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PAPEL DE LAS ESPECIES REACTIVAS DEL OXÍGENO (ERO) PRODUCIDAS POR LA MITOCONDRIA Y NADPH-OXIDASA (NOX) EN LA MUERTE NEURONAL

TESIS

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PRESENTA:
CAROLINA CID CASTRO

TUTOR PRINCIPAL
JULIO MORÁN ANDRADE
Instituto de Fisiología Celular

MIEMBROS DE COMITÉ TUTOR
Lourdes Massieu Trigo
Instituto de Fisiología Celular
Mahara Angélica Valverde Ramírez
Instituto de Investigaciones Biomédicas

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El comité tutoral que brindó asesoría durante el desarrollo de este trabajo estuvo conformado por:

Dr. Julio Morán Andrade

Dra. Lourdes Massieu Trigo

Dra. Mahara Valverde Ramírez

El jurado que revisó la tesis estuvo conformado por:

Dra. Perla Deyanira Maldonado Jiménez

Dra. María Eugenia Gonsebatt Bonaparte

Dra. Claudia Lydia Treviño Santa Cruz

Dr. Jesús Aguirre Linares

Dr. Jaime Iván Velasco Velásquez

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Abreviaturas.

K5: 5 mM de potasio

NGC: Neuronas granulares de cerebelo

K25: 25 mM de potasio

ST: Estaurosporina

DHE: Dihidroetidio

DPI: Difenil iodonio

MTT: Bromuro de 3-(4, 5-dimetiltiazol-2-il)-2, 5-difeniltetrazolio

NOX: NADPH Oxidasa

mtERO: Especies reactivas de oxígeno de la mitocondria

ctERO: Especies reactivas de oxígeno citoplásmicas

ERO: Especies reactivas de oxígeno

Drp1: Dynamin related protein 1

DIV: Días *in vitro*

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Resumen.

Introducción: Se ha descrito que las especies reactivas de oxígeno (ERO) participan en múltiples procesos fisiopatológicos. Existen diversas fuentes de ERO en la célula, entre ellas las NADPH oxidasas (NOX) y la mitocondria, ambas han demostrado ser importantes en los procesos fisiológicos y patológicos de las neuronas; sin embargo, no se conocen a detalle los mecanismos en los que participan. Una de las características que frecuentemente se reportan ante el aumento en los niveles de ERO es el cambio en la morfología mitocondrial, estos cambios son conocidos como dinámica mitocondrial e incluyen el alargamiento o el acortamiento de las redes mitocondriales. El acortamiento se conoce cómo fisión (o fragmentación) mitocondrial y se regula por la proteína Drp1. No se conoce si las ERO producidas por la mitocondria o por las NOX están relacionadas con la regulación de la actividad de Drp1 y si esto, cómo consecuencia participa en la progresión de la muerte neuronal. **Objetivo:** Conocer el papel de las ERO provenientes de la mitocondria sobre la muerte de neuronas granulares de cerebelo (NGC) y la regulación de la dinámica mitocondrial durante este proceso. **Estrategia experimental:** Se realizó un cultivo de NGC que se mantuvieron en un medio con 25mM de cloruro de potasio (K25) durante 7 días *in vitro*. Posteriormente, las neuronas se trataron con estaurosporina (ST, 0.5µM) o KCl 5mM (K5). Se determinó el nivel de las ERO citoplásmicas (ctERO) y las ERO mitocondriales (mtERO) durante distintos momentos del proceso de muerte utilizando, dihidroetidio (DHE) o MitoTracker Red CM-H_2XRos, respectivamente. La actividad metabólica se estimó mediante la reducción de bromuro de 3-(4, 5-dimetiltiazol-2-il)-2, 5-difeniltetrazolio (MTT) a lo largo de los estímulos y hasta 24 h después de los tratamientos. Para determinar si había cambios en la morfología mitocondrial, éstas se tiñeron con MitoTracker Green y se obtuvieron imágenes con un microscopio de epifluorescencia. Se determinaron los niveles de Drp1 total y fosforilado en el residuo Ser616 como indicativo de su activación, en distintos momentos después de los estímulos de K5 y ST, además de con NGC pretratadas con MitoTempo (10µM), un antioxidante mitocondrial. **Resultados:** Se observó un aumento en los niveles de ctERO y mtERO desde los primeros minutos del proceso de muerte, de igual manera los niveles de reducción de MTT comenzaron desde momentos tempranos del proceso y se mantuvieron hasta las 24h. La reducción de MTT se inhibió parcialmente en presencia de MitoTempo. Por otra parte, en ambas condiciones de muerte (ST y K5) se observaron mitocondrias fragmentadas y redondeadas y se observó que la fosforilación de Drp1(Ser616) aumentó en la condición de K5, pero disminuyó en las NGC tratadas con ST. En el caso de la fosforilación inducida por K5, observamos una disminución al pretratar con MitoTempo, un efecto que no se observó en las neuronas tratadas con ST. **Conclusiones:** estos resultados sugieren que la generación de mtERO es un evento muy temprano en el proceso de muerte neuronal, previo a la generación de ctERO y a la activación del programa apoptótico. Las mtERO parecen necesarias para el proceso de muerte neuronal, pero no para la fragmentación mitocondrial, aunque la fragmentación es inducida durante el proceso de muerte. La fragmentación mitocondrial y la muerte neuronal de las NGC parecen no estar mediadas por la fosforilación de Drp1 en el residuo Ser616, sugiriendo que la fragmentación mitocondrial se lleva a cabo por diferentes mecanismos, dependiendo de la condición apoptótica.

Abstract.

Introduction: Reactive oxygen species (ROS) have been described as participating in multiple pathophysiological processes. Exist various sources of ROS in the cell, including NADPH oxidases (NOX) and mitochondria, both of which have been shown to be important in the physiological and pathological processes of neurons; however, the mechanisms in which they participate are not known in detail. One of the characteristics that are frequently reported before the increase in ROS levels is the change in mitochondrial morphology, these changes are known as mitochondrial dynamics and include the hyperconnection or shortening of mitochondrial networks. The shortening is known as mitochondrial fission (or fragmentation) and is regulated by the Drp1 protein. It is not known whether the ROS produced by the mitochondria or by NOX are related to the regulation of Drp1 activity and if this, as a consequence, participates in the progression of neuronal death. **Objective:** Knowing the role of ROS from the mitochondria on the death of cerebellar granular neurons (NGC) and the regulation of mitochondrial dynamics during this process. **Experimental strategy:** A culture of NGC was carried out and kept in a medium with 25mM potassium chloride (K25) for 7 days in vitro. Subsequently, the neurons were treated with staurosporine (ST, 0.5µM) or 5mM KCl (K5). The level of cytoplasmic ROS (ctERO) and mitochondrial ROS (mtERO) was determined during different moments of the death process using dihydroetidium (DHE) or MitoTracker Red CM-H2XRos, respectively. The metabolic activity was estimated by the reduction of 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium (MTT) bromide throughout the stimuli and up to 24 h after the treatments. To determine if there were changes in mitochondrial morphology, these were stained with MitoTracker Green and images were obtained with an epifluorescence microscope. The levels of total and phosphorylated Drp1 in residue Ser616 were determined as indicative of its activation, at different times after K5 and ST stimuli, in addition to with NGC pretreated with MitoTempo (10 µM), a mitochondrial antioxidant. **Results:** An increase in the levels of ctERO and mtERO was observed from the first minutes of the death process, in the same way, the levels of reduction of MTT began from the early moments of the process and were maintained until 24h. The reduction of MTT was partially inhibited in the presence of MitoTempo. On the other hand, in both death conditions (ST and K5) fragmented and rounded mitochondria were observed and it was observed that the phosphorylation of Drp1 (Ser616) increased in the K5 condition, but decreased in the NGC treated with ST. In the case of K5-induced phosphorylation, we observed a decrease when pretreating with MitoTempo, an effect that was not observed in ST-treated neurons. **Conclusions:** These results suggest that the generation of mtERO is a very early event in the neuronal death process, prior to the generation of ctERO and the activation of the apoptotic program. MtEROs seem necessary for the neuronal death process, but not for mitochondrial fragmentation, although fragmentation is induced during the death process. Mitochondrial fragmentation and neuronal death of NGCs appear not to be mediated by phosphorylation of Drp1 at residue Ser616, suggesting that mitochondrial fragmentation is carried out by different mechanisms, depending on the apoptotic condition.

Introducción.

La muerte neuronal.

La muerte neuronal es un proceso que ocurre normalmente durante el desarrollo del sistema nervioso y es necesaria para completar todas las estructuras y circuitos requeridos para su adecuado funcionamiento (Fricker et al. 2018; Ikonomidou y Kaindl 2011). Por otra parte, la muerte neuronal también se observa de forma progresiva en procesos neurodegenerativos como las enfermedades de Parkinson y Alzheimer (Chi, Chang, y Sang 2018; Pozo Devoto y Falzone 2017).

Existen diversas formas de muerte neuronal inducidas por diferentes condiciones como la privación de factores tróficos o la excitotoxicidad, ésta última se caracteriza por una activación exacerbada de los receptores de glutamato. Estas formas de muerte incluyen, entre otras, a la necrosis, en la cual la membrana plasmática se rompe ocasionando la salida del contenido celular; la ferroptosis, que es una forma regulada de necrosis dependiente de hierro (Chi et al., 2018b; Fricker et al., 2018) y la muerte neuronal apoptótica que se ha descrito que es una parte trascendental del desarrollo del cerebro para el establecimiento de los circuitos neuronales (Hollville et al., 2019) y que también participa en procesos patológicos (Han et al. 2017; Li et al. 2013; Zsurka y Kunz 2013).

Uno de los tipos de muerte más estudiados es la muerte neuronal apoptótica (Lossi et al., 2005; Okouchi et al., 2007), que se caracteriza por cambios morfológicos y bioquímicos que incluyen la condensación de la cromatina, formación de núcleos picnóticos, la fragmentación del DNA, la exposición de fosfatidil-serina y la activación de proteasas conocidas como Caspasas (Coyoy et al., 2008; Maycotte et al., 2010; Ramiro-Cortés et al., 2011). Se ha descrito que la muerte neuronal apoptótica se lleva a cabo por dos vías, una de éstas es la vía intrínseca, también conocida como la vía mitocondrial. Esta vía está regulada por la familia de proteínas Bcl-2 que ejecutan la apertura del poro de la membrana mitocondrial (Mitochondrial Outer Membrane Pore, MOMP), lo que genera la salida de citocromo c de la mitocondria, esto dispara la formación del apoptosoma en el citosol al unirse a Aif-

1 (apoptotic protease-activating factor-1) que recluta y activa a la Caspasa 9, la cual se encarga a su vez de activar a la Caspasa efectora, la Caspasa 3 (Fricker et al., 2018; Okouchi et al., 2007). La activación de esta proteasa inicia el desmantelamiento ordenado y progresivo de los constituyentes de la célula.

Aumento de los niveles de ERO en procesos de muerte neuronal apoptótica.

Múltiples evidencias mencionan que hay un aumento en los niveles de ERO durante el desarrollo de los procesos apotóticos, independientemente de cuál sea el estímulo (Chen et al., 2009; Han et al., 2017; Li et al., 2018; Xu et al., 2017). Se sabe, además, que el empleo de antioxidantes inhibe la muerte neuronal apoptótica (Hu y Li, 2016; Valencia y Morán, 2001; Xu et al., 2017), por lo que se acepta que estas moléculas son reguladores importantes en el proceso apoptótico.

En las células, existe un gran número de fuentes de ERO (Holmström y Finkel, 2014); sin embargo, las más importantes y las más estudiadas en el contexto de la muerte neuronal son la mitocondria y las NADPH-oxidasas (NOX). Las NOX son una familia de proteínas que producen ERO. En mamíferos se conocen siete isoformas y se pueden encontrar en distintos espacios subcelulares, tales como: la membrana plasmática, la membrana del retículo endolásmico (RE) y la membrana externa de la mitocondria (Nayernia et al., 2014). Se sabe, por ejemplo, que las NOX participan en la muerte de neuronas corticales y dopaminérgicas (Chay et al., 2017; Choi et al., 2014). Además, las NOX participan en la muerte apoptótica de las neuronas granulares de cerebelo (NGC) (Coyoy et al., 2008). En particular, se sabe que el NOX2 participa en la muerte inducida por estaurosporina (ST) (Cho et al., 2012; Choi et al., 2014; Coyoy et al., 2008; Guemez-Gamboa y Morán, 2009). En NGC, la ausencia de NOX2 induce una disminución en los niveles de ERO y en los niveles de caspasa 3 activa (Guemez-Gamboa y Morán, 2009). Aunque en este trabajo se probó que la ausencia de NOX2 es importante en la muerte apoptótica, esta condición no fue capaz de evitar la muerte por completo, lo que sugiere que otra isoforma de NOX u otra fuente de ERO pudieran estar involucrados en el proceso (Guemez-Gamboa y Morán, 2009).

Aunque hay diferentes fuentes que producen ERO cómo productos secundarios de sus reacciones metabólicas (Angelova y Abramov, 2018), la mitocondria ha sido descrita como la fuente más importante de ERO dentro de la célula (Angelova y Abramov, 2018) y se encuentra íntimamente involucrada en los procesos de muerte celular. Las ERO provenientes de la mitocondria se producen en varios sitios dentro del organelo, los principales son el complejo I y el complejo III de la cadena respiratoria mitocondrial, ahí se produce el anión superóxido que posteriormente se convierte en H_2O_2 (Murphy, 2009). Una sobreproducción de ERO por la mitocondria se ha asociado a la disfunción mitocondrial, la caída del potencial de membrana mitocondrial y el daño celular, que consecuentemente lleva a la muerte neuronal (Hung et al., 2018; McManus et al., 2014).

En varios modelos celulares se ha demostrado que las dos principales fuentes de ERO, las NOX y la mitocondria, mantienen una interrelación en la que ambas fuentes participan produciendo ERO en distintos momentos del proceso de muerte. Por ejemplo, en una línea celular endotelial se probó que hay una relación entre el superóxido producido por NOX2 y las ERO producidas por la mitocondria (Nazarewicz et al., 2013a). En otro trabajo, ésta relación se establece entre la NOX1 y la mitocondria, probando nuevamente que la producción de mtERO y las provenientes de las NOX ocurren en distintos momentos del proceso de muerte celular (Seung et al., 2006), finalmente se encontró que las mtERO se producen de manera temprana al inducir estrés oxidante en células N27 y posteriormente se induce la actividad de NOX, además de que existe una relación entre la disminución de los niveles de actividad de NOX1 con la disminución de mtERO (Choi et al., 2014). Esta serie de trabajos indica que hay una diferencia temporal en la producción de ERO por ambas fuentes, además se comprobó que existe una interrelación entre la producción de ERO de ambas fuentes, en las que se ha mostrado que la mitocondria es la fuente de ERO inicial y esta a su vez induce a las NOX para elevar los niveles de ERO durante procesos de muerte, aunque, es importante señalar que esto no se ha demostrado en neuronas.

La dinámica mitocondrial.

Aunado a la elevación de los niveles de ERO, frecuentemente se observan cambios en la morfología mitocondrial (Lee et al., 2018; Park et al., 2015). Estos cambios morfológicos son consecuencia de la fusión y fisión de las membranas interna y externa de la mitocondria, que resulta en el rompimiento o la unión de las mitocondrias existentes. Este proceso se conoce cómo dinámica mitocondrial (Lackner 2014; Pernas y Scorrano 2016) y se encuentra finamente regulado por un complejo sistema de proteínas, entre las que se encuentra un grupo de GTPasas pequeñas, llamadas: Mitofusina 1 (Mfn1), Mitofusina 2 (Mfn2) y Optic atrophy protein 1 (Opa1), que están encargadas de la unión de la membrana externa e interna de la mitocondria. Mfn1 y Mfn2 están ancladas a la membrana externa de la mitocondria y ayudan a aproximar a las membranas para facilitar el proceso de fisión, Opa1 se encuentra en la membrana interna y su función en este contexto es la de mantener unidas entre ellas a las membranas mitocondriales internas. Por otra parte, la proteína dynamin like protein 1 (Drp1) se encarga de la escisión de ambas membranas, formando una estructura de anillo alrededor de la membrana externa de la mitocondria para generar la constricción de la misma y, producir de esta manera mitocondrias más cortas (Azzedine et al. 2012; Delettre et al. 2000; Westermann 2010; Yu y Pekkurnaz 2018).

Las proteínas de la dinámica mitocondrial se han involucrado en procesos como la biogénesis mitocondrial o el suministro de ATP en la sinapsis (Lackner, 2014). La fisión mitocondrial es importante para la correcta redistribución de organelos a lo largo de los axones (Chan, 2020). Además, se sabe que la expresión incorrecta de estas proteínas puede generar patologías del sistema nervioso (Cherubini et al., 2020; Delettre et al., 2000) y se han involucrado en los procesos responsables de la muerte neuronal (Han et al., 2017; Jahani-Asl et al., 2015; Zhou et al., 2008).

La dinámica mitocondrial en la muerte neuronal.

La regulación de la dinámica mitocondrial.

Las proteínas involucradas en la dinámica mitocondrial están encargadas de la constante fusión y fisión de membranas mitocondriales para mantener el balance entre estos dos procesos. La regulación de estas puede darse por diversos mecanismos, los más conocidos son las modificaciones postraduccionales como: la acetilación, ubiquitinación, SUMOilación y la fosforilación (Sabouny y Shutt, 2020). Se han reportado que un gran número de cinasas y fosfatasas están involucradas en la regulación de las proteínas de dinámica mitocondrial, como ERK1/2 que promueve la fisión activando Drp1 o inhibiendo la fusión al fosforilar Mfn1 (Pyakurel et al., 2015) y GSK3β que se ha relacionado con el aumento de la fisión mitocondrial a través de la activación de Drp1 (Yan et al., 2015).

Drp1 es una proteína cuya actividad puede modificarse a través de la fosforilación de varios de sus residuos. En neuronas se conocen varias cinasas y fosfatasas encargadas de su fosforilación (Tabla1). Drp1 puede encontrarse en el citoplasma y cuando se fosforila se transloca a la membrana externa de la mitocondria, para ejercer su función.

Tipo neuronal	Cinasas/Fosfatasas	Residuos fosforilados	Efecto en la fisión.	Referencia
Neuronas hipocampales	CAMKII/Akt	Ser616	induce	(Kim et al., 2016)
Neuronas corticales	----	Ser616	induce	(Cho et al., 2012)
Ht-22	Calcineurina (fosfatasa)	Ser637	inhibe	(Park et al., 2015)
Tejido de hipocampo	Calcineurina	Ser637	induce	(Yu et al., 2019)
Neuronas de PV	CDK5	Ser616	induce	(Kim y Kang, 2017)
Ht-22	Calcineurina	Ser637	inhibe	(Lee et al., 2018)
Células del giro dentado	ERK1/2	Ser616	induce	(Ko y Kang, 2017)
Neuronas granulares de cerebelo de ratón C1	CDK5	Ser585	induce	(Jahani-Asl et al., 2015)
Neuronas corticales	c-Abl	Tirosina 266, 368, 449	induce	(Zhou et al., 2017)
SH-SY5Y JNK/p38	CAMKII	Ser616	induce	(Yan y Zhao, 2020)
Neuronas hipocampales	GSK3β	Ser40, Ser44	Induce	(Yan et al., 2015)

La regulación de las proteínas involucradas en la fisión y la fusión mitocondrial en procesos de muerte apoptótica es compleja. Sin embargo, se ha propuesto que el aumento en los niveles de ERO que se da comúnmente durante estos procesos puede participar en la activación de las proteínas que regulan la dinámica mitocondrial, particularmente en Drp1 (Cid-Castro et al., 2018; Kim et al., 2016; Nakamura et al., 2010).

Antecedentes.

En el modelo de privación de potasio de las NGC se induce la muerte apoptótica de neuronas maduras por medio de la disminución de la concentración de K^+ (potasio) extracelular, a través del cambio de un medio de cultivo con una concentración despolarizante de KCl 5 mM (K25) a un medio con KCl 5mM (K5). Previamente se demostró que los altos niveles de K^+ extracelular promueven la supervivencia neuronal, aunque, no se conoce la causa de ello, se piensa que las altas concentraciones de K^+ actúan como un factor neurotrófico, promoviendo la supervivencia neuronal (D'Mello et al., 1993; Gallo et al., 1987), una vez que se disminuye el K^+ las NGC mueren después de 24 horas del inicio del tratamiento (Hu et al, 2013, Ramiro Cortes, Morán y Patel, 1989). Otra forma de inducir muerte neuronal es el tratamiento con ST, un inhibidor de proteína cinasas que se ha utilizado ampliamente para inducir apoptosis en varios tipos de células de mamíferos (Schwarz et al., 2020; Šimenc y Lipnik-Štangelj, 2012). En cultivos de neuronas se ha utilizado para dilucidar mecanismos de procesos neurodegenerativos y estudiar a detalle la muerte neuronal apoptótica (Ha et al., 2014; Maycotte et al., 2010; Ramiro-Cortés y Morán, 2009).

Ambos estímulos apoptóticos han mostrado ser inductores de un aumento de los niveles de ERO (Coyoy et al., 2008; Ramiro-Cortés et al., 2011; Zaragoza-Campillo y Morán, 2017) y en ambos modelos se ha demostrado la participación de una NOX como fuente de ERO y en el caso de la ST participa el homólogo NOX2 (Guemez-Gamboa y Morán, 2009). La producción de ERO en estas condiciones ocurre de forma temprana, entre las 3 y 5 h posteriores al estímulo, una vez pasado este tiempo las células mueren después de 12-48 h (Valencia y Morán, 2001).

Por otro lado, como se mencionó anteriormente, la mitocondria es una fuente de ERO que puede estar relacionada con la muerte apoptótica (Choi et al., 2013; Kim et al., 2016). Múltiples estudios también han mostrado que la morfología mitocondrial se ve afectada cuando se induce la muerte neuronal por diversos estímulos (Pyakurel et al., 2015; Yan et al., 2015; Yan y Zhao, 2020). En estas

condiciones, las mitocondrias se acortan o tienden a hacerse redondas, generando la pérdida de la red mitocondrial (Gao et al., 2017; Kim et al., 2019; Menges et al., 2017). Esta fragmentación mitocondrial ha mostrado estar mediada por la activación de Drp1 en varios modelos de muerte apoptótica (Barsoum et al., 2006; Guo et al., 2018; Kim et al., 2016; Nakamura et al., 2010). Sin embargo, en el modelo de K5 y ST no existe información relacionada con la participación de Drp1 en este proceso, ni de su relación con la generación de ERO en la muerte de estas células.

Planteamiento del problema.

Durante el proceso de muerte neuronal apoptótica existe aumento en los niveles de ERO. Este aumento puede proceder de distintas fuentes en la célula. En las NGC se sabe que las NOX están involucradas en este proceso, pero poco se conoce sobre el papel de la mitocondria, la producción de ERO y la muerte en este modelo. Además, se conoce poco sobre el papel de las ERO producidas durante la muerte en la dinámica mitocondrial. Debido a que el aumento en los niveles de ERO frecuentemente ocasiona anormalidades en la morfología mitocondrial, es posible que las condiciones apoptóticas como los modelos de K5 y ST produzcan modificaciones en la morfología y el tamaño de las mitocondrias, lo que se verá reflejado en su morfología y pueda contribuir al proceso de muerte. Por otro lado, estos cambios en la morfología mitocondrial podrían estar mediados por Drp-1. La relación entre el aumento en los niveles de ERO y el aumento en la fragmentación mitocondrial no se ha establecido claramente, y menos en el modelo de muerte en NGC en cultivo. Por lo anterior, este trabajo explora la relación que existe entre el aumento en los niveles de mtERO y ctERO, la fisión mitocondrial, la activación de Drp1 y la muerte de NGC inducida por K5 o ST.

Hipótesis.

El aumento en los niveles de mtERO inducirán fragmentación en las mitocondrias de NGC, estos cambios estarán mediados por la fosforilación de Drp1 en la Ser616 y serán determinantes en el proceso de muerte neuronal inducido por K5 y ST.

Objetivo general.

Conocer el papel de las ERO provenientes de la mitocondria sobre la muerte de NGC y su participación en la regulación de la dinámica mitocondrial durante este proceso.

Objetivos particulares.

Determinar los niveles temporales de las mtERO y las ctERO durante la fase inicial del proceso apoptótico inducido por K5 y ST.

Determinar el papel de los mtERO sobre la viabilidad y la producción de ctERO en condiciones apoptóticas.

Determinar el efecto de las condiciones apoptóticas sobre la morfología mitocondrial.

Determinar el efecto de las condiciones apoptóticas sobre la fosforilación de Drp1.

Determinar el papel de las mtERO en la morfología mitocondrial.

Determinar el papel de las mtERO sobre los niveles de fosforilación de Drp1.

Materiales y métodos.

Los animales empleados en este estudio fueron ratas Wistar (*Rattus novergicus*) de 7 a 8 días postnatales, proporcionados por el Bioterio del Instituto de Fisiología Celular (IFC) de la Universidad Nacional Autónoma de México (UNAM). Su manejo y tratamiento se realizó bajo los estándares de cuidado animal y procedimientos aprobados internacionalmente (NIH Publicación No. 8023, revisado en 1978) y localmente por el Comité Interno para el Uso de los Animales de Laboratorio (CICUAL) del IFC de la UNAM (Protocolo No. JMA72-15).

Cultivo de neuronas granulares de cerebelo (NGC).

Se obtuvo el cerebelo de ratas Wistar de 7 a 8 días postnatales y se disoció el tejido para obtener las NGC las cuales fueron cultivadas como se describió previamente (Morán y Patel, 1989). Las neuronas se sembraron a una densidad de $265x10^3$ células/cm^2 en placas de plástico previamente tratadas con poli-L-lisina (5µg/ml), el medio usado fue K25 que contenía medio basal Eagle suplementado con suero fetal bovino 10% (inactivado por calor), KCl 25 mM, glutamina 2 mM, estreptomicina 50 µg/ml y penicilina 50 U/ml. Después de 24 horas de haber realizado el cultivo, se agregó citosina arabinosa (10µM) para evitar la proliferación de células. Los cultivos se mantuvieron en incubación con este medio durante 7 días *in vitro* (DIV) a una temperatura de 37°C y una atmósfera humidificada de aire al 95% y CO_2 al 5%.

Viabilidad celular

Se evaluó la viabilidad midiendo la reducción de MTT (bromuro de 3-(4, 5-dimetiltiazol-2-il)-2, 5-difeniltetrazolio) que está basada en la habilidad que tiene la succinato deshidrogenasa mitocondrial de transformar el MTT en azul de formazán. La cantidad de formazán producido es directamente proporcional al número de células viables presentes en el cultivo. Brevemente, las NGC se incubaron con MTT 100µM por 15 minutos a 37°C, posteriormente los cristales de formazán formados se disolvieron en DMSO y se midieron en un espectrofotómetro a 570nn. Una vez obtenidos los valores de densidad óptica (DO), se considera a la DO de las células en el medio K25 como el 100% de capacidad de reducción de MTT, posteriormente se realizó el cálculo del porcentaje correspondiente a la DO obtenida en el resto de las condiciones estudiadas.

Determinación de ERO mitocondrial.

Las NGC se sembraron en cajas de Petri de 35 mm, y se mantuvieron durante 7 DIV en medio K25 y, después de esto, se incubaron durante 30 minutos con MitoTracker red CMH_2XRos (100 nM) a 37°C, después de este tiempo se hizo el cambio de medio K25 por el K5 o se agregó ST directamente a la caja de Petri. El

MitoTracker red CMH_2XRos no fue removido durante el experimento. Posteriormente, las neuronas se sometieron a las distintas condiciones de muerte y se obtuvieron las imágenes en un microscopio LSM710-Zeiss con un láser infrarrojo pulsado, Chameleon Ultra II a 740/599 nm de excitación/emisión, respectivamente. Se utilizó un objetivo de inmersión 63x/1. La intensidad de fluorescencia se determinó a partir de las imágenes obtenidas con el microscopio, se tomaron tres fotografías por condición, cuidando de que cada campo tuviera al menos 20 somas que se utilizaron para marcar una ROI (Region of interest) en cada uno y realizar la medición de la intensidad de fluorescencia utilizando Fiji ImageJ. Se normalizaron los valores de intensidad de fluorescencia tomando los valores de K25 como 1.

Determinación de ERO citoplásmico.

Las NGC se sembraron en medio K25 durante 7 DIV y posteriormente se trataron con medio K5 o ST. Después de los tiempos indicados, se incubaron con 3.2 µM de DHE (dihidroetido) por 30 min a 37°C. Después de este tiempo, las células se observaron en un microscopio de epifluorescencia Nikon Ti con un filtro de 488-515 nm. La intensidad de fluorescencia se determinó a partir de las imágenes obtenidas con el microscopio, utilizando Fiji ImageJ.

Obtención de imágenes de la morfología mitocondrial.

Las NGC se sembraron en medio K25 durante 7 DIV en placas de Petri de 35mm con fondo de vidrio (FluoroDish™), las neuronas se sometieron a las condiciones apoptóticas en los tiempos indicados en los resultados y se incubaron con MitoTracker Green 100nM por 30 min a 37°C. Después de ese tiempo, las células se lavaron 2 veces con medio Locke (NaCl 154 mM, KCl 25 ó 5 mM, $NaHCO_3$ 3.6 mM, $CaCl_2$ 2.3 mM, glucosa 5.6 mM y HEPES 10mM). Las imágenes se obtuvieron con un microscopio Eclipse-Ti-S Nikon con un objetivo 60x de inmersión en aceite con un filtro de 470/540 nm. La longitud mitocondrial se determinó con Fiji ImageJ.

Western blot.

Las NGC se sembraron en medio K25 por 7 DIV y después se trataron con medio K5 o ST a diferentes tiempos. Las células se lavaron 2 veces con PBS frío y se homogenizaron con buffer de lisis (Trizma 25 mM, NaCl 50 mM, Igepal 2%, SDS 0.2% y complete (Roche®), pH 7.4. Los homogenados se centrifugaron a 4500 rpm por 5 min y se recuperaron los sobrenadantes. La concentración de proteína se estimó con el método de Lowry. Los homogenados (30 µg por pozo) se corrieron en un gel SDS-PAGE al 10% y se transfirieron a una membrana de PVDF a 100V durante 1.5 h. Las membranas se bloquearon con 5% de leche libre de grasa disuelta en TTBS (Tris–HCl 100 mM, NaCl 150 mM y Tween 0.1%, pH 7.4) durante 1 h y después se incubaron durante toda la noche con los anticuerpos primarios, para Drp1 (Cell Signalling - 4E11B11), p-Drp1 (Cell Signalling - D9A1), GAPDH (Cell Signalling - 14C10). Después de los lavados, las membranas se incubaron con anticuerpos anti-ratón (Jackson Immuno Research code-115-035-003) o anti-conejo (Jackson Immuno Research code-211-032-171) conjugados con peroxidasa (1:10 000) durante 1 h a temperatura ambiente. Las bandas correspondientes a las proteínas de interés se visualizaron con un sistema de quimioluminiscencia y la exposición a placas fotosensibles Kodak BioMax-Light Film.

Actividad de NOX.

Las NGC sembradas en cajas de 35mm se trataron a los tiempos indicados con K5 o ST, o se pretrataron durante 30 min con DPI (Difenil iodonio) (520 nM). Posteriormente, se lavaron con PSB y se rasparon en un volumen de 100µl de PBS, se transfirieron a una placa de 96 pozos con paredes oscuras y, después se agregó una solución de NADPH (100 µM) y una de lucigenina (100 µM) hasta completar un volumen de 150 µl. La determinación de la luminiscencia se realizó en el lector de placas Synergy HT durante 60 min. La actividad de NOX se determina por medio de la luminiscencia emitida por la lucigenina que se reduce en presencia del anión superóxido producido por NOX, esta reacción se produce en presencia del NADPH

por lo que la cantidad de luminiscencia emitida es proporcional a la actividad de NOX.

Resultados.

Los tratamientos K5 y ST inducen un aumento en los niveles de las ERO.

Para determinar si las condiciones de K5 o ST generan cambios en la producción de ERO, realizamos un curso temporal para determinar los niveles de mtERO, tiñendo a las mitocondrias con MitoTracker red CMH_2XRos, que permite detectar las mtERO de manera específica y directa, este compuesto se acumula en el espacio intermembrana de la mitocondria y al oxidarse en presencia de ERO emite fluorescencia a 599nm (Kuznetsov et al., 2011) , además para determinar los niveles de ctERO se utilizó DHE, que se oxida en presencia de anión superóxido y forma el 2-hidroxietidio, una vez que el DHE se oxida en el citoplasma éste se intercala en el DNA y emite fluorescencia a 580nm (Wojtala et al., 2014). En el caso de los niveles de mtERO observamos un aumento después de 10 min del inicio de ambas condiciones apoptóticas. Interesantemente, la condición de privación de potasio indujo un aumento significativo en los niveles de mtERO en comparación a los producidos por ST (Fig. 1A). El tratamiento con ST mostró un aumento significativo en los niveles de mtERO del 65% en relación a la basal. Las imágenes de microscopia muestran que el incremento de mtERO ocurre principalmente a nivel del soma neuronal tanto con K5, como con ST.

Con respecto a los niveles de ctERO, encontramos que el tratamiento con K5 indujo un incremento significativo después de 45 min y 5 h, mientras que la ST causó un marcado aumento de ROS a las 5 h (Fig. 1B). La técnica no permite localizar el origen de las ERO producidas bajo estas condiciones. Estos datos nos sugieren que la mitocondria es la primera fuente que produce ERO en respuesta a una condición de muerte con una subsecuente producción de ERO en el citoplasma.

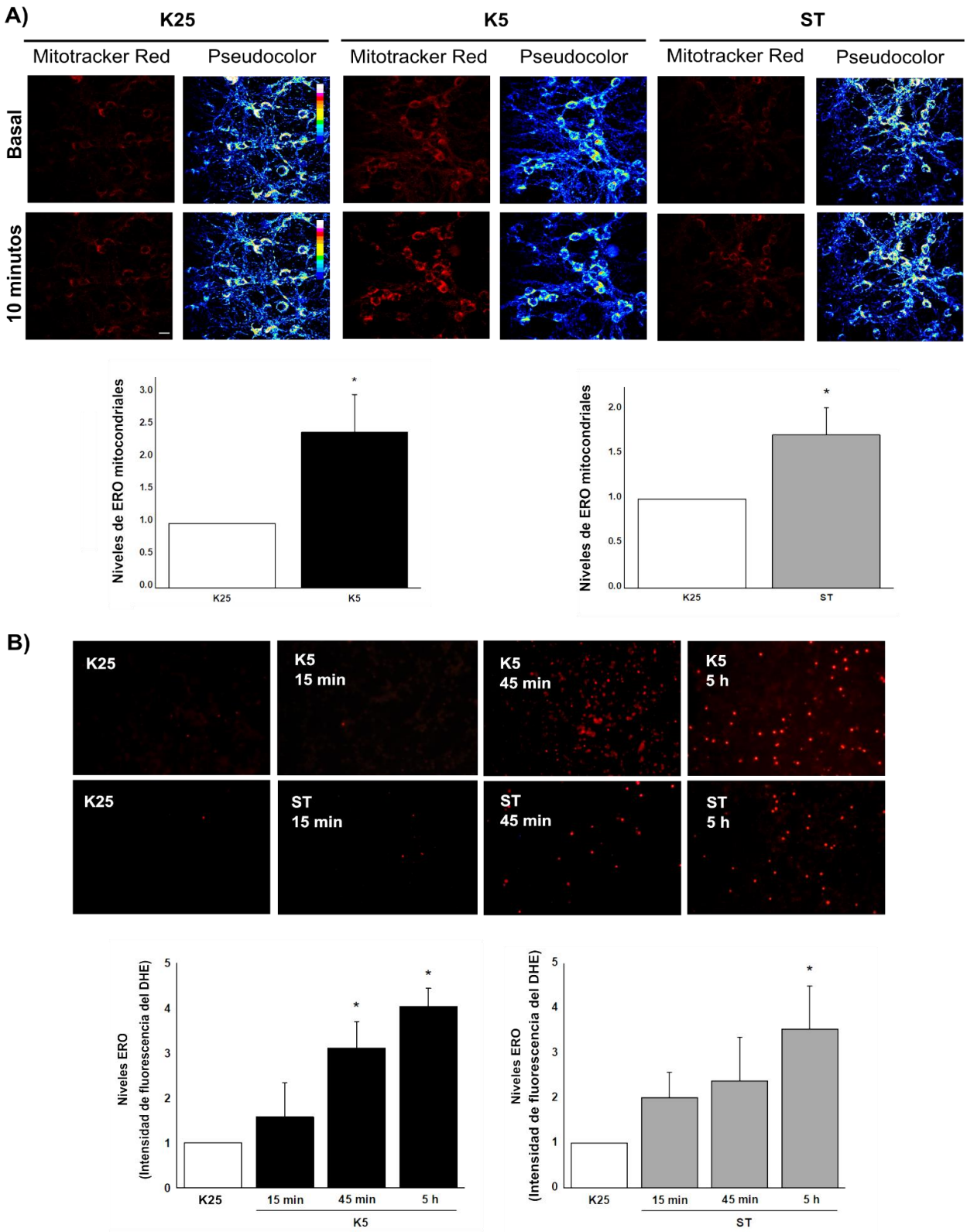

A)
K25
K5
ST
Mitotracker Red
Pseudocolor
Mitotracker Red
Pseudocolor
Mitotracker Red
Pseudocolor
Basal
10 minutos
Niveles de ERO mitocondriales
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
K25
K5
Niveles de ERO mitocondriales
2.0
1.5
1.0
0.5
0.0
*
K25
ST
B)
K25
K5
15 min
K5
45 min
K5
5 h
K25
ST
15 min
ST
45 min
ST
5 h
Niveles ERO
(Intensidad de fluorescencia del DHE)
5
4
3
2
1
0
*
*
K25
15 min
45 min
5 h
K5
Niveles ERO
(Intensidad de fluorescencia del DHE)
5
4
3
2
1
0
*
K25
15 min
45 min
5 h
ST

Figura 1. Condiciones apoptóticas induce un aumento en los niveles de mtERO y ctERO. Los niveles de mtERO y ctERO se midieron durante un curso temporal, después del tratamiento de privación de potasio (K5) o estaurosporina (ST). **A)** Las NGC se tiñeron con MitoTracker red y se tomaron fotografías después de 10 minutos para determinar los niveles de mtERO bajo las condiciones control (K25), y las condiciones apoptóticas (K5 y ST). Las gráficas muestran los niveles de mtERO, determinados por cambios en la intensidad de fluorescencia. **B)** Las NGC se tiñeron con DHE (dihidroetidio) bajo condiciones control (K25) y durante un curso temporal (15 min, 45 min y 5 h) de los tratamientos con K5 y ST. La escala de pseudocolor indica que entre más claro es el color (blanco), mayor es la intensidad de fluorescencia. La barra de escala corresponde a 10 µm. Las gráficas muestran los niveles de ctERO. Las barras son las medias ± ES de tres experimentos independientes. * $p < 0.05$ vs K25.

La disminución en la viabilidad correlaciona con el aumento de las ERO en neuronas tratadas con K5 y ST.

Se evaluó la viabilidad celular midiendo la capacidad de reducción del MTT de las NGC en un curso temporal, desde los 15 min y hasta las 8 horas, sometidas a K5 y ST. En estos experimentos se observó una disminución de 23% en la reducción del MTT, después de 30 min de tratamiento y esto continuó hasta las 8 h en cultivos tratados con K5 (Fig. 2A). Mientras que en neuronas tratadas con ST, la capacidad de reducción del MTT disminuyó 28% después de 15 min del estímulo y esta disminución continuó hasta las 8 h de tratamiento (Fig. 2B). Esto sugiere que la viabilidad está comprometida desde los primeros minutos del proceso de muerte inducida por ambos estímulos.

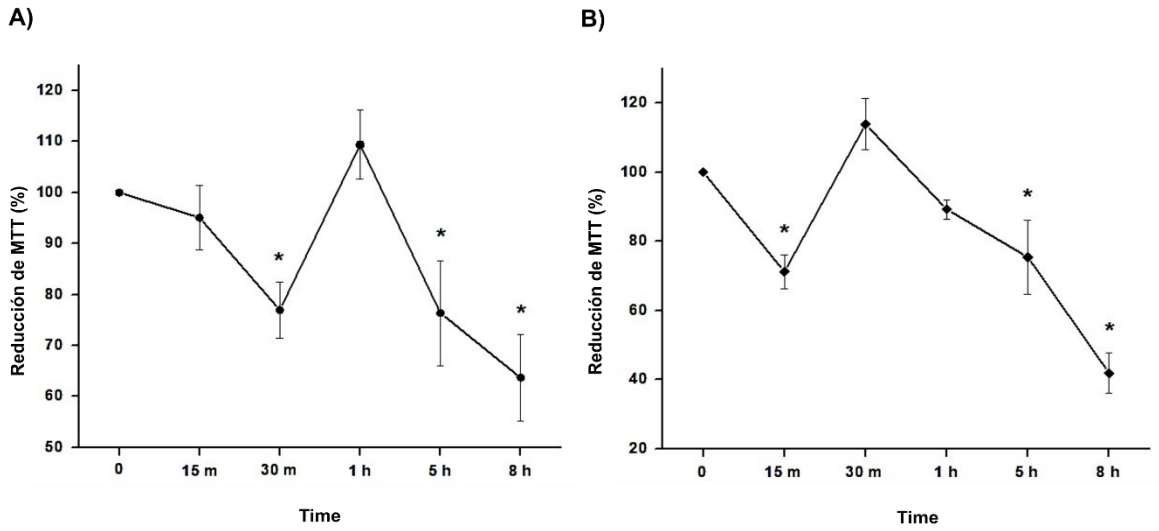


Figura 2. Curso temporal de la reducción de MTT de las NGC tratadas con K5 y ST. La viabilidad neuronal se evaluó por la reducción del MTT a los 15 min, 30 min, 1, 5 y 8 h, después diferentes tiempos en condiciones de K5 y ST. **A)** Curso temporal de la viabilidad de las NGC tratadas con K5 (●). **B)** Curso temporal de la viabilidad de las NGC tratadas con ST (♦). Los símbolos ● y ♦ muestran la media ± ES del porcentaje de viabilidad comparado con el control (K25) de tres experimentos independientes. * $p < 0.05$ vs tiempo 0 (K25).

Las mtERO participan en la muerte inducida por K5 y ST.

Para evaluar la contribución de las mtERO en la apoptosis de las NGC, estas células se trataron con MitoTEMPO, un antioxidante mitocondrial, y se determinó la viabilidad de las neuronas tratadas con K5 y ST. Después de 24 h de tratamiento, las células sometidas al tratamiento con K5 y ST redujeron su viabilidad a 58% y 45%, respectivamente. Cuando los cultivos tratados con K5 y ST se pretrataron durante 30 min con MitoTEMPO, se observó una reducción en la muerte de 20% (Fig. 3A) y 52% (Fig. 3B), respectivamente. Estos resultados sugieren que la producción de mtERO de la mitocondria son una señal temprana y determinante en el proceso de muerte.

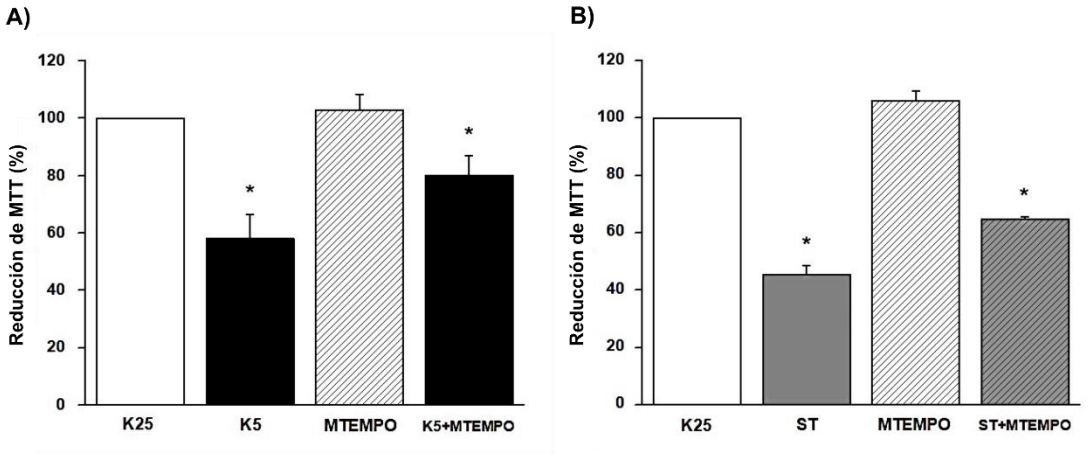


Figura 3. Las mtERO participan en la muerte de las NGC tratadas con K5 y ST. La viabilidad neuronal se evaluó por la reducción del MTT después de 24 h de la inducción de la muerte neuronal y en células pretratadas durante 30 min con el antioxidante mitocondrial MitoTEMPO 10 µM. **A)** Viabilidad de las NGC tratadas con K5. **B)** Viabilidad de NGC tratadas con ST. Las barras muestran la media ± ES del porcentaje de viabilidad comparado con el control (K25) de tres experimentos independientes. * $p< 0.05$ vs K25 $p<0.05$ vs K5+MTEMPO o ST+MTEMPO

Los tratamientos K5 y ST inducen cambios morfológicos en las mitocondrias.

En numerosos modelos de muerte neuronal se han reportado cambios morfológicos en las mitocondrias. Estos cambios se caracterizan por un hinchamiento, redondeo y acortamiento de las mitocondrias y se han identificado como un proceso de dinámica mitocondrial (Pernas y Scorrano, 2016; Westermann, 2010). En el modelo empleado en este estudio observamos que las NGC sometidas a K5 mostraron cambios morfológicos a las 8 h de tratamiento (Fig. 4A). Estas neuronas mostraron mitocondrias redondeadas y más cortas, comparadas con las que se mantuvieron condiciones control (K25). Además, la longitud de las mitocondrias se redujo un 18% a las 8 h post-tratamiento y 25% después de 24 h (Fig. 4B).

En el caso de las neuronas tratadas con ST las mitocondrias redondeadas se observaron a partir de las 8 h y se detectó un acortamiento de 11% en la longitud

de las mitocondrias después de 24 h del tratamiento (Fig. 4B). Estos resultados sugieren que una disminución en la longitud mitocondrial es un evento común durante la muerte neuronal inducida por diferentes condiciones.

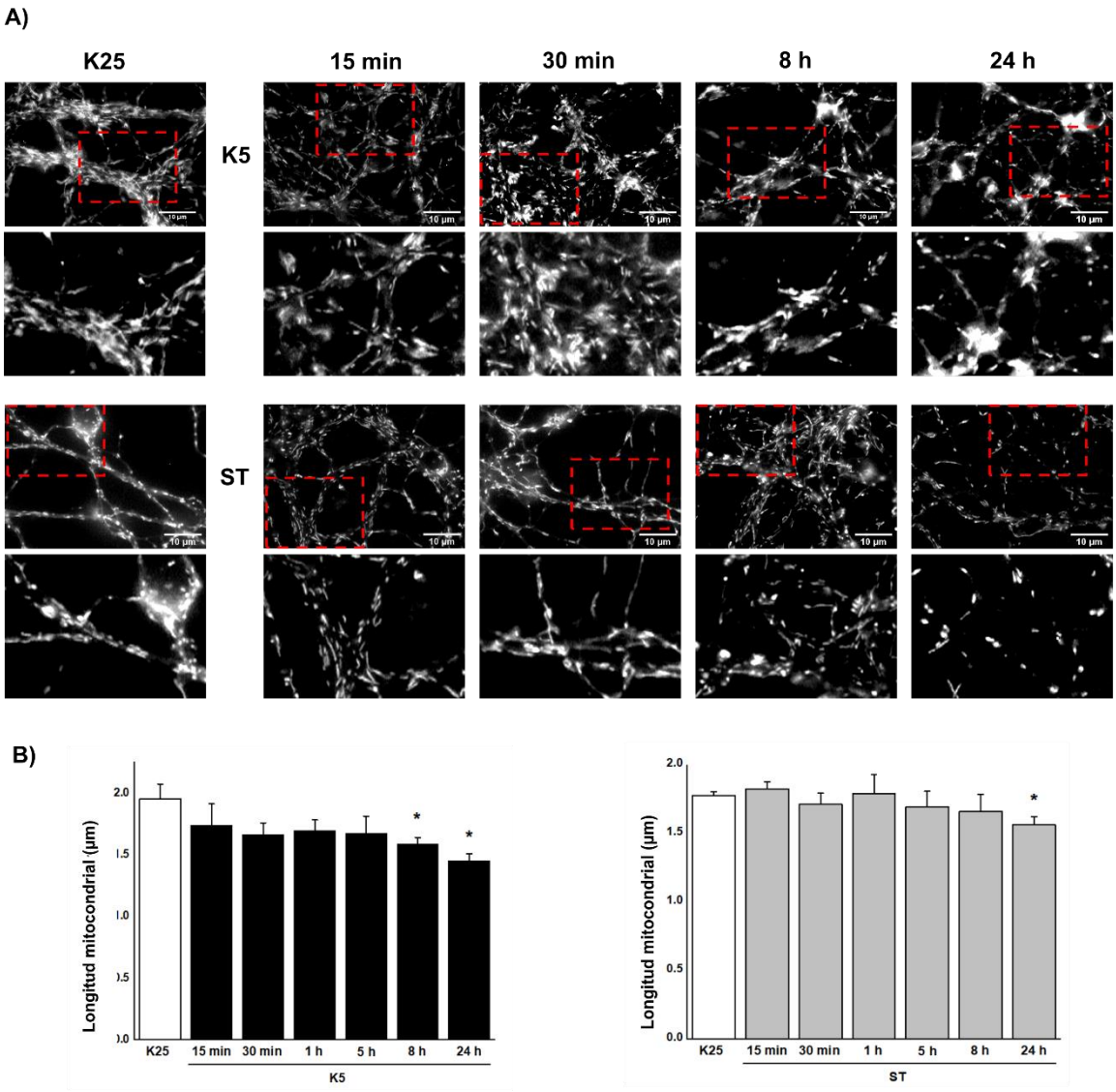


Figura 4. K5 y ST inducen cambios morfológicos en las mitocondrias de las NGC. Curso temporal de las NGC teñidas con MitoTracker green y tratadas con K5 y ST. **A)** Imágenes de la morfología mitocondrial de las NGC después de la privación de potasio o con ST durante un curso temporal. Los recuadros rojos se amplían en la parte de abajo para visualizar mejor la morfología mitocondrial. **B)** Las gráficas muestran la longitud mitocondrial en un curso temporal durante las condiciones apoptóticas. Las barras representan la media ± ES de tres experimentos individuales. * p<0.05 vs K25.

K5 induce la fosforilación de Drp1 de manera dependiente de las mtERO.

Con el fin de esclarecer el papel de la privación de potasio en el proceso de fisión mitocondrial, evaluamos la activación de Drp1, midiendo el nivel de fosforilación en la Ser616. Para ello, realizamos un curso temporal del tratamiento con K5 y observamos un aumento en los niveles de fosforilación de Drp1 después de 15 min de estimulación, el cual se mantuvo hasta las 8 h de tratamiento. Después de las 24 horas el nivel de fosforilación de Drp1 disminuyó (Fig. 5A). Como se mencionó anteriormente, se observó un incremento de los niveles de mtERO a los 10 min (Fig. 1A), por lo que evaluamos el efecto del MitoTEMPO sobre los niveles de fosforilación de Drp1 en ese periodo de tiempo. Los datos mostraron que el MitoTEMPO inhibe la fosforilación de Drp1 inducida por K5 desde los 15 min (Fig. 5B). Estos datos sugieren que las mtERO se requieren para la activación de Drp1 en el modelo de privación de potasio.

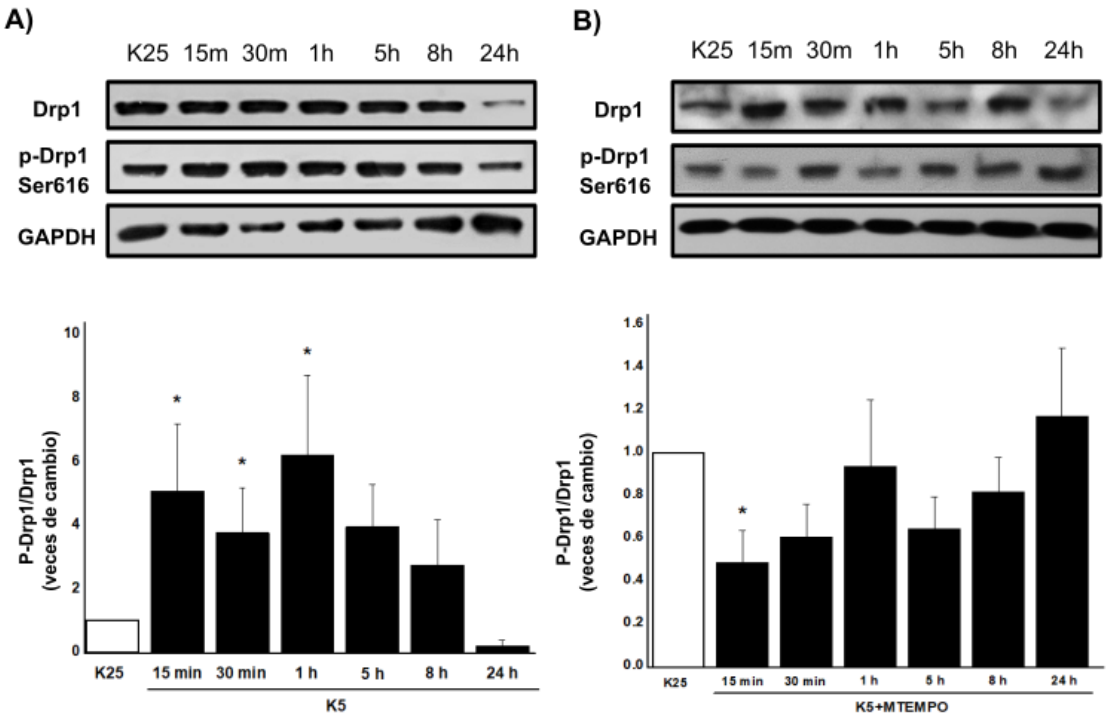


Figura 5. K5 induce la fosforilación de Drp1 de manera dependiente de mtERO. Los niveles de Drp1 total y Drp1 fosforilada en Ser616 (p-Drp1) se evaluaron en un curso temporal en lisados de NGC pretratadas durante 30 min con el antioxidante mitocondrial MitoTEMPO y bajo condiciones de muerte. Los niveles de proteína se determinaron por Western blot. **A)** Niveles de p-Drp1 (Ser616) en las NGC tratadas con K5. **B**) Niveles de p-Drp1 de las NGC pretratadas con MitoTEMPO y tratadas con K5. GAPDH se utilizó como control de carga. Las barras muestran la proporción densitométrica entre p-Drp1 y Drp1 que se normalizaron con el control, K25. Los valores son la media ± ES de tres experimentos individuales. * p<0.05 vs K25.

La ST disminuye los niveles de fosforilación de Drp1.

Exploramos el efecto de la ST sobre la fosforilación de Drp1 en un curso temporal y, contrario a lo observado con K5, observamos una disminución en la forma fosforilada de Drp1 a partir de 15 min y se mantiene así hasta las 24 h, cuando ya no es posible detectar la proteína (Fig. 6A). Inesperadamente, la disminución de la fosforilación de Drp1 no se modificó por el tratamiento con el MitoTEMPO (Fig. 6B).

Estos datos nos sugieren que la fragmentación mitocondrial inducida por el tratamiento de ST no está mediada por la fosforilación de Drp1 en el residuo de Ser616 (Fig. 4B).

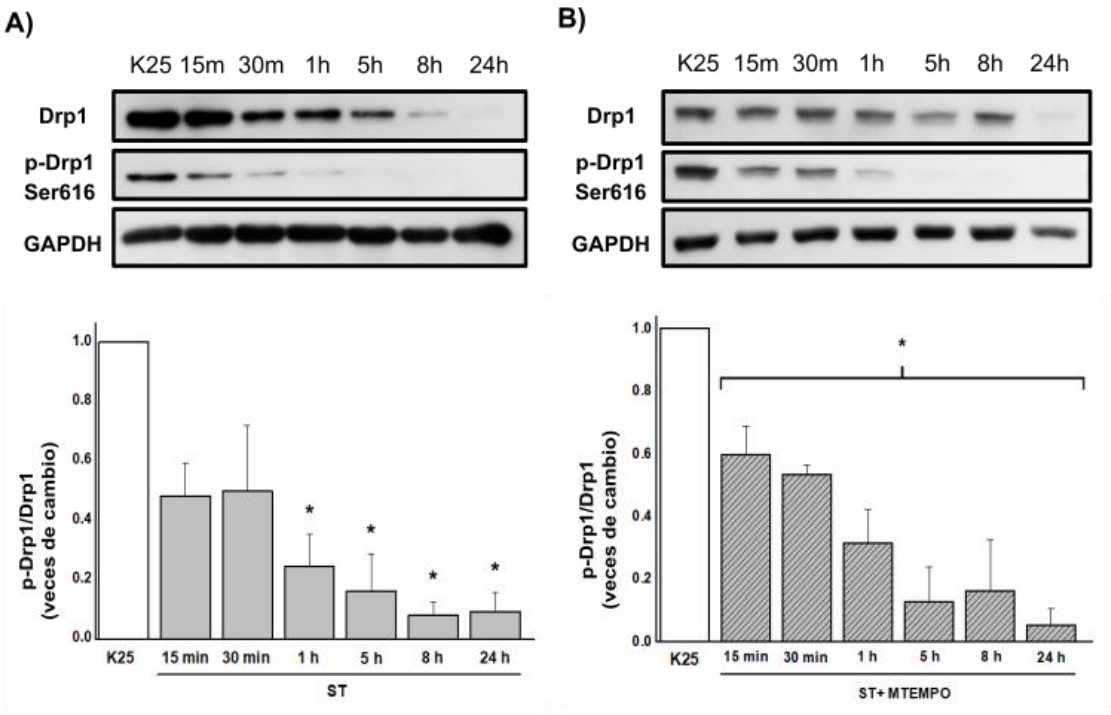


Figura 6. La ST disminuye los niveles de fosforilación de Drp1. Los niveles de Drp1 total y Drp1 fosforilado en Ser616 (p-Drp1) fueron evaluados en un curso temporal en lisados de las NGC pretratadas durante 30 min con el antioxidante mitocondrial MitoTEMPO, bajo condiciones de muerte. Los niveles de proteína se determinaron por Western blot. **A)** Niveles de p-Drp1 (Ser616) de las NGC tratadas con ST. **B**) Niveles de p-Drp1 de las NGC pretratadas con MitoTEMPO y tratadas con ST. GAPDH se utilizó como control de carga. Las barras muestran la proporción densitométrica entre p-Drp1 y Drp1 que se normalizaron con el control, K25. Los valores son la media ± ES de tres experimentos individuales. * p<0.05 vs K25.

La fisión mitocondrial inducida por las condiciones de muerte no se previene por el tratamiento con un antioxidante mitocondrial.

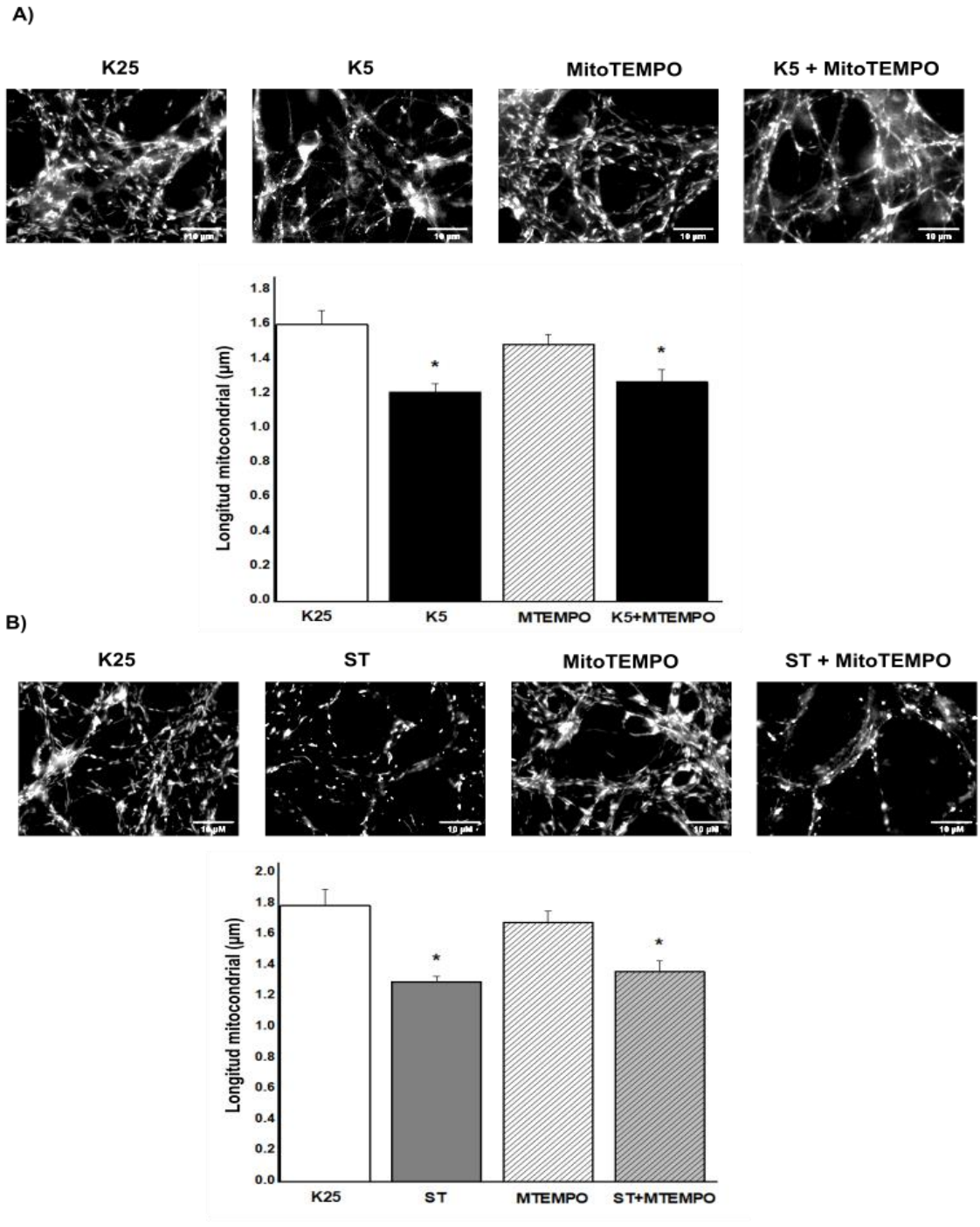


Figura 7. La fisión mitocondrial inducida por K5 y ST no se evita por el tratamiento con MitoTEMPO. Las NGC teñidas con MitoTracker green y pretratadas con MitoTEMPO (10 µM) se estimularon con K5 o ST y, posteriormente, se tomaron imágenes de las neuronas después de 24 h. **A)** Imagen de la morfología mitocondrial de las NGC después de la privación de potasio. **B**) Imagen de la morfología mitocondrial después del tratamiento con ST. Las barras muestran la longitud mitocondrial en µm, medidas después de 24 h de tratamiento con las condiciones de muerte. Las barras representan la media ± ES de tres experimentos individuales. * $p < 0.05$ vs K25.

Dado que las mtERO parecen mediar la fosforilación de Drp1 (Ser616) inducida por la privación de potasio, estudiamos si el MitoTEMPO modifica el efecto observado en la morfología mitocondrial; sin embargo, no observamos ningún efecto del MitoTEMPO sobre la disminución de la longitud mitocondrial inducida por K5 a las 24 h (Fig. 7A). De igual forma, en las neuronas estimuladas con ST tampoco observamos ningún efecto del MitoTEMPO en la reducción de longitud mitocondrial (Fig. 7B).

El MDiVi-1 no previene la pérdida de la viabilidad de las NGC sometidas a condiciones apoptóticas.

En la figura 5A se mostró que el Drp1 se fosforila al someter a las NGC a la privación de potasio o con ST; debido a esto se probó el efecto de la inhibición de Drp1 con MDiVi-1, un inhibidor de Drp1. En este experimento se observó que el pretratamiento con MDiVi-1 no evita la reducción del MTT de las NGC tratadas con K5 (Fig. 8A). Asimismo, al pretratar con MDiVi-1 a las NGC tratadas con ST, tampoco se observó ninguna mejora en la viabilidad (Fig. 8B). Es importante mencionar que el uso únicamente del MDiVi-1 induce una pérdida en la reducción del MTT del 20%, lo que podría sugerir que la actividad de Drp1 es necesaria para mantener la viabilidad de las NGC, sin embargo, no se observó un efecto aditivo en las neuronas tratadas con K5 o ST. Por otra parte, la ausencia de efecto en la reducción de MTT sugiere que la muerte inducida por K5 y ST es independiente de Drp1.

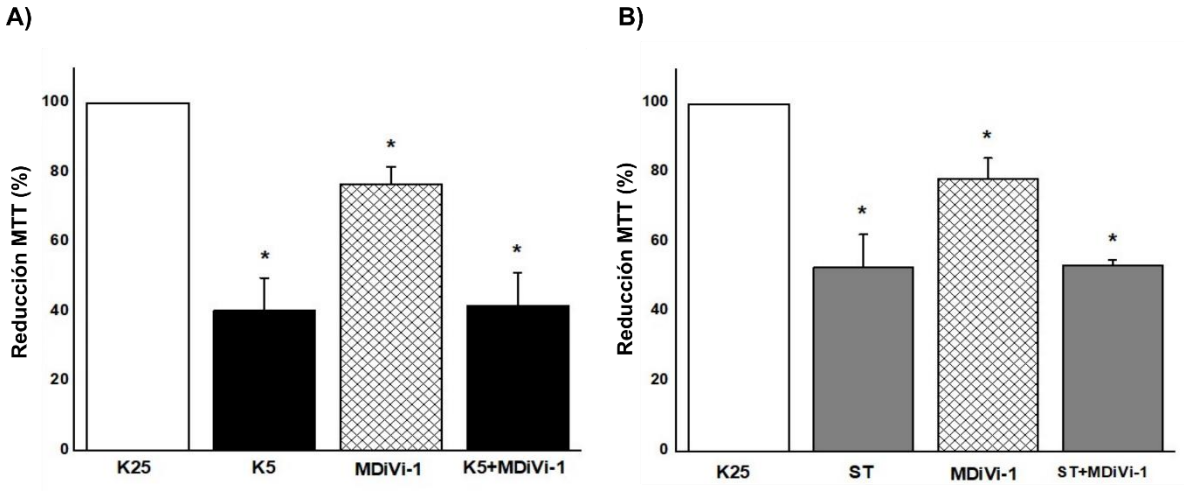
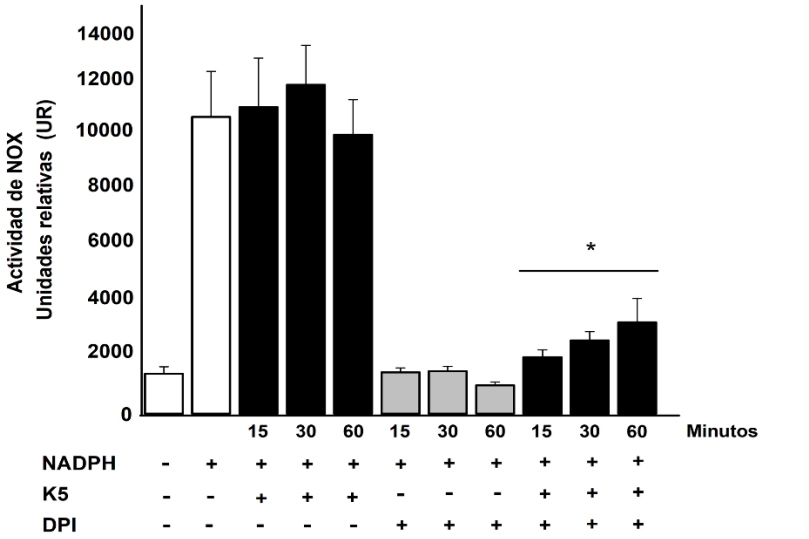


Figura 8. El MDivi-1 no evita la muerte de las NGC inducida por K5 y ST. Se evaluó la viabilidad de las NGC pretratadas con MDiVi-1 10 µM midiendo la capacidad de reducción de MTT después de 24h del tratamiento con K5 o ST. **A)** Viabilidad de las NGC tratadas con K5 en presencia de MDiVi-1 **B**) Viabilidad de las NGC tratadas con ST en presencia de MDiVi-1. Las barras muestran la media ± ES del porcentaje de viabilidad comparado con el control (K25) de tres experimentos independientes. * $p< 0.05$ vs K25.

El K5 y la ST inducen un aumento en la actividad de NOX en tiempos cortos.

Para determinar si NOX participa como fuente de ERO en la muerte inducida por K5 y ST se realizó un ensayo en el que se trataron a las NGC a distintos tiempos en presencia de K5 (Fig. 9A) y ST (Fig. 9B) y usando DPI como inhibidor de las NOX. En el caso de las células tratadas con K5 no se observó un aumento significativo en la actividad de NOX en ninguno de los tiempos observados, pero sí se observó una disminución en la actividad en presencia del DPI (Fig. 9A). En contraste, las neuronas tratadas con ST (Fig. 9B) mostraron un aumento en la actividad de NOX después de 30 min del tratamiento, aunque a los 60 min este aumento ya no se observó, sugiriendo una inducción transitoria de la actividad de NOX en los primeros 30 min del estímulo. En esta condición, nuevamente se observó que el DPI fue capaz de disminuir la actividad de NOX inducida por la ST. Estos datos sugieren que, a tiempos cortos, las NOX son una fuente de ERO inducida por ST, pero no por K5.

A)



B)

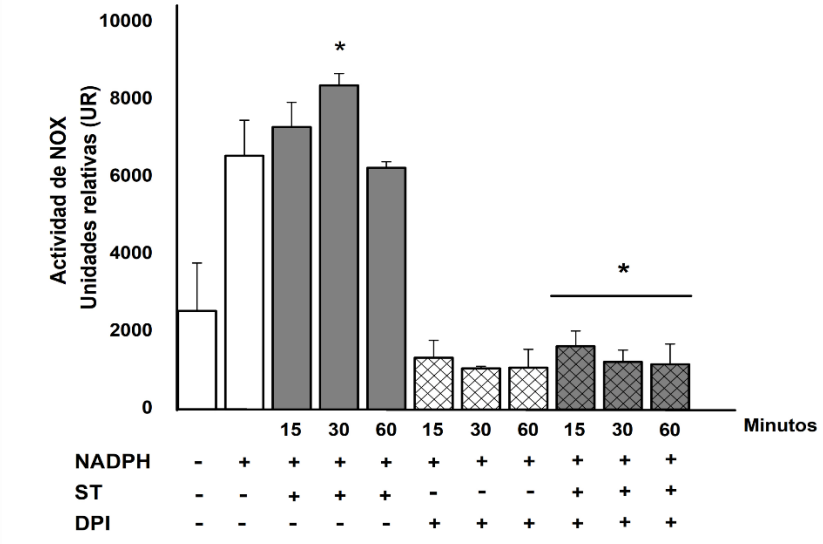


Figura 9. El K5 y la ST inducen un aumento en la actividad de NOX. Se determinó la actividad de NOX de NGC durante distintos tiempos de tratamiento de K5, ST y en presencia de DPI, un inhibidor de NOX. **A)** Actividad de NOX en NGC sometidas a privación de potasio desde 15 hasta 60 minutos. **B)** Actividad de NOX en NGC tratadas con ST desde 15 hasta 60 minutos. Las barras representan la media ± ES de la actividad de NOX. * $p < 0.05$ vs NGC con NADPH (K25).

Discusión.

En este estudio evaluamos la participación de las mtERO en el proceso de muerte neuronal inducida por K5 y ST. Se determinó que ambas condiciones inducen un aumento en los niveles de mtERO y ctERO. Un resultado interesante de este estudio fue la detección de un aumento temprano en los niveles de mtERO en las NGC en ambos modelos de muerte neuronal (Fig. 1A). Encontramos que ambas condiciones de muerte inducen un incremento en las mtERO a los 10 min del tratamiento y el cual es previo a la producción de ctERO que ocurrió alrededor de las 5 h y que corresponde a la activación de NOX como se publicó previamente (Guemez-Gamboa y Morán, 2009). Estos datos también coinciden con la actividad de NOX medida durante los primeros 60 min del estímulo, donde encontramos que, a diferencia de K5, la ST solo induce un ligero incremento en actividad la de NOX de forma transitoria a los 30 min post tratamiento.

Otro resultado interesante mostró que la inhibición de la producción temprana de mtERO redujo parcialmente la muerte neuronal 24 h después del tratamiento con las condiciones de muerte (Fig. 3). Estos resultados sugieren fuertemente que la mitocondria participa de forma crítica en el proceso de muerte y que la producción de mtERO es un acontecimiento temprano y necesario para este proceso que antecede a una serie de eventos relacionados, que incluye, entre otros, la activación de NOX después de 3-5 h y la producción de ctERO.

También encontramos una pérdida de la reducción de MTT desde los primeros minutos del proceso hasta perder el 50% después de 24 h, lo que se evitó parcialmente con MitoTEMPO.

Se ha demostrado que las ERO pueden actuar en la regulación de varios procesos fisiopatológicos. Nuestro grupo de investigación mostró previamente que las ERO son un determinante en el proceso de muerte apoptótica inducida por K5 y ST (Valencia y Morán, 2001; Coyoy et al., 2008; Maycotte et al., 2010; Ramiro-Cortés et al., 2011). El aumento de las ERO observado en las NGC después de 3-5 h se ha asociado con la progresión de la apoptosis, pero el mecanismo aún no ha sido

completamente dilucidado. Nuestro grupo de trabajo y otros grupos han mostrado que la NOX es una fuente de ERO crucial implicada en el proceso apoptótico (Choi et al., 2014; Coyoy et al., 2008; Guemez-Gamboa y Morán, 2009).

En el presente trabajo confirmamos que existe un aumento en los niveles de ctERO inducido por K5 y la ST después de 5 h (Fig. 1A y 1B), el cual se sabe que es necesario para que la muerte ocurra 12-24 h después. Esto se basó en el hecho de que la presencia de antioxidantes administrados justamente antes (2-3 h), pero no después del incremento de ROS (5-6 h), reduce marcadamente la muerte. Sin embargo, se desconocen los eventos moleculares que llevan a ese incremento de ctERO. Una posibilidad es que el aumento de las mtERO esté generando una respuesta que induzca a su vez un incremento mayor posterior en los niveles de ctERO. Una de las fuentes de ERO que pueden estar involucradas en este proceso son las NOX, estas últimas se activan de forma transitoria en el modelo de ST a los 30 min de tratamiento (Fig. 9B); sin embargo, es necesario explorar si a las 5 horas de tratamiento con los estímulos apoptóticos se presenta este aumento.

Estudios previos han mostrado que, bajo ciertas condiciones, existe una interrelación entre las ERO producidas por la mitocondria y las producidas por NOX (Choi et al., 2014), sugiriendo un mecanismo de retroalimentación entre ambas fuentes (Nazarewicz et al., 2013b). En nuestro modelo, observamos un aumento temprano de las mtERO y un aumento en la actividad de NOX en el modelo de ST a los 30 min y, posteriormente, un aumento en los niveles de ctERO (Fig. 9B), lo que apoyaría la idea de que ambas fuentes participan en el proceso. Sin embargo, en nuestro modelo no se estudió si existe la interrelación entre las mtERO y la ERO producidas por NOX.

En relación a lo anterior, nuestro grupo ha demostrado que, en respuesta a una condición de muerte como K5 o ST, ocurre una disminución temprana, en la concentración de calcio (Ca) del retículo endoplásmico y en el potencial de membrana mitocondrial (Benítez-Rangel et al., 2020). No se sabe si en el modelo de las NGC los cambios en las concentraciones de Ca observados pueden generar alteraciones a nivel de la morfología mitocondrial, que posteriormente pudieran

afectar la producción de mtERO o de las NOX y que éstos sean responsables del proceso de fragmentación mitocondrial.

En el presente trabajo evaluamos la viabilidad de las NGC tratadas con K5 y ST y observamos que ésta va disminuyendo desde los primeros 15 min y continua de esta forma hasta las 8 h post tratamiento (Fig. 2A y 2B). Esto indica que este es un proceso que se desencadena desde los primeros minutos posteriores al estímulo de muerte. Previamente, se reportó que un estímulo oxidante, como el H_2O_2, produce una disminución en la viabilidad de las NGC desde los primeros minutos (Hohnholt et al., 2015; Valencia y Morán, 2001), sugiriendo que las ERO pueden provocar la muerte de manera súbita en cultivos de NGC. Un dato interesante fue que en las NGC tratadas con K5 los niveles de reducción de MTT disminuyen significativamente a los 30 min; sin embargo, una hora después del estímulo se observa un aumento transitorio seguido de una disminución continua hasta las 8 h (Fig. 2A). Igualmente, en las neuronas tratadas con ST, a los 15 min hay una disminución y nuevamente a los 30 min un aumento transitorio, que va seguido de una disminución continua en la reducción de MTT hasta las 8 h (Fig. 2B). Aunque en la literatura es escasa la información acerca de qué pasa con la viabilidad celular en los primeros momentos del proceso de muerte, en las NGC se ha mostrado que existe una caída del potencial de membrana mitocondrial desde los primeros 10 min del tratamiento con K5 y ST (Benítez-Rangel et al., 2020). Esta caída del potencial de membrana se ha asociado con una disminución en la función mitocondrial y la muerte neuronal (Lim et al., 2020; Nishida et al., 2012), lo que nos sugiere que la función mitocondrial de las NGC se encuentra comprometida desde momentos tempranos del proceso. Aunque la información existente no permite explicar el incremento transitorio de la transformación de MTT observada, nosotros especulamos que el posible cambio en la función mitocondrial genera en las neuronas una respuesta celular para contender contra la muerte; sin embargo, ésta no es suficiente para evitar la pérdida de la viabilidad. Será necesario explorar el efecto del K5 y la ST en la función mitocondrial y la actividad metabólica de las NGC para conocer si existe algún mecanismo que se pueda relacionar con la recuperación transitoria de las NGC.

Aunque la inhibición de los niveles de ERO puede prevenir la muerte neuronal (Hwang y Kim, 2018; Lee et al., 2020), un estudio mostró que la inhibición de la producción de mtERO y las ERO de la NOX1 no tuvo un efecto aditivo en la prevención de la muerte inducida por la proteína β-amiloide en neuronas corticales (Hwang y Kim, 2018). Lo anterior sugiere que otros factores, además del aumento en los niveles de ERO, pueden participar en el proceso de muerte. Estos resultados son consistentes con lo observado en las neuronas pretratadas con MitoTEMPO, las cuales únicamente presentaron un efecto protector parcial en nuestros modelos de muerte apoptótica (Fig. 3A y 3B) y reafirma la idea de que otra fuente intracelular de ERO, como puede ser la NOX contribuye al proceso de muerte.

En este trabajo también se probó que hay un aumento en la actividad de NOX en NGC tratadas con ST (Fig. 9B), pero no con las tratadas con K5 (Fig. 9A). En trabajos anteriores se mostró que K5 y ST inducen la actividad de NOX después de 3 h de tratamiento en NGC de ratones (Guemez-Gamboa y Morán, 2009), en el actual trabajo no probamos los mismos tiempos, sin embargo, nuestros resultados son consistentes con la idea de que la otra fuente podría ser una NOX responsable de le generación de ctERO producidos posteriormente a las mtERO.

Por otra parte, se observó un aumento en la fragmentación mitocondrial con K5 y ST; sin embargo, sólo en el caso del K5 se observó un aumento en la fosforilación de Drp1 en el residuo Ser616. Los datos anteriores indican que el aumento de ctERO, mtERO y la fragmentación mitocondrial son parte del proceso de muerte inducido por K5 y ST, pero que no está mediada por la fosforilación de Drp1.

Se ha propuesto que los cambios detectados en la morfología mitocondrial son esenciales en la progresión de la muerte neuronal y en los procesos neurodegenerativos (Choi et al., 2012; Choi et al., 2013; Zhou et al., 2017). En este sentido, en coincidencia con estos estudios, nosotros observamos cambios morfológicos en las mitocondrias durante diferentes momentos posteriores al tratamiento con ambas condiciones apoptóticas. Las NGC que se mantuvieron en condiciones basales, mostraron mitocondrias interconectadas que después de

algunas horas de tratamiento con K5 o ST comienzan a hacerse más cortas y redondas en el área de los procesos neuronales (Fig. 4), lo cual también se ha reportado en otros modelos neuronales (Gray et al. 2013; Hung et al. 2018).

Cambios morfológicos en la mitocondria, generalmente, la fragmentación, se ha relacionado con un aumento en los niveles de ERO en algunas preparaciones (Deheshi et al., 2015; Hung et al., 2018; Yan y Zhao, 2020), dado que la reducción de los niveles de mtERO disminuyen su fragmentación (Hung et al., 2018; Lee et al., 2018). En nuestro trabajo, el uso de un antioxidante mitocondrial (Fig. 3) no previno la fragmentación mitocondrial en ninguna de las condiciones apoptóticas estudiadas (Fig. 7), descartando por lo tanto esta posibilidad.

Por otro lado, Drp1 es una de las proteínas principales involucradas en la regulación de la fisión mitocondrial (Cid-Castro et al. 2018; Hoppins et al. 2007). Para que la fisión ocurra se requiere que Drp1 se transloque del citoplasma a la membrana externa de la mitocondria. Esto requiere a su vez la activación de Drp1 y, aunque se ha reportado que distintas modificaciones postraduccionales, cómo la ubiquitinación y la sumoilación pueden regular su actividad (Rosdah et al., 2020), las más estudiadas en el contexto de muerte celular son: la fosforilación de Drp1 en el residuo Ser616 y Ser 637 (Cereghetti et al., 2008; Yu y Pekkurnaz, 2018). Por su parte, el balance redox se ha ligado a la regulación de las proteínas encargadas de la dinámica mitocondrial (Willems et al., 2015). Por ello, para evaluar la participación de las mtERO en la fisión mitocondrial, analizamos la activación de Drp1, mediante su fosforilación en el residuo Ser616 en NGC tratadas con K5 y ST.

En el caso de las neuronas privadas de potasio, encontramos un incremento en los niveles de fosforilación después de 15 min de tratamiento, lo cual correlaciona con el acortamiento de las mitocondrias (Fig. 4B), como se ha reportado en estudios previos (Kim et al., 2016; Yan y Zhao, 2020). Adicionalmente, observamos que el tratamiento con MitoTEMPO redujo de manera significativa el incremento de p-Drp1 inducido por K5 (Fig. 5B). Esto sugiere que la fosforilación de Drp1 puede estar mediada por las mtERO inducidas por la privación de potasio.

Por el contrario, en el modelo de ST, que indujo un aumento en las mtERO que correlaciona con la disminución de la longitud mitocondrial, no observamos una activación de Drp1, medida como la fosforilación del residuo Ser616. Bajo estas condiciones observamos una disminución de Drp1 total y Drp1 fosforilada desde tiempos muy tempranos (Fig. 6A). De acuerdo con estudios previos en los que se ha relacionado a la ST con la muerte neuronal, se ha reportado que ésta induce mitofagia a través de la E3 ubiquitin ligasa vía de Parkina (Ha et al., 2014), lo que se ha asociado a una degradación de Drp1 dependiente del sistema proteosomal (Tang et al. 2016; Wang et al. 2011). Estos antecedentes podrían explicar la reducción de los niveles de Drp1 total y Drp1 fosforilada en este modelo. Este resultado muestra que las mtERO y la Drp1 podrían no estar mediando la fisión mitocondrial inducida por ST en las NGC.

Es importante mencionar que, aunque el sitio de fosforilación de Drp1 más comúnmente reportado es el sitio Ser616 (Chang y Blackstone, 2010; Cho et al., 2012; Joshi et al., 2017), también se ha sugerido que una disminución en la fosforilación en el sitio Ser637 podría inducir la translocación de Drp1 a la mitocondria en neuronas de hipocampo (Kang et al., 2018; Park et al., 2015), contribuyendo así a la muerte neuronal (Wang et al., 2012). Con estas evidencias no podemos descartar que Drp1 fosforilada en Ser637 sea la responsable de la fragmentación mitocondrial en nuestro modelo; sin embargo, habrá que probar esta posibilidad en futuros experimentos.

Por otro lado, una posible explicación para las diferencias observadas en la fosforilación de Drp1 inducida por K5 y ST puede ser las distintas vías de señalización activadas por cada condición. La fragmentación mitocondrial dependiente de Drp1 se regula por varias cinasas, incluida CDK5 (Jahani-Asl et al., 2015), CaMKII (Ko y Kang, 2017; Park et al., 2015), ERK1/2, PKC, JNK y p38 en diferentes modelos (Kim et al., 2019; Ko y Kang, 2017; Yan y Zhao, 2020). Nuestro grupo de investigación reportó previamente que ambos modelos de muerte apoptótica muestran diferencias en el mecanismo molecular. Por ejemplo, K5 induce una reducción del Ca citoplásmico, mientras que la ST induce un aumento (Benítez-

Rangel et al. 2020; Moran y Patel 1989) y, aunque ambas condiciones activan a la NOX, únicamente ST induce la activación del homólogo NOX2 (Guemez-Gamboa y Morán, 2009).

Finalmente, en estudios previos encontramos una activación diferencial de las vías de señalización inducidas por K5 y ST durante el proceso de muerte neuronal apoptótica. Por ejemplo, el efecto de K5 está mediado por JNK y p38, mientras que ST requiere solo de la activación de p38 (Ramiro-Cortés et al. 2011). Nosotros hipotetizamos que las diferencias observadas en la fosforilación de Drp1 pueden ser responsables de las discrepancias encontradas en las condiciones de K5 y ST que, aunque producen muerte apoptótica, han mostrado diferencias en la parte inicial de su activación.

En este sentido, en este trabajo observamos una fragmentación mitocondrial en ambos modelos experimentales; sin embargo, en el caso de las neuronas sometidas a K5, la inhibición de los niveles de Drp1 fosforilado con el MitoTEMPO no fue suficiente para reducir la fisión mitocondrial. En el modelo de muerte inducida por ST, observamos un aumento en la fragmentación mitocondrial, incluso con bajos niveles de Drp1 total. Estos resultados sugieren que existe un mecanismo alternativo para la fragmentación mitocondrial inducida por los estímulos apoptóticos empleados. Se ha reportado que la actina y el retículo endoplásmico (RE) pueden participar en la fisión mitocondrial independiente de Drp1, probablemente por medio de un mecanismo de constricción física, directamente sobre la membrana mitocondrial (Stavru et al., 2013). Al respecto, se sabe que el citoesqueleto tiene una estrecha interacción con las mitocondrias en el espacio celular; la actina participa en la redistribución de mitocondrias durante la mitosis, mientras que los microtúbulos están involucrados en el transporte mitocondrial en axones y dendritas (Bartolák-Suki et al., 2017). Recientemente, se ha propuesto que el citoesqueleto de actina regula la morfología mitocondrial, ensamblándose alrededor de la membrana mitocondrial externa promoviendo la fisión (Moore et al., 2016; Rehklau et al., 2017) y también se ha involucrado en la regulación de la

muerte neuronal (Hoffmann et al. 2019), pero el papel del citoesqueleto en la muerte neuronal no se ha estudiado.

Un punto importante relacionado con el papel de Drp1 en este estudio es la falta de efecto del MDiVi-1 observado en la viabilidad de NGC en las condiciones estudiadas (Fig. 8). El MDiVi-1 es un inhibidor de Drp1 que tiene una acción reguladora de la fragmentación mitocondrial y de la muerte neuronal (Grohm et al., 2012; Xie et al., 2016). Esto sugiere que la activación de Drp1 no es determinante en la muerte inducida por K5 y ST. MDiVi-1 se ha utilizado como neuroprotector en varios modelos en los que se induce fragmentación mitocondrial y consecuentemente muerte neuronal (Grohm et al., 2012; Xie et al., 2016).

Es necesario aclarar que el MDiVi-1 también ha demostrado tener efectos independientes de la actividad de Drp1. Por ejemplo, en neuronas corticales ha mostrado inducir la despolarización neuronal y modular los niveles de Ca intracelular (Ruiz et al., 2018). Además, se encontró que este compuesto inhibe al complejo I de la cadena respiratoria mitocondrial, afectando los niveles de ERO (Bordt et al., 2018). Estos resultados indican que MDiVi-1 puede tener blancos distintos a Drp1 y que puede incidir en otros procesos además de la fragmentación mitocondrial, por lo que es necesario revisar sus efectos sobre la viabilidad de NGC.

Cabe señalar que la hipótesis planteada inicialmente solo se afirmó de manera parcial, ya que se comprobó que la mitocondria es una fuente importante de ERO que participa en el proceso de muerte de las NGC inducida por K5 y ST, además se comprobó que se generan cambios en la morfología mitocondrial; sin embargo, la fragmentación no se evita al inhibir los niveles de mtERO con MitoTEMPO. Por otra parte, los dos modelos de muerte neuronal utilizados se comportaron de maneras distintas con respecto a la fosforilación de Drp1, aunque, en el modelo de K5 se observó un aumento en la fosforilación de Drp1, también se estableció que la disminución de ésta en presencia de MitoTEMPO no es suficiente para evitar la fragmentación mitocondrial. Mientras que en el modelo de ST los niveles de fosforilación de Drp1 disminuyeron desde los primeros minutos del tratamiento, un

dato que indica nuevamente que los cambios morfológicos de las mitocondrias de las NGC no están mediados por Drp1.

En la figura 10 se muestra un resumen de los eventos más relevantes de cada modelo experimental utilizado en este trabajo.

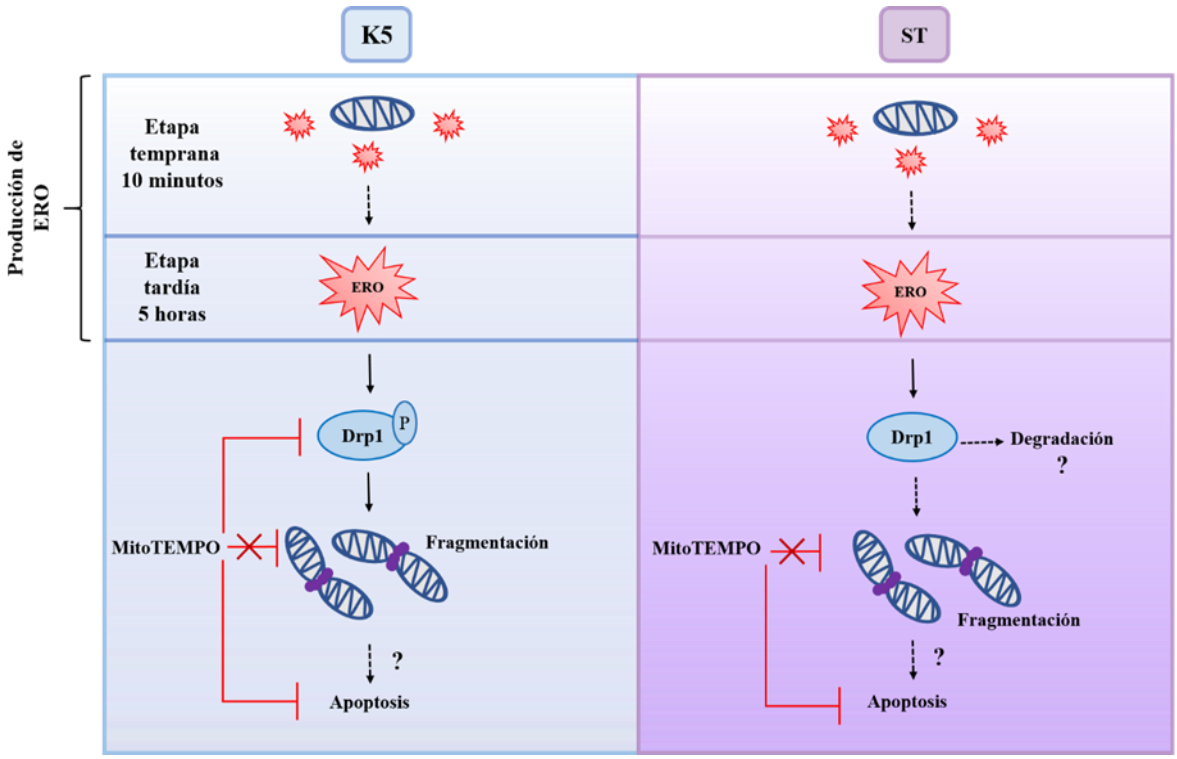


Figura 10. La fragmentación mitocondrial y la muerte neuronal inducidas por K5 y ST no están mediadas por la fosforilación de Drp1 en el residuo Ser616. El modelo propuesto para ambas condiciones apoptóticas incluye una etapa temprana de producción de mtERO, así como, una etapa más tardía de elevación de ctERO. En el modelo de K5 (panel izquierdo) se incrementó la fosforilación de Drp1 en la Ser61 en los primeros 15 min del tratamiento, posteriormente observamos una disminución en la fosforilación de Drp1 en presencia de MitoTEMPO, aunque este no pudo evitar la fragmentación mitocondrial. Por otra parte, el tratamiento con ST (panel derecho) también induce un incremento en mtERO y ctERO, encontramos que ST reduce los niveles de Drp1 total y los niveles de p-Drp1 Ser616, en NGC tratadas con ST también se indujo la fragmentación mitocondrial y tampoco se evitó con el uso del MitoTEMPO. Los estímulos de K5 y ST causaron muerte neuronal, a pesar de que el MitoTEMPO fue capaz de prevenir esta pérdida de viabilidad, no evitó la fragmentación mitocondrial, al igual que el MDiVi-1 que tampoco evitó la disminución en la viabilidad neuronal. Aunque la fosforilación de Drp1 es un determinante de la fisión mitocondrial en otros modelos de muerte neuronal, esto no ocurre en nuestros modelos, nuestros datos sugieren que la muerte de las NGC inducidas por K5 y ST no está mediada por la fragmentación causada por Drp-1 fosforilada en la Ser-616.

Conclusión.

Nuestros resultados sugieren que la generación de mtERO es un evento temprano en el proceso de muerte neuronal, previo a la generación de ctERO y a la activación del programa apoptótico. Además, las mtERO son necesarias para el proceso de muerte neuronal, pero no para la fragmentación mitocondrial. La fragmentación mitocondrial y la muerte neuronal de la NGC parecerían no estar mediadas por la fosforilación de Drp1 en el residuo Ser616. Nuestros datos sugieren que la fragmentación mitocondrial se lleva a cabo por diferentes mecanismos, dependiendo de la condición apoptótica.

Se requieren más experimentos para explorar la relación entre las mtERO y las ctERO y el papel que juegan en la dinámica mitocondrial y la muerte neuronal, así como los mecanismos moleculares y celulares finos involucrados en la regulación de la muerte neuronal.

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REVIEW PAPER

ROS as Regulators of Mitochondrial Dynamics in Neurons

Carolina Cid-Castro[1] · Diego Rolando Hernández-Espinosa[1] · Julio Morán[1]

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Abstract
Mitochondrial dynamics is a complex process, which involves the fission and fusion of mitochondrial outer and inner membranes. These processes organize the mitochondrial size and morphology, as well as their localization throughout the cells. In the last two decades, it has become a spotlight due to their importance in the pathophysiological processes, particularly in neurological diseases. It is known that Drp1, mitofusin 1 and 2, and Opa1 constitute the core of proteins that coordinate this intricate and dynamic process. Likewise, changes in the levels of reactive oxygen species (ROS) lead to modifications in the expression and/or activity of the proteins implicated in the mitochondrial dynamics, suggesting an involvement of these molecules in the process. In this review, we discuss the role of ROS in the regulation of fusion/fission in the nervous system, as well as the involvement of mitochondrial dynamics proteins in neurodegenerative diseases.

Keywords Mitochondrial dynamics · ROS · Neurons · Cell death · Neurodegenerative diseases

Introduction

Mitochondria have been characterized as the metabolic center of the cell. These organelles contain their own genome (Frezza 2017) and synthesize most of the cellular ATP, nucleotides, fatty acids, and iron-sulfur clusters (Lackner 2014). Additionally, mitochondria play a role in calcium and redox signaling during apoptosis (Galluzzi et al. 2014). Thus, mitochondria have been pointed out as central organelles in cellular function. In the last years, the morphology and structure of these organelles have been shown to be relevant for the physiology of the cell and are indicators of the cellular fate.

Mitochondrial networks are constantly undergoing remodeling via cycles of fission and fusion (Westernmann 2012). In different models, the core of proteins involved in the regulation of the mitochondrial morphology undergo changes of activity and/or expression level by a variety of intracellular signals and metabolic conditions (Wappler et al. 2013; Manczak et al. 2016; Twig et al. 2008). In this review, we discuss some evidences related to the mitochondrial dynamics, including the main components that participate in this process, and its role in some pathophysiological processes, with specific emphasis in the nervous system. Particularly, we discuss the importance of reactive oxygen species (ROS) as mediators of mitochondrial dynamics in neurons and the role of proteins involved in the mitochondrial fission/fusion in neurodegenerative diseases.

Mitochondrial Dynamics

Mitochondria are continuously dividing and fusing to control their size, morphology, and number. They may exist as individual organelles or as interconnected networks. These different forms of organization and structure depend on the tissue and cell type, as well as the metabolic state and developmental stages of the cell. Mitochondrial morphology is achieved by the constant cristae remodeling, as well as the fission and fusion of the mitochondrial membranes. Altogether, these processes are known as mitochondrial dynamics (Pernas and Scorrano 2016). The maintenance of the mitochondrial organization, as well as their function and morphology is a complex issue orchestrated by a

✉ Julio Morán
jmoran@ifc.unam.mx

Carolina Cid-Castro
ccid@email.ifc.unam.mx

Diego Rolando Hernández-Espinosa
dhernandez@email.ifc.unam.mx

[1] División de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-253, 04510 Mexico, DF, Mexico

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heterogeneous group of proteins that keep the equilibrium between form and function by coordinating their activities (Lee and Yoon 2016). The main mechanism of mitochondrial dynamics relies on proteolytic processing and post-translational modifications of the core of proteins involved in the process (Cho et al. 2012).

Mitochondrial fission participates in the control of the number and distribution of mitochondria, as well as in the response to changes in energetic cellular needs, the disposal of damaged mitochondria and the maintaining of the components of the respiratory chain, the cristae shape and the ATP production. On the other hand, fusion is an intricate process that involves the join of outer and inner mitochondrial membrane as an adaptation to facilitate communication between mitochondria and their host cells to maintain cell homeostasis (Pernas and Scorrano 2016). Fusion has been related to the preservation of the capacity of the mitochondria to maintain genetic and biochemical homogeneity, allowing the dissipation of ROS, the exchange of mutated DNA, and the repolarization of membranes to maintain mitochondrial functionality. Fission and fusion determines the structural and functional status of mitochondria (Santel and Frank 2008; Balog et al. 2016).

Fusion and Fission Machinery

Mitochondrial dynamics is highly regulated by at least four conserved dynamin-related GTPases that mediate the membrane remodeling through the join or scission of mitochondrial membranes (Westermann 2010). The most studied proteins are dynamin-related protein 1 (Drp1), which controls mitochondrial division, as well as mitofusins 1 and 2 (Mfn1 and Mfn2) and optic atrophy 1 (Opa1), which drive fusion. The general mechanism and main components are discussed below and are shown in Fig. 1.

The key protein involved in mitochondrial scission is the soluble Dynamin-Related Protein (DRP1, in humans), which controls division of the mitochondrial outer membrane (Ingerman et al. 2005; Mears et al. 2011; Nakamura et al. 2006; Karbowski et al. 2007; Chang and Blackstone 2007a, b; Cho et al. 2009; Chang et al. 2010). Their role in fission is conserved in all the characterized eukaryotes to date, including plants, and it is ubiquitously expressed in mammals. It is encoded by the DNM1L gene and its known as dynamin-1 (Dnm1) in yeast and there are other homologs in different species (Labrousse et al. 1999; Bleazard et al. 1999, 2013). It is noteworthy to note that Drp1 and some of its partners also mediate fission of peroxisomes (Bertholet et al. 2016). Drp1 undergoes post-translational modifications, which can affect its activity and cellular localization; the main modifications include S-nitrosylation (Nakamura et al. 2006; Karbowski et al. 2007; Chang and Blackstone

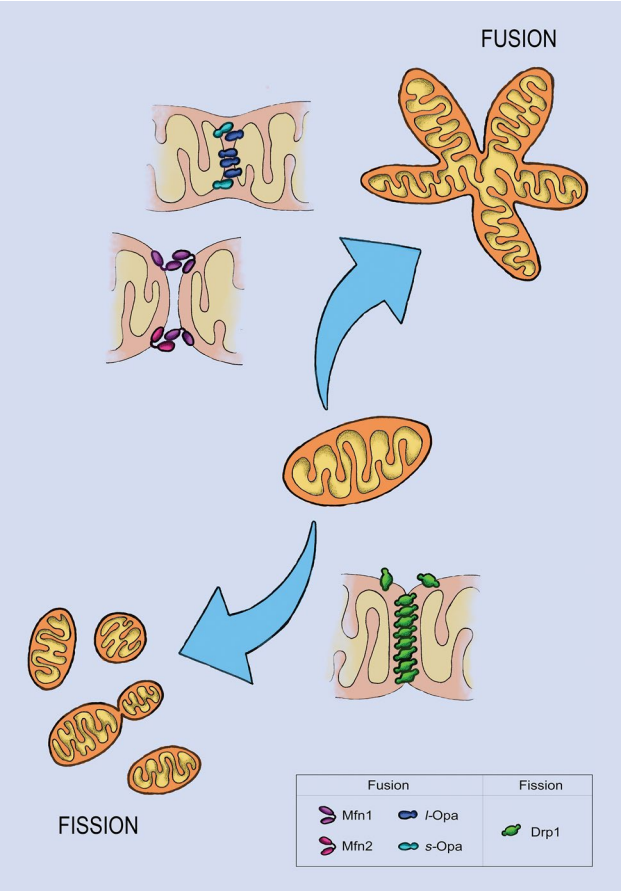

Fig. 1 Schematic representation of main components of mitochondrial dynamics machinery. Mitochondrial morphology is dependent on a proper balance between fusion and fission processes, which are coordinated by a systematized set of dynamin-related GTPases. Fusion of mitochondrial outer membrane is leading by Mfn1, Mfn2, which are anchored in the outer membranes and allows their close up and the fusion of membranes. Opa1 is in charge of the connection of the inner mitochondrial membrane and the cristae remodeling. Fusion of mitochondrial membranes produces an interconnected organelle. On the other hand, Drp1 is the master protein for mitochondrial fission and is initially positioned at the outer mitochondrial membranes by adaptor proteins. It leads membranes scission by forming a ring around the organelle to constrain the membranes producing mitochondrial shortening

2007a, b; Cho et al. 2009; Chang et al. 2010), phosphorylation (Taguchi et al. 2007; Han et al. 2008; Sesaki et al. 2014; Manczak et al. 2012), and sumoylation (Prudent et al. 2015).

During the fusion process, several proteins participate as mediators in the remodeling of outer and inner membranes. These include GTPases, Mfn1, Mfn2, and Opa1, among others. Mitofusins are known as Fzo (Fuzzy onions) in flies and yeast, as wells as Mfn1 and Mfn2 in humans (Mozdy and Shaw 2003). Regarding the structure, Mfn1 and Mfn2 share N-terminal regions, where the GTPase domains responsible for the binding and hydrolysis of GTP are located. (Huang et al. 2011; Palmer et al. 2011; Santel et al. 2003). The main

post-translational modifications reported for mitofusins are phosphorylation for the regulation of the mitochondrial fusion and ubiquitination to facilitate mitophagy, i.e., the mitochondria elimination by autophagy (Leboucher et al. 2012; Gegg et al. 2010; Park et al. 2009).

Opa1 is located at the inner mitochondrial membrane. Its biological relevance was established in a homozygous mouse model, which die in utero during embryogenesis. Heterozygous animals are viable, but exhibit loss of retinal ganglion cells and eventually a severe degeneration in nerve fiber layer (Alavi et al. 2007). Opa1 is synthesized in the cytoplasm and is processed in the mitochondrial matrix (Ishihara et al. 2006). It has eight different isoforms in humans and is enriched in retina, brain, testis, heart, and muscle (Delettre et al. 2001). Post-translational regulation is based on the proteolytic processing by mitochondrial metalloproteases that generate a long form that retain the N-terminal transmembrane domain (L-Opa1) and a short soluble isoform (S-Opa1) (Song et al. 2007; Ehses et al. 2009; Anand et al. 2014). Opa1 regulates the shape and the length of the mitochondrial cristae during apoptosis through the participation of oligomers of L-Opa and S-Opa (Frezza et al. 2006).

Physiological and Pathological Role of Mitochondrial Dynamics

In the physiological context, mitochondrial dynamics is particularly crucial for the regulation of the number of mitochondria, the elimination of organelles by mitophagy (Wu et al. 2018). It is also necessary for the distribution of mitochondria along the cells, since mitochondria are required to be accumulated in sites where high amount of energy or calcium buffering are needed (Otera and Mihara 2011). The importance of mitochondrial fission/fusion has been shown in different physiological processes including apoptosis, cell division, metabolism, and bioenergetics (Westermann 2010; Kanfer et al. 2017; Otera and Mihara 2011; Gomes et al. 2011; Chen et al. 2003; Amchenkova et al. 1988).

Recent studies have shown that aberrations in mitochondrial dynamics processes are associated with many human disorders (Huang et al. 2013; Itoh et al. 2013; Reddy 2011; Reddy and Shirendeb 2012; Cho et al. 2010; Knott et al. 2008). It is known that the loss of mitochondrial function, secondary to defects in mitochondrial dynamics, leads to an increase of ROS generation and a decrease in the ATP production (Guo et al. 2015).

Some human hereditary diseases are linked to defects in the activity of fusion and fission proteins. For example, it has been observed an inadequate function of Drp1 involved in a development delay, insensitivity to pain and microcephaly, as well as in syndromes such as sudden death (Waterham et al. 2007). Some types of lung cancer (Zhu et al. 2004; Chiang et al. 2009) have also been related to an altered function of Drp1, while spastic paraplegia syndrome and multiple sclerosis are associated with defects in Opa1 (Chao de la Barca et al. 2016). Similarly, recent evidence suggests the participation of mitochondrial dynamics proteins in acquired diseases. For example, alterations in mitofusins have been linked to diabetes mellitus, pulmonary hypertension, and breast cancer (Yu et al. 2009; Zhao et al. 2013; Sharp et al. 2014; Ryan et al. 2013) and Opa1 defects are observed in patients with hypertension (Jin et al. 2011).

Mitochondrial Dynamics in the Nervous System

Due to their high metabolic activity, neurons are particularly sensitive to changes in the mitochondrial function and are energetically demanding cells that require an adaptively maintenance of these organelles (Kann and Kovacs 2007). Moreover, as highly polarized cells containing complex neuritic processes, neurons also need a timely and appropriate transport and distribution of mitochondria to produce energy and regulate the calcium necessary for the neuronal activities, including synaptic transmission and vesicle recycling (Sheng and Cai 2012). Mitochondrial dynamics has also been related to neurogenesis during neuronal development and adult brain. Although the influence of the mitochondrial dynamics in this process has not been completely understood, it is evident that it results an important regulatory event for neuronal development (Khacho and Slack 2018).

The numerous structural profiles of mitochondria correlate with the different bioenergetics demands in several tissues, including the brain. Neurons depend on oxidative phosphorylation as primary source of energy production, which is vital to regulate complex dynamics that include the activity of pumps and transporters, the transport for long distances across neuritic extensions, as well as other processes such as fission and fusion that imply large ATP needs (Kuznetsov et al. 2009; Mironov 2009; Rolfe and Brown 1997).

In neurons, mitochondrial division is important to transport mitochondria to sites where high amount of energy is required, including synaptic terminals (Otera and Mihara 2011). Distribution of these organelles is particularly important in neurons due to the need to delivery and exchange of newly mitochondria along the processes. Thus, the biogenesis is crucial for the availability of healthy mitochondria (Schwarz 2013). Deficiencies in the mitochondrial dynamics are associated with the inability of neurons to maintain the ATP synthesis required for calcium regulation, neuronal electrical activity and axonal transport necessary for neuronal communication (Cuesta et al. 2002; Chen et al. 2003;

Wakabayashi et al. 2009; Shields et al. 2015; Dietrich et al. 2013).

During physiological conditions, it has been demonstrated that mitochondrial length is critical to define when a mitochondria should divide, but the motility is also determinant for fusion. This suggests that the equilibrium of the mitochondrial dynamics is finely regulated not only by a core of proteins, but also for other processes that affect the number and movement of mitochondria, which in turn exerts a feedback to control mitochondrial homeostasis in neurons (Cagalinec et al. 2013). In stress conditions, the length and shape of mitochondria usually adapting their shape form filamentous to short and round, showing the adaptability of these organelles for contend with the changing environment (Youle and van der Bliek 2012).

Nervous System Pathologies Associated with Mitochondrial Dynamics Defects

Despite the fact that different neuronal populations are affected in neurodegenerative diseases, a common condition in all cases is an abnormal mitochondrial structure and function. This suggests that the mitochondrial dynamics might not be involved in the selective vulnerability of specific neuronal populations, but rather in the mediation or amplification of mitochondrial dysfunction and neuronal death during the course of neurodegenerative or neuropsychiatric disorders (Jellinger 2009; Rezin et al. 2009). The pathologies associated with defects in fission and fusion proteins includes status epilepticus and schizophrenia in which activation of Drp1 is frequently reported (Flippo and Strack 2017; Kim and Kang 2017).

In several neurodegenerative diseases and disorders related to mitochondrial defects, the neurons show alterations in the oxidative phosphorylation, the homeostasis of intracellular ROS and the levels of calcium, as well as in the mitochondrial mobility, mitophagy and fusion/fission dynamics (Burte et al. 2015; Ryan et al. 2015). Deregulation of the mitochondrial fusion or fission has also been associated with defects in neuronal development and neuronal plasticity, both in ex vivo and in vivo models (Bertholet et al. 2016). In Drp1 mutant cultured neurons, abnormal mitochondrial distribution results in a compromised synapse formation. It is also known that lacking of Drp1 causes developmental abnormalities in mice, which die after embryonic day 12.5; these mutant embryos have a small body size and a heart and liver abnormal development (Ishihara et al. 2009).

Neuropathologies such as Alzheimer's, Parkinson's, and Huntington's diseases are characterized by a progressive loss of neuronal function and have been related to mitochondrial defects as an early sign of neurodegeneration (Gao et al. 2017; Correia et al. 2012; Itoh et al. 2013; Wilson et al. 2013). For example, in genetic models of Parkinson's disease, an overexpression of mutant α-synuclein leads to defects on axonal mitochondrial transport and an elevated mitochondrial fragmentation (Devoto et al. 2017; Ordonez et al. 2017), suggesting a close correlation between α-synuclein an mitochondrial distribution in this disease.

In postnatal mouse cortical neurons, apoptotic conditions decreased the expression of Drp1 and parkin and these effects were abolished by recovering the expression levels of parkin or Drp1, which enhanced neuronal viability and reestablished the mitochondrial morphology (Wang et al. 2013). It is known that mutations in the genes that codify for parkin are the cause of the autosomal recessive form of Parkinson's disease. Parkin recognizes proteins of the mitochondria in response to cellular insults and promotes the repair of mitochondria though autophagy and proteasomal mechanisms (Seirafi et al. 2015).

There are evidences suggesting that Drp1 and parkin work in a synergistic manner to maintain mitochondrial function and structure in the brain. Both molecules are critical when mitochondrial division is altered, which suggests that the initiation and progression of Parkinson's disease are related to a decrease in the mitochondrial division and depend on these molecules (Kageyama et al. 2015). The machinery that links Drp1 to the origin and evolution of Parkinson's disease is unclear; nevertheless, it has been demonstrated that Drp1 is closely modulated by different conditions that are also involved in Parkinson's disease. For example, Drp1 levels are quite sensitive to induction of autophagy. In cultured striatal neurons, mitochondrial fission and Drp1 levels are decreased after autophagy induction and the inhibition of autophagy induces high level of Drp1. Thus, It is possible that the observed fission in neurodegeneration could be counteracted by autophagy through a reduction in Drp1 (Purnell and Fox 2013).

Other conditions affecting Drp1 and parking modulation may also play a pivotal role in Parkinson's disease. This includes Drp1 and parkin sumoylation that interferes with mitochondrial fusion/fission by reducing the amount of parkin available for mitochondrial recruitment (Guerra de Souza et al. 2016). Finally, in a model of Parkinson's disease, it was shown that the S-nitrosylation of parkin leads to an increase in the levels of Drp1, but a reduction in its interaction with Drp1. This condition also induces the phosphorylation of Drp1 Ser616 and its recruitment to the mitochondria (Zhang et al. 2016).

Drp1 defects have also been observed in cells of Alzheimer's disease patients (Song et al. 2011; Kandimalla and Reddy 2016). In Alzheimer's disease, fibroblast and human neuroblastoma SH5YSY cells, both the expression of Drp1, and its interaction with mitochondrial adaptors are markedly increased by Aβ-42 (Kuruva et al. 2017). In contrast, the inhibition of Drp1 interaction with its adaptors reduces the

recruitment of Drp1 and prevents the mitochondrial fission and functional dysfunction induced by Aβ-42 (Joshi et al. 2017). On the other hand, in cultured cortical neurons, the amyloid peptide Aβ- 42 increases the expression of Drp1 and decreases the expression of Mfn1/2 and OPA- 1; the inhibition of DRP1 markedly reverts the observed disruption of mitochondrial membrane potential (Han et al. 2017).

Opa1 was identified as the human gene of autosomal dominant optic atrophy (ADOA) that is a hereditary optic neuropathy that causes progressive loss of vision (Delettre et al. 2000). Although initially Opa1 localization and function were unknown, it was later found a signal peptide sequence for mitochondrial localization, suggesting a mitochondrial function of this protein (Alexander et al. 2000). Mutations in Opa1 are responsible of a spectrum of diseases such as ADOA with deafness and multi-systemic syndromes, which involves neurological and neuromuscular symptoms (Amati-Bonneau et al. 2009). Additionally, there are evidences of abnormal cristae morphology in the striatum and cortex of murine models of Huntington's disease due to Opa1 defective oligomerization (Hering et al. 2017).

Regarding mitofusins, it has been known that mutations in Mfn2 are the most common cause of axonal Charcot-Marie-Tooth disease (CMT) type 2 (Züchner et al. 2004), which is a genetically heterogeneous disorder of peripheral neuropathies, characterized by distal muscle weakness and atrophy (Azzedine et al. 2012). In addition, in a model for idiopathic PD induced by paraquat, the observed mitochondrial fragmentation and dopaminergic neurodegeneration are markedly reduced by overexpression of Mfn2 (Zhao et al. 2017). In contrast to Mfn2, there are no reports showing a relation of Mfn1 to any neuropathology.

Neurons are particularly sensitive to alterations in mitochondrial dynamics, which seems to be important in the initiation and progression of neurodegenerative disorders; unfortunately, no much information exists about the mechanisms involving mitochondrial dynamics and the development of neuropsychiatric disorders. The mitochondrial fusion/fission represents a new scenario to explore the pathologies associated to nervous system, but more studies are needed to understand the complete role on these pathologies and their probable therapeutic approach. Some neuropathologies related to defects on mitochondrial dynamics and the role of fission and fusion in these processes are listed in Table 1.

Regulators of Mitochondrial Dynamics

Despite the experimental evidences about the post-translational regulation of mitochondrial dynamics proteins, the molecular mechanism is still not fully understood. In that regard, it is known that some signaling molecules influence the fusion and fission processes. Two conditions that seem to be mediators of fission and fusion in the nervous system include the intracellular levels of calcium and ROS levels.

Calcium is a ubiquitous cellular messenger involved in signaling pathways that regulate numerous processes. In the nervous system, calcium is critical for several events, including synaptic transmission (Jones and Smith 2016), cell migration (Komuro et al. 2015), and axonal guidance (Kaplan et al. 2014). The role of calcium in mitochondrial dynamics has been extensively reviewed. For example, it has been reported that an increase in the levels of calcium alters both the mitochondrial function and Drp1 activity (Hom et al. 2007). Other studies have demonstrated that the intracellular localization of Drp1 in neurons is regulated by calcium through the participation of calcineurin (Cereghetti et al. 2008; Cribbs and Strack 2007). In addition, under excitotoxic conditions, the levels of Drp1 and Opa1 are mainly affected by a rise in intracellular calcium (Wang et al. 2015; (Martorell-Riera et al. 2014; Jahani-Asl et al. 2011).

In addition to calcium, ROS are also important for the remodeling of mitochondrial architecture, probably by acting on some of the proteins responsible for the mitochondrial dynamics. In contrast to calcium, no much information is available about this topic in the nervous system.

Reactive Oxygen Species and Mitochondrial Dynamics

ROS are reactive metabolites of oxygen that can be radicals, such as superoxide anion and hydroxyl anion, or no-radicals, including hydrogen peroxide. All of them have a pivotal role in physiological and pathological processes. There are different ROS sources in the cell: xanthine oxidase, lipooxigenase, cyclooxygenase, and NADPH oxidase (NOX), among others (Nayernia et al. 2014, Phaniendra et al. 2015). Mitochondria also generate ROS, mainly as a byproduct of respiration. In all cases, ROS contribute to the redox signaling in the cell (Murphy 2009). Conventionally, mitochondrial complex I (NADH Coenzyme Q Oxidoreductase) and complex III (Ubiquinol-Cytochrome c reductase) are the major contributors of ROS production, but other enzymes in mitochondrial matrix have also been reported as noteworthy ROS producers (Andreyev et al. 2015; Angelova and Abramov 2016).

Experimental evidence shows that the redox signaling is important for the mitochondrial dynamics in several cell types and that the levels of ROS are closely related to the function of the proteins involved in fission or fusion. There is evidence relating the oxidative microenvironment to the modification of these proteins, as well as to the regulation of the mitochondrial dynamics (Mailloux et al. 2013). Thus, alterations in the ROS levels lead to deficiencies in the regulation of mitochondrial morphology and function (Willems

Table 1 Role of the core of proteins involved in mitochondrial dynamics in neuropathologies

Protein	Pathology	Action	References	Model
Drp1	Traumatic brain injury (TBI)	↑ fission	Fischer et al. (2016)	CCI of adult Sprague–Dawley rats
			Wu et al. (2018)	TBI in adult male ICR mice
	Amyotrophic lateral sclerosis (ALS)	↑ fission	Altanbyek et al. (2016)	Elav-gal4, Mhc-gal4, and D42-gal4 Drosophila line
			Joshi et al. (2017)	NSC34 cells stably expressing WT or G93A hSOD1
	Huntington's disease (HD)	↓ fusion	Song et al. (2011)	Neurons and fibroblasts of HD mice
			Shirendeb et al. (2012)	Mutant Htt expression in generated BACHD mouse
	Alzheimer's disease (AD)	↓ fusion	Kandimalla and Reddy (2016)	Drp1 heterozygote knockout mice and APP mice
			Kuruva et al. (2017)	AD neurons treated with DDQ
	Parkinson's disease (PD)	↑ Fission	Filichia et al. (2016)	MPTP administration regimen in C57BL/6 mice
			Ordonez et al. (2017)	Drosophila model of α-synucleinopathy phenotypes
Opa1	Leber's hereditary optic neuropathy (LHON)	↑ fission	Amati-Bonneau et al. (2009)	Eye-specific homozygous OPA1 Drosophila mutant
	Kjer's optic atrophy (KOA)		Schild et al. (2013)	Patients with heterozygous mutation in the OPA1
	Huntington's disease (HD)		Hering et al. (2017)	R6/2 transgenic mice (B6CBATg(HDexon1)62Gpb/1 J)
	Autosomal dominant optic atrophy (ADOA)		Delettre et al. (2000)	Patients (ex vivo) exhibited typical signs of DOA
			Zhang et al. (2017)	Lymphoblastoid cell lines carrying the OPA1 mutation
	Autosomal dominant optic atrophy and deafness (ADOAD)		Liguori et al. (2008)	A family with a unusual phenotype of ADOAD
			Amati-Bonneau et al. (2009)	Eye-specific homozygous OPA1 mice mutant
	Spastic paraplegia (SP)		Yu-Wai-Man and Chinnery (2011)	Blood 28-yo female with early-onset optic atrophy
			Pareyson et al. (2015)	Patients with OPA1 mutations in the North of England
	Leigh syndrome (LS)		Rubegni et al. (2017)	Muscle and skin punch biopsies
Mnf2	Alzheimer's disease (AD)	↓ fusion	Martín-Maestro et al. (2017)	Cell cultures of fibroblast cell lines from SAD
			Manczak et al. (2018)	Amyloid beta precursor protein mice (Tg2576 mice)
	Charcot-Marie-Tooth disease (CMT)		Azzedine et al. (2012)	Patients with MFN2 mutations and sensorineural hearing loss.
			Dankwa et al. (2018)	Blood samples of 6 family members - from a large CMT2 family
	Parkinson's disease (PD)		Gautier et al. (2016)	Human fibroblasts obtained from skin biopsies (PD patients)

et al. 2015). In spite of all this information, in the majority of the cases, the mechanism of this regulation is still unknown.

The mitochondrial fusion in HeLa cells and MEFs depends on the cellular oxidizing conditions. In these cells, oxidized glutathione (GSSH) stimulates this process. Furthermore, cysteine 684 seems to be important for the Mfn2 oligomer formation, since the mutant C684A resulted in a loss of GSSG-mediated oligomers disturbing mitochondrial network (Shutt et al. 2012). It is possible that local ROS production in mitochondria could be responsible for the modulation of the activity and/or expression of mitochondrial dynamic proteins. In other non-neuronal cell lines, it was found that ROMO1 (Reactive Oxygen Species Modulator 1), a mitochondrial key protein involved in the regulation

of ROS and cell death (Lee et al. 2010; Kim et al. 2010a, b), modulates the cristae morphology and the mitochondrial fusion. ROS regulate ROMO1 activity by the control of the redox sensitive cysteines, Cys15 and Cys79. Additionally, ROMO1 is essential for Opa1 oligomerization required for maintaining the integrity of cristae junctions and preventing the leakage of cytochrome C (Norton et al. 2014).

ROS as Regulators of Mitochondrial Dynamics in Neurons

In neurons, multiple conditions involved in physiological processes, such as proliferation, neurite outgrowth, differentiation, among others, have been related to oxidant conditions (Le Belle et al. 2011; Olguín-Albuerne and Morán 2015; Piras et al. 2016). In this context, ROS production has also been linked to the mitochondrial form and function in neurons. On the other hand, it has been reported abnormal forms of mitochondria in some models of neuropathological diseases, in which ROS production is involved.

The loss in the fusion and fission balance has been related to oxidative stress in neurons (Knott et al. 2008). Fission is probably the most studied event related to ROS production in neuronal models. In general, an elevation of ROS levels triggers mitochondrial fragmentation. This condition also leads to a modification of Drp1 activity. In cerebellar Purkinje cells, the loss of Drp1 causes neuronal damage, probably because mitochondrial division is necessary for their distribution in dendrites during neurite extension. In this regard, antioxidant treatment prevents mitochondrial morphological changes and cell death in KO Drp1 neurons, indicating that ROS production is involved in this process, and showing that mitochondrial fission capacity is important to avoid neurodegeneration (Kageyama et al. 2012).

There is evidence suggesting a connection between Drp1 and ROS (Cho et al. 2012). It has been observed that inducing Drp1 phosphorylation causes mitochondrial fission after 30 min, which also generates neuronal death (Zhou et al. 2017). On the other hand, amyloid β protein (Aβ) causes Drp1 phosphorylation mediated by Akt, which generates excessive mitochondrial fragmentation, elevation of ROS levels and subsequently neuronal apoptosis (Kim et al. 2016). In an Alzheimer's disease model, increased mitochondrial ROS levels lead to a shortening of mitochondria and to an increase in Drp1 activation by Ser616 phosphorylation (Cho et al. 2012).

Recently, it was described that in hippocampal neurons treated with Aβ the mitochondria take a granular shape, which is different to the typical spherical shape reported in the literature after an oxidant stimulus. Besides, the granular shape also depends on ROS, but the expression of mitochondrial dynamics proteins was not affected, suggesting a different mechanism from those described until now (Hung et al. 2018). The different stimuli that induce ROS levels have heterogeneous effects on mitochondrial fragmentation, as well as on the neuronal death process, but it is clear that a correlation exists between ROS increase and Drp1 activation, although the details in the mechanism have not been elucidated.

ROS production seems to exert also an effect on the fusion machinery. Specifically, it has been reported an influence of ROS over Mfn2 and Opa1. In cerebellar granule neurons, potassium deprivation and hydrogen peroxide induce mitochondrial fragmentation; however, under these conditions, the expression of Mfn2 reverts the mitochondrial shortening and prevents neuronal death, showing that Mfn2 overexpression promotes mitochondrial and neuronal viability (Jahani-Asl et al. 2007). In the same model of cerebellar neurons, the increase of ROS levels causes Opa1 cleavage at the N-terminal and the residue K301 is removed, leading to protein deactivation; finally, this condition results in mitochondrial fragmentation and dysfunction, as well as apoptosis, suggesting that mitochondrial fusion imbalance can compromise neuronal viability (Gray et al. 2013).

Interestingly, Opa1 deletion during early in vitro neuronal development also causes ROS increment and NRF2 translocation accompanied by a transitory mitochondrial hyperfilamentation, which correlates with the onset of synaptogenesis. Additionally, the lack of Opa1 induces a decrease of the expression of pre- and post-synaptic proteins and a reduction in the number of synapses (Bertholet et al. 2013). These results suggest that mitochondrial dynamics proteins expression related to ROS production is critical for the neuronal development. Consistently, $Opa1^{+/-}$ neurons are more sensitive to oxidative stress, probably because their antioxidant proteins suffer a reduced expression; these cells also show mitochondrial dysfunction, a decrease of oxygen consumption and cell death (Millet et al. 2016). Therefore, it seems that the defective expression of fusion proteins affects the response of cells against oxidant conditions, making them more susceptible to oxidation and subsequently to cell death.

It is clear from the literature that it is not totally understood the role of ROS in the mitochondrial dynamics and that more studies are needed to establish a relationship between these molecules and the expression and activity of Drp1, Opa1, and mitofusins. The main effect of ROS on the shape and function of neuronal mitochondria is depicted in Fig. 2.

Conclusions

Mitochondrial fusion and fission balance is critical to contend with the high energetic demand necessary to maintain the physiological cell functions, particularly in neurons.

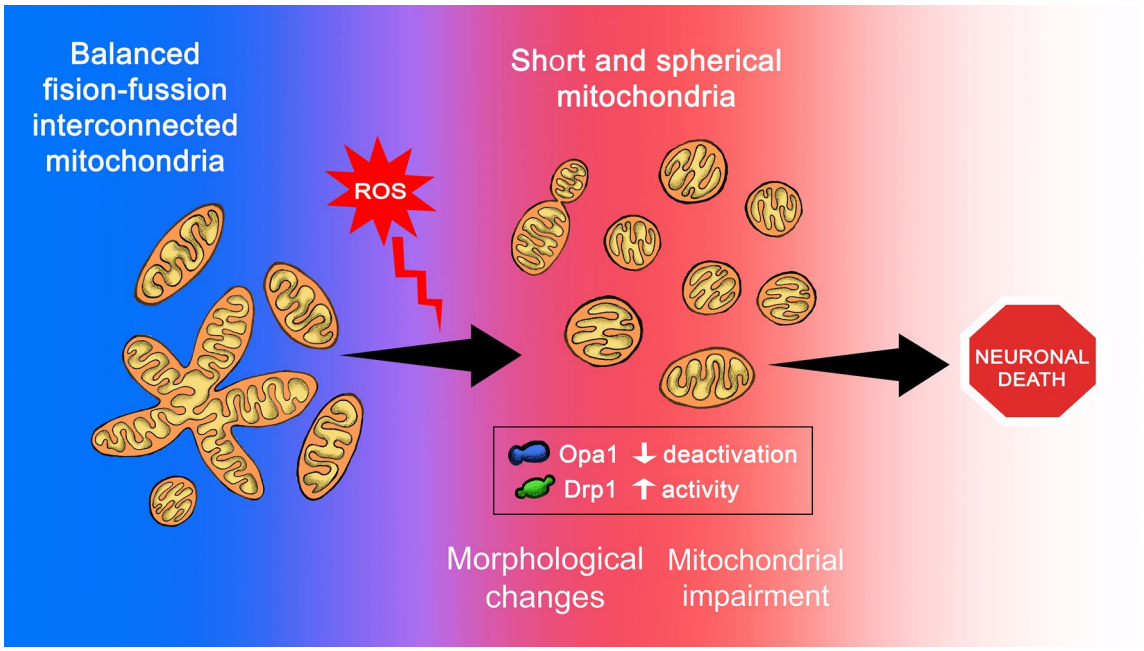

Fig. 2 Consequence of ROS levels on mitochondrial dynamics in neuronal cells. In neurons, mitochondria require a balance between fission and fusion to maintain cell homeostasis. This balance is lost in elevated ROS environments, which induces Drp1 activation, Opa1 deactivation, and mitochondrial fragmentation, leading eventually to neuronal death. Conversely, the reduction or scavenging of ROS by several conditions induces the elongation of mitochondrial network

In this context, most of the studies have been targeted to elucidate the contribution of the mitochondrial dynamics in some neuropathologies. ROS are important mediators in mitochondrial function and cellular health and have been point out as regulators of mitochondrial dynamics in several physiological and pathological processes. It is known that the loss of balance between fusion and fission is related to an increase of ROS production. This condition leads to a loss of mitochondrial membrane potential, a decoupling of the electron transport chain and the fall of ATP concentrations. The cellular ROS levels influence the expression and activity of Drp1, Opa1, and mitofusins, which in turn modulate the neuronal fate.

Future Challenges

The role of ROS in the regulation of mitochondrial dynamics is critical for several neurodegenerative disorders. One of the earliest signals in the pathophysiological process of neurodegeneration is an imbalance of ROS. It is therefore important to investigate the temporary course of ROS changes in relation to the proteins involved in fission or fission, as well as in the molecular pathways that are activated in this process. In addition, it would be particularly interesting to explore more exhaustively the action of ROS in the regulation of the mitochondrial dynamics process through redox modifications of specific amino acids. Thus, strategies to modify both the ROS production and abnormal mitochondrial dynamics may be an attractive therapeutic target for the treatment of neurodegenerative diseases. In this context, more studies are needed to understand the mechanisms responsible for the regulation of mitochondrial fission and fusion in pathological conditions. Progress exists in screening, identifying, and developing molecules as target therapies to reduce mitochondrial fission, maintaining mitochondrial fusion and cell survival; however, more information about neuronal physiological roles of mitochondrial dynamics is needed.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interests.

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Research Article

Differential ROS-Mediated Phosphorylation of Drp1 in Mitochondrial Fragmentation Induced by Distinct Cell Death Conditions in Cerebellar Granule Neurons

Carolina Cid-Castro and Julio Morán

División de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal, 70-253 Ciudad de México, Mexico

Correspondence should be addressed to Julio Morán; jmoran@ifc.unam.mx

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Reactive oxygen species (ROS) production has been associated with neuronal death. ROS are also involved in mitochondrial fission, which is mediated by Dynamin-related protein 1 (Drp1). The regulation of mitochondrial fragmentation mediated by Drp1 and its relationship to mitochondrial ROS (mtROS) in neuronal death have not been completely clarified. The aim of this study is to evaluate the role of mtROS in cell death and their involvement in the activation of Drp1 and mitochondrial fission in a model of cell death of cultured cerebellar granule neurons (CGN). Neuronal death of CGN induced by potassium deprivation (K5) and staurosporine (ST) triggers mitochondrial ROS production and mitochondrial fragmentation. K5 condition evoked an increase of Drp1 phosphorylation at Ser616, but ST treatment led to a decrease of Drp1 phosphorylation. Moreover, the death of CGN induced by both K5 and ST was markedly reduced in the presence of MitoTEMPO; however, mitochondrial morphology was not recovered. Here, we show that the mitochondria are the initial source of ROS involved in the neuronal death of CGN and that mitochondrial fragmentation is a common event in cell death; however, this process is not mediated by Drp1 phosphorylation at Ser616.

1. Introduction

Neuronal apoptotic death can be identified by multiple biochemical features [1–3] that involves the activation of several signaling pathways [3–6]. In addition to the classical biochemical changes, an elevation of ROS levels responsible for cell death is frequently reported [7–11]. The main sources of ROS implied in cell death are the mitochondria and the NADPH oxidases (NOX). In the first case, the elevation of ROS levels is caused by an impairment of the mitochondrial function and is mainly produced by complex I [12–15]. Depending on the cell death conditions, ROS are produced by the activation of different NOX homologues [5, 16, 17].

Additionally, the high levels of ROS observed during neuronal death have been associated with morphological changes of mitochondria [18, 19]. These alterations have been linked to a process known as mitochondrial dynamics that refers to a highly coordinated event responsible for the fusion and fission of the mitochondria [20–23]. This process is orchestrated by a family of GTPases called mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy 1 (Opa1) that are responsible for the fusion of the inner mitochondrial membranes. Other proteins, including Dynamin-related protein 1 (Drp1), are in charge of the scission of the outer and inner membranes [24–28]. An impairment in the expression or function of these proteins has been associated with pathologies of the nervous system such as Parkinson's disease, autosomal dominant optic atrophy, Charcot-Marie-Tooth disease, Leigh syndrome, and amyotrophic lateral sclerosis, among others [29–33].

Drp1 activation has been related to excessive mitochondrial fragmentation during neuronal death [19, 34–36]. The process involves the phosphorylation of Ser616, which generates the translocation of Drp1 from the cytoplasm to the

outer mitochondrial membrane resulting in the initiation of the shortening of the mitochondria. Mitochondrial fission is usually related to mitochondrial dysfunction and increased production of mitochondrial ROS [37–43]. Since mitochondria are one of the main sources of ROS and the pivot organelle of apoptotic death, the regulation of its fragmentation mediated by Drp1 and its relationship to mitochondrial ROS have been implicated to apoptotic neuronal death, but their association is not still completely elucidated.

In previous studies, it has been shown that cerebellar granule neurons (CGN) must be cultured under depolarizing conditions to survive, which can be attained by maintaining neurons in high potassium (25 mM, K25). Under these conditions, treatment of CGN with staurosporine (ST) or potassium deprivation (K5) induces an early NOX-mediated production of ROS, activation of JNK and p38 signaling pathways, and apoptotic death [3, 8, 11], but no information is available on the role of mitochondrial ROS in the cell death and their involvement in the activation of Drp1 and mitochondrial fission. In the present study, we, therefore, assessed the effect of two cell death conditions, K5 and ST, on the mitochondrial (mtROS) and cytoplasmic ROS (ctROS) production, as well as their participation in the Drp1 activation and mitochondrial morphology.

Here, we found that K5 and ST induced an early increase in mtROS and a decrease in mitochondrial length, as well as a rise of Drp1 phosphorylation at Ser616 for K5, but a reduction for ST. A mitochondrial antioxidant inhibited cell death and the phosphorylation of Drp1 induced by K5, suggesting that mtROS play a role in CGN death. Although mitochondrial fragmentation is a common process in neuronal death of CGN, Drp1 phosphorylation at Ser616 seems not to be involved in this process. These findings place mtROS as key regulators of neuronal death in a manner independent of mitochondrial fragmentation.

2. Materials and Methods

Fetal calf serum, penicillin/streptomycin, and basal Eagle's medium were purchased from GIBCO, Invitrogen (Carlsbad, CA, USA). Dihydroethidium (DHE), MitoTracker green, and MitoTracker red CMH_2XRos were purchased from Molecular Probes, Invitrogen (Carlsbad, CA, USA). Poly-l-lysine, trypsin, trypsin inhibitor, DNAse, cytosine arabinoside, DMSO (dimethyl sulfoxide), staurosporine, MitoTEMPO, and reagents for polyacrylamide gel electrophoresis (PAGE) were acquired from Sigma (St. Louis, MO, USA). Protease inhibitor cocktail tablets (Complete) were purchased from Roche (Mannheim, Germany), and phosphatase inhibitor minitablets were obtained from Thermo Scientific (Rockford, USA). ProSieve Quad Color Protein Marker was purchased from Lonza (Rockland, Maine, USA). Polyvinylidene fluoride (PVDF) membranes and Immobilon Western HRP substrate were acquired from Millipore (Concord Road, Billerica, MA, USA). Antibodies against Drp1, Drp1 (Ser616), and GAPDH were from Cell Signaling Technology (Danvers, MA, USA); peroxidase-conjugated anti-mouse was purchased from Jackson ImmunoResearch (West Grove, PA, USA).

2.1. Cell Culture. All animals used for the experimentation described in the present study were treated by the accepted standards of animal care and with the procedures approved by the local Committee of Research and Ethics of the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México (protocol number: JMA120-17). The protocol used followed the Guidelines for the Care and Use of Mammals in Neuroscience as well as guidelines released by the Mexican Institutes of Health Research and the National Institutes of Health guide for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Cerebellar granule neuron (CGN) cultures were prepared as previously described [44]. Briefly, cell suspensions dissociated from 8-day-old Wistar rat cerebellum were plated at a density of 265×10^3 cells/cm^2 in plastic dishes coated previously with poly-l-lysine (5 μg/mL). The culture medium contained basal Eagle's medium supplemented with 10% (*v*/*v*) heat-inactivated fetal calf serum, 2 mM glutamine, 25 mM KCl, 50 ?g/mL streptomycin, and 50 U/mL penicillin. The medium described previously is referred in the text as K25. Cytosine arabinoside (10 ?M) was added 24 h after seeding to prevent the proliferation of nonneuronal cells. The cultures were kept at 37°C in an atmosphere of CO_2 (5%) and saturated air with water vapor (95%). Cultures were maintained 7 days in vitro (DIV) in the depolarizing medium (K25), and cell death was induced by two different protocols: (1) the neurons were transferred to a serum-free medium containing 5 mM KCl (referred as K5 or potassium deprivation) or (2) cultures were added with 0.5 ?M of ST.

2.2. Determination of Cytoplasmic ROS Levels. CGN were cultured in K25 medium during 7 DIV and then treated with K5 medium or ST as previously described. After the indicated times, the CGN were incubated with 3.2 ?M of DHE for 30 min at 37°C and cells were observed in an epifluorescence microscope with a rhodamine filter. Cells were photographed, and fluorescence intensity was measured with the ImageJ platform.

2.3. Determination of Mitochondrial ROS Levels. CGN were cultured in 35 mm Petri dishes during 7 DIV, and cells were preincubated for 30 min with MitoTracker red CMH_2XRos (100 nM) at 37°C. Cells were then subjected to the cell death conditions for the indicated times, and pictures were collected by a LSM 710-Zeiss microscope at 740/599 nm excitation/emission, with a 63x immersion objective. Fluorescence intensity was measured with the Fiji ImageJ platform.

2.4. Mitochondrial Imaging. CGN were cultured on cover glass (FluoroDish™) in 35 mm Petri dishes, and after 7 DIV, the cells were treated with K5 medium or ST during the indicated times. Cells were then incubated with MitoTracker green (100 nM) for 30 min at 37°C, and cells were then washed twice with Locke medium (154 mM NaCl, 25 or 5 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, and 10 mM HEPES) and imaged with Eclipse-Ti-S Nikon by using a 63x oil objective with a fluorescein filter. The mitochondrial length was measured by using the Fiji

ImageJ platform by selecting 20 individual mitochondria per image. After calibrating the images with the objective 60x of the Eclipse-Ti-S Nikon microscope, we draw a line over the individual mitochondria and we measured the mitochondrial length.

2.5. Western Blot. CGN were cultured in a K25 medium for 7 DIV and then switched to K5 medium or treated with ST at different times. Cells were washed twice in ice-cold PBS and were homogenized in lysis buffer (25 mM Trizma, 50 mM NaCl, 2% Igepal, 0.2% SDS and complete protease inhibitors, pH 7.4). Homogenates were centrifuged at 4,500 rpm for 5 min, and the supernatants were recovered. The protein concentration of homogenates was estimated by the Lowry method. Cell homogenates (30 ?g protein per lane) were subjected to 10% SDS-PAGE and transferred to PVDF membranes at 100V for 1.5 h. The membranes were blocked with Tris-buffered saline (TBS)/Tween 20 (TTBS) buffer (100mM Tris-HCl, 150 mM NaCl, and 0.1% Tween, pH 7.4) containing 5% or 2.5% nonfat dry milk at 4°C per one hour and were incubated overnight at 4°C with the primary antibodies. After washing, the blots were incubated with peroxidase-conjugated anti-mouse (1 : 10,000) or peroxidase-conjugated anti-rabbit (1 : 10,000) for 1 h at room temperature. Bands were visualized using chemiluminescence according to the manufacturer's recommendations and exposed to Kodak BioMax-Light Film.

2.6. Viability. We evaluated cell viability by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction technique, which is based on the ability of mitochondrial succinate dehydrogenase to transform MTT to formazan blue. The amount of formazan produced is directly proportional to the number of viable cells present in the culture. The cells were incubated with MTT (100?M) for 15min at 37°C at the indicated times. Cells were then washed, and formazan blue crystals formed were dissolved with DMSO and measured in the spectrophotometer at 570nm.

2.7. Statistical Analysis. Data are presented as mean ± SE, and the statistical significance of the results was determined by one-way analysis of variance (ANOVA), followed by Fisher's test. p values less than 0.05 were considered statistically significant.

3. Results

3.1. K5 and ST Induce an Elevation of ROS Levels. In order to determine whether K5 or ST generated changes in ROS levels, we assessed a temporary course of mtROS and ctROS levels. First, we evaluated the effect of K5 and ST (0.5?M) on mtROS and we observed a ROS increase after 10min in both death conditions. Remarkably, K5 condition induced more than a twofold elevation in the levels of ROS (Figure 1(a)), while ST showed a significant increase in mtROS by about 65% (Figure 1(a)).

Regarding ctROS levels, we found, in a temporal course measurement, that both K5 and ST induced a significant increase after 45 min and 5 h, but not at 15 min (Figure 1(b)). These data indicate that the mitochondria are the first source of ROS during the apoptotic process and a subsequent increase of ROS levels occurs in the cytosol, as previously demonstrated [45].

3.2. Elevation of ROS Correlates with Loss of Viability in Neurons Treated with K5 and ST. Because ROS occurs at different times of neuronal death in both models, it is important to know whether ROS elevation correlates with the loss of viability. For this, we evaluated the ability of neurons to reduce MTT as a viability indicator, over a period of 15 min to 8 h of K5 and ST treatment. Under these conditions, we observed a decrease in MTT reduction of 23% after 30 min that continues decreasing for 8 h of K5 treatment (Figure 2(a)). Similarly, in neurons treated with ST (Figure 2(b)), the reduction in MTT decreased by 28% after 15 min of treatment that continued decreasing after 8 h of treatment. These results suggest that the viability is compromised from the first minutes of the process of cell death induced by both stimuli and that the initial loss of viability correlates with the early mtROS production and with the progressive rise of ctROS.

3.3. mtROS Are Involved in the Neuronal Death Induced by K5 and ST. To evaluate the contribution of mtROS in cell death of CGN, cultures were treated with MitoTEMPO and we measured cell viability in cells treated with K5 or ST. After 24 h, K5 and ST treatment reduced the neuronal viability to 58% and 45.45%, respectively. When cells were pretreated with MitoTEMPO for 30 min, the observed decrease in cell viability was prevented to 80.11% (Figure 3(a)) and 64.87% (Figure 3(b)), respectively. These results suggest that mtROS production is a critical early signal in neuronal death.

3.4. Cell Death Conditions Induce Changes in Mitochondrial Morphology. In numerous models of cell death, mitochondrial morphological changes have been reported. These changes are characterized by swelling, rounding, and shortening of mitochondria that has been identified as mitochondrial fragmentation [46–48]. Under our conditions, we observed that CGN subjected to K5 showed mitochondrial morphological changes at 8 h (Figure 4(a)). These neurons showed rounded and shorter mitochondria when compared to those observed in control conditions (K25); the average length of mitochondria was reduced by 18% at 8 h and 25% after 24 h of K5 treatment (Figure 4(b)). In the case of ST treatment, rounded mitochondria were observed starting at 8 h and a significant mitochondrial shortening of 11.86% was observed after 24 h of treatment (Figure 4(b)). When we quantified the number of mitochondria, we did not observe any difference between the evaluated conditions (not shown). These results demonstrate that a decrease in mitochondrial length is a common event during neuronal death induced by different apoptotic stimuli.

3.5. K5 Induces mtROS-Dependent Drp1 Phosphorylation. In order to clarify the role of potassium deprivation in the process of mitochondrial fission, we evaluated the activation of Drp1 measured as Drp1 phosphorylation at Ser616. We carried out a temporal course of K5 treatment, and we observed a rise in Drp1 phosphorylation after 15 min of stimulation,

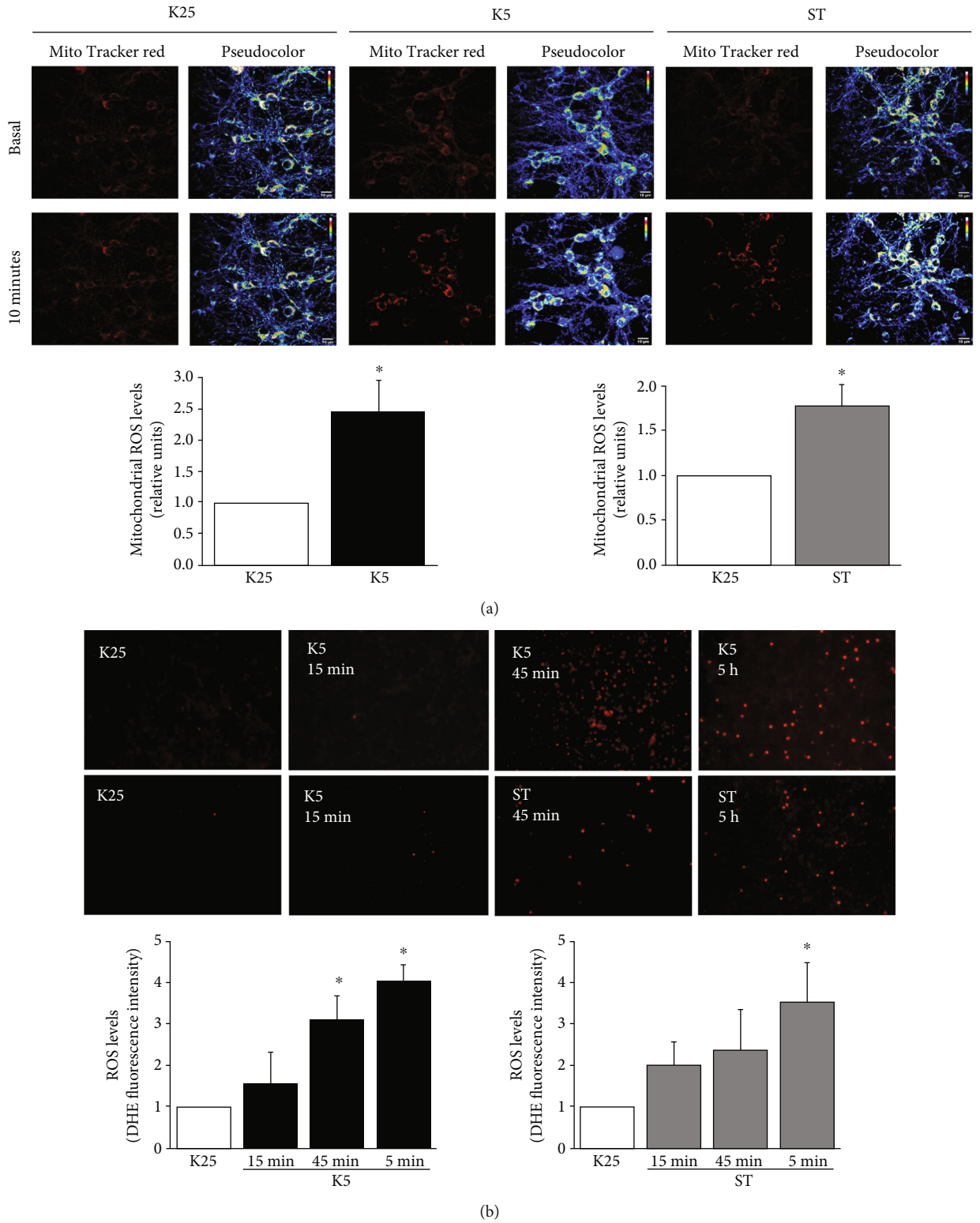

Figure 1: ROS levels induced by cell death conditions. The levels of mitochondrial and cytoplasmic ROS were measured at different times after potassium deprivation (K5) or staurosporine (ST) treatment. (a) CGN stained with MitoTracker red were imaged after 10 minutes to determine the mtROS levels under control (K25), K5, and ST treatments. The graphs show mitochondrial ROS production measured as indicated in Materials and Methods. (b) Cytoplasmic ROS were determined with DHE staining under control conditions (K25) or after 45 min and 5 h of K5 and ST treatment. The graphs show cytoplasmic ROS production measured as indicated in Materials and Methods. Bars are the means ± SE of three independent experiments. $^{*}p < 0.05$ vs. K25.

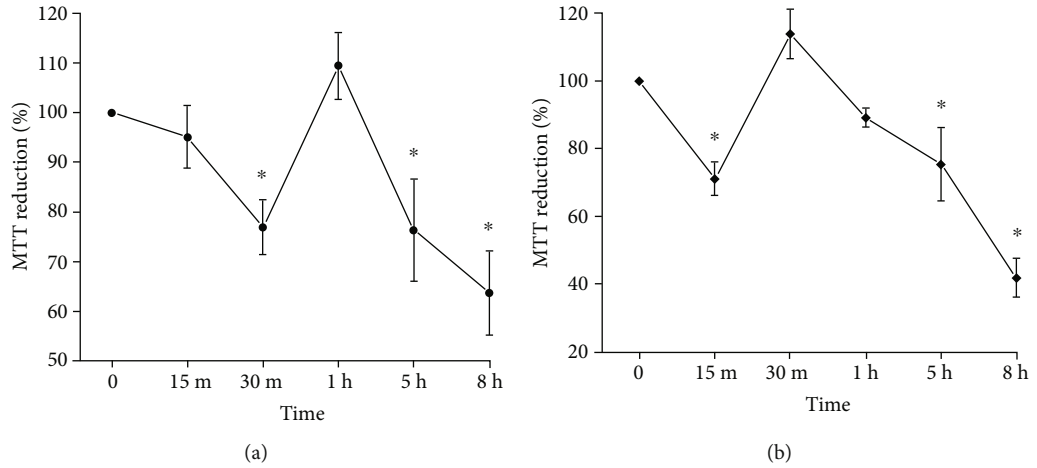

Figure 2: Time course of MTT reduction of CGN treated with K5 and ST. Cell viability was evaluated by MTT reduction after different times in cell death conditions. (a) Temporal course of the viability of CGN treated with K5 (●). (b) Viability of CGN treated with ST (✦). Symbols ● and ✦ show the mean ± SE of the percentage of viability compared with time 0 (K25) of three independent experiments. $^{*}p < 0.05$ vs. time 0 (K25).

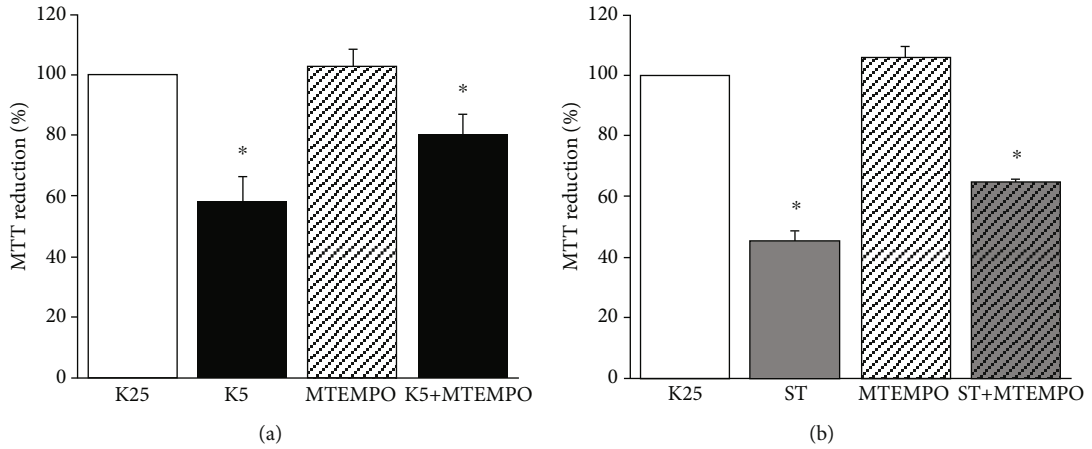

Figure 3: Role of mtROS in the viability of CGN treated with K5 and ST. Cell viability was evaluated by MTT reduction after 24 h of death induction in cells pretreated for 30 min with the mitochondrial antioxidant MitoTEMPO (10 μM). (a) Viability of CGN treated with K5. (b) Viability of CGN treated with ST. Bars show the mean ± SE of the percentage of viability compared with the control (K25) of three independent experiments. $^{*}p < 0.05$ vs. K25.

which remained constant for 8 h of treatment. After 24 h, Drp1 phosphorylation decreased (Figure 5(a)). As mentioned above, mtROS elevation was detected early during the cell death process (Figure 1(a)); thus, we evaluated the effect of the mitochondrial antioxidant MitoTEMPO on Drp1 phosphorylation in a temporal course. Data showed that MitoTEMPO inhibited the Drp1 phosphorylation induced by K5 from 15 min to 24 h of treatment (Figure 5(b)). These data suggest that mtROS are required for Drp1 activation, evidenced as Ser616 phosphorylation, during potassium deprivation.

3.6. ST Decreases Drp1 Phosphorylation Levels. Interestingly, when we explored the effect of ST on Drp1 phosphorylation in a time course assay, we found an early decrease in the phosphorylated form of Drp1 starting at 1 hour and further reducing after 5, 8, and 24 h (Figure 5(c)). The observed decrease in phosphorylation was not modified by treatment with MitoTEMPO (Figure 5(d)). These data suggest that mitochondrial fragmentation induced by ST treatment (Figure 5(c)) is not mediated by either Drp1 Ser616 phosphorylation, which is unrelated to mtROS production.

3.7. Mitochondrial Fission Induced by Cell Death Is Not Prevented by Treatment with a Mitochondrial Antioxidant. Since mtROS mediated the phosphorylation of Drp1 (Ser616) induced by potassium deprivation, we examined whether MitoTEMPO affected the observed effect of K5 on mitochondrial morphology; however, we did not observe any effect of MitoTEMPO on the decrease in mitochondrial length induced by K5 at 24 h (Figure 6(a)). Similar results were obtained for ST (Figure 6(b)). MitoTEMPO alone did not exert any effect on mitochondrial length (Figures 6(a) and 6(b)). Additionally, when cultures were incubated with

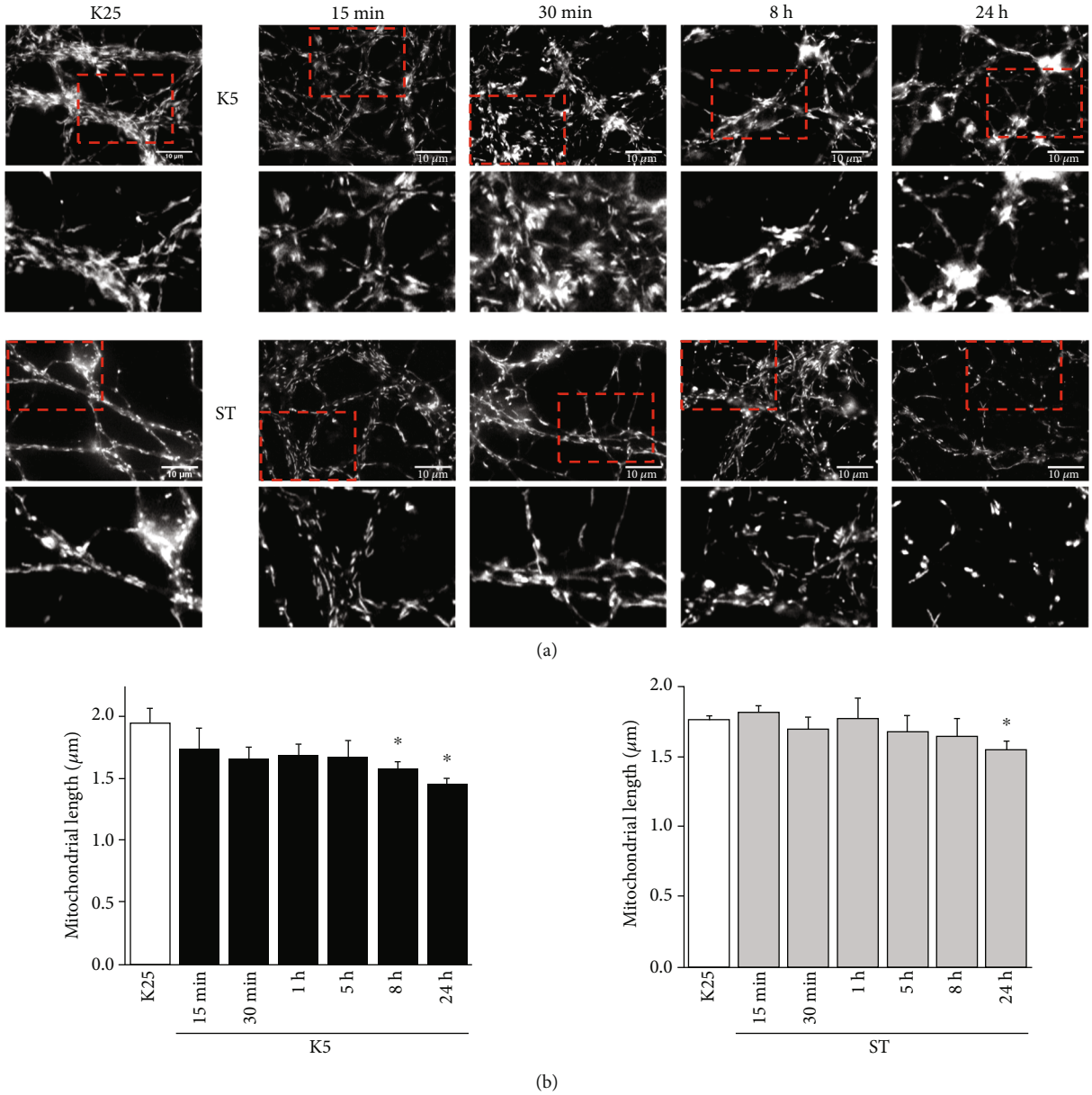

Figure 4: Morphological changes in mitochondria of CGN after cell death induction. Temporal course of CGN stained with MitoTracker green and treated with K5 and ST. (a) Image of mitochondrial morphology of CGN after potassium deprivation or ST at different times. The red arrows indicate the interconnected mitochondria in K25 condition and fragmented mitochondria after 8 and 24 h of treatment. (b) The graphs show the mitochondrial length in a temporal course during cell death. The bars represent the mean ± SE of three individual experiments. $^{*}p < 0.05$ vs. K25.

10 μM MDiVi-1, an inhibitor of Drp1, the cell death of CGN induced by K5 or ST was not reduced (Suppl. Fig. 1).

4. Discussion

One of the major findings in this study was the observation of an early increase of mtROS production in response to two different cell death conditions: K5 and ST (Figure 1(a)). Interestingly, the observation that mitochondrial ROS scavenging ameliorated neuronal viability under K5 and ST treatments indicates that the observed increase in mtROS is an event that contributes to neuronal death. This proposal is supported by previous studies in other experimental models where antioxidants improved mitochondrial function [49, 50]. The protective effect of MitoTEMPO on cell viability in both models was partial, suggesting that other sources of ROS are involved in the cell death process.

Mitochondria is a hub in many physiological functions and one of the main sources of ROS in the cell. There is a large body of evidence showing the role of mitochondrial ROS (mtROS) in the regulation of many physiological processes [51]. For example, mtROS are involved in neuronal differentiation [52] and cell proliferation [53]. Some of the mtROS actions are mediated by the regulation of calcium

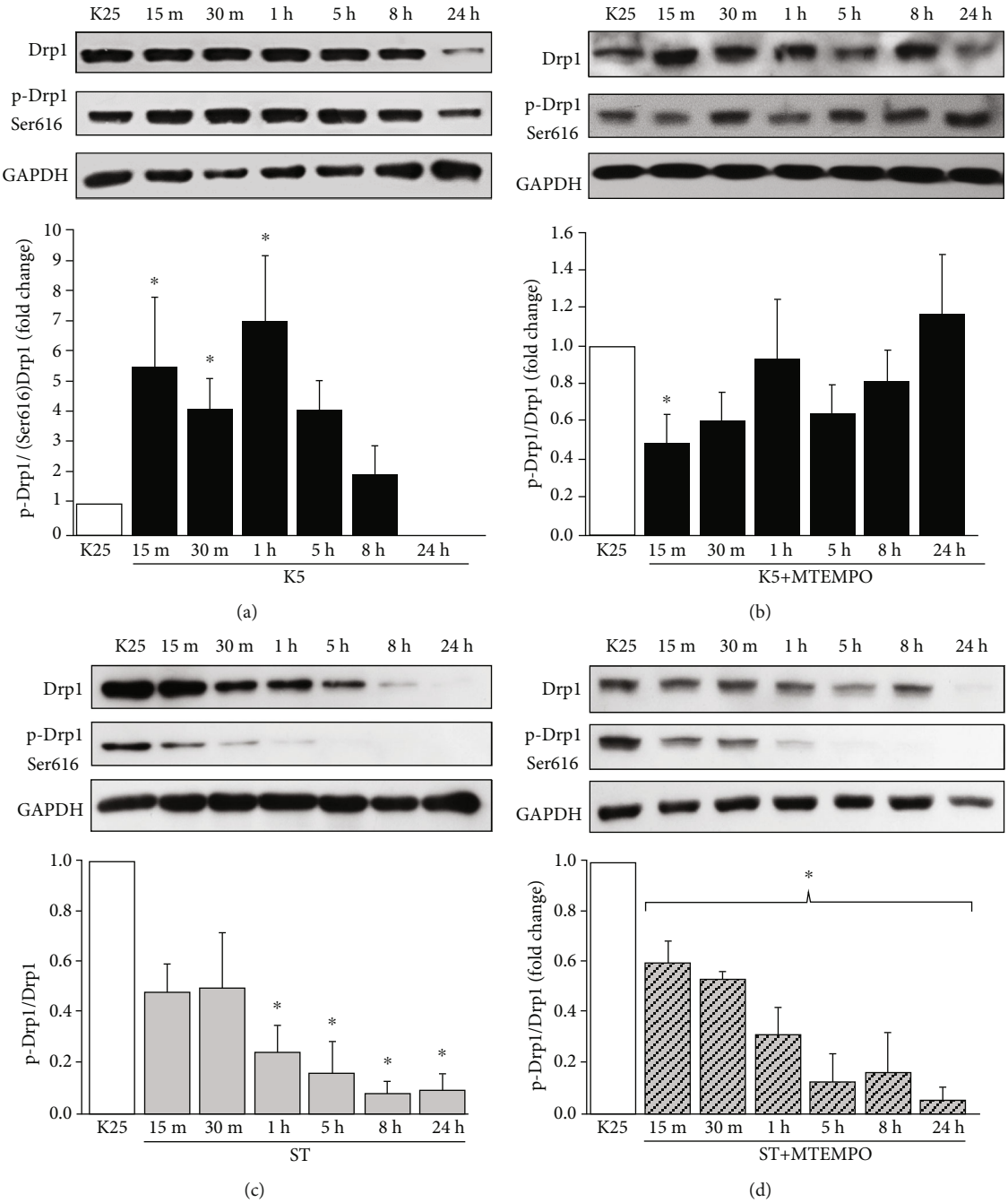

Figure 5: Effect of mtROS in the activation of Drp1 induced by K5 and ST. The levels of total Drp1 and Drp1 phosphorylated at Ser616 (p-Drp1) were evaluated in a temporal course in lysates of CGN pretreated for 30 min with the mitochondrial antioxidant MitoTEMPO under death conditions. The levels of the protein were determined by Western blot analysis as indicated in Materials and Methods. (a) Levels of p-Drp1 (Ser616) from CGN treated with K5. (b) Levels of p-Drp1 from CGN pretreated with MitoTEMPO and treated with K5. (c) Levels of p-Drp1 (Ser616) from CGN treated with ST. (d) Levels of p-Drp1 from CGN pretreated with MitoTEMPO and treated with ST. GAPDH was used as loading control. The bars show the densitometric ratio between p-Drp1 and Drp1 that were normalized to the control K25. Values are the mean ± SE of three individual experiments. $^*p < 0.05$ vs. K25.

transport into the cell and intracellular stores [54, 55]. Accordingly, deregulation of mtROS can lead to pathological conditions. It is known that the release of mtROS by mitochondrial permeability transition pore opening is a crucial step in the pathogenesis of diverse diseases [56–58]. Particularly, alterations in mtROS have been related to several neurodegenerative diseases [59]. For example, mtROS have been associated with an alteration of the long-term potentiation in an Alzheimer's disease (AD) model [60] and the use of mitochondrial antioxidants prevents the expression of the characteristics of AD in mice [61]. In addition, mitochondrial fission is related to an increased mtROS levels in an AD

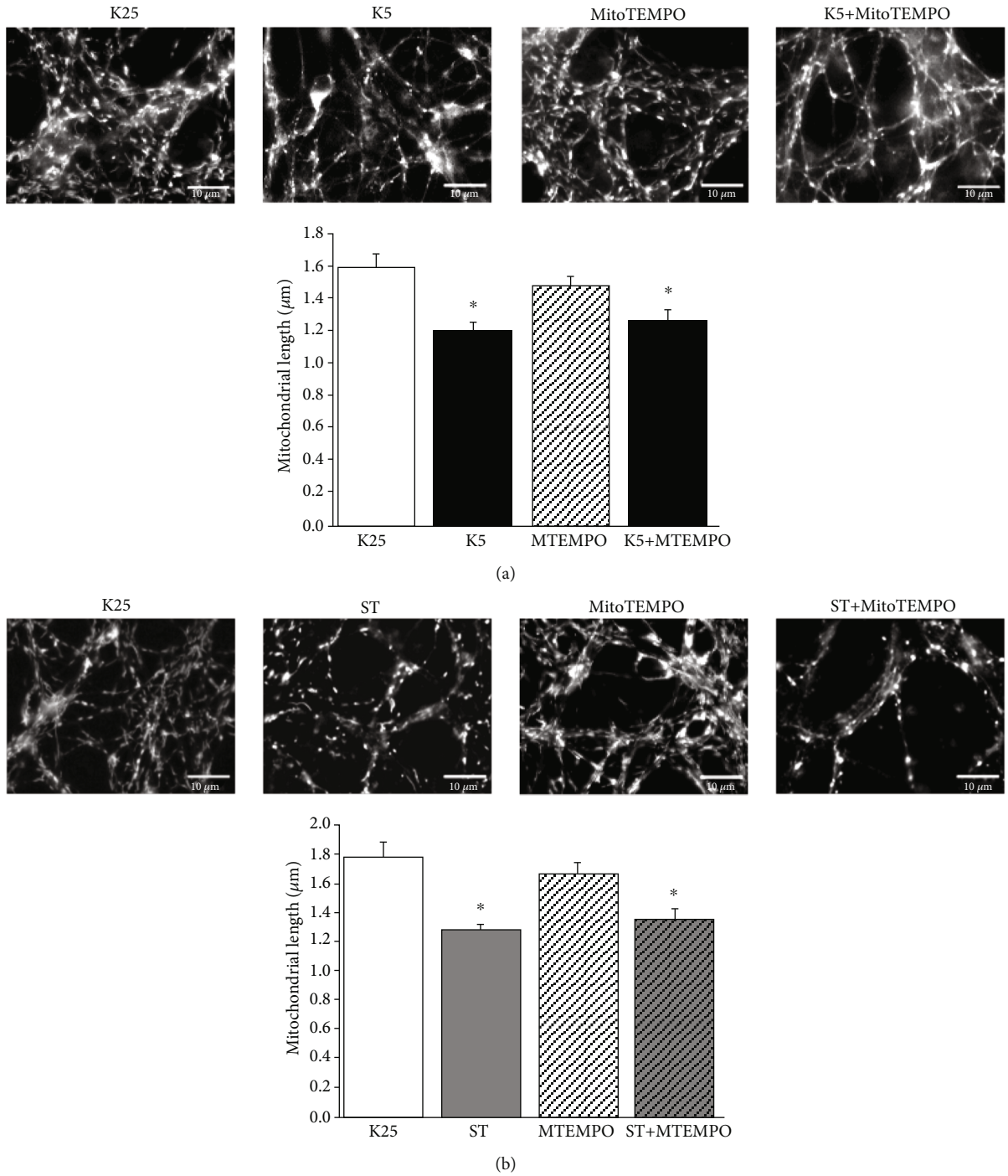

Figure 6: Role of mtROS in the morphological changes of mitochondria in CGN treated with cell death conditions. CGN stained with MitoTracker green and pretreated with MitoTEMPO (10 μM) were stimulated with K5 or ST, and neurons were imaged after 24 h. (a) Image of mitochondrial morphology of CGN after potassium deprivation. (b) Image of mitochondrial morphology after ST treatment. The bars show the mitochondrial length in μm measured after 24 h of treatment with the death conditions. The bars represent the mean ± SE of three individual experiments. $^*p < 0.05$ vs. K25.

model [39]. Other neuropathologies related to mtROS overproduction includes frontotemporal dementia [62] and Parkinson's disease [63], among others. Thus, mtROS are essential to maintain physiological homeostasis of the cell, but a misbalance can cause serious pathological alterations.

In this regard, our group previously demonstrated, and we corroborated here (Figure 1(b)), that cytoplasmic ROS elevation is a determinant process in neuronal death [3, 6, 64]. The observed increase in ROS levels has been associated with the promotion of apoptosis, but the specific mechanism remains elusive. We and others have shown that NOX is a

crucial ROS source implicated in apoptosis [8, 16, 65]. Here, we showed an early production of both mtROS and ctROS induced by K5 and ST. The initial ROS produced by mitochondria could be related to ctROS produced later by NOX. Previous studies have suggested an interrelationship between mtROS and NOX activation [65], and a feedback mechanism between mtROS and ROS generated by NOX has also been proposed [66].

We and others have demonstrated that an increase of ROS levels is related to the progression of cell death [8, 15, 45, 54]. Thus, we assessed the capacity of neurons to reduce MTT and we observed that K5 and ST induce a reduction in the viability from the first 15-30 min that continued decreasing for 8 h (Figure 2). These results suggest that the neuronal death process is an event triggered from the first minutes of the treatments as it occurs for the increase of ROS levels (Figures 1 and 2). Previously, the early impairment of viability has been reported in CGN under oxidant conditions [55], showing that ROS has a role in the sudden loss of viability and acts as a determinant to the neuronal fate. In our study, we confirmed that two death conditions induced mtROS and ctROS levels and this correlates with a rapid loss of viability.

There are evidence supporting the idea that altering mitochondrial function by K5 and ST is an early episode likely involved in the death of cerebellar granule neurons and that K5 and ST could have different actions in mitochondrial activity. For example, some studies suggest that apoptotic conditions alter the mitochondrial function early in the cell death process. For example, Jekabsons and Nicholls [67] showed a decrease in the oxygen consumption from the first few minutes of potassium deprivation.

A recent study has shown that the suppression of ROS production from both sources was not additive in preventing Aβ toxicity of cultured cortical neurons [68]. This result is in agreement with the observed partial protective effect of MitoTEMPO observed in our model (Figures 2(a) and 2(b)) and supports the idea that other intracellular signals besides mtROS contribute to the neuronal death.

Changes in mitochondrial morphology and their relationship to the process of neuronal death have gained relevance as an essential issue in the progression of neurodegeneration caused by harmful stimuli [39, 69, 70]. Interestingly, and consistent with previous studies, we observed morphological alterations in mitochondria at different times of treatment with both cell death conditions. CGN maintained in basal conditions showed highly connected mitochondria, which after several hours of treatment with K5 or ST became shorter and rounded along neurites (Figure 3), in agreement with previous studies [47, 50].

The impairment of mitochondrial morphology has been related to increased ROS levels in different experimental models [43, 46, 50, 71], including the use of hydrogen peroxide in neuroblastoma cells and cultured hippocampal neurons [50, 70]. Particularly, it has been reported that a reduction in mtROS decreased the mitochondrial fragmentation [50, 72]. Since in our study, the use of a mitochondrial antioxidant ameliorated the loss in cell viability of the neurons (Figure 2), we evaluated the role of mtROS on the mitochondrial morphology under cell death conditions; however, we found that MitoTEMPO did not prevent mitochondrial fragmentation in any of the cell death conditions studied (Figure 6). It remains to evaluate whether ctROS are involved in the morphological changes induced by K5 and ST.

The redox balance has been linked to the regulation of the core of the mitochondrial dynamics regulating proteins [73]. Drp1 is the main protein involved in the regulation of mitochondrial fission [26, 34, 74]. Mitochondrial fragmentation requires the translocation of Drp1 to the outer mitochondrial membrane [75], which involves its phosphorylation at several sites, including Ser616 and Ser637 [76, 77]. To further assess the role of mtROS in mitochondrial fission, we evaluated the activation of Drp1 mediated by its phosphorylation at Ser616 in neurons treated with K5 or ST. In the case of K5, we found an increase in Drp1 phosphorylation that correlated with the observed increase in mtROS and mitochondrial fission (Figure 4(a)), in agreement with previous studies [42, 71]. Moreover, we observed that MitoTEMPO significantly reduced the increase of p-Drp1 induced by K5 (Figure 4(b)), suggesting that Drp1 phosphorylation could be mediated by the mtROS induced by potassium deprivation.

In contrast to K5, although ST induced a rise in mtROS that correlated with decreased mitochondrial length, we did not observe any activation of Drp1 measured as phosphorylation at Ser616. In fact, we observed a marked decrease in both total Drp1 and p-Drp1 by ST (Figure 5(a)). In addition, MitoTEMPO did not modify the decrease in total Drp1 and p-Drp1 induced by ST (Figure 5(b)). Unexpectedly, we observed a decrease in Drp1 total levels from 15 min to 24 h in neurons treated with ST (Figures 5(c) and 5(d)). A decrease in phosphorylated Drp1 levels has been reported to promote mitochondrial elongation in the hippocampus [78]. Thus, these results show that Drp1 and mtROS do not mediate the mitochondrial fission induced by ST. It is possible that the observed Drp1 degradation by ST could be mediated by a mechanism dependent on the proteasome, as it has been observed in other models of neuronal death [79].

Alternatively, other Drp1 phosphorylation sites could be responsible for mitochondrial fission induced by ST, as it has also been suggested during neurodegeneration [80]. Although the most commonly reported phosphorylation of Drp1 is at Ser616 [29, 39, 81], a decrease in phosphorylation at Drp1 Ser637 has also been shown in hippocampal neurons [76, 82, 83]. In addition, the phosphorylation at Ser585 was related to an enhanced mitochondrial fission in CGN in response to excitotoxicity [84]. Thus, we cannot discard other possible sites of Drp1 phosphorylation involved in the process of mitochondrial fission.

A possible explanation for the observed differences in Drp1 phosphorylation by K5 and ST could be the distinct signaling pathways activated by each condition. Drp1-dependent mitochondrial fragmentation is regulated by several kinases, including CDK5 [84], CaMKII [82, 83], ERK1/2, PKC, JNK, and p38 in different models [71, 85, 86]. We have previously reported that both models of apoptotic death showed differences in their molecular mechanisms of action. K5 induces a reduction in cytoplasmic calcium, while ST induces an early increase of calcium [44,

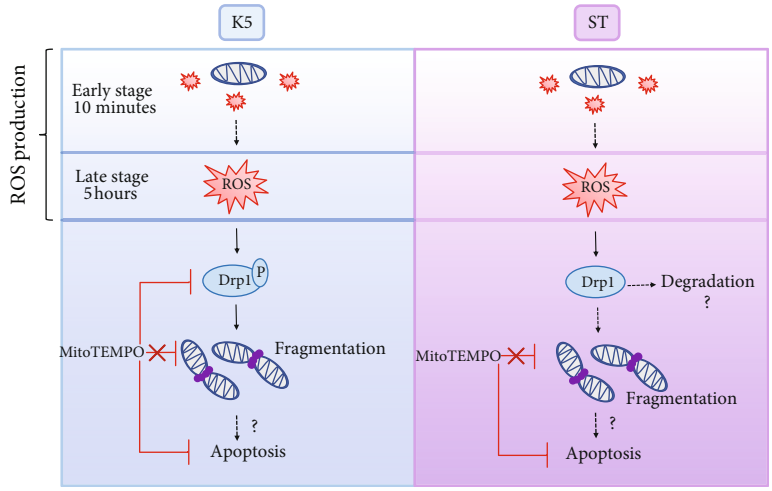

Figure 7: K5 and ST induce mtROS and mitochondrial fragmentation not mediated by Drp1 p-Ser616 during neuronal death. The model proposed for both apoptotic conditions comprises an early stage of ROS produced by mitochondria, which was shown in this study, as well as a late stage of ROS generated by NOX [16, 45]. Based on the finding that MitoTEMPO inhibited Drp1 phosphorylation, we propose that the mtROS are involved in the Ser616 phosphorylation of Drp1. Further, we observed mitochondrial fragmentation and finally neuronal death. However, the inhibition of Drp1 activation by MitoTEMPO did not reduce mitochondrial fragmentation; nevertheless, MitoTEMPO reduced neuronal death. This suggests that mitochondrial fragmentation mediated by Drp1 Ser616 is not involved in CGN apoptotic death. On the other hand, ST treatment (right panel) also induced an increase in both mitochondrial and cytoplasmic ROS levels. Here, we found that ST markedly reduced total Drp1 and Ser616 Drp1 phosphorylation levels and, as with K5, MitoTEMPO did not inhibit the mitochondrial fragmentation, but it prevented the neuronal death. As for K5, we observed that Drp1 phosphorylation at Ser616 is not related to either mitochondrial fragmentation or neuronal death induced by ST. Although Drp1 activation is a determinant of the mitochondrial fission in other models of death, this does not seem to occur in our model, at least through its phosphorylation at Ser616 Drp1, which does not play a role in mitochondrial fragmentation and cell death of CGN. Based on these findings, we propose that there exists an alternative mechanism that regulates the mitochondrial fragmentation in CGN.

87]; K5 evokes the release of K+, while ST produces a Cl- release [88], and although both conditions activate NOX, only ST induces the activation of NOX2 [16]. Finally, we have also highlighted the differential activation of signaling pathways by K5 and ST during apoptotic neuronal death; the effect of K5 was mediated by JNK, while ST required p38 activation [6]. We hypothesize that any of these differences could be responsible for the discrepancies in K5 and ST conditions in the Drp1 phosphorylation.

In the present study, we observed mitochondrial fragmentation in both experimental models. However, in the case of the neurons subjected to K5, the total abolition of the Drp1 phosphorylated levels by MitoTEMPO was not enough to reduce mitochondrial fission. In the ST model, we also observed a significant mitochondrial fragmentation even with very low levels of total and phosphorylated Drp1 at Ser616. It is worth mentioning that we do not observe any effect of the putative Drp1 inhibitor MDiVi-1 on the viability of CGN treated with K5 or ST suggesting that Drp1 could not be critical for cell death of CGN. It should be noted that in other models, including cell reprogramming [89] or cell proliferation in tumor growth [90], the mechanisms and consequences of mitochondrial fission might be different from those of neuronal death.

These results suggest alternative mechanisms to induce mitochondrial fission. It has been recently proposed that actin cytoskeleton modulates mitochondrial morphology changes [91, 92] that are involved in neuronal death [93], but the role of actin-cofilin has not been explored in neurons. Particularly, cofilin seems to participate in the mitochondrial fission and apoptosis through the dephosphorylation of Drp1 at Ser637 [94].

5. Conclusions

In conclusion, our findings suggest that mtROS are necessary for the process of neuronal death, but not for the mitochondrial fragmentation. However, the cell death conditions induce mitochondrial fragmentation in CGN. In addition, mitochondrial fragmentation and neuronal death of CGN seem not to be mediated by Drp1 phosphorylation at Ser616. Our data suggest that mitochondrial fragmentation is carried out by different mechanisms depending on the cell death condition. The details of the suggested mechanism are described in Figure 7.

More experiments are needed to explore the relationship among mtROS, mitochondrial dynamics, and cell death induced by different conditions, as well as the fine mechanisms involved, including the alternative sites of Drp1 phosphorylation. Future experiments may clarify how the different sources of ROS, including NOX, may induce mitochondrial fragmentation and cell death. Furthermore, other core proteins of the mitochondrial dynamic process, like Opa1 and mitofusins, could have a specific role in K5 and ST induction of cell death.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

The animal study was reviewed and approved by the Animal Care and Use Committee of the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México (protocol number JMA120-17).

Disclosure

Carolina Cid-Castro was the recipient of a doctoral fellowship from CONACYT (378149), and this study is part of her Ph.D. thesis in the Doctorado en Ciencias Bioquímicas, Universidad Nacional Autónoma de México.

Conflicts of Interest

All the authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

CC-C carried out the experiments, contributed to the analysis and interpretation of the data, and participated in the conception and design of the study and to the draft of the manuscript. JM conceived the study, participated in its design, and contributed to draft the manuscript. All authors approved the submitted version of the manuscript.

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Supplementary Materials

Supplementary Figure 1 Effect of MDiVi-1 on cell viability of CGN treated with K5 and ST. Cells were cultured for 7 DIV and treated with staurosporine (0.5 μM) (ST) or transferred to a 5 mM KCl-containing medium (K5) in the presence or absence of the Drp1 inhibitor MDiVi-1 (10 μM); the MDiVi-1 was preincubated 30 min before the corresponding treatment. Cell viability was evaluated as MTT transformation after 24 h. Values are *means* ± *SE* of three independent experiments. *Significantly different from control (K25) ($^*p < 0.05$). *(Supplementary Materials)*

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