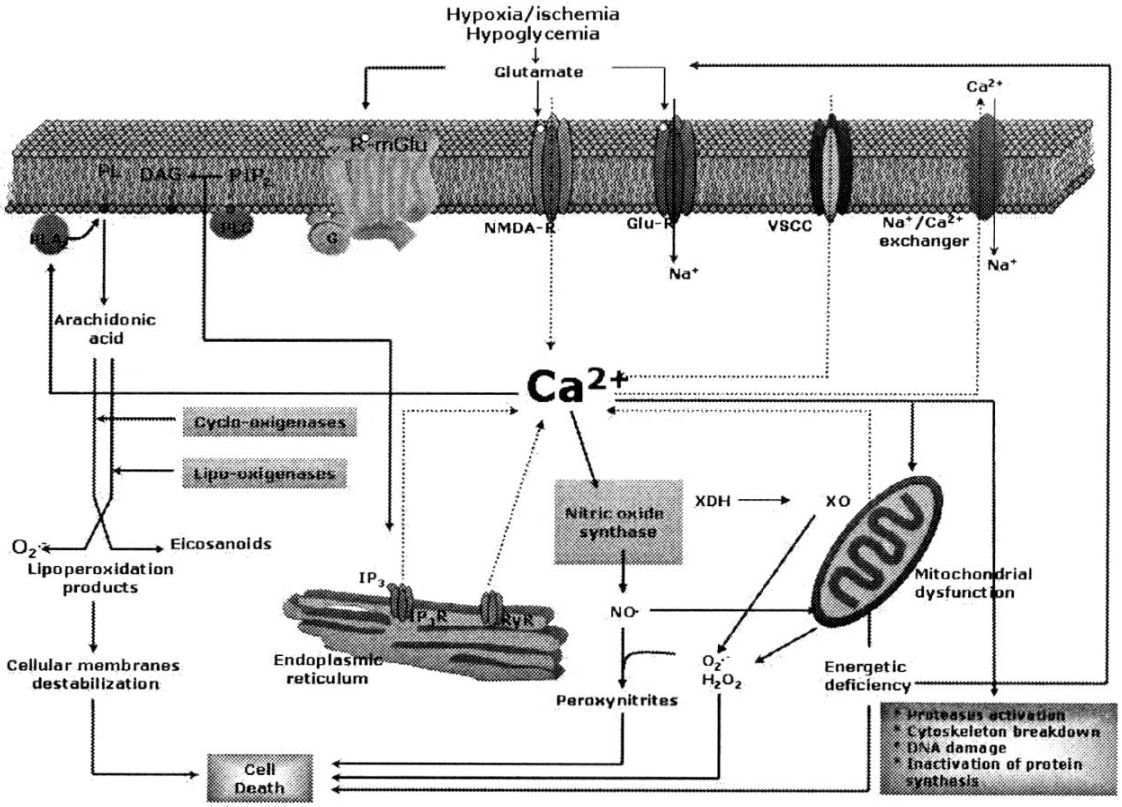


Figure 6. Possible metabolic pathways involved in the neuronal death induced by excitotoxicity during hypoxia/ischemia and hypoglycemia. The activation of the glutamatergic receptors and voltage-dependent Ca^{2+} channels induces massive Ca^{2+} influx. This initial increment produces the release of Ca^{2+} from intracellular stores or activation of enzymes, like phospholipase A_2 (PLA_2) and/or phospholipase C (PLC), this last produces IP_3 that induces the Ca^{2+} release from ER. The maintenance of the increase in intracellular Ca^{2+} concentration activates a series of proteolytic enzymes or enzymes that produce reactive oxygen and nitrogen species that contribute to neuronal damage.

has been implicated in neuronal death associated with ischemia and hypoglycemia [38, 40, 107, 108], as evidenced by the protective effect of CsA against neuronal damage in these conditions [109, 110, 111, 112]. Altogether, these evidences suggest that mitochondria have a crucial role in Ca^{2+} toxicity and ROS production during excitotoxic death.

The involvement of oxidative stress in glutamate-mediated neuronal damage is well documented. The production of $O_2^{\bullet-}$, $NO^{\bullet}$ and $ONOO^-$ after the exposure to glutamate or its analogues has been demonstrated in vitro [113, 114]. In vivo studies are more limited [115], but evidence suggests protection by antioxidants and radical scavengers against excitotoxic damage induced during ischemia and excitotoxin administration [97, 116]. Several in vitro studies have also shown protection of cell death induced by glutamate and glutamate receptor

agonists by antioxidants [113, 117], and it has been suggested that mitochondrial damage induced by glutamate might be the result of NO production, which in turn contributes to the decreased activity of complex IV of the electron transport chain, and ATP synthesis [118, 119, 120]. Moreover, in granular neurons from the cerebellum it has been observed that the loss of the Ca^{2+} regulation induced by glutamate, increases in $O_2^{\bullet -}$ concentration might result from phospholipase A_2 (PLA_2) activation [27]. The generation of $ONOO^-$ damages DNA, and actives directly or indirectly the nuclear poly-ADP-ribose polymerase (PARP-1) [121, 122]. PARP-1 activation catalyzes the hydrolysis of NAD^+ to nicotinamide and poly-ADP ribose, causing the loss of NAD^+ and as a consequence an energy deficit. PARP-1 activation also promotes ROS generation because NADPH is required by glutathione reductase as a cofactor for the conversion of oxidized glutathione (GSSG) to its reduced form (GSH). The loss of $NADP^+$ results in decreased levels of NADPH, and as consequence a reduction in GSH content. Moreover, it has been described that the mPTP is sensitive to the NADP/NADPH ratio and GSH content [13]. On the other hand, during oxidative conditions, the mitochondrial enzyme, NAD^+ glycosidase, hydrolyzes NAD^+ producing cyclic ADP-ribose, which will in turn induce the release of Ca^{2+} from the ER through the ryanodine receptor contributing to Ca^{2+} overload [123]. Altogether, these data suggest that ROS production might alter the energy state of the cell and disturb the intracellular Ca^{2+} homeostasis.

ROS production during mitochondrial Ca^{2+} overload might induce apoptotic cell death, since $ONOO^-$ can cause the opening of the mPTP pore [8] and the subsequent release of cytochrome *c* [124]. It has been recently demonstrated that cytochrome *c* released from the mitochondria binds to IP_3 receptors inducing Ca^{2+} release from the ER enhancing cell death [125]. On other hand, ROS can increase Ca^{2+} release from ER, probably through the oxidation of thiol groups in ryanodine and IP_3 receptors [126, 127]. A study in astrocytes shows that treatment with BAPTA-AM (an intracellular calcium chelator) and thapsigargin (an inhibitor of SERCA), prevent mitochondrial depolarization [128], suggesting that calcium release from intracellular Ca^{2+} stores induces mitochondrial depolarization, possibly through the opening of mPTP promoted by Ca^{2+} overload in this organelle. Theses evidences support the hypothesis of a space-time interrelation between mitochondria and ER.

In addition, ROS might alter the activity of diverse proteins involved in intracellular Ca^{2+} homeostasis, such as PAMCA and SERCA [94]. H_2O_2, $ONOO^-$, and $NO^{\bullet}$ among other reactive species, may alter the activity of some enzymes implicated in energy metabolism, like α-ketoglutarate dehydrogenase [129]. H_2O_2 can also oxidize the SH groups of cysteine 149 present in the active site of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase

(GADPH) inducing its inactivation [130, 131, 132]. These observations suggest that ROS can disrupt Ca^{2+} homeostasis, through the alteration of proteins involved in the mechanisms of storage and extrusion of Ca^{2+} ions, as well as enzymes involved in energy metabolism.

During conditions of severe hypoglycemia, when blood glucose levels drop to 1 μmol/ml or less, electric cerebral activity ceases leading to the isoelectric period or hypoglycemic coma [133]. During the isoelectric period there is an increase in the intracellular concentration of calcium [134], and $NADH/NAD^+$ and $NADPH/NADP^+$ ratios decrease, causing the elevation of the electron flow through the electron transport chain. Studies suggest that the hypoglycemic condition favors the generation of oxidative stress since the concentration of GSH decreases, as well as the activity of Mn-SOD and catalase, suggesting the presence of $O_2^{\cdot-}$ and H_2O_2 [135]. In addition, the presence of lipoperoxidation in brain and isolated mitochondria has been reported in hypoglycemic animals [136, 137].

On the other hand, the increase in the intracellular calcium concentration and ROS production favor the opening of the mPTP in neurons from hypoglycemic animals [45]. Electron microscopy studies show cell damage and swelling of the distal dendrites of granule cells in the hippocampal dentate gyrus, resulting from *in vivo* hypoglycemia. Treatment with high doses of CsA before the induction of the hypoglycemic period prevents mitochondrial swelling and cell damage in this region, which is highly vulnerable to hypoglycemia [38, 40]. Consistent with these results, oxygen and glucose deprivation induces mitochondrial dysfunction, ROS production, and glutathione oxidation in cultured cortical neurons [138]. A decreased in GSH content and glutathion peroxidase activity has also been reported in hippocampal neurons during these conditions [139]. Although these studies strongly suggest that increased intracellular calcium and ROS production is involved in neuronal damage associated with hypoglycemia, hypoglycemic cell death is still not well characterized, nor is the role of intracellular calcium and ER in these processes.

Conclusions

Regulation of cell Ca^{2+} homeostasis has a crucial role both in physiological processes and neuronal death. Strong evidences suggest a close relationship between increased intracellular Ca^{2+}, mitochondrial dysfunction and ROS production in excitotoxicity, associated with hypoxia/ischemia and hypoglycemia. During the last years, more studies have focused on the role of ER in the cell death process, since altered Ca^{2+} storage in this organelle leads to reticular stress, inducing the expression of proteins involved in cell damage. In addition, evidence suggests that the altered interaction between the ER and mitochondria is involved in the increase of calcium levels during the death process.

Acknowledgments

40306-M and U-49047 CONACYT grants to LM and MD-M, respectively and 167146 CONACYT and DGEP fellowships to KH-F supported this work.

References

1. Berridge, M.J., Bootmann, M.D. and Roderick, H.L. 2003, Nat. Rev. Mol. Cell. Biol., 4, 517.
2. Kristián, T. and Siesjö, B.K. 1998, Stroke, 29, 705.
3. Simpson, P.B. and Rusell, J.T. 1998, Brain Res. Rev., 26, 72.
4. Leist, M. and Nicotera, P. 1999, Calcium and Cell Death in cell death and diseases of the nervous system, V.E. Koliatosn and R.R. Ratan (Ed) Humana Press Inc., Totowa, New York.
5. Darley-Usmar, V.M., Rickwood, D. and Wilson, M.T. 1987, Mitochondria. A Practical Approach. Oxford, IRL.
6. Gunter, T.E. and Gunter, K.K. 2001, IUBMB Life, 52, 197.
7. Pozzan, T. and Rizzuto, R. 2000, Eur. J. Biochem., 267, 5269.
8. Jacobson, J. and Duchen, M.R. 2004, Mol. Cell. Biochem., 256, 209.
9. Kirochock, Y., Krapivinsky, G. and Clapman, D.E. 2004, Nature, 427, 360.
10. Rizzuto, R., Bernardi, P. and Pozzan, T. 2000, J. Physiol., 529, 37.
11. Beutner, G., Sharma, V.K., Giovannucci, D.R., Yule, D.I. and Sheu, S.S. 2004, J. Biol. Chem., 276, 21482.
12. Vandecasteele, G., György, S. and Rizzuto, R. 2001, IUBMB Life, 52, 213.
13. Crompton, M. 1999, Biochem. J., 341, 233.
14. Hajnoczky, G., Robb-Gaspers, L.D., Seitz, L.D. and Thomas, A.P. 1995, Cell, 82, 415.
15. Jouaville, L.S., Pjnton, P., Bastianutto, C., Rutter, G.A. and Rizzuto, R. 1999, Proc. Natl. Acad. Sci. USA, 96, 13807.
16. Territo, P.R., French, S.A., Dunleavy, M.C., Evans, F.J. and Balaban, R.S. 2001, J. Biol. Chem., 276, 2586.
17. He, L. and Lemasters, J.J. 2002, FEBS Lett., 512, 1.
18. Rohacs, T., Nagy, G. and Spat, A. 1997, Biochem. J., 322, 785.
19. Montero, M., Alonso, M.T., Carnicero, E., Cuchillo-Ibanez, I., Albillos, A., García, A.G., Garcia-Sancho, J. and Alvarez, J. 2000, Nat. Cell Biol., 2, 57.
20. Jouaville, L.S., Ichas, F., Holmuhamedov, E.L., Camacho, P. and Lechleiter, J.D. 1995, Proc. Natl. Acad. Sci. USA, 96, 13807.
21. Landolfi, B., Curci, S., Debelli, L., Pozzan, T. and Hofer, A. 1998, J. Cell Biol., 142, 1235.
22. Benveniste, H., Drejer, J., Schousboe, A. and Diemer, N.H. 1984, J. Neurochem., 43, 1369.
23. Choi, D.W., Maulucci-Gedde, M.A. and Kriegstein, A.R. 1987, J. Neurosci., 8, 185.
24. Schinder, A.F, Olson, E.C., Spitzer, N.C. and Montal, M. 1996, J. Neuroscience, 16, 6125.
25. Griffiths, T.E., Ocampo, C.J., Savage, J.S., Rutter, G.A., Hansford, R.G., Stern, M.D. and Silverman, H.S. 1998, Cardiovasc. Res., 39, 423.
26. Budd, S.L. and Nicholls, D.G. 1996, J. Neurochem., 67, 2282.

27. Vesce, S., Kirk, L. and Nicholls, D.G. 2004, J. Neurochem., 90, 683.
28. García, O. and Massieu, L. 2001. J. Neurosci. Res., 64, 418.
29. Massieu, L., Gómez-Román, N. and Montiel T. 2000, Exp. Neurobiol., 165, 257.
30. Massieu, L., Haces, M.L., Montiel, M. and Hernández-Fonseca, K. 2003, Neurosci., 120, 365.
31. Duchen, M.R. 2000, J. Physiol., 529, 57.
32. Halestrap, A., McStay, G.P. and Clarke, S.J. 2002, Biochim., 84, 153.
33. Chalmers, S. and Nicholls, D.G. 2003, J. Biol. Chem., 279, 19602.
34. Krieger, C. and Duchen, M.R. 2002, Eur. J. Pharmacol., 447, 177.
35. He, L. and Lemasters, J.J. 2002, FEBS Lett., 512,1.
36. Vieira, H.L., Haouzi, D., Hamel, C.E., Jacotot, E., Belzacq, A.S., Brenner, C. and Kroemer, G. 2000, Cell Death Differ., 7, 1146.
37. Friberg, H., Connern, C., Halestrap, A.P. and Wieloch, T. 1999, J. Neurochem., 72, 2488.
38. Ferrand-Drake, M., Zhu, C., Gido, G., Hansen, A., Karlsson, J.O., Bahr, B.A., Zamzami, N., Kroemer, G., Chan, P.H., Wieloch, T. and Blomgren, K. 2003, J. Neurochem., 85, 1431.
39. Uchino, H., Elmér, E., Uchino, K., Lindvall, O. and Siesjö. 1995, Acta. Physiol. Scand., 155, 469.
40. Friberg, H., Ferrand-Drake, M., Bengtsson, F., Halestrap, A.P. and Wieloch, T. 1998, J. Neurosci., 18, 5151.
41. Nieminen, A.L., Petrie, T.G., Lemasters, J.J. and Seman, W.R. 1996, Neuroscience, 75, 993.
42. Petersen, A., Castillo, R.F., Hansson, O., Wieloch, T. and Brundin, P. 2000, Brain Res., 857, 20.
43. Korge, P. and Weiss, J.N. 1999, Eur. J. Biochem., 265, 273.
44. Du, C, Fang, M., Li, Y., Li, L. and Wang, X. 2000. Cell, 102, 33.
45. Friberg, H. and Wieloch, T. 2002, Biochimie., 84, 241.
46. Antonsson, B., Montessuit, S., Sánchez, B. and Martinou, J.C. 2001, J. Biol. Chem., 276, 11615.
47. Verkhratsky, A. and Petersen, O.H. 2002, Eur. J. Pharmacol., 447, 141.
48. Rao, R.V., Ellerby, H.M. and Bredesen, D.E. 2004, Cell Death Diff., 11, 372.
49. Groenendyk, J. and Michalak, M. 2005, Act. Biochim. Pol., 52, 381.
50. Verkhratsky, A. 2002, Cell Calcium, 32, 393.
51. Vangheluwe, P., Raeymaekers, L., Dode, L. and Wuytack, F. 2005, Cell Calcium. 38, 291.
52. Mattson, M.P., La Ferla, F.M., Chan, S.L. and Leissring, M.A. 2000, Trends Neurosci., 23, 222.
53. Galione, A. and Ruas, M. 2005, Cell Calcium, 38, 273.
54. de Meis, L., Arruda, A.P. and Carvalho, D.P. 2005, Biosci Rep., 25,181.
55. Toyoshima, C., Asahi, M., Sugita, Y., Khanna, R., Tsuda, T. and MacLennan, D.H. 2003, Proc. Natl. Acad. Sci. USA, 100, 467.
56. Buffy, J.J., Buck-Koehntop, B.A., Traaseth, N.J., Thomas, D.D. and Viglia, G. 2006, J. Mol. Biol., 358, 420.
57. Berridge, M.J. 1993, Nature, 361, 315.
58. Bilmen, J.G. and Michelgeli, F. 2002, Cell Signal., 14, 955.
59. Worley, P.F., Baraban, J.M., Colvin, J.S and Snyder, S.H. 1987, Nature, 325, 159.

60. Furuichi, T., Futurama, D., Hakamata, Y., Nakai, J., Takeshima, H. and Mikoshiba, K. 1994, J. Neurosci., 14, 4794.
61. Danoff, S.K., Ferris, C.D., Donath, C., Fisher, G.A., Munemitsu, S., Ulrich, A., Snyder, S.H. and Ross, C.A. 1991, Proc. Natl. Acad. Sci. USA., 88, 2951.
62. Patterson, R.L., Boehning, D. and Snyder, S.H. 2004, Annu Rev Biochem., 73, 437.
63. Patterson, R.L., Van Rossum, D.B., Kaplin, A.I., Barrow, R.K. and Snyder, S.H. 2005, Proc. Natl. Acad. Sci. USA., 102, 1357.
64. Brading, A.F. 2002, Novartis Found Symp., 246, 24.
65. Sedlak, T.W. and Snyder, S.H. 2006, JAMA., 295, 81.
66. Spassova, M.A., Soboloff, J., He, L.P., Hewavitharana, T., Xu, W., Venkatachalam, K., Van Rossum, D.B., Patterson, R.L. and Gill, D.L. 2004, Biochim Biophys Acta., 1742, 9.
67. Miyamoto, S., Izumi, M., Hori, M., Kobayashi, M., Ozaki, H., and Karki, H. 2000, Br. J. Pharmacol., 130, 650.
68. Hernández-Fonseca, K. and Massieu, L. 2005, J. Neurosci. Res., 82, 196.
69. Takahashi, M., Tanzawa, K. and Takahashi, S. 1994, J. Biol. Chem., 269, 369.
70. Cifuentes, F., González, C.E., Fiordelisio, T., Guerrero, G., Lai, F.A., Hernández-Cruz, A. 2001, Cell. Signal., 13, 353.
71. Gerasimenko, O. and Gerasimenko, J. 2004, J. Cell. Sci., 117, 3087.
72. Martínez-Merlos, T., Cañedo-Merino, R. and Díaz-Muñoz, M. 1996, Int. J. Biochem. Cell. Biol., 29, 529.
73. Vázquez-Martínez, O., Cañedo-Merino, R., Díaz-Muñoz, M. and Riesgo-Escovar, J. 2003, J. Cell. Sci., 116, 2483.
74. Fill, M. and Copello, J.A. 2002, Physiol. Rev., 82, 893.
75. Morales-Tlalpan, V., Arellano, R.O. and Díaz-Muñoz, M. 2005, Cell Calcium, 37, 203.
76. Protasi, F. 2002, Front. Biosci., 7, d650.
77. Trueta, C., Sánchez-Armass, S., Morales, M.A. and De-Miguel, F.F. 2004, J. Neurobiol., 61, 309.
78. George, C.H., Higgs, G.V., Mackrill, J.J. and Lai, F.A. 2003, J. Biol. Chem., 278, 28856.
79. Szabadkai, G. and Rizzuto, R. 2004, FEBS Lett., 567, 111.
80. Díaz-Muñoz, M., Dent, M.A.R., Granados-Fuentes, D., Hall, A.C., Hernández-Cruz, A., Harrington, M.E. and Aguilar-Roblero, R. 1999, Neuroreport, 10, 481.
81. Zhao, W., Meiri, N., Xu, H., Cavallaro, S., Quattrone, A., Zhang, L. and Alkon, D.L. 2000, FASEB J., 14, 290.
82. Mejia-Gervacio, S., Hounsgaard, J. and Díaz-Muñoz, M. 2003, Neuroscience, 123, 123.
83. Sampieri, L., Díaz-Muñoz, M., Antaramián, A. and Vaca, L. 2005, J. Biol. Chem., 280, 24804.
84. Wagenknecht, T. and Radermacher, M. 1997, Curr. Opinion Struct. Biol., 7, 258.
85. Serysheva, I.I., Hamilton, S.L., Chiu, W. and Ludtke, S.J. 2005, J. Mol. Biol., 345, 427.
86. Zhao, M., Li, P., Li, X., Zhang, L., Winkfein, R.J. and Wayne Chen, S.R. 1999, J. Biol. Chem., 274, 25971.
87. Wayne Chen, S.R., Ebizawa, K., Li, X. and Zhang, L. 1998, J. Biol. Chem., 273, 14675.

88. Eisner, D.A., Díaz, M.E., O´Neill, S.C. and Trafford, A.W. 2004, Cell Calcium, 35, 583.
89. Paschen, W. and Mengesdorf, T. 2005, Cell Calcium, 38, 409.
90. Oyadomari, S. and Mori, M. 2004, Cell. Death Diff., 11, 381.
91. Hu, B.R., Janelidze, S., Ginsberg, M.D., Busto, R., Perez-Pinzon, M., Sick, T.J., Siësjo, B.K. and Liu, C.L. 2001, J. Cereb. Blood Flow Metab., 21, 865.
92. Paschen, W., Gissel, C., Linden, T., Althausen, S. and, Doutheil, J. 1998, Brain Res., 60, 115.
93. Kumar, R., Azam, S., Sullivan, J.W., Owen, C., Cavener, D.R., Zhang, P., Ron, D., Harding, H.P., Chen, J.J., Han, A., White, B.C., Krause, G.S. and DeGracia, D.J. 2001, J. Neurochem., 77, 1418.
94. Kourie, J. 1998, Am. J. Physiol., 275, C1.
95. Coyle, J.T. and Puttfarcken, P. 1993, Science, 262, 689.
96. Nordberg, J. and Arnér, E.S.J. 2001, Free Rad. Biol. Med., 31, 1287.
97. Schulz, J.B., Henshaw, D.R., Siwek, D., Jenkins, B.G., Ferrante, R.J., Cipolloni, P.B., Kowall, N.W., Rosen, B.R. and Beal, M.F. 1995, Neurochem., 64, 2239.
98. Ishige, K., Schubert, D. and Sagara, Y. 2001, Free Rad. Biol. Med., 30, 433.
99. Rego, A.C., Santos, M.S. and Oliveira, C.R. 1999, Free Rad. Biol. Med. 26, 1405.
100. Olney, J.W. 1978, Science, 164, 719.
101. Paschen, W. 2000, Brain Res. Bull., 53, 409.
102. Sattler, R., Charlton, M.P., Haftner, M. and Tymianski, M. 1998, J. Neurochem. 71, 2349.
103. Puka-Sundvall, M., Gajkowska, B., Cholewinski, M., Blomgren, K., Lazarewicz, J.W. and Hagberg, H. 2000, Brain Res. Dev. Brain Res., 125, 31.
104. Schild, L., Huppelsberg, J., Kahlert, S., Keilhoff, G. and Reiser, G. 2003, J. Biol. Chem., 278, 25454.
105. Cheng, Y. and Sun, A.Y. 1994, Neurochem. Res., 19, 1557.
106. Almeida, A., Allen, K.L., Bates, T.E. and Clark, J.B. 1995, J. Neurochem., 65, 1698.
107. Sims, N.R. and Anderson, M.F. 2002, Neurochem. Int., 40, 511.
108. Blomgren, K., Zhu, C., Hallin, U. and Hagberg, H. 2003, Biochem. Biophys. Res. Commun., 304, 551.
109. Kuroda, S., Janelidze, S. and Siesjö, B.K. 1999, Brain Res., 843, 148.
110. Yoshimoto, T. and Siesjö, B.K. 199. Brain Res., 839, 283.
111. Ruiz, F., Álvarez, G., Ramos, M., Hernández, M., Bogonez, E. and Satrústegi, J. 2000, Eur. J. Pharmacol., 404, 29.
112. Fiskum, G., Murphy, A.N. and Beal, M.F. 1999, J. Cereb. Blood Flow Metab. 19, 351.
113. Lafon-Cazal, M., Petri, S., Culcasi, M. and Bockaert, J. 1993, Nature, 364, 535.
114. Bondy, S.C. and Lee, D, K. 1993, Brain. Res., 610, 229.
115. Hammer, B., Parker, W.D. and Bennet, J.P. 1993, Neuroreport, 5, 72.
116. Phillis, J.W. and Clough-Helfman, C. 1990, Neurosci. Lett., 116, 315.
117. Dykens, J.A., Stern, A. and Trenkner, E. 1987, J. Neurochem., 49, 1222.
118. Bolaños, J.P. and Almeida, A. 1999, Biochem. Biophys. Acta., 1411, 415.
119. Almeida, A. and Bolaños, J.P. 2001, J. Neurochem., 77, 676.
120. Dawson, V.L., Dawson, T.M. and London, E.D. 1991. Proc. Natl. Acad. Sci. USA. 88, 6368.

121. Szabo, C. and Dawson, V.L. 1998, Trends Pharmacol. Sci., 19, 287
122. Virag, L. Salzman, A.L. and Szabo, C. 1999, J. Inmunol., 161, 3753.
123. Chakraborti, T., Das, S., Mondal, M., Roychoudhury, S. and Chakraborti, S. 1999, Cell Signal., 11, 77.
124. Brookes, P.S. and Darley-Usmar, V.M. 2004, Am. J. Physiol. Heart Cir. Physiol. 386, H39.
125. Boehning, D., Patterson, R.L., Sedaghat, L., Glebova, N.O., Kurosaki, T., Snyder, S.H. 2003, Nat. Cell Biol., 12, 1051.
126. Abramson, J.J. and Salama, G. 1988, Mol. Cell Biochem., 82, 81.
127. Suzuki, Y.J. and Ford, G.D. 1999, J. Mol. Cell Cardiol., 31, 435.
128. Jacobson, J. and Duchen, M.R. 2002, J. Cell Science, 115, 1175.
129. Gibson, G.E and Huang, H.M. 2004, J. Bioenerg. Biomembr., 36, 335.
130. Schuppe-Koistinen, I., Moldeus, P., Bergman, T. and Cotgreave. 1994, FEBS Lett., 221, 1033.
131. Lind, C., Gerdes, R., Schuppe-Koistinen, I. and Cotgreave, I.A. 1998, Biochim. Biophys. Res. Commun., 247, 481.
132. Colussi, C., Alberini, M.C., Coppola, S., Rovidati, S., Galli, F. and Ghibelli, L. 2000, FASEB J., 14, 2266.
133. Auer, R.N., Wieloch, T., Olsson, Y. and Siesjö, B.K. 1984, Acta Neropathol. 64, 177.
134. Harris, R.J., Wieloch, T., Symon, L. and Siesjö, B.K. 1984, J. Cereb. Blood Flow Metab., 4, 187.
135. Bhardwarj, S.K., Sharma, M.L., Gulati, G., Chhbra, A., Kaushik, R. and Sharma, P. 1998, Mol. Chem. Neuropatol., 34, 157.
136. Ballesteros, J.R., Mishra, O.P. and McGowan, J.E. 2003, Biol. Neonate, 84,159.
137. Singh, P., Jain, A. and Kaur, G. 2004, Mol. Cel. Biochem., 260, 153.
138. Almeida, A., Delgado-Esteban, M., Bolaños, J.P. and Medina, J.M. 2002, J. Neurochem., 81, 207.
139. Jiang, X., Mu, D., Manabat, C., Kosky, A.A., Christein, S., Täuber, M.G., Vexler, Z.S. and Ferreiro, D.M. 2004, Exp. Neurol., 190, 224.

Estrés oxidativo y muerte excitotóxica

Los radicales libres son átomos o moléculas extremadamente reactivas, debido a que en su último orbital tienen uno o más electrones sin aparear. Esta inestabilidad les confiere una avidez física por un electrón de cualquier otra molécula de su entorno, ocasionando que la molécula afectada quede inestable. Dentro de los radicales libres encontramos a las especies reactivas de oxígeno (EROS), como el anión radical superóxido ($O_2^{\bullet -}$), el singulete de oxígeno ($^1O_2$), el peróxido de hidrógeno (H_2O_2), el radical hidroxilo ($^{\bullet}OH$), y el ácido hipocloroso (HOCl) que son producto del metabolismo oxidativo (Kourie, 1998). Las EROS están implicadas en varios procesos fisiológicos y patofisiológicos, como la mitogénesis, la apoptosis, la regulación de la expresión de varios genes (Kourie, 1998), la isquemia y en enfermedades neurodegenerativas (Coyle y Pufftarcken, 1993). El estrés oxidativo se refiere a las consecuencias citotóxicas de las EROS, cuando las defensas antioxidantes son rebasadas por el incremento en la producción de EROS, por la disminución o pérdida de los mecanismos antioxidantes, o por ambas (Coyle y Puttfarcken, 1993). En estas condiciones se pueden presentar alteraciones en el DNA, peroxidación de lípidos y modificación de proteínas. La mitocondria es la principal fuente de EROS intracelulares. Bajo condiciones de reposo, entre el 2 - 5 % del oxígeno consumido en la mitocondria es parcialmente reducido por la cadena transportadora de electrones formando $O_2^{\bullet -}$ y subsecuentemente peróxido de hidrógeno (Jacobson y Duchen, 2004).

El incremento en la generación de EROS durante la muerte excitotóxica está bien documentado. Estas pueden producirse por la entrada de Ca^{2+} a través de los receptores NMDA o por la disfunción mitocondrial, ocurriendo tal vez de manera simultánea. La generación de $O_2^{\bullet -}$, de oxido nítrico ($NO^{\bullet}$) y peroxinítritos ($ONOO^-$) inducida por glutamato se ha demostrado *in vitro* (Lafon-Cazal et al., 1993; Bondy y Lee, 1993). El estrés oxidativo tiene un papel crítico en un gran número de estados patológicos, y ha sido claramente establecida su contribución a la muerte en condiciones de isquemia-reperfusión y en enfermedades neurodegenerativas (Jacobson y Duchen, 2002). En neuronas

granulares de cerebelo se ha observado que la falla en la regulación de la concentración intracelular del Ca^{2+} inducida por glutamato incrementa la concentración de $O_2^{\bullet-}$, y este incremento está relacionado con la actividad de la fosfolipasa A_2 (PLA_2) (Vesce, et al, 2004), que induce liberación de ácido araquidónico en la membrana (Williams et al., 1996; Kim et al., 1995; Saluja et al., 1887). A partir del ácido araquidónico se sintetizan prostaglandinas y leucotrinos con la concomitante producción de $O_2^{\bullet-}$ (Kramer y Sharp, 1997).

Se ha observado que el tratamiento con moléculas y enzimas antioxidantes previene el daño inducido por glutamato (Ishige et al., 2001; Vesce et al., 2004), NMDA, Kainato, AMPA (Shulz et al., 1995) e isquemia química (Rego et al., 1999). Por otra parte se ha demostrado que en condiciones de privación de oxígeno y glucosa hay disfunción mitocondrial, producción de EROS y un incremento en la concentración de glutatión oxidado en cultivos corticales de neuronas (Almeida et al., 2002). Otro estudio *in vitro* en un modelo de privación de oxigeno y glucosa muestra una disminución en el contenido de glutatión (GSH) y de la actividad de la glutatión peroxidasa (GPx) en neuronas de hipocampo (Jiang et al., 2004).

Estrés oxidativo y falla energética

Diversos trabajos han sugerido que el estrés oxidativo es un factor asociado al daño neuronal hipoglucémico. Algunos de estos estudios han demostrado la producción de EROS en diversos modelos de falla metabólica, como son cultivos de retina e hipocampo incubados con el inhibidor glucolítico yodoacetato; células PC12 sometidas a periodos de ausencia de glucosa y mitocondrias aisladas sometidas a hipoglucemia inducida por insulina (Rego et al., 1999; Liu et al., 2003; Sing et al., 2004; Mc Gowan et al., 2006).

En otros trabajos se ha observado que la hipoglucemia induce lipoperoxidación tanto de membranas plasmáticas como mitocondriales (Bhardwarj et al., 1998; Ballesteros et al., 2003; Singh et al., 2004; Patockova et al., 2003). Al igual que en el daño producido por glutamato, el inducido por la

inhibición glucolítica es prevenido con antioxidantes (Rego et al., 1999). La vitamina E previene el daño ocasionado por yodoacetato en células de retina y en cultivos primarios de neuronas de hipocampo (Rego et al., 1998; Massieu et al., 2003).

La producción de EROS en las mitocondrias es regulada por enzimas antioxidantes dentro cuales se incluyen: la superóxido dismutasa de manganeso (SOD-Mn), la catalasa, la GPx; así como por la concentración de glutatión reducido (GSH). Se ha observado que en estados patológicos, como la hipoglucemia, hay una disminución en la concentración de GSH y un incremento en la actividad de la SOD-Mn y de la catalasa, sugiriendo indirectamente la presencia de $O_2^{\bullet -}$ y H_2O_2. Sin embargo, el papel de las EROS en el daño hipoglucémico no se ha determinado, ni se conoce el papel del calcio intracelular en la producción de EROS en estas condiciones.

De lo que sí tenemos conocimiento, es que las EROS pueden afectar a los sistemas de almacenamiento de Ca^{2+} intracelular y la función de enzimas que participan en el metabolismo energético, dando lugar a un círculo vicioso. Se ha observado que las EROS incrementan la probabilidad de liberación de Ca^{2+} de pozas intracelulares del RE, probablemente por modificación de grupos tioles importantes en la actividad de los receptores a rianodina e IP_3 (Abramson y Salama, 1988; Suzuki y Ford, 1999). Por otra parte, estudios han sugerido que las EROS pueden afectar la actividad de diversas proteínas involucradas en la homeostasis del Ca^{2+} intracelular, como la ATPasa de la membrana plasmática (PMCA) y la ATPasa del retículo endoplásmico (SERCA) (Kourie, 1998). Además el H_2O_2, el $ONOO^-$, el $NO^{\bullet}$ entre otras especies reactivas, disminuyen la actividad de la α-cetoglutarato deshidrogenasa (Gibson y Huang, 2004), y estudios han demostrado que el H_2O_2 puede oxidar los grupos SH de la cisteína 149 del sitio activo de la enzima glucolítica, gliceraldehído-3-fosfato deshidrogenada (GAPDH) induciendo su inactivación (Schuppe-Koistinen, 1994; Lind et al., 1998; Colussi et al., 2000). A la fecha, el papel de la mitocondria en la regulación del Ca^{2+} y la producción de EROS durante la excitotoxicidad está bien

descrito (Budd et al., 1996; Schinder et al., 1996; Castilho et al., 1999), sin embargo el papel del RE en estos procesos no ha recibido tanta atención.

VI. ANTECEDENTES

Inhibición glucolítica y muerte neuronal

Durante décadas se había considerado que la enzima gliceraldehído-3-fosfato deshidrogenasa (GAPDH) es una de las enzimas cuya función principal es su participación en la vía glucolítica. Recientemente se ha demostrado que la GAPDH es una proteína multifuncional (Sirover, 1999; Chuang et al., 2005), ya que participa modulando el empaquetamiento de microtúbulos (Huitorel y Pantalón, 1985; Volker y Knull, 1997; Kumagai y Sakai, 1983), en el transporte de RNA nuclear (Singh y Green, 1993), contribuye a la fusión membranal (Glaser y Gross, 1995) y tiene actividad de fosfotransferasa/cinasa (Kawamoto y Caswell, 1986).

En diversos estudios se ha reportado una interacción proteína-proteína entre la GAPDH y el dominio citoplásmico del precursor de la proteína β-amiloide (Shulze et al. 1993) y de la proteína huntingtina (Burke et al. 1996), involucradas en la enfermedad de Alzheirmer y Huntigton, respectivamente; sugiriendo una disminución en su actividad (Roses, 1996). También se ha observado interacción con la atrofina (Burke et al. 1996), la ataxina (Koshy et a., 1996) y el receptor de andrógenos (Koshy et a., 1996).

Uno de los modelos *in vitro* más utilizados para mimetizar una condición hipoglucémica, es la administración de yodoacetato (un inhibidor irreversible de la enzima glucolítica, gliceraldehído 3-fosfato deshidrogenasa). El yodoacetato inhibe a la enzima uniéndose de manera covalente a un grupo sulfihidrilo localizado en su sitio activo (Fig. 8).

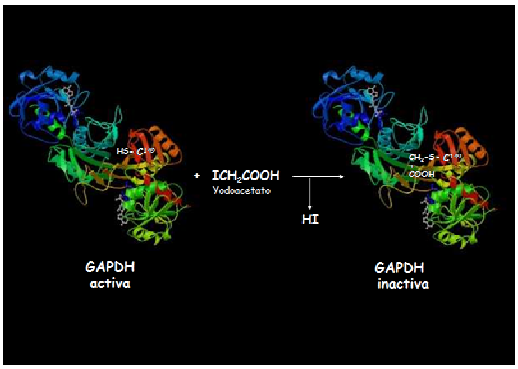


Figura 8. Inhibición de la GAPDH por la unión covalente del yodoacetato al sulfihidrilo de la cisteína 149 del sitio activo de la enzima.

En un estudio realizado por Uto (1995) en cultivos de células hipocampales de la región CA1 y de corteza cerebral, la presencia de 1 o 10 mM de KCN y arsenato (que inhiben el metabolismo aeróbico), produce una reducción en la sobrevivencia del 50%. En tanto que, las mismas concentraciones de yodoacetato producen la muerte de la totalidad de las células. Por otra parte, Matthews y colaboradores (1997) demostraron que la administración de yodoacetato directamente en el estriado de la rata produce muerte neuronal y sugiere que ésta puede ser de tipo excitotóxico asociada a la producción de $^{\bullet}$OH. Además, experimentos de microdiálisis *in vivo* han mostrado que la inhibición de la glucólisis con yodoacetato produce la elevación de los niveles extracelulares de aspartato y glutamato, lo cual podría dar lugar a la muerte neuronal (Sandberg et al. 1985; Massieu et al. 2000). La administración *in vivo* de 5 mM de yodoacetato induce un incremento de 3.5 veces y de 35 veces de los niveles extracelulares de glutamato y aspartato, respectivamente, y se acompaña de muerte neuronal en la región CA1 y en el giro dentado del hipocampo (Sandberg et al. 1985; Massieu et al., 2000), mimetizando lo que ocurre durante un periodo de hipoglucemia, en el cual la liberación de aspartato es mayor que la de glutamato (Sandberg et al. 1986).

Diversos estudios *in vitro* han mostrado que el yodoacetato induce la liberación de glutamato y aspartato aparentemente a través del funcionamiento inverso de sus transportadores (Madl y Burgesser, 1993; Gemba et al., 1994; Ogata et al., 1995; Longuemare y Swanson, 1995). Un estudio *in vivo* de nuestro grupo apoya esta hipótesis (Camacho et al., 2007).

Nuestro grupo de investigación ha mostrado que la toxicidad del glutamato se exacerba en presencia de yodoacetato (Massieu et al., 2000; Massieu et al., 2003; Hernández-Fonseca y Massieu, 2005), y que el daño inducido en condiciones de inhibición glucolítica puede deberse a un mecanismo excitotóxico, ya que el MK-801, un antagonista del receptor NMDA previene la muerte neuronal (Zeevalk y Nicklas, 1992; Massieu et al. 2003).

Estudios previos en nuestro laboratorio sugieren que la muerte neuronal inducida en condiciones de inhibición glucolítica es dependiente de la presencia

de calcio externo y de la regulación de la concentración intracelular de calcio por el retículo endoplásmico (Hernández-Fonseca y Massieu, 2005, ver apéndice).

Tanto la ausencia del Ca^{2+} extracelular, como la presencia de Bapta-AM (quelante de calcio intracelular), así como la de un antagonista de los receptores a glutamato de tipo NMDA, el MK-801, previenen significativamente la muerte neuronal y mantienen la integridad morfológica de los cultivos de hipocampo. También se demostró que las pozas intracelulares de calcio del RE participan en la muerte inducida por la inhibición glucolítica, ya que el bloqueo de los receptores a rianodina por dantroleno y por rianodina a una concentración relativamente baja (1 μM), reducen la muerte de las neuronas al igual que el bloqueo de los receptores a IP3 del retículo endoplásmico con xetospongina C. En concordancia con esta observación agonistas de los receptores a rianodina como la cafeína y rianodina a altas concentraciones (25 μM) exacerban la muerte neuronal (Hernández-Fonseca y Massieu, 2005, ver apéndice). Estas observaciones sugieren que la pérdida de la homeostasis de Ca^{2+} intracelular durante la inhibición glucolítica favorece la muerte neuronal.

Por otra parte, otros estudios *in vitro* sugieren que el daño inducido por yodoacetato puede deberse a la presencia de radicales libres. En estos estudios realizados en cultivos de neuronas de la región CA1 del hipocampo, en cultivos de células granulares de cerebelo, y en retina de pollo, se observó que el MK-801 no tuvo un efecto protector sobre la muerte, mientras que la administración de diferentes antioxidantes mostró un claro efecto protector (Uto et al. 1995; Malcolm et al, 2000; Rego et al., 1999). Estos resultados han llevado a sugerir que el mecanismo de muerte inducido por la administración de yodoacetato no es de tipo excitotóxica, sino que el daño está mediado por la producción de radicales libres (Uto et al., 1995; Malcolm et al., 2000; Rego et al., 1999). Cabe mencionar que en estos estudios se utilizaron usando altas concentraciones de yodoacetato, lo que induce una deficiencia energética severa. De acuerdo a lo anterior, a la fecha, no está claro el mecanismo de muerte inducido por yodoacetato. Los estudios realizados en la presente tesis contribuyeron a desentrañar dicho mecanismo.

VII. PLANTEAMIENTO DEL PROBLEMA

Los estudios descritos en la sección anterior sugieren que la inhibición glucolítica induce la muerte de las neuronas por un mecanismo dependiente de Ca^{2+} en el que participa la producción de EROS. Sin embargo, el papel de la activación de los receptores a glutamato de tipo NMDA en el aumento en la concentración de Ca^{2+} intracelular y en el mecanismo de muerte, hasta el momento no había quedado claro. Tampoco se conocía cual era la relación entre la activación de dichos receptores y la producción de EROS. Con base en lo anterior, en la primera parte de la tesis nos planteamos conocer si el aumento en la concentración de Ca^{2+} intracelular está relacionado con la producción de EROS y si la activación de los receptores NMDA interviene en ambos procesos.

Los resultados de los experimentos realizados para contestar a estas preguntas se presentan en el artículo: Hernández-Fonseca K., Massieu L. 2008. **"Calcium-dependent production of reactive oxygen species is involved in neuronal damage induced during glycolysis inhibition in cultured hippocampal neurons"**. J. Neurosci. Res., DOI: 10.1002/jnr.21634

Por otra parte, se ha mencionado en la introducción que los cuerpos cetónicos pueden utilizarse como sustratos alternativos a la glucosa. En estudios previos demostramos que el AcAc es capaz de prevenir eficientemente la disminución de ATP y la muerte producida por yodoacetato en neuronas cultivadas de hipocampo (Massieu et al., 2003). En el presente trabajo se estudió si el efecto protector de los cuerpos cetónicos podría estar mediado por una reducción en la producción de EROS. Con el fin de conocer si dicho efecto podría deberse al restablecimiento de la falla energética por los cuerpos cetónicos o a otras acciones de dichos compuestos, utilizamos los isómeros D- y L- del β-hidroxibutirato. El isómero D tiene un papel fisiológico ya que es transformado a acetil-CoA por la β-hidroxibutirato deshidrogenasa, mientras que el isómero L tiene un papel fisiológico mucho menor.

Los resultados de los experimentos diseñados para contestar estas preguntas se presentan en la sección de resultados en el artículo (sólo los

experimentos *in vitro* presentados en este artículo forman parte de esta tesis). Haces M.L., Hernández-Fonseca K., Medina-Campos O.N., Montiel T., Pedraza-Chaverri J., Massieu L. 2008. "**Antioxidant capacity contributes to protection of ketone bodies against oxidative damage induced during hypoglycemic conditions".** Exp. Neurol. DOI:10.1016/expneurol.2007.17.029.

VIII. HIPÓTESIS

La excitotoxicidad y el estrés oxidativo participan en la muerte neuronal inducida por la inhibición glucolítica. El incremento en la concentración intracelular de calcio favorecerá la producción de EROS las cuales participarán en el mecanismo de muerte neuronal.

Los cuerpos cetónicos tendrán un efecto protector de la muerte neuronal al actuar como sustratos energéticos y/o al reducir la producción de EROS.

IX. OBJETIVOS

- Estudiar el efecto de diferentes concentraciones del inhibidor glucolítico, yodoacetato, sobre la actividad de la GAPDH, los niveles de ATP y la sobrevivencia de neuronas cultivadas de hipocampo

- Estudiar los cambios en la concentración de Ca^{2+} intracelular en condiciones de inhibición glucolítica

- Conocer la dependencia de Ca^{2+} extracelular e intracelular en la muerte producida en condiciones de inhibición glucolítica

- Conocer el papel de la excitotoxicidad en la muerte inducida en condiciones de inhibición glucolítica

- Conocer el papel de la activación de los receptores a NMDA sobre la concentración intracelular de Ca^{2+} en condiciones de inhibición glucolítica

- Conocer si hay producción de EROS en condiciones de inhibición glucolítica y su papel en la muerte neuronal

- Estudiar el efecto del yodoacetato sobre la actividad de algunas enzimas antioxidantes en neuronas cultivadas de hipocampo

- Estudiar el efecto de los cuerpos cetónicos sobre la muerte neuronal y la producción de EROS

X. METODOLOGÍA

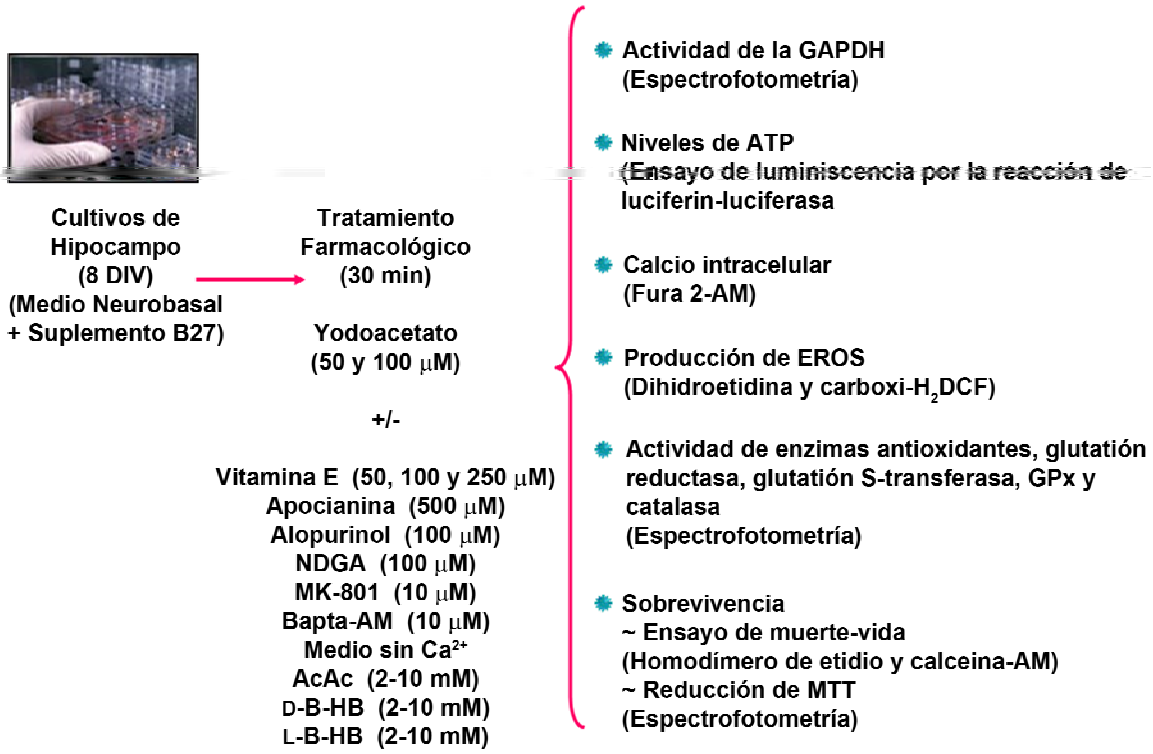


Figura 9. Diagrama de flujo de la metodología empleada (descrita con detalle en los artículos correspondientes). Los cultivos de hipocampo de rata fueron obtenidos a partir de embriones de rata de 17 días de gestación, cultivados en medio Neurobasal suplementado con B27. Después de 8 días in vitro (DIV) los cultivos fueron tratados durante 30 min con las diferentes concentraciones de yodoacetato en presencia o ausencia de diferentes fármacos. La actividad enzimática de la GAPDH y las enzimas antioxidantes fue determinada una hora después del tratamiento. Los niveles de ATP y la producción de EROS se determinaron a diferentes tiempos después del tratamiento. La concentración de Ca^{2+} intracelular fue monitoreada cada minuto durante 3 horas. La sobrevivencia fue determinada 4 y 24 horas después del tratamiento por medio de los ensayos de muerte vida y de reducción de MTT, respectivamente. Cabe aclarar, que no todos los fármacos fueron estudiados con todos los ensayos experimentales.

XI. RESULTADOS

Artículo 2

"Calcium-dependent production of reactive oxygen species is involved in neuronal damage induced during glycolysis inhibition in cultured hippocampal neurons"

(Journal of Neuroscience Research)

Journal of Neuroscience Research 00:000–000 (2008)

Calcium-Dependent Production of Reactive Oxygen Species Is Involved in Neuronal Damage Induced During Glycolysis Inhibition in Cultured Hippocampal Neurons

Karla Hernández-Fonseca,[1] Noemí Cárdenas-Rodríguez,[2] Jose Pedraza-Chaverri,[2] and Lourdes Massieu[1]*

[1]Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México (UNAM), México D.F., México
[2]Facultad de Química, Departamento de Biología, Universidad Nacional Autónoma de México, México D.F., México

Neuronal damage associated with in vivo hypoglycemia has been suggested to be excitotoxic due to the release of excitatory amino acids and the protective effect of glutamate receptor antagonists. The production of reactive oxygen species (ROS) has been also implicated in hypoglycemic damage. Excitotoxicity involves oxidative stress, insofar as the influx of calcium through N-methyl-D-aspartate (NMDA) receptors stimulates ROS production. We have studied the participation of NMDA receptors and intracellular calcium in ROS production and cell death triggered during moderate and severe glycolysis inhibition in cultured hippocampal neurons. Iodoacetate (IOA), an inhibitor of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), dose dependently reduces ATP levels and cell survival and increases the intracellular concentration of calcium. During mild glycolysis inhibition, the increases in intracellular calcium, ROS production, and cell death are dependent on NMDA receptor activation. In contrast, during severe glycolysis, these processes are not inhibited by NMDA receptor blockade. BAPTA-AM and vitamin E efficiently reduce ROS generation and cell death under both conditions. Results suggest that calcium influx through NMDA receptors is involved in ROS production and neuronal damage resulting from moderate energy depletion, whereas intracellular calcium increase and ROS generation during severe glycolysis inhibition are more related to energy depletion.

Key words: calcium; reactive oxygen species; neuronal damage; glycolysis inhibition; hippocampal neurons

The brain is particularly vulnerable to oxidative damage induced by reactive oxygen species (ROS), because it has an extremely high rate of oxygen consumption, a high content of oxidizable polyunsaturated fatty acids, and a weak antioxidant defense (Braughler and Hall, 1989). Oxidative stress plays an important role in different pathological conditions and has been considered as an important contributor to cell death associated with ischemia (Tominaga et al., 1993; Bolaños and Almeida, 1999; Jacobson and Duchen, 2002; Abramov et al., 2007) and several neurodegenerative diseases (Beal and Matthews, 1997; Butterfield et al., 1999, 2007).

Glutamate-mediated excitotoxic damage is thought to contribute to neuronal death in a variety of pathological conditions, including ischemia and hypoglycemia, because of the release of excitatory amino acids and subsequent activation of the N-methyl-D-aspartate (NMDA) receptors, which triggers the influx of calcium (Wieloch, 1985; Sandberg et al., 1986; Gill et al., 1988; Hillered et al., 1989). The increase in ROS production during excitotoxic death is well documented. The influx of calcium through NMDA receptors as well as mitochondrial dysfunction associated with glutamate receptor activation stimulates ROS production. The formation of superoxide anion ($O_2^{-\bullet}$), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^-$) induced by glutamate has been demonstrated in several in vitro studies (Dawson et al.,

Contract grant sponsor: CONACYT; Contract grant number: 48645-Q (to L.M.); Contract grant number: 48812-M (to J.P.-C.); Contract grant number: 167146 (to K.H.-F.); Contract grant sponsor: DGEP (to K.H.-F.).

*Correspondence to: Lourdes Massieu, Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México, D.F. 04510, AP 70-253 México.
E-mail: lmassieu@ifc.unam.mx

Received 8 August 2007; Revised 23 November 2007; Accepted 24 November 2007

Published online 00 Month 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21634

1991; Lafon-Cazal et al., 1993; Bondy and Lee, 1993; Vesce et al., 2004; Kahlert et al., 2005).

Studies show that energy failure induced by chemical ischemia in the retina stimulates ROS production and that neuronal injury is prevented by antioxidants (Rego et al., 1999). Similarly, during glucose and oxygen deprivation in cultured neurons, mitochondrial dysfunction, ROS production, and increased oxidized glutathione have been observed (Almeida et al., 2002; Abramov et al., 2007). The mechanisms leading to neuronal death induced by in vitro glucose deprivation have received less attention, but it has been suggested that ROS generation plays a role (Liu et al., 2003; Suh et al., 2007). During glycolysis inhibition induced by the exposure to iodoacetate (IOA), an inhibitor of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ROS are produced and neuronal damage is prevented by antioxidants (Uto et al., 1995; Rego et al., 1999; Malcolm et al., 2000). In vitro studies have shown that IOA-induced neuronal damage is of an excitotoxic nature and thus is prevented by NMDA receptor antagonists (Zeevalk and Nicklas, 1990; Massieu et al., 2003a). However, other studies suggest that the activation of NMDA receptors is not involved, insofar as antagonists of this receptor do not prevent neuronal death, whereas antioxidants are very efficient (Uto et al., 1995; Rego et al., 1999; Malcolm et al., 2000). These studies suggest increased ROS production after IOA exposure; however, the role of intracellular calcium and NMDA receptor activation in this process has not been elucidated.

Therefore, we investigated whether ROS production and cell death induced by IOA are related to NMDA receptor activation and increased intracellular calcium, in cultured hippocampal neurons. We also studied whether the contribution of an excitotoxic mechanism to neuronal death is related to the severity of the energy failure. Results suggest that increased intracellular $[Ca^{2+}]_i$, ROS production, and neuronal damage induced by moderate glycolysis inhibition involve the influx of extracellular calcium and NMDA receptor activation, whereas, during severe glycolysis inhibition, the increase in the intracellular Ca^{2+} concentration and ROS production are related more to energy failure.

MATERIALS AND METHODS

Trypsin, DNAase, gentamicin, L-glutamine, glutamate, poly-L-lysine, cytosine arabinoside, iodoacetate, vitamin E [(+)-α-tocopherol], 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nordihydroguaiaretic acid (NDGA), allopurinol, 4′-hydroxy-3′-methoxyacetophenone (apocynin), and acetoacetate were purchased from Sigma-RBI (St. Louis, MO). Soybean trypsin inhibitor, Neurobasal media, and B27 supplement (Minus AO) were from Gibco/Life Technologies (Rockville, MD), and BAPTA-AM was obtained from Alomone Labs (Jerusalem, Israel). Dizolcipine(+)-5-methyl-10,11-dihydroxy-5H-dibenzo(a,d)-cyclohepten-5,10-imine maleate (MK-801) was purchased from Tocris (Ellisville, MO). Dihydroethidium, 5-(and 6-)carboxy-2′,7′-dichlorodihydrofluorescein diacetate, luciferin-luciferase assay kit, calcein-AM, and ethidium homodimer (live/dead kit) were purchased from Molecular Probes (Eugene, OR). Fura2-AM was from TEF Labs (Austin, TX).

Cell Cultures

Primary cultures of hippocampal neurons were prepared from Wistar rat embryos 17–18 days of gestation as previously described (Hernández-Fonseca and Massieu, 2005). Cells were suspended in Neurobasal culture medium (Brewer et al., 1993) supplemented with B27, 0.5 mM L-glutamine, and 20 μg/ml gentamicin and were plated at a density of 260–290 $\times 10^3/cm^2$ (1.5×10^6 cells/ml/well) in Costar 24-well plates (Cambridge, MA) precoated with poly-L-lysine (5 μg/ml). Cells were cultured for 8–9 days in vitro at 37°C in a humidified 5% CO_2/95% air atmosphere. Four days after plating, glucose (5 mM) and cytosine arabinoside (10 μM) were added. Animals were handled and cared for according to the NIH *Guide for care and use of laboratory animals*, with approval of the local animal care committee. All efforts were made to minimize animal suffering.

Drug Exposure

Culture medium was withdrawn, and neuronal cultures were exposed for 0.5 hr to 50 or 100 μM IOA in Ringer Krebs medium containing (in mM): 154 NaCl, 5.6 KCl, 2.3 $CaCl_2$, 1.2 KH_2PO_4, 5.0 HEPES, 3.6 $NaHCO_3$, and 5.6 glucose. Ringer Krebs medium was changed by the culture medium previously withdrawn, and neuronal viability was monitored 1, 4, 6, 8, 12, and 24 hr later by the MTT reduction assay (Berridge and Tan, 1993), as previously described (Massieu et al., 2003b). Data are expressed as percentage of control. Neuronal viability after the different treatments was corroborated by the fluorescent markers calcein-AM (green) and ethidium homodimer (red) for live and dead neurons, respectively, 5.5 hr after IOA exposure, as previously described (García and Massieu, 2001). Cells were grown on coverslips and the numbers of dead neurons expressed as the percentage of red-fluorescent cells (positive to ethidium) relative to the total (red- and green-fluorescent). About 200 cells were counted per coverslip in five different fields randomly selected.

The protective effect of vitamin E (50, 100, and 250 μM), apocynin (500 μM), allopurinol (100 μM), NDGA (100 μM), calcium-free medium, the calcium chelator BAPTA-AM (10 μM), the NMDA receptor antagonist MK-801 (10 μM), and the ketone body acetoacetate (10 mM) was tested. BAPTA-AM was preincubated for 15 min before IOA exposure. Cells were exposed to IOA during 30 min in Ringer Krebs medium in the presence or absence of the different compounds. Calcium-free medium and BAPTA-AM were present only during IOA exposure, whereas the rest of the drugs were added again to culture wells after IOA removal for the following 24 hr.

Determination of GAPDH Activity

GAPDH activity was determined 1 hr after the onset of IOA (50 or 100 μM) exposure as previously reported, with some modifications (Ikemoto et al., 2003). Cells were washed twice with prewarmed Locke's solution, and extracted in 100 μl Tris-HCl, pH 8.5. They were homogenized, and GAPDH activity was monitored in a reaction mixture (1 ml total volume) containing (in mM): 1.7 arsenic acid, 20 sodium fluoride, 1 NAD^+, and 5 KH_2PO_4. The reaction was initiated by the addition of glyceraldehyde-3-phosphate (final concentration 1 mM). Activity was determined by the production of NADH at 340 nm during the first 60 sec, when the reaction is linear. A molar extinction coefficient of 2.07 was used to calculate the amount NADH formed in micromoles. Data are expressed as μmol NADH/min/mg protein.

Determination of Antioxidant Enzymes Activity

Cells were extracted and homogenized as described above, and enzyme activities were measured after incubation of cell homogenates with IOA (50 or 100 μM or 1 mM) for 5 min at room temperature. In the case of glutathione peroxidase (GPx), enzyme activity was also determined in the presence of vitamin E (50, 100, or 250 μM). Catalase activity was assayed at 240 nm by a method based on the disappearance of hydrogen peroxide (Pedraza-Chaverri et al., 2001). Results are expressed in k/mg protein where k is the first-order reaction rate constant. Glutathione peroxidase activity was assayed at 340 nm using H_2O_2, reduced glutathione (GSH), glutathione reductase, and NADPH in a coupled reaction as previously described (Pedraza-Chaverri et al., 2001). One unit of GPx was defined as the amount of enzyme that oxidizes 1 μmol NADPH/min. Glutathione reductase (GR) activity was assayed at 340 nm, measuring the rate of oxidation of NADPH by oxidized glutathione (GSSG), according to Carlberg and Mannervik (1975). One unit of GR was defined as the amount of enzyme that oxidizes 1 μmol NADPH/min. Glutathione-S-transferase (GST) activity was determined at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) and GSH by the method of Habig et al. (1974). One unit of GST is defined as the amount of enzyme that produces 1 nmol GSH-CDNB conjugate/min. For glutathione peroxidase, glutathione reductase, and glutathione-S-transferase, data are expressed as U/mg protein.

ATP Determination

ATP levels were determined at 0.25, 0.5, 1, 2, 4, 6, 8, and 24 hr after IOA (50 or 100 μM) exposure by means of a luminometer through the luciferin-luciferase quimioluminescent assay as previously described (Massieu et al., 2003b). Protein concentration was measured by Bradford's method, and data are expressed as pmol/μg protein. When the effect of acetoacetate (10 mM) or vitamin E (250 μM) was tested, acetoacetate was incubated during and after IOA exposure and ATP determined at 4 hr, whereas vitamin E was added during IOA exposure and ATP determined 0.5 hr after the onset the IOA incubation.

Intracellular Calcium Measurement

Intracellular calcium concentration was monitored using the fluorescent calcium indicator fura-2-AM in an emission fluorescence spectrofluorophotometer (Shimadzu RF-5301 PC, Kyoto, Japan) as previously described (Itou et al., 1998). Cells grown on coverlips were loaded with 5 μM fura-2/AM and 0.02% pluronic F-127 for 1 hr in Ringer Krebs medium and washed twice. Coverlips were placed in a cuvette and positioned inside the spectrofluorophotometer. Cells were perfused during 30 min with Ringer Krebs medium containing IOA (50 or 100 μM) in the presence or the absence of MK-801 (10 μM); after this time, medium was changed for Ringer Krebs with or without MK-801. Cells were alternatively illuminated with a Xe lamp at 340 nm and 380 nm excitation, and emission fluorescence was monitored at 510 nm. Fluorescence signals were continuously monitored during 120 min, and $[Ca^{2+}]_i$ was calculated from the ratio (R) of 340 nm to 380 nm excitation wavelength, using the following equation (Grynkiewicz et al., 1985): $[Ca^{2+}]_i = b\ Kd\ (R - R_{min})/(R_{max} - R)$, where Kd is the dissociation constant of fura-2/AM,b is the ratio of fluorescent signals at 380 nm for Ca^{2+}-free and Ca^{2+}-saturated dye, R_{min} is R in the absence of external Ca^{2+}, and R_{max} is R in saturating $[Ca^{2+}]_i$. These parameters were determined by in vitro calibration using free calcium medium plus EGTA or ionomycin.

ROS Determination

Cells were plated on coverslips, and the presence of ROS was determined by oxidation-sensitive fluorescent markers at 0.25, 0.5, 1, 4, and 6 hr after the onset of IOA (50 or 100 μM) exposure. The effect of vitamin E, MK-801, calcium-free medium, BAPTA-AM, and acetoacetate on ROS production was determined 3.5 hr after 30 min incubation with IOA. Afterward, the fluorescent markers dihydroethidium (DHE) 3.2 μM, and 5-(and 6-)carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) 5 μM were loaded in Ringer Krebs during 20 min.

DHE has been used as a marker of $O_2^{\bullet-}$ because of its relative specificity for this ROS producing the fluorescent products ethidium (Et) and 2-hydroxyethidium (OH-et; Bindokas et al., 1996; Zhao et al., 2003). However, it can also be oxidized by H_2O_2 by nonspecific peroxidases, oxidases, cytochrome c, and other reactive species, such as $ONOO^-$ and hydroxyl radical ($OH^{\bullet}$), providing an index of the production of reactive nitrogen species (RNS) and ROS (Zhao et al., 2003; Gomes et al., 2005; Robinson et al., 2006). DHE enters the cells, and, after its oxidation to Et in the cytosol, it is retained within the cell nucleus because of its interaction with DNA, staining the nucleus with bright red fluorescence (Bindokas et al., 1996). Carboxy-H_2DCFDA is deacetylated, oxidized by ROS and RNS, and converted to the fluorescent compound 5-(and 6-)carboxy-2′,7′-dichlorofluorescein (carboxy-DCF), staining the cell cytoplasm with bright green fluorescence (Hockenbery et al., 1993). After incubation, cells were washed with Ringer Krebs and the coverslips mounted in a perfusion chamber. Cells were examined under a fluorescence microscope equipped with an argon laser (488 nm excitation and 530 nm emission for carboxy-DCF, and 488 nm

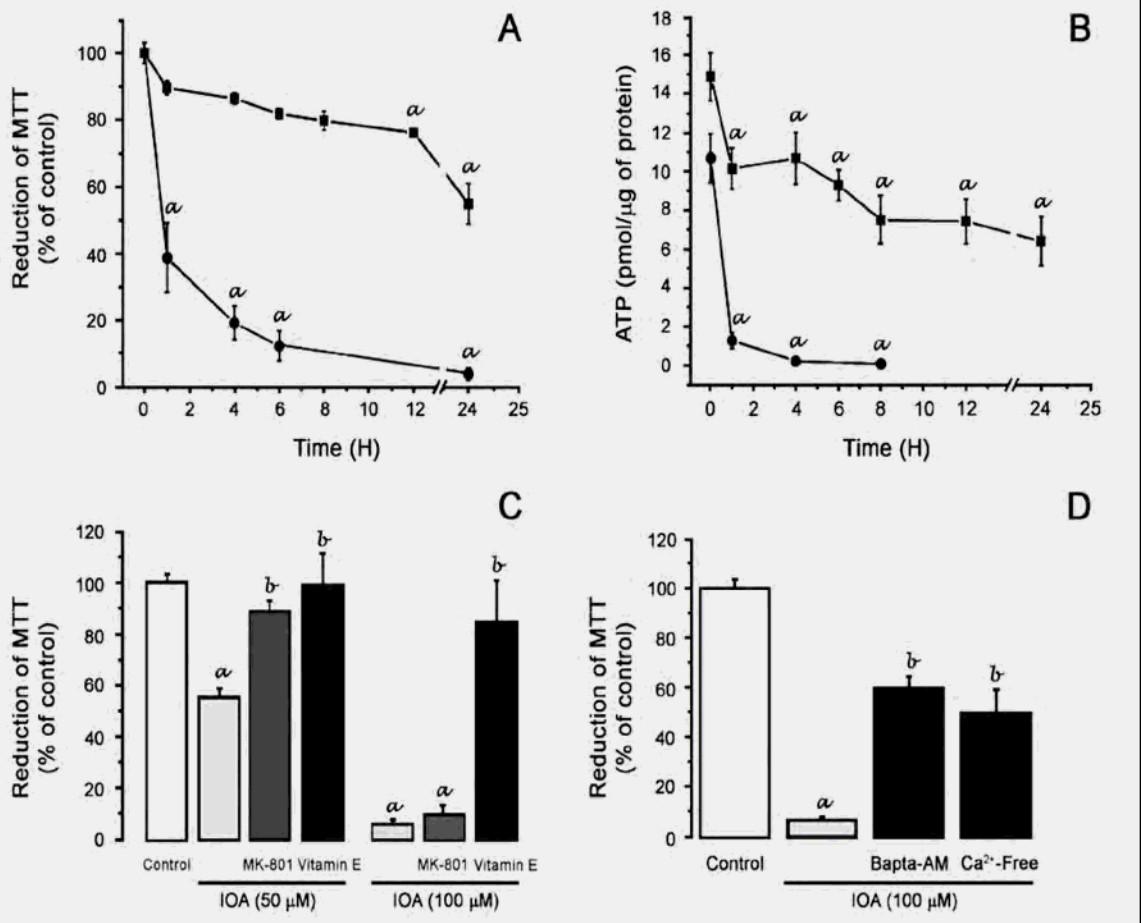


Fig. 1. Time course of changes in cell survival (A) and ATP levels (B) induced by iodoacetate (IOA) in hippocampal cultured neurons. **A,B:** Cells were exposed to IOA (50 µM, squares; 100 µM, circles) during 30 min, and MTT reduction and ATP content were determined at different times. Data are means ± SEM from four or five (A) and five to seven (B) independent experiments. $^{a}P < 0.0001$ relative to control (time 0). **C,D:** Protective effect of MK-801 (10 µM), vitamin E (250 µM), Ca^{2+}-free medium, and BAPTA-AM (10 µM) against neuronal damage induced by IOA, as evaluated by MTT reduction 24 hr later. Data are means ± SEM from five or six independent experiments. $^{a}P < 0.0001$ relative to control, $^{b}P < 0.001$ relative to IOA-treated cultures.

excitation and 535 nm emission for Et). The intensity of Et or carboxy-DCF fluorescence was measured in 10 different fields per coverslip per condition in at least four independent experiments, by means of the AxioVision AC 4.4 image analyzer (Carl Zeiss Imaging Systems). Fluorescence in the control condition was normalized to 1 unit, and the changes in fluorescence expressed were as -fold increase relative to control. In a different series of experiments, the increase in Et and carboxy-DCF fluorescence was monitored spectrofluorometrically. Cells grown on coverslips were loaded with both fluorescent markers in Ringer Krebs medium during 20 min. They were washed and coverslippped, placed in a cuvette, and positioned inside the spectrofluorophotometer. Cells were perfused during 30 min with Ringer Krebs medium containing IOA (100 µM). After this period, medium was changed for Ringer Krebs, and the fluorescence signal was followed during 150 min. Cells were alternatively illuminated with a Xe lamp at 470 and 480 nm of excitation and 590 and 530 nm of emission for Et and carboxy-DCF, respectively.

Statistical Analysis

In all cases, data were analyzed by one-way ANOVA, followed by a Fisher's multiple-comparisons test. $P < 0.05$ was considered statistically significant.

RESULTS

In previous studies, we have shown that 0.5 hr exposure of cultured hippocampal neurons to 50 and 100 µM IOA induces a 50% and 80% reduction in cell survival, respectively, as assessed by the MTT method 24 hr after the treatment (Massieu et al., 2003b). We have now studied the relationship between the decline

TABLE I. GADPH Activity, MTT Reduction, ATP Levels, and ROS Production in Cultured Hippocampal Cells Exposed to IOA in the Presence or Absence of Acetoacetate*

				ROS production	
	GADPH activity	MTT reduction	ATP levels	Et fluorescence	Carboxy-DCF fluorescence
Control	0.582 ± 0.02	100 ± 6.48	10.95 ± 0.93	1 ± 0.08	1 ± 0.04
IOA 50 μM	0.420 ± 0.06[a]	33.19 ± 9.60	5.83 ± 0.85	5.46 ± 0.20[g]	9.39 ± 1.57[f]
IOA 100 μM	0.184 ± 0.02[b]	10.29 ± 2.40	3.84 ± 1.16	13.15 ± 2.20[g]	20.10 ± 1.8[g]
IOA 50 μM + AcAc	ND	62.06 ± 8.59[c]	12.11 ± 1.39[e]	1.26 ± 0.11[i]	2.43 ± 0.84[h]
IOA 100 μM + AcAc	ND	58.66 ± 11.83[d]	12.59 ± 1.95[e]	4.29 ± 0.56[h]	7.69 ± 1.11[f,i]

*Hippocampal cultured cells were exposed to IOA 50 μM or 100 μM in the presence or the absence of acetoacetate (AcAc) during 0.5 hr. GADPH activity, ATP content, ROS production, and MTT reduction were determined at 1 hr (GADPH activity), 4 hr (ATP content and ROS production), and 24 hr (MTT reduction). Data are expressed as nmol NADH/min/mg protein, % of control, pmol/μg protein, and fluorescence intensity, for GADPH activity, MTT reduction, ATP levels, and ROS production, respectively. Data are means ± SEM.
GADPH activity ($^aP < 0.005$ and $^bP < 0.001$ relative to control, n = 5–8), MTT reduction ($^cP < 0.01$ and $^dP < 0.005$ relative to IOA alone, n = 4–6), ATP levels ($^eP < 0.005$ relative to IOA alone, n = 3–5), ROS production ($^fP < 0.005$ and $^gP < 0.0005$ relative to control, and $^hP < 0.005$ and $^iP < 0.0001$ relative to IOA alone, n = 3). ND, not determined.

TABLE II. Effect of IOA Exposure on the Activity of Antioxidant Enzymes in Hippocampal Cell Homogenates*

	Glutathione peroxidase	Glutathione reductase	Glutathione S-transferase	Catalase
Control	0.110 ± 0.014	0.078 ± 0.005	0.053 ± 0.006	0.020 ± 0.002
IOA 50 μM	0.078 ± 0.009[a]	0.081 ± 0.009	0.046 ± 0.007	0.020 ± 0.003
IOA 100 μM	0.072 ± 0.006[b]	0.088 ± 0.010	0.043 ± 0.006	0.015 ± 0.002
IOA 1 mM	0.016 ± 0.007[c]	0.043 ± 0.002[c]	0.025 ± 0.002[c]	0.006 ± 0.003[c]

*Enzyme activity was measured after incubation of cell homogenate with iodoacetate (50 or 100 μM or 1 mM) for 5 min at room temperature. Enzyme activity was measured as described in Materials and Methods. Data are expressed as k/mg protein for catalasa and as U/mg protein for glutathione peroxidase, glutathione reductase, and glutathione-S-transferase. Data are means ± SEM. From four or five independent experiments.
$^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ relative to control.

in MTT reduction and ATP depletion induced by the two concentrations of IOA.

Thirty minutes of exposure to 50 μM IOA induced a slow decline in MTT reduction, accompanied by a progressive decrease in ATP levels, as previously reported (Massieu et al., 2003b; Hernández-Fonseca and Massieu, 2005). In contrast, 30 min of exposure to 100 μM IOA induced a rapid decline in MTT reduction to 38.7% of control values at 1 hr and a further decrease to 3.7% of control at 24 hr. In this condition, ATP rapidly declined to 11.8% of control values 1 hr after the onset of the treatment and was completely depleted at 4 hr (Fig. 1A,B). These observations correlated with the degree of inhibition of GAPDH activity, which was 27.8% and 68.4% inhibited after a 30 min exposure to 50 and 100 μM IOA, respectively (Table I). These observations suggest that 100 μM IOA severely inhibits the glycolytic pathway, causing a rapid energy deficit and cell death, whereas 50 μM IOA induces a partial energy deficit and a slow and progressive neuronal death. Incubation with the energy substrate acetoacetate completely prevented the decline in ATP levels induced by both IOA concentrations and significantly reduced the decrease in MTT reduction (Table I), as previously reported (Massieu et al., 2003b).

To test the specificity of IOA inhibition for GADPH activity at the concentrations tested, we studied the effect of this inhibitor on the activity of some other enzymes, including glutathione peroxidase, glutathione reductase, glutathione S-transferase, and catalase. Enzymatic activity was monitored in cell homogenates after a 5-min incubation with IOA. We observed that at 50 and 100 μM IOA had no effect on the activity of glutathione reductase, glutathione S-transferase, and catalase. Only at a 1 mM concentration was the activity of these enzymes significantly inhibited in 44.8%, 52.8%, and 70%, respectively (Table II). Glutathione peroxidase activity was significantly inhibited in 29.0%, 34.5%, and 85.4% by 50, 100, and 1,000 μM IOA, respectively (Table II).

To study the participation of an excitotoxic mechanism in neuronal damage, cells were incubated in the presence of MK-801 (10 μM), an antagonist of NMDA glutamate receptors. The reduction in cell survival induced by IOA 50 μM was completely prevented by MK-801, whereas that induced by IOA 100 μM was not (Fig. 1C). In contrast to MK-801, vitamin E effectively prevented the decline in MTT reduction induced by 50 μM and 100 μM IOA, restoring MTT reduction capacity to 89.5% of control values (Fig. 1C). The effect

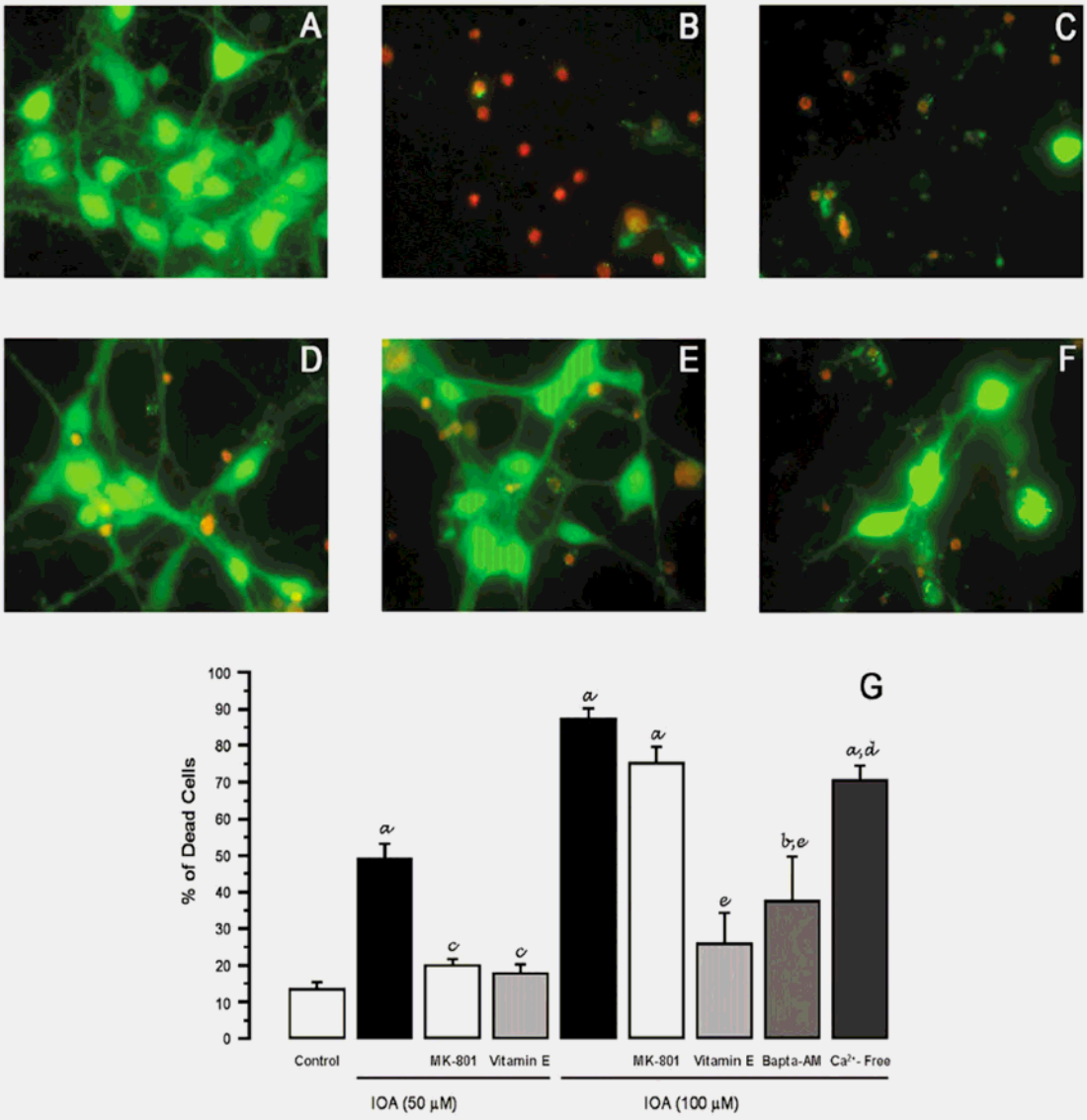


Fig. 2. A–F: Representative photographs showing the effect of MK-801 (**C**), vitamin E (**D**), BAPTA-AM (**E**), and Ca-free medium (**F**) against IOA-induced (100 µM) neuronal death (**B**) monitored by the fluorescent markers calcein-AM (green) and ethidium homodimer (red). Control is shown in **A**. **G:** Graph shows the percentage of dead cells (red) from the total of cells (red and green) counted in coverslips from five different fields randomly selected, 5.5 hr after IOA exposure (50 and 100 µM during 30 min) in the presence or absence of the different treatments. Data are means ± SEM from five independent experiments. $^{a}P < 0.0001$ and $^{b}P < 0.001$ relative to control, $^{c}P < 0.001$ relative to IOA 50 µM, $^{d}P < 0.01$, and $^{e}P < 0.0001$ relative to IOA 100 µM.

of MK-801 and vitamin E on neuronal death was verified by the calcein-AM/ethidum homodimer fluorescent method. As can be observed in Figure 2, MK-801 effectively reduced the number of dead cells only in the 50 µM condition, whereas vitamin E prevented cell damage induced by both IOA concentrations.

To study whether the notable effect of vitamin E was mediated by its antioxidant activity and not by interfering with the inhibitory effect of IOA on GADPH activity, we monitored enzyme activity in cell homogenates after a 5-min incubation with IOA in the presence or absence of vitamin E (250 µM). Under these conditions, GADPH inhibition by IOA was not affected by the presence of vitamin E (not shown), nor was the inhibition of GPx activity by IOA on (not shown). The effect of vitamin E on IOA-induced ATP depletion was also studied. For this purpose, cells were exposed for 30 min to 100 µM IOA with or without vitamin E

TABLE III. Protective Effect of Different Antioxidant Compounds on Neuronal Death Induced by Iodoacetate*

	MTT reduction (% of survival relative to control)
IOA (100 μM)	1.494 ± 0.976
+ Vitamin E (50 μM)	22.37 ± 7.41[a]
+ Vitamin E (100 μM)	25.14 ± 5.50[b]
+ Vitamin E (250 μM)	35.13 ± 10.94[c]
+ Apocynin (1 mM)	13.91 ± 5.0
+ Allopurinol (100 μM)	9.15 ± 1.71
+ NDGA (100 μM)	33.04 ± 9.48[b]

*Hippocampal cultured cells were exposed to 100 μM during 0.5 hr in the presence or the absence of vitamin E (50, 100, and 250 μM), apocynin, allopurinol, and NDGA. Cell survival was measured as MTT reduction 24 hr after the exposure. Data are expressed as percentage survival relative to control from four independent experiments.
[a]$P < 0.01$, [b]$P < 0.005$, [c]$P < 0.0005$ relative to IOA alone.

(250 μM) and ATP levels monitored immediately after. Results show that vitamin E does not affect the reduction in ATP levels (in pmol/μg protein, control = 47.52 ± 3.41, IOA = 17.38 ± 3.19, $P < 0.005$, IOA + vitamin E = 18.15 ± 7.06, $P < 0.005$, n = 3). The effect of lower concentrations of vitamin E on cell survival (as determined by the MTT reduction assay) was also assessed. Results show that vitamin E at 50, 100, and 250 μM elicited very similar protection (Table III). We also tested the effect of the antioxidants apocynin (NADPH oxidase inhibitor), allopurinol (xanthine/xanthine oxidase inhibitor), and NDGA (scanvenger of several reactive oxygen and nitrogen species; Papadopoulus et al., 1997; Floriano-Sanchez et al., 2006; Suh et al., 2007). Coincubation of vitamin E and NDGA effectively prevented neuronal death, whereas apocynin and allopurinol showed no significant protection (Table III).

We next sought to know whether oxidative stress is related to excitotoxicity and to increased intracellular calcium. We first examined the calcium dependence of neuronal death and the effect of IOA on intracellular calcium levels. In a previous study, we showed that IOA (50 μM)-induced cell death in hippocampal cultured neurons is calcium dependent, in that it is prevented in calcium-free media or in the presence of the calcium chelator BAPTA-AM (Hernández-Fonseca and Massieu, 2005). Similarly, neuronal death induced by severe glycolytic inhibition (100 μM IOA) was significantly reduced in calcium-free media or in the presence of BAPTA-AM (Fig. 1D). The effect of the calcium-free and the BAPTA-AM conditions was corroborated by using the calcein-AM/ethidium homodimer method to evaluate cell viability (Fig. 2).

Changes in the $[Ca^{2+}]_i$ during and after IOA exposure were then studied. As can be observed in Figure 3A, $[Ca^{2+}]_i$ was not changed during the 30-min exposure to 50 μM IOA but slowly increased thereafter. Changes in $[Ca^{2+}]_i$ were statistically different with

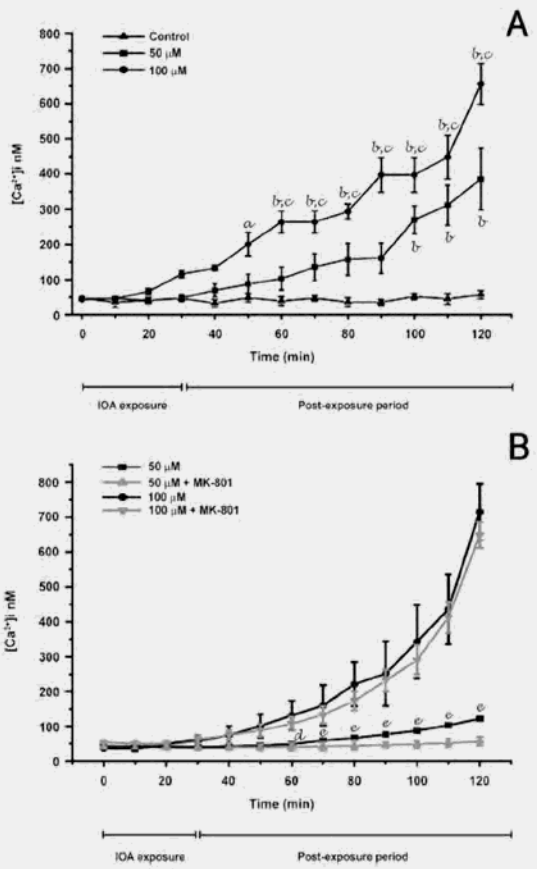


Fig. 3. **A:** Time course of changes in the intracellular Ca^{2+} concentration in cells exposed to IOA (50 μM; squares), IOA (100 μM; circles), or Ringer Krebs (triangles). **B:** Effect of MK-801 (10 μM) on the changes in the intracellular Ca^{2+} concentration induced by IOA. IOA (50 μM; squares), IOA (50 μM) + MK-801 (upward triangles), IOA (100 μM; circles), and IOA (100 μM) + MK-801 (downward triangles). Cells were preincubated with fura-2/AM (5 μM) and perfused with IOA (50 or 100 μM) in the presence or the absence of MK-801 for 30 min (IOA exposure). After this time, cells were continuously perfused with Ringer Krebs containing or not MK-801 during the following 90 min (postexposure period). In the control condition, cells were continuously exposed to Ringer medium. Data are means ± SEM from four independent experiments. [a]$P < 0.05$, [b]$P < 0.0001$ relative to the control condition and [c]$P < 0.005$, [d]$P < 0.05$, and [e]$P < 0.0001$ relative to IOA 50 μM alone.

respect to control values from 50–90 min after IOA incubation, reaching concentrations of 385 nM 90 min after IOA removal (Fig. 3A). In the 100 μM condition, the increase in $[Ca^{2+}]_i$ was faster, and immediately after the exposure intracellular calcium levels increased 2.6-fold relative to basal values (from 46.3 nM to 115.8 nM). Intracellular calcium levels were significantly elevated 20 min after IOA removal, reaching concentrations up to 200 nM and increasing further to 667 nM after 90 min (Fig. 3A).

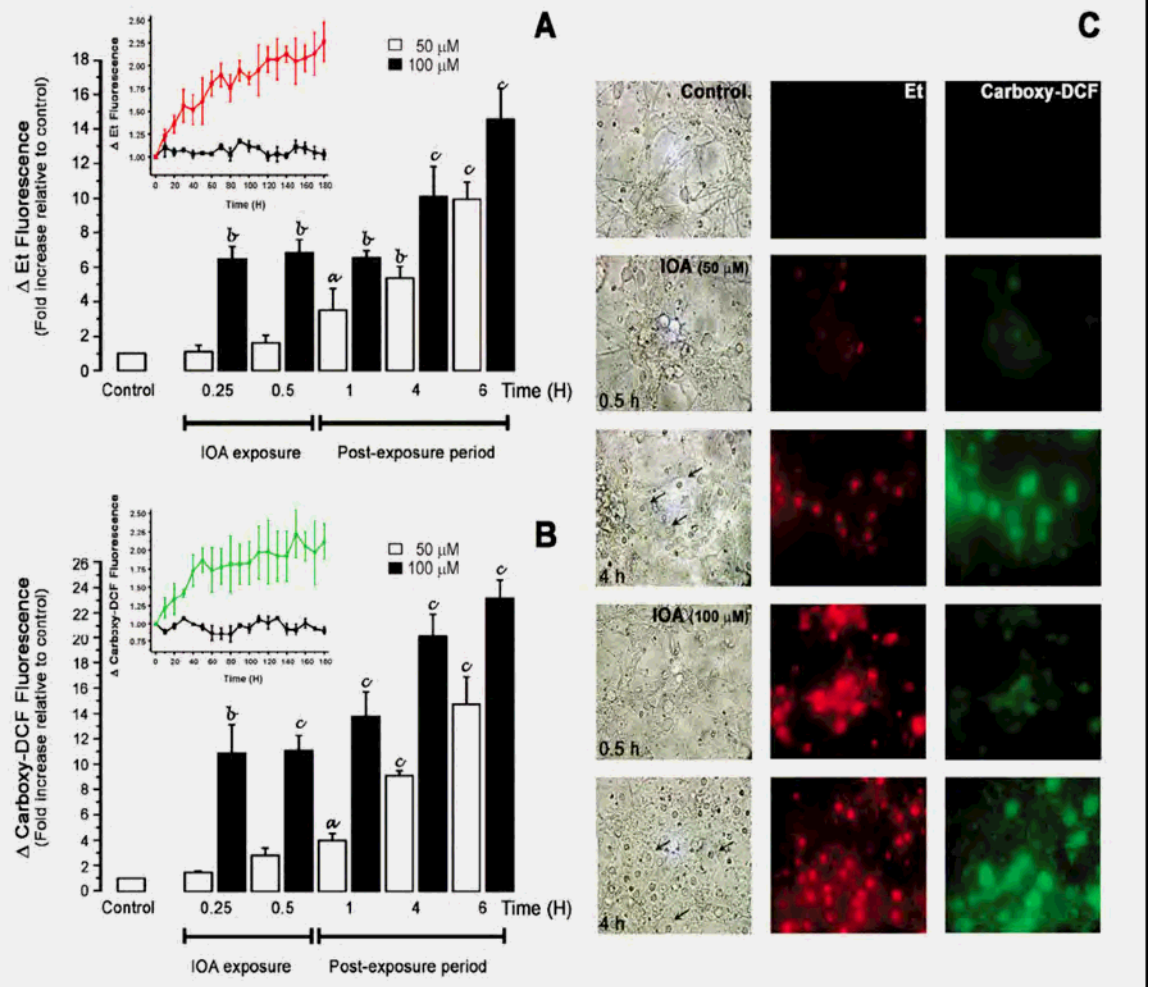


Fig. 4. Time course of ROS production induced by the exposure to IOA (50 or 100 μM). ROS formation was measured as the change in Et (**A**) and carboxy-DCF (**B**) fluorescence (-fold increase) relative to control. Culture medium was withdrawn and cells were exposed to IOA in Ringer Krebs medium during 30 min (IOA exposure). Medium was replaced by the culture medium previously withdrawn (postexposure period). Cells were incubated with fluorescent probes, and fluorescence intensity was measured. Data are means ± SEM from four independent experiments. $^{a}P < 0.05$, $^{b}P < 0.001$, and $^{c}P < 0.0001$ relative to control. **Inset** in A,B shows the change in Et (in red) or carboxy-DCF (in green) fluorescence relative to control (in black) as measured spectrofluorometrically, as described in Materials and Methods. Data are means ± SEM from four independent experiments. **C:** Micrographs of representative experiments showing ROS production induced by IOA (50, 100 μM) at 0.5 and 4 hr. Immediately after or 3.5 hr after IOA removal, cells were incubated with DHE and carboxy-H_2DCFDA and photographs were taken. Arrows show the presence of swollen cells with condensed nuclei.

To study the role of NMDA receptors on the rise of intracellular calcium levels, we tested the effect of MK-801. Results show that the $[Ca^{2+}]_i$ increase induced by 50 μM IOA was efficiently prevented by MK-801, although that induced by 100 μM IOA was not (Fig. 3B). These results agree with those of cell survival, showing protection by MK-801 only under the IOA 50 μM condition.

Two oxidation-sensitive fluorescent markers were used to study ROS production during glycolytic inhibition. A significant increase in the fluorescence of both markers was observed 1 hr after the onset of the incubation with IOA 50 μM (3.5-fold for Et and 3.9-fold for carboxy-DCF), which increased further during the following 5 hr (Fig. 4A,B). Severe glycolysis inhibition induced by 100 μM IOA promoted a rapid increase in Et and carboxy-DCF fluorescence as soon as 15 min after the onset of IOA exposure, which progressively increased up to 14.5-fold for Et and 23.2 for carboxy-DCF at 6 hr (Fig. 4A,B). To corroborate these results, we followed the changes in the fluorescent signal produced by the oxidation of DHE and carboxy-H_2DCFDA by means of a spectrofluorometer during 180 min. With this method, we observed that Et fluorescence increased during IOA (100 μM) exposure (first 30 min in graph) and continued to increase up to 2.2-fold

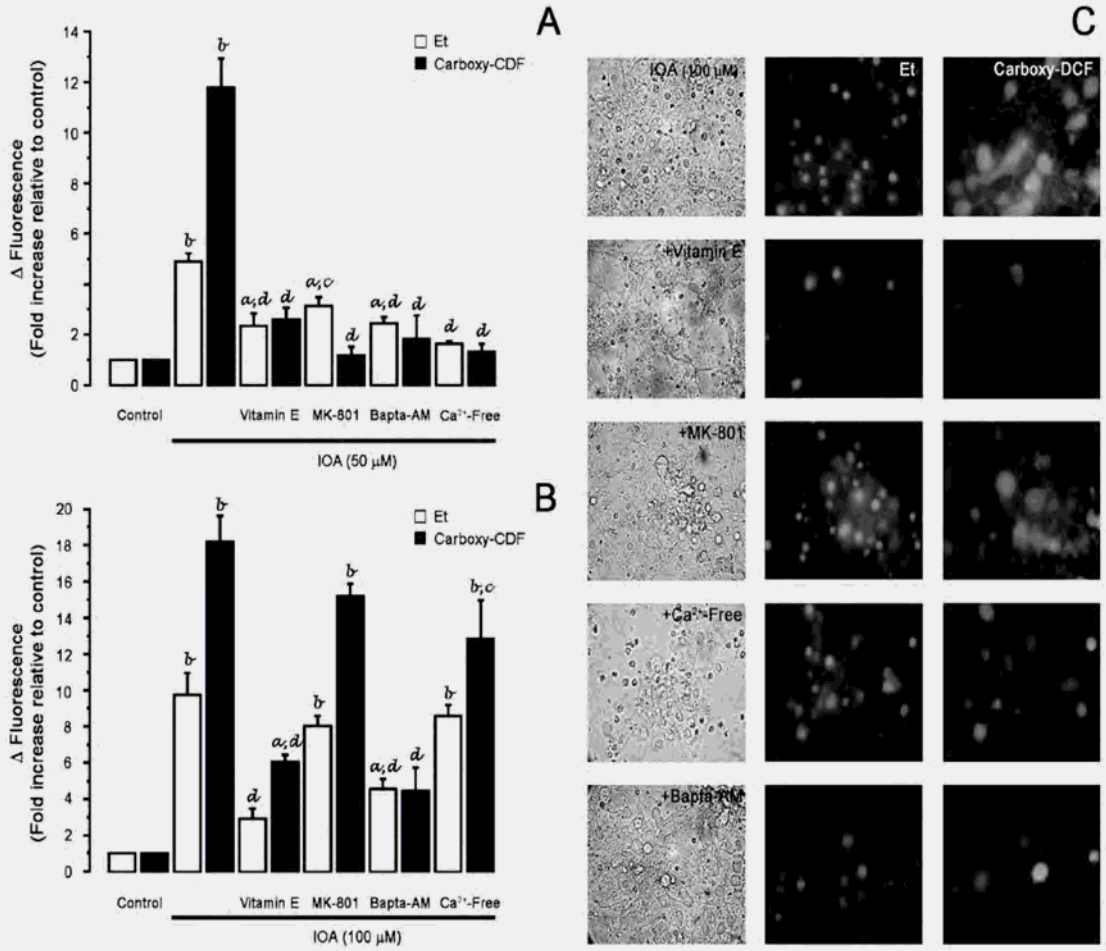


Fig. 5. Protective effect of MK-801 (10 μM), vitamin E (250 μM), BAPTA-AM (10 μM), and Ca^{2+}-free medium against ROS production induced by IOA 50 μM (**A**) or 100 μM (**B**). Cells were treated as described for Figure 2. They were incubated with the fluorescent probes 3.5 hr after IOA removal, and Et or carboxy-DCF fluorescence intensity was measured. Fluorescence was measured in 10 different fields per coverslip for each experimental condition. Data are means ± SEM from five independent experiments. $^{a}P < 0.005$ and $^{b}P < 0.0001$ relative to control cultures, $^{c}P < 0.001$ and $^{d}P < 0.0001$ relative to IOA-treated cultures. **C:** Micrographs of representative experiments showing the effect of vitamin E, MK-801, Ca^{2+}-free medium, and BAPTA-AM on ROS production induced by IOA 100 μM.

at 3 hr. In the case of carboxy-DFC, the fluorescent signal increased, though not significantly, during IOA exposure and continued to be augmented during the postexposure period (inset in Fig. 4A,B).

Figure 4C depicts micrographs of representative experiments showing that, immediately after a 30-min exposure to 50 μM IOA, only a few red-fluorescent nuclei positive for Et are detected; meanwhile, at this time, numerous red-fluorescent nuclei can be observed in the case of 100 μM IOA. At 4 hr, numerous positive cells are present under both conditions, although more red cells are observed under the 100 μM condition. Similar results are observed when ROS are monitored with carboxy-DCF (Fig. 4C, right panel). Brightfield micrographs in the left panel of Figure 4C show the morphological changes induced by IOA. At 4 hr, most of the cells appear swollen with condensed nuclei and light cytoplasm under the 100 μM condition (arrows in the left bottom picture). These changes are also visible at 4 hr after 50 μM IOA incubation (arrows in left panel, third row). To study the effect of energy depletion on ROS production, acetoacetate was added during and after IOA exposure, and ROS were monitored 3.5 hr later. Results indicate that acetoacetate significantly reduced the increase in the intensity of the fluorescent signal produced by the oxidation of both markers under the 50 μM and 100 μM IOA conditions (Table I).

To study the relationship between excitotoxicity and ROS generation, the effect of MK-801 on ROS production was tested. Figure 5A shows that the increase

in Et and carboxy-DCF fluorescence significantly decreased in the presence of MK-801 as assessed at 4 hr in cultures treated with 50 μM IOA. In contrast, MK-801 did not prevent ROS production induced by severe glycolysis inhibition in the presence of 100 μM IOA. Vitamin E effectively reduced the increase in fluorescence of both markers in the 50 μM and 100 μM IOA conditions (Fig. 5A,B). Micrographs in Figure 5C show the differential effects of MK-801 and vitamin E on ROS production induced by 100 μM IOA. The brightfield micrographs in the left panel show that, in the presence of vitamin E, cells are better preserved compared with IOA 100 μM, whereas, in the presence of MK-801, cells appear swollen and nuclei condensed.

To study the calcium dependence of ROS generation, cultures were exposed to IOA in the absence of extracellular Ca^{2+} or in the presence of BAPTA-AM. Results show that ROS production induced by moderate glycolysis inhibition is dependent on the presence of extracellular calcium, in that fluorescence of Et and carboxy-DCF was significantly reduced in calcium-free medium (Fig. 5A,B). In contrast, in the 100 μM condition, only carboxy-DCF fluorescence was reduced in the calcium-free condition (Fig. 5A,B). BAPTA-AM efficiently prevented ROS production induced by moderate and severe glycolysis inhibition as monitored by both fluorescent markers (Fig. 5A,B). Micrographs in Figure 5C (last two rows) show the effects of calcium-free medium and BAPTA-AM on ROS production induced under the 100 μM condition. Brightfield micrographs in the left panel show that cells in cultures treated with BAPTA-AM are well preserved, whereas those in cultures treated with Ca-free medium are not. These observations are in accordance with the micrographs shown in Figure 2.

DISCUSSION

Several studies have implicated oxidative stress in neuronal damage induced by in vivo ischemia or by glucose and oxygen deprivation in cultured neurons (Oliver et al., 1990; Li et al., 1999; Candelario-Jalil et al., 2001; Almeida et al., 2002; Abramov et al., 2007). Previous in vitro (Uto et al., 1995; Rego et al., 1999; Malcolm et al., 2000) and in vivo (Matthews et al., 1997) experiments suggest that neuronal damage induced by IOA is associated with ROS production, but the relationship among NMDA receptor activation, the increase in intracellular calcium, and ROS generation by this compound has not been elucidated.

As previously reported (Uto et al., 1995; Rego et al., 1999; Malcolm et al., 2000), a very efficient protection of neuronal damage by vitamin E was observed under both experimental conditions, suggesting that oxidative stress is a common mechanism involved in the cell death cascade triggered by moderate and severe energy deficit. This result is supported by the observation of increased ROS production after IOA exposure, which was dependent on the severity of the energy deficit. At 100 μM, IOA-induced ROS production was significantly faster and larger compared with the 50 μM condition.

The production of ROS is highly dependent on the presence of extracellular calcium and the activation of NMDA receptors in the 50 μM condition, suggesting that calcium influx through NMDA receptors promotes the generation of ROS. The inhibitory effect of MK-801 on the intracellular calcium increase induced by 50 μM IOA favors this hypothesis. In addition, BAPTA-AM efficiently reduced the fluorescent signal of Et and carboxy-DCF, suggesting the contribution of intracellular calcium to the generation of ROS. In a previous study, we observed that exposure to 50 μM IOA enhances only slightly the extracellular concentration of glutamate or aspartate. Thus, activation of NMDA receptors in this condition might result from a secondary excitotoxic mechanism from energy depletion (Novelli et al., 1988; Zeevalk and Nicklas, 1992).

In contrast to the 50 μM IOA condition, ROS production during exposure to 100 μM IOA was not sensitive to MK-801 and was only slightly reduced in the absence of extracellular calcium. In contrast, it was effectively inhibited by vitamin E and BAPTA-AM. Accordingly, MK-801 had no effect on the increase in intracellular calcium concentration observed in these conditions. These observations suggest that, during abrupt and severe energy depletion, calcium influx through NMDA receptors is not involved in ROS generation. In addition, we observed that in this condition ROS are efficiently reduced by the ketone body acetoacetate, which also prevented the decline in ATP levels and in MTT reduction, suggesting that energy depletion promotes ROS generation. This result agrees with other studies showing that treatment with ketone bodies prevents neuronal death associated with hypoxia, ischemia (Suzuki et al., 2001,2002; Masuda et al., 2005), and excitotoxicity induced during metabolic inhibition (García and Massieu, 2001; Massieu et al., 2003) and in models of Alzheimer's and Parkinson's diseases (Kashiwaya et al., 2000; Tieu et al., 2003). The effect of acetoacetate might result from the improvement of mitochondrial metabolism through its conversion to acetyl-CoA. The ATP-dependent systems responsible for the intracellular calcium loading and calcium extrusion might be impaired during severe glycolysis inhibition and contribute to the increase in intracellular calcium ions. Undering these conditions, buffering intracellular free calcium with BAPTA-AM still exerts a protective effect against ROS production and cell death, suggesting that the systems responsible for maintaining the intracellular homeostasis of calcium are overwhelmed.

Results show a correlation among increased intracellular calcium, ATP decline, and ROS production. In the case of severe glycolysis inhibition, calcium levels start to rise and ROS production to increase during IOA incubation. Furthermore, ATP is already diminished in 92% and MTT reduction in 89% as soon as 30 min after the onset of IOA incubation. In the case of mild glycolysis, inhibition intracellular calcium slowly

increases after IOA removal, a time at which a significant change in Et and carboxy-DCF fluorescence is observed and a significant 30% reduction in ATP levels occurs. Both ATP levels and MTT reduction capacity progressively decrease, whereas intracellular calcium and ROS production continue to increase during the postincubation period. These results suggest that a partial (30–50%) but sustained decline in ATP levels is sufficient to induce the production of ROS.

The role of ROS in neuronal death is evidenced by the protective effect of vitamin E and NDGA. NDGA is a potent scavenger of $ONOO^-$, singlet oxygen ($^1O_2$), $OH\bullet$, $O_2^{-}\bullet$ and hypochlorous acid (HOCl; Floriano-Sánchez et al., 2006). Treatment with NDGA in diabetic rats significantly increases GSH levels and superoxide dismutase and catalase activities and reduces malondialdehyde production (Anjaneyulu and Chopra, 2004). Vitamin E had no effect on the inhibitory action of IOA on GADPH and GPx activities, nor on the IOA-induced decline in ATP levels. These results suggest that protection by vitamin E is due mainly to its antioxidant activity. In addition to its lipophilic radical-quenching antioxidant effect in cell membranes, tocopherol can react directly with several reactive oxidative species, including $^1O_2$, alkoxyl radical, $ONOO^-$, nitrogen dioxide, ozone and $O_2^{-}\bullet$ (Wang and Quinn, 1999). However, other actions of vitamin E cannot be discarded. Vitamin E supplementation in animal and human individuals induces an increase in lymphocyte proliferation and IL-2 expression and reduces the production of the immunosuppressive factor PGE_2 (Meydani et al., 1986, 1990). In addition, α-tocopherol modulates two major signal transduction pathways, protein kinase C and phosphatidylinositol 3-kinase, which are related to cell proliferation, platelet aggregation, and stimulation of the phosphoprotein phosphatase 2A. In addition, vitamin E inhibits NADPH-oxidase activation (Azzi et al., 2004). A recent study suggests that the cytoprotective effect of the vitamin E observed in human hepatocytes treated with D-galactosamine is related to the reduced activation of nuclear factor-κB and expression of the inducible nitric oxide synthase (Gonzalez et al., 2007).

The mechanisms leading to ROS production in the present experimental conditions require further study; mitochondrial calcium loading as well as the activation of Ca-dependent systems involved in ROS or RNS production, such as nitric oxide synthase, phospholipase A_2, and the subsequent araquidonic acid metabolism leading to $O_2^{-}\bullet$ production, might have a role. From the present results it is not possible to identify whether the Ca-dependent mechanisms leading to ROS production during the two conditions tested are different. However, we can be certain that the mechanisms leading to the increase in intracellular calcium concentration are not identical. During moderate glycolysis, ROS production and cell death are linked to calcium influx through NMDA receptors, suggesting the involvement an excitotoxic mechanism. In contrast, during severe glycolysis inhibition, neuronal death and ROS production do not involve NMDA receptors activation but are directly related to the increase in intracellular calcium. Because MK-801 and free calcium are much less effective in reducing ROS generation and cell damage induced by severe glycolysis inhibition, the release of calcium from intracellular stores might play significant role under these conditions. Additionally, energy failure might also have an important contribution to the loss of calcium homeostasis.

According to the present results, ROS production is not likely the consequence of inhibition of the antioxidant enzymes glutathione reductase, glutathione-S-transferase, and catalase. The inhibition of GPx by IOA might contribute to oxidative stress because of the accumulation of H_2O_2. Although the mechanisms of ROS production during inhibition of glycolysis by iodoacetate are still elusive, the present data shed more light on bioenergetic failure-related cell death mechanisms, such as those associated with ischemia and hypoglycemia. Results suggest that, even during moderate inhibition of glucose oxidation through the glycolytic pathway, ROS are produced and that their production predispose neurons to cell death.

ACKNOWLEDGMENTS

The authors thank Mrs. Teresa Montiel for her technical assistance.

REFERENCES

Abramov AY, Scorziello A, Duchen MR. 2007. Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. J Neurosci 27:1129–1138.

Almeida A, Delgado-Esteban M, Bolaños JP, Medina JM. 2002. Oxygen and glucose deprivation induces mitochondrial dysfunction and oxidative stress in neurons but not in astrocytes in primary culture. J Neurochem 81:207–217.

Anjaneyulu M, Chopra K. 2004. Nordihydroguaiaretic acid, a lignin, prevents oxidative stress and the development of diabetic nephropathy in rats. Pharmacology 72:42–50.

Azzi A, Gysin R, Kempna P, Munteanu A, Negis Y, Villacorta L, Visarius T, Zingg JM. 2004. Vitamin E mediates cell signaling and regulation of gene expression. Ann N Y Acad Sci 1031:86–95.

Beal MF, Matthews RT. 1997. Coenzyme Q10 in the central nervous system and its potential usefulness in the treatment of neurodegenerative diseases. Mol Aspects Med 18:S169–S179.

Berridge MV, Tan AS. 1993. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular lozalization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys 303:474–482.

Bindokas V, Jordan J, Lee C, Miller R. 1996. Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. J Neurosci 16:1324–1336.

Bolaños JP, Almeida A. 1999. Roles of nitric oxide in brain hypoxia-ischemia. Biochim Biophys Acta 1411:415–436.

Bondy SC, Lee DK. 1993. Oxidative stress induced by glutamate receptor agonists. Brain Res 610:229–233.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Braughler JM, Hall ED. 1989. Central nervous system trauma and stroke. I. Biochemical considerations for oxygen radical formation and lipid peroxidation. Free Radic Biol Med 6:289–301.

Brewer GJ, Torricelli JR, Evege EK, Price PJ. 1993. Optimized survival of hippocampal neurons in B27-suplemented neurobasal, a new serum-free medium combination. J Neurosci Res 35:567–576.

Butterfield DA, Howard B, Yatin S, Koppal T, Drake J, Hensley K, Aksenov M, Aksenova M, Subramaniam R, Varadarajan S, Harris-White ME, Pedigo NW Jr, Carney JM. 1999. Elevated oxidative stress in models of normal brain aging and Alzheimer's disease. Life Sci 65:1883–1892.

Butterfield DA, Reed TT, Perluigi M, De Marco C, Coccia R, Keller JN, Markesbery WR, Sultana R. 2007. Elevated levels of 3-nitrotyrosine in brain from subjects with amnestic mild cognitive impairment: Implications for the role of nitration in the progression of Alzheimer's disease. Brain Res 1148:243–238.

Candelario-Jalil E, Mhadu NH, Al-Dalain SM, Martínez G, León OS. 2001. Time course of oxidative damage in different brain regions following transient cerebral ischemia in gerbils. Neurosci Res 41:233–241.

Carlberg I, Mannervik B. 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J Biol Chem 250:5475–5480.

Dawson VL, Dawson TM, London ED, Bredt DS, Snyder SH. 1991. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc Natl Acad Sci U S A 88:6368–6371.

Floriano-Sanchez E, Villanueva C, Medina-Campos ON, Rocha D, Sanchez-Gonzalez DJ, Cardenas-Rodriguez N, Pedraza-Chaverri J. 2006. Nordihydroguaiaretic acid is a potent in vitro scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion and hypochlorous acid and prevents in vivo ozone-induced tyrosine nitration in lungs. Free Radic Res 40:523–533.

García O, Massieu L. 2001. Strategies for neuroprotection against L-trans-2,4-pyrrolidine dicarboxylate-induced neuronal damage during energy impairment in vitro. J Neurosci Res 64:418–428.

Gill R, Iversen SE, Woodruff GN. 1988. MK-801 is neuroprotective in gerbils when administered during the post-ischemic period. Neuroscience 25:847–855.

Gomes A, Fernandes E, Lima JL. 2005. Fluorescence probes used for detection of reactive oxygen species. J Biochem Biophys Methods 65:45–80.

Gonzalez R, Collado JA, Nell S, Briceno J, Tamayo MJ, Fraga E, Bernardos A, Lopez-Cillero P, Pascussi JM, Rufian S, Vilarem MJ, Mata Mde L, Brigelius-Flohe R, Maurel P, Muntane J. 2007. Cytoprotective properties of alpha-tocopherol are related to gene regulation in cultured d-galactosamine-treated human hepatocytes. Free Radic Biol Med 43:1439–1452.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Habig WH, Pabst MJ, Jakoby WB. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139.

Hernández-Fonseca K, Massieu L. 2005. Disruption of endoplasmic reticulum calcium stores is involved in neuronal death induced by glycolysis inhibition in cultured hippocampal neurons. J Neurosci Res 82:196–205.

Hillered L, Hallstrom A, Segersvard S, Persson L, Ungerstedt U. 1989. Dynamics of extracellular metabolites in the striatum after middle cerebral artery occlusion in the rat monitored by intracerebral microdialysis. J Cereb Blood Flow Metab 9:607–616.

Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ. 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75:241–251.

Ikemoto A, Bole DG, Ueda T. 2003. Glycolysis and glutamate accumulation into synaptic vesicles. Role of glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase. J Biol Chem 278:5929–5940.

Itou M, Kishimoto S, Kawata H, Ozaki M, Sakurai H, Itoh F. 1998. Development of an (X, eX) spectrometer for measuring the energies of the scattered photon and recoil electron. J Synchrotron Radiat 5:676–678.

Jacobson J, Duchen MR. 2002. Mitochondrial oxidative stress and cell death in astrocytes-requirement for stored Ca^{2+} and sustained opening of the permeability transition pore. J Cell Sci 115:1175–1188.

Kahlert S, Zundorf G, Reiser G. 2005. Glutamate-mediated influx of extracellular Ca^{2+} is coupled with reactive oxygen species generation in cultured hippocampal neurons but not in astrocytes. J Neurosci Res 79:262–271.

Kashiwaya Y, King MT, Veech RL. 1997. Substrate signaling by insulin: a ketone bodies ratio mimics insulin action in heart. Am J Cardiol 80:50A–64A.

Lafon-Cazal M, Petri S, Culcasi M, Bockaert J. 1993. NMDA-dependent superoxide production and neurotoxicity. Nature 364:535–537.

Li PA, Liu GJ, He QP, Floyd RA, Siesjo BK. 1999. Production of hydroxyl free radical by brain tissues in hyperglycemic rats subjected to transient forebrain ischemia. Free Radic Biol Med 27:1033–1040.

Liu Y, Song XD, Liu W, Zhang TY, Zuo J. 2003. Glucose deprivation induces mitochondrial dysfunction and oxidative stress in PC12 cell line. J Cell Mol Med 7:49–56.

Malcolm CS, Benwell KR, Lamb H, Bebbington D, Porter RHP. 2000. Characterization of iodoacetate-mediated neurotoxicity in vitro using primary cultures of rat cerebellar granule cells. Free Radic Biol Med 28:102–107.

Massieu L, Montiel T, del Río P, Hernández K, Haces ML, García O, Camacho A, Mejia J. 2003a. Role of energy metabolism in neuronal death associated with cerebral ischemia and neurodegenerative diseases and its prevention by energy substrates. In: Pandalai SG, editor. Recent research developments in neurochemistry. Kerala, India: Research Singpost. p 81–104.

Massieu L, Haces ML, Montiel M, Hernández-Fonseca K. 2003b. Acetoacetate protects hippocampal neurons against glutamate-mediated neuronal damage during glycolysis inhibition. Neuroscience 120:365–378.

Masuda R, Monahan JW, Kashiwaya Y. 2005. D-beta-hydroxybutyrate is neuroprotective against hypoxia in serum-free hippocampal primary cultures. J Neurosci Res 80:501–509.

Matthews RT, Ferrante RJ, Jenkins BG, Browne SE, Goetz K, Berger S, Chen IY, Beal MF. 1997. Iodoacetate produces striatal excitotoxic lesions. J Neurochem 69:285–289.

Meydani SN, Meydani M, Verdon CP, Shapiro AA, Blumberg JB, Hayes KC. 1986. Vitamin E supplementation suppresses prostaglandin E1(2) synthesis and enhances the immune response of aged mice. Mech Ageing Dev 34:191–201.

Meydani SN, Barklund MP, Liu S, Meydani M, Miller RA, Cannon JG, Morrow FD, Rocklin R, Blumberg JB. 1990. Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects. Am J Clin Nutr 52:557–563.

Novelli A, Reilly JA, Lysko PG, Henneberry RC. 1988. Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. Brain Res 451:205–212.

Oliver CN, Starke-Reed PE, Stadtman ER, Liu GJ, Carney JM, Floyd RA. 1990. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. Proc Natl Acad Sci U S A 87:5144–5147.

Papadopoulus MC, Koumenis IL, Dugan LL, Giffard RG. 1997. Vulnerability of glucose deprivation injury correlates with glutathione levels in astrocytes. Brain Res 748:151–156.

Pedraza-Chaverri J, Granados-Silvestre MA, Medina-Campos ON, Maldonado PD, Olivares-Corichi IM, Ibarra-Rubio ME. 2001. Post-transcriptional control of catalase expression in garlic-treated rats. Mol Cell Biochem 216:9–19.

Rego AC, Santos MS, Oliveira CR. 1999. Influence of the antioxidants vitamin E and idebenone on retinal cell injury mediated by chemical ischemia, hypoglycemia, or oxidative stress. Free Radic Biol Med 26:1405–1407.

Robinson KM, Janes MS, Pehar M, Monette JS, Ross MF, Hagen TM, Murphy MP, Beckman JS. 2006. Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. Proc Natl Acad Sci U S A 103:15038–15043.

Sandberg M, Butcher SP, Hagberg H. 1986. Extracellular overflow of neuroactive amino acids during severe insulin-induced hypoglycemia: in vivo dialysis of the rat hippocampus. J Neurochem 47:178–184.

Suh SW, Gum ET, Hamby AM, Chan PH, Swanson RA. 2007. Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. J Clin Invest 1174:910–918.

Suzuki M, Suzuki M, Sato K, Dohi Sato T, Matsuura A, Hiraide A. 2001. Effect of betahydroxybutyrate, a cerebral function improving agent, on cerebral hypoxia, anoxia and ischemia in mice and rats. Jpn J Pharmacol 87:143–150.

Suzuki M, Suzuki M, Kitamura Y, Mori S, Sato K, Dohi S, Sato T, Matsuura A, Hiraide A. 2002. β-Hydroxybutyrate, a cerebral function improving agent, protects rat brain against ischemic damage caused by permanent and transient focal cerebral ischemia. Jpn J Pharmacol 89:36–43.

Tieu K, Perier C, Caspersen C, Teismann P, Wu DC, Yan SD, Naini A, Vila M, Jackson-Lewis V, Ramasamy R, Przedborski S. 2003. D-beta-hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease. J Clin Invest 112:892–901.

Tominaga T, Sato S, Ohnishi T, Ohnishi ST. 1993. Potentiation of nitric oxide formation following bilateral carotid occlusion and focal cerebral ischemia in the rat: in vivo detection of the nitric oxide radical by electron paramagnetic resonance spin trapping. Brain Res 614:342–346.

Uto A, Dux E, Kusumoto M, Hossmann KA. 1995. Delayed neuronal death after brief histotoxic hypoxia in vitro. J Neurochem 64:2185–2191.

Vesce S, Kirk L, Nicholls DG. 2004. Relationships between superoxide levels and delayed calcium deregulation in cultured cerebellar granule cells exposed continuously to glutamate. J Neurochem 9:683–693.

Wang X, Quinn PJ. 1999. Vitamin E and its function in membranes. Prog Lipid Res 38:309–336.

Wieloch T. 1985. Hypoglycemia-induced neuronal damage prevented by an N-methyl-D-aspartate antagonist. Science 230:681–683.

Zeevalk GD, Nicklas WJ. 1990. Chemically induced hypoglycemia and anoxia: relationship to glutamate receptor-mediated toxicity in retina. J Pharmacol Exp Ther 253:1285–1292.

Zeevalk GD, Nicklas WJ. 1992. Evidence that loss of the voltage-dependent Mg^{2+} block at the N-methyl-D-aspartate receptor underlies receptor activations during inhibition of neuronal metabolism. J Neurochem 59:1211–1220.

Zhao H, Kalivendi S, Zhang H, Joseph J, Nithipatikom K, Vasquez-Vivar J, Kalyanaraman B. 2003. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. Free Radic Biol Med 34:1359–1368.

Artículo 3

"Antioxidant capacity contributes to protection of ketone bodies against oxidative damage induced during hypoglycemic conditions"

(Experimental Neurology)

Available online at www.sciencedirect.com
ScienceDirect
Experimental Neurology xx (2008) xxx–xxx
Experimental Neurology
www.elsevier.com/locate/yexnr

Antioxidant capacity contributes to protection of ketone bodies against oxidative damage induced during hypoglycemic conditions

María L. Haces [a,1], Karla Hernández-Fonseca [a,1], Omar N. Medina-Campos [b], Teresa Montiel [a], José Pedraza-Chaverri [b], Lourdes Massieu [a,*]

[a] Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México (UNAM), CP. 04510, Apartado Postal 70-253, México D.F., Mexico.
[b] Departamento de Biología, Facultad de Química, Edificio F Segundo Piso, Laboratorio 209, Universidad Nacional Autónoma de México (UNAM), CP 04510, México D.F., Mexico.

Received 1 September 2007; revised 10 December 2007; accepted 29 December 2007

Abstract

Ketone bodies play a key role in mammalian energy metabolism during the suckling period. Normally ketone bodies' blood concentration during adulthood is very low, although it can rise during starvation, an exogenous infusion or a ketogenic diet. Whenever ketone bodies' levels increase, their oxidation in the brain rises. For this reason they have been used as protective molecules against refractory epilepsy and in experimental models of ischemia and excitotoxicity. The mechanisms underlying the protective effect of these compounds are not completely understood. Here, we studied a possible antioxidant capacity of ketone bodies and whether it contributes to the protection against oxidative damage induced during hypoglycemia. We report for the first time the scavenging capacity of the ketone bodies, acetoacetate (AcAc) and both the physiological and non-physiological isomers of β-hydroxybutyrate (D- and L-BHB, respectively), for diverse reactive oxygen species (ROS). Hydroxyl radicals were effectively scavenged by D- and L-BHB. In addition, the three ketone bodies were able to reduce cell death and ROS production induced by the glycolysis inhibitor, iodoacetate (IOA), while only D-BHB and AcAc prevented neuronal ATP decline. Finally, in an *in vivo* model of insulin-induced hypoglycemia, the administration of D- or L-BHB, but not of AcAc, was able to prevent the hypoglycemia-induced increase in lipid peroxidation in the rat hippocampus. Our data suggest that the antioxidant capacity contributes to protection of ketone bodies against oxidative damage in *in vitro* and *in vivo* models associated with free radical production and energy impairment.

Keywords: Iodoacetate; Hypoglycemia; Oxidative stress; Acetoacetate; β-hydroxybutyrate; Brain; Antioxidant capacity; Scavenger

Introduction

Ketone bodies, β-hydroxybutyrate (BHB) and acetoacetate (AcAc) constitute the main brain energy substrates during the suckling period. Upon maturation, as diet changes, blood levels of ketone bodies decrease and the adult brain becomes more dependent on glucose as the main energy fuel (for review see Nehlig and Pereira de Vasconcelos, 1993). However, the capacity to transport and use ketone bodies as alternative energy substrates is preserved in the adult brain (Hawkins et al., 1971). Whenever ketone bodies' concentration increases, such as in prolonged starvation, an exogenous infusion or as a result of a ketogenic diet, their oxidation rate in the brain rises (Owen et al., 1967; Hawkins and Biebuyck, 1979; Yudkoff et al., 2001). These strategies have been conducted to evaluate the potential protective effect of these compounds against diverse neurotoxic insults (reviewed by Smith et al., 2005). The ketogenic diet has been used effectively in the treatment of refractory epilepsy (Nordli et al., 2001; Yudkoff et al., 2001), and recently against neurotoxicity induced during hypoglycemia in young rats (Yamada et al., 2005). In addition, ketotic infusion prevents neuronal death associated with hypoxia, ischemia (Suzuki et al., 2001, 2002; Masuda et al., 2005), excitotoxicity

* Corresponding author. Fax: +52 55 56 22 56 07.
E-mail address: lmassieu@ifc.unam.mx (L. Massieu).
[1] These authors contributed equally to this work.

0014-4886/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2007.12.029

induced during metabolic inhibition (Garcia and Massieu, 2001; Massieu et al., 2003), and in models of Parkinson's and Alzheimer's diseases (Kashiwaya et al., 2000; Tieu et al., 2003). In all these studies either AcAc or D-β-hydroxybutyrate (D-BHB), the physiological isomer of BHB, was used and their neuroprotective effect attributed mainly to their incorporation into the Krebs cycle favoring ATP supply, leaving unexplored other neuroprotective mechanisms. We have recently reported that administration of D-BHB reduces the levels of lipoperoxidation and neuronal damage induced by glutamate in the rat striatum (Mejia-Toiber et al., 2006), and in cultured neurons AcAc has been shown to decrease glutamate-mediated production of reactive oxygen species (ROS) and cell death (Noh et al., 2006), suggesting an antioxidant effect of ketone bodies.

Neuronal damage induced during conditions of energy failure, such as ischemia and glycolysis inhibition involves oxidative damage (Love, 1999; Adibhatla and Hatcher, 2006; Tomizawa et al., 2005; Uto et al., 1995; Rego et al., 1999; Malcolm et al., 2000), and ketone bodies can prevent neuronal death induced under these circumstances (Suzuki et al., 2001, 2002; Massieu et al., 2003; Izumi et al., 1998; Yamada et al., 2005). However, the contribution of a potential antioxidant effect to neuroprotection exerted by ketone bodies has not been elucidated. Recently, ROS production has been implicated in hypoglycemic neuronal death (Suh et al., 2003, 2007), and peroxidation of brain lipids has been observed during insulin-induced hypoglycemia (Patočková et al., 2003; Ballesteros et al., 2003; Singh et al., 2004). The present study was conducted to investigate the scavenging capacity of ketone bodies against various ROS and its contribution to the prevention of oxidative damage. We performed the study in two experimental conditions known to involve oxidative damage and energy failure: *in vivo* insulin-induced hypoglycemia and *in vitro* glycolysis inhibition by iodoacetate (IOA), an inhibitor of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). We tested the effect of AcAc and of D- and L-BHB (the physiological and non-physiological isomers of BHB, respectively) on lipid peroxidation in the hippocampus after insulin administration. We also studied the effect of ketone bodies against ROS production and neuronal death in cultured hippocampal neurons exposed to IOA.

Materials and methods

Several concentrations of D-BHB, L-BHB, and AcAc were used throughout this study (BHB sodium salt and AcAc lithium salt; Sigma-Aldrich, St. Louis, MO, USA).

In vitro determination of ROS scavenging capacity of ketone bodies

Hydrogen peroxide (H_2O_2) scavenging assay

A 50 μM solution of H_2O_2 was mixed with different concentrations of either ketone body (1:1 v/v) and incubated for 4 h at room temperature. Immediately after, H_2O_2 was measured as described previously (Medina-Campos et al., 2007) using the FOX reagent (xylenol orange, ammonium ferrous sulfate and H_2SO_4). The concentration of H_2O_2 was recorded at 560 nm. Sodium pyruvate was used as a reference compound showing an $IC_{50}=1.466\pm0.062$ mM.

Hydroxyl radical ($^{\bullet}OH$) scavenging assay

The ability of either ketone body to scavenge $^{\bullet}OH$ was monitored in the Fe^{3+}–EDTA–H_2O_2–deoxyribose system (Floriano-Sánchez et al., 2006). The extent of deoxyribose degradation by the $^{\bullet}OH$ formed was measured directly in the aqueous phase by the thiobarbituric acid reactive substances (TBARS) assay at 532 nm. Mannitol was used as a reference compound showing an $IC_{50}=3.30\pm0.19$ mM.

Hypochlorous acid (HOCl) scavenging assay

The HOCl scavenging capacity of ketone bodies was tested as previously described (Medina-Campos et al., 2007). This method is based on the inhibition of thio-2-nitrobenzoic acid oxidation to 5.5′-dithiobis(2-nitrobenzoic acid) (DTNB) induced by HOCl. DTNB concentration was measured at 412 nm. Lipoic acid was used as a reference compound showing an $IC_{50}=0.858\pm0.085$ mM.

Peroxynitrite ($ONOO^-$) scavenging assay

The $ONOO^-$ used in the assay was synthesized as previously described (Medina-Campos et al., 2007) mixing an acidic solution of H_2O_2 with KNO_2. Concentrations of $ONOO^-$ were determined before each experiment at 302 nm using a molar extinction coefficient of 1670 M^{-1} cm^{-1}. The $ONOO^-$ scavenging capacity of ketone bodies was performed spectrophotometrically at 500 nm according to a previously described method (Pedraza-Chaverri et al., 2007) monitoring the oxidation of dihydrorhodamine-123 (DHR 123) to rhodamine. Penicillamine was used as a reference compound showing an $IC_{50}=0.0306\pm0.002$ mM.

Singlet oxygen ($^1O_2$) scavenging assay

The production of $^1O_2$ by sodium hypochlorite and H_2O_2 was determined using a spectrophotometric method (Medina-Campos et al., 2007). The $^1O_2$ scavenging assay is based on *N, N*-dimethyl-*p*-nitrosoaniline (DMNA) bleaching in the presence of $^1O_2$. The extent of $^1O_2$ production and the effect of the ketone bodies on it were determined by measuring the decrease in the absorbance of DMNA at 440 nm. Lipoic acid was used as a reference compound showing an $IC_{50}=3.87\pm0.23$ mM.

Superoxide radical ($O_2^{\bullet-}$) scavenging assay

The xanthine–xanthine oxidase system was used to determine the $O_2^{\bullet-}$ scavenging capacity of ketone bodies (Medina-Campos et al., 2007). The $O_2^{\bullet-}$ production and xanthine oxidase activity were measured as the reduction of nitro-blue tetrazolium (NBT) (at 560 nm) and uric acid production (at 295 nm), monitored every minute during a 3-minute period. Vitamin C was used as a reference compound with an $IC_{50}=0.0187\pm0.0021$ mM.

IC_{50} calculation

The ROS scavenging capability of ketone bodies was expressed as 50% of the inhibitory concentration (IC_{50}) as previously

described (Floriano-Sánchez et al., 2006). IC_{50} values denote the concentration of D-BHB, L-BHB or AcAc (mM) required for a 50% decrease in (a) NBT reduction (for $O_2^{\bullet -}$), (b) H_2O_2 concentration (for H_2O_2), (c) TBARS production (for $^{\bullet}OH$), (d) the bleaching of DMNA (for $^1O_2$), (e) TNB oxidation (for HOCl), and (f) DHR 123 oxidation (for $ONOO^-$), relative to the tube without sample. IC_{50} was calculated by the least square method. The lower the IC_{50} value the higher the scavenging capacity of the compound.

Iodoacetate-induced neuronal damage

All animals used throughout this study were handled according to the Rules for Research in Health Matters (México) and with the local animal care committee approval. All efforts were made to minimize the number of animals used and their suffering. Wistar rats were obtained from the local animal house at Instituto de Fisiología Celular, UNAM. Rats were housed under controlled conditions of temperature and light (12 h cycle) with ad libitum access to food and water, unless otherwise stated.

Primary cultures of hippocampal neurons were prepared from Wistar rats embryos of 17–18 days of gestation as previously described (Hernández-Fonseca and Massieu, 2005). Briefly, after dissection 10–12 hippocampi were chopped into 300 μm cubes, incubated in 0.25% trypsin solution, and dispersed by trituration in a DNAase and soybean trypsin inhibitor containing solution (0.08% and 0.52%, respectively). Cells were suspended in Neurobasal culture medium (Gibco/Life Technologies, Rockville, MD, USA) supplemented with B27 (Minus AO; Gibco/Life Technologies), 0.5 mM L-glutamine, 20 μg/ml gentamicin, and 0.2 mM glutamate, and plated at a density of $260–290 \times 10^3$/cm^2 (1.5×10^6 cells/ml/well) in Costar 24-well plates (Cambridge, MA, USA), precoated with poly-L-lysine (5 μg/ml). Cells were cultured for 8 days *in vitro* at 37 °C in a humidified 5% CO_2/95% air atmosphere. Glucose (5 mM) and cytosine arabinoside (10 μM) were added to cultures 4 days after plating. Neuronal population in these cultures was 95% as determined by immunocytochemistry against microtubule-associated protein 2 (MAP-2) and glial fibrillary acidic protein (GFAP), neuronal and astrocytes cell markers, respectively (not shown).

Drug exposure and viability assays

Neuronal cultures were exposed to IOA (Sigma-Aldrich) during 30 min, at 50 or 100 μM concentrations in Ringer Krebs medium containing (in mM): NaCl 154; KCl 5.6; $CaCl_2$ 2.3; KH_2PO_4 1.2; HEPES 5.0; $NaHCO_3$ 3.6 and glucose 5.6, in the presence or absence of L-BHB, D-BHB or AcAc at different concentrations. After this period, medium was changed for conditioned medium containing the ketone bodies and neuronal survival was monitored at 24 h using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) reduction assay (Mosmann, 1983; Berridge and Tan, 1993) as previously described (Hernández-Fonseca and Massieu, 2005). Results are expressed as percent of control. The effect of IOA (100 μM) and ketone bodies on cell survival was corroborated by the live/dead kit (Invitrogen/Molecular Probes; Carlsbad, CA, USA) as previously described (García and Massieu, 2001). Briefly, cells were grown on coverslips and exposed for 30 min to IOA in the presence or absence of the ketone bodies, 4 h later cells were incubated for 10 min with the fluorescent markers calcein-AM (green) and ethidium homodimer (red) for live and dead cells, respectively. Around 200 cells were counted per condition in ten different fields, randomly selected. The number of living neurons is expressed as the percentage of green-fluorescent cells (positive to calcein-AM) relative to the total number of cells (red- and green-fluorescent) in at least 3 independent experiments.

ATP determination

ATP levels were determined 4 h after IOA (50, 100 μM) exposure in the presence or absence of D-BHB, L-BHB or AcAc. ATP determinations were performed using previously described methodology (Massieu et al., 2003). Briefly, cells were washed twice with pre-warmed Locke's solution and lysed by incubation in 125 μl somatic cell ATP releasing agent (Sigma). Lysate (15 μl) were diluted in 85 μl distilled water and placed in polyethylene tubes in a luminometer. The luminometer injected a 400 μl volume of luciferin–luciferase reaction mixture (Invitrogen/Molecular Probes). The luminometer records chemiluminescence values in millivolts and ATP concentrations were calculated from readings obtained from an ATP standard curve (6.5–250 pmol). Aliquots of cell homogenates were kept for protein determination (Bradford, 1976), and data are expressed as pmol ATP/μg protein.

ROS determination

The presence of ROS was determined by means of oxidative-sensitive fluorescent markers. For this purpose dihydroethidium (HE; Invitrogen/Molecular Probes) and 5-(and-6) carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA; Invitrogen/Molecular Probes) were used. HE is oxidized by $O_2^{\bullet -}$ to 2-hydroxyethidium (OH-Et) and to ethidium (Et) by nonsuperoxide-dependent cellular processes, such as H_2O_2 through the activity of non-specific peroxidases, oxidases and cytochromes or by other ROS, such as $ONOO^-$ and $^{\bullet}OH$ (Zhao et al., 2003; Gomes et al., 2005; Robinson et al., 2006). Et binds within the cells' DNA, staining the nucleus with a bright-red fluorescence (Bindokas et al., 1996). Carboxy-H_2DCFDA is deacetylated, oxidized by ROS and converted to the fluorescent compound 5-(and-6-) carboxy-2′,7′-dichlorofluorescein (carboxy-DCF), that stains the cell cytoplasm with a bright-green fluorescence (Hockenbery et al., 1993). Cells plated on coverslips were incubated with IOA and ketone bodies as previously described. 4 h later cells were exposed for 20 min to 3.2 μM of HE and 5 μM of carboxy-H_2DCFDA in Ringer Krebs medium. Cells were examined under an epifluorescence microscope equipped with argon laser (488 nm excitation and 535 nm emission for Et; 488 nm excitation and 530 nm emission for carboxy-DCF). Positive cells to both fluorescent markers were counted in ten different fields per condition in at least 4 independent experiments. The total number of cells was counted from bright-field images (~200 cells per coverslip) and results are expressed

as the percent of cells positive to each one of the oxidative-sensitive fluorescent markers.

Insulin-induced in vivo hypoglycemia

In order to induce a hypoglycemic condition, male Wistar rats (320–380 g) were fasted overnight and received an i.p. injection of 30 U bovine insulin (Sigma). Blood glucose concentration was measured with a glucometer (Abbott Lab, Bedford, MA, USA) before and every hour after insulin administration. Animals received a single i.p. injection of 500 mg/kg (250 mg/ml) of either D-BHB, L-BHB, or AcAc 1 h after insulin treatment. The final concentration of the solution was 1.98 mM for D-BHB and L-BHB and 2.3 mM for AcAc. Animals received between 0.65 and 0.76 ml of ketone body solution. We have previously shown that the i.p. administration of similar doses of AcAc or pyruvate are able to prevent glutamate-induced neuronal damage in the hippocampus *in vivo* (Massieu et al., 2001, 2003). In a different group of animals a second administration of ketone body was given 30 min after the first one. Control animals received the vehicle solution (phosphate buffer). Approximately 2 h after insulin administration, when the rats lost the righting reflex and were close to the hypoglycemic coma, they were killed and their hippocampi dissected for lipoperoxidation and 4-hydroxy-2-nonenal (HNE) protein adducts determinations. We have previously determined that the loss of the righting reflex occurs minutes before the appearance of the isoelectric period as assessed electroencephalographically (not shown).

TBARS assay

We assessed the production of TBARS, which are products of lipid peroxidation, in tissue homogenates according to Gluck et al. (2000). Briefly, hippocampi were homogenized in 0.5 ml of 1.15% KCl/0.4 mM sodium azide and incubated at 37 °C for 15 min. Then, 20% trichloroacetic acid was added and samples were centrifuged at 14,000 ×*g* for 10 min. Supernatants were collected and mixed with an equal volume of 0.75% thiobarbituric acid (Merck, Darmstadt, Germany) and samples were incubated at 50 °C for 20 min. Optical density (OD) was read at 532 nm and TBARS levels were calculated using a standard curve of malondialdehyde bis-dimethyl acetal. Data are expressed as nmol of TBARS/mg protein. The protein concentration was calculated with 20 µl of the homogenates according to Bradford (1976) using albumin as standard.

Determinations of HNE-protein adducts

HNE adducts were analyzed by Western blot. For this purpose the hippocampi were homogenized in phosphate buffer containing 1% Triton, 1 mM EDTA and 0.2% of a cocktail of protease inhibitors (Complete, Roche Diagnostics, Mannheim, Germany), and stored at −70 °C until used. After determination of protein concentration, 50 µg per sample were resolved in 7.5% acrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA). Membranes were blocked and then incubated overnight with either HNE (1:1500, Alpha Diagnostic Intl., San Antonio, TX, USA) or α-actin (1:2500, Sigma) antibodies. Peroxidase-conjugated anti-rabbit IgG (1:4000, Amersham Bioscience, Little Chalfont, Buckinghamshire, UK) or anti-mouse IgG (1:4000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used as secondary antibodies, respectively. Chemiluminescence reaction using the ECL kit (Amersham Bioscience) was carried out for 1 min followed by exposure to Konica Minolta medical film (Tokyo, Japan). For the densitometric analysis of HNE-protein adducts, bands with a molecular weight between 60 and 150 kDa were considered and results are expressed as OD HNE/OD α-actin.

Determination of ketone bodies' plasma levels

D-BHB and AcAc levels were determined by enzymatic assays in plasma of control and hypoglycemic rats treated with each ketone body. For this purpose, blood was collected in heparinized tubes, 10 min after administration of the ketone body or at the time the animals lost their righting reflex (~2 h after insulin administration), and left on ice until an equal volume of 10% perchloric acid was added. Samples were centrifuged at 3400 ×*g* for 10 min and the supernatant collected and stored at −20 °C until measured. Ketone body levels were determined according to Williamson and Mellanby (1965) and Mellanby and Williamson (1965), as previously reported (Massieu et al., 2003). Plasma levels of D-BHB and AcAc were also examined in a control group non-treated with insulin, 10 min after ketone body i.p. administration. In order to discard a possible hepatic conversion of L-BHB to D-BHB, as suggested previously (Lincoln et al., 1987), D-BHB plasma levels were determined in L-BHB treated animals.

Statistical analyses

All data are expressed as means ± SEM and were analyzed by one-way ANOVA followed by a Fisher's least multiple comparison test.

Results

In vitro ROS scavenging capacity of ketone bodies

In vitro experiments were carried out to determine the ROS scavenging capacity of ketone bodies. For this purpose, different concentrations of each ketone body were used and the maximal concentration tested in each assay is shown in Table 1. Results show that both isomers of BHB were able to effectively neutralize $^{\bullet}OH$, showing L-BHB a lower IC_{50} value relative to that shown by D-BHB. The IC_{50} values of L-BHB and D-BHB were similar to that of mannitol (3.30 ± 0.19 mM), which was used as reference compound (Fig. 1A). The $^{\bullet}OH$ scavenging capacity of AcAc was much lower (Fig. 1A). Both BHB isomers were ineffective for all the other ROS tested: H_2O_2, HOCl, $O_2^{\bullet-}$, $^1O_2$, $ONOO^-$, while AcAc was also capable of scavenging $^1O_2$, HOCl, and $ONOO^-$ (Fig. 1B), although to a lesser extent (Table 1 and Fig. 1B).

Table 1
Scavenging capacity of D-BHB, L-BHB and AcAc for various ROS

Maximal concentration tested (mM)					IC_{50} (mM)		
ROS	D-BHB	L-BHB	AcAc	Scavenging capacity	D-BHB	L-BHB	AcAc
H_2O_2	75	20	40	None	–	–	–
$^{\bullet}OH$	15	14	60	All	3.2±0.44	2±0.47	31.1±5.47
HOCl	150	40	80	AcAc	–	–	66.8±0.71
$ONOO^-$	150	40	80	AcAc	–	–	49.0±2.94
$^1O_2$	75	20	40	AcAc	–	–	20.4±3.8
$O_2^{\bullet -}$	150	20	80	None	–	–	–

The radical scavenging capacity of ketone bodies was determined by *in vitro* assays. Data represent means±SEM from 4 independent experiments.

Effect of ketone bodies on cell viability

To test the neuroprotective effect of these compounds we used an *in vitro* pharmacological model of hypoglycemia, exposing cultured hippocampal neurons to the glycolysis inhibitor IOA in the presence or absence of 10 mM of ketone bodies. Two concentrations of IOA (50 and 100 μM) were used in order to mimic moderate and severe hypoglycemic conditions, respectively, and cell viability was assessed 24 h after IOA exposure. As shown in Fig. 2, both concentrations of IOA caused substantial neuronal death. Cell survival was reduced in 60.7 and 92.2% after the exposure to 50 and 100 μM IOA, respectively. The non-physiological isomer of BHB, L-BHB, and AcAc were similarly effective in preventing neuronal death induced during moderate glycolysis inhibition, restoring cell viability to 59.1 and 62%, respectively; while in the presence of the physiological isomer D-BHB, 80.4% of the neuronal population was still viable. Co-incubation with both isomers of BHB prevented completely IOA-induced cell death restoring MTT reduction to control levels. In cultures treated with 100 μM IOA, L-BHB showed a significant protective effect ($p<0.030$ vs IOA-treated) but D-BHB and AcAc were more effective ($p<0.002$ for D-BHB and $p<0.003$ for AcAc vs IOA-treated). Co-incubation with both isomers of BHB showed better neuroprotection relative to that shown by L-BHB, and no significant difference was observed between the effects of D-BHB and D-BHB+L-BHB. The protective effect of ketone bodies, against

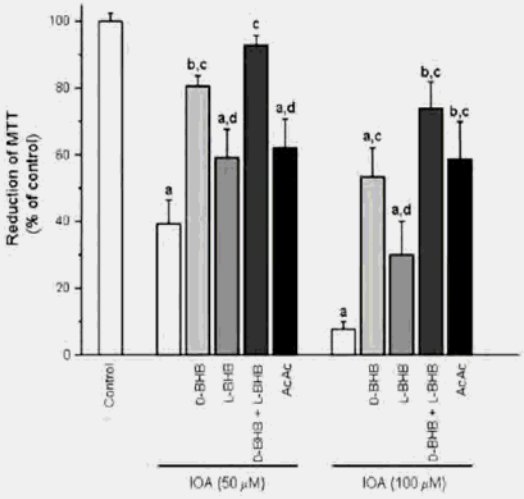


Fig. 2. Protective effect of ketone bodies against neuronal damage induced by IOA evaluated by the MTT reduction assay 24 h later. Cells were exposed to IOA for 30 min either alone or in the presence of the ketone bodies (10 mM). Ringer Krebs was replaced by culture media containing D-BHB, L-BHB, AcAc or D-BHB+L-BHB until viability was determined. Data are means± SEM from 4 to 7 independent experiments. a $p<0.001$, b $p<0.02$ vs control cultures, c $p<0.001$, and d $p<0.03$ vs IOA-treated cultures.

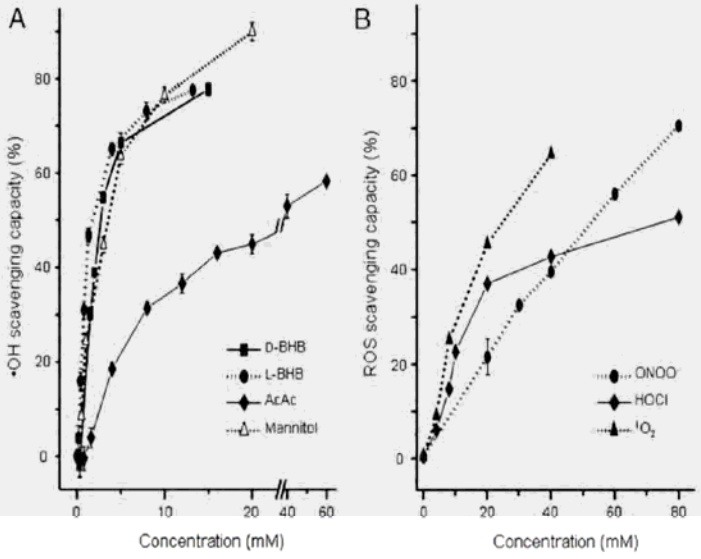


Fig. 1. ROS scavenging capacity of ketone bodies. (A) $^{\bullet}OH$ scavenging capacity of ketone bodies. Mannitol was used as a reference compound. (B) ROS scavenging capacity of AcAc. IC_{50} values were calculated from both curves and are depicted in Table 1. Data represent the mean±SEM of at least 4 independent experiments. In some points, the SEM values were too small thus the SEM bars are not visible.

100 μM IOA, was maintained even if their concentration was lowered to 5 and 2.5 mM (data not shown). At these concentrations ketone bodies showed no direct effect on MTT reduction in control conditions (in OD: control=0.215±0.003; D-BHB(10 mM)=0.198±0.003; L-BHB(10 mM)=0.194±0.002; AcAc(10 mM)=0.225±0.002, n=4). Concentrations of 20 and 30 mM of AcAc, which are close to its IC_{50} to scavenge $^{\bullet}OH$ anions were also tested, however they showed no protection against IOA-induced neuronal death and were even toxic for control cultures reducing cell viability to 51.05±5.88 and 38.15±4.96%, respectively (n=4 independent experiments).

Cell viability was also evaluated using fluorescent markers for living and dead cells 4 h after the exposure to 100 μM IOA. Dead cells incorporate Et into DNA and nuclei appear red-fluorescent, while living cells incorporate the permeable fluorescent dye, calcein, and appear green-fluorescent. As shown in Fig. 3, control cultures show numerous living cells, while 4 h after IOA exposure the number of red cells notably increases with a concomitant reduction in the number of green cells. In agreement with MTT reduction data, cultures exposed to IOA plus D-BHB, L-BHB or AcAc (10 mM) showed a significant higher number of living green-fluorescent cells relative to the IOA condition. Similar results were obtained when cells were incubated with 5 mM of each ketone body (data not shown).

Effect of ketone bodies on ATP levels

To assess the possible metabolic effect of each one of the ketone bodies, we evaluated the contribution of L-BHB, D-BHB or AcAc to the restoration of ATP levels during IOA exposure. Both moderate and severe glycolytic inhibition significantly reduced ATP levels. As expected, D-BHB and AcAc effectively prevented the decrease in ATP in both conditions restoring ATP levels to control values (Fig. 4). In contrast, L-BHB did not show any effect on IOA-induced decrease in ATP concentration.

Effect of ketone bodies on ROS levels

The effect of ketone bodies on the presence of intracellular ROS was tested. For this purpose, cultures were treated with

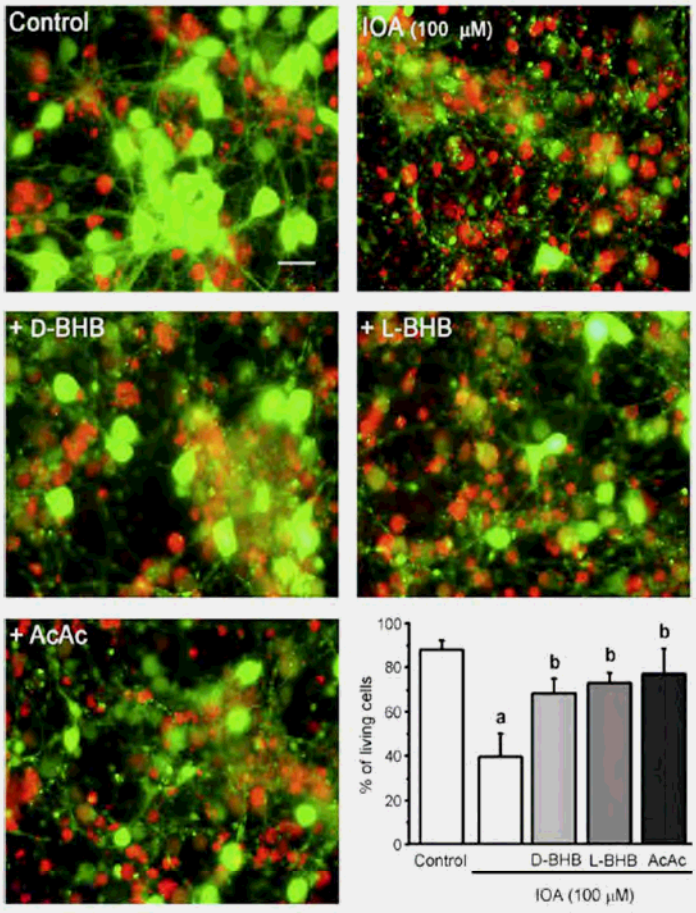


Fig. 3. The protective effect of 10 mM D-BHB, L-BHB and AcAc against neuronal damage induced by 30 min exposure to 100 μM IOA evaluated 4 h later by live/dead kit. Living cells incorporate calcein and appear green, while the nucleus in dead cells is red-labeled due to Et incorporation into DNA. Scale bar=20 μm. Data are expressed as means±SEM from at least 4 independent experiments. Quantification of living cells in 4 independent experiments is shown. a $p<0.02$ vs control cultures, and b $p<0.03$ vs IOA-treated cultures.

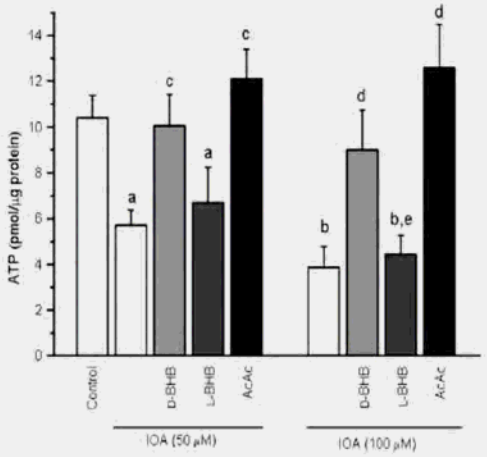


Fig. 4. Changes in ATP levels induced by 30 min exposure to IOA (50 or 100 μM) in cultured hippocampal cells as evaluated 4 h after the exposure, and effect of ketone bodies (10 mM) treatment. Data are means±SEM of 4–8 independent experiments. ATP levels were determined by the luciferin–luciferase assay. a $p<0.01$; b $p<0.001$ vs control cultures; c $p<0.01$ vs 50 μM IOA-treated cultures; d $p<0.005$ vs 100 μM IOA-treated cultures; and e $p<0.05$ vs 100 μM IOA + D-BHB-treated cultures.

either concentration of IOA in the presence or absence of ketone bodies, and the presence of ROS was monitored 4 h later by means of the fluorescent dyes HE and carboxy-H_2DCFDA. After oxidation of HE to Et it concentrates in the cell nucleus giving a red-bright fluorescence, while oxidation of carboxy-H_2DCFDA to carboxy-DCF leads to green-fluorescent cells. As shown in Fig. 5A, cultures treated with 50 μM IOA showed a significant increase in the number of positive cells to both dyes (50.2% for Et and 38.2% for carboxy-DCF). The number of positive cells was significantly reduced, although not completely, in the presence of the three ketone bodies. After exposure to 100 μM IOA, ROS production was significantly enhanced since the number of cells positive to Et and carboxy-DCF increased up to 76.5% and 60.4%, respectively. Even in these conditions, ketone bodies reduced the number of cells positive to both markers (Et: 29.5–33.5%; carboxy-DCF: 24.5–27.8%) (Fig. 5B). A substantial effect of ketone bodies was observed during severe glycolysis inhibition, but a significant number of positive cells still remained. A representative experiment of cultures treated with 100 μM IOA is shown in Fig. 6. The same microscopic field is observed in the three panels. Bright-field micrographs (left panel) show that neuronal cultures are severely affected after IOA exposure, neurons appear swollen with condensed nucleus (arrowheads), while the cytoplasm of many cells is no longer visible with only debris remaining. It can be observed, in the middle and right panels, that the number of positive cells to both ROS markers is markedly increased after IOA exposure. Some of the swollen cells were positive to both fluorescent markers (arrows). Control cultures show normal appearing neurons and are negative to both markers. Treatment with ketone bodies prevented cell swelling particularly D-BHB, and reduced the number of positive cells to Et and carboxy-DCF.

In vivo effect of ketone bodies on oxidative damage during hypoglycemia

In order to study the antioxidant action of ketone bodies in an *in vivo* model, an i.p. dose of 500 mg/kg of either D-BHB, L-BHB or AcAc was administered 1 h after inducing a hypoglycemic state through an insulin injection, and levels of lipoperoxidation in the rat hippocampus were evaluated. As shown in Fig. 7A, lipoperoxidation levels were 2-fold increased in the hippocampus of hypoglycemic animals as compared to control rats. D-BHB or L-BHB treatment prevented the increase in lipoperoxidation observed in the hypoglycemic animals (Fig. 7A). A similar effect was obtained in animals receiving two doses (the first 1 h and the second 1.5 h after insulin injection) of either isomer of BHB (in pmol TBARS/mg protein: control = 0.091 ± 0.012; hypoglycemic group = 0.183 ± 0.074; D-BHB-treated = 0.103 ± 0.019; L-BHB =

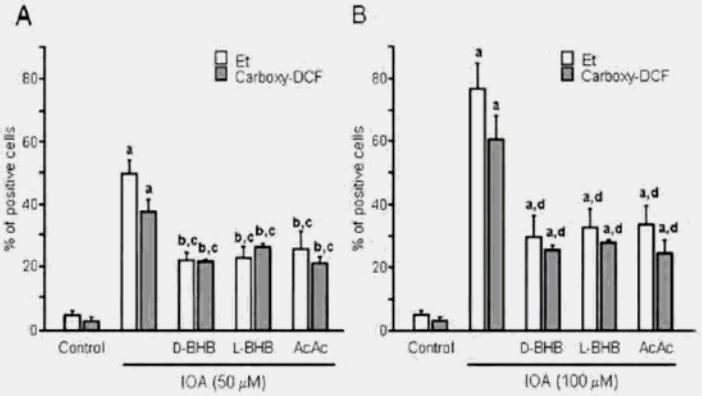


Fig. 5. Effect of ketone bodies on ROS levels induced during IOA treatment. White and gray bars represent the percent of cells positive to Et ($O_2^{\bullet-}$) or to carboxy-DCF, respectively. Cultures were exposed to IOA 50 μM (A) or 100 μM (B) in Ringer Krebs medium for 30 min in the presence or absence of D-BHB, L-BHB and AcAc (10 mM). Ringer Krebs was removed and replaced by conditioned media containing the ketone bodies until fluorescent markers were added 4 h later. Ten fields per condition were counted of at least 4 independent experiments. Data are expressed as means±SEM. a $p<0.001$, b $p<0.05$ vs control values, c $p<0.05$ vs cultures treated with IOA 50 μM, and d $p<0.0001$ vs cultures treated with IOA 100 μM.

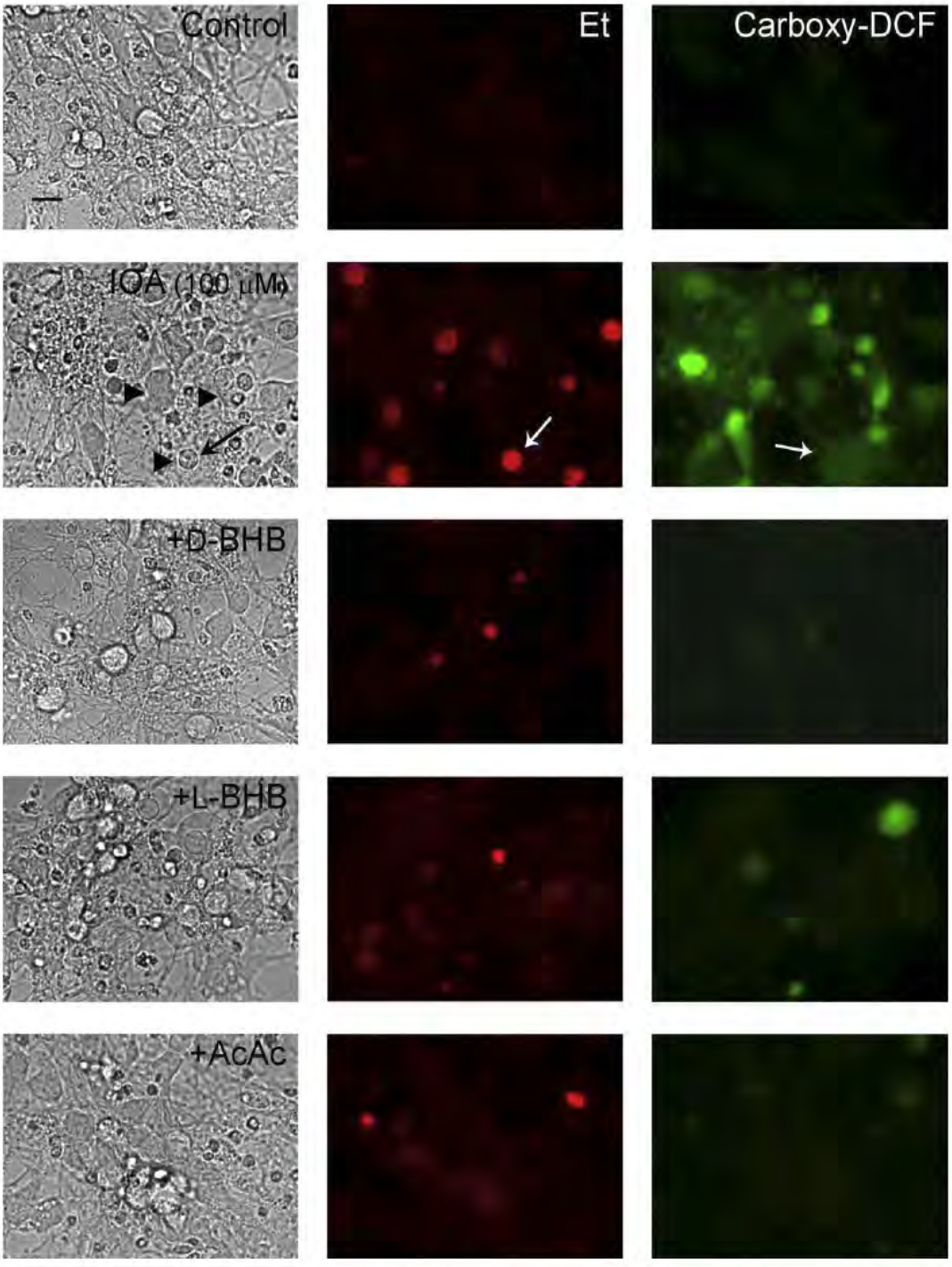


Fig. 6. Images from a representative experiment showing the effect of ketone bodies on ROS levels during IOA exposure. Bright-field images are shown in the left panel and Et and carboxy-DCF images in the middle and right panels, respectively. The same field is shown in each condition. Arrowheads (left panel) show swollen cells, some of them are positive to both oxidation-sensitive markers (arrows in middle and right panels). Scale bar=20 μm.

0.065±0.005, n=3–6), despite a sustained increase in D-BHB plasma levels was observed at the time of sacrifice (Table 2). At this time D-BHB plasma levels were significantly higher relative to the control group in D-BHB-treated rats, and if determined earlier, 10 min after D-BHB administration, higher levels were obtained (Table 2), suggesting that D-BHB is removed from blood during the hypoglycemic period. When D-BHB was administered to intact animals levels of D-BHB were even higher,

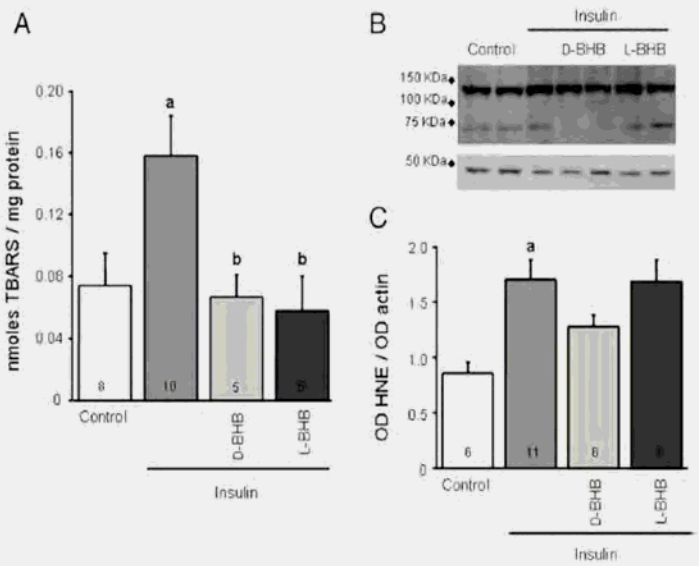


Fig. 7. *In vivo* effects of BHB on lipoperoxidation levels and HNE-protein adducts during insulin-induced hypoglycemia. 1 h after insulin injection rats received an i.p. injection (500 mg/kg) of either isomer of BHB. Rats were killed when they lost the righting reflex and were close to coma, and their hippocampi dissected. (A) Lipoperoxidation levels in the different animal groups. Data are means ± SEM of at least 5 animals. a $p<0.05$ vs control animals; and b $p<0.05$ vs hypoglycemic animals. (B) Representative Western blot showing HNE-protein adducts in the different animal groups. HNE-protein adducts of different molecular weights are shown in the upper panel while α-actin immunoreactivity used as a loading control, is shown at the bottom. (C) Quantification of HNE-protein adducts. For the OD analysis of HNE adducts, bands with a molecular weight between 60 and 150 kDa were considered. Data are means ± SEM of at least 5 animals. a $p<0.05$ vs control animals.

further suggesting that in hypoglycemic animals, ketone bodies are rapidly removed from blood. On the other hand L-BHB-treated animals did not show any increase in D-BHB plasma level discarding a possible conversion of the L-isomer into D-BHB (Table 2).

AcAc treatment had no effect on lipoperoxidation levels in hypoglycemic rats either after the administration of 1 or 2 doses (in pmol TBARS/mg protein, control = 0.024 ± 0.006; hypoglycemic group = 0.065 ± 0.006; AcAc-treated (one dose) = 0.075 ± 0.016; AcAc-treated (2 doses) = 0.084 ± 0.035, $n = 4–7$). Failure of AcAc to reduce lipoperoxidation cannot be attributed to the lack of increase of the ketone body concentration in blood, since rats receiving AcAc showed significantly higher plasma levels of this ketone body as determined 10 min and ~2 h after insulin administration (Table 2). Control animals receiving AcAc showed increased levels of this ketone body as compared to hypoglycemic animals (Table 2), suggesting AcAc removal from blood during the hypoglycemic period, as observed in BHB-treated animals.

Table 2
Effect of ketone bodies (KB, either AcAc or D-BHB) administration on KB plasma levels in control and insulin-treated rats

Treatment	D-BHB (mM)	AcAc (mM)
Control	0.060 ± 0.009	0.051 ± 0.010
KB#	0.600 ± 0.060^{a,b}	0.613 ± 0.038^{a,b}
Insulin	0.092 ± 0.013	0.077 ± 0.019
Insulin + KB#	0.371 ± 0.080^{a,b,d}	0.358 ± 0.062^{a,b,d}
Insulin + KB*	0.186 ± 0.035^{a,c}	0.218 ± 0.044^{a,b,c}
Insulin + KB 2 doses*	0.326 ± 0.079^{a,b,d}	0.244 ± 0.062^{a,b,c}
Insulin + L-BHB#	0.098 ± 0.001	–

Either D-BHB and AcAc levels were determined by enzymatic assays in plasma of control and hypoglycemic rats treated with each ketone body 10 min after the administration (#), or when animals lost their righting reflex (*). Data represent means ± SEM from least 3 independent animals. a $p<0.05$ vs control; b $p<0.05$ vs insulin-treated animals; c $p<0.05$ vs all determinations performed 10 min after KB administration; and d $p<0.05$ vs intact animals that received KB.

Administration of either isomer of BHB or AcAc had no effect on the development of the hypoglycemic state. The latency to the loss of the righting reflex was not different in treated and control animals (data not shown). Consistently, there were no differences in glucose levels at the time of sacrifice between ketone body-treated groups and hypoglycemic animals. All rats that received insulin showed glucose levels ≤ 20 mg/dl at time of sacrifice.

Furthermore, we studied whether some proteins were modified by HNE, a lipoperoxidation product capable of reacting with thiol groups, lysine, histidine, serine and tyrosine residues of proteins (Esterbauer et al., 1991). As shown in Figs. 7B and C, hypoglycemia significantly increased HNE adducts in some proteins which was not prevented by treatment with D-BHB and L-BHB. The trend to diminish HNE products induced by D-BHB was not significant ($p = 0.075$). AcAc had no effect on the production of HNE adducts (data not shown).

Discussion

The full understanding of the mechanisms responsible for the neuroprotective effect of ketone bodies is of great relevance considering their therapeutic potentiality. The present study demonstrates that ketone bodies are capable of directly scavenging some ROS, and that this ability is preserved when they are administered to cultured neurons or to the animal *in vivo*, at least for BHB. In accordance, recently ketone bodies have been

shown to counteract ROS production induced by H_2O_2 (Maalouf et al., 2007; Kim et al., 2007). We also provide new evidence suggesting that the antioxidant capacity of ketone bodies is involved in neuroprotection. To our knowledge this is the first study showing that the non-physiological isomer of BHB is capable of preventing oxidative damage independent of a metabolic effect, according to *in vitro* experiments. The mechanism by which ketone bodies scavenge directly several ROS is not clear at present. We are tempted to speculate that the hydroxyl group present in L-BHB and D-BHB may contribute to their scavenging capacity. In fact, the $^{\bullet}OH$ scavenging capacity of D-BHB and L-BHB was clearly higher than that of AcAc, which lacks the hydroxyl group.

Previous studies have shown that metabolic inhibition induced by IOA leads to an important decrease in ATP levels, both in cultured neurons and retina (Zeevalk and Nicklas, 1990; Uto et al., 1995; Rego et al., 1997; Massieu et al., 2003), or when locally administered into the hippocampus (Massieu et al., 2003). On the other hand, it has been suggested that ROS production is an important component of neurotoxicity induced during this condition, since treatment with antioxidants effectively prevents neuronal death (Uto et al., 1995; Rego et al., 1999; Malcolm et al., 2000; Sperling et al., 2003). In previous studies we have shown that AcAc prevents ATP depletion in hippocampal cultured neurons exposed to IOA (Massieu et al., 2003), and in an *in vivo* model of excitotoxicity we have recently observed that D-BHB prevents neuronal damage and reduces lipoperoxidation levels in the rat striatum (Mejia-Toiber et al., 2006). In an attempt to elucidate the contribution of other mechanisms of neuroprotection additional to their metabolic action, the effect of L-BHB on neuronal damage, ROS levels and lipoperoxidation induced during hypoglycemic-mimicking conditions, were studied.

Our results show that L-BHB promoted neuronal survival during both moderate and severe glycolysis inhibition, despite that it did not have any effect on ATP levels. We have determined that GAPDH is inhibited to a higher extent during the exposure to 100 μM relative to 50 μM IOA (Hernández-Fonseca et al., in press), suggesting that energy impairment is more pronounced in the former condition. Consistently, neuronal death induced by 100 μM IOA is more extensive than that observed after 50 μM treatment. Nevertheless, L-BHB is effective in both conditions, suggesting a contribution of the scavenging capacity of the L-isomer to neuroprotection.

According to the results, there is a good correlation between cell death and ROS production. During exposure to 100 μM IOA a higher percentage of cells positive to the fluorescent markers was found compared to the 50 μM condition. We observed that all three ketone bodies are equally effective in reducing the percentage of positive cells to fluorescent markers in both conditions, but are not equally effective in preventing neuronal death. It can be concluded from these results that the metabolic action of D-BHB and AcAc might influence ROS production through the preservation of mitochondrial metabolism, while L-BHB will mainly have a role as ROS scavenger. The protective effect of AcAc against ROS production and neuronal death might be attributed to its rapid transformation to acetyl-CoA in mitochondria, efficiently restoring ATP levels. However, its capacity to scavenge various ROS cannot be discarded; *in vitro* assays show that at a 10 mM, D-BHB will scavenge close to 70% of the generated $^{\bullet}OH$, while AcAc will neutralize this radical in 31%, but will also scavenge other radicals in different proportions: 25% for $^1O_2$, 22% for HOCl, and 12% for $ONOO^-$. On the other hand, it has been observed that at high concentrations AcAc but not BHB stimulates the production of oxygen radicals, increases lipoperoxidation and decreases GSH levels in human eritrocytes and endothelial cells *in vitro* (Jain et al., 1998; Jain and McVie, 1999). Accordingly, we found that AcAc at 20 and 30 mM concentrations was toxic for control cultures.

Two fluorescent markers were used to monitor the presence of ROS, and ketone bodies were able to reduce the number of cells positive to both markers. Since the scavenging capacity of BHB is mainly for $^{\bullet}OH$, we can conclude that this radical is importantly produced during glycolysis inhibition, however, it might not be the only radical generated and involved in cell damage, since none of the isomers of BHB prevented completely ROS production. The antioxidant capacity of ketone bodies might also be attributed to other actions such as the stimulation of glutathione peroxidase activity (Ziegler et al., 2003), an increase in the CoQ/CoQH ratio with a subsequent reduction in free radical production (Sato et al., 1995; Kashiwaya et al., 1997), and a decrease in the NAD and NADP couples (Kashiwaya et al., 1997; Veech et al., 2001) favoring glutathione reduction. In addition, ketone bodies can reduce NADPH-oxidase activity decreasing $O_2^{\bullet-}$ production (Sato et al., 1992).

Consistent with *in vitro* observations, *in vivo* results show a reduction in lipoperoxidation in rats treated with either one of the BHB isomers. Since no conversion of L-BHB to the D-isomer was observed under our experimental conditions, results suggest that the effect of L-BHB is mainly due to its radical scavenging capacity. In the case of AcAc, no significant reduction of lipoperoxidation was observed probably because of its higher IC_{50} value for $^{\bullet}OH$. Lipoperoxidation was used as an index of early oxidative damage in hypoglycemic animals before neuronal death takes place as a consequence of the hypoglycemic coma; thus the scavenger capacity rather than the metabolic action of ketone bodies is evaluated in this condition. Our results are consistent with those of other authors suggesting increased oxidative stress in the hypoglycemic brain after insulin administration before the onset of coma (Patočková et al., 2003; Ballesteros et al., 2003; Singh et al., 2004; McGowan et al., 2006).

The beneficial effects of the ketogenic diet in the treatment of refractory epilepsy have been widely documented (Pan et al., 1999; Vining, 1999; Yudkoff et al., 2001; Nordli et al., 2001; Kossoff et al., 2002), but the mechanism involved remains elusive. The present results suggest that the $^{\bullet}OH$ scavenging capacity of D-BHB could play a role, since the plasma concentrations of ketone bodies after the ketogenic diet (3–5 mM) are close to the IC_{50} value of BHB for $^{\bullet}OH$. $^{\bullet}OH$ is known to initiate peroxidation, and both initiation and propagation of lipoperoxidation have been suggested to play a role in epileptogenesis (Willmore, 1990).

Ketone bodies were ineffective in preventing the increase in HNE-modified proteins observed during the hypoglycemic condition. Among all the byproducts of lipid peroxidation, HNE is the most reactive compound (Esterbauer et al., 1991). It is cytotoxic and capable of modifying the activity of several proteins (Petersen and Doorn, 2004), including glucose-6-phosphate dehydrogenase, GAPDH and glutathione reductase (Szweda et al., 1993; Uchida and Stadtman 1993; Vander Jagt et al., 1997). Here we show, for the first time, increased levels of HNE-protein adducts in the rat hippocampus during the hypoglycemic condition. The potential contribution of HNE to neurotoxicity is not well understood, in addition to its direct effect on proteins, an inhibitory effect on proteosome activity has been described leading to a reduced clearance of altered proteins followed by their accumulation (Friguet and Szweda, 1997; Okada et al., 1999; Shringarpure et al., 2000; Bulteau et al., 2001; Ferrington and Kapphahn, 2004). The lack of effect of ketone bodies on HNE levels might be related to the temporality of their production. Possibly, HNE-protein adducts are produced early after insulin injection, before ketone body administration and poorly cleared, limiting the effect of ketone bodies.

In conclusion, the present results show for the first time a direct ROS scavenging capacity of ketone bodies, providing new evidence about the mechanisms of neuroprotection of these molecules. Results are of relevance due to the therapeutic potentiality of ketone bodies against damage associated with diverse pathological conditions including hypoxia, ischemia, epilepsy and some neurodegenerative diseases.

Acknowledgments

This work was supported by IN213507 PAPIIT and 48645-Q CONACyT grants to L. Massieu; IN227103 PAPIIT and 48812 CONACyT grants to J. Pedraza-Chaverri. M.L. Haces and K. Hernández-Fonseca were supported by 181312 and 167146 CONACyT scholarships, respectively.

References

Adibhatla, M.R., Hatcher, J.F., 2006. Phospholipase A2, reactive oxygen species, and lipid peroxidation in cerebral ischemia. Free Radic. Biol. Med. 40, 376–387.

Ballesteros, J.R., Mishra, O.P., McGowan, J.E., 2003. Alterations in cerebral mitochondria during acute hypoglycemia. Biol. Neonate 84, 159–163.

Berridge, M.V., Tan, S., 1993. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch. Biochem. Biophys. 303, 474–482.

Bindokas, V.P., Jordán, J., Chong, C.L., Miller, R.J., 1996. Superoxide production in rats hippocampal neurons: selective imaging with hydroethidine. J. Neurosci. 16, 1324–1336.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Bulteau, A.L., Lundberg, K.C., Humphries, K.M., Sadek, H.A., Szweda, P.A., Friguet, B., Szweda, L.I., 2001. Oxidative modification and inactivation of the proteasome coronary during occlusion/reperfusion. J. Biol. Chem. 276, 30057–30063.

Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. 11, 1–128.

Ferrington, D.A., Kapphahn, R.J., 2004. Catalytic site-specific inhibition of the 20S proteasome by 4-hydroxynonenal. FEBS Lett. 578, 217–223.

Floriano-Sánchez, E., Villanueva, C., Medina-Campos, O.N., Rocha, D., Sánchez-González, D.J., Cárdenas-Rodríguez, N., Pedraza-Chaverri, J., 2006. Nordihydroguaiaretic acid is a potent in vitro scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion and hypochlorous acid and prevents in vivo ozone-induced tyrosine nitration in lungs. Free Radic. Res. 40, 523–533.

Friguet, B., Szweda, L.I., 1997. Inhibition of the multicatalytic proteinase (proteasome) by 4-hydroxy-2-nonenal cross-linked protein. FEBS Lett. 405, 21–25.

García, O., Massieu, L., 2001. Strategies for neuroprotection against L-trans-2, 4-pyrrolidine dicarboxylate-induced neuronal damage during energy impairment in vitro. J. Neurosci. Res. 64, 418–428.

Gluck, M.R., Jayatilleke, E., Shaw, S., Rowan, A.J., Haroutunian, V., 2000. CNS oxidative stress associated with kainic acid rodent model of experimental epilepsy. Epilepsy Res. 39, 63–71.

Gomes, A., Fernandes, E., Lima, J.L., 2005. Fluorescence probes used for detection of reactive oxygen species. J. Biochem. Biophys. Methods 65, 45–80.

Hawkins, R.A., Williamson, D.H., Krebs, H.A., 1971. Ketone-body utilization by adult and suckling rat brain in vivo. Biochem. J. 122, 13–18.

Hawkins, R.A., Biebuyck, J.F., 1979. Ketone bodies are selectively used by individual brain regions. Science 205, 325–327.

Hernández-Fonseca, K., Massieu, L., 2005. Disruption of endoplasmic reticulum calcium stores is involved in neuronal death induced by glycolysis inhibition in cultured hippocampal neurons. J. Neurosci. Res. 82, 196–205.

Hernández-Fonseca, K., Cárdenas-Rodríguez, N., Pedraza-Chaverri, J., Massieu, L., in press. Calcium-dependent production of reactive oxygen species is involved in neuronal damage induced during glycolysis inhibition in cultured hippocampal neurons. J. Neurosci. Res. doi:10.1002/jnr.21634.

Hockenbery, D.M., Oltvai, Z.N., Yin, X.-M., Milliman, C.L., Korsmeye, S.J., 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75, 241–251.

Izumi, Y., Ishii, K., Katsuki, H., Benz, A.M., Zorumski, C.F., 1998. beta-Hydroxybutyrate fuels synaptic function during development. Histological and physiological evidence in rat hippocampal slices. J. Clin. Invest. 101, 1121–1132.

Jain, S.K., Kannan, K., Lim, G., 1998. Ketosis (acetoacetate) can generate oxygen radicals and cause increased lipid peroxidation and growth inhibition in human endothelial cells. Free Radic. Biol. Med. 25, 1083–1088.

Jain, S.K., McVie, R., 1999. Hyperketonemia can increase lipid peroxidation and lower glutathione levels in human erythrocytes in vitro and in type I diabetic patients. Diabetes 48, 1850–1855.

Kashiwaya, Y., King, M.T., Veech, R.L., 1997. Substrate signaling by insulin: a ketone bodies ratio mimics insulin action in heart. Am. J. Cardiol. 80, 50A–64A.

Kashiwaya, Y., Takeshima, T., Mori, N., Nakashima, K., Clarke, K., Veech, R.L., 2000. D-beta-Hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. Proc. Natl. Acad. Sci. U. S. A. 97, 5440–5444.

Kim, Y., Davis, L.M., Sullivan, P.G., Maalouf, M., Simeone, T.A., van Brederode, J., Rho, J.M., 2007. Ketone bodies are protective against oxidative stress in neocortical neurons. J. Neurochem. 101, 1316–1326.

Kossoff, E.H., Pyzik, P.L., McGrogan, J.R., Vining, E.P., Freeman, J.M., 2002. Efficacy of the ketogenic diet for infantile spasms. Pediatrics 109, 780–783.

Lincoln, B.C., Des Rosiers, C., Brunengraber, H., 1987. Metabolism of S-3-hydroxybutyrate in the perfused rat liver. Arch. Biochem. Biophys. 259, 149–156.

Love, S., 1999. Oxidative stress in brain ischemia. Brain Pathol. 9, 119–131.

Maalouf, M., Sullivan, P.G., Davis, L., Kim, D.Y., Rho, J.M., 2007. Ketones inhibit mitochondrial production of reactive oxygen species production following glutamate excitotoxicity by increasing NADH oxidation. Neuroscience 145, 256–264.

Malcolm, C.S., Benwell, K.R., Lamb, H., Bebbington, D., Porter, R.H., 2000. Characterization of iodoacetate-mediated neurotoxicity in vitro using primary cultures of rat cerebellar granule cells. Free Radic. Biol. Med. 28, 102–107.

Massieu, L., Del Río, P., Montiel, T., 2001. Neurotoxicity of glutamate uptake inhibition in vivo: correlation with succinate dehydrogenase activity and prevention by energy substrates. Neuroscience 106, 669–677.

Massieu, L., Haces, M.L., Montiel, T., Hernández-Fonseca, K., 2003. Acetoacetate protects hippocampal neurons against glutamate-mediated neuronal damage during glycolysis inhibition. Neuroscience 120, 365–378.

Masuda, R., Monahan, J.W., Kashiwaya, Y., 2005. D-beta-hydroxybutyrate is neuroprotective against hypoxia in serum-free hippocampal primary cultures. J. Neurosci. Res. 80, 501–509.

McGowan, J.E., Chen, L., Gao, D., Trush, M., Wei, C., 2006. Increased mitochondrial reactive oxygen species production in newborn brain during hypoglycemia. Neurosci. Lett. 399, 111–114.

Medina-Campos, O.N., Barrera, D., Segoviano-Murillo, S., Rocha, D., Maldonado, P.D., Mendoza-Patino, N., Pedraza-Chaverri, J., 2007. S-allylcysteine scavenges singlet oxygen and hypochlorous acid and protects LLC-PK(1) cells of potassium dichromate-induced toxicity. Food Chem. Toxicol. 45, 2030–2039.

Mejía-Toiber, J., Montiel, T., Massieu, L., 2006. D-β-Hydroxybutyrate prevents glutamate-mediated lipoperoxidation and neuronal damage elicited during glycolysis inhibition in vivo. Neurochem. Res. 31, 1399–1408.

Mellanby, J., Williamson, D.H., 1965. Acetoacetate. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 454–457.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assay. J. Immunol. Methods 65, 55–63.

Nehlig, A., Pereira de Vasconcelos, A., 1993. Glucose and ketone bodies utilization by the brain of neonatal rats. Prog. Neurobiol. 40, 163–221.

Noh, H.S., Hah, Y.S., Nilufar, R., Han, J., Bong, J.H., Kang, S.S., Cho, G.J., Choi, W.S., 2006. Acetoacetate protects neuronal cells from oxidative glutamate toxicity. J. Neurosci. Res. 83, 702–709.

Nordli, D.R., Kuroda, M.M., Carroll, J., Koenigsberger, D.Y., Hirsch, L.J., Bruner, H.J., Seidel, W.T., De Vivo, D.C., 2001. Experience with ketogenic diet in infants. Pediatrics 108, 129–133.

Okada, K., Wangpoengtrakul, C., Osawa, T., Toyokuni, S., Tanaka, K., Uchida, K., 1999. 4-Hydroxy-2-nonenal-mediated impairment of intracellular proteolysis during oxidative stress. Identification of proteasomes as target molecules. J. Biol. Chem. 274, 23787–23793.

Owen, O.E., Morgan, A.P., Kemp, H.G., Sullivan, J.M., Herrera, M.G., Cahill Jr., G.F., 1967. Brain metabolism during fasting. J. Clin. Invest. 46, 1589–1595.

Pan, J.W., Bebin, E.M., Chu, W.J., Hetherington, H.P., 1999. Ketosis and epilepsy: 31P spectroscopic imaging at 4.1 T. Epilepsia 40, 703–707.

Patočková, J., Marhol, P., Tumova, E., Krsiak, M., Rokyta, R., Stipek, S., Crkovska, J., Andel, M., 2003. Oxidative stress in the brain tissue of laboratory mice with acute post insulin hypoglycemia. Physiol. Res. 52, 131–135.

Pedraza-Chaverri, J., Medina-Campos, O.N., Segoviano-Murillo, S., 2007. Effect of heating on peroxynitrite scavenging capacity of garlic. Food Chem. Toxicol. 45, 622–627.

Petersen, D.R., Doorn, J.A., 2004. Reactions of 4-hydroxynonenal with proteins and cellular targets. Free Radic. Biol. Med. 37, 937–945.

Rego, A.C., Santos, M.S., Oliveira, C.R., 1997. Adenosine triphosphate degradation products after oxidative stress and metabolic dysfunction in cultured retinal cells. J. Neurochem. 69, 1228–1235.

Rego, A.C., Santos, M.S., Oliveira, C.R., 1999. Influence of the antioxidants vitamin E and idebenone on retinal cell injury mediated by chemical ischemia, hypoglycemia, or oxidative stress. Free Radic. Biol. Med. 26, 1405–1417.

Robinson, K.M., Janes, M.S., Pehar, M.P., Monette, J.S., Ross, M.F., Hagen, T.M., Murphy, M.P., Beckman, J.S., 2006. Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. Proc. Natl. Acad. Sci. 103, 15038–15043.

Sato, N., Shimizu, H., Shimomura, Y., Suwa, K., Mori, M., Kobayashi, I., 1992. Mechanism of inhibitory action of ketone bodies on the production of reactive oxygen intermediates (ROIS) by polymorphonuclear leukocytes. Life Sci. 51, 113–118.

Sato, K., Kashiwaya, Y., Keon, C.A., Tsuchiya, N., King, M.T., Radda, G.K., Chance, B., Clarke, K., Veech, R.L., 1995. Insulin, ketone bodies, and mitochondrial energy transduction. FASEB J. 9, 651–658.

Shringarpure, R., Grune, T., Sitte, N., Davies, K.J., 2000. 4-Hydroxynonenal-modified amyloid-beta peptide inhibits the proteasome: possible importance in Alzheimer's disease. Cell. Mol. Life Sci. 57, 1802–1809.

Singh, P., Jain, A., Kaur, G., 2004. Impact of hypoglycemia and diabetes on CNS: correlation of mitochondrial oxidative stress with DNA damage. Mol. Cell. Biochem. 260, 153–159.

Smith, S.L., Heal, D.J., Martin, K.F., 2005. KTX 0101: a potential metabolic approach to cytoprotection in major surgery and neurological disorders. CNS Drug Rev. 11, 113–140.

Sperling, O., Bromberg, Y., Oelsner, H., Zoref-Shani, E., 2003. Reactive oxygen species play an important role in iodacetate-induced neurotoxicity in primary rat neuronal cultures and in differentiated PC12 cells. Neurosci. Lett. 351, 137–140.

Suh, S.W., Aoyama, K., Chen, Y., Garnier, P., Matsumori, Y., Gum, E., Liu, J., Swanson, R.A., 2003. Hypoglycemic neuronal death and cognitive impairment are prevented by poly(ADP-ribose) polymerase inhibitors administered after hypoglycemia. J. Neurosci. 23, 10681–10690.

Suh, S.W., Gum, E.T., Hamby, A.M., Chan, P.H., Swanson, R.A., 2007. Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. J. Clin. Invest. 117, 910–918.

Suzuki, M., Suzuki, M., Sato, K., Dohi, Sato, T., Matsuura, A., Hiraide, A., 2001. Effect of beta-hydroxybutyrate, a cerebral function improving agent, on cerebral hypoxia, anoxia and ischemia in mice and rats. Jpn. J. Pharmacol. 87, 143–150.

Suzuki, M., Suzuki, M., Kitamura, Y., Mori, S., Sato, K., Dohi, S., Sato, T., Matsuura, A., Hiraide, A., 2002. β-hydroxybutyrate, a cerebral function improving agent, protects rat brain against ischemic damage caused by permanent and transient focal cerebral ischemia. Jpn. J. Pharmacol. 89, 36–43.

Szweda, L.I., Uchida, K., Tsai, L., Stadtman, E.R., 1993. Inactivation of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. Selective modification of an active-site lysine. J. Biol. Chem. 268, 3342–3347.

Tieu, K., Perier, C., Caspersen, C., Teismann, P., Wu, D.C., Yan, S.D., Naini, A., Vila, M., Jackson-Lewis, V., Ramasamy, R., Przedborski, S., 2003. D-beta-hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease. J. Clin. Invest. 112, 892–901.

Tomizawa, S., Imai, H., Tsukada, S., Simizu, T., Honda, F., Nakamura, M., Nagano, T., Urano, Y., Matsuoka, Y., Fukasaku, N., Saito, N., 2005. The detection and quantification of highly reactive oxygen species using the novel HPF fluorescence probe in a rat model of focal cerebral ischemia. Neurosci. Res. 53, 304–313.

Uchida, K., Stadtman, E.R., 1993. Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase. A possible involvement of intra- and intermolecular cross-linking reaction. J. Biol. Chem. 268, 6388–6393.

Uto, A., Dux, E., Kusumoto, M., Hossmann, K.A., 1995. Delayed neuronal death after brief histotoxic hypoxia in vitro. J. Neurochem. 64, 2185–2192.

Vander Jagt, D.L., Hunsaker, L.A., Vander Jagt, T.J., Gomez, M.S., Gonzales, D.M., Deck, L.M., Royer, R.E., 1997. Inactivation of glutathione reductase by 4-hydroxynonenal and other endogenous aldehydes. Biochem. Pharmacol. 53, 1133–1140.

Veech, R.L., Chance, B., Kashiwaya, Y., Lardy, H.A., Cahill Jr., G.F., 2001. Ketone bodies, potential therapeutic uses. IUBMB Life 51, 241–247.

Vining, E.P.G., 1999. Clinical efficacy of the ketogenic diet. Epilepsy Res. 37, 181–190.

Williamson, D.H., Mellanby, J., 1965. D(-)-β-hydroxybutyrate. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic press, New York, pp. 459–461.

Willmore, L.J., 1990. Post-traumatic epilepsy: cellular mechanisms and implications for treatment. Epilepsia 31, S67–S73.

Yamada, K.A., Rensing, N., Thio, L.L., 2005. Ketogenic diet reduces hypoglycemia-induced neuronal death in young rats. Neurosci. Lett. 385, 210–214.

Yudkoff, M., Daikhin, Y., Nissim, I., Lazarow, A., Nissim, I., 2001. Ketogenic diet, amino acid metabolism and seizure control. J. Neurosci. Res. 66, 931–940.

Zeevalk, G.D., Nicklas, W.J., 1990. Chemically induced hypoglycemia and anoxia: relationship to glutamate receptor-mediated toxicity in retina. J. Pharmacol. Exp. Ther. 253, 1285–1292.

Zhao, H., Kalivendi, S., Zhang, H., Joseph, J., Nithipatikom, K., Vásquez-Vivar, J., Kalyanaraman, B., 2003. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. Free Radic. Biol. Med. 34, 1359–1368.

Ziegler, D.R., Ribeiro, L.C., Hagenn, M., Siqueira, I.R., Araújo, E., Torres, I.L., Gottfried, C., Netto, C.A., Gonçalves, C.A., 2003. Ketogenic diet increases glutathione peroxidase activity in the rat hippocampus. Neurochem. Res. 28, 1793–1797.

XII. DISCUSIÓN

Diversos estudios han relacionado al estrés oxidativo con el daño neuronal inducido por isquemia cerebral *in vivo*, o por privación de oxígeno y glucosa en cultivos neuronales (Oliver et al., 1990; Li et al., 1999; Candelario-Jalil et al., 2001; Almeida et al., 2002; Abramov et al., 2007). Estudios previos *in vitro* (Uto et al., 1995; Rego et al., 1999; Malcolm et al., 2000) e *in vivo* (Matthews et al., 1997) sugieren que el daño neuronal provocado por yodoacetato está asociado a la producción de EROS. Sin embargo, la relación entre la activación del receptor NMDA, el incremento en la concentración de Ca^{2+} intracelular y la generación de EROS posterior a la inhibición glucolítica con yodoacetato, no se había estudiado hasta el momento.

Previamente se había reportado que el tratamiento con vitamina E previene eficientemente la muerte neuronal inducida por yodoacetato, sugiriendo la participación del estrés oxidativo en la cascada de muerte celular disparada por el déficit energético (Uto et al., 1995; Malcolm et al., 2000; Rego et al., 1999). Los resultados del presente trabajo apoyan esta hipótesis, ya que se observó un incremento en la producción de EROS después de la exposición al yodoacetato, el cual depende de la severidad del déficit energético. Durante la exposición a 100 µM de yodoacetato, la producción de EROS fue significativamente mayor y más rápida, que la observada durante la exposición a yodoacetato 50 µM.

La producción de EROS es altamente dependiente de la presencia de Ca^{2+} extracelular y de la activación del receptor a NMDA en la condición de 50 µM, apuntando a que la entrada de Ca^{2+} a través de estos receptores promueve la generación de EROS. El efecto inhibidor del MK-801 sobre el incremento en la concentración de Ca^{2+} intracelular y la producción de EROS apoya esta hipótesis. Además, el Bapta-AM reduce eficientemente la señal fluorescente del Et y la carboxi-DCF, sugiriendo la contribución del Ca^{2+} intracelular a la generación de EROS.

Por otra parte, en estudios previos hemos observado que la exposición a 50 µM de yodoacetato aumenta sólo ligeramente la concentración extracelular de

glutamato y aspartato, lo que sugiere que en esta condición, la activación de los receptores a NMDA podría ser la consecuencia un mecanismo excitotóxico secundario, resultante de la falla energética (Novelli et al., 1988; Zeevalk y Nicklas, 1992).

En contraste con la condición de yodoacetato 50 μM, la producción de EROS durante la exposición a 100 μM de yodoacetato no fue sensible al MK-801, y sólo parcialmente reducida en ausencia de Ca^{2+} extracelular. Por el contrario, fue eficientemente inhibida por vitamina E y Bapta-AM. Estas observaciones concuerdan con la falta de efecto del MK-801 sobre el aumento en la concentración intracelular de Ca^{2+} observada en estas condiciones. Los resultados apuntan a que en condiciones de deficiencia energética abrupta y severa, la entrada de Ca^{2+} a través de los receptores a NMDA no está involucrada en la producción de EROS, pero sí la falla en los sistemas de regulación de Ca^{2+} intracelular.

Los presentes resultados muestran que en condiciones de inhibición glucolítica tanto moderada como severa, la producción de EROS es eficientemente reducida por el tratamiento con los cuerpos cetónicos, AcAc y el isómero fisiológico del β-HB, los cuales evitaron la disminución de los niveles de ATP y la reducción del MTT. Estas observaciones sugirieren que la deficiencia energética promueve la generación de EROS. Este resultado concuerda con otros estudios que muestran que el tratamiento con cuerpos cetónicos previene la muerte neuronal asociada con la hipoxia, la isquemia (Suzuki et al., 2001,2002; Masuda et al., 2005), la excitotoxicidad inducida durante la inhibición metabólica (García y Massieu, 2001; Massieu et al., 2003; Mejía-Toiber et al., 2006) y en modelos de las enfermedades de Alzheimer y Parkinson (Kashiwaya et al., 2000; Tieu et al., 2003).

El efecto del AcAc y del D-β-HB podría ser resultado de la suplementación metabólica mitocondrial a través de su transformación a acetil-CoA restaurando los niveles de ATP. Los sistemas dependientes de ATP responsables de la captura y extrusión del Ca^{2+} intracelular podrían estar alterados durante la inhibición glucolítica severa y contribuir al incremento en la concentración de

Ca^{2+} intracelular. En estas condiciones, el atrapamiento del Ca^{2+} intracelular libre con Bapta-AM reduce la producción de EROS y la muerte celular, sugiriendo la falla de los sistemas responsables del mantenimiento de la homeostasis intracelular de Ca^{2+} durante un déficit energético severo. Es posible que los cuerpos cetónicos al suplementar el metabolismo mitocondrial pudieran contribuir a restaurar la homeostasis de Ca^{2+} intracelular.

La participación de EROS en el proceso de muerte neuronal es evidenciada por el efecto neuroprotector de la vitamina E y del ácido nordihidroguaiaretico (NDGA). El NDGA es un potente atrapador de $^{\bullet}OH$ (principalmente), $ONOO^{-}$, $^{1}O_2$, $O_2^{\bullet-}$ y ácido hipocloroso (HOCl) (Floriano-Sánchez et al., 2006). Un estudio previo *in vivo* utilizando un atrapador de $^{\bullet}OH$, sugirió la producción de este radical después de la inyección de yodoacetato en el estriado (Matthews et al., 1997). A su vez, se ha observado que el tratamiento con NDGA en ratas diabéticas incrementa significativamente los niveles de GSH, la actividad de la superóxido dismutasa y de la catalasa, y reduce la producción de malondialdehído (Anjaneyulu y Chopra, 2004). La vitamina E no alteró el efecto inhibidor del yodoacetato sobre la actividad de la GAPDH, y ni de la GPx. Tampoco modificó la disminución de los niveles de ATP inducida por yodoacetato. Estos resultados sugieren que la protección de la vitamina E se debe principalmente a su actividad antioxidante. Además de su efecto lipofílico antioxidante de atrapador de radicales en la membrana celular, el tocoferol puede reaccionar directamente con varias especies reactivas de oxígeno, incluyendo $^{1}O_2$, radical alkoxil, $ONOO^{-}$, dióxido de N_2, ozono y $O_2^{\bullet-}$ (Wang y Quinn, 1999).

Estos resultados nos permiten concluir que la producción de EROS durante la inhibición glucolítica es un factor determinante en la cascada metabólica asociada en la muerte neuronal. Esta observación es relevante ya que la participación del estrés oxidativo en el daño neuronal se ha sugerido en el caso de la isquemia cerebral; sin embargo en la mayoría de los modelos de isquemia existe reperfusión, lo cual induce la generación de EROS después del incremento abrupto en los niveles de oxígeno. En el caso de la hipoglucemia, la

tensión de O_2 no cambia y sin embargo hay producción de EROS, de aquí que es importante identificar los mecanismos de generación de EROS y su participación en la muerte neuronal.

Los resultados que reportamos descartan que la producción de EROS se debe a un posible efecto alquilante del yodoacetato sobre algunas enzimas de la defensa antioxidante, ya que no modificó la actividad de la catalasa, la glutatión reductasa ni de la glutatión S-transferasa. Únicamente inhibió en aproximadamente un 30% la actividad de la glutatión peroxidasa, pudiendo contribuir a la acumulación de H_2O_2. La identificación de otros mecanismos que llevan a la producción de EROS en las presentes condiciones experimentales requiere de estudios adicionales. La participación de sistemas productores de EROS activados por calcio como la óxido nítrico sintasa, la fosfolipasa A_2 y el subsecuente metabolismo del ácido araquidónico que conlleva a la producción de $O_2^{\bullet -}$; la actividad de la xantina oxidasa, la participación de EROS mitocondriales y la producción de especies reactivas de nitrógeno (ERNS), no fueron estudiados en el presente trabajo.

Con base en los presentes resultados podemos concluir que los mecanismos responsables del incremento en la concentración de Ca^{2+} intracelular en condiciones de falla energética severa y moderada, no son idénticos. Durante la inhibición glucolítica moderada la producción de EROS y la muerte neuronal están asociadas a la entrada de Ca^{2+} a través de los receptores NMDA, sugiriendo un mecanismo excitotóxico. Por el contrario, durante la inhibición glucolítica severa la muerte neuronal y la producción de EROS no están relacionadas con la activación de los receptores a NMDA, pero sí con el incremento en la concentración de Ca^{2+} intracelular. De allí que el MK-801 y el medio libre de Ca^{2+} son menos efectivos en reducir la generación de EROS y el daño neuronal inducido durante inhibición glucolítica severa. En esta última condición, la liberación de Ca^{2+} de pozas intracelulares podría tener una participación importante. Estudios previos indican que el bloqueo de la salida de Ca^{2+} del retículo endoplásmico con dantroleno previene la muerte neuronal, mientras que la inducción de su liberación, la exacerba (Hernández-Fonseca y

Massieu, 2005). Sería interesante conocer si la falla en la regulación del Ca^{2+} por el retículo endoplásmico, y de los sistemas responsables de la extrusión de Ca^{2+} intracelular, como la ATPasa de Ca^{2+} membranal y el intercambiador Na^{+}/Ca^{2+}, sistemas que son directa o indirectamente dependientes de energía, influyen en la generación de EROS.

El efecto neuroprotector del β-HB hasta el momento se ha atribuido a su acción metabólica suplementando el metabolismo mitocondrial al ser transformado en el cerebro a acetil-CoA, a través de la enzima mitocondrial β-HB deshidrogenasa. De la misma manera, se ha considerado que el isómero D- era el único con efectos neuroprotectores en el cerebro, ya que el isómero L- es inerte metabólicamente. Estudios recientes han demostrado que el β-HB es capaz de contrarrestar la producción de EROS inducida por H_2O_2 (Maalouf et al., 2007; Kim et al., 2007). En este trabajo observamos que el isómero no metabolizable del β-HB, el L-β-HB, es capaz de prevenir el daño oxidativo independientemente de un efecto metabólico, ya que no tiene efecto alguno sobre los niveles de ATP. El mecanismo por el cual los cuerpos cetónicos atrapan directamente las EROS aún no está claro. Es posible que los grupos hidroxilo presentes en L- y el D-β-HB contribuyan a su capacidad atrapadora. De hecho, la capacidad de atrapar el $^{\bullet}OH$ del L-β-HB y D-β-HB fue claramente mayor que la del AcAc, el cual carece de grupos hidroxilo. Por otra parte, se ha sugerido la producción de $^{\bullet}OH$ por yodoacetato en un estudio *in vivo* (Matthews et al., 1997); nuestros resultados apoyan esta hipótesis ya que tanto el NDGA y como el β-HB, que atrapan eficientemente esta especie, muestran un efecto protector importante. El efecto protector del acetoacetato contra la producción de EROS y la muerte neuronal podría atribuirse a su rápida transformación a acetil-CoA en la mitocondria restaurando eficientemente los niveles de ATP. Su efecto atrapador de EROS, aunque es pobre también podría contribuir a la protección de la muerte.

XIII. CONCLUSIONES

Basándonos en los presentes resultados podemos concluir que la producción de EROS no es consecuencia de la inhibición de las enzimas antioxidantes, glutatión reductasa, glutatión-S-transferasa y catalasa por el yodoacetato. La inhibición de la GPX por yodoacetato podría contribuir al estrés oxidativo por la acumulación de H_2O_2, la cual a su vez podría favorecer la producción de $^{\bullet}OH$. El o los mecanismos de producción de EROS durante la inhibición de la glucólisis no fueron identificados en este trabajo; sin embargo, los datos reportados contribuyen al conocimiento de los mecanismos responsables de muerte celular producida en condiciones de falla bioenergética, la cual se asocia con la isquemia y la hipoglucemia.

Los resultados sugieren la participación de diferentes mecanismos involucrados en la muerte neuronal dependiendo de la severidad del déficit energético. Durante la inhibición glucolítica moderada un mecanismo excitotóxico está claramente involucrado tanto en la generación de EROS como en la muerte, mientras que durante un déficit energético severo, el estrés oxidativo tiene un papel preponderante en la muerte y la producción de EROS está aparentemente relacionada con la falla en la regulación de la homeostasis de calcio intracelular. El efecto del Ca^{2+} sobre la producción de EROS podría explicarse por la activación de enzimas dependientes de calcio productoras de radicales como la xantina oxidasa, la óxido nítrico sintasa y fosfolipasa A_2. La producción de EROS mitocondriales también podría tener una papel importante. Los posibles mecanismos que participan en la generación de EROS y en la muerte neuronal se esquematizan en la figura 10.

Por otra parte, los presentes resultados muestran la capacidad antioxidante de los cuerpos cetónicos, dando nuevas evidencias acerca de los mecanismos de neuroprotección de estas moléculas. Podemos concluir que la acción metabólica del D- β-hidroxibutirato y del acetoacetato pueden reducir la producción de EROS a través del mantenimiento del metabolismo mitocondrial,

mientras que en el caso del el D- β-HB la acción protectora también estaría mediada por su efecto como atrapador de EROS.

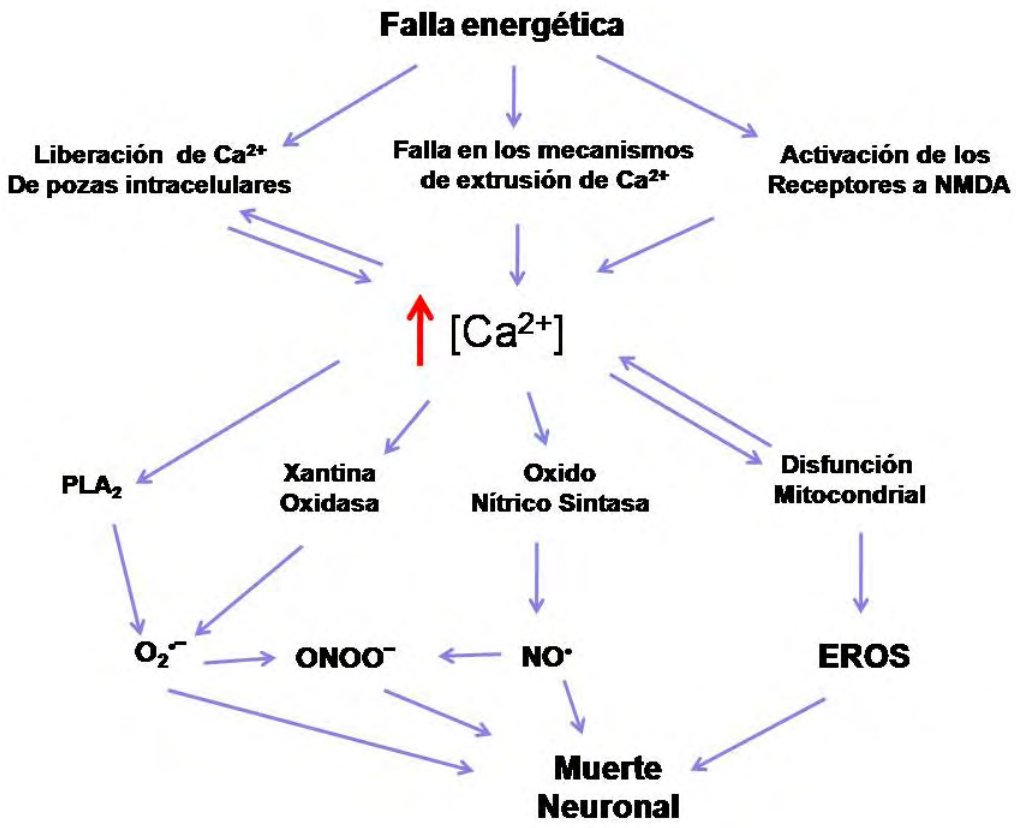


Figura 10. Posibles mecanismos involucrados en la muerte neuronal inducida por falla energética. El déficit energético induce un aumento de la concentración intracelular de Ca^{2+} debido a la activación de los receptores a NMDA, y a la falla en los mecanismos de homeostasis de Ca^{2+} intracelular (liberación de Ca^{2+} de pozas intracelulares y falla en los mecanismos de extrusión de Ca^{2+} membranales). La sobrecarga de Ca^{2+} induce la activación de diversas enzimas productoras de EROS, como la fosfolipasa A_2 (PLA_2), la xantina oxidasa y la oxido nítrico sintasa. Así mismo, induce una sobre carga de calcio mitocondrial, llevando a la disfunción mitocondrial, la producción de EROS mitocondriales, y a un subsecuente estado de estrés oxidativo. Tanto el incremento en la concentración de Ca^{2+} intracelular, como la producción de EROS participan en la cadena de muerte neuronal.

XIV. REFERENCIAS

Abramov AY, Scorziello A, Duchen MR. 2007. Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. J Neurosci, 27:1129-1138.

Abramson JJ, Salama G. 1988. Sulfhydryl oxidation and Ca^{2+} release from sarcoplasmic reticulum. Mol Cell Biochem, 82:81-84.

Allen KL, Almeida A, Bates TE, Clark JB. 1995. Effect of reperfusion following cerebral ischemia on the activity of the mitochondrial respiratory chain in the gerbil brain. J Neurochem, 65:1698-1703.

Allen NJ, Rossi DJ, Attwell, D. 2004. Sequential release of GABA by exocytosis and reversed uptake leads to neuronal swelling in simulated ischemia of hippocampal slices. J Neurosci, 24:3837-3849.

Almeida A, Delgado-Esteban M, Bolaños JP, Medina JM. 2002. Oxygen and glucose deprivation induces mitochondrial dysfunction and oxidative stress in neurons but not in astrocytes in primary culture. J Neurochem, 81:207-217.

Alt A, Weiss B, Ogden AM, Knauss JL, Oler J, Ho K, Large TH, Bleakman D. 2004. Pharmacological characterization of glutamatergic agonists and antagonists at recombinant human homomeric and heteromeric kainate receptors in vitro. Neuropharmacology, 46:793-806.

Anjaneyulu M, Chopra K. 2004. Nordihydroguaiaretic acid, a lignin, prevents oxidative stress and the development of diabetic nephropathy in rats. Pharmacology 72:42-50.

Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera P. 1995. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron, 15:961-973.

Arriza JL, Eliasof S, Kavanaugh MP, Amara SG. 1997. Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proc Natl Acad Sci U S A, 94:4155-4160.

Attwell D, Laughling SB. 2001. An energy budget for signaling in the grey matter of the brain. J. Cereb. Blood Flow Metab, 21:1133-1145.

Ballesteros JR, Mishra OP, McGowan JE. 2003. Alterations in cerebral mitochondria during acute hypoglycemia. Biol Neonate, 84,159-163.

Beal MF, Brouillet E, Jenkins BG, Ferrante RJ, Kowall NW, Miller JM, Storey E, Srivasrava R, Rosen BR, Hyman BT. 1993. Neurochemical and histologic

characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acids. J Neurosci, 13:4181-4192.

Benveniste H, Jorgensen MB, Sanberg M, Christenten B, Hangberg H, Diemer NH. 1989. Ischemic damage in hippocampal CA1 is depending on glutamate release and intact innervation from CA3. J Cereb Blood Flow Metab, 9:629-693.

Bhardwaj SK, Sharma ML, Gulati G, Chhbra A, Kaushik R, Sharma P. 1998, Effect of starvation and insulin-induced hypoglycemia on oxidative stress scavenger system and electron transport chain complexes from rat brain, liver, and kidney. Mol Chem Neuropatol, 34:157-168.

Birnbaum MJ, Haspel HC, Rosen OM. 1986. Cloning and characterization of a cDNA encoding the rat brain glucose-transporter protein. Proc Natl Acad Sci U S A, 83:5784-5788.

Bleakman D, Gates MR, Ogden A M, Mackowiak M. 2002. Kainate receptor agonists, antagonists and allosteric modulators. Curr Pharm, 8:873-885.

Bondy SC, Lee DK. 1993. Oxidative stress induced by glutamate receptor agonists. Brain Res, 610:229-233.

Bough KJ, Yao SG, Eagles DA. 2000. Higher ketogenic diet ratios confer protection from seizures without neurotoxicity. Epilepsy Res, 38:15-25.

Bowie D, Mayer ML. 1995. Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. Neuron, 15:453-462.

Broer S, Brookes N. 2001. Transfer of glutamine between astrocytes and neurons. J Neurochem, 77:705-719.

Budd SL, Nicholls DG. 1996. Mitochondrial calcium regulation and cute glutamate excitotoxicity in cultured cerebellar granule cells. J. Neurochem, 67:2282-2291.

Burke JR, Enghild JJ, Martin ME, Jou Y-S, Myers RM, Roses AD, Vance JM, Strittmatter WJ. 1996. Huntingtin and DRPLA proteins selectively interact with the enzyme GAPDH, Nature Med, 2:347-350.

Bustamante E, Pedersen PL. 1980. Mitochondrial hexokinase of rat hepatoma cells in culture: solubilization and kinetic properties. Biochemistry, 19:4972-4977.

Camacho A, Montiel T, Massieu L. 2007. Sustained metabolic inhibition induces an increase in the content and phosphorylation of the NR2B subunit of N-methyl-D-aspartate receptors and a decrease in glutamate transport in the rat hippocampus in vivo. Neuroscience, 145,873-886.

Candelario-Jalil E, Mhadu NH, Al-Dalain SM, Martínez G, León OS. 2001. Time course of oxidative damage in different brain regions following transient cerebral ischemia in gerbils. Neurosci Res, 41:233-241.

Canevari L, Kuroda S, Bates TE, Clark JB., Siesjo BK. 1997 Mitochondrial dysfunction after transient focal ischaemic in the rat is not related to a decreased activity of respiratory chain enzymes. J Cereb Blood Flow Meta, 17:1166-1169.

Castilho RF, Ward MW, Nicholls DG. 1999. Oxidative stress, mitochondrial function, and acute glutamate excitotoxicity in cultured cerebellar granule cells. J Neurochem. 72:1394-1401.

Cecchini G. 2003. Function and structure of complex II of the respiratory chain. Annu Rev Biochem, 72:77-109.

Chan PH, Fishman RA. 1982. Alterations of membrane integrity and cellular constituents by arachidonic acid in neuroblastoma and glioma cells. Brain Res, 248:151-157.

Chance B. 1977. Electron transfer: pathways, mechanisms, and controls. Annu Rev Biochem, 46:967-980.

Chen JC, Hsu-Chou H, Lu JL, Chiang YC, Huang HM, Wang HL, Wu T, Liao JJ, Yeh TS. 2005. Down-regulation of the glial glutamate transporter GLT-1 in rat hippocampus and striatum and its modulation by a group III metabotropic glutamate receptor antagonist following transient global forebrain ischemia. Neuropharmacology, 49:703-714.

Choi DW. 1988. Glutamate neurotoxicity and diseases of the nervous system. Neuron, 1:623-634.

Chuang DM, Hough C, Senatorov VV. 2005. Glyceraldehyde-3-phosphate dehydrogenase, apoptosis, and neurodegenerative diseases. Annu Rev Pharmacol Toxicol, 45:269–290.

Collingridge GL, Lester RA. 1989. Excitatory amino acid receptors in the vertebrate central nervous system. Pharmacol Rev, 41:143-210.

Colussi C, Albertini MC, Coppola S, Rovidati S, Galli F, Ghibelli L. 2000. H2O2-induced block of glycolysis as an active ADP-ribosylation reaction protecting cells from apoptosis. FASEB J, 14:2266-2276.

Contractor A, Swanson GT, Sailer A, O'Gorman S, Heinemann SF. 2000. Identification of the kainate receptor subunits underlying modulation of excitatory synaptic transmission in the CA3 region of the hippocampus. J Neurosci, 20:8269-8278

Coyle JT, Puttfarcken P. 1993. Oxidative stress, glutamate, and neurodegenerative disorders. Science, 62:689-95.

Crofts AR. 2004. The cytochrome bc1 complex: function in the context of structure. Annu Rev Physiol, 66:689-733.

Cull-Candy SG, Leszkiewicz DN. 2004. Role of distinct NMDA receptor subtypes at central synapses. Sci STKE, 255:re16.

Curtis DR, Watkins JC. 1960. The excitation and depression of spinal neurones by structurally related amino acids. J Neurochem, 6:117-141.

Danbolt NC. 2001. Glutamate uptake. Prog Neurobiol, 65:1-105.

Duelli R, Kuschinsky W. 2001. Brain glucose transporters: relationship to local energy demand. News Physiol Sci, 16:71–6

Dunaway GA. 1983. A review of animal phosphofructokinase isozymes with an emphasis on their physiological role. Mol Cell Biochem, 52:75-91.

Dwyer DS, Vannucci SJ, Simpson IA. 2002. Expression, regulation, and functional role of glucose transporters (GLUTs) in brain. Int Rev Neurobiol, 51:159–88.

Erecinska M, Cherian S, Silver IA. 2004. Energy metabolism in mammalian brain during development. Prog Neurobiol, 73:397-445.

Erecinska M, Silver IA. 1990. Metabolism and role of glutamate in mammalian brain. Prog Neurobiol, 35:245-296.

Fairman WA, Vandenberg RJ, Arriza JL, Kavanaugh MP, Amara SG. 1995. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. Nature, 375:599-603.

Floriano-Sánchez E, Villanueva C, Medina-Campos ON, Rocha D, Sánchez-González DJ, Cárdenas-Rodríguez N, Pedraza-Chaverrí J. 2006. Nordihydroguaiaretic acid is a potent in vitro scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion and hypochlorous acid and prevents in vivo ozone-induced tyrosine nitration in lungs. Free Radic Res, 40:523-533.

Fonnum F. 1984. Glutamate: a neurotransmitter in mammalian brain. J Neurochem, 42:1-11.

Foster AC, Gill R. Woodruff. 1988. Neuroprotective effects of MK-801 in vivo: selectivity and evidence for delayed degeneration mediated by NMDA receptor activation. J Neurosci, 8:4745-4754.

Freeman JM, Vining EPG, Pillas DJ et al 1998. The efficacy of the ketogenic diet-1998. A prospective evaluation of intervention in 150 children. Pediatrics, 102:1358-1363.

García O, Massieu L. 2001. Strategies for neuroprotection against L-trans-2,4-pirrolidine dicarboxylate-induced neuronal damage during energy impairment in vitro. J Neurosci Res, 64:418-428.

Gascon S, Deogracias R, Sobrado M, Roda JM, Renart J, Rodríguez-Pena A, Díaz-Guerra M. 2005. Transcription of the NR1 subunit of the N-methyl-D-aspartate receptor is down-regulated by excitotoxic stimulation and cerebral ischemia. J Biol Chem, 280:5018-35027.

Gemba T, Oshima T, Ninomiya M. 1994. Glutamate efflux via the reversal of the sodium-dependent glutamate transporter caused by glycolitic inhibition in rat cultured astrocytes. Neurosci, 63:789-795.

Gerhart DZ, Broderius MA, Borson ND, Drewes LR. 1992. Neurons and microvessels express the brain glucose transporter protein GLUT3. Proc Natl Acad Sci U S A, 89:733-737.

Gerhart DZ, Leino RL, Taylor WE, Borson ND, Drewes LR. 1994. GLUT1 and GLUT3 gene expression in gerbil brain following brief ischemia: an in situ hybridization study. Brain Res Mol Brain Res, 25:313-322.

Gibson GE, Huang HM. 2004. Mitochondrial enzymes and endoplasmic reticulum calcium stores as targets of oxidative stress in neurodegenerative diseases. J Bioenerg Biomembr, 36:335-340.

Gjedde A, Crone CH. 1975. Induction processes in blood–brain transfer of ketone bodies during starvation. Am J Physiol, 229:1165–1169.

Glaser PE, Gross RW. 1985. Rapid plasmenylethanolamine-selective fusion of membrane bilayers catalyzed by an isoform of glyceraldehyde-3-phosphate dehydrogenase: discrimination between glycolytic and fusogenic roles of individual isoforms, Biochemistry, 341:2193-12203.

Greene JG, Greenamyre JT. 1995. Exacerbation of NMDA, AMPA and glutamate excitotoxicity by succinate dehydrogenase inhibitor malonate. J. Neurochem, 64:2332-2338.

Grewer C, Rauen T. 2005. Electrogenic glutamate transporters in the CNS: molecular mechanism, pre-steady-state kinetics, and their impact on synaptic signaling. J Membr Biol, 203:1-20.

Guzmán M, Blázquez C. 2001. Is there an astrocyte-neuron ketone body shuttle. Trends Endocrinol Metab, 12:169-173.

Hatefi Y. 1985. The mitochondrial electron transport and oxidative phosphorylation system. Annu Rev Biochem, 54:1015-1069.

Hawkins RA, Mans AM, Davis DW. 1986. Regional ketone body utilization by rat brain in starvation and diabetes. Am J Physiol, 250:E169-E178.

Henneberry RC. 1989. The role of neuronal energy in the neurotoxicity of excitatory amino acids. Neurobiol. Aging, 10:611-613.

Hernandez-Fonseca K, Massieu L. 2005. Disruption of endoplasmic reticulum calcium stores is involved in neuronal death induced by glycolysis inhibition in cultured hippocampal neurons. J Neurosci Res, 82:196-205.

Hertz L, Dringen R, Schousboe A, Robinson SR. 1999. Astrocytes: glutamate producers for neurons. J Neurosci Res, 57:417-428

Hollmann M, Heinemann S. 1994. Cloned glutamate receptors. Ann Rev Neurosci, 17:31-108.

Hori A, Tandon P, Holmes GL, Stafstrom CE. 1997. Ketogenic diet: effects on expression of kindled seizures and behavior in adult rats. Epilepsia, 38:750-758.

Huitorel P, Pantaloni D. 1985. Bundling of microtubules by glyceraldehyde-3-phosphate dehydrogenase and its modulation by ATP, Eur J Biochem, 150:265–269.

Hyder F, Patel AB, Gjedde A, Rothman DL, Behar K L, Shulman RG. 2006. Neuronal-glial glucose oxidation and glutamatergic-GABAergic function. J Cereb Blood Flow Metab, 26:865-77.

Ishige K, Schubert D, Sagara Y. 2001. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. Free Radic Biol Med, 30:433-446.

Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M, et al. 1993. Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. J Biol Chem, 268: 2836-2843.

Izumi Y, Ishii K, Katsuki H, Benz AM, Zorumski CF. 1998. Beta-hydroxybutyrate fuels synaptic function during development. Histological and physiological evidence in rat hippocampal slices. J Clin Invest, 101:1121-1132.

Jacobson J, Duchen MR. 2004. Interplay between mitochondria and cellular calcium signalling. Mol Cell Biochem, 256-257:209-18

Jiang X, Mu D, Manabat C, Kosky AA, Christein S, Täuber MG, Vexler ZS, Ferreiro DM. 2004, Differential vulnerability of immature murine neurons to oxygen-glucose deprivation. Exp Neurol, 190:224-232.

Kahlert S, Reiser G. 2000. Requirement of glycolytic and mitochondrial energy supply for loading of $Ca(^{2+})$ stores and InsP(3)-mediated $Ca(^{2+})$ signaling in rat hippocampus astrocytes. J Neurosci Res, 61:409-420.

Kanai Y, Hediger, MA. 1992. Primary structure and functional characterization of a high-affinity glutamate transporter. Nature, 360:467-471.

Kashiwaya Y, Takeshima T, Mori N, Nakashima K, Clarke K, Veech RL. 2000. D-beta-hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. Proc Natl Acad Sci U S A.,97:5440-5444.

Kasten TP, Dunaway GA. 1993. Fructose 2,6-bisphosphate: changes during neonatal maturation and aging of rat and potential role in regulation of glucose utilization. Mech Ageing Dev, 68:37-45.

Kawamoto RM, Caswell AH. 1986. Autophosphorylation of glyceraldehydephosphate dehydrogenase and phosphorylation of protein from skeletal muscle microsomes, Biochemistry, 25:656-661.

Kemp JA, McKernan RM. 2002. NMDA receptor pathways as drugs targets. Nature neuroscience, 5:1039-1042.

Kew JN, Kemp JA. 2005. Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology (Berl), 179: 4-29.

Kew JN, Richards JG, Mutel V, Kemp JA. 1998. Developmental changes in NMDA receptor glycine affinity and ifenprodil sensitivity reveal three distinct populations of NMDA receptors in individual rat cortical neurons. J Neurosci, 18:1935-1943.

Kim DK, Rordorf G, Nemenoff RA, Koroshetz WJ, Bonventre JV. 1995. Glutamate stably enhances the activity of two cytosolic forms of phospholipase A2 in brain cortical cultures. Biochem J, 310:83-90.

Kim do Y, Davis LM, Sullivan PG, Maalouf M, Simeone TA, van Brederode J, Rho JM. 2007. Ketone bodies are protective against oxidative stress in neocortical neurons. J Neurochem, 101:1316-1326.

Kim DS, Kwak SE, Kim JE, Jung JY, Won MH, Choi SY, Kwon OS, Kang T C. 2006. Transient ischemia affects plasma membrane glutamate transporter, not vesicular glutamate transporter, expressions in the gerbil hippocampus. Anat Histol Embryol, 35:265-270.

Kirsch JR, D'Alecy LG. 1984. Hypoxia induced preferential ketone utilization by rat brain slices. Stroke, 15:319–323.

Koshy B, Matilla T, Burright EN, Merry DE, Fischbeck KH, Orr HT, Zoghbi HY. 1996. Spinocerebellar ataxia type-1 and spinobulbar muscular atrophy gene products interact with glyceraldehyde-3-phosphate dehydrogenase. Hum Mol Genet, 5:1311-1318.

Kourie JI. 1998. Interaction of reactive oxygen species with ion transport mechanisms. Am J Physiol, 275:C1-24.

Kramer RM, Sharp JD. 1997. Structure, function and regulation of Ca2+-sensitive cytosolic phospholipase A2 (cPLA2). FEBS Lett, 410:49-53.

Kumagai H, Sakai H. 1983. A porcine brain protein (35 K protein) which bundles microtubules and its identification as glyceraldehyde 3-phosphate dehydrogenase, J Biochem (Tokyo), 93:1259–1269.

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M. 1992. Molecular diversity of the NMDA receptor channel. Nature, 358:36-41.

Lafon-Cazal M, Culcasi M, Gaven F, Pietri S, Bockaert J. 1993. Nitric oxide, superoxide and peroxynitrite: putative mediators of NMDA-induced cell death in cerebellar granule cells. Neuropharmacology, 32:1259-66.

Lee WH, Bondy CA. 1993. Ischemic injury induces brain glucose transporter gene expression. Endocrinology, 133:2540-2544.

Lerma J. 2006. Kainate receptor physiology. Curr Opin Pharmacol, 6:89-97.

Li PA, Liu GJ, He QP, Floyd RA, Siesjo BK. 1999. Production of hydroxyl free radical by brain tissues in hyperglycemic rats subjected to transient forebrain ischemia. Free Radic Biol Med, 27:1033-1040.

Lind C, Gerdes R, Schuppe-Koistinen I, Cotgreave IA. 1998. Studies on the mechanism of oxidative modification of human glyceraldehyde-3-phosphate dehydrogenase by glutathione: catalysis by glutaredoxin. Biochem Biophys Res Commun, 247:481-486.

Liu Y, Song XD, Liu W, Zhang TY, Zuo J. 2003. Glucose deprivation induces mitochondrial dysfunction and oxidative stress in PC12 cell line. J Cell Mol Med, 7:49-56.

Longuemare MC, Swanson RA. 1995. Excitatory amino acids release from astrocytes during energy failure by reversal of sodium-dependent uptake. J Neurosci Res, 40:379-386.

Lucas DR, Newhouse JP 1957. The toxic effect of sodium L-glutamate on the inner layers of the retina. Arch Ophthal, 58:193-201.

Lynch DR.Guttmann RP. 2002. Excitotoxicity: Perspectives based on N-Methyl-D-Aspartate receptor subtypes. JPET, 300:717-123.

Maalouf M, Sullivan PG, Davis L, Kim DY, Rho JM. 2007. Ketones inhibit mitochondrial production of reactive oxygen species production following glutamate excitotoxicity by increasing NADH oxidation. Neuroscience. 145:256-264.

Madl JE, Burgesser K. 1993. Adenosine triphosphate depletion reverses sodium-dependent neuronal uptake of glutamate in rat hippocampal slices. J Neurosci, 13:4429-4444.

Magistretti PJ, Pellerin L, Rothman DL, Shulman RG. 1999. Energy on demand. Science, 283:496-497.

Maher F, Davies-Hill TM, Simpson IA. 1996. Substrate specificity and kinetic parameters of GLUT3 in rat cerebellar granule neurons. Biochem J, 315:827-831.

Maher F, Vannucci S, Takeda J, Simpson IA. 1992. Expression of mouse-GLUT3 and human-GLUT3 glucose transporter proteins in brain. Biochem Biophys Res Commun, 182:703-711.

Malcolm CS, Benwell KR, Lamb H, Bebbington D, Porter RH. 2000. Characterization of iodoacetate-mediated neurotoxicity in vitro using primary cultures of rat cerebellar granule cells. Free Radic Biol Med, 28:102-107.

Massieu L, Del Rio P, Montiel T. 2001. Neurotoxicity of glutamate uptake inhibition in vivo: correlation with succinate dehydrogenase activity and prevention by energy substrates. Neuroscience, 106:669-677.

Massieu L, Gomez-Roman N, Montiel T. 2000. In vivo potentiation of glutamate-mediated neuronal damage after chronic administration of the glycolysis inhibitor iodoacetate. Exp Neurol, 165:257-267.

Massieu L, Haces ML, Montiel T, Hernández-Fonseca K. 2003. Acetoacetate protects hippocampal neurons against glutamate-mediated neuronal damage during glycolysis inhibition. Neuroscience, 120:365-378.

Massieu L, Tapia R. 1988. Relationship of dihydropyridine binding sites with calcium-dependent neurotransmitter release in synaptosomes. J Neurochem, 51:1184-1189.

Massieu L. 1998. Isquemia y excitotoxicidad. Gac Méd Méx vol. 134 No. 6: 690-693.

Masuda R, Monahan J, Kashiwaya Y. 2005. D-beta-Hydroxybutyrate is neuroprotective against hypoxia in serum-free hippocampal primary cultures. J Neurosci Res, 80:501-509.

Matthews RT, Ferrante RJ, Jenkins BG, Browne SE, Goetz K, Berger S, Chen IY, Beal MF. 1997. Iodoacetate produces striatal excitotoxic lesions. J Neurochem, 69:285-289.

McBain CJ, Mayer ML. 1994. N-methyl-D-aspartic acid receptor structure and function. Physiol Rev, 74:723-760.

McCulloch JM, Bullock R, Teadsdale GM. 1991. Excitatory amino acids antagonists: opportunities for the treatment of ischemic brain damage in man. In: Excitatory amino acids antagonists (ed. Meldrum B.S.) Blackwell Scientific Publications. Oxford, pp 257-326.

McEwen BS, Reagan LP. 2004. Glucose transporter expression in the central nervous system: relationship to synaptic function. Eur J Pharmacol, 490: 13-24.

McGowan JE, Chen L, Gao D, Trush M, Wei C. 2006. Increased mitochondrial reactive oxygen species production in newborn brain during hypoglycemia. Neurosci Lett, 399:111-114.

Mejía-Toiber J, Montiel T, Massieu L. 2006. D-beta-hydroxybutyrate prevents glutamate-mediated lipoperoxidation and neuronal damage elicited during glycolysis inhibition in vivo. Neurochem Res, 31:1399-1408.

Mhaskar Y, Dunaway GA. 1995. Alteration of PFK subunit protein, synthesis, and mRNA during neonatal brain development. Brain Res Dev Brain Res, 85:54-57.

Michaelis EK. 1997. Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. Prog. Neurobiol, 54:369-415.

Mitani A, Tanaka K. 2003. Functional changes of glial glutamate transporter GLT-1 during ischemia: an in vivo study in the hippocampal CA1 of normal mice and mutant mice lacking GLT-1. J Neurosci, 23:7176-7182

Mitchell P, Moyle J. 1965. Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphatase systems of rat liver mitochondria. Nature, 208:147-151.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH. 1992. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science, 256:1217-1221.

Mueckler M, Caruso C, Baldwin SA, Panico M, Blench I, Morris HR, Allard WJ, Lienhard GE, Lodish HF. 1985. Sequence and structure of a human glucose transporter. Science, 229:941-945.

Nagamatsu S, Sawa H, Kamada K, Nakamichi Y, Yoshimoto K, Hoshino T. 1993. Neuron-specific glucose transporter (NSGT): CNS distribution of GLUT3 rat glucose transporter (RGT3) in rat central neurons. FEBS Lett, 334:289-295.

Nagamatsu S. Kornhauser JM, Burant CF, Seino S, Mayo KE, Bell GI. 1992. Glucose transporter expression in brain. cDNA sequence of mouse GLUT3, the brain facilitative glucose transporter isoform, and identification of sites of expression by in situ hybridization. J Biol Chem, 267:467-472.

Nakanishi S. 1992. Molecular diversity of glutamate receptors and implications for brain function. Science, 258:597-603.

Navarro A, Boveris A. 2007. The mitochondrial energy transduction system and the aging process. Am J Physiol Cell Physiol, 292:C670-686.

Nehlig A. 1996. Respective roles of glucose and ketone bodies as substrates for cerebral energy metabolism in the suckling rat. Dev Neurosci, 18:426–433.

Nishi M, Hinds H, Lu HP, Kawata M, Hayashi Y. 2001. Motoneuron-specific expression of NR3B, a novel NMDA-type glutamate receptor subunit that works in a dominant-negative manner. J Neurosci, 21:RC185.

Noh HS, Hah YS, Nilufar R, Han J, Bong JH, Kang SS, Cho GJ, Choi WS. 2006. Acetoacetate protects neuronal cells from oxidative glutamate toxicity. J Neurosci Res, 83:702-709.

Novelli A, Reilly JA, Lysko PG, Henneberry RC. 1988. Glutamate becomes neurotoxic via the NMDA receptor when intracellular energy levels are reduced. Brain. Res, 451:205-212.

Ogata T, Nakamura Y, Tsuji K, Shibata T, Kataoka K. 1995. A possible mechanisms for the hypoxia-hipoglycemia-induced release of excitatory amino acids from cultured hippocampal astrocytes. Neurochem. Res, 20:737-743.

Oliver CN, Starke-Reed PE, Stadtman ER, Liu GJ, Carney JM, Floyd RA. 1990. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. Proc Natl Acad Sci USA, 87:5144-5147.

Olney JW. 1969. Glutamate-induced retinal degeneration in neonatal mice. Electron microscopy of the acutely evolving lesion. J Neuropathol Exp Neurol, 28:455-474.

Ottersen OP, Zhang N, Walberg F. 1992. Metabolic compartmentation of glutamate and glutamine: morphological evidence obtained by quantitative immunocytochemistry in rat cerebellum. Neuroscience, 46:519-534.

Owen OE, Morgan AP, Kemp HG, Sullivan JM, Herrera MG, Cahill GF Jr. 1967. Brain metabolism during fasting. J Clin Invest, 46:1589-1595.

Ozawa S, Kamiya H, Tsuzuki K. 1998. Glutamate receptors in the mammalian central nervous system. Prog Neurobiol, 54:581-618.

Palaiologos G, Hertz L, Schousboe A. 1988. Evidence that aspartate aminotransferase activity and ketodicarboxylate carrier function are essential for biosynthesis of transmitter glutamate. J Neurochem, 51:317-320.

Palmieri L, Pardo B, Lasorsa FM, del Arco A, Kobayashi K, Iijima M, Runswick MJ, Walker JE, Saheki T, Satrustegui J, Palmieri F. 2001. Citrin and aralar1 are Ca(2+)-stimulated aspartate/glutamate transporters in mitochondria. Embo J, 20:5060-5069.

Pang Z, Geddes JW. 1997. Mechanisms of cell death induced by the mitochondrial toxin 3-nitropropionic acid: acute excitotoxic necrosis and delayed apoptosis. J Neurosci, 17:3064-3073.

Patel AB, de Graaf RA, Mason GF, Rothman DL, Shulman RG, Behar KL. 2005. The contribution of GABA to glutamate/glutamine cycling and energy metabolism in the rat cortex in vivo. Proc Natl Acad Sci U S A, 102:5588-5593.

Patocková J, Marhol P, Tůmová E, Krsiak M, Rokyta R, Stípek S, Crkovská J, Andel M. 2003. Oxidative stress in the brain tissue of laboratory mice with acute post insulin hypoglycemia. Physiol Res, 52:131-135.

Pellerin L, Magistretti PJ. 1994. Glutamate uptake into astrocytes stimulates aespecies reactivas de oxígenobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci U S A, 91:10625-10629.

Perry TL, Hansen S, Jones K. 1987. Brain glutamate deficiency in amyotrophic lateral sclerosis. Neurology, 37:1845-1848.

Pines G, Danbolt NC, Bjoras M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI. 1992. Cloning and expression of a rat brain L-glutamate transporter. Nature, 360:464-467.

Pollay M, Stevens A. 1980. Starvation-induced changes in transport of ketone bodies across the blood–brain barrier. J Neurosci Res, 5:163–172.

Quintana P, Alberi S, Hakkoum D, Muller D. 2006. Glutamate receptor changes associated with transient anoxia/hypoglycaemia in hippocampal slice cultures. Eur J Neurosci, 23:975-983.

Qutub AA, Hunt CA. 2005. Glucose transport to the brain: a systems model. Brain Res Brain Res Rev, 49:595-617.

Raghavendra Rao VL, Rao AM, Dogan A, Bowen KK, Hatcher J, Rothstein, JD, Dempsey RJ. 2000. Glial glutamate transporter GLT-1 down-regulation precedes delayed neuronal death in gerbil hippocampus following transient global cerebral ischemia. Neurochem Int, 36:531-537.

Rao VL, Bowen KK, Dempsey RJ. 2001a. Transient focal cerebral ischemia down-regulates glutamate transporters GLT-1 and EAAC1 expression in rat brain. Neurochem Res, 26:497-502.

Rao VL, Dogan A, Bowen KK, Todd KG, Dempsey RJ. 2001b. Antisense knockdown of the glial glutamate transporter GLT-1 exacerbates hippocampal neuronal damage following traumatic injury to rat brain. Eur J Neurosci, 13:119-128.

Reed LJ. 2001. A trail of research from lipoic acid to alpha-keto acid dehydrogenase complexes. J Biol Chem, 276: 38329-38336.

Rego AC, Santos MS, Oliveira CR. 1999. Influence of the antioxidants vitamin E and idebenone on retinal cell injury mediated by chemical ischemia, hypoglycemia, or oxidative stress. Free Rad Biol Med, 26:1405-1407.

Rodriguez-Moreno A. 2006. The role of kainate receptors in the regulation of excitatory synaptic transmission in the hippocampus. Rev Neurol, 42:282-287.

Rogawski MA, Gryder D, Castaneda D, Yonekawa W, Banks MK, Lia H. 2003. GluR5 kainate receptors, seizures, and the amygdala. Ann N Y Acad Sci, 985:150-162.

Rosenmund C, Stern-Bach Y, Stevens CF. 1998. The tetrameric structure of a glutamate receptor channel. Science, 280:1596-1599.

Roses AD. 1996. From genes to mechanisms to therapies: lessons to be learned from neurological disorders. Nat Med, 2:267-269.

Rossi DJ. Oshima T, Attwell D. 2000. Glutamate release in severe brain ischemia is mainly by reversed uptake. Nature, 403:316-321.

Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW. 1994. Localization of neuronal and glial glutamate transporters. Neuron, 13:713-725.

Saluja I, Song D, O'Regan MH, Phillis JW. 1997. Role of phospholipase A2 in the release of free fatty acids during ischemia-reperfusion in the rat cerebral cortex. Neurosci Lett, 233:97-100.

Sanchez-Carbente MR, Massieu L. 1999. Transient inhibition of glutamate uptake in vivo induces neurodegeneration when energy metabolism is impaired. J Neurochem, 72:129-138.

Sandberg M, Butcher SP, Hagberg H. 1986. Extracellular overflow of neuroactive amino acids during severe insulin-induced hypoglycemia: in vivo dialysis of the rat hippocampus. J Neurochem, 47:178-184.

Sandberg M, Nystrom B, Hamberger A. 1985. Metabolically derived aspartate--elevated extracellular levels in vivo in Iodoacetate poisoning. J Neurosci Res, 13:489-495.

Schinder AF, Olson EC, Spitzer NC, Montal M. 1996. Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J Neurosci, 16:6125-6133.

Schmitt A, Asan E, Lesch KP, Kugler P. 2002. A splice variant of glutamate transporter GLT1/EAAT2 expressed in neurons: cloning and localization in rat nervous system. Neuroscience, 109:45-61.

Schulz JB, Matthews RT, Beal MF. 1995. Role of nitric oxide in neurodegenerative diseases. Curr Opin Neurol, 8:480-486.

Schulze H, Schuler A, Stuber Dobeli H, Langen H, Hubert G. 1993. Rat brain glyceraldehyde-3-phosphate dehydrogenase interacts with the recombinant cytoplasmic domain of Alzheimer's beta-amyloid precursor protein. J Neurochem, 60:1915-1922.

Schuppe-Koistinen I, Moldeus P, Bergman T, Cotgreave. 1994. S-thiolation of human endothelial cell glyceraldehyde-3-phosphate dehydrogenase after hydrogen pespecies reactivas de oxígenoxide treatment. FEBS, 221:1033-1037.

Seeburg PH, Hartner J. 2003. Regulation of ion channel/neurotransmitter receptor function by RNA editing. Curr Opin Neurobiol, 13:279-283.

Shulman RG, Rothman DL, Behar KL, Hyder F. 2004. Energetic basis of brain activity: implications for neuroimaging. Trends Neurosci, 27:489-495.

Sibson NR, Dhankhar A, Mason GF, Behar KL, Rothman DL, Shulman RG. 1997. In vivo 13C NMR measurements of cerebral glutamine synthesis as evidence for glutamate-glutamine cycling. Proc Natl Acad Sci U S A, 94:2699-2704.

Silver IA, Deas J, Erecinska M. 1997. Ion homeostasis in brain cells: differences in intracellular ion responses to energy limitation between cultured neurons and glial cells. Neuroscience, 78:589-601.

Siman R, Noszek JC. 1988. Excitatory amino acids activate calpain I and induce structural protein breakdown in vivo. Neuron, 1:279-287.

Simon RP, Swan JH, Griffith T, Meldrum BS. 1984. Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. Science, 226:850-852.

Simpson IA, Vannucci SJ, DeJoseph MR, Hawkins RA. 2001. Glucose transporter asymmetries in the bovine blood-brain barrier. J Biol Chem, 276: 12725-12729.

Simpson IA, Carruthers A, Vannucci SJ. 2007. Supply and demand in cerebral energy metabolism: role of nutrient transporters. J Cereb Blood Flow Metab, 27:1766-1791.

Singh P, Jain A, Kaur G. 2004 Impact of hypoglycemia and diabetes on CNS: correlation of mitochondrial oxidative stress with DNA damage, 260:153-159

Singh R, Green MR. 1993. Differential vulnerability of immature murine neurons to oxygen-glucose deprivation.3. Sequence-specificity binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase. Science, 259:365-368.

Sirover MA. 1999. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim Biophys Acta 1432:159–184.

Sokoloff L. 1973. Metabolism of ketone bodies by the brain. Annu Rev Med, 24:271–280.

Sonders MS, Quick M, Javitch JA. 2005. How did the neurotransmitter cross the bilayer? A closer view. Curr Opin Neurobiol, 15:296-304.

Stone TW, Addae JI. 2002. The pharmacological manipulation of glutamate receptor and neuroprotection. Eur J Pharmacol, 447:285-296

Storck T, Schulte S, Hofmann K, Stoffel W. 1992. Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain. Proc Natl Acad Sci U S A, 89:10955-10959.

Storey E, Hyman BT, Jenkins B, Brouillet E, Miller JM, Rosen BR, Beal MF. 1992. 1-Methyl-4-phenylpyridinium produces excitotoxic lesions in rat striatum as result of impairment of oxidative metabolism. J Neurochem, 58:1975-1978.

Storm-Mathisen J, Leknes AK, Bore AT, Vaaland JL, Edminson P, Haug FM, Ottersen OP. 1983. First visualization of glutamate and GABA in neurons by immunocytochemistry. Nature, 301:517-520.

Sutcu R, Altuntas I, Eroglu E, Delibas N. 2005. Effects of ischemia-reperfusion on NMDA receptor subunits 2a and 2b level in rat hippocampus. Int J Neurosci, 115:305-314.

Suzuki M, Suzuki M, Kitamura Y, Mori S, Sato K, Dohi S, Sato T, Matsuura A, Hiraide A. 2002. β-Hydroxybutyrate, a cerebral function improving agent, protects rat brain against ischemic damage caused by permanent and transient focal cerebral ischemia. Jpn J Pharmacol, 89:36-43.

Suzuki M, Suzuki M, Sato K, Dohi S, Sato T, Matsuura A, Hiraide A. 2001. Effect of bhydroxybutyrate, a cerebral function improving agent, on cerebral hypoxia, anoxia and ischemia in mice and rats. Jpn J Pharmacol, 87:143-50 38.

Suzuki YJ, Ford GD. 1999, Redox regulation of signal transduction in cardiac and smooth muscle. J Mol Cell Cardiol, 31:435.

Swanson CJ, Bures M, Johnson MP, Linden AM, Monn JA, Schoepp DD. 2005. Metabotropic glutamate receptors as novel targets for anxiety and stress disorders. Nat Rev Drug Discov, 4:131-144.

Tabernero A, Vicario C, Medina JM. 1996. Lactate spares glucose as a metabolic fuel in neurons and astrocytes from primary culture. Neurosci Res, 26: 369-376.

Tieu K, Perier C, Caspersen C, Teismann P, Wu DC, Yan SD, Naini A, Vila M, Jackson-Lewis V, Ramasamy R, Przedborski S. 2003. D-b-Hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease. J Clin Invest, 112:892-901.

Uldry M, Thorens B. 2004. The SLC2 family of facilitated hexose and polyol transporters. Pflugers Arch, 447: 480-489.

Uto A, Dux E, Kusumoto M, Hossmann KA. 1995. Delayed neuronal death after brief histotoxic hypoxia in vitro. J Neurochem, 64:2185-2192.

Vannucci SJ, Maher F, Simpson IA. 1997a Glucose transporter proteins in brain: delivery of glucose to neurons and glia. Glia 21:2–21

Vannucci RC, Brucklacher RM, Vannucci SJ. 1997b. Effect of carbon dioxide on cerebral metabolism during hypoxia-ischemia in the immature rat. Pediatr Res, 42:24-29.

Vannucci SJ, Gibbs EM, Simpson IA. 1997c. Glucose utilization and glucose transporter proteins GLUT-1 and GLUT-3 in brains of diabetic (db/db) mice. Am J Physiol, 272: E267-274.

Vannucci SJ, Seaman LB, Brucklacher RM, Vannucci RC. 1994. Glucose transport in developing rat brain: glucose transporter proteins, rate constants and cerebral glucose utilization. Mol Cell Biochem, 140:177-84.

Vesce S, Kirk L, Nicholls DG. 2004. Relationships between superoxide levels and delayed calcium deregulation in cultured cerebellar granule cells exposed continuously to glutamate. J Neurochem, 90:683-93.

Volker KW, Knull A. 1997. Glycolytic enzyme binding domain on tubulin, Arch. Biochem. Biophys. 338:237–243.

Wadiche JI, Amara SG, Kavanaugh MP. 1995. Ion fluxes associated with excitatory amino acid transport. Neuron, 15:721-728

Waldvogel D, van Gelderen P, Muellbacher W, Ziemann U, Immisch I, Hallett M. 2000. The relative metabolic demand of inhibition and excitation. Nature 406:995-998.

Walker JE, Collinson IR, Van Raaij MJ, Runswick MJ. 1995. Structural analysis of ATP synthase from bovine heart mitochondria. Methods Enzymol, 260:163-190.

Wang X, Quinn PJ. 1999. Vitamin E and its function in membranes. Prog Lipid Res 38:309-336.

Watanabe M, Mishina M, Inoue Y. 1994. Distinct distributions of five NMDA receptor channel subunit mRNAs in the brainstem. J Comp Neurol, 343:520-531.

Watkins JC, Olverman HJ. 1987. Agonist and antagonists for excitatory amino acids receptors. Trends Neurosci, 100:265-272.

Watzke N, Bamberg E, Grewer C. 2001. Early intermediates in the transport cycle of the neuronal excitatory amino acid carrier EAAC1. J Gen Physiol 117: 547-562.

Wieloch T, Engelsen B, Westerberg E, Auer R. 1985. Lesions of the glutamatergic cortico-striatal projections in the rat ameliorate hypoglycemic brain damage in the striatum. Neurosci Lett. 58:25-30.

Williams RJ, Maus M, Stella N, Glowinski J, Premont J. 1996. Reduced glucose metabolism enhances the glutamate-evoked release of arachidonic acid from striatal neurons. Neuroscience, 74:461-8.

Wilson JE. 1980. Brain hexokinase, the prototype ambiquitous enzyme. Curr Top Cell Regul, 16:1-54.

Wilson JE. 2003. Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. J Exp Biol, 206:2049-2057.

Yeh TH, Hwang H M, Chen JJ, Wu T, Li AH, Wang HL. 2005. Glutamate transporter function of rat hippocampal astrocytes is impaired following the global ischemia. Neurobiol Dis 18, 476-483.

Yudkoff M, Daikhin Y, Nissim I, Lazarow A, Nissim I. 2001. Ketogenic diet, amino acid metabolism, and seizure control. J Neurosci Res, 66:931–940.

Yudkoff M, Nissim I, Daikhin Y, Lin ZP, Nelson D, Pleasure D, Erecinska M. 1993. Brain glutamate metabolism: neuronal-astroglial relationships. Dev Neurosci 15, 343-350.

Zaidan E, Sims NR. 1994. The calcium content of mitochondria from brain subregions following short-term forebrain ischemia and recirculation in the rat. J. Neurochem, 63:1812-1819.

Zeevalk GD, Nicklas WJ. 1990. Chemically induced hypoglycemia and anoxia: relationship to glutamate receptor-mediated toxicity in retina. J Pharmacol Exp Ther, 253:1285-1292.

Zeevalk GD, Nicklas WJ. 1992. Evidence that loss of the voltage-dependent Mg2+ block at the N-methyl-D-aspartate receptor underlies receptor activations during inhibition of neuronal metabolism. J. Neurochem, 59:1211-1220.

Zhang L. Hsu JC, Takagi N, Gurd J W, Wallace M C, Eubanks J H. 1997. Transient global ischemia alters NMDA receptor expression in rat hippocampus: correlation with decreased immunoreactive protein levels of the NR2A/2B subunits, and an altered NMDA receptor functionality. J Neurochem, 69:1983-1994.

XV. APÉNDICE

ARTICULO 4

"Disruption of Endoplasmic Reticulum Calcium Stores Is Involved in Neuronal Death Induced by Glycolysis Inhibition in Cultured Hippocampal Neurons"

(Journal of Neuroscience Research. 2005, 82:196-205)

Journal of Neuroscience Research 82:196–205 (2005)

Disruption of Endoplasmic Reticulum Calcium Stores Is Involved in Neuronal Death Induced by Glycolysis Inhibition in Cultured Hippocampal Neurons

Karla Hernández-Fonseca and Lourdes Massieu*

Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México

Disturbances in neuronal calcium homeostasis have been implicated in a variety of neuropathological conditions, including cerebral ischemia, hypoglycemia, and epilepsy, and possibly constitute part of the cell death process associated with chronic neurodegenerative disorders. We investigated if endoplasmic reticulum (ER) calcium stores participate in neuronal death triggered by moderate glycolysis inhibition induced by iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, in cultured hippocampal neurons. Results show that exposure to iodoacetate leads to a slow partial decrease in cell survival, which is significantly prevented in the absence of Ca^{2+} or in the presence of the calcium chelator BAPTA-AM. Treatment with caffeine and a low (1 μM) concentration of ryanodine, which activates the ryanodine receptor (RyR), exacerbates neuronal death, whereas dantrolene and 25 μM ryanodine, which antagonizes RyR, prevents damage. Xestospongin C (XeC), an antagonist of the inositol-3-phosphate (IP_3) receptor (IP_3R) also prevents neuronal damage. Inhibitors of the ER calcium ATPase (sarcoendoplasmic reticulum Ca^{2+} ATPase; SERCA) have no effect. The decrease in ATP levels induced by iodoacetate is potentiated by caffeine and prevented by dantrolene. Although only a slight increase in glutamate extracellular levels is observed 3.5 hr after iodoacetate exposure, the *N*-methyl-D-aspartate (NMDA) glutamate receptor antagonist, MK-801, efficiently prevents neuronal damage. Taken together, the data suggest that neuronal death induced during moderate glycolysis inhibition involves calcium influx through NMDA receptors and calcium release from intracellular ER stores. These results might be relevant to the understanding the mechanisms involved in neuronal damage related to aging and chronic neurodegenerative diseases, which have been associated with decreased glucose metabolism.

Key words: calcium; endoplasmic reticulum; excitotoxic death; iodoacetate

Cytosolic calcium concentration at rest is kept around 10,000 times lower than its concentration in the extracellular space. The elevation of intracellular calcium is regulated by its extrusion to the extracellular medium or its storage in intracellular organelles, such as the endoplasmic reticulum (ER) and mitochondria. Some of the mechanisms maintaining intracellular calcium homeostasis are energy dependent. Plasma membrane calcium ATPase is fueled by ATP, and Na^+/Ca^{2+} exchangers extrude Ca^{2+} at the expense of the Na^+ gradient, driven by the Na^+/K^+ ATPase. Calcium is loaded in ER through the activity of the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) or in mitochondria through a process depending on the maintenance of the proton gradient across the inner mitochondrial membrane. Impairment of Ca^{2+}-extruding or calcium-loading mechanisms leads to excessive intracellular calcium and might trigger cell death (Paschen, 2003).

Disturbances in neuronal calcium homeostasis have been implicated in some neuropathologic conditions, including cerebral ischemia, hypoglycemia, and epilepsy (Siesjö and Bengtsson, 1989; Kahlert and Reiser, 2004; Yao and Haddad, 2004). The cell death process associated with these disorders involves an excitotoxic mechanism mediated by the activation of *N*-methyl-D-aspartate (NMDA) glutamate receptors (Siesjö and Bengtsson, 1989; Choi, 1995; Arundine and Tymianski, 2003), which are highly permeable to calcium (Mayer and Westbrook, 1987). An excitotoxic component in neurodegeneration associated with Huntington's and Alzheimer's diseases has also been suggested (Schwarcz et al., 1983; Young et al., 1988, Csernansky et al., 1996). Excessive calcium causes the overactivation of diverse signals such as proteases, phospholipases, and DNAses contributing to cell death (Choi, 1992; Kristián and Siesjö, 1998; Siesjö et al.,

Contract grant sponsor: CONACYT; Contract grant number: 40306-M, 167146; Contract grant sponsor: DGEP.

*Correspondence to: Lourdes Massieu, Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México, D.F. 04510, AP 70-257.
E-mail: lmassieu@ifc.unam.mx

Received 17 March 2005; Revised 17 June 2005; Accepted 20 July 2005

Published online 20 September 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20631

1999). Lately, a role of mitochondria in excitotoxic cell death has been raised. Mitochondrial calcium overload causes depolarization of the mitochondrial membrane, generation of reactive oxygen species, opening of the mitochondrial permeability transition pore, ATP depletion, and eventually the release of cytochrome C and caspase activation (Schinder et al., 1996; Vesce at al., 2004).

The role of ER in Ca^{2+}-mediated neuronal death has received less attention; however, it has been suggested that prolonged depletion of Ca^{2+} from ER is a critical component of neuronal damage (Paschen and Doutheil, 1999). Various studies suggest the participation of intracellular pools of Ca^{2+} in excitotoxic death due to the protective effect of dantrolene, an antagonist of the ryanodine receptor (RyR), against neuronal damage induced by NMDA (Frandsen and Schousboe, 1992; Gepdiremen et al., 2001), ischemia (Zhang et al., 1993; Nakayama et al., 2002), and oxygen and glucose deprivation (Wang et al., 2002). Antagonists of metabotropic glutamate receptors coupled to phospholipase C, however, prevent hypoxic-mediated death in hippocampal cultures (Opitz and Reymann, 1991). All together, these studies suggest the role of intracellular calcium pools of ER in excitotoxicity.

Previous studies show that glutamate-mediated excitotoxic death is favored during conditions of energy deficit (Novelli et al., 1988; Zeevalk and Nicklas, 1992; Massieu et al., 2000; García and Massieu, 2003). We have reported that glycolysis inhibition induced by iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase (GADPH), induces neuronal death, sensitive to blockade of the NMDA glutamate receptor subtype (Massieu et al., 2003a). The aim of the present study was to investigate whether ER calcium stores participate in neuronal death induced by glycolysis inhibition in cultured hippocampal neurons. During iodoacetate exposure, there is a progressive decrease in energy levels (Massieu et al., 2003a), and thus the mechanisms responsible for the extrusion and the storage of intracellular calcium might be perturbed. We have exposed neurons to iodoacetate in the presence and absence of drugs that alter calcium storage and release from ER, through the activation or inhibition of RyR, inositol-3-phosphate receptors (IP_3R) and the SERCA pump. In a variety of cell types, including neurons and astrocytes, it is well documented that activation of RyR and IP_3R induces an increase in the intracellular concentration Ca^{2+}, whereas inhibition of these receptors prevents it. Similarly, depletion of ER Ca^{2+} stores by inhibition of the SERCA pump is well known, as well as the Ca^{2+}-chelating effect of EGTA and BAPTA-AM (for review, see Kahlert and Reiser, 2000, 2004; Hajnóczky and Csordás, 2002; Verkhratsky, 2002; Arundine and Tymianski, 2003; Yao and Haddad, 2004). Results suggest that disruption of Ca^{2+} storage in ER contributes to the cell death process.

MATERIALS AND METHODS

Trypsin, DNAse, gentamicine, L-glutamine, glutamate, poly-L-lysine, cytosine arabinoside, iodoacetate, dantrolene, xestospongin C (XeC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-RBI (St. Louis, MO). Soybean trypsin inhibitor, Neurobasal medium, and B27 supplement (Minus AO) were from Gibco/Life Technologies (Rockville, MD), and caffeine, ryanodine, TMB-8, cyclopiazonic acid (CPA), thapsigargin (TG), and BAPTA-AM were obtained from Alomone Labs (Jerusalem, Israel). Luciferin-luciferase assay kit was purchased from Molecular Probes (Eugene, OR).

Cell Cultures

Primary cultures of hippocampal neurons were prepared from Wistar rat embryos at 17–18 days of gestation as described by Brewer et al. (1993) with some modifications. Briefly, after dissection, 10–12 hippocampi were chopped into 300-μm cubes, incubated in 0.25% trypsin solution, and dispersed by trituration in a DNAse and soybean trypsin inhibitor containing solution (0.08% and 0.52%, respectively). Cells were suspended in Neurobasal culture medium (Brewer et al., 1993) supplemented with B27, 0.5 mM L-glutamine, 20 μg/ml gentamicine, and 0.2 mM glutamate, and plated at a density of 260–290 $\times$ $10^3/cm^2$ (1.5 $\times$ 10^6 cells/ml/well) in Costar 24-well plates (Cambridge, MA), precoated with poly-L-lysine (5 μg/ml). Cells were cultured for 8 days in vitro at 37°C in a humidified 5% CO_2/95% air atmosphere. Glucose (5 mM) and cytosine arabinoside (10 μM) were added to cultures 4 days after plating. Neuronal population in these cultures was 95% as determined by immunocytochemistry against microtubule-associated protein 2 (MAP-2) and glial fibrillary acidic protein (GFAP), neuronal and glial cell markers, respectively. Experiments were carried out at 7–8 days in vitro (DIV). Animals were handled and cared according to the NIH guide for care and use of laboratory animals, with approval of the local animal care committee. All efforts were made to minimize animal suffering.

Drug Exposure

Neuronal cultures were exposed during 30 min to 50 μM IOA in Ringer-Kreb's medium containing (in mM): NaCl 154; KCl 5.6; $CaCl_2$ 2.3; KH_2PO_4 1.2; HEPES 5.0; $NaHCO_3$ 3.6; and glucose 5.6. After this period, the medium was changed to conditioned medium and neuronal survival was monitored at different times as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction (Mosmann, 1983; Berridge and Tan, 1993), as described previously (Massieu et al., 2003a). At 24 hr after the exposure, a 50% reduction in cell survival was observed. This iodoacetate concentration therefore was used to test the protective effect of ryanodine (25 μM) and dantrolene (antagonists of RyR), XeC (antagonist of IP_3R), calcium-free medium, the calcium chelator BAPTA-AM, and the NMDA receptor antagonist, MK-801. Ryanodine shows a biphasic effect on RyR: at low concentrations (10 nM–1 μM) it behaves as an agonist, whereas at high concentrations (10–100 μM) it shows antagonistic effects (Fill and Copello, 2002).

According to a dose–response curve determined previously (Massieu et al., 2003a), 30 min exposure to 25 μM iodoacetate induces a 20–30% reduction in cell survival at 24 hr. This concentration therefore was used to study the

putative exacerbating effect of caffeine, 1 μM ryanodine (agonists of RyR), TG (irreversible antagonist of SERCA), CPA, and TMB-8 (reversible antagonists of SERCA) on iodoacetate toxicity. The effect of these drugs on cell survival was assessed at 24 hr by MTT assay.

BAPTA-AM and dantrolene were preincubated during 15 and 30 min, respectively, before IOA exposure. Cells were exposed to 50 or 25 μM iodoacetate during 30 min in the presence or absence of calcium-free medium, BAPTA-AM (10 μM), dantrolene (25 μM), ryanodine (1 or 25 μM), caffeine (5 mM), TG (100 nM), CPA (10 μM), TMB-8 (10 μM), XeC (2 μM), and MK-801 (10 μM). Calcium-free medium and BAPTA-AM were present only during iodoacetate exposure; they were removed and conditioned medium was added again for the following 24 hr. The rest of the drugs used were added to individual culture wells after iodoacetate removal for the following 24 hr.

Determination of Amino Acid Extracellular Concentration

Amino acid concentration in the medium was measured by high-performance liquid chromatography (HPLC) immediately and 3.5 hr after a 30-min exposure to iodoacetate in Ringer-Kreb's medium. When the content of amino acids was studied at 3.5 hr, Ringer-Kreb's containing iodoacetate was changed for conditioned medium after iodoacetate exposure, and 3 hr later aliquots were taken for amino acid determination. When amino acid levels were determined immediately after iodoacetate exposure, aliquots from Ringer-Kreb's medium were taken. Aliquots from conditioned medium were deproteinized with perchloric acid (7%) and neutralized with KOH. Amino acid content was determined by HPLC according to Antonie et al. (1999), with slight modifications. Briefly, 100-μl fractions were derivatized with the same volume of *o*-phthalaldehyde and 3 min later a 10-μl volume of this mixture was injected into an HPLC system (Waters 600, Milford, MA) equipped with an ODS column (25 cm × 4 mm internal diameter; Supelco Inc., Bellefonte, PA). The mobile phase consisted of 18% methanol: 22% acetonitrile: 14% isopropanol: 46% phosphate buffer (60 mM, pH 6.65)/phosphate buffer (60 mM, pH 6.65). A flux rate of 1 ml/min was used in a linear gradient of 33 min duration from 10 to 90% solvent mixture. Amino acid concentration was calculated by comparison with a standard mixture of amino acids equally processed. Data represent means ± standard error of the mean (SEM) of the number of animals indicated in figure legends.

ATP Determination

ATP levels were determined at 1 and 8 hr after iodoacetate exposure in either the presence or absence of caffeine or dantrolene. ATP determinations were carried out using previously described methodology (Massieu et al., 2003a). Briefly, cells were washed twice with prewarmed Locke's solution containing (in mM): 154 NaCl; 5.6 KCl; 3.6 $NaHCO_3$; 2.3 $CaCl_2$; 5 HEPES; and 5.6 glucose; pH 7.4. Cells were then lysed by incubation in 25 μl somatic cell ATP-releasing agent (Sigma). Lysate (15 μl) was diluted in 85 μl distilled water and the 100 μl volume was added to a polyethylene tube and placed in a luminometer. The luminometer injected a 400-μl volume of luceferin-luciferase reaction mixture. The luminometer records quimioluminescence values in millivolts and ATP concentrations were calculated from readings obtained from an ATP standard curve (from 6.5 to 250 pmol). Aliquots of cell homogenates were kept for protein determination by Bradford's (1976) method and data are expressed as picomoles per microgram of protein.

Statistics

Data were analyzed by one-way analysis of variance (ANOVA) followed by a Fisher's multiple comparison test. Data on MTT are expressed as percent of control, but were analyzed statistically using optical density (OD) values.

RESULTS

Exposure of hippocampal neurons to iodoacetate (50 μM) during 30 min, leads to a slow progressive reduction in cell survival as evaluated by the MTT reduction assay at different times. A 15–20% decrease in cell survival was observed 4–12 hr after iodoacetate treatment and it became statistically significant at 12 hr. After 24 hr, cell survival was reduced in 46% (Fig. 1).

To test the role of calcium on iodoacetate-induced cell death, exposure to the GADPH inhibitor was carried out either in the absence of extracellular calcium or in the presence of the calcium chelator BAPTA-AM. Results show that iodoacetate-induced neuronal death was calcium-dependent because in the presence of BAPTA-AM or calcium-free medium neuronal survival, as assessed by the MTT reduction assay, was significantly improved (Fig. 2). None of these manipulations, however, completely prevented iodoacetate-induced neuronal damage (Fig. 2). Figure 3 shows the morphologic appearance of hippocampal cultured neurons 24 hr after 30-min exposure to 50 μM iodoacetate. Compared to controls, cultures exposed to iodoacetate show the presence of many bright and refringent cells indicative of death cell bodies and reduced number of well-preserved dark somata. Neurites are also reduced in number and those remaining are thin and fragmented (Fig. 3). Cultures exposed to iodoacetate in the presence of either BAPTA-AM or calcium-free medium are better preserved. Many dark and round somata are observed and neurites are thick, similar to those present in control cultures. These results suggest that iodoacetate toxicity is related to increased intracellular calcium.

To assess the role of intracellular calcium storage in ER on iodoacetate-induced neuronal damage, we first studied the effect of different inhibitors of the calcium ATPase of ER (SERCA). For these experiments, we used a 25-μM concentration of iodoacetate because a potentiating effect of neuronal damage was expected in the presence of SERCA inhibitors, TG, CPA, and TMB-8. TG (100 nM), and irreversible inhibitor of SERCA induced a 40% reduction in cell survival when incubated

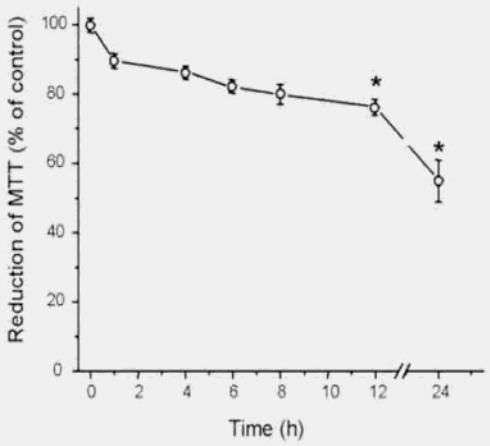


Fig. 1. Time–response curve of cell survival after exposure to iodoacetate in hippocampal cultured neurons. Cells were exposed during 30 min to iodoacetate (50 μM) in Ringer-Kreb's medium and cell survival was assessed by MTT reduction at the indicated times. Data are expressed by means ± standard error of the mean (SEM) of five independent experiments. Data were analyzed by one-way ANOVA followed by a Fisher's least significant difference test. $^{*}P < 0.001$ relative to control cultures.

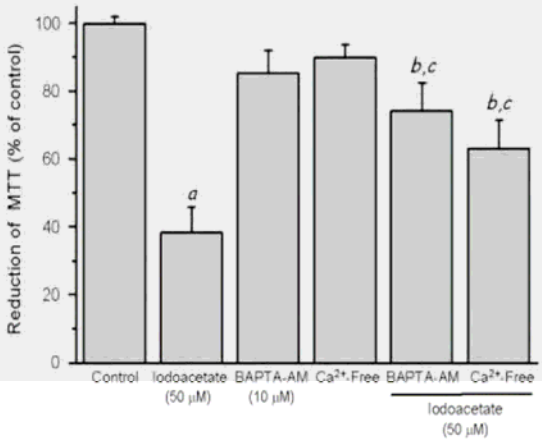


Fig. 2. Protective effect of BAPTA-AM and Ca^{2+}-free medium against neuronal damage induced by iodoacetate (50 μM). Cells were exposed to iodoacetate in Ringer-Kreb's without calcium or with calcium containing BAPTA-AM (10 μM). Medium was removed and replaced for conditioned medium, and cell viability was assessed 24 hr later. Data are expressed as means ± standard error of the mean (SEM) of four or five independent experiments. Data were analyzed by one-way ANOVA followed by a Fisher's least significant difference test. $^{a}P < 0.001$ relative to control, $^{b}P < 0.05$ relative to control, and $^{c}P < 0.01$ relative to iodoacetate-treated cultures.

alone, and it did not potentiate iodoacetate toxicity (Fig. 4). Lower doses of TG (10 and 50 nM) did not exacerbate the effect of iodoacetate either. We also incubated TG before iodoacetate exposure either to deplete ER from calcium and increase its intracellular concentration

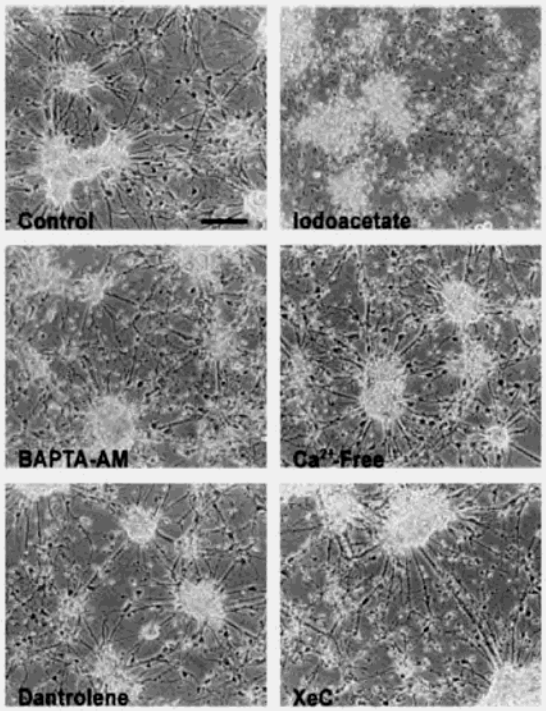


Fig. 3. Protective effect of BAPTA-AM (10 μM), calcium-free medium, dantrolene (25 μM), and xestospongin C (XeC; 2 μM) against cell damage induced by iodoacetate in cultured hippocampal neurons. Cultures were incubated in Ringer-Kreb's medium containing the different compounds or in Ca-free medium. Medium was removed after iodoacetate exposure and replaced for conditioned medium. Dantrolene and XeC were added again for the following 24 hr. Scale bar = 100 μM. Photomicrographs show a representative experiment from four to six independent assays.

before glycolysis was inhibited; however, in these conditions neuronal damage was neither exacerbated (data not shown). The reversible inhibitors of SERCA, CPA, and TMB-8 had no effect on neuronal survival when incubated alone and did not potentiate iodoacetate-induced neuronal damage (Fig. 4).

To induce calcium release from ER and study its effect on neuronal death induced by iodoacetate, two agonists of RyR were used: ryanodine (1 μM) and caffeine (5 mM). Individual treatments with caffeine and ryanodine slightly reduced cell survival and incubation of neurons with either compounds during and after iodoacetate treatment potentiated the iodoacetate toxic effect (Fig. 5). Treatment with a low concentration 100 nM of ryanodine did not exacerbate neuronal damage (data not shown).

To study further the role of RyR and IP_3R on neuronal damage induced during glycolysis inhibition, the protective effect of antagonists of RyR (dantrolene and ryanodine at 25 μM) and IP_3R (XeC) was tested. As shown in Figure 6, in the presence of dantrolene MTT reduction capacity was restored substantially to 83% of that of control cultures, whereas in the presence

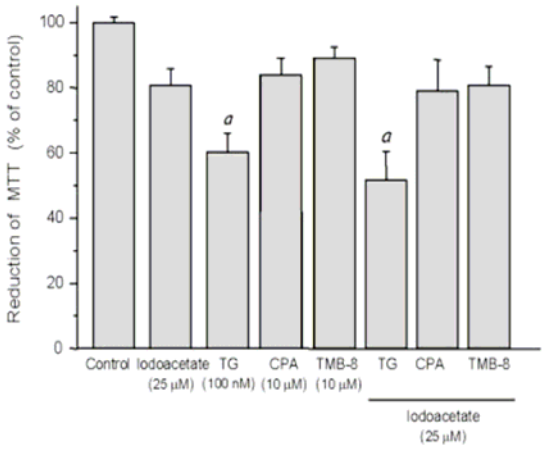


Fig. 4. Effect of sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitors on iodoacetate-induced neurotoxicity. Cultures were exposed to iodoacetate (25 μM) in Ringer-Kreb's solution containing thapsigargin (TG; 100 nM), cyclopiazonic acid (CPA; 10 μM), and TMB-8 (10 μM) for 30 min. After this time, the medium was removed and replaced by conditioned media containing TG, CPA, and TMB-8. Cell viability was assessed at 24 hr. Data are means ± standard error of the mean (SEM) of four or five independent experiments. Data were analyzed by one-way ANOVA followed by a Fisher's least significant difference test. $^{a}P < 0.01$ relative to control.

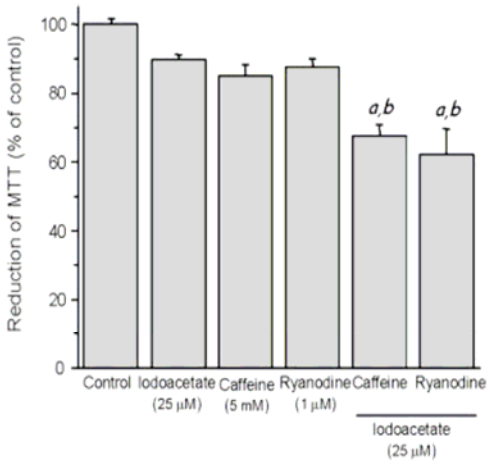


Fig. 5. Agonists of the ryanodine receptor (RyR) exacerbate neuronal damage induced by iodoacetate. Cultures were exposed to iodoacetate (25 μM) in Ringer-Kreb's solution for 30 min in the presence of ryanodine (1 μM) and caffeine (5 mM). The medium was removed and replaced by conditioned medium containing ryanodine and caffeine. Cell viability was assessed 24 hr later. Data are means ± standard error of the mean (SEM) of eight to ten independent experiments. Data were analyzed by one-way ANOVA followed by a Fisher's least significant difference test. $^{a}P < 0.01$ relative to control cultures, $^{b}P < 0.05$ relative to iodoacetate-treated cultures.

of ryanodine and XeC it was significantly restored to 70%. The protective effect of dantrolene and XeC as assessed morphologically is shown in Figure 3. As can be observed, cultures exposed to iodoacetate in the presence

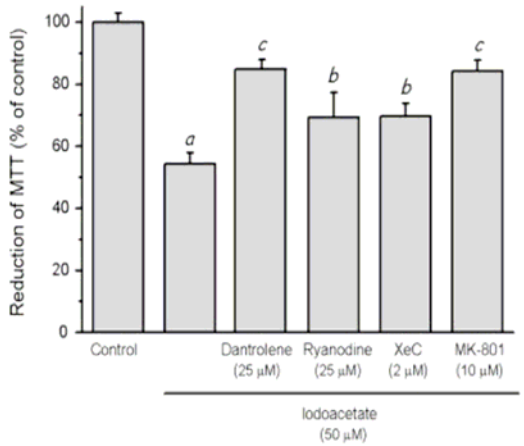


Fig. 6. Protective effect of antagonists of ryanodine receptor (RyR), inositol-3-phosphate receptor (IP_3R), and *N*-methyl-D-aspartate (NMDA) receptors against iodoacetate-induced neuronal damage. Cultures were exposed to iodoacetate (50 μM) for 30 min in Ringer-Kreb's solution containing dantrolene (25 μM), ryanodine (25 μM), xestospongin C (XeC; 2 μM), and MK-801 (10 μM). The medium was removed and replaced by conditioned medium containing the different compounds. Cell viability was assessed 24 hr later. Data are means ± standard error of the mean (SEM) of four to nine independent experiments. Data were analyzed by one-way ANOVA followed by a Fisher's least significant difference test. $^{a}P < 0.001$ relative to control cultures, $^{b}P < 0.05$ relative to iodoacetate-treated cultures, and $^{c}P < 0.01$ relative to iodoacetate-treated cultures.

of dantrolene and XeC are better preserved, as evidenced by the presence of many dark and round cells with thick neurites, indicative of healthy neurons.

The protective effect of these drugs was compared to that of MK-801, an antagonist of the NMDA glutamate receptor subtype. In the presence of MK-801, MTT reduction capacity was restored to 84%, suggesting that neuronal death induced by glycolysis inhibition is of the excitotoxic type. In contrast, 30-min exposure to a higher iodoacetate concentration (100 μM) leads to a severe decrease in cell survival (90%), which is not prevented by MK-801 (5.7 ± 2.5% cell survival without MK-801 and 3.4 ± 1.5% with MK-801; $n = 3$). The extracellular concentration of the excitatory amino acids glutamate and aspartate was examined in the medium immediately and 3.5 hr after the exposure to iodoacetate. For this purpose, cultures were incubated during 0.5 hr with different concentrations of iodoacetate (5–100 μM). After a 30-min exposure, no significant increase in glutamate or aspartate levels was observed except for a 70% increase in aspartate levels at 100 μM iodoacetate (Table I). After 3.5 hr, no significant increases in excitatory amino acids were found except for a slight increase in glutamate when 50 μM iodoacetate was used (Table I).

In previous experiments, we showed that iodoacetate exposure induces a decrease in ATP levels (Massieu et al., 2003a). We therefore aimed to study the effect of

TABLE I. Extracellular Levels of Amino Acids (μM) and Their Changes Induced by Exposure to Iodoacetate in Hippocampal Cultured Neurons‡

Iodoacetate (μM)	30 min		4 hr	
	Glu	Asp	Glu	Asp
0	3.60 ± 0.70	3.77 ± 0.63	1.53 ± 0.16	1.81 ± 0.34
5	3.05 ± 0.57	4.51 ± 0.734	1.86 ± 0.16	1.53 ± 0.25
10	2.43 ± 0.44	2.48 ± 0.49	1.72 ± 0.29	1.66 ± 0.25
25	3.19 ± 0.62	3.75 ± 0.94	1.58 ± 0.26	2.24 ± 0.40
50	4.18 ± 0.51	5.72 ± 0.55	2.96 ± 0.72*	1.43 ± 0.23
100	4.75 ± 0.86	6.38 ± 0.77*	1.71 ± 0.24	1.62 ± 0.43

‡Hippocampal neuronal cultures were exposed during 30 min to different concentrations of iodoacetate and amino acid levels were determined by HPLC in aliquots collected from the medium at 30 min and 4 hr after the onset of iodoacetate exposure. Data are expressed as means ± SEM of 4–6 independent experiments. Glutamate (Glu), aspartate (ASP).
*$P < 0.01$ relative to control values.

TABLE II. Effect of Caffeine and Dantrolene on the ATP Levels During Glycolysis Inhibition*

Condition	ATP levels (pmol/μg protein)	
	1 hr	8 hr
Control	8.08 ± 0.83	8.08 ± 0.83
IOA	2.13 ± 0.55[a]	3.32 ± 0.37[a]
IOA + caffeine	4.97 ± 0.85[b,c]	0.75 ± 0.53[a,d]
IOA + dantrolene	2.03 ± 0.59[a]	5.94 ± 1.59[d]

*ATP levels were determined at 1 and 8 hr after 30 min exposure to iodoacetate (50 μM) in the presence or absence of caffeine (5 mM) or dantrolene (10 μM) in cultured hipocampal neurons. Data are means ± SEM of three independent experiments. ATP levels were determined by the luciferin-luciferase assay. Data were analyzed by one-way ANOVA followed by a Fischer's least significant difference test. Iodoacetate (IOA).
[a]$P < 0.001$ relative to control ATP levels.
[b]$P < 0.01$ relative to control ATP levels.
[c]$P < 0.02$ relative to IOA at 1 hr.
[d]$P > 0.04$ relative to IOA at 8 h.

RyR activation or inhibition on ATP levels 1 and 8 hr after the exposure. Cultures were treated with iodoacetate during 0.5 hr in the presence or absence of caffeine (5 mM) or dantrolene (10 μM). It was observed that 1 hr after exposure, caffeine (5 mM) prevented the iodoacetate-induced decrease in ATP levels whereas dantrolene had no effect. In contrast, at 8 hr caffeine notably potentiated (by 77%) the decrease in ATP levels, whereas in the presence of dantrolene ATP concentration was preserved (Table II).

DISCUSSION

Many studies suggest that disruption of calcium homeostasis is a critical event in neuronal death induced by ischemia and other pathologic conditions (Orrenius and Nicotera, 1994; Choi, 1995; Kristián and Siesjö, 1998; Kahlert and Reiser, 2004; Yao and Haddad, 2004). It has been postulated that during ischemia, one of the initial events involved in neuronal damage is calcium influx through glutamate receptors, particularly of the NMDA subtype (Kristián and Siesjö, 1998; Arundine and Tymianski, 2003). More recently, it has been proposed that disruption of intracellular calcium homeostasis due to the impairment of Ca^{2+} storage in ER also contributes to neuronal damage induced by ischemia and other pathologic states (Wei and Perry, 1996; Paschen and Doutheil, 1999; Siesjö et al., 1999; Kahlert and Reiser, 2000; Paschen, 2000). The present results suggest the involvement of increased intracellular calcium in neuronal death induced by glycolysis inhibition, because in the presence of BAPTA-AM or in the absence of extracellular calcium, MTT reducing capacity was preserved and hippocampal cultures maintained their morphologic integrity. None of these maneuvers prevented cell death completely, however, probably because BAPTA-AM or calcium-free medium was present only during the exposure to iodoacetate. EGTA or BAPTA-AM was not added to the medium after iodoacetate removal, because in these conditions substantial neuronal death was observed (data not shown). Higher concentrations of BAPTA-AM (25 and 50 μM) were tested, but they significantly decreased neuronal survival was also observed (data not shown). Previous studies have also shown toxicity of BAPTA-AM at high concentrations, which is attributed to an imbalance of calcium in intracellular organelles (Wang et al., 2002). Our results agree with previous studies showing a protective effect of BAPTA-AM against glutamate-induced neuronal damage both in vitro and in vivo (Tymianski et al., 1993; Tymianski, 1995). The protective effect induced by incubation in Ca^{2+}-free medium suggests a role of extracellular calcium influx in cell death, probably through NMDA glutamate receptors, because MK-801 substantially prevented neuronal damage. Previous studies have shown an increase in intracellular calcium during exposure to millimolar concentrations of iodoacetate in astrocytes (Kahlert and Reiser, 2000), although in cultured CA1 hippocampal neurons, a previous study showed no increase in intracellular calcium at 100 μM iodoacetate and no protection by Ca^{2+}-free medium or the addition of MK-801 (Uto et al., 1995). According to the present results, no significant protection by MK-801 was observed when 100 μM iodoacetate was used. In contrast, we have shown that vitamin E efficiently prevents damage in these conditions (Massieu et al., 2003b). Apparently, an excitotoxic component of cell death is associated with a moderate energy deficit, whereas during acute glycolysis inhibition additional mechanisms might be involved, such as oxidative stress. In agreement, in cultured cerebellar granule neurons cell damage induced by 30 μM iodoacetate is not sensitive to glutamate receptor antagonists but induces prominent toxicity, suggesting the involvement of mechanisms additional to excitotoxicity in neuronal damage (Malcolm et al., 2000).

According to the present results, the extracellular levels of glutamate and aspartate were not altered either immediately or 3.5 hr after iodoacetate exposure, except

for a slight increase in glutamate levels at 3.5 hr. At this time, a small decrease in MTT reduction was observed. Increased glutamate levels might result from leakage from damaged neurons or deficient removal by glutamate transporters, which are energy dependent. Several studies have shown that treatment with high concentrations of iodoacetate (1–5 mM), resulting in large ATP deficit, substantially increase glutamate and aspartate levels in the extracellular medium both in vivo (Sandberg et al., 1985; Massieu et al., 2000) and in vitro (Madl and Burgesser, 1993; Gemba et al., 1994; Ogata et al., 1995). Excitatory amino acid release during these conditions results from deficient uptake or even inverse activation of glutamate transporters (Madl and Burgesser, 1993; Gemba et al., 1994; Ogata et al., 1995). Although it is not known if there is further accumulation of excitatory amino acids in the extracellular medium beyond 4 hr, a secondary excitotoxic mechanism of cell death may still be favored by energy impairment conditions. During ATP-limiting conditions, a small increase in glutamate or aspartate levels, or even physiologic concentrations of these amino acids, can induce excitotoxic cell death through activation of NMDA receptors after the extrusion of Mg^{2+} from the receptor channel resulting from partial plasma membrane depolarization (Novelli et al., 1988; Zeevalk and Nicklas, 1992). Accordingly, exposure to 50 μM iodoacetate induces a decline in ATP levels (Massieu et al., 2003a).

Results show that blockade of Ca^{2+} storage in ER through inhibition of SERCA, either reversibly by CPA or TMB-8 or irreversibly by TG, did not potentiate neuronal damage induced by iodoacetate. Incubation with TG alone substantially reduced cell survival. A toxic effect of TG has been reported previously and induction of apoptosis by this inhibitor has been suggested (Wei and Perry, 1996; Takadera and Ohyashiki, 1998). Similarly, TG and CPA induce neuronal death in the SH-SY5Y cell line (Nguyen et al., 2002). Our results agree with a previous study showing that TG-releasable calcium is reduced considerably during glucose and oxygen deprivation (Wang et al., 2002). It is possible that in the present experimental conditions, there is sufficient ATP derived from mitochondria to fuel SERCA. However, a recent study showed that in hippocampal astrocytes glycolysis inhibition induced by iodoacetate or 2-deoxyglucose completely abolished calcium release from CPA-sensitive ER pools. The authors suggested that glycolysis is the main source of energy used to fuel Ca^{2+} loading into ER pools, and that increased intracellular calcium during glycolysis inhibition, is mainly the consequence of the disruption of calcium storage (Kahlert and Reiser, 2000). Further studies are needed to investigate if SERCA-supported calcium loading in ER is impaired in our experimental conditions.

Promoting calcium release from RyR by caffeine and ryanodine (1 μM) exacerbated neuronal damage induced by iodoacetate. These results agree with a previous study suggesting the involvement of calcium release through activation of RyR, in the elevation of intracellular calcium during oxygen/glucose deprivation (Pisani et al., 2000). The role of calcium release mediated by RyR is suggested further by the protective effect of dantrolene, a blocker of RyR-mediated Ca^{2+} release and of 25 μM ryanodine. Dantrolene has been shown to efficiently inhibit the elevation of cytosolic Ca^{2+} and neurotoxicity induced by NMDA, glutamate, or potassium depolarization (Bouchelouche et al., 1989; Frandsen and Schousboe; 1991, 1992; Simpson et al., 1993; Mody and MacDonald, 1995; Gepdiremen et al., 2001). Others studies have demonstrated a significant neuroprotective effect of dantrolene against ischemic neuronal damage (Zhang et al., 1993; Wei and Perry, 1996; Nakayama et al., 2002). The more efficient neuroprotective effect of dantrolene relative to that of ryanodine against iodoacetate-induced cell death might be attributed to additional actions of dantrolene. Some studies suggest that dantrolene prevents cell death and increased cytosolic Ca^{2+} induced by thapsigargin (Wei and Perry, 1996; Nguyen et al., 2002), as well as calcium release induced by IP_3 microinjection in myenteric neurons (Turner et al., 2001). These observations indicate that the effect of dantrolene is not limited to blockade of RyR. Accordingly, it was proposed recently that dantrolene inhibits Ca^{2+} influx through the NMDA receptor (Makarewicz et al., 2003). In addition, the protective effect of dantrolene against 3-hydroxykynurenine in PC12 and GT1-7 cells is related to the increased expression of the Bcl-2 protein (Wie et al., 2000), and some studies suggest an antioxidant action of dantrolene (Büyükokuroglu et al., 2001). It has been reported, however, that RyR2, which is abundantly expressed in brain, is not a target for dantrolene, in contrast to RyR1 and RyR3 (Zhao et al., 2001). The three subtypes of RyRs, however, have been observed in the hippocampus of rabbit and mouse (Furuichi et al., 1994; Giannini et al., 1995). Alternatively, dantrolene and ryanodine might be acting at different sites on RyR, because it is suggested that their binding sites are pharmacologically distinct and might represent different molecular entities (Palnitkar et al., 1997). Consistent with this hypothesis, It has been shown that dantrolene has no effect on [^{3}H]ryanodine binding in cerebellar and cortical membranes (Smith and Nahorski, 1993; Rosa et al., 1997).

Nonetheless, the protective effect of dantrolene and the dose-dependent effect of ryanodine on neuronal death, suggest that RyR strongly contributes to neuronal damage induced during glycolysis inhibition. Calcium release from RyR might be mediated by calcium influx after activation of NMDA receptors. Previous studies show that stimulation of NMDA receptors induces the release of calcium via RyR both in vivo and in vitro (Frandsen and Schousboe, 1992; Lazarewicz et al., 1998; Makarewicz et al., 2000, 2003).

Neuronal damage induced by iodoacetate might result from the disruption of ATP-dependent calcium storage and extrusion mechanisms. The observed potentiation of neuronal damage by RyR agonists and its protection by dantrolene therefore might be related to intracellular ATP levels. According to the results, the release

of calcium from ER shows a dual effect. At early times after iodoacetate exposure, caffeine induces an increase in ATP levels, whereas the opposite effect is observed at 8 hr. Increased ATP might result from the stimulation of mitochondrial metabolism due to activation of Ca^{2+}-dependent dehydrogenases (Hajnóczky and Csórdas, 2002). The rate of ATP synthesis is influenced by mitochondrial Ca^{2+} uptake, and a physiologic elevation in intracellular calcium concentration causes an increase in the mitochondrial NADH/NAD^+ ratio in different cell types (Jacobson and Duchen, 2004). Moreover, Rizzuto et al. (1994) demonstrated a correlation between increased mitochondrial calcium after IP_3R activation and augmented NAD(P)H levels, which presumably reflects the activation dehydrogenases in the mitochondrial matrix. At 8 hr after iodoacetate exposure, caffeine potentiated the iodoacetate-induced decrease in ATP levels. This result might be attributed to an increased energy requirement for the extrusion of cytosolic calcium or impairment of mitochondrial metabolism due to Ca^{2+} overload. Consistent with this result, inhibition of RyR with dantrolene preserved ATP levels. Taken together, the results suggest that release of Ca^{2+} from ER contributes to ATP depletion during glycolysis inhibition and that preservation of ATP levels during this condition prevents neuronal death (Massieu et al., 2003a).

The role of calcium release from IP_3R in neuronal damage is suggested by the protective effect of XeC, which specifically inhibits IP_3R (Gafni et al., 1997), and has been proven to inhibit Ca^{2+} release mediated by IP_3 in diverse preparations (Miyamoto et al., 2000; Ozaki et al., 2002). Only partial protection of neuronal death was observed in the presence of XeC, suggesting that calcium release from this receptor is involved at least in part in cell death. Although the activation of IP_3R in the present experimental conditions is not clear, it might result from the activation of metabotropic glutamate receptors by accumulated glutamate. Alternatively, it has been suggested that calcium-induced calcium release from IP_3R can occur in some circumstances even in the absence of IP_3 (Yang et al., 2002). Nevertheless, additional experiments are needed to clarify this issue.

In summary, the present study suggests that moderate glycolysis inhibition leads to cell death in cultured hippocampal neurons by a mechanism involving excitotoxicity and disruption of intracellular calcium storage. It remains to be determined if the release of calcium from ER is related to calcium influx through NMDA receptors, to diminished glycolytic ATP or both. Decreased glucose metabolism is related to aging (Eberling et al., 1995) and chronic neurodegenerative diseases (Mielke et al., 1998; Slosman et al., 2001; Planel et al., 2004), and reduced GAPDH activity is observed in brain and fibroblasts from Alzheimer's and Huntington's disease patients (Kish et al., 1998; Mazzola and Sirover, 2001, 2003). The present results might contribute to the understanding of the mechanisms involved in cell death related to these conditions. Moderate but progressive alterations in energy metabolism during the lifetime of patients might disturb systems involved in the extrusion and storage of intracellular Ca^{2+} and predispose neurons to excitotoxicity.

ACKNOWLEDGMENTS

This work was supported by CONACYT (grant 40306-M to L.M. and 167146 to K.H.-F.) and DGEP (fellowships to K.H.-F.).

We thank Mrs. Teresa Montiel for her technical assistance.

REFERENCES

Antoine FR, Wei CI, Littell RC, Marshall MR. 1999. HPLC method for analysis of free amino acids in fish using o-phthaldialdehyde precolumn derivatization. J Agric Food Chem 47:5100–5107.

Arundine M, Tymianski M. 2003. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium 34:325–337.

Berridge MV, Tan AS. 1993. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys 303: 474–482.

Bouchelouche P, Belhage B, Frandsen A, Drejer J, Schousboe A. 1989. Glutamate receptor activation in cultured cerebellar granule cells increases cytosolic free Ca^{2+} by mobilization of cellular Ca^{2+} and activation of Ca^{2+} influx. Exp Brain Res 76:281–291.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Brewer GJ, Torricelli JR, Evege EK, Price PJ. 1993. Optimized survival of hippocampal neurons in B27-supplemented neurobasal, a new serum-free combination. J Neurosci Res 35:567–576.

Büyükokuroglu ME, Gülcin I, Oktay M, Küfreviogle ÖI. 2001. In vitro antioxidant properties of dantrolene sodium. Pharmacol Res 44:491–494.

Choi DW. 1992. Cell death. J Neurobiol 23:1261–1276.

Choi DW. 1995. Calcium: still center-stage in hypoxic-ischemic neuronal death. Trends Neurosci 18:58–60.

Csernansky JG, Bardgett ME, Sheline YI, Morris JC, Olney JW. 1996. CSF excitatory amino acids and severity of illness in Alzheimer's disease. Neurology 46:1715–1720.

Eberling JL, Nordahl TE, Kusubov N, Reed BR, Budinger TF, Jagust WJ. 1995. Reduced temporal lobe glucose metabolism in aging. J Neuroimaging 5:178–182.

Fill M, Copello JA. 2002. Ryanodine receptor calcium release channels. Physiol Rev 82:893–922.

Frandsen A, Schousboe A. 1991. Dantrolene prevents glutamate cytotoxicity and Ca^{2+} release from intracellular stores in cultured cerebral cortical neurons. J Neurochem 56:1075–1078.

Frandsen A, Schousboe A. 1992. Mobilization of dantrolene-sensitive intracellular calcium pools is involved in the cytotoxicity induced by quisqualate and N-methyl-D-aspartate but not by 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate and kainate in cultured cerebral cortical neurons. Proc Natl Acad Sci USA 89:2590–2594.

Furuichi T, Furutama D, Hakamata Y, Nakai J, Takeshima H, Mikoshiba K. 1994. Multiple types of ryanodine receptor/Ca^{2+} release channels are differentially expressed in rabbit brain. J Neurosci 14:4794–4805.

Gafni J, Munsch JA, Lam TH, Catlin MC, Costa LG, Molinski TF, Pessah IN. 1997. Xestospongins: potent membrane permeable blockers of the inositol 1, 4,5-trisphosphate receptor. Neuron 19:723–733.

Garcia O, Massieu L. 2003. Glutamate uptake inhibitor L-trans-pyrrolidine 2, 4-dicarboxylate becomes neurotoxic in the presence of subthreshold concentrations of mitochondrial toxin 3-nitropropionate:

involvement of mitochondrial reducing activity and ATP production. J Neurosci Res 74:956–966.

Gemba T, Oshima T, Ninomiya M. 1994. Glutamate efflux via the reversal of the sodium-dependent glutamate transporter caused by glycolytic inhibition in rat cultured astrocytes. Neuroscience 63:789–795.

Gepdiremen A, Düzenli S, Hacimüftüoglu A, Süleyman H., Öztas S. 2001. The effects of dantrolene alone or in combination with nimodipine in glutamate-induced neurotoxicity in cerebellar granule cell culture of rats pups. Pharmacol Res 43:241–244.

Giannini G, Conti A, Mammarella S, Scrobogna M. 1995. The ryanodine receptor/calcium channel genes are widely and differentially expressed in murine brain and peripheral tissues. J Cell Biol 12:893–904.

Hajnóczky G, Csordás M. 2002. Old players in a new role: mitochondria-associated membranes, VDAC, and ryanodine receptors as contributors to calcium signal propagation from endoplasmic reticulum to the mitochondria. Cell Calcium 32:363–377.

Jacobson J, Duchen MR. 2004. Interplay between mitochondria and cellular calcium signaling. Mol Cell Biochem 256/257:209–218.

Kahlert S, Reiser G. 2000. Requirement of glycolytic and mitochondrial energy supply for loading of Ca^{2+} stores and $InsP_3$-mediated Ca^{2+} signaling in rat hippocampus astrocytes. J Neurosci Res 61:409–420.

Kahlert S, Reiser G. 2004. Glial perspectives of metabolic states during cerebral hypoxia-calcium regulation and metabolic energy. Cell Calcium 36:295–302.

Kish SJ, Lopes-Cendes I, Guttman M, Furukawa Y, Pandolfo M, Rouleau GA, Ross BM, Nance M, Schut L, Ang L, DiStefano L. 1998. Brain glyceraldehyde-3-phosphate dehydrogenase activity in human trinucleotide repeat disorders. Arch Neurol 55:1299–12304.

Kristián T, Siesjö BK. 1998. Calcium in ischemic cell death. Stroke. 29:705–718.

Lazarewicz JW, Rybkowski W, Sadowski M, Ziembowicz A, Alaraj M, Wegiel J, Wisniewski HM. 1998. N-methyl-D-aspartate receptor-mediated, calcium-induced calcium release in rat dentate gyrus/CA4 in vivo. J Neurosci Res 51:76–84.

Madl JE, Burgesser K. 1993. Adenosine triphosphate depletion reverses sodium-dependent, neuronal uptake of glutamate in rat hippocampal slices. J Neurosci 13:4429–4444.

Makarewicz D, Salinska E, Puka-Sundvall M, Alaraj M, Ziembowicz A, Skangiel-Kramska J, Jablonska B, Bona E, Hagberg H, Lazarewicz JW. 2000. NMDA-induced ^{45}Ca release in the dentate gyrus of newborn rats: in vivo microdialysis study. Neurochem Int 37:307–316.

Makarewicz D, Zieminska E, Lazarewicz . 2003. Dantrolene inhibits NMDA-induced ^{45}Ca uptake in cultured cerebellar granule neurons. Neurochem Int 43:273–278.

Malcolm CS, Benwell KR, Lamb H, Bebbington D, Porter RH. 2000. Characterization of iodoacetate-mediated neurotoxicity in vitro using primary cultures of rat cerebellar granule cells. Free Radic Biol Med 28:102–127.

Massieu L, Gómez-Román N, Montiel T. 2000. In vivo Potentiation of glutamate-mediated neuronal damage after chronic administration of the glycolysis inhibitor Iodoacetate. Exp Neurol 165:257–267.

Massieu L, Haces ML, Montiel M, Hernández-Fonseca K. 2003a. Acetoacetate protects hippocampal neurons against glutamate-mediated neuronal damage during glycolysis inhibition. Neuroscience 120:365–378.

Massieu L, Montiel T, del Río P, Hernández K, Haces ML, García O, Camacho A, Mejia J. 2003b. Role of energy metabolism in neuronal death associated with cerebral ischemia and neurodegenerative diseases, and its prevention by energy substrates. Rec Res Dev Neurochem 6:1–24.

Mayer ML, Westbrook GL. 1987. Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurons. J Physiol 394:501–527.

Mazzola JL, Sirover MA. 2001. Reduction of glyceraldehyde-3-phosphate dehydrogenase activity in Alzheimer's disease and in Huntington's disease fibroblasts. J Neurochem 76:442–449.

Mazzola JL, Sirover MA. 2003. Subcellular alteration of glyceraldehyde-3-phosphate dehydrogenase in Alzheimer's disease fibroblasts. J Neurosci Res 71:279–285.

Mielke R, Kessler J, Szelies B, Herholz K, Wienhard K, Heiss WD. 1998. Normal and pathological aging—findings of positron-emission-tomography. J Neural Transm 105:821–837.

Miyamoto S, Izumi M, Hori M, Kobayashi M, Ozaki H, Karaki H. 2000. Xestospongin C, a selective and membrane-permeable inhibitor of IP_3 receptor, attenuates the positive inotropic effect of alpha-adrenergic stimulation in guinea-pig papillary muscle. Br J Pharmacol 130:650–654.

Mody I, MacDonald JF. 1995. NMDA receptor-dependent excitotoxicity: the role of intracellular Ca^{2+} release. Trends Pharmacol Sci 16:356–359.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63.

Nakayama R, Yano T, Ushijima K, Abe E, Terasaki H. 2002. Effects of dantrolene on extracellular glutamate concentration and neuronal death in the rat hippocampal CA1 region subjected to transient ischemia. Anesthesiology. 96:705–710.

Nguyen HN, Wang C, Perry DC. 2002. Depletion of intracellular calcium stores is toxic to SH-SY5Y neuronal cells. Brain Res 924:159–166.

Novelli A, Reilly JA, Lysko PG, Henneberry RC. 1988. Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. Brain Res 451:205–212.

Ogata T, Nakamura Y, Tsuji K, Shibata T, Kataoka K. 1995. A possible mechanism for the hypoxia-hypoglycemia-induced release of excitatory amino acids from cultured hippocampal astrocytes. Neurochem Res 20: 737–743.

Opitz T, Reymann KG. 1991. Blockade of metabotropic glutamate receptors protects rat CA1 neurons from hypoxic injury. Neuroreport 2:455–457.

Orrenius S, Nicotera P. 1994. The calcium ion and cell death. J Neural Transm Suppl 43:1–11.

Ozaki H, Hori M, Kim YS, Kwon SC, Ahn DS, Nakazawa H, Kobayashi M, Karaki H. 2002. Inhibitory mechanism of xestospongin-C on contraction and ion channels in the intestinal smooth muscle. Br J Pharmacol 137:1207–1212.

Palnitkar SS, Mickelson JR, Louis CF, Parness J. 1997. Pharmacological distinction between dantrolene and ryanodine binding sites: evidence from normal and malignant hyperthermia-susceptible porcine skeletal muscle. Biochem J 326:847–852.

Paschen W. 2000. Role of calcium in neuronal cell injury: which subcellular compartment is involved? Brain Res Bull 53:409–413.

Paschen W. 2003. Mechanisms of neuronal cell death: diverse roles of calcium in the various subcellular compartments. Cell Calcium 34:305–130.

Paschen W, Doutheil J. 1999. Disturbance of endoplasmic reticulum functions: a key mechanism underlying cell damage? J Cereb Blood Flow Metab 19:1–18.

Pisani A, Bonsi P, Centonze D, Giacomini P, Calabresi P. 2000. Involvement of intracellular calcium stores during oxygen/glucose deprivation in striatal large aspiny interneurons. J Cereb Blood Flow Metab 20:839–846.

Planel E, Miyasaka T, Launey T, Chui DH, Tanemura K, Sato S, Murayama O, Ishiguro K, Tatebayashi Y, Takashima A. 2004. Alterations in glucose metabolism induce hypothermia leading to tau hyperphosphorylation through differential inhibition of kinase and phosphatase activities: implications for Alzheimer's disease. J Neurosci 24:2401–2411.

Rizzuto R, Bastianutto C, Brini M, Murgia M, Pozzan T. 1994. Mitochondrial Ca^{2+} homeostasis in intact cells. J Cell Biol 126:1183–1194.

Rosa R, Sanfeeliu C, Rodríguez-Farré E, Frandsen A, Schousboe A, Suñol C. 1997. Properties of ryanodine receptors in cultured cerebellar granule neurons: effects of hexachlorocyhexane isomers and calcium. J Neurosci Res 47:23–33.

Sandberg M, Nystrom B, Hamberger A. 1985. Metabolically derived aspartate-elevated extracellular levels in vivo in iodoacetate poisoning. J Neurosci Res 13:489–495.

Schinder AF, Olson EC, Spitzer NC, Montal M. 1996. Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J Neuroscience 16:6125–6133.

Schwarcz R, Whetsell WO, Mangano RM. 1983. Quinolic acid: an endogenous metabolite that produces axon-sparing lesions in rat brain. Science 219:316–318.

Siesjö BK, Bengtsson F. 1989. Calcium fluxes, calcium antagonists, and calcium-related pathology in brain ischemia, hypoglycemia, and spreading depression: a unifying hypothesis. J Cereb Blood Flow Metab 9: 127–140.

Siesjö BK, Hu B, Kristián T. 1999. Is the cell death pathway triggered by the mitochondrion or the endoplasmic reticulum? J Cereb Blood Flow Metab 19:19–26.

Simpson PB, Challiss RA, Nahorski SR. 1993. Involvement of intracellular stores in the Ca^{2+} responses to N-methyl-D-aspartate and depolarization in cerebellar granule cells. J Neurochem 61:760–763.

Slosman DO, Ludwig C, Zerarka S, Pellerin L, Chicherio C, de Ribaupierre A, Annoni JM, Bouras C, Herrmann F, Michel JP, Giacobini E, Magistretti PJ. 2001. Brain energy metabolism in Alzheimer's disease: 99mTc-HMPAO SPECT imaging during verbal fluency and role of astrocytes in the cellular mechanism of 99mTc-HMPAO retention. Brain Res Brain Res Rev 36:230–240.

Smith SM, Nahorski SR. 1993. Characterization and distribution of inositol polyphosphate and ryanodine receptors in rat brain. J Neurochem 60:1605–1614.

Takadera T, Ohyashiki T. 1998. Apoptotic cell death and CPP32-like activation induced by thapsigargin and their prevention by nerve growth factor in PC12 cells. Biochim Biophys Acta 1401:63–71.

Turner DJ, Segura BJ, Cowles RA, Zhang W, Mulholland MW. 2001. Functional overlap of IP_3- and cADP-ribose-sensitive calcium stores in guinea pig myenteric neurons. Am J Physiol Gastrointest Liver Physiol 281:208–215.

Tymianski M. 1995. Neuroprotection in vitro and in vivo by cell membrane-permeant Ca^{2+} chelator. Clin Exp Pharmacol Physiol 22:299–300.

Tymianski M, Wallace MC, Spigelman I, Uno M, Carlen PL, Tator CH, Charlton MP. 1993. Cell-permeant Ca^{2+} chelators reduce early excitotoxic and ischemic neuronal injury in vitro and in vivo. Neuron 11: 221–235.

Uto A, Dux E, Kusumoto M, Hossmann KA. 1995. Delayed neuronal death after brief histotoxic hypoxia in vitro. J Neurochem 64:2185–2191.

Verkhratsky A, Petersen OH. 2002. The endoplasmic reticulum as an integrating signalling organelle: from neuronal signalling to neuronal death. Eur J Pharmacol 447:141–154.

Vesce S, Kirk L, Nicholls DG. 2004. Relationships between superoxide levels and delayed calcium deregulation in cultured cerebellar granule cells exposed continuously to glutamate. J Neurochem 9:683–693.

Wang C, Nguyen HN, Maguire JL, Perry DC. 2002. Role of intracellular calcium stores in cell death from oxygen-glucose deprivation in a neuronal cell line. J Cereb Blood Flow Metab 22:206–214.

Wei H, Perry DC. 1996. Dantrolene is cytoprotective in two models of neuronal cell death. J Neurochem 67:2390–2398.

Wie H, Leeds P, Chen RW, Wie W, Leng Y, Bredesen DE, Chuang DM. 2000. Neuronal apoptosis induced by pharmacological concentration of 3-hydroxykynurenine: characterization and protection by dantrolene and Bcl-2 overexpression. J Neurochem 75:81–90.

Yao H, Haddad GG. 2004. Calcium and pH homeostasis in neurons during hypoxia and ischemia. Cell Calcium 36:247–255.

Yang J, McBride S, Mak DO, Vardi N, Palczewski K, Haeseleer F, Foskett JK. 2002. Identification of a family of calcium sensors as protein ligands of inositol trisphosphate receptor Ca^{2+} release channels. Proc Natl Acad Sci USA. 99:7711–7716.

Young AB, Greenamyre JT, Hollingsworth Z, Albin R, D'Amato C, Shoulson I, Penney JB. 1988. NMDA receptor losses in Putamen from patients with Huntington's disease. Science 241:981–983.

Zeevalk GD, Nicklas WJ. 1992. Evidence that loss of the voltage-dependent Mg^{2+} block at the N-methyl-D-aspartate receptor underlies receptor activation during inhibition of neuronal metabolism. J Neurochem 59:1211–1220.

Zhang L, Andou Y, Masuda S, Mitani A, Kataoka K. 1993. Dantrolene protects against ischemic delayed neuronal death in gerbil brain. Neurosci Lett 158:287–288.

Zhao F, Li P, Chen SR, Louis CF, Fruen BR. 2001. Dantrolene inhibition of ryanodine receptor Ca^{2+} release channels. J Biol Chem 276:13810–13816.