UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS BIOMÉDICAS

FACULTAD DE MEDICINA

EVALUACIÓN DE LOS EFECTOS DE LA 3-HIDROXIKINURENINA EN EL ESTRIADO DE ROEDORES

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

ANA LAURA COLÍN GONZÁLEZ

TUTORA PRINCIPAL: DRA. ANAHÍ CHAVARRÍA KRAUSER
FACULTAD DE MEDICINA, UNAM.

COMITÉ TUTOR: DRA. VERONICA GUARNER LANS
FACULTAD DE MEDICINA, UNAM.
DR. GABRIEL GUTIÉRREZ OSPINA
INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

MÉXICO, CIUDAD DE MÉXICO, FEBRERO, 2017

La ciencia será siempre una búsqueda, jamás un descubrimiento real. Es un viaje, nunca una llegada.

Karl Popper

Quiero agradecer a todas las maravillosas personas que me han ayudado en este viaje:

- A Rocío González, por ser mi apoyo en los momentos más difíciles, por su paciencia y cariño.
- A Gerardo Colín, por inculcar en mi la semilla del estudio.
- Al Dr. Santamaría por dirigirme, por su amistad y por enseñarme a no rendirme en situaciones difíciles.
- A los dos Albertos que encontré en el camino, por escucharme, por su compañía y apoyo incondicional.
- A la Dra. Chavarría por aceptarme como alumna.
- A Jorge Muños por todas esas pláticas que me hicieron más agradable el recorrido.
- A todos los integrantes del Laboratorio de Aminoácidos Excitadores por entender y aceptar mi locura.
- A la UNAM y al CONACyT por darme la oportunidad de vivir esta experiencia.
- Y finalmente, a todas las piedras que, en su intento por detenerme, me hicieron más fuerte.

Muchas Gracias…

ÍNDICE

1. **RESUMEN**

El triptófano es un aminoácido esencial que participa en múltiples eventos fisiológicos. El triptófano es convertido en nicotinamida adenina dinucleótido (NAD^+) a través de la vía de la kinurenina (VK), durante esta transformación varios metabolitos con actividad biológica son producidos. En enfermedades neurodegenerativas y psiquiátricas el funcionamiento de la vía se altera y se producen niveles incrementados de 3-hidroxikinurenina (3-HK) y ácido quinolínico (QUIN). Los efectos del QUIN en el sistema nervioso central (SNC) han sido ampliamente estudiados mientras que el papel de la 3-HK en estos desórdenes se desconoce. Por un lado, la 3-HK se relaciona con el daño oxidante y la muerte neuronal y por otro, parece ser un antioxidante natural. En este trabajo se realizaron estudios *in vitro* e *in vivo* para caracterizar los efectos tóxicos y/o antioxidantes de la 3-HK en el estriado de roedores.

Los resultados indican que la 3-HK modula el ambiente redox mediante la activación del factor nuclear eritroide-2 (Nrf2, del inglés, ***N****uclear Factor Erythroid* ***2****- **r**elated **f**actor*) y de enzimas antioxidantes, y estos eventos previenen el daño oxidante causado por QUIN, ácido 3-nitropropiónico (3-NP) y $FeSO_4$.

Al no ser comprobada la toxicidad de la 3HK, en este trabajo se incluyeron los estudios realizados en paralelo con el QUIN con el objetivo de proveer información sobre los efectos de este metabolito derivados de la 3-HK y conocer más sobre el papel de la VK en procesos neurodegenerativos. En esta parte, se evaluó el efecto del QUIN sobre la activación del Nrf2 y se investigó el posible efecto sinérgico que puede haber entre esta molécula excitotóxica y los ácidos orgánicos que se acumulan en acidemias metabólicas.

El QUIN estimuló la activación del Nrf2 de forma transitoria como un mecanismo de compensación, y favoreció un patrón tóxico sinérgico con los ácidos orgánicos, sugiriendo que esta molécula altamente tóxica podría ser responsable de las acciones nocivas causadas par alteraciones de la VK. Además, el sinergismo del QUIN con otros metabolitos tóxicos ayuda a comprender los mecanismos por los cuales la neurodegeneración ocurre y, por lo tanto, ayuda a la

identificación de mecanismos útiles para el diseño de estrategias terapéuticas más efectivas.

2. **ABSTRACT**

Tryptophan is an essential amino acid involved in multiple physiological events. It is converted to nicotinamide adenine dinucleotide (NAD^+) through the kynurenine pathway (KP), during this transformation several metabolites with biological activity are produced. In neurodegenerative and psychiatric diseases, the KP function is altered and increased levels of 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN) are observed. QUIN effects in the CNS have been studied extensively while the role of 3-HK in these disorders is unknown. On the one hand, 3-HK is related to oxidative damage and neuronal death, and on the other hand, it appears to be a natural antioxidant. In this work, *in vitro* and *in vivo* studies were performed to characterize the toxic and/or antioxidant effects of 3-HK in the striatum of rodents. The toxicity of the molecule was impossible to prove.

The results indicate that 3-HK modulates the redox environment by activation of Nuclear Factor 2- Erythroid related factor (Nrf2) and antioxidant enzymes, and these events prevent oxidative damage induced by QUIN, 3-nitropropionic acid (3-NP) and $FeSO_4$.

Because the 3-HK toxicity was not proven, studies in parallel with QUIN were performed in this work in order to know more about the effects of this 3-HK metabolite and learn more about the role of KP in neurodegenerative processes. In this part, the effect of QUIN on Nrf2 activation was assessed and the possible synergistic effect of QUIN and organic acids, accumulate in metabolic acidemias, was investigated.

QUIN transiently stimulated the activation of Nrf2 as a compensation mechanism, and favored a synergistic toxic pattern with organic acids, suggesting that this highly toxic molecule may be responsible for the harmful actions of altered KP. Moreover, the QUIN synergism with other toxic metabolites helps to understand the mechanisms by which neurodegeneration occurs. Therefore, it will be useful for designing more effective therapeutic strategies.

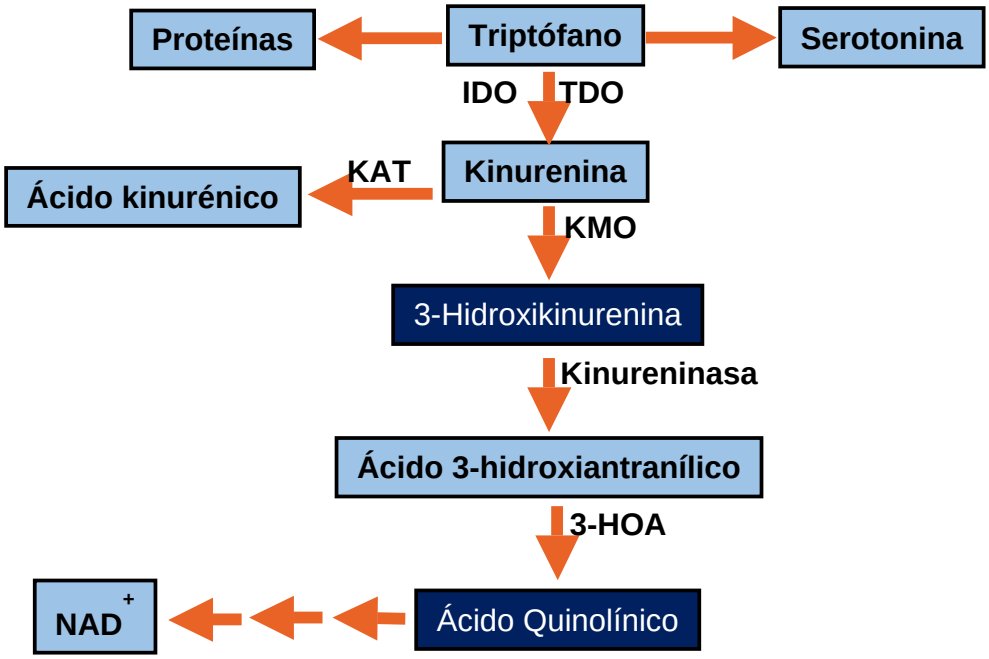
3. **INTRODUCCIÓN**

Las enfermedades neurodegenerativas causan grandes desafíos económicos y sociales a nivel mundial. La investigación de los mecanismos involucrados en los procesos de daño y muerte celular observados en estos desordenes contribuirá al desarrollo de estrategias terapéuticas. Estos padecimientos comparten mecanismos tóxicos conocidos, entre ellos la excitotoxicidad y el estrés oxidante. La excitotoxicidad es un proceso caracterizado por la activación sostenida de receptores de N-metíl-D-aspartato (rNMDA). La sobre-activación de los rNMDA aumenta la concentración de Ca^{2+} intracelular y, posteriormente, desencadena una activación masiva de enzimas que contribuyen a la muerte celular. La activación enzimas pro-oxidantes altera en el balance entre la formación de especies reactivas de oxígeno/nitrógeno (ERO/ERN) y la defensa antioxidante. Como resultado se producen modificaciones celulares en lípidos, ácidos nucleicos y proteínas, lo que compromete la integridad celular (Halliwell, 2006; Dasuri et al., 2013; Niranjan, 2014). Por lo anterior, el estudio de procesos bioquímicos relacionados con estos dos mecanismos ayudará a comprender mejor los procesos de muerte observados en enfermedades neurodegenerativas. Una de las vías metabólicas que se altera en estas condiciones es la vía de la kinurenina (VK).

3.1. LA VÍA DE LA KINURENINA

La VK es la principal ruta metabólica que transforma al triptófano en nicotinamida adenina dinucleótido (NAD^+). Durante esta transformación, varios metabolitos con actividad biológica son producidos. Se han demostrado que alteraciones en el metabolismo de la VK y cambios en los niveles de sus metabolitos pueden participar en la patogénesis de varias enfermedades neurodegenerativas, desórdenes depresivos y esquizofrenia (Schwarcz et al., 2012; Tan et al., 2012; Amaral et al., 2013).

Las enzimas limitantes de la VK son la indolamino-2,3-dioxigenasa (IDO) y la triptófano 2,3-dioxigenasa (TDO), su activación produce la formación de kinurenina a partir de triptófano. La vía tiene dos brazos, en uno de ellos, la kinurenina forma ácido kinurénico mediante una transaminación irreversible catalizada por la kinurenina aminotransferasa (KAT). En el otro brazo, la kinurenina es convertida en 3-hidroxikinurenina (3-HK) por la acción de la kinurenina 3-monooxigenasa (KMO). La 3-HK es transformada por la kinureninasa en ácido 3-hidroxiantranílico y éste es convertido en ácido quinolínico (QUIN) por la enzima 3-hidroxiantranilato oxigenasa (3-HAO). Finalmente, el QUIN es transformado a NAD^+. En la **Figura 1** se muestra una representación simple de la vía.



Algunos metabolitos de la VK con actividad biológica son: el QUIN, una neurotoxina y un agonista glutamatérgico (Schwarcz et al., 1984; Guidetti et al., 2006); el ácido kinurénico, un agente neuroprotector y un antagonista glutamatérgico (Schwarcz et al., 1983; Yu et al., 2004); la 3-HK y el ácido 3-hidroxiantranílico, dos moléculas con actividad redox (Goldstein et al., 2000; Braidy et al., 2009; Colín-González., 2013).

3.2. LA 3-HIDROXIKINURENINA

Los niveles de la 3-HK aumentan en pacientes con enfermedades neurodegenerativas y psiquiátricas. En un principio, este efecto se relacionó con la disfunción y muerte neuronal que se encontró en estos desórdenes. Más tarde, estudios *in vitro* demostraron que la 3-HK también podría actuar como un antioxidante (Colín-González., 2013).

La dualidad de la 3-HK se debe a su naturaleza química, ya que en condiciones fisiológicas, la molécula se auto-oxida formando una *o*-aminoquinona (agente oxidante) y ERO ($O_2^{\cdot -}$, H_2O_2, $^{\cdot}OH$) (Eastman y Guilarte, 1990; Ishii et al., 1992; Okuda et al., 1996; Hiraku et al., 1995; Vazquez et al., 2000). Además, la 3-HK participa en la formación de QUIN, el cual es responsable de causar daño celular mediado principalmente por excitotoxicidad y estrés oxidante (Schwarcz et al., 1984). El efecto deletéreo de la 3-HK se observó en cultivos de neuronas de cerebelo, estriado, hipocampo y en líneas tumorales (Eastman y Guilarte, 1989; Okuda et al., 1998; Jeong et al., 2004; Smith et al., 2009). Actualmente no existen reportes que hayan demostrado su toxicidad *in vivo* (Pinelli et al., 1984; Nakagami et al., 1996; Guidetti y Schwarcz, 1999).

En contraste, la 3-HK es un agente reductor que actúa como antioxidante. Este metabolito puede atrapar $O_2^{\cdot -}$, $^{\cdot}OH$, radicales peroxilo y $^{\cdot}NO$; consecuentemente, la presencia de la 3-HK previene la oxidación y la nitración de diversas biomoléculas (Goshima et al., 1986; Christen et al., 1990; Goda et al., 1999; Leipnitz et al., 2007; Backhaus et al., 2008). Al respecto, los efectos protectores de este metabolito se han demostrado en varios modelos como homogenados de corteza cerebral, células de glioma C6 y en el cristalino donde

aumenta la capacidad antioxidante, inhibe la lipoperoxidación y disminuye las reacciones cruzadas entre proteínas (Luthra y Balasubramanian, 1992; Leipnitz et al., 2007).

Por su parte, la acidemia glutárica tipo I es un desorden autosómico recesivo relacionado con el metabolismo de lisina, hidroxilisina y triptófano. Pacientes con esta enfermedad muestran atrofia cerebral, macrocefalia y degeneración estriatal (Viau et al., 2012). La adición de 3-HK en homogenados de corteza de rata previno la producción de radicales libres producidos por el ácido glutárico, el principal metabolito que se acumula en esta enfermedad (Leipnitz et al., 2007). Todo lo anterior sugiere que la 3-HK actúa como antioxidante en procesos patológicos y en neuronas aisladas genera la producción de especies reactivas. Por lo tanto, el efecto tóxico de la 3-HK reportado en otros trabajos bien podría ser mediado por su conversión en QUIN, una hipótesis que no ha sido demostrada.

3.3. EL ÁCIDO QUINOLÍNICO

En condiciones patológicas, el QUIN es responsable de causar daño celular mediado principalmente por excitotoxicidad y estrés oxidante (Schwarcz et al., 1984; Santamaría et al., 2001; Stone et al., 2003). El QUIN es un agonista de los rNMDA que se une en las subunidades NR2A y NR2B (Schwarcz et al., 2012). La sobreactivación de estos receptores produce el aumento de los niveles de Ca^{2+} intracelular y la activación de numerosas enzimas (proteasas, sintasas de óxido nítrico, fosfolipasas, endonucleasa, etc.) que disparan una secuencia de eventos destructivos asociados con procesos neurodegenerativos.

El QUIN se ha utilizado como modelo para producir excitotoxicidad y estrés oxidante en estudios *in vitro* e *in vivo*. Su inyección intraestriatal produce: 1) la acumulación de metales de transición que en presencia de oxígeno contribuyen a la formación de $O_2^{\cdot -}$, H_2O_2, y $^{\cdot}OH$; 2) modifica los perfiles de antioxidantes endógenos; 3) aumenta el estrés nitrosante mediante la producción de NO, $ONOO^{-\cdot}$ y 4) produce disfunción mitocondrial (Perez de la Cruz, et al., 2012).

El QUIN se asocia con la patogénesis de varias enfermedades infecciosas, inflamatorias, neurodegenerativas, autoinmunes y psiquiátricas. Los efectos del QUIN en estos desórdenes dependen de su concentración y son relacionados con alteraciones en el citoesqueleto, la producción de estrés oxidante, daño excitotoxicidad, disfunción mitocondrial, inflamación, autofagia y apoptosis (Jong-Mi et al., 2016). El QUIN también podría participar en los mecanismos de disfunción y muerte celular observados en las acidemias orgánicas, un grupo de enfermedades neurodegenerativas (Varadkar y Surtees, 2004).

3.4. LAS ACIDEMIAS ORGÁNICAS

Las acidemias orgánicas son desórdenes metabólicos hereditarios producidos por la deficiencia de enzimas que participan en el catabolismo de aminoácidos, lípidos y ácidos grasos. Esta deficiencia produce la acumulación de uno o más ácidos orgánicos (Chalmer, 1989). El cerebro de personas con una acidemia orgánica desarrolla neurodegeneración aguda relacionada con la acumulación de los siguientes metabolitos tóxicos: ácido glutárico (GA), ácido 3-hidroxiglutárico (3-OHGA), ácido metilmalónico (AMM), ácido propiónico (AP) y ácido 3-metilglutárico (AMG), por mencionar a unos cuantos.

Las acidemias metilmalónica y propiónica son causadas por la deficiencia de la mutasa metilmalonil-CoA (EC 5.4.99.2) y la carboxilasa de propil-CoA (EC 6.4.1.4), respectivamente. En la acidemia metilmalónica se acumula el AMM (1-2.5 mmol/L) y en la acidemia propiónica el AP (5 mm/L) en sangre. Las manifestaciones clínicas de estos dos desordenes incluyen macrocefalia, retraso mental, convulsiones, vómito, encefalopatía, coma y muerte (Deodato et al., 2006; Hauser et al., 2011).

La acidemia glutarica tipo I (AG I) es una AO que produce la muerte de neonatos, causada por la deficiencia de la enzima glutaril-CoA deshidrogenasa (GDD, McKusick 23167; OMIM # 231670). Los pacientes con esta enfermedad tienen concentraciones aumentadas de AG (500-5000 µmol/L) y 3-OHGA (40-200 µmol/L) en fluidos biológicos y en el SNC (Kölker et al., 2004; Sauer et al., 2006). Los niños afectados con este desorden al nacer desarrollan atrofia cortical y entre

los 6 meses y 4 años de edad daño agudo en el caudado/putamen (Amir et al., 1987; Hoffmann y Zschocke, 1999). Estudios *in vitro* e *in vivo* sugieren que la acumulación de ácidos orgánicos puede inducir excitotoxicidad, estrés oxidante y alteraciones del metabolismo (de Oliveira Marques et al., 2003; Ferreira et al., 2007; Flott-Rahmel et al., 1997; Kölker et al., 2004; Latini et al., 2000; Rosa et al., 2004; Sauer et al., 2006; Wajner et al., 2004). El ratón *knockout* para la glutaril-CoA deshidrogenasa (*gcdh*, gen de la enzima responsable para la degradación de AG) constituye un modelo la AG I.

Los macrófagos/monocitos de pacientes con AG I, en episodios de encefalopatía y necrosis estriatal, presentan un aumento en los niveles γ-interferon que induce la activación de la IDO, la primera enzima de la VK y; consecuentemente, genera un aumento en la producción de kinurenina. La kinurenina sintetizada periféricamente puede cruzar la barrera hematoencefálica mediante un transportador de aminoácidos neutros. Una vez dentro del cerebro, puede ser catabolizada por la microglia a 3-HK y después a QUIN (Heyes et al., 1996). El QUIN sintetizado no es removido efectivamente y concentraciones tóxicas se pueden acumular en la hendidura sináptica (Cao et al., 2002).

El daño causado por AG *per se* no es suficiente para explicar el daño celular observado en los pacientes. Por lo que se sugiere la presencia de otro factor que amplifique o exacerbe el daño (Kölker et al., 2004; Varadkar y Surtees, 2004). El QUIN cumple con las características necesarias para participar en estos mecanismos, generando la potencia suficiente para inducir la necrosis estriatal observada en este desorden.

4. **PLANTEAMIENTO DEL PROBLEMA**

Según el Instituto Nacional de Estadística y Geografía (INEGI) la esperanza de vida en México en 1930 era de 34 años, en el 2015 en este valor aumento a 76 y para el 2050 se prevé que sean 85 años. La población está envejeciendo, para el 2050 una de cada cuatro personas tendrá más de 60 años. Los individuos de la tercera edad tienen mayor probabilidad de desarrollar enfermedades degenerativas, trastornos mentales y depresión, desórdenes que causarán grandes estragos económicos y sociales. El estudio de los mecanismos que se activan en condiciones patológicas ayudará a encontrar estrategias terapéuticas o herramientas de diagnóstico temprano, y de esta forma se aumentará la calidad de vida.

Las enfermedades neurodegenerativas comparten varios mecanismos en común: la excitotoxicidad, el estrés oxidante, la inflamación y las alteraciones metabólicas. Entre las alteraciones del metabolismo se pueden mencionar los cambios observados en el funcionamiento de la VK. Niveles incrementados de la 3-HK, un metabolito que se produce en esta ruta, son relacionados con neurodegeneración en condiciones experimentales y clínicas. Una alternativa para explicar la neurotoxicidad asociada a la 3-HK puede ser a través de su metabolito de degradación, el QUIN, el cual es una potente neurotoxina en concentraciones elevadas. Actualmente no hay estudios *in vivo* que definan el verdadero papel de la 3-HK en el SNC. Por lo cual, el estudio de los efectos que producen estas dos moléculas en diferentes condiciones experimentales ayudará a definir el papel de la VK en las enfermedades neurodegenerativas.

5. **HIPÓTESIS**

Si la 3-HK es un metabolito que al oxidarse produce la formación de especies reactivas que pueden modificar el funcionamiento celular, entonces su aplicación en modelos *in vitro* e *in vivo* inducirá mecanismos de neurotoxicidad que comprometerán la viabilidad celular.

Si la 3-HK es precursor del QUIN, un metabolito relacionado con disfunción y muerte celular, entonces uno de los mecanismos que podría participar en la toxicidad de la 3-HK es su transformación a moléculas más tóxicas.

6. **OBJETIVOS**

6.1. OBJETIVO GENERAL

- Caracterizar los mecanismos de acción de la 3-HK y de su metabolito, el QUIN, en el estriado de roedores.

6.2. OBJETIVOS PARTICULARES

- Caracterizar los efectos tóxicos y/o antioxidantes de la 3-HK en ensayos *in vitro* e *in vivo* en estriado de roedores.
- Evaluar los efectos de la 3-HK y del QUIN en la modulación temprana del factor Nrf2.
- Describir los mecanismos mediante los cuales el QUIN magnifica los procesos tóxicos que se observan en desórdenes relacionados con el metabolismo de aminoácidos.

7. **MATERIAL Y MÉTODOS**

Ver la descripción detallada en los artículos presentados en la sección de resultados.

8. DISEÑO EXPERIMENTAL

8.1. LA 3-HIDROXIKINURENINA

Este trabajo fue diseñado para investigar si la 3-HK es tóxica y si su presencia es capaz de alterar el ambiente redox en tejido estriatal. Se utilizaron condiciones *in vitro* (rebanadas estriatales incubadas con la molécula) e *in vivo* (administración intraestriatal). En los dos casos se hizo un curso temporal y una curva dosis respuesta con el objetivo de encontrar una concentración y un tiempo en el cual la presencia de la 3-HK altere el ambiente celular. Se hizo particular énfasis en marcadores de función mitocondrial y estrés oxidante en estudios *in vitro* (**Figura 2**).

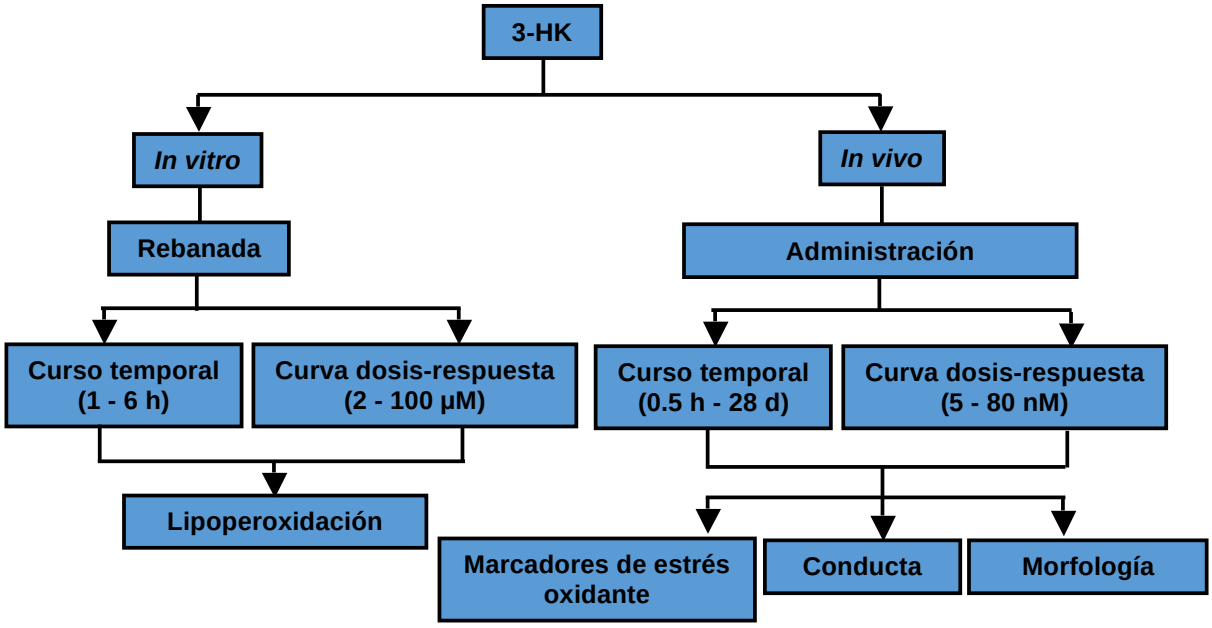


Figura 2. Diseño experimental utilizado para caracterizar la posible toxicidad de la 3-hidroxikinurenina (3-HK) en estriado de roedores.

La presencia de la 3-HK modificó el ambiente redox en las diferentes condiciones estudiadas. Con el objetivo de evaluar si esta reacción potenciaba o disminuía los efectos neurotóxicos de compuestos conocidos [QUIN (1 mM), ácido

3-nitropropiónico (3-NP, 1 mM) y $FeSO_4$ (50 µM)], se co-incubaron rebanadas estriatales con la 3-HK más cada una de las toxinas por 6 h para ejemplificar un ambiente excitotóxico, con disfunción energética y oxidante, respectivamente (**Figura 3**).

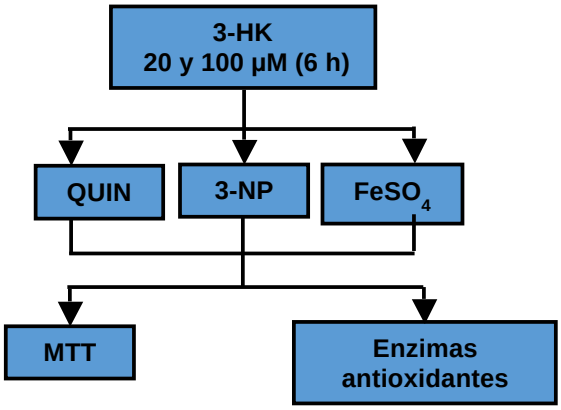


Figura 3. Diseño experimental utilizado para caracterizar el posible efecto protector *in vitro* de la 3-hidoxikinurenina (3-HK) en presencia de moléculas conocidas [QUIN (1 mM), ácido 3-nitropropiónico (3-NP, 1 mM) y $FeSO_4$ (50 µM)].

8.2. EL ÁCIDO QUINOLÍNICO

La falta de toxicidad de la 3-HK, aunada a su capacidad de modular el ambiente redox mediante el Nrf2 y su posible efecto neuroprotector sugirieren que los efectos tóxicos reportados para la 3-HK pueden explicarse a través de la producción de QUIN. Por lo tanto, la realización de estudios en paralelo con este metabolito fue pertinente para tratar de explicar, en términos fisiopatológicos, los efectos de producen las alteraciones de la VK.

Lo que primero se caracterizó fue la capacidad del QUIN para inducir daño oxidante en lípidos, usando rebanadas estriatales de ratones. Se estableció una curva concentración-respuesta. En este ensayo, la incubación de las rebanadas con QUIN a una concentración de 50 µM produjo los niveles más altos de lipoperoxidación observados, utilizando esta concentración se realizó un curso temporal de la inducción del Nrf2 y de enzimas antioxidantes (**Figura 4**).

Con base a la hipótesis de que parte de la toxicidad del QUIN es ejercida vía estrés oxidante, se emprendió un abordaje adicional para demostrar si su patrón neurotóxico es sensible a un agente antioxidante de amplio espectro. El ácido cafeico es un compuesto fenólico que induce la expresión del Nrf2 en el núcleo. Se ha sugerido que esta molécula podría unirse a Keap1, una proteína que secuestra al Nrf2 en el citoplasma, lo que produce la disociación y la activación transcripcional del Nrf2 (Pang et al., 2016). Este estudio demostró el efecto protector del ácido cafeico en rebanadas y en animales inyectados intraestriatalmente con QUIN. Las rebanadas estriatales fueron incubadas en presencia de ácido cafeico (30-300 µM) y/o QUIN (100 µM) y se evaluó un marcador de estrés oxidante. En los animales, el ácido cafeico (20 mg/kg) fue administrado *i.p*. cinco días antes de la inyección del QUIN (240 nmol/µL) y siete días después se realizaron pruebas conductuales. (**Figura 4**).

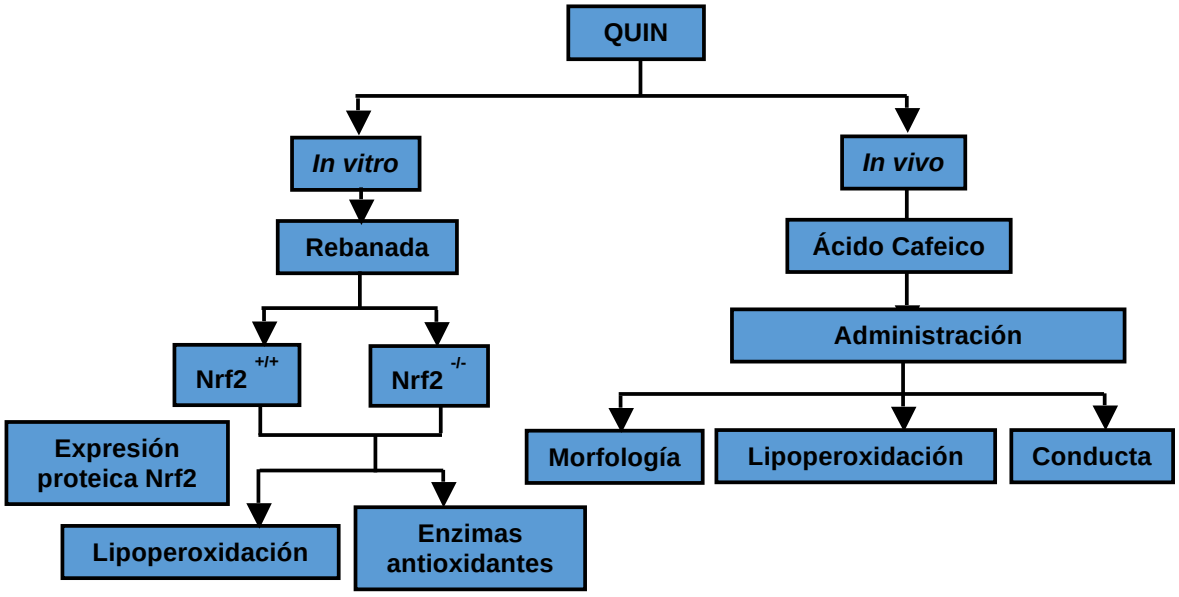


Figura 4. Diseño experimental utilizado para evaluar el efecto del ácido quinolínico (QUIN) en la modulación del Nrf2 y el posible efecto protector del ácido cafeico.

Adicionalmente, se determinó si el QUIN magnifica los efectos de los ácidos orgánicos como parte de su espectro tóxico. En este experimento se utilizaron sinaptosomas tratados con concentraciones subtóxicas de varios ácidos orgánicos

(AG, 3-OHGA, AMM, AP, AMG) en presencia y ausencia del QUIN. Los sinaptosomas fueron pre-incubados con agentes con mecanismos de acción conocidos: 1) ácido kinurénico (AK), un antagonista de los rNMDA; 2) S-alilcisteina (SAC), un antioxidante de amplio espectro; y 3) L-nitroarginina metilester (L-NAME), un inhibidor de la sintasa de óxido nítrico (**Figura 5**).

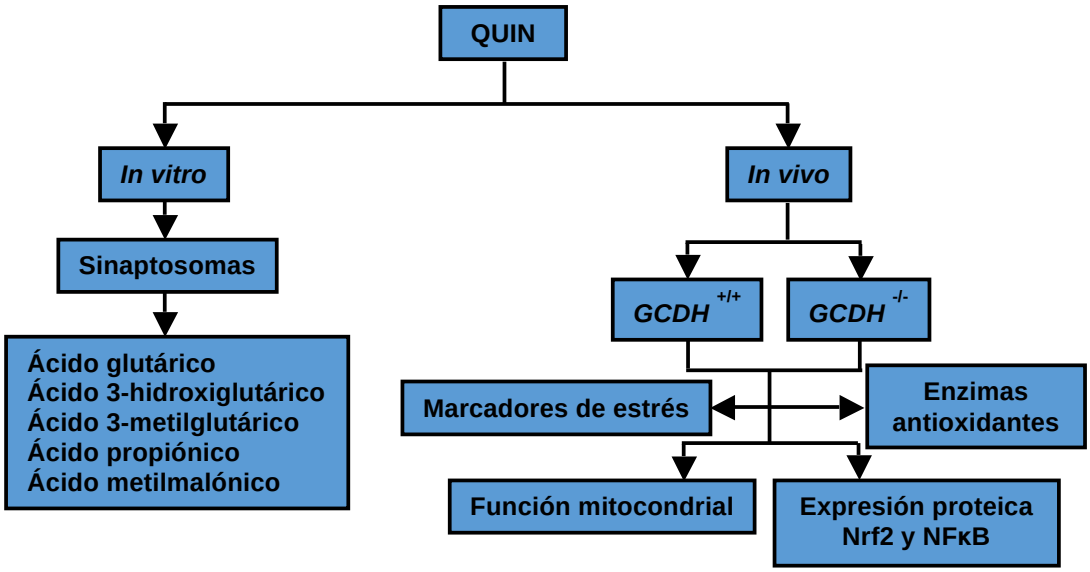


Figura 5. Estrategia experimental desarrollada *in vitro* e *in vivo* para determinar el posible un posible efecto tóxico sinérgico del ácido quinolínico (QUIN) con metabolitos acumulados en acidemias metabólicas.

Finalmente, se evaluó el efecto sinérgico del AG y el QUIN en un modelo de AG I. Animales *knockout* para el gen *GCDH* (la enzima que degrada el AG) fueron inyectados intraestriatalmente con el QUIN y alimentados con una dieta alta en lisina para estimular la producción de AG. Este modelo constituye la mejor aproximación experimental para evidenciar el posible sinergismo entre el QUIN y los ácidos orgánicos que se acumulan en las acidemias metabólicas mediante marcadores de estrés oxidante, actividad metabólica y energética, y actividad transcripcional (**Figura 5**).

9. **RESULTADOS**

9.1. LA 3-HK MODIFICA EL AMBIENTE REDOX ESTRIATAL DE ROEDORES SIN MOSTRAR TOXICIDAD

La concentración y el tiempo de exposición de la 3-HK alteraron los niveles de lipoperoxidación en rebanadas estriatales. La 3-HK fue pro-oxidante en concentraciones bajas (5-20 µM) y antioxidante en concentraciones altas (100 µM). Sin embargo, en ninguno de los dos casos la molécula alteró la función mitocondrial. Además, su presencia previno los efectos tóxicos del QUIN, 3-NP y $FeSO_4$, por lo que se asume que su efecto estimulante sobre la peroxidación es más una respuesta compensatoria que estimula los sistemas antioxidantes.

La 3-HK es un antioxidante directo e indirecto. Su efecto como antioxidante directo se demostró con un ensayo de FRAP (Del inglés: *ferric reducing ability*), cuyos resultados indican que la molécula tiene un alto poder antioxidante o reductor. Por otro lado, la 3-HK estimula al Nrf2, un factor que regula la expresión de enzimas antioxidantes y detoxificantes, propuesto como herramienta para disminuir la progresión de eventos neurodegenerativos. La activación del Nrf2 se comprobó con el aumento de las actividades enzimáticas de la glutatión-S-tranferasa y la superóxido dismutasa, así como con el aumento en la expresión de la hemo-oxigenasa-1 y la γ-glutamilcisteina ligasa.

Al encontrar que la 3-HK en ninguna de las concentraciones probadas alteró la función mitocondrial ni la viabilidad celular, se decidió entonces explorar sus efectos *in vivo*. En tiempos cortos (horas), la 3-HK produjo un aumento moderado en los niveles de lipoperoxidación y de oxidación de proteínas. Este efecto, sin embargo, fue correlacionado con el aumento en las actividades de las enzimas glutatión reductasa y glutatión-S-transferasa, consolidando el concepto de la peroxidación de lípidos inducida por la 3-HK como una estrategia compensatoria para estimular la respuesta antioxidante. En ninguna de las concentraciones probadas (5-80 nmol/µL) se encontraron cambios conductuales o morfológicos.

Todo lo anterior sugiere que la 3-HK induce un estado de estrés moderado que induce una respuesta oxidante endógena y el resultado final puede ser inocuo o incluso parcialmente neuroprotector. Por lo tanto, la presencia de esta molécula puede disminuir el daño celular y en estas condiciones, no puede ni debe considerarse como un metabolito neurotóxico.

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Research Report

The Janus faces of 3-hydroxykynurenine: Dual redox modulatory activity and lack of neurotoxicity in the rat striatum

Ana Laura Colín-González[a,1], *Marisol Maya-López*[a], *José Pedraza-Chaverrí*[b], *Syed F. Ali*[c], *Anahí Chavarría*[d], *Abel Santamaría*[a,*]

[a]*Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, SSA, Mexico City 14269, Mexico*
[b]*Departamento de Biología, Facultad de Química, Universidad Nacional Autónoma de México, Mexico City, Mexico*
[c]*Division of Neurotoxicology, National Center for Toxicological Research, FDA, Jefferson, AR, USA*
[d]*Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico City, Mexico*

ARTICLE INFO

Article history:
Accepted 14 September 2014
Available online 22 September 2014

Keywords:
Redox activity
Oxidative stress
Antioxidant systems
Cell preservation
Kynurenine pathway

ABSTRACT

3-Hydroxykynurenine (3-HK), an intermediate metabolite of the kynurenine pathway, has been largely hypothesized as a neurotoxic molecule contributing to neurodegeneration in several experimental and clinical conditions. Interestingly, the balance in literature points to a dual role of this molecule in the CNS: *in vitro* studies describe neurotoxic and/or antioxidant properties, whereas *in vivo* studies suggest a role of this metabolite as a weak neurotoxin. This work was designed to investigate, under different experimental conditions, whether or not 3-HK is toxic to cells, and if the redox activity exerted by this molecule modulates its actions in the rat striatum. In order to evaluate these effects, 3-HK was administered *in vitro* to isolated striatal slices, and *in vivo* to the striatum of rats. In striatal slices, 3-HK exerted a concentration- and time-dependent effect on lipid peroxidation, inducing both pro-oxidant actions at low (5–20) micromolar concentrations, and antioxidant activity at a higher concentration (100 μM). Interestingly, while 3-HK was unable to induce mitochondrial dysfunction in slices, at the same range of concentrations it prevented the deleterious effects exerted by the neurotoxin and related metabolite quinolinic acid (QUIN), the mitochondrial toxin 3-nitropropionic acid, and the pro-oxidant compound iron sulfate. These protective actions were related to the stimulation of glutathione S-transferase (GST) and superoxide dismutase (SOD) activities. In addition, 3-HK stimulated the protein content of the transcription factor and antioxidant regulator Nrf2, and some of its related proteins. Accordingly, 3-HK, but not QUIN, exhibited reductive properties at high concentrations. The striatal tissue of animals infused with 3-HK exhibited moderate levels of lipid and protein oxidation at short times post-lesion (h), but these endpoints were substantially decreased at longer times (days). These effects were

*Corresponding author.
E-mail address: absada@yahoo.com (A. Santamaría).
[1]Doctoral Program in Biomedical Sciences, Universidad Nacional Autónoma de México (UNAM).

http://dx.doi.org/10.1016/j.brainres.2014.09.034

correlated with an early increase in glutathione reductase (GR) and GST activities. However, these changes were likely to be merely compensatory as 3-HK-infused animals did not display behavioral (rotation) alterations or morphological changes in their injected striata. Altogether, these findings suggest that, despite 3-HK might exert pro-oxidant actions under certain conditions, these changes serve to evoke a redox modulatory activity that, in turn, could decrease the risk of cell damage. In light of this evidence, 3-HK seems to be more a redox modulatory molecule than a neurotoxic metabolite.

1. Introduction

Extensive research has demonstrated that neurodegenerative diseases share several deleterious mechanisms including excitotoxicity – a toxic process characterized by a sustained stimulation of excitatory amino acid receptors – , oxidative stress – which results of an impaired balance between the formation of reactive oxygen species (ROS) and antioxidant defenses – and inflammation – leading to a breakdown of the blood-brain barrier, cell infiltration and release of chemical mediators such as cytokines, chemokines and lipid mediators – , all of them ultimately leading to cell death (Halliwell, 2006; Dasuri et al., 2013; Niranjan, 2014).

The kynurenine pathway (KP) is a metabolic route in which the essential amino acid tryptophan is primarily metabolized, hence producing relevant intermediates for neuronal integrity and redox balance (Stone et al., 2007; Massudi et al., 2012; Schwarcz et al., 2012). Recent reports have shown that alterations of the KP metabolism and changes in the brain levels of its metabolites may play a key role in the pathogenesis of some neurodegenerative diseases, depressive disorders and schizophrenia (Schwarcz et al., 2012; Tan et al., 2012; Amaral et al., 2013). In addition, the KP has been associated with inflammatory responses in different neurological disorders, and this mechanism, which may be inherent to some KP metabolites, could contribute to the neurodegenerative pattern associated with these diseases (Chen et al., 2010; Tan et al., 2012). Some neuroactive metabolites are formed in the KP, including the well-known neurotoxin and glutamate agonist quinolinic acid (QUIN) (Schwarcz et al., 1984; Guidetti et al., 2006), the neuroprotectant and glutamate antagonist kynurenic acid (KYNA) (Schwarcz et al., 1983; Yu et al., 2004), and the redox active metabolites 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid (3-HAAO) (Goldstein et al., 2000; Braidy et al., 2009; reviewed by Colín-González et al., 2013).

Increased levels of 3-HK have been found in early stages of Huntington's disease (HD) patients (Schwarcz et al., 1984; Reynolds and Pearson, 1989; Pearson et al., 1995; Guidetti and Schwarcz, 2003), in Alzheimer's disease (AD) – in which 3-HK is assumed to be relevant for memory alterations (Savvateeva et al., 2000; Duleu et al., 2010) – and in the putamen of Parkinson's disease (PD) patients (Ogawa et al., 1992; reviewed by Colín-González et al., 2013). Also, transgenic mice models (R6/2 mice, YAC 128, and the chimeric HdhQ) have shown a substantial elevation of the 3-HK content (Guidetti et al., 2000; Guidetti et al., 2006; Sathyasaikumar et al., 2010). Moreover, genetic models of HD using invertebrates have shown an increased activity of kynurenine 3-monooxygenase, the enzyme responsible for 3-HK formation (Giorgini et al., 2005; Ramaswamy et al., 2007; Campesan et al., 2011).

Under physiological conditions, 3-HK undergoes auto-oxidation, forming an o-aminoquinone – a stronger oxidant agent that can be responsible for exacerbated oxidative damage – which can be responsible for the formation of different reactive oxygen species ($O_2^{\cdot-}$, H_2O_2, $^{\cdot}OH$) (Eastman and Guilarte, 1990; Ishii et al., 1992; Okuda et al., 1996; Hiraku et al., 1995; Vazquez et al., 2000). As a metabolic precursor, 3-HK might also account for QUIN formation, which in turn is responsible for cellular damage mainly mediated by excitotoxicity and oxidative damage (Schwarcz et al., 1984). In addition, 3-HK has also shown to be a generator of reactive species, acting as a potential endogenous neurotoxin in cerebellar granule, striatal and hippocampal neurons, as well as in neuronal hybrid cell lines, human neuroblastoma SH-SY5Y, PC-12 pheochromocytoma cells, and GT1-7 hypothalamic neurosecretory cells (Eastman and Guilarte, 1989; Okuda et al., 1998; Jeong et al., 2004; Smith et al., 2009). However, to our knowledge, there are only three studies using 3-HK in *in vivo* models in the CNS, but none of them has characterized in a detailed manner its mechanisms of toxicity. The intraventricular administration of 3-HK (634.21 mg/rat) was responsible for convulsive attacks in rats (Pinelli et al., 1984), whereas the intrastriatal injection of 3-HK (50 nmol) induced only tissue damage around the injected site, without any abnormal behavior in rats (Nakagami et al., 1996). Finally, it was suggested that 3-HK potentiates QUIN toxicity by means of a possible synergic interaction comprising a combination of direct NMDA receptor activation and free radical production (Guidetti and Schwarcz, 1999).

In contrast to these toxic features, under certain circumstances 3-HK is known to be a potent reductive agent that donates electrons, sometimes acting as an antioxidant agent. This behavior of 3-HK, which is opposite to its toxic character, has been described in several reports, some of which discuss a scavenging activity for this molecule, showing that 3-HK is able to scavenge $O_2^{\cdot-}$, $^{\cdot}OH$, peroxyl radicals, and $^{\cdot}NO$, also acting as an endogenous natural antioxidant (Goshima et al., 1986; Christen et al., 1990; Goda et al., 1999; Leipnitz et al., 2007; Backhaus et al., 2008). Still, its precise role in the CNS remains uncertain. Hence, 3-HK is an intriguing and puzzling compound found at increased levels in pathological conditions; consequently, characterizing and identifying the precise physiological and/or physiopathological roles exerted by

this tryptophan metabolite in animal models will help to elucidate the mechanisms involved in normal cell redox regulation and/or cell dysfunction and death in neurodegenerative diseases.

Therefore, the aim of this work was to investigate the precise role exerted by 3-HK in nerve (striatal) tissue of rats in order to characterize, identify and infer mechanistic actions of this metabolite, thereby contributing to our actual knowledge on its actions and relevance. For this purpose, we evaluated the redox activity induced by 3-HK, assessing concentration– and time–response curves of oxidative damage in rat striatal slices, as well as the possible morphological, behavioral and oxidative alterations induced by 3-HK after its intrastriatal infusion to rats. Our results suggest that 3-HK, under different conditions, can exert both pro- and antioxidant effects in striatal tissue of rats, but when this agent induces oxidative stress, this effect does not yield cell damage. Thus, 3-HK seems to act more like a redox modulator than a neurotoxin.

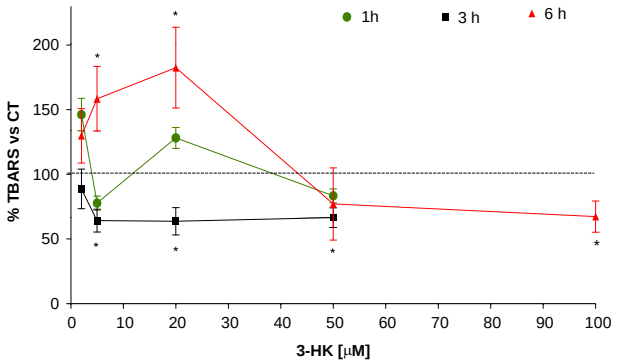

Fig. 1 – Time–(1–6 h) and concentration (2–100 μM)–response effects of 3-hydroxykynurenine (3-HK) on lipid peroxidation (percent of TBARS formed *vs.* control) in rat striatal slices. Data are expressed as mean values ± S.E.M. of $n=6$ experiments per group. One-way ANOVA followed by *post hoc* Tukey's test for multiple comparisons. Asterisks denote statistically significant differences (*$P<0.05$) *vs.* control.

2. Results

2.1. *Concentration and exposure time influenced the 3-HK-induced pro- and antioxidant effects in striatal slices*

Striatal slices were incubated in the presence of increasing concentrations of 3-HK (2–100 μM), and during different times of exposure (1, 3 and 6 h) (Fig. 1). While at 1 h of incubation the molecule (2–50 μM) evoked an oscillatory effect which mostly remained close to the baseline, at 3 h all the tested concentrations (2–50 μM) showed a consistent inhibitory action on lipoperoxidation (45–47% below baseline from 5 to

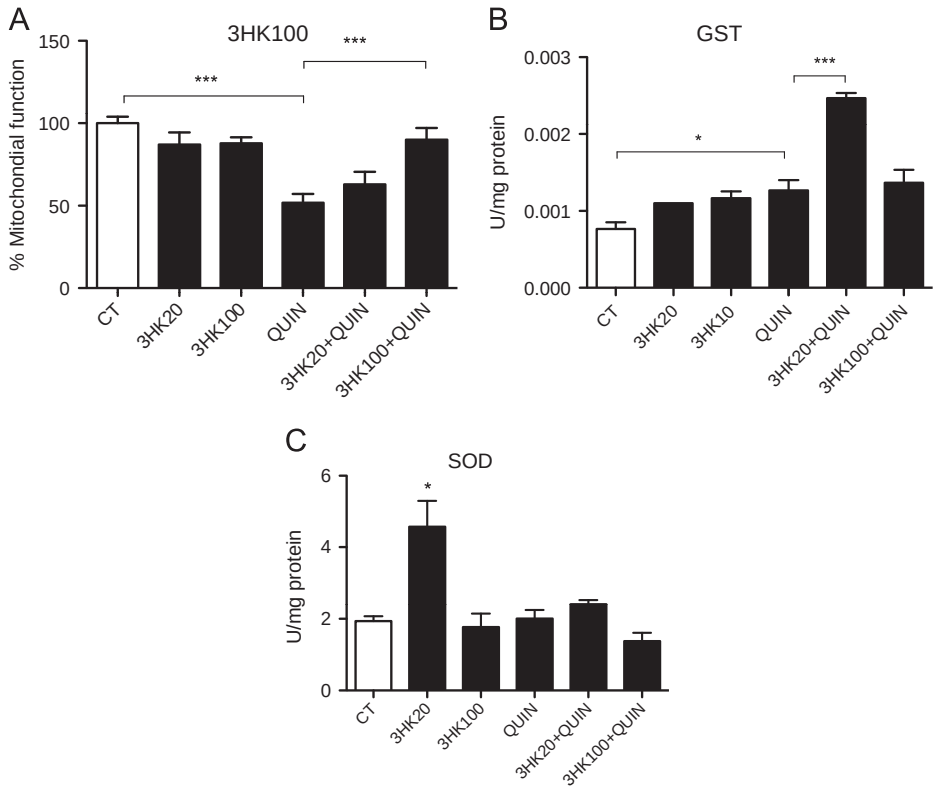

Fig. 2 – Effects of 3-hydroxykynurenine (3-HK) and/or quinolinic acid (QUIN) on mitochondrial function (A), glutathione S-transferase (GST) (B) and superoxide dismutase (SOD) (C) activities in rat striatal slices. All endpoints were estimated at 6 h of incubation with two concentrations of 3-HK (20 or 100 μM) and one of QUIN (1 mM). Data are expressed as mean values ± S.E.M. of $n=6$ experiments per group. One-way ANOVA followed by *post hoc* Tukey's test for multiple comparisons. Asterisks denote statistically significant differences (*$P<0.05$ and ***$P<0.001$).

50 μM; $P<0.05$). In contrast, at 6 h of exposure, striatal slices underwent a pro-oxidant effect (54% and 78% above baseline at 5 and 20 μM, respectively; $P<0.05$), except for the 50 and 100 μM concentrations, which induced an antiperoxidative effect. In fact, the 50 μM concentration resulted in levels of lipid peroxidation below the baseline at all times tested. The 100 μM concentration of 3-HK was tested at 6 h in order to know whether this extreme condition would be responsible for some damage.

2.2. *3-HK reduced the toxicant-induced mitochondrial dysfunction by stimulating the activity of antioxidant enzymes in striatal slices*

In order to know whether the dual redox actions of 3-HK observed this far confer neurotoxic or neuroprotective properties to the molecule when challenged by other well-known toxicants, two different concentrations of this molecule (20 and 100 μM) were added to cultured striatal slices 30 min before de addition of QUIN (1 mM), 3-nitropropionic acid (3-NP; 1 mM) or iron sulfate ($FeSO_4$; 50 μM) (Figs. 2–4). These conditions were maintained for 6 h and simulated excitotoxic events, mitochondrial dysfunction and oxidative stress, respectively. In addition, the activity of two antioxidant enzymes, glutathione S-transferase (GST) and superoxide dismutase (SOD) were determined in order to correlate the cell damage evidenced by the mitochondrial dysfunction with oxidative stress evidenced by the altered enzyme activities.

3-HK *per se*, at 20 and 100 μM, did not alter the levels of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltettazolium (MTT) reduction when compared with control slices (9% and 10% below the baseline, respectively). In contrast, QUIN reduced by 55% the baseline mitochondrial function. Surprisingly, in the presence of QUIN, 20 and 100 μM 3-HK increased MTT reduction (42% and 7% below the control value, respectively) in the slices (Fig. 2A).

Additionally, 3-HK, at 20 and 100 μM concentrations, stimulated GST activity by 40% and 45% above the control, respectively (Fig. 2B). QUIN increased GST activity by 57%. Also, 20 μM 3-HK plus QUIN exerted an additive effect, increasing the enzyme activity by 213% when compared with control values, while 100 μM 3-HK plus QUIN had no effect when compared with QUIN *per se* (63% above the control).

SOD activity was intensely stimulated by 20 μM 3-HK (120% above the Control; Fig. 2C). In contrast, neither 100 μM 3-HK nor QUIN exerted stimulatory actions on SOD (5% below and 3% above Control, respectively). In the presence of QUIN, 20 μM 3-HK significantly reduced SOD (33% above the baseline levels).

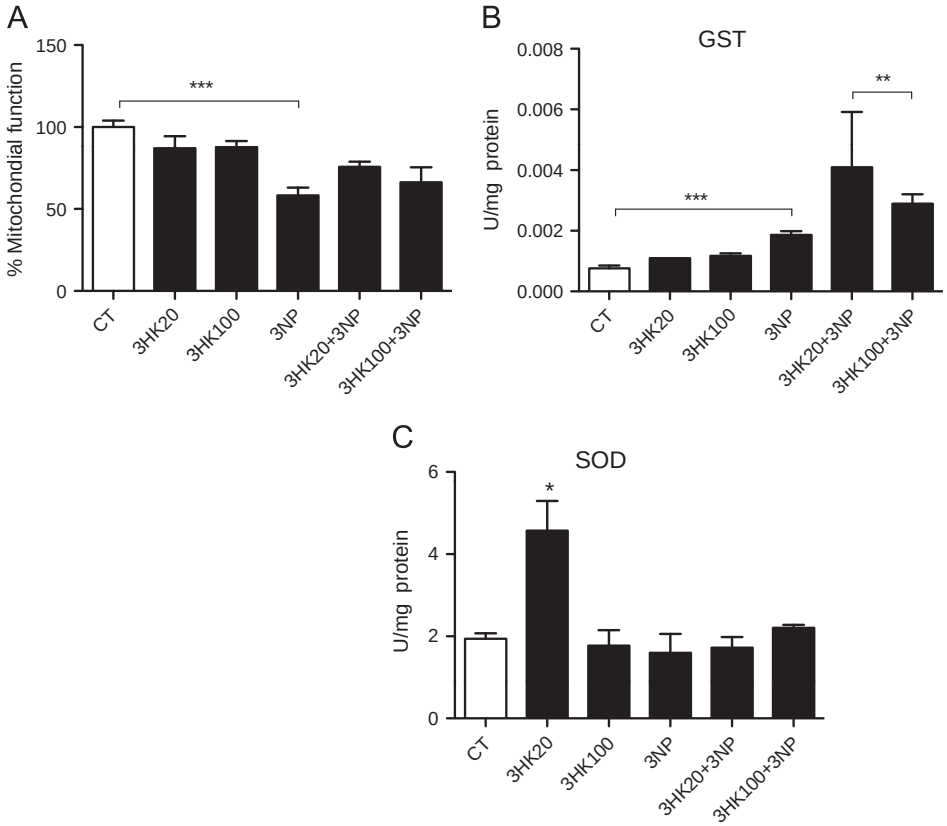

Fig. 3 – Effects of 3-hydroxykynurenine (3-HK) and/or 3-nitropropionic acid (3-NP) on mitochondrial function (A), glutathione S-transferase (GST) (B) and superoxide dismutase (SOD) (C) activities in rat striatal slices. All endpoints were estimated at 6 h of incubation with two concentrations of 3-HK (20 or 100 μM) and one of 3-NP (1 mM). Data are expressed as mean values ± S.E.M. of $n=6$ experiments per group. One-way ANOVA followed by *post hoc* Tukey's test for multiple comparisons. Asterisks denote statistically significant differences (*$P<0.05$, **$P<0.01$ and ***$P<0.001$).

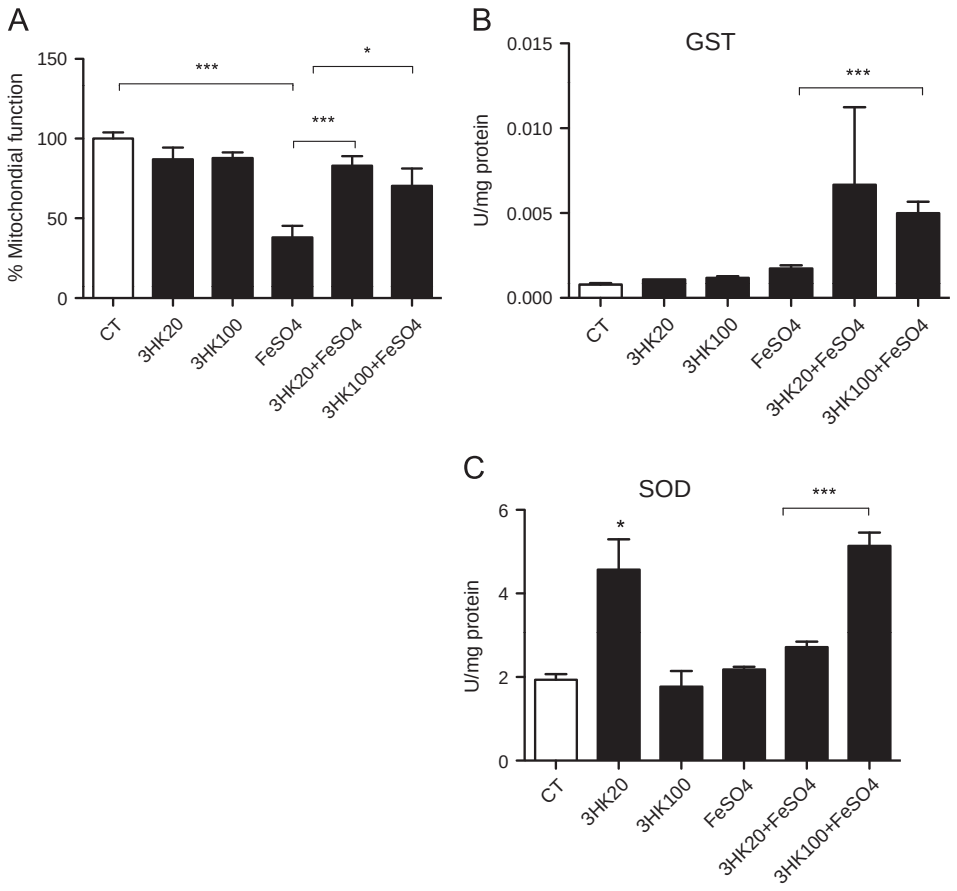

Fig. 4 – Effects of 3-hydroxykynurenine (3-HK) and/or iron sulfate ($FeSO_4$) on mitochondrial function (A), glutathione S-transferase (GST) (B) and superoxide dismutase (SOD) (C) activities in rat striatal slices. All endpoints were estimated at 6 h of incubation with two concentrations of 3-HK (20 or 100 μM) and one of $FeSO_4$ (50 μM). Data are expressed as mean values ± S.E.M. of $n = 6$ experiments per group. One-way ANOVA followed by *post hoc* Tukey's test for multiple comparisons. Asterisks denote statistically significant differences (*$P < 0.05$ and ***$P < 0.001$).

Fig. 3A shows the effect of 3-HK on 3-NP-induced mitochondrial dysfunction. 3-NP reduced MTT reduction by 42% when compared with control levels. Twenty μM, but not 100 μM 3-HK, attenuated the effect of 3-NP, leading the mitochondrial function to 25% below the baseline level (29% above 3-NP *per se*).

Furthermore, 3-NP increased GST baseline activity by 85%. The addition of 3-NP to the slices incubated with 20 μM 3-HK substantially increased the baseline enzyme activity by 500% (91% above 3-NP *per se*), whereas the 100 μM 3-HK plus 3-NP condition exhibited an increase in GST activity of 328% above the Control (36% above 3-NP alone; Fig. 3B).

Fig. 3C depicts the effects of 3-HK and 3-NP on SOD activity in striatal slices. No effect of 3-NP *per se* on SOD activity was found (13% below the control). The addition of 3-NP to slices incubated with 20 μM 3-HK returned the enzyme activity to basal levels (9% below the baseline). The combination of 100 μM 3-HK plus 3-NP had no effect on SOD activity.

In Fig. 4A, $FeSO_4$ significantly reduced the basal mitochondrial function by 58%, whereas the pre-incubation of the slices with 20 and 100 μM 3-HK preserved the MTT reduction index by 18% and 30% below the baseline (95% and 67% above $FeSO_4$ *per se*, respectively).

Fig. 4B describes the effects of 3-HK and $FeSO_4$ on GST activity. $FeSO_4$ increased the baseline enzyme activity by 92%. Noteworthy, pretreatment of the $FeSO_4$-treated slices with 20 and 100 μM 3-HK enhanced the GST activity by 543% and 493% above the Control, respectively (424% and 368% above FeSO4 *per se*).

In Fig. 4C, neither $FeSO_4$, nor 20 μM 3-HK plus $FeSO_4$ treatments induced any significant change in SOD activity when compared with baseline levels (8% and 27% above Control, respectively). In contrast, the pre-incubation of $FeSO_4$-treated slices with 100 μM 3-HK reached a prominent stimulation of the enzyme activity (163% when compared with Control, and 138% when compared with $FeSO_4$ *per se*).

2.3. *3-HK stimulated nuclear factor erythroid-2-related (Nrf2), heme-oxygenase 1 (HO-1) and gamma-glutamyl cysteine-ligase (γ-GCL-C) expression in striatal slices*

After exposed to 3-HK (20 μM) for 6 h, rat striatal slices increased the expression of the transcription factor Nrf2 (42% above the baseline), and the antioxidant enzymes

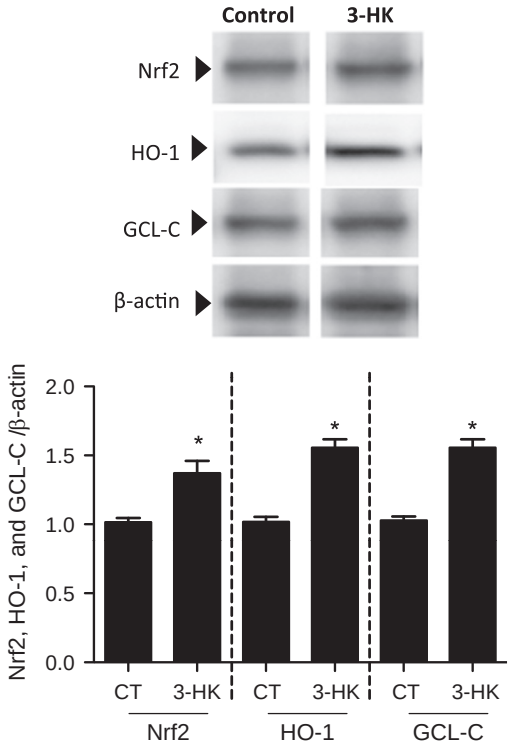

Fig. 5 – Effect of 3-hydroxykynurenine (3-HK, 20 μM) on the total Nrf2, heme-oxygenase (HO-1), superoxide dismutase (SOD) and Gamma-glutamyl-cysteine ligase-C (γ-GCL-C) levels at 6 h of incubation in rat striatal slices. Protein levels are expressed as the ratio of protein/β-actin. Data are expressed as mean values ± S.E.M. of $n = 6$ experiments per group. Student's *t*-test for comparison among treatments. Asterisks denote statistically significant differences ($^{*}P < 0.05$).

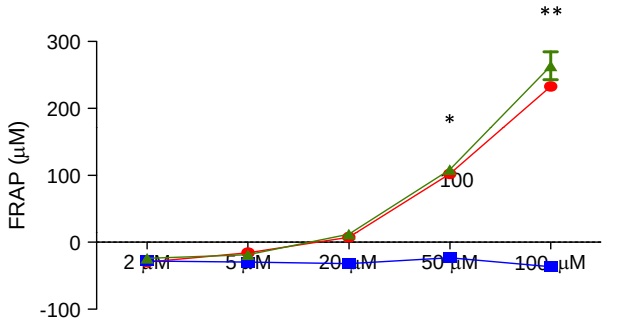

Fig. 6 – Effects of increasing concentrations (2–100 μM) of 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN) on iron reductive capacity (FRAP) as an index of antioxidant potential. One-way ANOVA followed by *post hoc* Tukey's test for multiple comparisons. Asterisks denote statistically significant differences ($^{*}P < 0.05$ and $^{**}P < 0.01$) vs. normalized control.

HO-1 and γ-GCL-C (53% and 52% above the control, respectively; Fig. 5). The rationale for the use of 20 μM 3-HK (instead of 100 μM) for the estimation of these antioxidants is based on the fact that this concentration produced higher levels of lipid peroxidation, but also depicted the most prominent responses in SOD activity; therefore, it was fundamental for us to search for possible explanations on why the pro-oxidant concentration is unable to induce cell damage through these endpoints.

2.4. 3-HK, but not QUIN, exhibited a concentration–response reductive capacity

The ferric reductive ability of plasma (FRAP) constitutes an accurate measure of the antioxidant power tested for certain molecules (Benzie and Strain, 1996). We used this technique to evidence any possible reductive capacity of 3-HK *per se*, once we found that this metabolite exerted antioxidant properties under some experimental circumstances.

The reductive capacity of 3-HK (2–100 μM) was compared with that of QUIN (Fig. 6). Low concentrations of 3-HK did not show reductive activity, whereas at 50 and 100 μM concentrations, this metabolite exhibited an intense activity (106% and 219% above the control, respectively; red line). In contrast, QUIN, at the same concentrations, never displayed a reductive capacity, as expected (blue line). The addition of increasing concentrations of 3-HK to 5 μM QUIN resulted in a reductive activity similar to that exerted by 3-HK alone (green line).

2.5. The intrastriatal infusion of 3-HK to rats increased the pro-oxidant activity shortly after its injection, but decreased after days

Once we found that the dual actions of 3-HK on redox activity did not compromise mitochondrial function and cell viability in striatal slices, we were interested in exploring the effects of 3-HK when the metabolite is infused in the striatum of rats.

Fig. 7A depicts the effect of 3-HK (80 nmol) on TBARS formation in the rat striatum at different times post-lesion. 3-HK induced increased levels of lipid peroxidation at 6 and 24 h post-lesion (29% and 35% above the control), but these effects were not statistically significant. In contrast, at 7 days post-lesion, 3-HK decreases the baseline lipoperoxidation (22% below the control). This dual effect was found also when the formation of carbonyl groups was tested as a marker of oxidative activity in the same samples (Fig. 7B). In this case, the levels of protein oxidation were increased by 3-HK only at 6 h post-lesion (36% above the baseline), but not at 24 h. Again, at 7 days post-lesion, there was a consistent decrease in oxidative activity when 3-HK was infused (21% below the baseline).

2.6. Glutathione reductase (GR) and GST activities were stimulated by 3-HK at short times after its intrastriatal injection

The activities of GR, glutathione peroxidase (GPx), GST and glucose 6-phosphate dehydrogenase (G6PD) were all monitored at 0.5 and 2 h after the intrastriatal infusion of 3-HK to

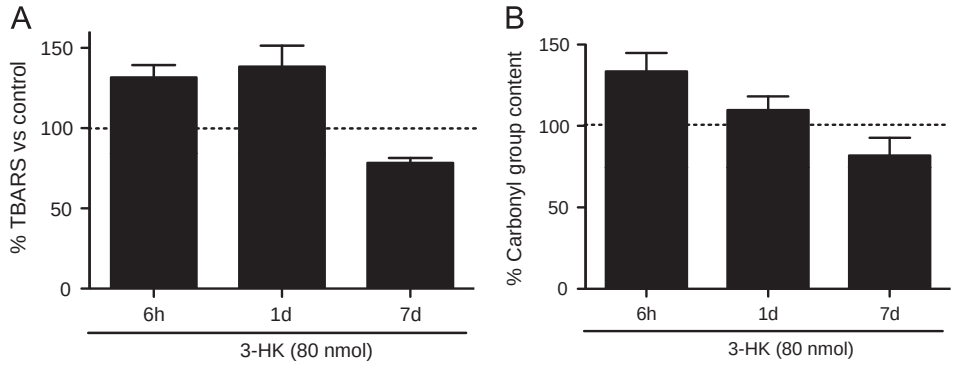

Fig. 7 – Time-course effect of the intrastriatal injection of 3-hydroxykynurenine (3-HK, 80 nmol/μl) to rats on the striatal levels of lipid peroxidation (A) and protein carbonylation (B). Data were normalized to baseline levels and are expressed as mean values ± S.E.M. of $n = 10$ rats per group. One-way ANOVA. No statistical changes were found.

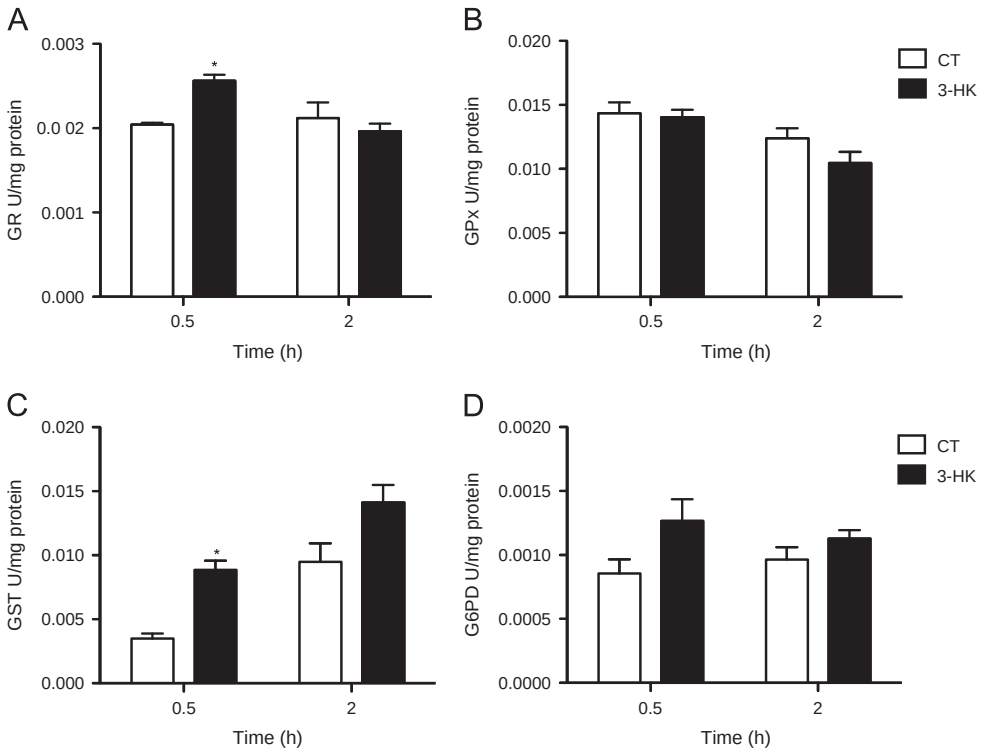

Fig. 8 – Early time-course effect of the intrastriatal infusion of 3-hydroxykynurenine (3-HK) on the activities of glutathione reductase (GR) (A), glutathione peroxidase (GPx) (B), glutathione S-transferase (GST) (C) and glucose 6-phosphate dehydrogenase (G6PD) (D). Data were normalized to baseline and are expressed as mean values ± S.E.M. of $n = 10$ rats per group. Student's t-test for comparison among treatments. Asterisks denote statistically significant differences (*$P < 0.05$).

rats in order to investigate whether the glutathione (GSH) system is involved in the early oxidative activity exerted by this metabolite, and if its possible changes are related to 3-HK-induced redox patterns.

GR and GST activities were found increased at 0.5 h (27% and 166% above the control, respectively), suggesting an increased use of GSH by the striatum in the presence of 3-HK (Fig. 8A and C). This observation was reinforced by increased tendencies of GST and G6PD activities stimulated by 3-HK (40% and 56% above the control at 2 and 0.5 h, respectively; Fig. 8C and D).

2.7. 3-HK produced no behavioral or morphological alterations after its striatal infusion to rats

In order to know whether the redox changes induced by 3-HK could be responsible for striatal damage and degeneration, coronal striatal sections or rats injected with 3-HK were stained with H&E and observed in an optic microscope at 7, 14 and 21 days post-lesion. Fig. 9 shows representative micrographs (40×) of sham and 3-HK-treated animals at 7 and 21 days post-lesion. Fields corresponded to areas of the dorsal striatum distant to the injection site by 100 μm. In general

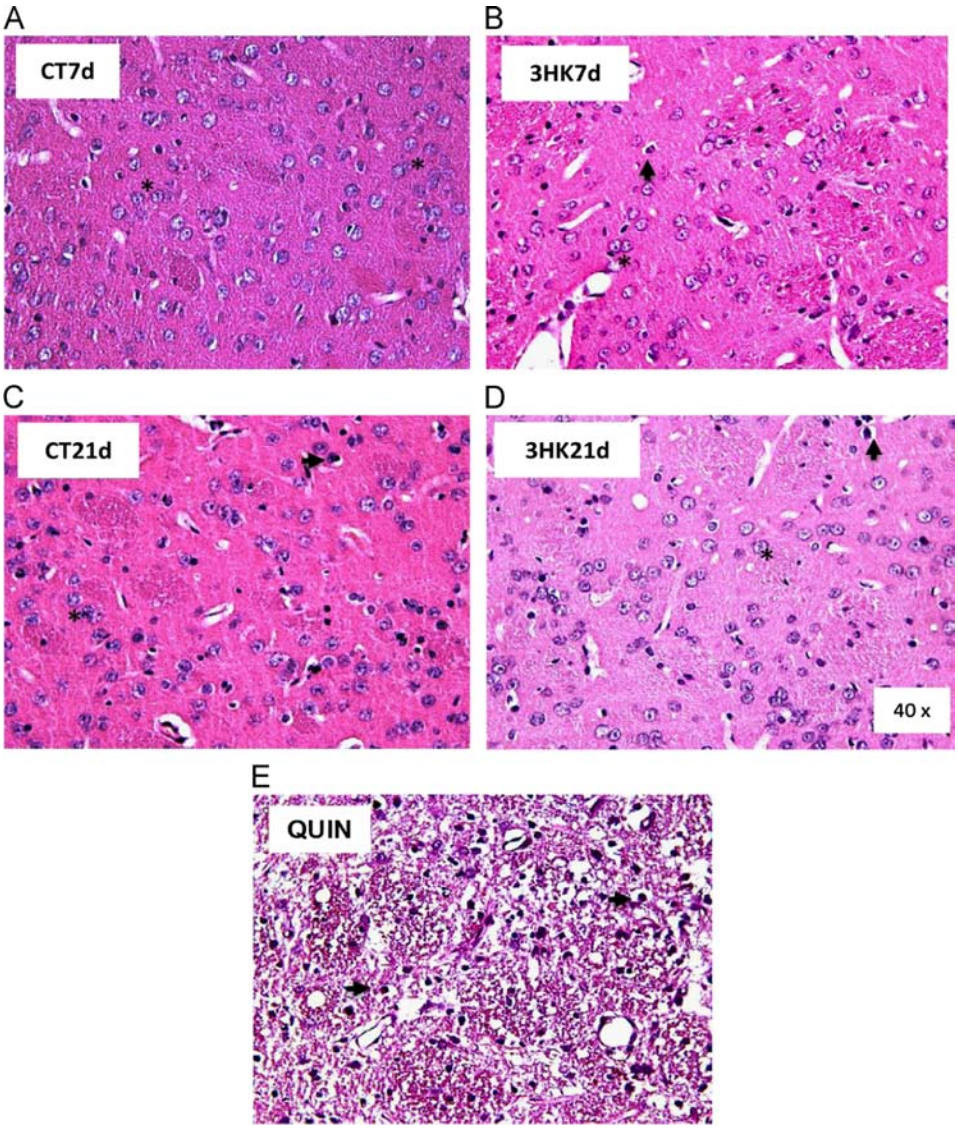

Fig. 9 – Time-course effect of the intrastriatal infusion of 3-hydroxykynurenine (3-HK) on striatal morphological changes estimated through the hematoxylin and eosin stain. Sham at 7 (A) and 21 (C) days post-lesion are contrasted with images of 3-HK-treated animals at 7 (B) and 21 (D) days post-lesion. No morphological changes were found. In (E), for comparative purposes, we included an image of the striatum of a QUIN-lesioned rat, where pyknosis, edema and massive cell death are appreciated. Scale, 40 ×. Representative images of all groups are presented.

terms, the appearance of the sham and 3-HK-treated striata at 7 and 21 days post-lesion were similar, exhibiting a well-preserved neuropil, with a majority of healthy cells (indicated by asterisks) and only a limited number of damaged cells (indicated by arrows). The proportion of preserved cells in sham and 3-HK-lesioned rats were also similar. No edema or vacuolization were seen in 3-HK-injected sections. This lack of morphological changes was congruent with the absence of apomorphine-challenged rotation behavior estimated in 3-HK-lesioned animals at 6 days post-lesion when compared with sham rats (data not shown). For comparative purposes, we included an image of the striatum of a rat lesioned with the neurotoxin QUIN at 7 days post-lesion (Fig. 9E), where it is evident the damage exerted on neuropil and cells, comprising edema and vacuolization.

3. Discussion

In this manuscript we explored the effects exerted by the kynurenine pathway metabolite 3-HK on oxidative damage and mitochondrial function in striatal slices, as well as on biochemical, behavioral and morphological endpoints in the striata of infused animals. At the beginning, our original hypothesis was oriented to declare this metabolite as a pro-oxidant and neurotoxic molecule, on the basis of previous reports and upon the assumption that the series of biochemical, behavioral and morphological tests developed in this study will indicate so. However, the experimental evidence collected in this study suggested a dual role of 3-HK either as a pro-oxidant or an antioxidant molecule, depending on the experimental conditions tested. Moreover, we found that

even under stimulated oxidative activity, 3-HK was unable not only to induce cell and tissue damage, but also prevented some damage elicited by other well-known toxins.

First, we described the time- and concentration-dependent effects of this metabolite in striatal slices (see Fig. 1). The most revealing condition was the incubation of slices in the presence of 3-HK for 6 h. It seems clear that it takes some time to the molecule to activate mechanisms of oxidative damage at relatively low concentrations (2–20 μM), and this was inferred because at 1 h of incubation, the effect of 3-HK remained close to baseline, whereas at 3 h of incubation an antioxidant effect was present at all concentrations tested. Noteworthy, at concentrations above 20 μM (50–100) the antioxidant effect prevailed at all times tested. The explanation of this dual behavior of 3-HK could be related to its redox properties and the balance between its reductive and oxidative precursor features. In this regard, the reductive, scavenging and antioxidant properties of this metabolite have been described at concentrations ranging those used by us in this study (Christen et al., 1990; Leipnitz et al., 2007; reviewed by Colín-González et al., 2013). However, if under certain conditions 3-HK preferentially undergoes auto-oxidation to form highly oxidant quinones, then its reductive properties could be surpassed by its nature as a precursor of free radicals and quinones. Thus, its effect seems to be a question of a delicate balance between antioxidant/reductive and pro-oxidant species, mostly depending on the experimental conditions employed. The concentration-dependent reductive character of 3-HK was clearly demonstrated by the FRAP assay (see Fig. 6).

Interestingly, 3-HK, at conditions promoting either oxidative damage to lipids (20 μM) or antioxidant actions (100 μM; see Fig. 1), was unable to induce mitochondrial dysfunction (cell damage) in the slices. Furthermore, contrary to what we expected, its co-incubation, at both concentrations, with well-known toxic agents not only did not potentiate its effect, but served to prevent the cell compromise exerted by QUIN, 3-NP and $FeSO_4$ (see Figs. 2–4), in mechanisms likely involving the compensatory activation of the antioxidant enzymes GST and SOD. The fact that 3-HK was more effective against a typical pro-oxidant model ($FeSO_4$) emphasizes its nature as a redox modulatory agent. Another possible contributing factor to the apparently protective action of 3-HK against different toxic models was suggested by our simple experiments presented in Fig. 5. The nuclear factor related to NF-E2, also known as Nuclear factor (erythroid-derived 2)-like 2, or simply Nrf2, is a transcription factor responsible for regulating the expression of detoxifying phase 2 enzymes, and is also known as the "master coordinator" of antioxidant responses (Kensler et al., 2007). The fact that 3-HK was able to induce an increased expression of Nrf2 accompanied by HO-1 and GCL-C protein levels in striatal slices is indicative of a concerted and complementary strategy to counteract the deleterious actions of reactive oxygen/nitrogen species (ROS/RNS). Hence, for the case of this metabolite, its reductive capacity, together with the recruitment of Nrf2 activation as part of its action pattern, seem to represent an integral antioxidant and potentially protective response. Although Nrf2 can also be activated by pro-oxidant toxins like QUIN (Colín-González et al., 2014), for the case of 3-HK and unlike QUIN, this effect seems to be sufficient to prevent or counteract cell damage. Nonetheless, the role of Nrf2 and its regulation by 3-HK deserves further and more detailed investigation.

For us, it was surprising to find a lack of effect of 3-HK on mitochondrial function. Since MTT reduction is accepted as an index of mitochondrial function and cell viability, we expected to find decreased levels induced by 3-HK on this endpoint. This expectation was based on evidence demonstrating that 3-HK, at the same concentration range employed by us is capable to induce cell death in different neuronal cell cultures, including cerebellar, striatal, hippocampal and cortical neurons, as well as in a human neuroblastoma SH-SY5Y cell line (Eastman and Guilarte, 1989; Okuda et al., 1998; Chiarugi et al., 2001; Jeong et al., 2004; Smith et al., 2009; reviewed by Colín-González et al., 2013). So, how to explain the toxic effects in these cultures vs. the lack of effect of 3-HK in the striatal slices? Probably the clue to solve this discrepancy is laying in evidence collected from experiments in glioma C6 cells exposed to 3-HK, where this metabolite did not produce cell death (Leipnitz et al., 2007). Since the KP takes place in glial cells (mostly astrocytes and microglia), and these cells naturally possess the enzymes necessary to degrade KP metabolites (Schwarcz et al., 2012), it seems obvious that these cells could be resistant to the actions of 3-HK, in contrast to isolated neuronal cells in culture that do not regularly possess these catalytic proteins. Therefore, a possible role of glial cells to handle 3-HK should not be discarded in this paradigm as glial cells also represent the first line of defense of the CNS against oxidative and inflammatory insults (Alarcón-Aguilar et al., 2014). We can then hypothesize that more integral biological preparations, such as tissue slices containing both neuronal and glial cells, could behave differently to primary cultures and cell lines – represented by a single type of cells – when exposed to 3-HK. Thus, the buffering and/or homeostatic role of glial cells might contribute to generate differential actions of 3-HK and other metabolites in the CNS.

At this point, our observations in striatal slices suggested that different experimental conditions rule the faith of the actions of this metabolite; however, what about the *in vivo* effects of 3-HK? Would the molecule exert toxic events *per se* in the striatum if infused to animals? Only a few reports about the toxic effects exerted by 3-HK under *in vivo* conditions are available in the literature. One of them, published by Nakagami et al. (1996), showed striatal degeneration at doses ranging 1–50 nmol/μl; though, the lack of images showing the control condition, and the fact that the image corresponding to the 3-HK-induced lesion depicts a limited area of damage remitted to the injection site, prompted to more detailed studies. Shortly thereafter, another article was published by Guidetti and Schwarcz (1999). This report established that 3-HK *per se* is unable to produce striatal damage, but when co-injected with its toxic relative QUIN at subtoxic doses, it potentiates the QUIN-induced degeneration. A possible contribution of 3-HK as a metabolic precursor of QUIN to exert tissue damage was discarded as 3-HK was unable to increase QUIN levels. Despite these reports support a toxic role of 3-HK under *in vivo* conditions, the question on whether 3-HK can be toxic *per se* for the striatal tissue remains unclear.

We therefore tested the effects of this metabolite when infused to the striatum of rats on different endpoints. The maximum dose of 3-HK tested was 80 nmol/μl, which

corresponded to the maximum concentration that we were able to dissolve. At such dose, the dual actions of 3-HK were evident once again as the levels of lipid and protein oxidation were increased at short times (6 and 24 h) and drastically decreased at longer times (7 days; see Fig. 7). These findings were in agreement not only with the biphasic action of this metabolite in striatal slices (see again Fig. 1), but also with the early stimulation of GR and GST activities (see Fig. 8). In this regard, the increased activity of the glutathione-related enzymes suggest that the early use of glutathione by the glutathione system in the striatum is being effectively used to reduce the risk of tissue damage, which was confirmed by the lack of behavioral alterations (data not shown), as well as the most conclusive evidence presented in this report: the lack of tissue damage in the striata of 3-HK-treated animals that were infused with a high dose of the metabolite (80 nmol; see Fig. 9). Even allowing the metabolite to act for 21 days, no major morphological changes were seen.

Altogether, this evidence suggest that the presence of 3-HK in the striatum at concentrations as high as those we tested here, does not represent *per se* a risk for neurodegeneration, and this might probably be due to either a limited and transitory permanence of the metabolite in this tissue – which in turn could be rapidly metabolized to other non-toxic forms – , or its intense redox activity oriented to activate antioxidant systems in an effective manner. Whatever the explanation, this topic deserves deeper investigation, with special regard to those conditions that could be responsible for the induction of 3-HK toxicity, if any, and whether they could take place under human pathological conditions.

3.1. *Concluding remarks*

In a recent review, we have compiled and revised evidence that 3-HK is augmented in some neurodegenerative disorders (reviewed by Colín-González et al., 2013). In principle, this evidence suggests that 3-HK may be playing a role in those neurodegenerative processes, thus accounting for neuronal disorders, and this possible role cannot be ruled out at all. However, considering the experimental evidence collected in this and other studies (Leipnitz et al., 2007), it is also reasonable to consider that the real role that 3-HK could be playing in different physiological and pathological conditions would be more related to a redox modulatory activity. This consideration points to compensatory actions exerted by this metabolite once inflammatory, oxidative and/or excitotoxic events have begun in the CNS, but these compensatory responses could not be sufficient to counteract the already-in-progress degeneration. This general concept is strengthened by the observation that the oxidative damage that 3-HK exerted in this study was insufficient to compromise cells, and this effect was probably due to the stimulation of antioxidant systems and the recruitment of the Nrf2 protective system. Thus, oxidative activity seems to be part of the strategy of 3-HK to compensate possible harmful signals in the striatum.

On the other hand, its potential role as a contributing factor to cell damage still remains open as, under certain circumstances, 3-HK can stimulate the formation of aggressive pro-oxidant molecules and could be also stimulating the formation of excitotoxic metabolites in KP as a precursor, but when this toxic character is adopted by 3-HK during neurodegenerative processes?

Another possibility, in light of our findings, is that the augmented levels of 3-HK in neurological disorders could be merely reflecting an increased activity of glial cells during the progression of neuronal cell damage. Therefore, clarifying its precise role in human pathologies is more than complicated, given the dual effects that this molecule is exerting. Further and more detailed studies are needed to respond the many questions raised by this and other reports. In the meantime, the results obtained in this work support the concept that 3-HK is not capable to exert toxicity *per se* and that its physiological role is more oriented to a redox modulatory activity. A schematic representation of the actions exerted by this metabolite in the CNS is presented in Fig. 10.

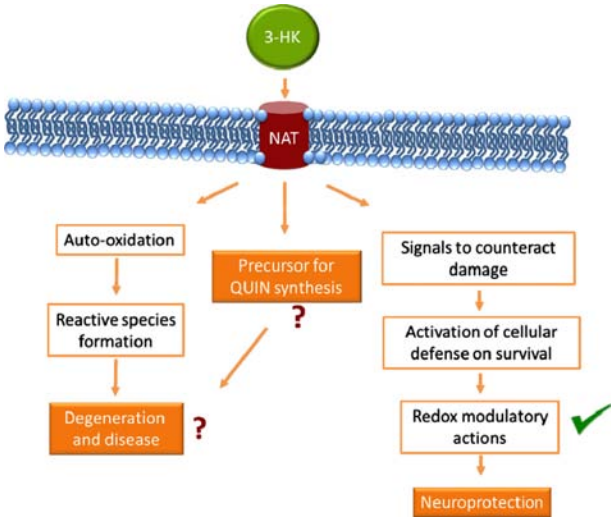

Fig. 10 – Schematic representation of the hypothetical mechanisms involved in the 3-hydroxykynurenine (3-HK) actions in the rat striatum. 3-HK would be entering cells through neutral amino acid transporters (NAT), and once in the cytoplasm it could follow different paths. One of them is related with auto-oxidation and further formation of reactive oxygen species (ROS), which might be responsible or cell damage and degeneration (not supported by findings of this work), or simply activating antioxidant survival mechanisms. The other path, which is more according to our findings, could be responsible for antioxidant defense signaling and neuroprotection. The effects exerted by 3-HK in this work clearly supports the activation of survival and protective mechanisms *via* the stimulation of adaptive and protective responses to counteract oxidative stress.

4. Experimental procedures

4.1. *Reagents*

3-HK, QUIN, 3-NP, thiobarbituric acid (TBA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glucose-6-phosphate and other reagents were obtained from Sigma/Aldrich

Co. (St. Louis, MO, USA). All other chemicals were obtained from other well-known commercial sources.

4.2. FRAP assay

For the FRAP assay, we followed the method described by Benzie and Strain (1996), with modifications. We employed this method to investigate the reductive potency of 3-HK *per se*. Briefly, the FRAP reagent (A) consisted of a 10:1:1 mixture containing 300 mM acetate buffer (3.1 g of sodium acetate plus 16 ml of acetic acid for 1 l)+20 mM $FeCl_3$+10 mM TPTZ (2,4,6-tripiridyl-s-triazine)+40 mM HCl, was prepared at 50 °C and used throughout the experiments. The reaction solution (C) consisted of 480 ml of deionized H_2O (B) plus 500 ml of reagent A. Solution C was incubated at 37 °C for 10 min and immediately added with 20 μl of the solution containing 3-HK at different concentrations. Samples were re-incubated for 30 min and optical density was recorded by triplicate at 593 nm in a Thermo Spectronic Genesys 8 Spectrometer. A standard curve was constructed using increasing concentrations of $FeSO_4$ (50–2000 μM).

4.3. Animals

Male Wistar rats weighing 270–310 g (60 animals total, 5–10 per group), bred-in-house strain, were used throughout the study. Animals were housed five per cage in acrylic cages and provided with Rodent Chow (Purina, St. Louis, MO, USA) and water *ad libitum*. Animals were maintained under conditions of constant temperature (25±3 °C), humidity (50±10%) and lighting (12:12 1ight:dark cycle). All experimental manipulations were performed according to the "Guidelines for the Use of Animals in Neuroscience Research" from the Society of Neuroscience, the local Ethical Committees, and in compliance of the ARRIVE guidelines.

4.4. Isolation of striatal slices (in vitro experiments)

Striatal slices were obtained and collected according to a procedure previously described (Colín-González et al., 2014). Rats were decapitated and their brains were rapidly placed in ice-cold Krebs-bicarbonate dissection buffer pH 7.4 (120 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM glucose and 200 mM sucrose). Chamber solutions were pre-bubbled with 95% O_2/5% CO_2 gas mixture, for at least 45 min before slice immersion, to ensure O_2 saturation. Striata were quickly dissected, glued down leaning vertically against agar blocks in a small chamber, submerged in cold oxygenated dissection buffer and sectioned in 200 μm thick transverse slices using vibratome (TS1000 Leica; Heidelberg, Germany). Cutting parameters were 0.5 mm/s, 60 Hz and 0.8 mm amplitude. Immediately after vibratome sectioning, the slices were transferred to a vial of sucrose-free dissection buffer, and bubbled with 95% O_2/5% CO_2 at room temperature for 30 min to recover from slicing trauma. Then, slices were exposed to different concentrations of 3-HK (2–100 μM), for different times of incubation in a shaking water bath at 37 °C. Immediately after incubated, slices were placed on ice, collected and scheduled for measurement of different experimental parameters. In additional experiments, some toxic agents were added to the slices (1 mM 3-NP and QUIN, and 50 μM $FeSO_4$) and tested in the presence of 3-HK.

4.5. Lipid peroxidation

Lipid peroxidation was determined as the formation of thiobarbituric acid-reactive substances (TBARS), according to a previous report (García et al., 2008). Fifty μL aliquots of the homogenates obtained from striatal slices were added to 100 μL of the TBA reagent (0.75 g of TBA+15 g of trichloroacetic acid+2.54 mL of HCl) and incubated at 100 °C for 20 min. A pink chromophore was produced in samples in direct proportion to the amount of peroxidized products. Samples were then kept on ice for 5 min and centrifuged at 3000*g* for 15 min. The optical density from the supernatants was measured in a Thermo Spectronic Genesys 8 Spectrometer at 532 nm. Final amounts of TBA-RS – mostly malondialdehyde (MDA) – were calculated by interpolation of values in a constructed MDA standard curve, and results were calculated as nmoles of MDA per mg protein. This method was used for estimation of lipid peroxidation both in slices and striatal tissue.

4.6. Functional assessment of striatal slices by MTT reduction assay

In order to assess the viability of cells from the striatal slices, MTT reduction was measured as a current index of the functional status of the respiratory chain and mitochondrial function. This endpoint was estimated in striatal slices, according to a method previously described (Pérez-De La Cruz et al., 2007). Briefly, the slices already exposed to different treatments were added with 8 μl of MTT (5 mg/ml), and re-incubated at 37 °C for 60 min. The samples were then centrifuged at 15,300*g* for 15 min, and pellets were resuspended in 1 ml of isopropanol. During this first step of centrifugation, the aqueous phase was discarded. A second step of centrifugation was then performed at 1700*g* for 3 min. Quantification of formazan was estimated in supernatants in a Thermospectronic Genesys 8 spectrometer (Cole-Parmer, Vernon Hills, Ill., USA) by measuring optical density at a 570-nm wavelength. The results were expressed as the percentage of MTT reduction with respect to control values.

4.7. Surgical lesion technique and treatment (in vivo experiments)

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). 3-HK was dissolved in distilled water and adjusted to pH 7.4. Single unilateral injections were made with a Hamilton syringe into the right striatum at the stereotaxic coordinates 0.5 mm anterior to bregma, 2.6 mm lateral to bregma and 4.5 mm ventral to the dura, according to the brain Atlas of 3-HK was injected at different doses (5, 10, 20, and 80 nmol/μl). The needle was left in place for another 2 min and then, slowly withdrawn. Control animals were similarly injected with isotonic saline solution. Animals were sacrificed at 0.5 and 2 h; 7, 14 and 21 days.

4.8. Rotation behavior test

Motor alterations, assessed as rotation behavior, were evaluated in animals from all experimental groups, according to previous reports (Silva-Adaya et al., 2008). Six days after 3-HK (80 nmol) infusion, animals were administered with apomorphine (1 mg/kg, s.c.) and separated into individual acrylic box cages. Five minutes later, the number of ipsilateral rotations to the lesioned side was recorded for 1 h. Each rotation was evaluated as a complete turn (360°). Data are expressed as the total number of ipsilateral turns in 1 h.

4.9. Histological examination

Seven, 14 and 21 days after performing the striatal lesions, animals from all groups were anesthetized i.p. with 0.5 mL of sodium pentobarbital and perfused transcardially with 0.9% saline solution containing heparin (200/1 v/v), followed by 4% paraformaldehyde at 4 °C. Brains were removed, post-fixed in 4% p-formaldehyde for 7 days and embedded in paraffin. Fixed tissues were serially sectioned in an 820 HistoSTAT microtome (American Instrument Exchange, Inc., Haverhill, MA, USA). Striatal sections (5 μm-thick) were obtained every 100 μm, covering a total distance of 300 μm (100 μm anterior and 100 μm posterior to the needle tract). All sections were stained with hematoxylin-eosin (H&E) to visualize cell bodies, using an image analyzer IM100 (Leica Cambridge, UK).

4.10. Protein carbonyl content

As an index of protein oxidation, protein carbonyl content in the striatal tissue (2 and 6 h, and 1 and 7 days post-lesion) was determined as previously described (Chevion et al., 2000). Assessment of carbonyls formation was done on the basis of formation of protein hydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH). Supernatants were incubated with 10% streptomycin sulfate to remove nucleic acids overnight, and centrifuged at 21,000*g* at 4 °C for 20 min. Then, supernatants were treated with 10 mM DNPH (in 2.5 M HCl) for 1 h at room temperature, and 10% TCA was added and centrifuged at 2500*g* at 4 °C for 10 min. Pellets were washed three times with ethanol/ethyl acetate (1:1), dissolved with 6 M guanidine hydrochloride (in phosphate buffer 20 mM, pH 7.4), and centrifuged at 5000*g* at 4 °C for 3 min to remove insoluble material. Absorbance was measured at 370 nm. Protein carbonyl content is expressed as nmol DNPH/mg protein, using the molar absorption coefficient of DNPH (22,000 $M^{-1}\,cm^{-1}$). Total protein concentration was obtained by reading optical density at 280 nm in blank tubes prepared in parallel (treated only with 2.5 M HCl), using a standard curve of bovine serum albumin (0.25–2 mg/ml) prepared in 6 M guanidine hydrochloride.

4.11. Antioxidant enzymes activity assays

Striatal samples were homogenized in 500 ml of lysis buffer pH 7.4 (containing 10 mM Tris–HCl, 15 mM NaCl, 0.25 mM sucrose and proteases inhibitors), and centrifuged at 13,000*g* for 30 min. The supernatants were used to determine gluthatione peroxidase (GPx), glutathione reductase (GR), gluthatione-S-transferase (GST), and glucose 6-phosphate dehydrogenase activities (G6PD).

4.11.1. Assessment of GPx activity

Reaction mixture consisted of 50 mM potassium phosphate (pH 7.0), 1 mM EDTA, 1 mM Sodium azide, 0.2 mM NADPH, 1 U/ml of glutathione reductase, and 1 mM GSH. One hundred microliters of homogenates were added to 0.8 ml of mixture and allowed to incubate for 5 min at room temperature before initiation of the reaction by the addition of 0.1 ml 0.25 mM H_2O_2 solution. Absorbance at 340 nm was recorded for 5 min, and the activity was calculated from the slope of these lines as moles of NADPH oxidized per min. Blank reactions with homogenates replaced by distilled water were subtracted from each assay. GPx activity was expressed as U/mg protein.

4.11.2. Assessment of GST Activity

In order to evaluate this enzyme activity, we employed the method described by Habig et al. (1974), with modifications. One hundred microliters of homogenates were added 50 mM potassium phosphate (pH 6.5), 20 mM 50 mM GSH, and 1-chloro-2,4-dinitrobenzene (CDNB) as substrate in a total volume 1 ml. Optical density was detected at 340 nm. The results were expressed as nmol CDNB conjugate formed/min/mg protein.

4.11.3. Assessment of GR Activity

The reaction mixture consisted of 100 mM potassium phosphate (pH 7.6), 0.5 mM EDTA, 1 mM NADPH, and 1.1 mM GSSG. One hundred microliters of homogenates were added to 0.9 ml of mixture. Absorbance at 340 nm was recorded for 3 min, and the activity was calculated from the slope of these lines as moles of NADPH oxidized per min. Blank reactions with homogenates replaced by distilled water were subtracted from each assay. GR activity was expressed as U/mg protein.

4.11.4. Assessment of G6PD Activity

The reaction mixture consisted of 50 mM Tris–HCl buffer (pH 7.8) containing 33 mM $MgCl_2$, 6 mM nicotinamide adenine dinucleotide phosphate (NADP), and 100 mM G6P. One hundred fifty microliters of homogenates were added to 0.85 ml of mixture. Absorbance at 340 nm was recorded for 6 min, and the activity was calculated from the slope of these lines as moles of NADP per min. Blank reactions with homogenates replaced by distilled water were subtracted from each assay. G6PD activity was expressed as U/mg protein.

4.12. Statistical analysis

Results were expressed as mean values ± S.E.M. All data were statistically analyzed using one-way analysis of variance (ANOVA) for repeated measures, followed by *post hoc* Tukey's. All analytical procedures were performed using the scientific statistic software GraphPad Prism 5 (GraphPad Scientific, San Diego, CA, USA). Differences of $P<0.05$ were considered as statistically significant.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by CONACyT Grant 205648 (A.S.). Ana Laura Colín-González is scholarship holder of CONACyT-Mexico (239954). Authors wish to thank Dr. Irma González-Herrera for her technical support and to Dr. Enrique Pinzón and Dr. Ismael Torres for the technical assistance with the experimental animals.

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9.2. El QUIN ESTIMULA LA ACTIVACIÓN DEL NRF2 DE FORMA TRANSITORIA

Siguiendo el razonamiento expuesto anteriormente que postula a la 3-HK como una molécula inductora de respuestas antioxidantes y a los reportes que indican que es una neurotoxina, se creó la siguiente hipótesis: El QUIN es una molécula que en concentraciones elevadas induce efectos tóxicos en el SNC. La 3-HK se transforma en QUIN, por lo que los deletéreos observados podrían ser consecuencia de una mayor producción de QUIN.

El QUIN (μM) estimuló la translocación nuclear del Nrf2 de forma transitoria y aumentó la expresión proteica de la hemo-oxigenasa-1. A diferencia de lo ocurrido con la 3-HK, estos eventos no fueron suficientemente fuertes para disminuir el daño oxidante. Esto sugiere que el QUIN genera ambiente que estimula la activación del Nrf2 en un intento para disminuir su toxicidad, sin tener éxito.

Se ha sugerido la modulación del Nrf2 como estrategia terapéutica en diversos modelos tóxicos a través del empleo de antioxidantes y/o electrófilos. En el siguiente estudio demostró el efecto protector del ácido cafeico, un antioxidante de amplio espectro, en rebanadas incubadas con QUIN y en animales inyectados intraestriatalmente con la misma toxina. Como se esperaba, el ácida cafeico previno las alteraciones conductuales causadas por la administración de QUIN, mientras que en rebanadas estriatales disminuyó el daño oxidante.

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EARLY MODULATION OF THE TRANSCRIPTION FACTOR NRF2 IN RODENT STRIATAL SLICES BY QUINOLINIC ACID, A TOXIC METABOLITE OF THE KYNURENINE PATHWAY

A. L. COLÍN-GONZÁLEZ,[a†] A. LUNA-LÓPEZ,[b] M. KÖNIGSBERG,[c] S. F. ALI,[d] J. PEDRAZA-CHAVERRÍ[e] AND A. SANTAMARÍA[a*]

[a] *Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, S.S.A., Mexico City, Mexico*

[b] *Instituto Nacional de Geriatría, S.S.A., Mexico City, Mexico*

[c] *Departamento de Ciencias de la Salud, DCBS, Universidad Autónoma Metropolitana Iztapalapa, Mexico City, Mexico*

[d] *Division of Neurotoxicology, National Center for Toxicological Research, FDA, Jefferson, AR, USA*

[e] *Departamento de Biología, Facultad de Química, Universidad Nacional Autónoma de México, Mexico City, Mexico*

Abstract—Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor involved in the orchestration of antioxidant responses. Although its pharmacological activation has been largely hypothesized as a promising tool to ameliorate the progression of neurodegenerative events, the actual knowledge about its modulation in neurotoxic paradigms remains scarce. In this study, we investigated the early profile of Nrf2 modulation in striatal slices of rodents incubated in the presence of the toxic kynurenine pathway metabolite, quinolinic acid (QUIN). Tissue slices from rats and mice were obtained and used throughout the experiments in order to compare inter-species responses. Nuclear Nrf2 protein levels and oxidative damage to lipids were compared. Time– and concentration–response curves of all markers were explored. Nrf2 nuclear activation was corroborated through phase 2 antioxidant protein expression. The effects of QUIN on Nrf2 modulation and oxidative stress were also compared between slices of wild-type ($Nrf2^{+/+}$) and Nrf2 knock-out ($Nrf2^{-/-}$) mice. The possible involvement of the N-methyl-D-aspartate receptor (NMDAr) in the Nrf2 modulation and lipid peroxidation was further explored in mice striatal slices. In rat striatal slices, QUIN stimulated the Nrf2 nuclear translocation. This effect was accompanied by augmented lipid peroxidation. In the mouse striatum, QUIN *per se* exerted an induction of Nrf2 factor only at 1 h of incubation, and a concentration–response effect on lipid peroxidation after 3 h of incubation. QUIN stimulated the striatal content of phase 2 enzymes. $Nrf2^{-/-}$ mice were slightly more responsive than $Nrf2^{+/+}$ mice to the QUIN-induced oxidative damage, and completely unresponsive to the NMDAr antagonist MK-801 when tested against QUIN. Findings of this study indicate that: (1) Nrf2 is modulated in rodent striatal tissue in response to QUIN; (2) $Nrf2^{-/-}$ striatal tissue was moderately more vulnerable to oxidative damage than the Wt condition; and (3) early Nrf2 up-regulation reflects a compensatory response to the QUIN-induced oxidative stress in course as part of a general defense system, whereas Nrf2 down-regulation might contribute to more intense oxidative cell damage.

Key words: kynurenine pathway, oxidative stress, antioxidant defense, excitotoxicity, Nrf2.

INTRODUCTION

Neurodegenerative processes represent the major cause of neurological disorders in human beings (Coyle and Puttfarcken, 1993; Santamaría and Jiménez, 2005). Disorders coursing with neurodegeneration, such as Huntington's disease (HD), share common key triggering factors that activate cell damage. These factors include excitotoxicity, mitochondrial energy depletion, oxidative stress and inflammation (Zádori et al., 2012). Together, these components establish a complex toxic scenario that is responsible for neuronal cell degeneration and death. Excitotoxicity, a toxic event defined as a persistent stimulation of membrane receptors in neuronal cells (Olney, 1990), is characterized by a cascade of processes comprising increased levels of intracellular calcium in response to a continuous opening of Ca^{2+} channels associated to glutamate receptors activation after sustained exposure to excitatory amino acids (reviewed by Essa et al. (2013), Mehta et al. (2013)). In turn, enhanced intracellular Ca^{2+} levels trigger lethal metabolic pathways, further leading to continuous enzyme activation, enhanced reactive oxygen and nitrogen species (ROS/RNS) formation, mitochondrial

*Corresponding author. Address: Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, S.S.A., Mexico City 14269, Mexico. Tel: +52-55-5606-3822x2013.
E-mail address: absada@yahoo.com (A. Santamaría).

† Doctoral Program in Biomedical Sciences, Universidad Nacional Autónoma de México (UNAM).

Abbreviations: γ-GCL-C, gamma-glutamylcysteine ligase-C; ARE, antioxidant response element; DMF, dimethylfumarate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; HD, Huntington's disease; HEPES, hydroxyethyl piperazineethanesulfonic acid; HO-1, heme oxygenase-1; KP, Kynurenine pathway; MDA, malondialdehyde; MK-801, dizocilpine; NAD(P)H, nicotinamide adenine dinucleotide phosphate-oxidase; NMDAr, N-methyl-D-aspartate receptor; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; QUIN, quinolinic acid; ROS, reactive oxygen species; SAC, S-allyl cysteine; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; tBHQ, tert-butylhydroquinone.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.12.025

dysfunction (Rami et al., 1997), and necrotic or apoptotic cell death.

Kynurenine pathway (KP), a metabolic pathway involved in tryptophan degradation to synthesize NAD^+, has gained increasing attention because some of its intermediary metabolites possess neuroactive properties, exerting either excitatory or inhibitory actions in the CNS (reviewed by Pérez-De La Cruz et al. (2007)). Some KP metabolites have been involved in pathological conditions, triggering factors for degenerative events in neurological disorders (reviewed by Schwarcz et al. (2010, 2012)). One of these metabolites, quinolinic acid (QUIN or 2,3-pyridine dicarboxylic acid), is an endogenous glutamate agonist acting on selective populations of N-methyl-D-aspartate receptors (NMDAr) (Stone et al., 2003). QUIN has been recognized as an experimental tool to produce excitotoxicity and oxidative stress under *in vitro* and *in vivo* conditions, and its toxic pattern includes enhanced intracellular Ca^{2+} levels, GABA depletion, increased ROS formation, decreased activity and expression of antioxidant systems, oxidative stress, increased protease activity and cell death (Rios and Santamaría, 1991; Rodríguez-Martínez et al., 2000; Braidy et al., 2009, 2010; Pérez-De La Cruz et al., 2010). Several studies have demonstrated that QUIN toxicity can be reduced or prevented by the use of antioxidants, including ebselen (Rossato et al., 2002), L-carnitine (Silva-Adaya et al., 2008), S-allylcysteine (Pérez-Severiano et al., 2004), guanosine (Dobrachinski et al., 2012), probucol (Colle et al., 2012), caffeic acid (Kalonia et al., 2009), polyphenolic compounds (Braidy et al., 2010), and the combination of non-effective concentrations of glutamatergic modulators plus antioxidants (Dobrachinski et al., 2012). Considering that increased levels of this toxic metabolite have been reported in several neurodegenerative disorders (Schwarcz et al., 2010), its role as a pathogenic factor in the brain is a matter under continuous investigation; therefore, the study of the pro-oxidant effects and mechanisms exerted by QUIN in the CNS is of major importance for biomedical research.

The nuclear transcription factor related to NF-E2 (also known as Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)) constitutes a promising tool to counteract the deleterious effects of oxidative stress through the up-regulation of endogenous antioxidant genes. Nrf2 is considered a master regulator of redox homeostasis because it up-regulates the expression of more than 100 genes (phase 2 enzymes) involved in xenobiotic and ROS detoxification (Itoh et al., 1999; Kensler et al., 2007). Phase 2 enzymes share a common promoter enhancer known as antioxidant response element (ARE) that is regulated by Nrf2. Among many proteins encoded by this gene are heme oxygenase-1 (HO-1), superoxide dismutase (SOD), NAD(P)H quinone oxidoreductase 1 (NQO-1), glutathione peroxidase (GPx), glutathione reductase (GR), and γ-glutamyl cysteine ligase (γ-GCL), just to mention a few (Itoh et al., 1999; Johnson et al., 2002; Lee et al., 2003; Kensler et al., 2007).

This study was designed to investigate how Nrf2, a well-known orchestrator of antioxidant responses in mammals, is regulated by QUIN at early times of incubation. Its development emerges from the need to offer more accurate information on this topic because: (1) QUIN is a toxic metabolite with relevance for the explanation of neurodegenerative events in neurological disorders (Schwarcz et al., 2012); and (2) to date, there is only limited information available on this emerging issue. Precisely, one of the fewest reports dealing with this issue was published by our group (Tasset et al., 2010). In that report, we described positive actions of the antioxidant and well-known Nrf2 inducer, tert-butylhydroquinone (tBHQ) in this toxic model. tBHQ exerted protective effects in different markers of oxidative damage induced by QUIN. We also described a depleting effect of QUIN on the Nrf2 levels in rat striatal slices. However, this first approach was inaccurate because we described a form of Nrf2 corresponding to 57 kDa. Recently, a report by Lau et al. (2013) discussed evidence pointing out that the real biologically relevant molecular weight of mammalian Nrf2 is around 95–110 kDa, and this is due to the fact that multiple acidic residues in Nrf2 promote its gain in molecular weight. Derived from this observation, herein we characterized the effect of QUIN on the striatal levels of the ~98 kDa Nrf2 form, together with some related functional markers of oxidative stress. Therefore, striatal slices obtained from adult male rats and mice were challenged with this toxicant and further used throughout the study for comparative purposes.

EXPERIMENTAL PROCEDURE

Chemicals

QUIN, MK-801, HEPES, thiobarbituric acid (TBA), malondialdehyde (MDA), 2,3,5-triphenyltetrazolium chloride (TTC) and other reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were obtained from other commercial sources. Antibodies employed along the study are described forward (in Section ''Immunoblotting'').

Animals – rats

Adult male Wistar rats (250–300 g) were used throughout the first part of the study. Animals (n = 25) were housed five per cage in acrylic cages and provided with food and water *ad libitum*. The housing rooms at the vivarium (facilities of the Instituto Nacional de Neurología y Neurocirugía) were maintained under constant conditions of temperature (25 ± 3 °C), humidity and light cycles (12:12 light:dark schedule). All experimental manipulations were performed according to the ''Guidelines for the Use of Animals in Neuroscience Research'' from the Society of Neuroscience, the local Ethics Committees, and in compliance with the ARRIVE guidelines.

Animals – mice

All animal protocols with mice followed Institutional and European guidelines (86/609/EEC, 2003/65/EC European Council Directives). Experimentation with mice was also in compliance with the ARRIVE guidelines. Mice ($n = 24$) were housed at room temperature under a 12-h light–dark cycle. Food and water were provided *ad libitum*. Mouse genotyping was done according to previous reports (Itoh et al., 1997; Tsuchihashi et al., 2006). Six-month-old male wild-type C57BL/6 mice ($n = 6$ per group) and Nrf2-knockout littermates ($n = 6$ per group) were kindly provided by Dr. Antonio Cuadrado and used throughout the study.

Isolation of striatal slices and treatments

Striatal slices were collected strictly according to procedures previously described (Rojo et al., 2008). Rats and mice were decapitated and their brains were rapidly dissected out. Striata were then isolated, sectioned using a vibratome (TS1000 Leica; Heidelberg, Germany), and kept in Krebs solution until the beginning of the experiments. Five slices per probe (250–300 μm thickness) were used. Depending on the experimental design, slices were exposed to different concentrations of QUIN (25–100 μM), MK-801 (50 μM), H_2O_2 (100 μM), $FeSO_4$ (50 μM) or Krebs solution for different times of incubation in a shaking water bath at 37 °C. Immediately after incubation, slices were placed on ice, collected and scheduled for measurement of different experimental parameters.

Nuclear fractions preparation

To estimate nuclear Nrf2, striatal slices were washed once with cold PBS and lysed on ice with cold buffer A (250 mM sucrose, 20 mM HEPES (pH 7.0), 0.15 mM EDTA, 0.015 mM EGTA, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM Na_3VO_4 and 1 μg/mL leupeptin plus 1% Nonidet P-40). Homogenates were centrifuged at 500*g* for 5 min. Nuclear pellets were washed in cold buffer B (10 mM HEPES (pH 8.0), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 0.1 M NaCl, 1 μg/mL leupeptin and 1 mM Na_3VO_4 plus 25% glycerol). After centrifugation at 500*g* for 5 min, nuclei were resuspended in RIPA buffer (50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4). Proteins of both fractions were resolved by SDS–PAGE and immunoblotted with the indicated antibodies (Espada et al., 2010).

Immunoblotting

Striatal slices were washed once with cold PBS and lysed on ice with lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris–HCl (pH 7.5), 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4). Cell lysates were pre-cleared by centrifugation and the protein concentration was quantified by Bradford's method (Bradford, 1976); then, protein extracts were resolved by SDS–PAGE using 80 μg (nuclear fraction) of protein per lane (see below the assay for separation of fractions), and transferred to Immobilon-P membranes (PVDF, Millipore, Corporation, Billerica, MA, USA). Blots were analyzed with the appropriate primary antibodies (1:1,000): anti-Nrf2, anti-Lamin B, anti-Actin, anti-Histone 1 and anti-GAPDH, all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against antioxidant enzymes (gamma-glutamylcysteine ligase-catalytic subunit (γ-GCL-C), HO-1 and SOD) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Peroxidase-conjugated secondary antibodies (1:10,000) were used to detect the proteins of interest by an enhanced chemiluminescence kit. Secondary antibodies were goat anti-rabbit HRP from Zymed (San Francisco, CA, USA) and goat anti-mouse HRP from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Lipid peroxidation

Lipid peroxidation was determined as the formation of thiobarbituric acid-reactive substances (TBARS), according to a previous report (García et al., 2008). Two hundred-μL aliquots of the homogenates obtained from striatal slices were added to 500 μL of the TBA reagent (0.75 g of TBA + 15 g of trichloroacetic acid + 2.54 mL of HCl) and incubated at 100 °C for 30 min. A pink chromophore was produced in samples in direct proportion to the amount of peroxidized products. Samples were then kept on ice for 5 min and centrifuged at 3000*g* for 15 min. The optical density from the supernatants was measured in a Thermo Spectronic Genesys 8 Spectrometer at 532 nm. Final amounts of TBARS—mostly MDA—were calculated by interpolation of values in a constructed tetramethoxypropane standard curve, and results were calculated as nmoles of MDA per mg protein.

Statistical analysis

Results were expressed as mean values ± one S.E.M. All data were statistically analyzed using a one-way analysis of variance (ANOVA) for repeated measures, followed by *post hoc* Tukey's test. All analytical procedures were performed using the scientific statistic software GraphPad Prism 5 (GraphPad Scientific, San Diego, CA, USA). Differences of $P < 0.05$ were considered as statistically significant.

RESULTS

QUIN induced an early increase of nuclear Nrf2 levels in rat striatal slices

First, we explored the modulation of the ~98-kDa Nrf2 form reported by Lau et al. (2013). We wanted to investigate whether this phenomenon is dependent on the presence of QUIN and if it can be modulated by tBHQ, a well-known Nrf2 modulator. For this purpose, rat striatal slices were incubated in the presence of

100 μM QUIN at 6 h (short-term effect) and/or 25 or 50 μM tBHQ. QUIN exerted a stimulatory effect on nuclear Nrf2 levels, reaching significant levels compared with control (42% of increase; Fig. 1A, B). Cytoplasmic Nrf2 levels were slightly stimulated by QUIN (18% above the control). In turn, tBHQ (25 μM) increased the nuclear and cytoplasmic Nrf2 levels *per se* (25% and 43% above the control, respectively). Interestingly, when co-incubated with QUIN, 25 μM tBHQ stabilized Nrf2 levels to basal values, but at the 50 μM concentration, a significant stimulation in the nuclear levels of Nrf2 levels was induced by the coordinated action of these two agents (58% above the control).

QUIN increased lipid peroxidation in a concentration-dependent manner in rat striatal slices

Since nuclear Nrf2 translocation is also known to obey pro-oxidant stimuli, in order to further know if the changes in nuclear levels of Nrf2 induced by QUIN were related to markers of oxidative cell damage, we estimated the concentration–response effect of QUIN on lipid peroxidation (Fig. 2) in rat striatal slices at 6 h of incubation. The toxin exerted a concentration–response effect on lipid peroxidation, achieving a peak with the 100 μM concentration (71% of increase vs. control values), and this effect seems to be correlated with the increase in the levels of nuclear Nrf2 observed in Fig. 1. The changes in lipid peroxidation induced by QUIN in a concentration-dependent manner clearly suggest that the pro-oxidant environment in our experiments represent a potential triggering factor for the transactivation of Nrf2.

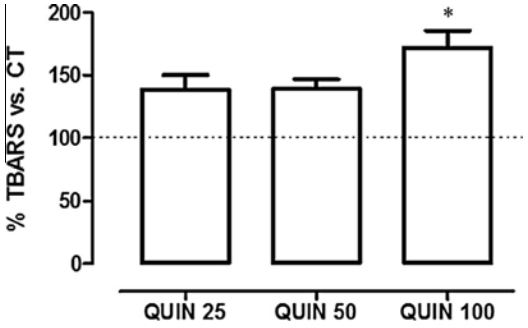

Fig. 2. Concentration–response effect of QUIN on lipid peroxidation in rat striatal slices. Lipid peroxidation was estimated at 6 h of incubation with increasing concentrations of QUIN (25–100 μM). Data are expressed as mean values ± S.E.M. of $n = 6$ experiments per group. One-way ANOVA followed by *post hoc* Tukey's test for comparisons against the control. Asterisks denote statistically significant differences ($^{*}P < 0.05$) vs. control.

QUIN enhanced lipid peroxidation in striatal slices of wild-type (Wt) C57BL/6 mice

We then started our work with mice tissue. First, we characterized the ability of QUIN to induce oxidative damage to lipids in striatal slices of Wt mice that were incubated for 3 h in the presence of the toxin in order to establish a concentration–response effect. QUIN induced a maximum peroxidative effect at 50 μM (233% above the control). The toxin also enhanced oxidative damage to lipids at 100 μM (108% above the control). A positive control of oxidative damage (50 μM $FeSO_4$) was included in the experiment, but the extent of lipid peroxidation induced by this condition was only moderate (Fig. 3).

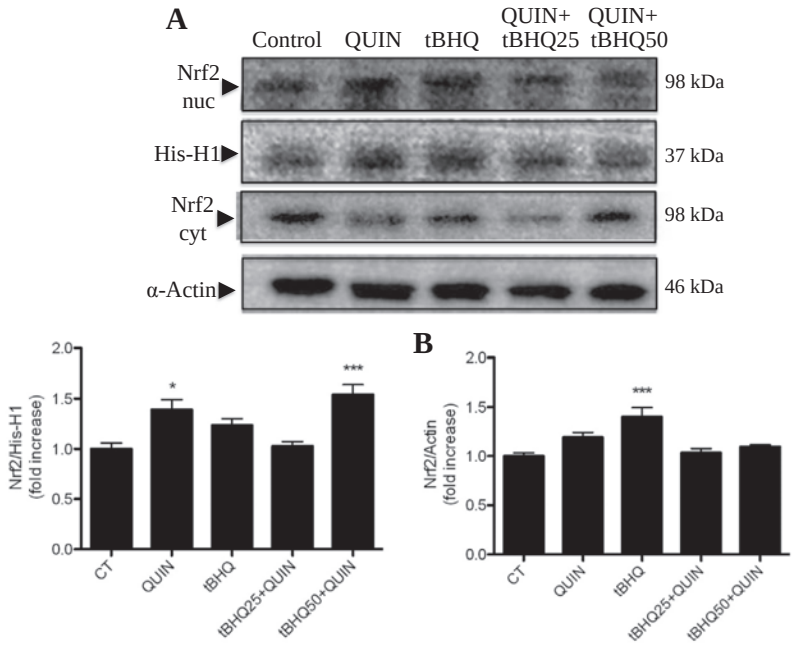

Fig. 1. Effects of QUIN (100 μM) and tBHQ (25 and 50 μM) on nuclear and cytoplasmic Nrf2 levels in rat striatal slices. In (A), immunoblot for nuclear Nrf2 after 6 h of incubation with QUIN and/or tBHQ. In (B), densitometric analysis of immunoblot from (A). In (B), data are expressed as mean values ± S.E.M. of $n = 3$ experiments per group. One-way ANOVA followed by *post hoc* Tukey's test for multiple comparisons. Asterisks denote statistically significant differences ($^{*}P < 0.05$ and $^{***}P < 0.01$) vs. control.

QUIN augmented nuclear Nrf2 levels in an early manner in mice striatal slices

The striatal content of Nrf2 induced at different times of incubation of the slices in the presence of QUIN (50 μM) is presented in Fig. 4. The time-course of Nrf2 induction by QUIN revealed a transitory and significant stimulation of this factor at short times of incubation (0.5–4 h), peaking at 1 h (231% above the control), and then returning to baseline. Thus, the early and transitory increase of Nrf2 induced by QUIN followed by its partial fall could be interpreted as an early compensatory attempt of cells to counteract oxidative toxicity already in course.

QUIN induced changes in HO-1, SOD and GCL-C protein levels in striatal slices

We further investigated whether the toxin *per se* could be able to induce redox alterations characterized by increased expression of representative phase 2 antioxidant enzymes (HO-1, SOD and GCL-C) in direct correlation to its stimulatory role in the Nrf2 regulation observed in Fig. 4. For this purpose, striatal slices were incubated for 3 h in the presence of QUIN, a time that was assumed to involve an early transactivation of Nrf2. QUIN (50 μM) enhanced the HO-1 levels compared to control (1.5-fold). In the case of SOD regulation, QUIN stimulated the enzyme levels by 50% above the control. Under the same experimental conditions, QUIN induced an increase of GCL-C levels (28%) compared with the control (Fig. 5).

MK-801 inhibited the lipid peroxidation induced by QUIN in striatal slices of C57BL/6 Wt, but not in Nrf2$^{-/-}$ mice

We then initiated experiments with Nrf2$^{+/+}$ (Wt) and Nrf2$^{-/-}$ mice, also investigating the possible contribution of the NMDAr to the toxic action mediated by QUIN, either in the presence or the absence of Nrf2, through

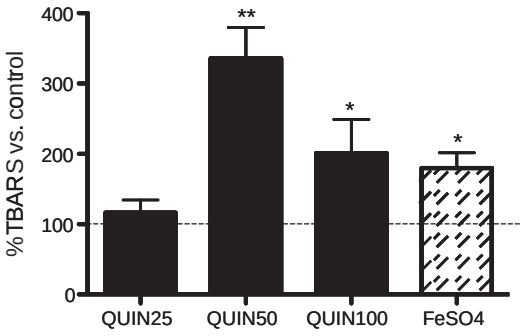

Fig. 3. Concentration–response effects of QUIN on lipid peroxidation in striatal slices from Wt C57BL/6 mice. Levels of lipid peroxidation generated by QUIN (25–100 μ) were compared against a positive control induced by 50 μM $FeSO_4$. Lipid peroxidation is expressed as the percent of TBARS formation vs. control line. Bars correspond to mean values ± S.E.M. of $n = 6$ experiments per group. One-way ANOVA followed by *post hoc* Tukey's test for comparisons among treatments. Asterisks denote statistically significant differences ($^{*}P < 0.05$ and $^{**}P < 0.01$) vs. control.

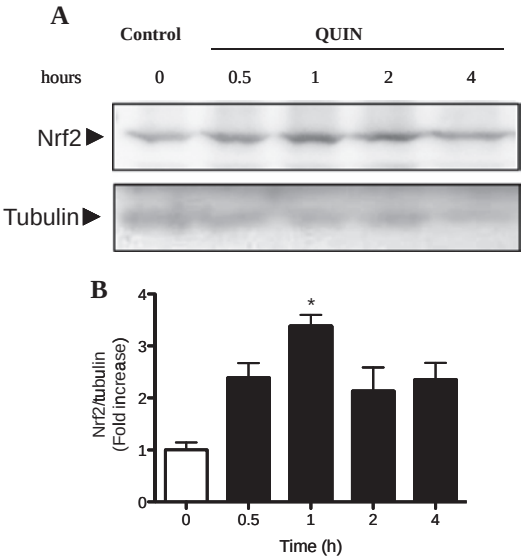

Fig. 4. Time-course of Nrf2 induction by QUIN (50 μM) in striatal slices of Wt C57Bl/6 mice. In (A), immunoblots for nuclear Nrf2 levels. In (B), densitometric analysis of immunoblots from (A). Also in (B), data are expressed as mean values ± S.E.M. of $n = 6$ experiments per group. One-way ANOVA followed by *post hoc* Tukey's test for comparisons among treatments. Asterisks denote statistically significant differences ($^{*}P < 0.05$) vs. control (0).

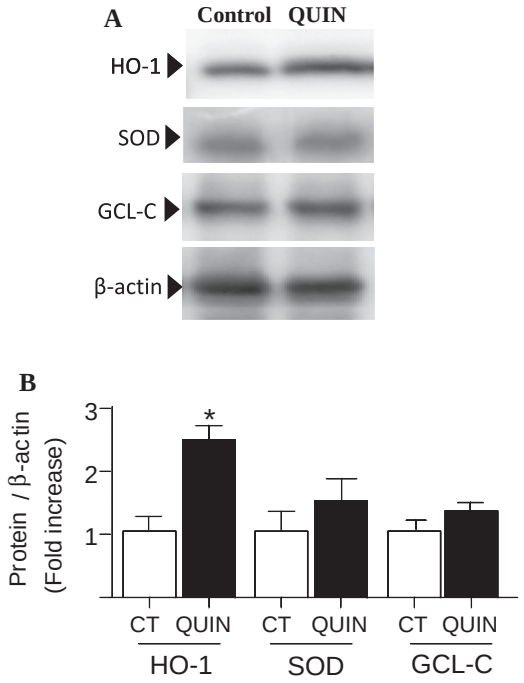

Fig. 5. Effect of QUIN (50 μM) on the HO-1, SOD and γ-GCL-C protein levels at 3 h of incubation in striatal slices of Wt C57BL/6 mice. Protein levels (A) and (B) are expressed as the ratio of protein/β-actin. Data are expressed as mean values ± S.E.M. of $n = 6$ experiments per group. One-way ANOVA followed by *post hoc* Tukey's test for comparisons among treatments. Asterisks denote statistically significant differences ($^{*}P < 0.05$) vs. control.

the actions of MK-801. For this purpose, striatal slices from Wt and Nrf2$^{-/-}$ mice were incubated for 3 h in the

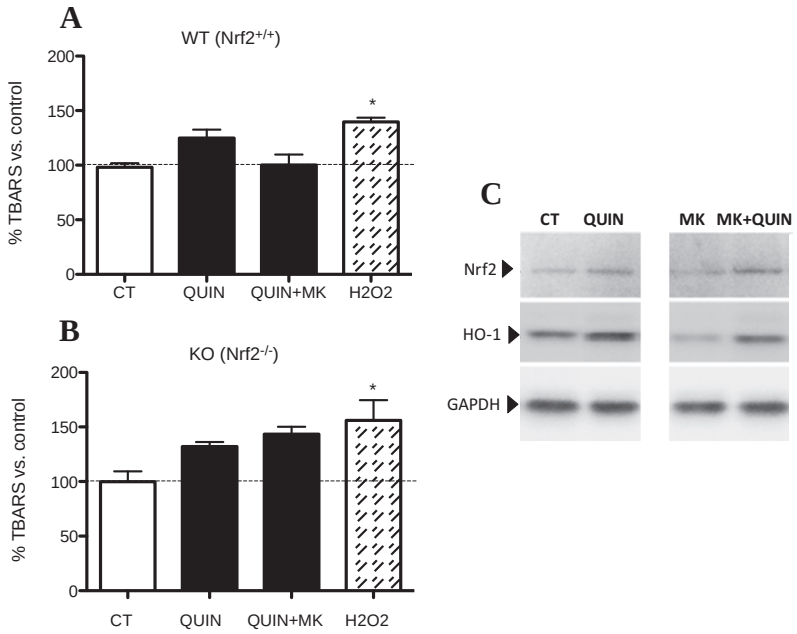

Fig. 6. Effect of the NMDAr antagonist MK-801 (50 μM) on the QUIN (50 μM)-induced lipid peroxidation in striatal slices of Wt (A) and Nrf2$^{-/-}$ (B) C57BL/6 mice at 3 h of incubation. Levels of lipid peroxidation were compared against a positive control (100 μM H_2O_2). Lipid peroxidation is expressed as the percent of TBARS formation vs. control. In (C), the effects of QUIN and/or MK-801 on the Nrf2 and HO-1 protein levels. Data are expressed as mean ± S.E.M. of $n = 6$ experiments per group. One-way ANOVA followed by *post hoc* Tukey's test for comparisons among treatments. Asterisks denote statistically significant differences ($^*P < 0.05$) vs. control.

presence of QUIN (50 μM) and/or MK-801 (50 μM), and then lipid peroxidation was estimated as the levels of TBARS formation (Fig. 6A, B). QUIN increased lipoperoxidation in slices from Nrf2$^{+/+}$ and Nrf2$^{-/-}$ mice (25% and 32% vs. control, respectively). The positive control (H_2O_2) exerted a pro-oxidant effect in the Wt and the Nrf2$^{-/-}$ conditions. In turn, MK-801 *per se* produced no effects on lipid peroxidation in mice striatal slices (data not shown). However, when combined, QUIN + MK-801 returned the levels of lipid peroxidation induced by the toxicant alone to baseline in Wt mice (Fig. 6A), but not in the Nrf2$^{-/-}$ condition (Fig. 6B). In slices from Wt mice, QUIN also induced increased protein levels of Nrf2 and HO, and MK-801 was unable to attenuate this effect (Fig. 6C). Once again, MK-801 *per se* produced no changes in these proteins.

DISCUSSION

Studying the role of the KP metabolite and reported neurotoxin QUIN in the transcription factor Nrf2 regulation in the brain is relevant because variations in the levels of QUIN are known to account for the etiological explanation of HD and other disorders; however, evidence available on this topic remains scarce. To date, some groups have described Nrf2 regulation patterns in HD experimental models. For instance, Li et al. (2007) overexpressed Nrf2 in astrocytes as an antioxidant gene therapy to counteract the lesion produced by the mitochondrial complex II inhibitor malonate in the striatum. In a further study, Nrf2 target genes were up-regulated in PC12 cells expressing mutant huntingtin, possibly as a protective/compensatory mechanism (van Roon-Mom et al., 2008). In parallel, these authors discovered alterations in some genes resulting in increased oxidative stress and damage. Shortly thereafter, Stack et al. (2010) showed that the triterpenoids CDDO-ethyl amide and CDDO-trifluoroethyl amide were able to improve the behavioral phenotype and brain pathology in the transgenic N171-82Q mouse model of HD. Both triterpenoids up-regulated Nrf2 and induced neuroprotective genes. In the same context, the fumaric acid ester dimethylfumarate (DMF), a drug commonly employed as a therapy for relapsing–remitting multiple sclerosis, exerted neuroprotective effects via induction of Nrf2 and detoxification pathways (Ellrichmann et al., 2011). The effects of DMF in R6/2 and YAC128 HD transgenic mice revealed a significant improvement in different physiological markers (body weight, motor impairment, striatal morphology, etc.). DMF also increased the Nrf2 immunoreactivity in neuronal subpopulations. More recently, in STHdh (Q111/Q111) striatal cells, the mHtt expression resulted in reduced activity of Nrf2, whereas the activation of the Nrf2 pathway by the oxidant tBHQ was significantly impaired (Jin et al., 2013). Altogether, these studies provide biomedical evidence supporting the concept that Nrf2 transactivation and phase 2 antioxidant up-regulation in the brain represent a neuroprotective strategy with clinical relevance for HD therapy, although the role of KP metabolites in this regulation deserves more attention.

During the first part of this study, we demonstrated that rat striatal slices exposed to QUIN are sensitive to

the toxin. In contrast to our previous report (Tasset et al., 2010), the levels of Nrf2 were increased in response to QUIN. We assume that this event represents a compensatory action of the biological system to the toxicant, but not necessarily recruiting a major antioxidant strategy. Interestingly, also in rat striatal slices, QUIN exerted a concentration–response effect on an endpoint of oxidative damage, supporting our previous appreciation that, despite Nrf2 exhibited a tendency to increase after QUIN treatment, the pro-oxidant environment is propitious to exert oxidative cell damage beyond any effort to activate the endogenous antioxidant defense. Thus, compensatory mechanisms could involve adaptive responses of some specific cell types to oxidative modifications in an attempt to ''reorganize'' and ''redirect'' the redox activity if a toxic insult is present, albeit they could not be sufficient to counteract damage already in progress. Meanwhile, the consistent concentration-dependent response of the rat striatal slices to QUIN demonstrates the suitability of this preparation to explore the Keap-1/Nrf2/ARE axis using this toxic paradigm in this species.

We also explored Nrf2-associated responses in mice striatal slices. In this preparation, QUIN evoked a robust peroxidative action at 50 μM concentration, with a more moderate peroxidative action at 100 μM concentration. This result clearly establishes differences in the susceptibility of striatal tissue of these two different species if considering that concentrations typically employed by some groups (including us) for the induction of neurotoxic events in rat striatal slices are between 0.1 and 1 mM (Pérez-De La Cruz et al., 2010; Colle et al., 2012). The effect of QUIN on the striatal levels of Nrf2 in this preparation was stimulated at all times tested and reached a peak at 60 min, pointing out the differential responses to this toxic paradigm depending on the explored species. Nonetheless, this stimulation of Nrf2 by QUIN was likely to be sufficient to induce increased levels of phase 2 enzymes, including HO-1, SOD, and in a less extent, γ-GCL-C. Noteworthy, the magnitude of HO-1 protein levels clearly corresponded to the extent of Nrf2 induction by QUIN. Although Nrf2 is known to regulate the expression of HO-1 and SOD (Tufekci et al., 2011; Zhang et al., 2012), it is also known that HO-1 can be regulated by other factors, including the transcriptional activators NF-κB and AP-1, and the transcription repressors BTB and CNC homolog 1 (Bach1), all controlling the inducible HO-1 gene expression (Paine et al., 2010). In turn, SOD and γ-GCL-C levels induced by QUIN were clearly more moderate, but still in line with an early and transitory induction of Nrf2.

Regarding the experiments in striatal slices of Wt and $Nrf2^{-/-}$ mice, our findings revealed interesting features of the early oxidative pattern exerted by QUIN: while moderate differences in the QUIN-induced lipid peroxidation were found among $Nrf2^{+/+}$ and $Nrf2^{-/-}$ slices, the $Nrf2^{-/-}$, but not the $Nrf2^{+/+}$ slices, were completely insensitive to MK-801, suggesting that Nrf2 regulation might also be subordinated to NMDAr. Altogether, these findings suggest that Nrf2 could be more relevant as a concurrent physiological redox sensor and potentially concurrent mechanism of resistance against oxidative damage than a physiologically relevant first line of defense, where NMDAr might, somehow, regulate Nrf2 expression and function. These findings also support the concept that most of the actions exerted by QUIN are mediated by NMDAr (Stone et al., 2003).

The attenuation or mitigation of the deleterious actions of QUIN and other toxic KP metabolites emerges as a priority for basic and clinical research because this pathway has gain attention as a potential source of metabolites driving redox and neurochemical alterations in the CNS when the metabolic pathway is altered (Moroni, 1999; Chen and Guillemin, 2009; Zádori et al., 2012). The use of antioxidants with different profiles along several studies using QUIN as the paradigm of choice has demonstrated that its toxic effects can be reduced or even blocked by these agents (reviewed by Pérez-De La Cruz et al. (2012)). While an initial assumption on these findings has suggested that these agents are mostly acting as direct ROS scavengers, our actual perspective is changing to consider an additional, promising and not excluding mechanism: transcriptional Nrf2 regulation. Of note, one of the agents that have been successfully tested against QUIN toxicity is S-allyl cysteine (SAC), an antioxidant compound obtained from the aged garlic extract (Pérez-Severiano et al., 2004). SAC was shown to prevent the QUIN-induced oxidative damage and neurotoxicity, suggesting that oxidative stress is a major component of its toxic pattern. Whether SAC or other antioxidants already tested in the QUIN model can modulate the Nrf2 system to contribute to neuroprotection is a question to explore in further investigations. In addition, another well-known Nrf2 inducer, curcumin, has been recently tested against several markers of the toxic model induced by QUIN in the rat striatum (Carmona-Ramírez et al., 2013), thereby supporting the concept that Nrf2 modulation can constitute a key tool for the mitigation of the noxious actions of QUIN if properly stimulated, and a tool for consideration in the design of therapeutic strategies for those central and peripheral disorders exhibiting alterations in the KP metabolism.

CONCLUDING REMARKS

Evidence presented herein suggests that QUIN can exert early redox modifications involving alterations in the Nrf2 modulation. These alterations depend on the time of exposure, the tested concentrations, and the extent of oxidative damage accompanying Nrf2 changes. In turn, these modifications can recruit the up-regulation of phase 2 enzymes, although this effect could merely represent a compensatory response induced by QUIN to the toxic events already in course. Two additional relevant points of this study are: (1) The moderate contribution of the $Nrf2^{-/-}$ condition to stimulate oxidative damage, albeit the striatal slices from these mutant animals were more sensitive to the toxic actions of QUIN; and (2) the possible contribution of NMDAr to

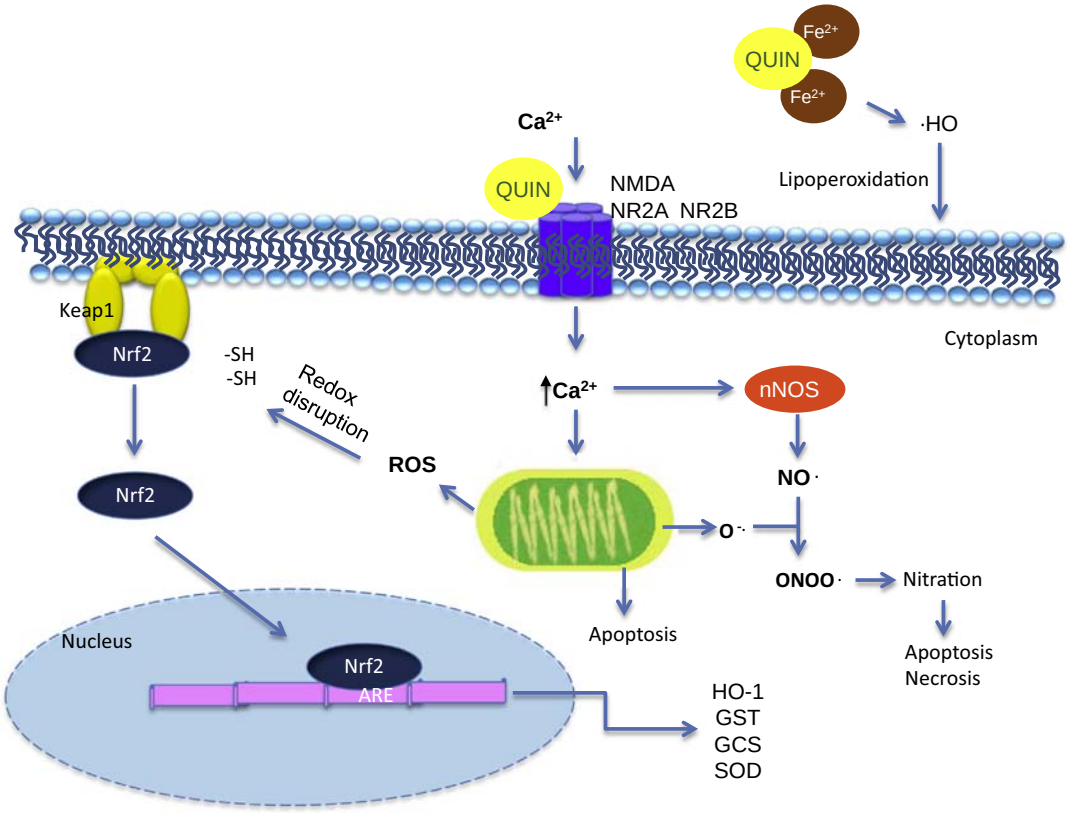

Fig. 7. Schematic representation of the hypothetical mechanisms involved in the Nrf2 regulation by QUIN in the striatum of rodents. QUIN, an NMDAr agonist, is acting mostly through NMDAr overactivation, but also through direct stimulation of ROS formation, thereby accounting for extracellular oxidative damage. Increased levels of intracellular Ca^{2+} induced by opening of the NMDAr-channel complex will be responsible for persistent activation of Ca^{2+}-dependent enzymes, including proteases, phospholipases, ATPases and neuronal nitric oxide synthase (NOS). These events will create a scenario of exacerbated intracellular ROS/RNS formation, which will contribute to an altered redox status and might be responsible for an early compensatory Nrf2 nuclear translocation after its dissociation from Keap-1. This effect, however, could not be enough to induce Nrf2 transactivation in terms of a lasting stimulation of phase two enzyme synthesis for cell defense, thereby surrendering to oxidative cell damage and death.

the modulation of the oxidative stress and the Nrf2-mediated responses, as well as its active role in the $Nrf2^{+/+}$, but not in the $Nrf2^{-/-}$ condition. This first approach opens up more questions than unequivocal conclusions, but it serves as a platform to initiate more detailed studies on the effects of QUIN on the Nrf2 transcription factor, and its potential role as a therapeutic tool to reduce neurodegenerative events involving oxidative damage. A schematic representation of the mechanisms likely occurring in this study is summarized in Fig. 7.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

Acknowledgments—This work was supported by CONACyT Grant 205648 (A.S.). We thank Dr. Antonio Cuadrado (Autonomous University of Madrid, Spain) for technical help to conduct the experiments with striatal mice slices. We are also grateful to Dr. Ismael Torres and Dr. Enrique Pinzón for the technical support with the experimental animals. Ana Laura Colín-González is scholarship holder of CONACyT-Mexico (239954).

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(Accepted 11 December 2013)
(Available online 19 December 2013)

Ashdin Publishing
Journal of Drug and Alcohol Research
Vol. 4 (2015), Article ID 235959, 5 pages
doi:10.4303/jdar/235959

Research Article

Protective Effects of Caffeic Acid on Quinolinic Acid-Induced Behavioral and Oxidative Alterations in Rats

Ana Laura Colín-González,[1] Sayde Sánchez-Hernández,[1] Maria Eduarda de Lima,[2] Syed F. Ali,[3] Anahí Chavarría,[4] Juana Villeda,[5] and Abel Santamaría[1]

[1]*Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, Mexico City 14269, Mexico*
[2]*Universidade Federal do Pampa, Uruguaiana, Brazil*
[3]*Neurochemistry Laboratory, Division of Neurotoxicology, National Center for Toxicological Research/FDA, Jefferson, AR 72079, USA*
[4]*Unidad de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico City 04510, Mexico*
[5]*Laboratorio de Patología Experimental, Instituto Nacional de Neurología y Neurocirugía, Mexico City 14269, Mexico*
Address correspondence to Abel Santamaría, absada@yahoo.com

Received 30 October 2015; Revised 20 November 2015; Accepted 9 December 2015

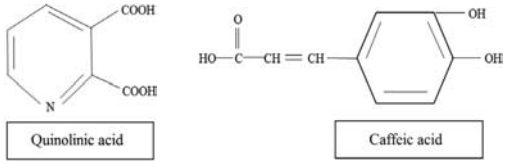
Abstract *Study design.* The protective effects of the natural antioxidant caffeic acid (CA) on behavioral tasks and lipid peroxidation were tested in an excitotoxic model produced by unilateral intrastriatal injection of quinolinic acid (QUIN), and in striatal slices incubated in the presence of the same toxin. CA (20 mg/kg) was administered intraperitoneally to rats every day for five days; then, rats received QUIN (240 nmol/μL). Six days later, motor asymmetry was quantified by the preferential use of forelimbs and the circling behavior tests. Rat striatal slices (300 μm thick) were incubated in the presence of CA (30–300 μM) and/or QUIN (100 μM) to estimate oxidative stress. *Results.* QUIN induced motor asymmetry in lesioned rats and increased lipid peroxidation in striatal slices when compared to control values. CA prevented the QUIN-induced toxic endpoints in a concentration-dependent manner. *Conclusion.* Our results support the neuroprotective role of CA in neurotoxic paradigms recruiting excitotoxic events.

Keywords motor asymmetry; antioxidant defense; excitotoxicity; oxidative stress; corpus striatum; caffeic acid; quinolinic acid

1. Introduction

The kynurenine pathway for tryptophan degradation is responsible for the formation of neuroactive metabolites in the CNS [1]. The alteration in the levels of these metabolites is involved in different neurological disorders [2]. One of these metabolites is quinolinic acid (QUIN), a glutamate agonist acting on NMDA receptors (NMDAr) [3]. Through excitotoxic events, QUIN induces oxidative stress, increased intracellular Ca^{2+} levels, enhanced levels of extracellular glutamate, augmented protease activity, and stimulated deadly cascades under different experimental conditions [4, 5]. In turn, excitotoxicity can be defined as a toxic mechanism affecting neurons that are continuously stimulated via overactivation of NMDAr and further increased intracellular Ca^{2+} levels triggering deadly cascades [6].

Caffeic acid (3,4-dihydroxycinnamic acid or CA) is a natural phenolic compound that has been shown to exert neuroprotective actions against different neurotoxic insults, including ischemia and excitotoxic damage in rodents, and these effects have been related with its antioxidant and anti-inflammatory properties [7, 8, 9, 10]. Although some positive effects of CA on endpoints of behavioral (motor activity) and redox status (reduced glutathione/oxidized glutathione) alterations in the excitotoxic model induced by QUIN in rats have already been reported [9], key behavioral and oxidative stress markers denoting neuroprotection are still needed as complementary evidence. In particular for this toxic model, motor asymmetry and lipid peroxidation as an index of oxidative damage in the striatum are required. Therefore, the present study aims to evaluate the effects of CA on QUIN-induced behavioral and biochemical alterations in the rat brain. For comparative purposes, the effect of CA was also tested in the 3-nitropropionic acid (3-NP) model of striatal toxicity. The chemical structures of both CA and QUIN are represented in Figure 1.



(a) (b)

Figure 1: Schematic representation of the chemical structures of QUIN (a) and CA (b).

2. Materials and methods

2.1. Reagents

Apomorphine, CA, QUIN, 3-NP, thiobarbituric acid (TBA), and other reagents were obtained from Sigma-Aldrich

(St. Louis, MO, USA). Other chemicals, including buffers, were obtained from other commercial sources.

2.2. Animals and treatments

Twenty eight bred-in-house male Wistar rats (280–320 g) were randomly separated into four groups (seven animals per group). Animals were placed into acrylic cages and provided with Rodent Chow (Purina, St. Louis, MO, USA) and water ad libitum. Constant conditions of temperature (25 ° ± 1 °C), humidity (50 ± 10%), and lighting (12:12 light-dark cycle) were maintained throughout the experiments. All experimental procedures with animals were strictly carried out according to the "Guidelines for the Use of Animals in Neuroscience Research" from the Society for Neuroscience, the local ethical committees, and in compliance of the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

The dose employed for CA was close to that used in a previous report [9]. The experimental groups were injected intraperitoneally (IP) with sterile water as vehicle or CA (20 mg/kg) every day for five consecutive days. Shortly after the last CA administration (30 min), the animals were anesthetized with sodium pentobarbital (50 mg/kg, IP) and 30 min later infused for 2 min with a single intrastriatal injection of QUIN (240 nmol/μL). The dose employed for QUIN has been previously reported [11]. The striatal infusion was performed at the following stereotaxic coordinates: 0.5 mm anterior to bregma, −2.6 mm lateral to bregma, and 4.5 mm ventral to the dura [12]. Control rats received sterile saline intrastriatally and sterile water (pH 7.4) IP. Six days after the striatal lesion, animals from all groups were subjected to behavioral tests.

2.3. Behavioral tests

2.3.1. Circling behavior test

Rotation behavior was evaluated in rats from all groups, following a protocol previously reported by us [13]. Six days after the intrastriatal QUIN infusion, all rats were administered with apomorphine subcutaneously (1 mg/kg, SC) and placed in individual acrylic cages. The number of ipsilateral rotations (complete 360 ° turns) to the lesioned side was recorded for 60 min in periods of 5 min. Results are expressed as the total number of ipsilateral turns per 60 min. Largely known as a morphine decomposition product, apomorphine is a nonselective dopamine agonist that activates both D1 and D2 dopamine receptors.

2.3.2. Cylinder test

The cylinder test is a behavioral method providing accurate information on locomotor asymmetry in rodent models. Animals were placed into an open-top, clear plastic cylinder, and monitored in regard to their forelimb activity while rearing against the wall. The use of forelimb was defined as the preferential placement of the whole palm of a given limb on the wall of the device, therefore indicating its use for body support while rearing. The number of ipsilateral and contralateral forelimb contacts was calculated and expressed as the count in 5 min.

2.4. Isolation of striatal slices

Rat brains were collected after animal decapitation and placed in ice-cold Krebs-bicarbonate buffer pH 7.4 (120 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM glucose, and 200 mM sucrose). Striatal slices were dissected, glued down against agar blocks in small chambers, submerged in cold oxygenated dissection buffer, and sectioned in 300 μm thick transverse slices using a vibratome (TS1000 Leica; Heidelberg, Germany). Thereafter, the slices were transferred to a sucrose-free dissection buffer, and bubbled with 95% O_2/5% CO_2 at room temperature for 30 min to recover from the slicing procedure. Then, slices were incubated for 30 min, exposed to different concentrations of CA (30, 100 or 300 μM) or vehicle for 30 min more, and added with QUIN (100 μM) for 3 h in a shaking water bath at 37 °C. Immediately after incubation, slices were placed on ice, collected, and scheduled for measurement of oxidative damage to lipids. For comparative purposes, a positive control consisting of slices incubated with the mycotoxin 3-nitropropionic acid (3-NP, 1 mM) and preincubated with CA (100 μM) was ran in parallel.

2.5. Assay of lipid peroxidation

The formation of thiobarbituric acid-reactive substances (TBARS) as an index of lipid peroxidation was determined in striatal slices, according to a previous report [14]. Briefly, 50 μL aliquots of the homogenates were added to 100 μL of the TBA reagent (0.75 g of TBA + 15 g of trichloroacetic acid + 2.54 mL of HCl) and incubated at 100 °C for 20 min. The pink chromophore produced after this reaction denoted the amount of peroxidized lipid products. Samples were kept on ice for 5 min and centrifuged at $3,000 \times$ g for 15 min. The optical density was measured in the supernatants in a CYT3MV Biotek Cytation 3 Imaging Reader at 532 nm. The amount of TBA-RS was calculated by interpolation of values in a constructed malondialdehyde (MDA) standard curve. Results were originally calculated as nmol of MDA per mg protein, and finally expressed as percent of lipid peroxidation versus control. The content of protein in samples was determined by Bradford's method [15].

2.6. Statistical analysis

Results were expressed as mean values ±SEM. Behavioral data were analyzed by nonparametric ANOVA (Kruskal-Wallis) followed by comparison with Mann-Whitney's test. Biochemical data were analyzed with

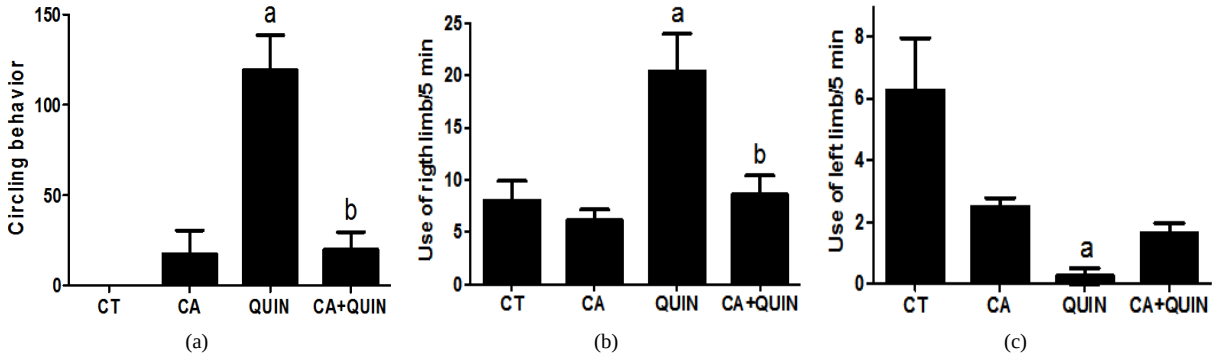


Figure 2: Effect of CA (20 mg/kg, IP) on the behavioral alterations induced by QUIN (240 nmol/μL) in rats. Rats were administered with CA for five consecutive days prior a single infusion of QUIN into the right striatum. Both behavioral markers of motor asymmetry were explored six days after QUIN injection. The preferential use of forelimbs ((b) and (c)) was monitored for 5 min by placing the animals into a plastic cylinder. Immediately thereafter, rats were administered with apomorphine (1 mg/kg, SC) and the number of ipsilateral turns was recorded every 5 min for 60 min. Each bar represents mean values $\pm$ one SEM of seven rats per group. $^{a}P < .05$ different of control, $^{b}P < .05$ different of QUIN. Data analysis was performed with a nonparametric ANOVA (Kruskal-Wallis) followed by a comparison with Mann-Whitney's test.

a one-way analysis of variance (ANOVA) for repeated measures, followed by post-hoc Tukey's test. The analytical procedures were performed using the scientific statistic software GraphPad Prism 5 (GraphPad Scientific, San Diego, CA, USA). Differences of $P < .05$ were considered as statistically significant.

3. Results

3.1. CA prevented the QUIN-induced behavioral alterations in rats

Figure 2 shows the behavioral results obtained from rats subjected to the rotation behavior test (Figure 2(a)) and the cylinder test (Figures 2(b) and 2(c)).

In Figure 2(a), the control animals—receiving apomorphine six days after intrastriatally infused with vehicle—displayed no rotations, although they exhibited excitement and hyperactivity during the evaluation time (data not shown). The group receiving CA displayed a moderate number of ipsilateral rotations (18 ± 9). The QUIN-lesioned rats displayed a considerable number of rotations in 60 min (121 ± 16; $P < .05$ vs. control) in response to apomorphine, whereas the group pretreated with CA and further infused with QUIN displayed a moderate number of rotations, similar to the CA group (22 ± 6; 82% below QUIN; $P < .05$ vs. QUIN).

Figures 2(b) and 2(c) depict the preferential use of forelimbs in the cylinder test for 5 min. Control rats showed, more or less, and equal use of the right (7.5 ± 2.3 times) versus the left forelimb (6.2 ± 1.7 times). This tendency was not significantly changed in animals receiving CA (6.1 ± 1.2 for the right forelimb vs. 2.3 ± 0.2 for the left forelimb). In contrast, the group receiving QUIN exhibited a remarkable increased preference of the right forelimb (21.0 ± 3.3 times; 180% above the control; $P < .05$) versus the left forelimb (0.4 ± 0.2; 83% below the control; $P < .05$). These tendencies were prevented in QUIN-lesioned rats by the pretreatment with CA (7.8 ± 2.1 for the right forelimb vs. 1.7 ± 0.3 for the left forelimb; 63% below and 325% above QUIN, resp.; $P < .05$ against QUIN for both cases).

3.2. CA reduced the QUIN-induced oxidative damage to lipids in rat striatal slices in a dose-dependent manner

Figure 3 depicts the curve-response effects (as percent values vs. the control) of CA on QUIN-induced striatal lipid peroxidation. QUIN per se produced a significant increase in oxidative damage to lipids when compared to control (49.5 ± 7.6% above the control; $P < .05$). CA per se, administered at different concentrations (30, 100, and 300 μM), reduced the levels of lipid peroxidation below the control levels (39, 10, and 19% below the control, resp.), although none of these changes were statistically significant. When preadministered to QUIN-treated slices, CA reduced the oxidative damage to lipids by 18, 40 ($P < .05$) and 87% ($P < .05$) versus QUIN, respectively, in a concentration-dependent manner.

In the upper right panel, 3-NP per se increased the levels of lipid peroxidation by 735% above the control ($P < .05$). Once again, CA per se did not affect the oxidative damage when compared to the control (19% below the control), whereas when preadministered to 3-NP-treated slices, it reduced the 3-NP-induced lipid peroxidation by 32% ($P < .05$).

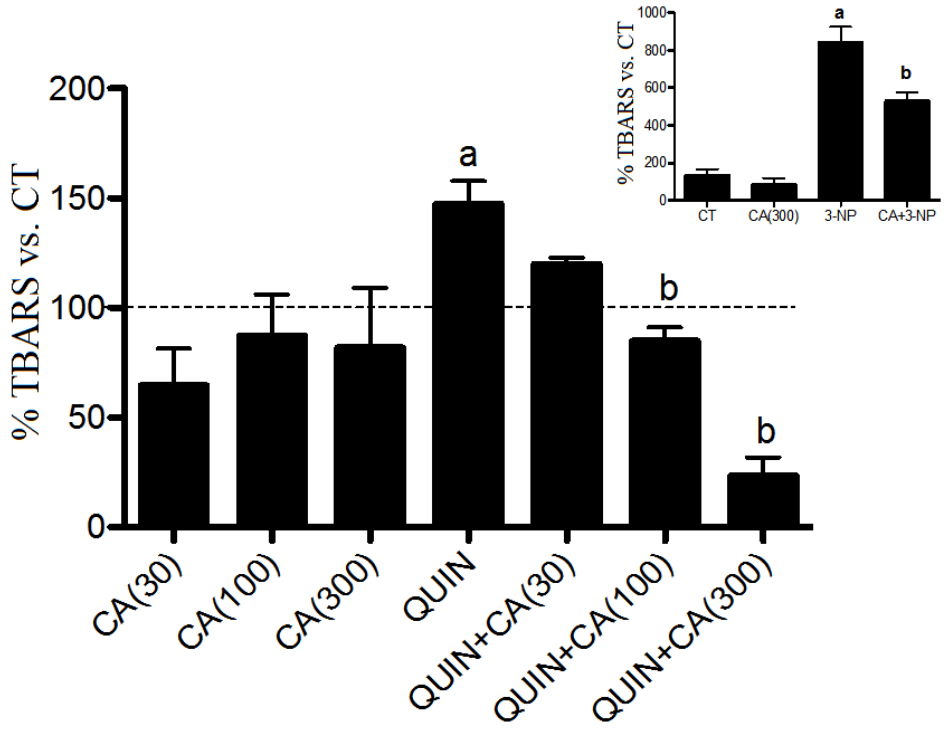


Figure 3: Curve-response effect of CA (30–300 μM) on the striatal oxidative damage to lipids (TBARS formation) induced by QUIN (100 μM) in tissue slices. For comparative purposes, the effect of CA (100 μM) was also tested on the lipoperoxidative effect induced by 3-nitropropionic acid (3-NP, 1 mM) in additional groups (small graph in the right corner). Bars represent mean values $\pm$SEM of seven experiments per group. $^{a}P < .05$ statistically different of control, $^{b}P < .05$ statistically different of QUIN or 3-NP. Data analysis was performed with a one-way ANOVA followed by Tukey's test.

4. Discussion

In this simple study, we found evidence that the natural phenolic compound CA exerted neuroprotective properties against the excitotoxic damage induced by QUIN in the rat striatum. We achieved this conclusion through the positive effects of CA observed on both behavioral and biochemical endpoints of striatal toxicity induced by QUIN. CA was able to significantly attenuate the QUIN-induced circling behavior and the use of the right forelimb, thus confirming that enough degree of neuroprotection is achieved through this agent to prevent the motor asymmetry evoked by the neurotoxin. In addition, it can also be assumed that the preventive effect exerted by CA on QUIN- and 3-NP-induced striatal lipid peroxidation—a major indicator of oxidative damage—is due to the antioxidant properties previously reported for this phenolic compound [7, 8, 9, 10], and this effect might strongly account for its neuroprotective action in the brain.

Our results are complementary to previous findings reported by Kalonia et al. [9], who demonstrated that CA administered per orally (5 mg/kg and 10 mg/kg, PO) was able to prevent alterations in locomotor activity in animals intrastriatally lesioned with QUIN. Despite the obvious experimental differences between Kalonia's report and ours (PO vs. IP administration of CA, range of dosage for CA, and the dose of QUIN employed [300 nmol vs. 240 nmol], all in regard to the in vivo approach), CA demonstrates to be a protective agent that can reduce behavioral and biochemical alterations in the toxic model evoked by QUIN.

Few reports have previously deal with the concept that CA and QUIN are opposite in their effects. Hirai and coworkers [16] described the effects of some natural iron chelators and derivatives on in vitro oxygen consumption rates and superoxide radical formation. Among the agents tested, QUIN and CA were compared. These authors found that QUIN repressed oxygen consumption, whereas CA accelerated it. In turn, these opposite effects establish relevant chemical basis to understand the mechanistic nature of these two different molecules in regard to the induction of superoxide formation (for the case of QUIN) or its repression (for the case of CA). More recently, Minakata et al. [17] tested the capacity of QUIN and CA to modulate the formation of radicals in the reaction mixtures of rat liver microsomes in the presence of ADP,

Fe^{3+}, and NADPH in order to provide information on the protective actions of CA and the neurotoxic actions of QUIN through a redox approach. They demonstrated that while CA inhibited the radical formation, QUIN enhanced their production. The conclusion achieved from these observations is that oxidative stress is part of the toxic pattern elicited by QUIN in the progression of several pathological conditions, whereas antioxidant activity is part of the protective profile of CA and other natural polyphenols. In addition, other groups have provided direct evidence on the in vivo protective effect of CA in other toxic paradigms. For example, Kumar et al. [10] demonstrated that CA is able to reduce different endpoints of oxidative damage and mitochondrial dysfunction in a murine model of chronic fatigue, once again emphasizing the relevance of the antioxidant profile of CA to exert its protective actions. Like this, other studies have provided relevant evidence that CA can prevent different deleterious events linked to oxidative stress and inflammation in chronic pathological conditions.

Of final consideration, phenolic acids, such as CA, may present or combine with other acids in its natural form. Through its combination with other acids, CA yields the formation of another important antioxidant compound, chlorogenic acid (CGA), and such biotransformation occurs by esterification of CA with a cyclic alcohol-acid, quinic acid [18]. Noteworthy, similar to CA, CGA has been also described to exert protective actions in a number of inflammatory and oxidative events [19]. CGA is the most abundant polyphenol found in food and plants [18, 19], thus it can be hypothesized that the presence of both of these agents might induce a simultaneous action, thereby enhancing the protective effect of CA. Of course, this speculation deserves further and more detailed investigation.

In summary, CA is a promising antioxidant tool to investigate the role of oxidative stress in toxic models of neurodegenerative disorders coursing with excitotoxic events. However, more detailed studies are needed to characterize the precise mechanisms underlying the antioxidant and neuroprotective properties of this naturally occurring agent.

Acknowledgments This work was supported by CONACyT grant 205648 (A.S., Mexico). Ana Laura Colín-González is a doctoral student from the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship 239954 from CONACyT.

Conflict of interest The authors declare that they have no conflict of interest.

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9.3. EXISTE UN SINERGISMO TÓXICO ENTRE EL QUIN Y LOS METABOLITOS QUE SE ACUMULAN EN LAS ACIDEMIAS ORGÁNICAS

¿Puede el QUIN generar sinergismos tóxicos con otras moléculas para inducir neurotoxicidad y favorecer así procesos patológicos que expliquen diversas enfermedades neurodegenerativas? La incubación de sinaptosomas (terminales nerviosas) con ácidos orgánicos en concentraciones subtóxicas no produjo efectos deletéreos; sin embargo, la co-incubación de estos metabolitos con QUIN disminuyó la función mitocondrial, aumentó la formación de especies reactivas y la lipoperoxidación. Interesantemente, para todos los casos este efecto fue parcialmente prevenido por el ácido kinurénico, un antagonista de los rNMDA, y por la L-NAME, un inhibidor de la sintasa de óxido nítrico, lo que sugiere que este nuevo modelo sinérgico involucra mecanismos de excitotoxicidad y de estrés nitrérgico. Más aún, los efectos tóxicos fueron completamente prevenidos por la acción antioxidante de la SAC, proporcionando un papel central al estrés oxidante en este modelo. En general, estos hallazgos sugieren que el daño causado un aumento en los niveles de ácidos orgánicos en acidemias metabólicas puede ser magnificado por la presencia del QUIN, un proceso que es principalmente mediado por estrés oxidante.

En apoyo a estos resultados, se realizaron experimentos *in vivo* para confirmar el posible sinergismo encontrado *in vitro*. La administración intraestriatal de QUIN a animales gcdh$^{-/-}$ alimentados con una dieta alta en lisina produjo una disminución en la actividad de la creatina cinasa y del complejo IV de la cadena respiratoria, aumentó marcadores de estrés (lipoperoxidación, formación de nitrito/nitrato), y disminuyó los niveles de glutatión reducido. Todos estos cambios pueden deberse al efecto aditivo del QUIN con AG y 3-OHAG, y confirman el sinergismo tóxico y el papel central del estrés oxidante en este modelo combinado.

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TOXIC SYNERGISM BETWEEN QUINOLINIC ACID AND ORGANIC ACIDS ACCUMULATING IN GLUTARIC ACIDEMIA TYPE I AND IN DISORDERS OF PROPIONATE METABOLISM IN RAT BRAIN SYNAPTOSOMES: RELEVANCE FOR METABOLIC ACIDEMIAS

A. L. COLÍN-GONZÁLEZ,[a†] A. L. PAZ-LOYOLA,[a†] I. SERRATOS,[b] B. SEMINOTTI,[c†] C. A. J. RIBEIRO,[c†] G. LEIPNITZ,[c] D. O. SOUZA,[c] M. WAJNER[c,d*] AND A. SANTAMARÍA[a*]

[a] *Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, S.S.A., Mexico City, Mexico*

[b] *Departamento de Química, Universidad Autónoma Metropolitana-Iztapalapa, Mexico City, Mexico*

[c] *Departamento de Bioquímica, Instituto de Ciências Básicas da Sáude, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*

[d] *Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil*

Abstract—The brain of children affected by organic acidemias develop acute neurodegeneration linked to accumulation of endogenous toxic metabolites like glutaric (GA), 3-hydroxyglutaric (3-OHGA), methylmalonic (MMA) and propionic (PA) acids. Excitotoxic and oxidative events are involved in the toxic patterns elicited by these organic acids, although their single actions cannot explain the extent of brain damage observed in organic acidemias. The characterization of co-adjuvant factors involved in the magnification of early toxic processes evoked by these metabolites is essential to infer their actions in the human brain. Alterations in the kynurenine pathway (KP) – a metabolic route devoted to degrade tryptophan to form NAD^+ – produce increased levels of the excitotoxic metabolite quinolinic acid (QUIN), which has been involved in neurodegenerative disorders. Herein we investigated the effects of subtoxic concentrations of GA, 3-OHGA, MMA and PA, either alone or in combination with QUIN, on early toxic endpoints in rat brain synaptosomes. To establish specific mechanisms, we pre-incubated synaptosomes with different protective agents, including the endogenous N-methyl-D-aspartate (NMDA) receptor antagonist kynurenic acid (KA), the antioxidant S-allylcysteine (SAC) and the nitric oxide synthase (NOS) inhibitor nitro-L-arginine methyl ester (L-NAME). While the incubation of synaptosomes with toxic metabolites at subtoxic concentrations produced no effects, their co-incubation (QUIN + GA, +3-OHGA, +MMA or +PA) decreased the mitochondrial function and increased reactive oxygen species (ROS) formation and lipid peroxidation. For all cases, this effect was partially prevented by KA and L-NAME, and completely avoided by SAC. These findings suggest that early damaging events elicited by organic acids involved in metabolic acidemias can be magnified by toxic synergism with QUIN, and this process is mostly mediated by oxidative stress, and in a lesser extent by excitotoxicity and nitrosative stress. Therefore, QUIN can be hypothesized to contribute to the pathophysiology of brain degeneration in children with metabolic acidemias.

Key words: organic acidemias, excitotoxicity, oxidative stress, mitochondrial dysfunction, cell damage, toxic synergism.

INTRODUCTION

The hereditary metabolic disorders known as organic acidemias (OA) are characterized by a blockage of the aberrant catabolism of amino acids and lipids due to a deficient activity of specific enzymes. These alterations are responsible for the accumulation and high urinary excretion of potentially toxic organic acids (Bodamer et al., 2006). Neurological symptoms and brain abnormalities are seen in patients suffering from OA. Glutaric acidemia type I (GA I), methylmalonic acidemia (MMAcidemia) and propionic acidemia (PAcidemia) have a relatively high prevalence in the population, all with a severe clinical presentation in the neonatal period.

GA I is known to be caused by a deficiency of glutaryl-CoA dehydrogenase (GDD, McKusick 23167; OMIM # 231670) activity, resulting in the accumulation of glutaric (GA, 500–5000 μmol/L) and 3-hydroxyglutaric (3-OHGA, 40–200 μmol/L) acids in the CNS (Kölker et al., 2004; Sauer et al., 2006). Among its pathological features are

*Corresponding authors. Address: Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Tel: +55-5133085571; fax: +55-5133085540 (M. Wajner). Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, SSA, Mexico City 14269, Mexico. Tel: +52-55-5606-3822x2013 (A. Santamaría).

E-mail addresses: mwajner@ufrgs.br (M. Wajner), absada@yahoo.com (A. Santamaría).

† These authors equally contributed to this work.

Abbreviations: GA, glutaric acid; GA I, glutaric acidemia type I; GDD, glutaryl-CoA dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KA, kynurenic acid; KP, kynurenine pathway; L-NAME, L-nitro-L-arginine methyl ester; MDA, Malondialdehyde; MMA, methylmalonic acid; MMAcidemia, methylmalonic acidemia; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, N-methyl-D-aspartate; NMDAr, NMDA receptors; NOS, nitric oxide synthase; OA, organic acidemias; PA, propionic acid; PAcidemia, propionic acidemia; QUIN, quinolinic acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAC, S-allylcysteine; TBA, thiobarbituric acid; 3-OHGA, 3-hydroxyglutaric acid.

http://dx.doi.org/10.1016/j.neuroscience.2015.09.002
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a fronto-temporal cortical atrophy at birth, progressive spongy formation, leukoencephalopathy and acute damage of the caudate/putamen occurring between 6 months and 4 years of age (Amir et al., 1987; Hoffmann and Zschocke, 1999). Experimental evidence suggests that accumulating organic acids induce excitotoxicity, oxidative stress and energy metabolism impairment (Flott-Rahmel et al., 1997; Latini et al., 2002; de Oliveira Marques et al., 2003; Kölker et al., 2004; Wajner et al., 2004; Sauer et al., 2005; Latini et al., 2005a,b; Ferreira et al., 2007; Rosa et al., 2007), although the precise pathogenic mechanisms occurring in GA I have not been fully described.

In turn, MMAcidemia and PAcidemia are caused by severe deficiencies of methylmalonyl-CoA mutase (EC 5.4.99.2) and propionyl-CoA carboxylase (EC 6.4.1.3) activities, respectively. MMAcidemia is biochemically characterized by accumulation of methylmalonic acid (MMA) (1-2.5 mmol/L), whereas PAcidemia by propionic acid (PA) (5 mmol/L) in blood. Clinical manifestations of these two OA comprise lethargy, psychomotor delay/mental retardation, focal and generalized convulsions, vomiting, dehydration, hepatomegaly, hypotonia, and encephalopathy further leading to coma and death (Deodato et al., 2006; Hauser et al., 2011). Disrupted myelination revealing progressive cortical atrophy, as well as histopathological injury of the basal ganglia can be observed (Brismar and Ozand, 1994; Chemelli et al., 2000; Harting et al., 2008). For both acidemias, brain damage has been related to the toxic actions produced by their corresponding accumulating metabolites. This suggestion is based on experimental evidence demonstrating that MMA can cause brain mitochondrial energy metabolism disruption, as well as redox status and glutamatergic transmission alterations (Kölker et al., 2006; Sauer et al., 2006, 2010; Stellmer et al., 2007), whereas PA has also been shown to exert toxic effects in the rat brain (Wyse et al., 1998; Brusque et al., 1999; de Mattos-Dutra et al., 2000; Fontella et al., 2000; Pettenuzzo et al., 2002; Trindade et al., 2002; Rigo et al., 2006; Ribas et al., 2010a,b).

Tryptophan catabolism and NAD^+ synthesis occur in cells from different tissues through the kynurenine pathway (KP). This metabolic route is relevant for biomedical research as neuroactive intermediary metabolites are synthesized throughout (reviewed by Pérez-De La Cruz et al. (2007)), some of which are involved in pathogenic processes of neurological disorders, including Huntington's disease (HD) (reviewed by Schwarcz et al., 2010, 2012). One of these KP metabolites, quinolinic acid (QUIN or 2,3-pyridine dicarboxylic acid) is an endogenous N-methyl-D-aspartate receptor (NMDAr) agonist (Stone et al., 2003). QUIN induces excitotoxicity in animal models and cell cultures, provoking enhanced intracellular $[Ca^{2+}]$, augmented levels of extracellular glutamate, increased reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation, decreased activity and expression of antioxidant systems, oxidative stress, stimulated protease activity and cell death (Rios and Santamaría, 1991; Rodríguez-Martínez et al., 2000; Tavares et al., 2000; Braidy et al., 2009, 2010; Pérez-De La Cruz et al., 2010). Moreover, QUIN could exert a pathogenic role in different neurodegenerative disorders since increased levels of this metabolite have been described in these pathological conditions (Schwarcz et al., 2010).

When considered separately, the toxic profiles characterized at the experimental level for the organic acids accumulating in OA and for QUIN in human neurological disorders could be not sufficient to explain the extent of cell and tissue damage produced by them *per se*, yielding the assumption that additional and additive mechanisms could account for the toxic profiles of these metabolites. Therefore, the aim of this work was to investigate whether GA, 3-OHGA, MMA or PA can exert synergic toxic effects with QUIN when tested in rat brain synaptosomes at subtoxic concentrations, upon the hypothesis that QUIN might eventually contribute to neurodegenerative processes in OA.

EXPERIMENTAL PROCEDURES

Reagents

GA, MMA, PA, QUIN, HEPES, thiobarbituric acid (TBA), kynurenic acid (KA), L-nitro-L-arginine methyl ester (L-NAME), malondialdehyde (MDA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other reagents were obtained from Sigma–Aldrich Chemical Co. (St Louis, MO, USA). Dr. Ernesto Brunet (Universidad Autónoma de Madrid, Spain) kindly supplied 3-OHGA. Other reagents were obtained from other well-known commercial sources. S-allylcysteine (SAC) was synthesized according to previous reports (García et al., 2008, 2014).

Animals

Male Wistar adult (250–300 g) rats were used throughout the study. Animals ($N = 40$) were obtained from the vivarium of the Universidad Nacional Autónoma de México. All rats were housed five per cage and provided with food and water *ad libitum* under constant conditions of temperature (25 ± 3 °C), humidity and light (12:12-h light:dark schedule). All animal manipulations were carried out following the "Guidelines for the Use of Animals in Neuroscience Research" from the Society of Neuroscience, the local Ethics Committees, and in compliance with the ARRIVE guidelines.

Isolation of brain synaptosomal P2 fractions and treatments

Isolation of synaptosomal P2 fractions from rat brains was carried out according to Lopachin et al. (2009), with modifications (Rangel-López et al., 2015). All brains (without cerebellum) were surgically removed, weighted, transferred to ice-cooled PBS (pH 7.4), and homogenized in 10 volumes (g/ml) of sucrose (0.32 M). The cerebellum was excluded because this brain region is generally not altered in GA I, MMAcidemia and PAcidemia, whose accumulating metabolites were tested in our work. Homogenates were centrifuged for 10 min at 1073×*g* (4 °C) and

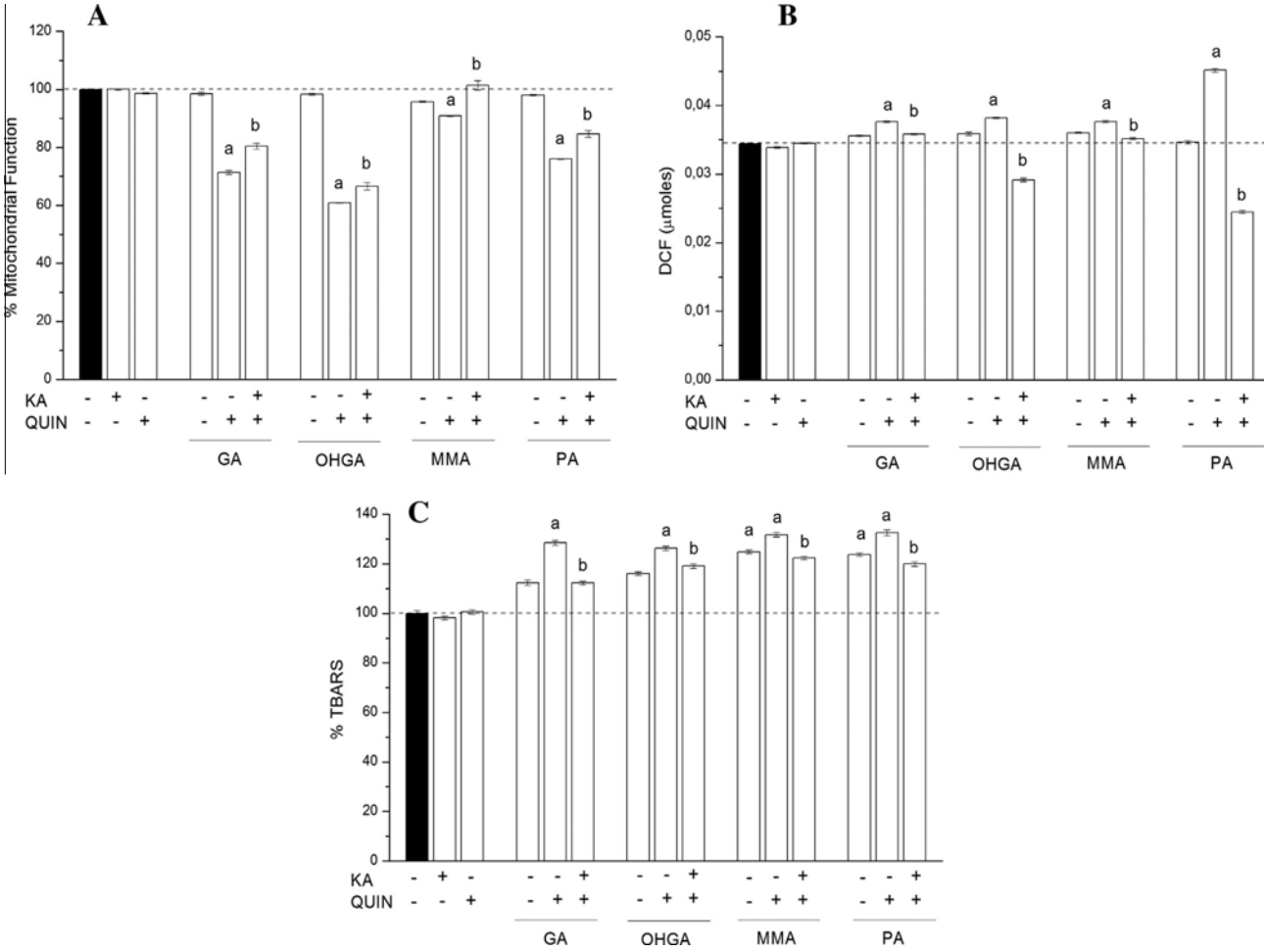

Fig. 1. Effects of kynurenic acid (KA, 50 μM) on the quinolinic acid (QUIN, 50 μM) plus glutaric acid (GA, 500 μM)-, 3-hydroxyglutaric acid (3-OHGA)-, methylmalonic acid (MMA)- or propionic acid (PA)-induced changes in mitochondrial function (A), reactive oxygen species (ROS) formation (B) and lipid peroxidation (C) in rat brain synaptosomal fractions. KA was added to synaptosomes in incubation 30 min before the simultaneous addition of QUIN and/or organic acids. The total incubation time was 60 min. All data are expressed as mean values ± S.E.M. of $n = 6–8$ experiments per group. One-way ANOVA followed by *post hoc* Duncan's test for comparisons among treatments. Symbols denote statistical differences vs. control ($^{a}P < 0.05$), and vs. QUIN plus each organic acid ($^{b}P < 0.05$).

the supernatants were re-centrifuged for 15 min at 17,000×*g* (4 °C). The resulting pellets were resuspended in 40 volumes (ml) of HEPES-buffer containing 0.1 M NaCl, 0.001 M NaH_2PO_4, 0.005 M $NaHCO_3$, 0.001 M $CaCl_2$, 0.006 M glucose, and 0.01 M HEPES (pH 7.4). Aliquots were briefly stored at −70 °C until employed for the experiments. Total protein quantifications were estimated using the technique reported by Bradford (1976).

Synaptosomal fractions were pre-incubated for 30 min at 37 °C with O_2 supply, and immediately thereafter co-incubated for 30 min with GA, 3-OHGA, MMA or PA (all at 500 μM) plus QUIN (50 μM). These concentrations were assumed to be subtoxic as revealed by the estimation of toxic endpoints with the different agents added separately. Effective concentrations of other agents (50 μM KA, an endogenous NMDAr antagonist; 100 μM SAC, a potent antioxidant; 100 μM L-NAME, a nitric oxide synthase (NOS) inhibitor; 0.05 U/mL catalase (CAT), an antioxidant and detoxifying enzyme; and 500 μM creatine (CREAT), a well-known metabolic precursor) were added as pretreatments 30 min before the addition of toxic metabolites in order to explore possible mechanisms involved in these models since a pharmacological perspective. Data of 6–8 experiments per group (three probes per condition per experiment) were collected for each endpoint evaluated. After exposed to the mentioned treatments, synaptosomal fractions were assigned to the different analytical procedures described as follows.

The assay of ROS formation

The formation of ROS was estimated according to previous reports (Santamaría et al., 2001; Rangel-López et al., 2015). After incubating in the presence of the different treatments, the synaptosomal fractions were diluted in nine volumes of 40 mM Tris plus HEPES buffer. Then, samples were incubated with 5 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 60 min at 37 °C. Fluorescent signals were recorded at 488 nm of excitation and

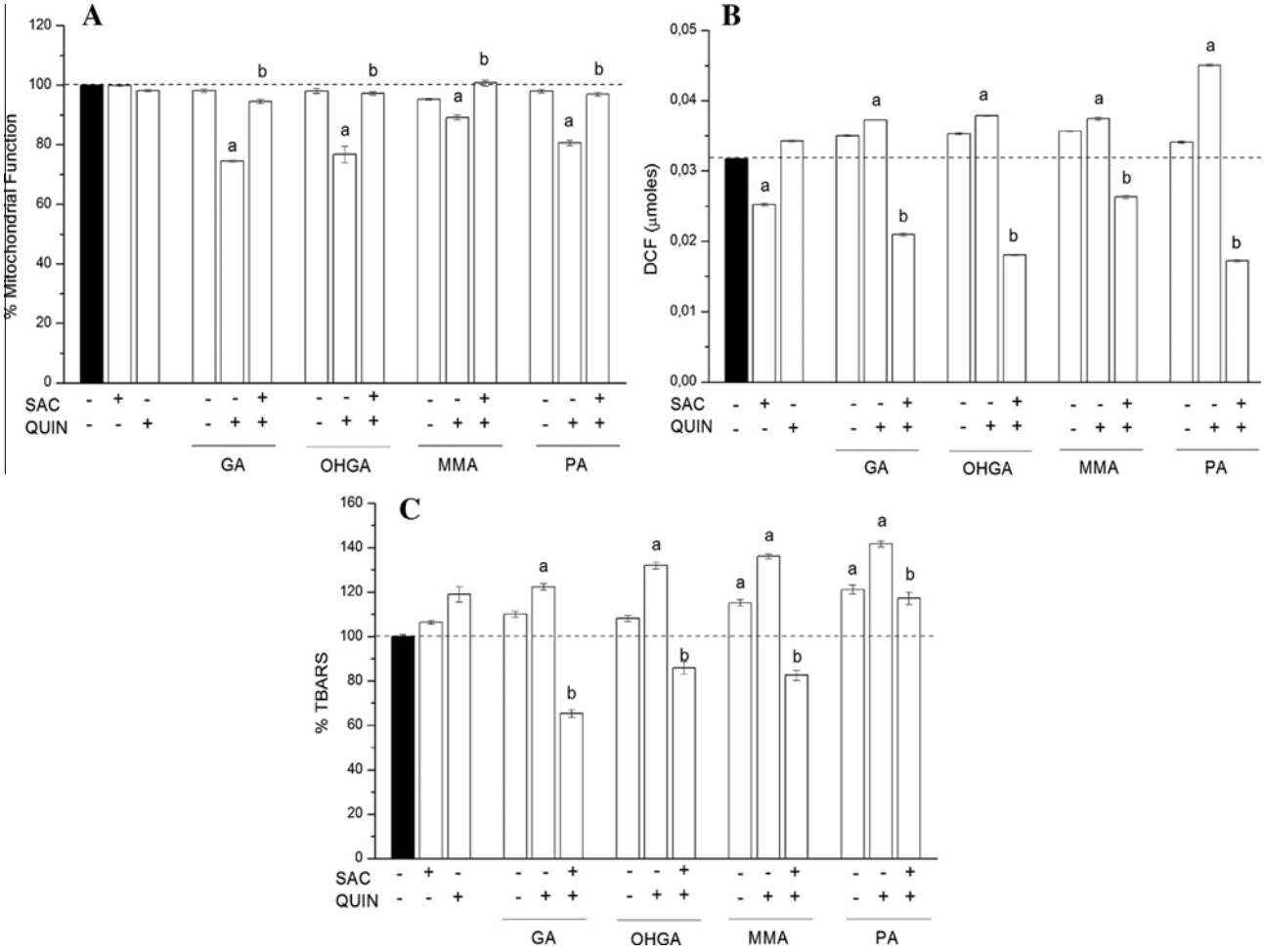

Fig. 2. Effects of S-allyl cysteine (SAC, 100 μM) on the quinolinic acid (QUIN, 50 μM) plus glutaric acid (GA, 500 μM)-, 3-hydroxyglutaric acid (3-OHGA)-, methylmalonic acid (MMA)- or propionic acid (PA)-induced changes in mitochondrial function (A), reactive oxygen species (ROS) formation (B) and lipid peroxidation (C) in rat brain synaptosomal fractions. SAC was added to synaptosomes in incubation 30 min before the simultaneous addition of QUIN and/or organic acids. The total incubation time was 60 min. All data are expressed as mean values ± S.E.M. of n = 6–8 experiments per group. One-way ANOVA followed by *post hoc* Duncan's test for comparisons among treatments. Symbols denote statistical differences vs. control ($^{a}P < 0.05$), and vs. QUIN plus each organic acid ($^{b}P < 0.05$).

525 nm of emission wavelengths in a CYT3MV Biotek Cytation 3 Imaging Reader. Results were expressed as micromoles of 2′,7′-dichlorofluorescein (DCF)/g wet tissue.

Assay of lipid peroxidation

The formation of thiobarbituric acid-reactive substances (TBARS) in synaptosomes was used as an index of lipid peroxidation, according to a previous report (García et al., 2008). After homogenized, the synaptosomal fractions (200 μL) were added with 500 μL of the TBA reagent containing 0.75 g of TBA + 15 g of trichloroacetic acid + 2.54 mL of HCl. The pink chromophore indicating the amount of peroxidized lipids was formed in samples after incubated in a water bath at 100 °C for 30 min. To stop the reaction, samples were kept on ice for 5 min and further centrifuged at 3000×g for 15 min. A CYT3MV Biotek Cytation 3 Imaging Reader was used to estimate the optical density of the supernatants at 532 nm. A standard curve was constructed in parallel with tetramethoxypropane and served for interpolation and calculation of the amounts of TBARS – mostly MDA – formed in samples. Final results were estimated as nanomoles of MDA per mg protein, and finally expressed as the percent of lipid peroxidation vs. control.

The MTT reduction assay for functional assessment of synaptosomes

The functional status of the respiratory chain and mitochondrial function was estimated in synaptosomes by the MTT reduction assay, according to a method previously described (Rangel-López et al., 2015). Briefly, the synaptosomes were added with 8 μl of MTT (5 mg/ml) and re-incubated for 60 min at 37 °C. Samples were centrifuged at 15,300×g for 15 min and the pellets resuspended in 1 ml of isopropanol. The aqueous phase was discarded after the first centrifugation. The second centrifugation was performed at 1700×g for 3 min. A CYT3MV Biotek Cytation 3 Imaging Reader was used to estimate the content of formazan at a 570 nm wavelength.

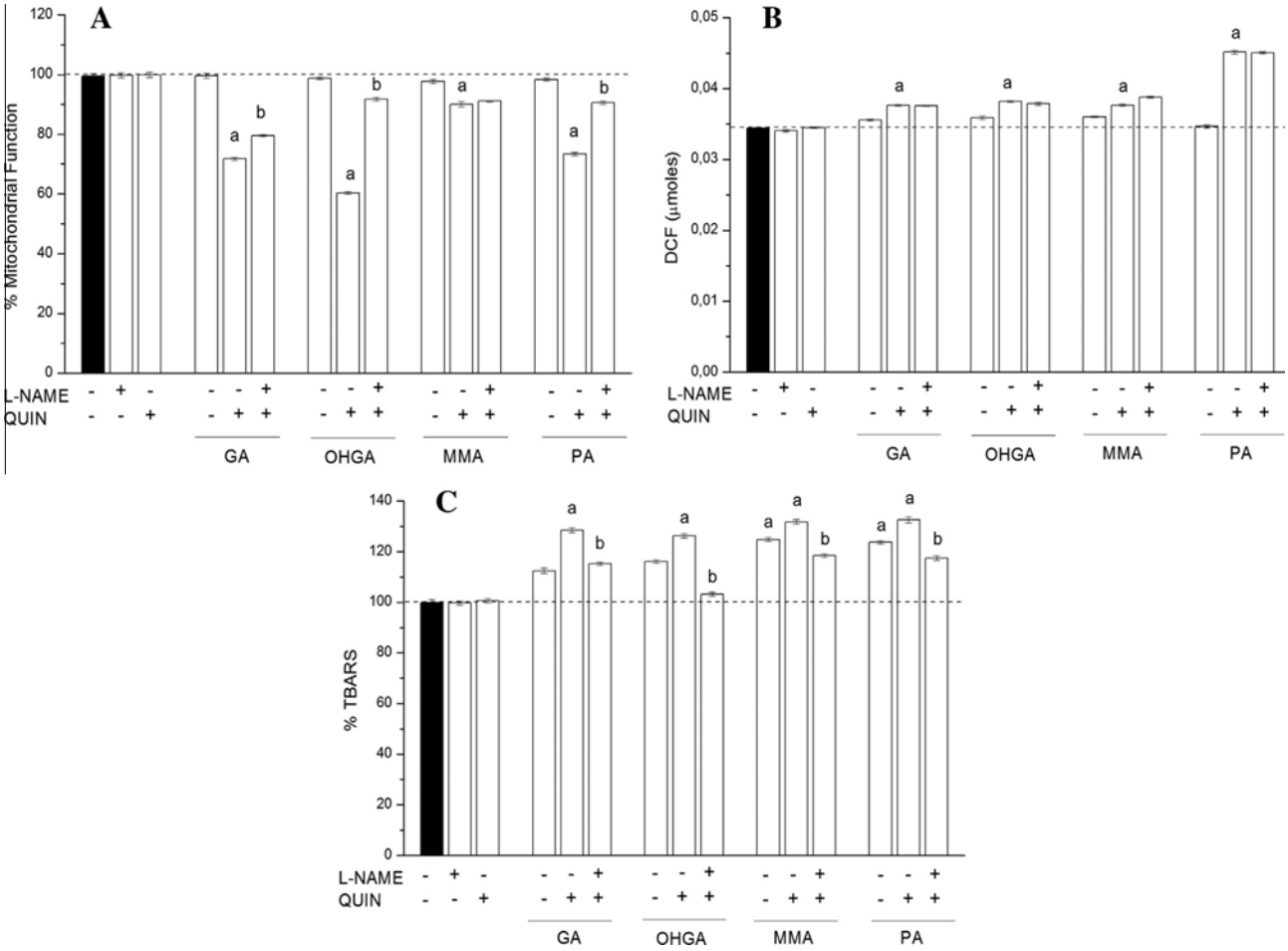

Fig. 3. Effects of L-nitro-arginine methyl ester (L-NAME, 100 μM) on the quinolinic acid (QUIN, 50 μM) plus glutaric acid (GA, 500 μM)-, 3-hydroxyglutaric acid (3-OHGA)-, methylmalonic acid (MMA)- or propionic acid (PA)-induced changes in mitochondrial function (A), reactive oxygen species (ROS) formation (B) and lipid peroxidation (C) in rat brain synaptosomal fractions. L-NAME was added to synaptosomes in incubation 30 min before the simultaneous addition of QUIN and/or organic acids. The total incubation time was 60 min. All data are expressed as mean values ± S.E.M. of $n = 6–8$ experiments per group. One-way ANOVA followed by *post hoc* Duncan's test for comparisons among treatments. Symbols denote statistical differences vs. control ($^{a}P < 0.05$), and vs. QUIN plus each organic acid ($^{b}P < 0.05$).

Results were expressed as the percent of MTT reduction vs. control values.

Statistical analysis

Data are expressed as mean values ± S.E.M. All results were statistically analyzed by a one-way analysis of variance (ANOVA), followed by *post hoc* Duncan's test. Statistical significance was assigned to comparison of treatments reaching values of $P < 0.05$. The statistical procedures were performed using the scientific statistic software GraphPad Prism 5 (GraphPad Scientific, San Diego, CA, USA).

RESULTS

GA, 3-OHGA, MMA and PA exerted a synergism with QUIN on different toxic endpoints

As above mentioned, in this study we used concentrations of organic acids that could be considered as subtoxic on the basis of previous studies and our own experience. The 500 μM concentration of GA, 3-OHGA, MMA and PA used herein represents the half of their toxic concentration (1 mM) previously used in synaptosomal fractions in a recent study (Colín-González et al., 2015).

While most of the toxic metabolites tested (QUIN, GA, 3-OHGA, MMA and PA) did not produce *per se* significant changes in mitochondrial function (3% below the control for QUIN, 6% below the control for GA, 5% below the control for 3-OHGA, 8% below the control for MMA, and 3% below the control for PA), ROS formation (2% below the control for QUIN, 3% above the control for GA, 4% above the control for 3-OHGA, 3% above the control for MMA, and 1% above the control for PA) or lipid peroxidation (2% above the control for QUIN, 10% above the control for GA, 14% above the control for 3-OHGA, 22% above the control for MMA [$P < 0.05$], and 20% above the control for PA [$P < 0.05$]) (Figs. 1–3), the simultaneous incubation of synaptosomes with QUIN and the organic acids produced a toxic synergism evidenced by decreased levels of MTT reduction (30% below the control for QUIN + GA [$P < 0.05$], 42% below the control for QUIN + 3-OHGA [$P < 0.05$], 9%

below the control for QUIN + MMA [$P < 0.05$], and 22% below the control for QUIN + PA [$P < 0.05$]), moderate but still significant changes in ROS formation (8% above the control for QUIN + GA [$P < 0.05$], 9% above the control for QUIN + 3-OHGA [$P < 0.05$], 7% above the control for QUIN + MMA [$P < 0.05$] and 29% above the control for QUIN + PA [$P < 0.05$]), and more prominent increases in lipid peroxidation (32% above the control for QUIN + GA [$P < 0.05$], 28% above the control for QUIN + 3-OHGA [$P < 0.05$], 34% above the control for QUIN + MMA [$P < 0.05$], and 31% above the control for QUIN + PA [$P < 0.05$]) (Figs. 1–3).

The toxic markers stimulated by the co-incubation of synaptosomes with QUIN + organic acids were partially sensitive to KA

The pre-conditioning of synaptosomal fractions for 30 min with the NMDAr antagonist KA resulted in partial and moderate but still significant prevention of mitochondrial dysfunction induced by QUIN + GA (10% above the toxic treatment and 20% below the control [$P < 0.05$]), QUIN + 3-OHGA (6% above the toxic treatment and 36% below the control [$P < 0.05$]), QUIN + MMA (13% above the toxic treatment and 3% above the control [$P < 0.05$]), and QUIN + PA (7% above the toxic treatment and 15% below the control [$P < 0.05$]). When tested *per se* or when challenging each toxic metabolite separately, KA had no effect on MTT reduction (Fig. 1A).

KA also moderately but significantly reduced the ROS formation induced by QUIN + GA (8% below the toxic treatment [$P < 0.05$] and 6% above the control), QUIN + 3-OHGA (26% below the toxic treatment and 17% below the control [$P < 0.05$]), QUIN + MMA (5% below the toxic treatment [$P < 0.05$] and 3% above the control), and in a more prominent manner by QUIN + PA (53% below the toxic treatment and 37% below the control [$P < 0.05$]) (Fig. 1B).

In regard to oxidative damage to lipids, this marker was moderately but still significantly reduced by KA (19% below, comparing KA + QUIN + GA vs. QUIN + GA [$P < 0.05$]; 5% below, comparing KA + QUIN + 3-OHGA vs. QUIN + 3-OHGA [$P < 0.05$]; 15% below, comparing KA + QUIN + MMA vs. QUIN + MMA [$P < 0.05$]; 14% below, comparing KA + QUIN + PA vs. QUIN + PA [$P < 0.05$]) (Fig. 1C). In addition, KA moderately reduced the levels of lipoperoxidation induced by the metabolic acids *per se* (data not shown).

The effects exerted by the combination of QUIN + organic acids were prevented by SAC

The antioxidant SAC, added as pretreatment to synaptosomes, completely prevented the mitochondrial dysfunction induced by the combination of QUIN plus all organic acids (30% above and 5% below when comparing SAC + QUIN + GA vs. QUIN + GA [$P < 0.05$] and Control, respectively; 23% above and 3% below when comparing SAC + QUIN + 3-OHGA vs. QUIN + 3-OHGA [$P < 0.05$] and Control, respectively; 17% above and 2% above when comparing SAC + QUIN + 3-MMA vs. QUIN + MMA [$P < 0.05$] and Control, respectively; 14% above and 3% below when comparing SAC + QUIN + 3-PA vs. QUIN + PA [$P < 0.05$] and Control, respectively) (Fig. 2A).

When SAC was tested in the ROS formation assay, the antioxidant was able to significantly reduce the QUIN + organic acids-induced DCFH oxidation even below the control values (44% below and 38% below when comparing SAC + QUIN + GA vs. QUIN + GA and Control, respectively [$P < 0.05$]; 55% below and 47% below when comparing SAC + QUIN + 3-OHGA vs. QUIN + 3-OHGA and Control, respectively [$P < 0.05$]; 28% below and 19% above when comparing SAC + QUIN + 3-MMA vs. QUIN + MMA and Control, respectively; 72% below and 59% below when comparing SAC + QUIN + 3-PA vs. QUIN + PA and Control, respectively [$P < 0.05$]) (Fig. 2B).

The TBARS formation induced by QUIN + organic acids was decreased by SAC in all cases (50% below and 39% below when comparing SAC + QUIN + GA vs. QUIN + GA and Control, respectively [$P < 0.05$]; 37% below and 15% below when comparing SAC + QUIN + 3-OHGA vs. QUIN + 3-OHGA and Control, respectively [$P < 0.05$]; 45% below and 21% below when comparing SAC + QUIN + 3-MMA vs. QUIN + MMA and Control, respectively [$P < 0.05$]; 21% below and 15% above when comparing SAC + QUIN + 3-PA vs. QUIN + PA and Control, respectively [$P < 0.05$]) (Fig. 2C).

L-NAME exerted differential effects on the toxic endpoints stimulated by QUIN + organic acids

The NOS inhibitor L-NAME slightly improved the QUIN + GA- and QUIN + PA-induced MTT reduction (14% and 20% above, respectively [$P < 0.05$]). More prominent prevention of mitochondrial dysfunction was produced by L-NAME on QUIN + 3-OHGA (55% above [$P < 0.05$]) (Fig. 3A). No effect of L-NAME was found on QUIN + MMA. L-NAME *per se* had no effect on MTT reduction. L-NAME *per se* did not induce any effect on MTT reduction.

Despite that L-NAME did not prevent the levels of ROS formation induced by none of the QUIN + organic acids conditions (Fig. 3B), this agent was able to significantly reduce the levels of DCFH oxidation induced by all toxic metabolites *per se* (data not shown).

Regarding lipid peroxidation (Fig. 3C), L-NAME reduced the oxidative damage induced by QUIN + GA (13% below, [$P < 0.05$]), QUIN + 3-OHGA (18% below [$P < 0.05$]), QUIN + MMA (21% below [$P < 0.05$]), and QUIN + PA (20% below [$P < 0.05$]). Finally, L-NAME *per se* did not induce any effect on TBARS production (data not shown).

Neither CAT, nor CREAT, was able to prevent the mitochondrial dysfunction induced by QUIN + organic acids

The antioxidant and metabolic modulators CAT and CREAT were tested in synaptosomes challenged with the combination of QUIN + organic acids. The only endpoint evaluated was MTT reduction capacity. None

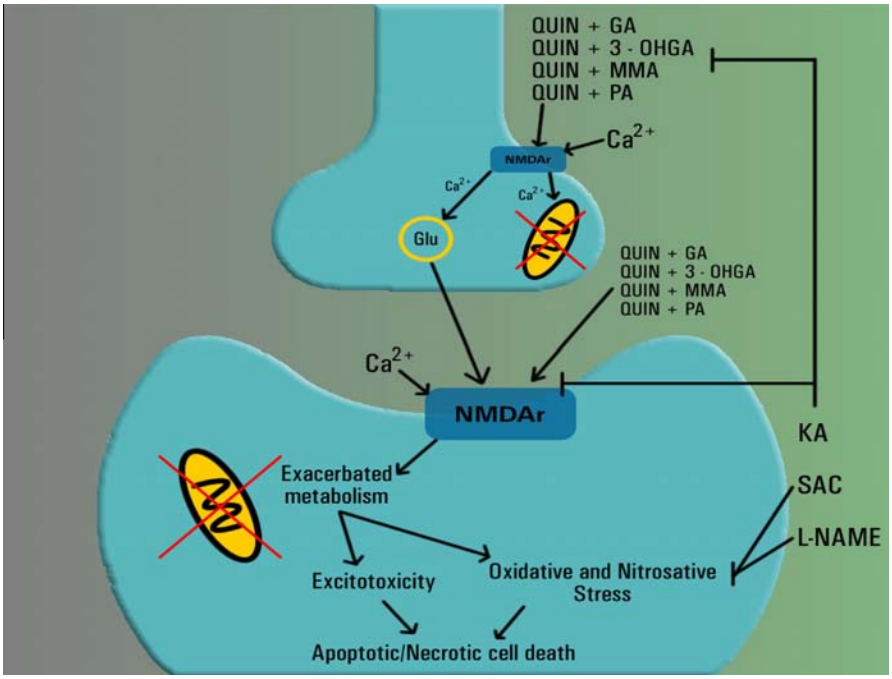

Fig. 4. Schematic representation of the toxic events stimulated by the synergic action of glutaric acid (GA), 3-hydroxyglutaric acid (3-OHGA), methylmalonic acid (MMA) or propionic acid (PA) plus quinolinic acid (QUIN) in the rat brain synaptosomes, and their prevention by agents with different protective profiles like kynurenic acid (KA, endogenous NMDAr antagonist), S-allyl cysteine (SAC, antioxidant and redox modulatory agent) and L-nitro-arginine methyl ester (L-NAME, NOS inhibitor). All toxic metabolites *per se* can stimulate excitotoxic processes, reactive oxygen/nitrogen species (ROS/RNS) formation and mitochondrial respiratory chain disruption, further leading to cell death via apoptotic/necrotic events. When simultaneously present at subtoxic concentrations, metabolic acids and QUIN might potentiate toxic events by synergic actions, as demonstrated herein with synaptosomal fractions. The efficacy that the different protective agents tested exerted on these models suggests that these synergic patterns can be interrupted at different levels, thus revealing the active role of several toxic mechanisms in these models.

of these agents prevented at all the alterations produced by the toxic combinations (data not shown). In light of this lack of effects, no further endpoints were tested for these agents.(see Fig. 4).

DISCUSSION

To date, the precise mechanisms underlying neurodegeneration in GA I remain poorly unknown, despite the many studies available that have been published so far investigating the pathophysiology of this disorder.

Based on a previously proposed hypothesis (Varadkar and Surtees, 2004), our group has recently addressed the issue of a possible active role of the KP in the neuropathology of GA I through the concerted action of two pathological events comprising the *in vivo* knockout of GDD (Gcdh, the key degradation enzyme for GA) followed by an intrastriatal infusion of QUIN in mice (Seminotti et al., 2015). These animals ($Gcdh^{-/-}$) were fed a high lysine diet to stimulate GA production. QUIN exerted an acute synergic action with the stimulated $Gcdh^{-/-}$ condition to decrease energetic markers like creatine kinase activity and the respiratory chain complex IV, while it increased oxidative stress endpoints like oxidative damage to lipids, nitrite/nitrate formation and depleted levels of reduced glutathione in striatal tissue. Despite that this first contribution clearly establishes hard basis to support the hypothesis that the KP (through QUIN) may be involved in the neuronal damage observed in GA I and other acidemias, specific toxic mechanisms underlying neuronal degeneration and occurring at the level of synaptic structures (the nerve communication functional unit) are still needed. Therefore, in the present study we aimed to challenge synaptic terminals with QUIN and different toxic organic acids (GA, 3-OHGA, MMA and PA) accumulating in common OA, and evaluated endpoints of oxidative toxicity and mitochondrial dysfunction to provide more specific information on the role of synergic actions between these molecules as initiators of deleterious events in the brain. The sensitivity of the markers evaluated to different pharmacological agents acting at different mechanistic levels was also assessed to complement the *in vitro* acute toxic paradigms developed.

Our results revealed that there is an acute toxic synergism exerted by QUIN + organic acids in all the three toxic endpoints evaluated herein, but this effect was differentially expressed among the organic metabolite tested: in the case of mitochondrial dysfunction, the order of magnitude was QUIN + 3-OHGA > QUIN + GA = QUIN + PA > QUIN + MMA. This result clearly suggests that the first three models recruit in a more prominent manner mitochondrial alterations than that produced by the combination of QUIN + MMA. In contrast, regarding ROS formation, the order of magnitude for this effect was as follows: QUIN + PA > QUIN + 3-OHGA = QUIN + GA = QUIN + MMA. These tendencies suggest that the toxic model exerted by QUIN + PA is more prompt to oxidative

damage; however, when the biological consequence of ROS formation was estimated (oxidative damage to lipids), the order of magnitude tells another story: QUIN + GA = QUIN + MMA = QUIN + PA = QUIN + 3-OHGA. Therefore, it seems evident that, although all models involved the active formation of precursors for oxidative damage in a differential manner, the levels of oxidative damage in all of them are more or less similar, thus supporting the concept that oxidative stress is a major component of the toxic pattern induced by these synergic models.

We also found a moderate, but still significant prevention of mitochondrial dysfunction induced by KA in all the toxic models tested, which contrasted with a potent reduction of ROS formation for all cases – including the toxins *per se* – reaching values even below the basal levels. Once again, the response represented by ROS formation seems to be disproportional when compared to its biological consequence, lipid peroxidation, which for the case of KA, was modestly but still significantly reduced in all models. Altogether, these results clearly suggest that there is a glutamatergic component involved in the initiation of the toxic events produced in all the synergic models tested, but the precise contribution of this component remains to be characterized in further studies.

While KA was employed in these models to evidence the degree of participation of the glutamatergic component (as above mentioned), SAC was used as a tool to emphasize the oxidative component involved in these models. In contrast to KA, the effect of SAC on the mitochondrial dysfunction induced by QUIN + organic acids was more intense. The order of magnitude of protection by SAC on the toxic models was QUIN + GA = QUIN + 3-OHGA = QUIN + PA > QUIN + MMA. In addition, SAC was a potent inhibitor of ROS formation, not only in all synergic models, but also when tested against the toxins *per se*, as well as in the basal condition. The order of magnitude of protection induced by SAC in the synergic models was QUIN + PA > QUIN + 3-OHGA = QUIN + GA > QUIN + MMA. This effect was confirmed through the action of SAC on the QUIN + organic acids-induced lipid peroxidation, in which SAC not only reduced this marker even below the control levels in all synergic models, but also when tested against the toxins *per se*. The order of magnitude of SAC efficacy among the synergic models was QUIN + GA > QUIN + MMA = QUIN + 3-OHGA > QUIN + PA. The relevance of these findings is laying in the evidence collected that oxidative stress is playing a major role in the alterations evoked by the combined actions of QUIN + all organic acids, as evidenced by the robust inhibitory effects that SAC exerted on all toxic endpoints, which in magnitude were more prominent than those of KA, thus leaving the glutamatergic component as an convergent mechanism to the already-in-progress damage induced by oxidative stress.

Moreover, as part of the oxidative damage in course, another converging subordinated mechanism is nitrosative stress. The characterization of the actions of nitric oxide and other nitrogen species on diverse markers of cell dysfunction and oxidative damage is crucial for the estimation of the degree of participation of nitrosative stress in these models. We made this approach through the use of the NOS inhibitor L-NAME, which produced partial prevention of mitochondrial dysfunction in three of the four synergic models. The order of magnitude of the preventive action of L-NAME among the models was QUIN + 3-OHGA > QUIN + PA > QUIN + GA. Noteworthy, despite L-NAME reduced the levels of ROS/RNS formation induced by all the toxins *per se* even below the control levels, it was unable to reduce this endpoint at all in the synergic models tested. In contrast, L-NAME partially reduced lipid peroxidation in all the synergic models. The order of magnitude of this effect among the models was QUIN + 3-OHGA > QUIN + GA = QUIN + MMA = QUIN + PA. In addition, this agent reduced the oxidative damage to lipids when tested against all organic acids *per se*. Therefore, the effects of L-NAME on these markers and models suggest that, as expected, nitrosative stress is also participating in the acute pattern of toxicity elicited by the synergic condition produced by QUIN and the organic acids, being responsible of part of the mitochondrial dysfunction and oxidative damage to lipids, but not in ROS formation.

QUIN-induced neurodegeneration has been shown to involve the activation of different signaling pathways and transcription factors. So far, the most relevant mechanism recruited by QUIN in the CNS is excitotoxicity, an overstimulation of glutamatergic NMDAr mainly acting on subunits NR2A and NR2B, thus leading to an increased Ca^{2+} influx through and the consequent pathological cascade resulting in neuronal death (Pérez-De La Cruz et al., 2012). Recent data of our group show that QUIN stimulates the mitochondrial dysfunction *in vivo* in GDD knockout ($Gcdh^{-/-}$) mice subjected to a high Lys dietary intake, which is probably occurring because of the additive effect of QUIN, GA and 3-OHGA (Seminotti et al., 2015). Therefore, although the precise mechanisms by which QUIN and the metabolic acids exerted a synergic action in this study remain to be solved in further studies, in our previous work we found support to our present findings since $Gcdh^{-/-}$ mice under a high Lys dietary intake were more susceptible to the effects of QUIN. It can be hypothesized that increased amounts of the accumulating organic acids GA and 3-OHGA may be participating in these effects. A clue to solve this issue appeared recently in a report showing that NMDAr, specifically NR2A and NR2B subunits, are highly expressed in $Gcdh^{-/-}$ animals receiving a high Lys overload (Lagranha et al., 2014). Given that QUIN stimulates these NMDA receptor subunits, as some of the organic metabolites are supposed to do, it can be suggested that the increased stimulation of these receptors by these neurotoxic metabolites in a synergic action may play a crucial role in both mutant mice and synaptosomes.

Of major consideration is the fact that the concept of synergism is not new at all for metabolic acids. Recently, a toxic interaction between azaspiracid (50 nM) – a toxin found in shellfish harvested in Ireland

– and GA (1 mM) was shown to produce a significant inhibition of sodium channels in *in vitro* experiments, while when added separately, these two compounds had no effects on these channels (Chevallier et al., 2015). Moreover, GA was found as a component of the same shellfish used in the study. Furthermore, the synergic action of QUIN and glutamate to induce and exert neurotoxicity has been well documented through the stimulation of glutamate release from the presynaptic terminals and the inhibition of reuptake by astrocytes, both leading to a recruited excitotoxicity (Tavares et al., 2002). It can be then assumed that chemical or biological interactions like those described above can potentiate the toxic features of endogenous toxic agents, thus giving support to our hypothesis that an interaction of QUIN and the organic acids involved in acidurias at the biological, chemical and/or molecular levels may contribute to the understanding of toxic mechanisms occurring in these and other neurological disorders coursing with neurodegeneration. In addition, our study might also have implications for cancer research and could bring an additional dimension of translational relevance since it has been shown that both GA metabolism (Quincozes-Santos et al., 2010; Vissers et al., 2011) and kynurenine metabolism (Sahm et al., 2013; Adams et al., 2014) through events like QUIN uptake by tumor cells (Saito et al., 1993; Müller and Schwarz, 2007), have been demonstrated to be involved partially in tumor growth in gliomas.

CONCLUDING REMARKS

Our present results show that the combination of QUIN plus GA, 3-OHDA PA or MMA impairs mitochondrial function and enhances oxidative damage in rat brain synaptic terminals. In addition, we found that this damage was primarily linked to ROS formation, and to a lesser extent, to RNS formation and acute excitotoxicity. As proposed in a previous study of our group (Seminotti et al., 2015), our present results also support the hypothesis that increased concentrations of QUIN produced after the KP activation occurring during inflammatory and other toxic events, could play a crucial role in the magnification of the striatal degeneration that follows alterations in patients affected by GA I (Varadkar and Surtees, 2004), MMAcidemia and PAacidemia through a synergic action with the accumulating metabolites GA, 3-OHGA, MMA or PA, which may turn the striatum more vulnerable during pathologic episodes commonly occurring in OA. Hence, the identification of new toxic mechanisms recruiting the deleterious actions produced by neurotoxins with different profiles represent an alternative with potential application for the design of more effective therapeutic approaches.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

Acknowledgements—This work was supported by CNPq Grant 404883/2013-3 (M.W., Brazil) and CONACYT Grant 205648 (A. S., Mexico). Ana Laura Colín-González is scholarship holder of CONACyT-Mexico (239954). We appreciate the generosity of Drs. Enrique Pinzón and Ismael Torres (Departamento de Bioterio, UNAM) for supplying animals for some experiments.

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(Accepted 1 September 2015)
(Available online 4 September 2015)

Published online: 09 June 2016

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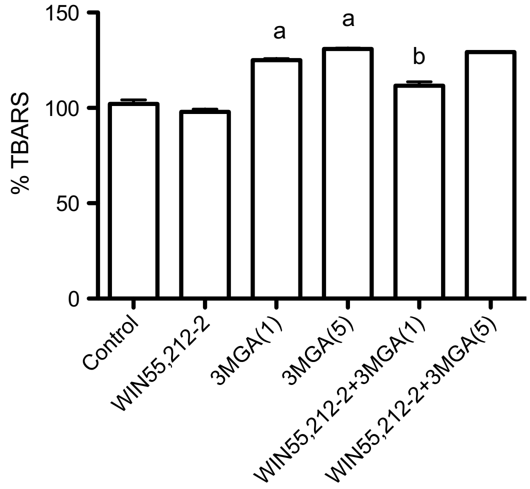
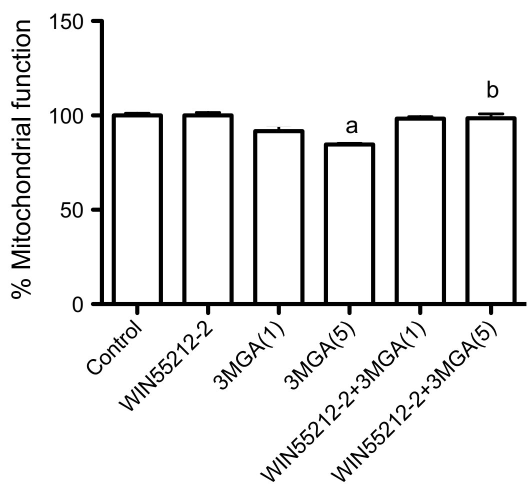
Y kWbV Y Vq L Uf] d, F uh r 1 Ud- S07 T- □ dh mg lk o □□□ k hU, □U Y Uf Y be e Y bU dq e hdgq Y [g □la □ ph □be f lk- Na □ bkgdU Y kqf Uhlgkge Ud Uf Y e hdgWdgf Y bUd, f bWa Y [UW lbgf k o □□□ bf WmVU Y bf la □ h □k f W g[la □ □; □U] gf bkd Q DH44 101,1 (0 □G) [g □2/ e bf Uf Y.g □2G B □ (0 g □4 e G) [g □la □f □pl 2/ e bf - ERH □ (4/ □G) □M □ □ (0// □G) Uf Y F, H □G □ (0// □G) o □□□ Udkg mk Y bf kge □ ph □be f lk lg l □kl la □bf ngdh e f l g[HG □ □□□ la □ gd □ g[gpbYUbn □ kl □kk Uf Y la □ hU lbWh Ubgf g[f lbWgpbY □ (HI) kh W k bf la □ [[W k dbW Y Vq la □ lgpbWkqf □] bke h gYmWY Vq 2G B □ (0 e G) □ KODH (4/ g □0// □G) - ERH □ □M □ □ Uf Y F, H □G □ o □□□ Udkg UYY Y lg kqf Uhlgkge □k 2/ e bf V [g □□ la □ UYY dbgf g[la □ lgpbWU f lk- Na □ kUe □bf WmVUbgf W gf Yh, lbgf k o □□□ mk Y la □ gm] agm la □ klmYq [g □ Udde Uc □ k- Na □ □□□□□□F □□□□□□□□□□□



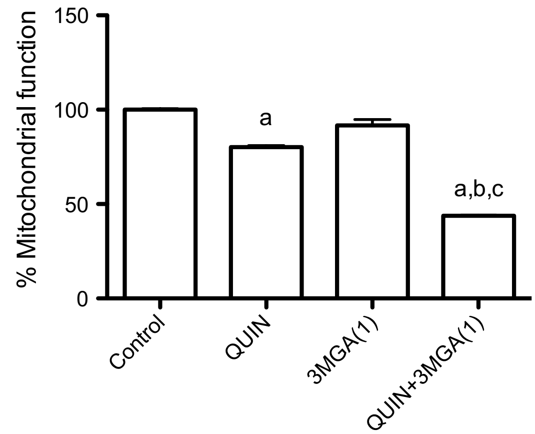
□N□□ □WVTih dW2,b Vi□naYa□iRg T RTU (2F B; □0 RcU 4 b F) RcU dg P DG44□101,1 (0 □F) dc a□e□Ue Vgd mUR□dc (M<; K□aVkVah) □ gRi SgR□c hncReidhdb Ra RcU b idT□dcUgRa, VcgT□VU VgRTi□dch- □RiR RgV VmegVhhVU Rh b VRc kRa□Vh □□□F dWc □5p7 VmeVg□b Vcih eVg Ygd□e- □□□□□□□ UVcdiV h□Rc□h□TRa U□WVgVcTVh kh- Tdcigda ([R]I □/-/4) RcU RYR□chi 2F B; (0) igVRib Vci ([S]I □/-/4)- M d,1 Rn ; GHO; Waadl VU Sn □□cTRcdh iVhi



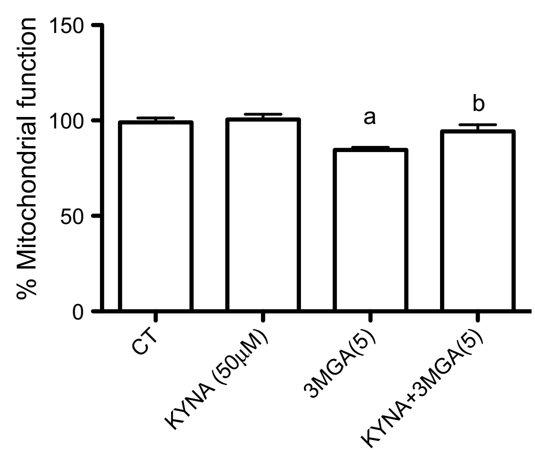
□N□□ P DG44□101,1 (0 □F) egVkVciVU i□V 2,b Vi□naYa□iRg T RTU (2F B; □0 RcU 4 b F), □cUTVU T□RcYVh □c b idT□dcUgRa WcTi□dc (F MMgVU□Ti□dc) □c SgR□c hncReidhdb Ra RcU b idT□dcUgRa, VcgT□VU VgRTi□dch- □RiR RgV VmegVhhVU Rh b VRc kRa□Vh □□□F dWc □5p7 VmeVg□b Vcih eVg Ygd□e- □□□□□□□ UVcdiV h□Rc□h□TRa U□WVgVcTVh kh- Tdcigda ([R]I □/-/4) RcU RYR□chi 2F B; ([S]I □/-/4)- M d,1 Rn ; GHO; Waadl VU Sn □□cTRcdh iVhi

□B □1 4c□I □□□GEdS□N□□ b □□DFA□ □S B □□□□□S□□GQ3 d□MS□□□S □S 2 □GS EdSGU□□□□ □□

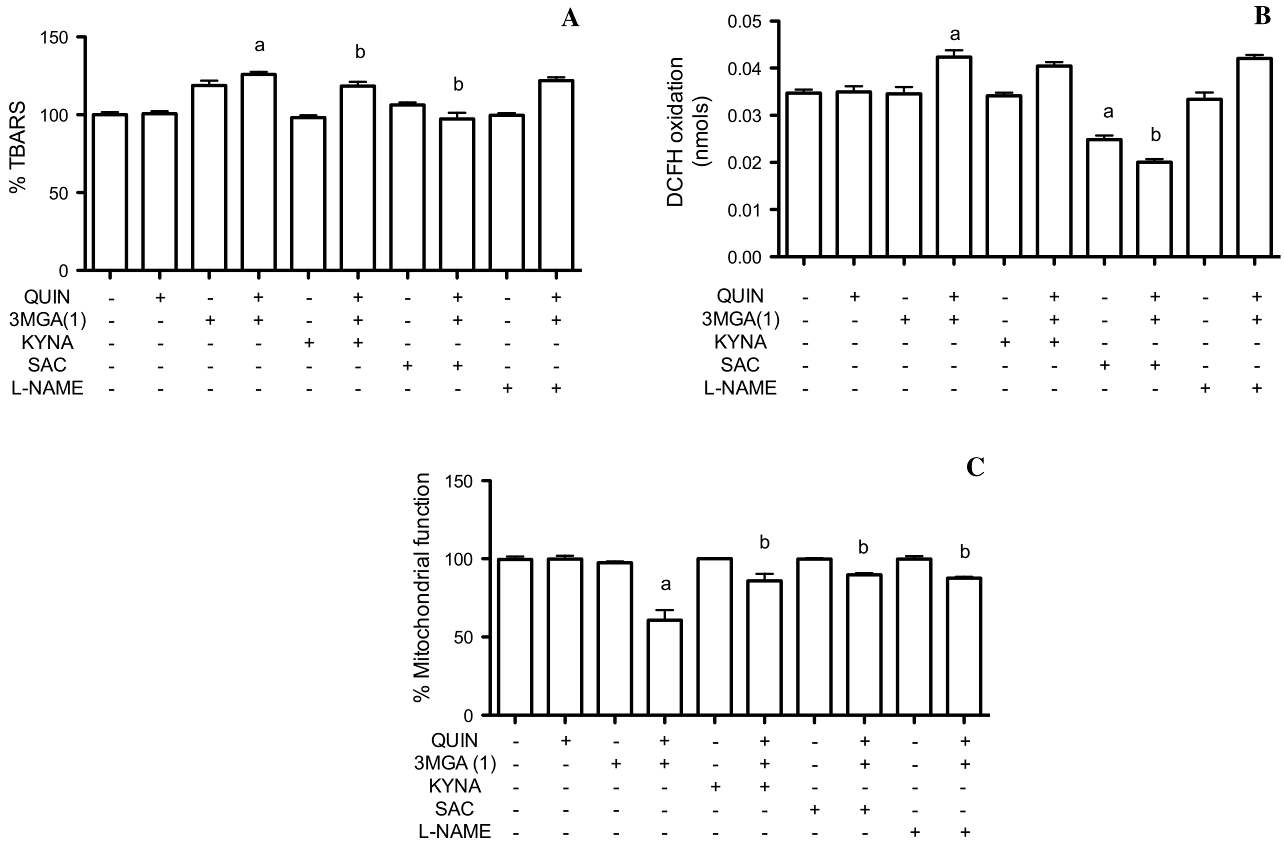
Dc dgUVg id iVhi l □Vi□Vg 2F B; RcU □NDG b Rn VmVgi hncVgYT idmTRTi□dch l □Vc RUUVUidYVi□Vg id □cT SRiVU hncReidhdb Ra □, □□□ □-□□-, □.□□□, .□□□□□□.□□□-, □□□□.□□□□□□, □□□□□□□ egVeRgRi□dc l i□ RidmT TdcTVcigRi□dc dW□NDG (0// □F) eaRh R cdc,idmT TdcTVcigRi□dc dW2F B; (0 b F)-; h VmeVTiVU□ l □Vc iVhi□cY i□V VWWTi dW□NDG Ri R idmT TdcTVcigRi□dc dc b idT□dcUgRa WcTi□dc (A□Y- 2) i□h b VRSda□iV TRhVU eVg hVhdb VaVkVa dWb idT□dcUgRa UnhWcTi□dc (07% SVadl i□V Tdcigda9I □/-/4)- Dc Tdcig Rhi 2F B; eVghVRi 0 b F TdcTVc, □□□-, □□□□, -□□□□, □□□, □□□□□□, □□□□ □-□□-, □□□□□□, □□-, □□ □dl VkVg l □Vc hncReidhdb Vh l VgV Td,□cT SRiVU l i□ □NDG RcU 2F B; i□V UVYgVV dWb idT□dcUgRa UnhWcTi□dc l Rh hnc, VgYhi□TRan Vc□RcTVU (28 RcU 36 % SVadl □NDG RcU 2F B; eVghV gVhVTi□kVan9I □/-/4)- Dc WgiVg VmeVg□b Vcih (A□Y- 4) l V cVkV□YRiVU l □Vi□Vg Rc ReeRgVcian h□SidmT TdcTVcigRi□dc dW□NDG (4/ □F) l Rh RSaV id kd□Vh hncVgYhb l i□ 2F B; -



□N□□ MdmT hncVgYhb SVil VVc 2F B; (0 b F) RcU f □cda□c□T RTU (□NDG □0// □F) □c b idT□dcUgRa WcTi□dc (F MMgVU□Ti□dc) □c gRi SgR□c hncReidhdb Vh- □RiR RgV VmegVhhVU Rh b VRc kRa□Vh □□□F dW c □5p7 VmeVg□b Vcih eVg Ygd□e- □□□□□□□ UVcdiV h□Rc□h□TRa U□WVgVcTVh kh- Tdcigda ([R]I □/-/4) RcU RYR□chi i□V □NDG ([S]I □/-/4) dg 2F B; ([T]I □/-/4) igVRib Vcih- M d,1 Rn ; GHO; Waadl VU Sn □□cTRcdh iVhi



□N□. Enc□gVc□T RTU (EQG; □4/ □F) eRgi□Ran egVkVciVU i□V T□RcYVh □c b idT□dcUgRa WcTi□dc (F MMgVU□Ti□dc) TRhVU Sn 2F B; □c gRi SgR□c hncReidhdb Vh- □RiR RgV VmegVhhVU Rh b VRc kRa□Vh □□□F dW c □5p7 VmeVg□b Vcih eVg Ygd□e- □□□□□□□ UVcdiV h□Rc□h□TRa U□WVgVcTVh kh- Tdcigda ([R]I □/-/4) RcU RYR□chi i□V 2F B; igVRib Vci ([S]I □/-/4)- M d,1 Rn ; GHO; Waadl VU Sn □□cTRcdh iVhi


A
% TBARS
150
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□]khW 3U □We diHS□W □]S□ F MM hWkU]ed mSi □WhWSiW□To □]WUec T]dS]ed eY1F A9 Sd□□NCG (2□% TWem □]WUed heb)□Tk□de□To 1F A9 eh □NCG fWiW DPG9 □L9; Sd□E,G9F □fhWWd□W]d S i]c]bSh c Sd, dWi □]W 1F A9 □□NCG,]dkUW c]eU[ed h]Sb □oiYkdU,]ed (14□20 Sd□16% STelW□]W1F A9 □□NCG hWSc Wd□ hWfWU]lWbo□I □. -. 3)- □]dShbo□dedWeY□]WWW hk im□[fe Wd□]Sbfhe WU]lWSU]edi (DPG9 □L9; ehE,G9F □)□[S□ WWWU fWiWed c]eU[ed h]SbYkdU]ed-

□□□□□□□□□□□□

Gd □]i ik□o mW[SlWUehbWUW W]□WdUWikffeh]d□□]W UedUW□□[S□□]YWWWd□Sd□iodWi□]Uden]eki c W[Sd]ic i Uekb□TWfSh□eY□]W en]UfS□Wd WbjU]W□To 1F A9]d □]W ThS]d-M[hek□[i]c fbWWfWc Wd□Sb Sffhe SU[W Sffb]W□e iodSf eiec Sb Sd□c]eU[ed h]Sb,Wdh]U[W YhSU]edi □]□mSi □We diHS□W □]S□1F A9 WiWi□en]U]o□]hek□[□]WWShbo SU]lS]ed eYc W[Sd]ic bjaWen]□S]lWihWi (KHL Yehc S,]ed Sd□bjf]□fWen]□S]ed) Sd□c]eU[ed h]Sb□oiYkdU]ed (F MM hWkU]ed)- MemSh□□]WWWd□fe]di□S USddST]de]□, iWi]]lWUec fedWd□mSi hWWSbW□To □]WiodW]U;:h S□ed ji OGG33 0 0,0 i]dUW□]i Uec fekd□fhWWd□W iec W eY□]WWWWUi WiWiW□To 1F A9 - Gd S□□]ed□SaodkhWd]dW Uec fedWd□Ykh□]Wi]d□]US]d□S□hk Sc S□W□]U Uec fedWd□ mSi hWWSbW□To □]WiodW□]USU]ed WiWiW□To 1F A9 Sd□ □]WWU]e en]d□NCG ed □]W en]Uc ShaWi□Si mWbSi □]W fhWWd□lWSU]edi eY□]WGF □9h Sd□S ed ji□DPG9 ed □]W1F A9,]dkUW□en]U]o-M[WSU]lWfSh□]UfS]ed eY□]W en]□S]lWUec fedWd□]d □]WiodW□]Uc e Wb fhe kUW□To □□□□□□□□□□□□

ZWiWd j d[R D ZW Wo]ipWo] oc] cjsdZ]aa]Zcn ZWpn][Yt oc] WZZph pgWd]i ja oc]n] ZWnYjstgdZWZd[n, D W[dod]i)d Wjoc]mncp[t jajpmbmjnpk U/4V)r] cWq] []hjinomWo][W cjsdZntij]mbdhh jZZpmodb Y]or]]ijnjh] ja oc]n] jmbWdZ WZd[n W[LPD) npkkjmodb WbgpoWiWo]mbdZ Zjhkjij]io d oc]n] ijq]gcjsdZhj[]gn, Oc] ZWiWd j d[Zjhkjij]io cWiWgnj Y]]i ZcWnWZo]mdu][d oc] cjsdZhj[]gkmjqjf][Yt LPD k]mn] U/6V: gcjb]oc]moc]n] ncp[d]nZgWgt npbb]no ocWo]sZdcjcjsdZdt) jsd[Wdq] [WiWo] W[h dcjZcji[mdWg [tnapiZodji Zjpg[pi[]ngd] oc] cjsdZdt ja 1HB: W[dcn mjgWo][ZWnYjstgdZWZd[n[pmdb oc] kmjnbmjmndji ja jmbWdZ WZd[pmdWid Zcdg[mjigWjdbcji]pmjn[]b]i]mWdji, Apmoc]m hjmj)dor Wic]mj[]hjinomWo][ocWoc]n]]q]icnZjpg[Y] Wo]ipWo][ocjpbc oc] kcWnh WZjgibdZWgh Wdkpgdji ja oc] FKW[oc] □□N,

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□□, JO□ead A□) Ka□S 8 ROI) □SWaidO: □) RS Jaf□O□□) □S□U□) RS □cSWQdI □) BacRO□B□□-. -(DWaWOWRPZa□□dSWfcSdWRf□SR Pi bWa□QbWSgWWW□cSQdSdWRSZOO□WaZSgfZWWRS□iRcQeQdS O□R FO□)C□8KHQdS O□eeWWW□cQe PcOW, H□e□O□aZ9W□S□ 9S□Qg62755k6.

□0, JQfScJM) G□f□B□) □cW□Sc□) □O□cWUSc8) □fZZScA): c□WDI) □f□ZQfdS□:) □aTT□O□□□□) □acdeSc□) □aaR□O□JA) □QeRWU: G) CaSZZSc□□) CaZ□ScJ □□-- 3(A□eO□ScSPcOZO□□f□fZQeW□aT □3□□□□□□□□□□

Mol Neurobiol
DOI 10.1007/s12035-015-9548-9

Oxidative Stress, Disrupted Energy Metabolism, and Altered Signaling Pathways in Glutaryl-CoA Dehydrogenase Knockout Mice: Potential Implications of Quinolinic Acid Toxicity in the Neuropathology of Glutaric Acidemia Type I

Bianca Seminotti[1] · Alexandre Umpierrez Amaral[1] · Rafael Teixeira Ribeiro[1] · Marília Danyelle Nunes Rodrigues[1] · Ana Laura Colín-González[2] · Guilhian Leipnitz[1] · Abel Santamaría[2] · Moacir Wajner[1,3]

Received: 23 June 2015 / Accepted: 17 November 2015

Abstract We investigated the effects of an acute intrastriatal QUIN administration on cellular redox and bioenergetics homeostasis, as well as on important signaling pathways in the striatum of wild-type ($Gcdh^{+/+}$, WT) and knockout mice for glutaryl-CoA dehydrogenase ($Gcdh^{-/-}$) fed a high lysine (Lys, 4.7 %) chow. QUIN increased lactate release in both $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice and reduced the activities of complex IV and creatine kinase only in the striatum of $Gcdh^{-/-}$ mice. QUIN also induced lipid and protein oxidative damage and increased the generation of reactive nitrogen species, as well as the activities of the antioxidant enzymes glutathione peroxidase, superoxide dismutase 2, and glutathione-S-transferase in WT and $Gcdh^{-/-}$ animals. Furthermore, QUIN induced DCFH oxidation (reactive oxygen species production) and reduced GSH concentrations (antioxidant defenses) in $Gcdh^{-/-}$. An early increase of Akt and phospho-Erk 1/2 in the cytosol and Nrf2 in the nucleus was also observed, as well as a decrease of cytosolic Keap1caused by QUIN, indicating activation of the Nrf2 pathway mediated by Akt and phospho-Erk 1/2, possibly as a compensatory protective mechanism against the ongoing QUIN-induced toxicity. Finally, QUIN increased NF-κB and diminished IκBα expression, evidencing a pro-inflammatory response. Our data show a disruption of energy and redox homeostasis associated to inflammation induced by QUIN in the striatum of $Gcdh^{-/-}$ mice submitted to a high Lys diet. Therefore, it is presumed that QUIN may possibly contribute to the pathophysiology of striatal degeneration in children with glutaric aciduria type I during inflammatory processes triggered by infections or vaccinations.

Keywords Glutaric acidemia · Quinolinic acid · Inflammatory response · Redox homeostasis · Energy metabolism · Signaling pathways

✉ Moacir Wajner
mwajner@ufrgs.br

[1] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos N° 2600-Anexo, CEP 90035-003 Porto Alegre, RS, Brazil

[2] Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, Manuel Velasco Suárez, SSA, México, DF, México

[3] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

Introduction

Glutaric acidemia type I (GA I) is a neurometabolic recessive disease caused by glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) deficiency, which participates in the catabolism of lysine, hydroxylysine, and tryptophan. Blockage of this enzyme activity leads to accumulation of high amounts of glutaric (GA) and 3-hydroxyglutaric (3HGA) acids in tissues and biological fluids (blood, urine, and cerebrospinal fluid) of the affected patients [1, 2]. GA I is considered a cerebral organic acidemia because affected individuals present predominantly neurological symptoms. At birth, macrocephaly and frontotemporal cortical atrophy are observed. Between 3 and 36 months of age, patients suffer acute bilateral striatal degeneration during or following encephalopathic episodes triggered by catabolic events, such as infections, fever, or prolonged fasting, in which the concentrations of the accumulating metabolites dramatically increase reaching millimolar concentrations [3]. Thereafter, they present dyskinesia and

Published online: 25 November 2015

dystonia, hypotonia, seizures, muscle stiffness, and spasticity [2, 4–6].

Inflammatory processes contribute to the appearance and progression of various neurodegenerative disorders. Neuroinflammation can change various parameters of cerebral energy metabolism and induce oxidative stress and excitotoxicity in these diseases [7–9]. Although excessive release of inflammatory cytokines by macrophages and microglia in the central nervous system (CNS) has been associated with the pathogenesis of neurodegeneration, the exact role of these molecules is not yet well established [10]. The kynurenine pathway (KP), the most important route of tryptophan catabolism, is stimulated by inflammatory cytokines resulting in the production of metabolites capable of modulating different redox systems in various physiological functions. In the brain, some metabolites formed in this pathway, including kynurenic acid, kynurenine, and quinolinic acid (QUIN), are important for normal functioning [11–13]. However, when in excess, they may play a toxic role in CNS disorders mainly associated to excitotoxicity, such as Parkinson's and Huntington's diseases and in GA I [13–15]. QUIN (2,3-pyridine-dicarboxylic acid) is a metabolite of KP that is capable of inducing neurotoxic effects by different mechanisms [16]. The primary mechanism exerted by this excitotoxin in the CNS has been largely related with overactivation of N-methyl-D-aspartate receptors (NMDAr) and increased cytosolic Ca^{2+} concentrations that may be accompanied by free radical formation and oxidative damage, mitochondrial dysfunction, cytochrome c release, and ATP exhaustion [17, 18].

As regards to GA I, various in vitro and in vivo studies demonstrated that GA and 3HGA, the metabolites that most accumulate in this disorder, induce excitotoxicity, oxidative stress, and impairment of cellular energy metabolism in rat brain [19–41]. Despite the great deal of work carried out with these toxic organic acids, the pathomechanisms underlying the acute degeneration of the striatum that occurs in the affected patients during episodes of metabolic decompensation are not yet well established. However, it has been postulated that QUIN, a key KP metabolite whose biosynthesis is stimulated during these situations, may potentially contribute to striatal GA I pathogenesis and potentially precipitate severe neurological symptoms during these crises [15]. Indeed, the symptomatology of glutaric acidemic patients suddenly worsens during or after infection or immunization. So, it is possible that the high accumulation of organic acids (GA and 3HGA) during crises, associated with increased concentrations of QUIN, may play an important role in the pathophysiology of striatal damage in GA I patients.

Therefore, in the present study, we investigated the effects of QUIN intrastriatal administration on cellular bioenergetics and redox homeostasis in the brain of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice submitted to a high dietary lysine (Lys) chow. Central components of mitochondrial energy production, transfer and utilization, as well as important parameters of redox homeostasis and finally signaling pathways were evaluated in the striatum of 30-day-old *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice. Lactate production, the activities of the electron transfer chain complexes and creatine kinase (CK), as well as malondialdehyde (MDA) levels, sulfhydryl content, 2′,7′-dihydrodichlorofluorescein (DCFH) oxidation, nitrite and nitrate generation, reduced glutathione (GSH) concentrations, and the activities of glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), glucose-6-phosphate dehydrogenase (G6PDH), and glutathione-S-transferase (GST) were determined. We also assessed the protein content of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), Kelch-like ECH-associated protein 1 (Keap 1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), NF-kappa-B inhibitor alpha (IκBα), protein kinase B (Akt), and Erk 1/2 (extracellular signal-regulated kinase).

Material and Methods

Chemicals

All chemicals were of analytical grade and purchased from Sigma (St Louis, MO, USA) unless otherwise stated. Solutions were prepared on the day of the experiments and the pH was adjusted to 7.2–7.4 in the appropriate buffer for each technique.

Animals

Gcdh$^{+/+}$ and *Gcdh*$^{-/-}$ littermates, both of C129SvEv background, were generated from heterozygotes and maintained at Unidade Experimental Animal of the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil). The animals were maintained on a 12:12-h light/dark cycle (lights on 07:00–19:00 h) in air-conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20 % (*w*/*w*) protein commercial chow containing 0.9 % Lys (SUPRA, Porto Alegre, RS, Brazil). Thirty-day-old *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice were used in all experiments.

Ethical Statement

This study was performed in strict accordance with the Principles of Laboratory Animal Care, National Institute of Health of United States of America, NIH, publication no. 85-23, revised in 2011, the International Guiding Principles for Biomedical Research Involving Animals and approved by the Ethical Committee for the Care and Use of Laboratory Animals of the Hospital de Clínicas de Porto Alegre (n° 26967). All efforts were made to minimize suffering, discomfort,

stress, and the number of animals necessary to produce reliable scientific data.

High Dietary Lys Treatment and QUIN Striatal Infusion

Thirty-day-old *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ animals were submitted to a high dietary Lys intake (4.7 % Lys) for 48 h. This diet was shown to provoke an increase of GA and 3HGA similar to those found in GA I patients and lead to striatal damage in *Gcdh*$^{-/-}$ mice [42]. Animals (six per group) were randomly assigned to four experimental groups: groups I (*Gcdh*$^{+/+}$-NaCl) and III (*Gcdh*$^{-/-}$-NaCl) received a single intrastriatal infusion of 50 nmol NaCl (1 μL) dissolved in phosphate-buffered saline (PBS, pH 7.4), whereas groups II (*Gcdh*$^{+/+}$-QUIN) and IV (*Gcdh*$^{-/-}$-QUIN) received 50 nmol QUIN (1 μL) dissolved in PBS, pH 7.4 (dose selected on the basis of the study of Ignarro and coworkers [43].

After 48 h of Lys overload, NaCl or QUIN was injected into the striatum of the mice previously anesthetized with isoflurane (45 mg/kg, i.p.) and placed on a stereotaxic frame (Stoelting Co., Wood Dale IL, USA), using the following coordinates: 0.0 mm anterior to bregma, +2.6 mm lateral to bregma, and −2.4 mm ventral to the dura [44]. Drugs were injected for 3 min using a 10-μL Hamilton microsyringe. The needle was left in place for an additional 1 min after the administration and then slowly withdrawn. *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice were euthanized 24 h after QUIN infusion, and the striatum was immediately removed for the determination of the biochemical parameters (oxidative stress and bioenergetics). After QUIN injection, the animals received a moderately high dietary Lys (2.8 %) chow. Samples for immunoblotting experiments were obtained from animals killed 30 min after QUIN intrastriatal infusion and prepared as described below.

Bioenergetics Parameters

Striatum Preparation for Lactate Production

The striatum was dissected, weighed, and cut into 400 μm slices, which were incubated in Krebs–Ringer bicarbonate buffer, pH 7.4. Briefly, 50 mg of striatal slices was added to small flasks (11 cm^3), pre-incubated at 37 °C for 15 min in Krebs–Ringer bicarbonate buffer, pH 7.4, followed by the addition of 5 mM glucose. After 60 min incubation at 37 °C in a metabolic shaker, the reaction was stopped by the addition of one volume of 0.6 N perchloric acid to the medium. Striatal slices were then homogenized and the excess of perchloric acid was precipitated as a potassium salt with the addition of one volume of 3 M potassium bicarbonate. After centrifugation at 10,000×*g* for 5 min at 4 °C, lactate concentrations were measured in the supernatants by the lactate peroxidase method [45]. Results were expressed as micromole lactate/hour/gram tissue.

Striatal Preparation for Respiratory Chain Complex and Creatine Kinase (CK) Activities

For the determination of the respiratory chain complex and CK activities, the striatum was homogenized in 19 volumes (1:20, *w*/*v*) of SETH buffer (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base, and 50 UI mL^{-1} heparin), pH 7.4. Homogenates were centrifuged at 800×*g* for 10 min at 4 °C to discard nuclei and cell debris. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and used to measure these parameters.

Respiratory Chain Complex I–IV Activities

The activities of the complexes of the respiratory chain were measured in the presence of approximately 30 μg of protein. Succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate:cytochrome c oxidoreductase (complex II-III) activities were determined according to Fischer et al. [46]. Cytochrome c oxidase (complex IV; COX) was assayed according to Rustin et al. [47]. The activities were calculated as nanomole/minute.milligram protein.

Creatine Kinase (CK) Activity

CK activity was measured in total homogenates according to Hughes [48] with slight modifications. Briefly, the reaction mixture consisted of 50 mM Tris buffer, pH 7.5, containing 7.0 mM phosphocreatine, 7.5 mM $MgSO_4$, and 0.5–1.0 μg protein in a final volume of 0.1 mL. The reaction was started by addition of 4.0 mM ADP and stopped after 10 min by addition of 50 mM p-hydroxy-mercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes. The color was developed by the addition of 0.1 mL of 20 % α-naphtol and 0.1 mL of 20 % diacetyl in a final volume of 1.0 mL and read after 20 min at λ = 540 nm. Results were calculated as micromole of creatine/minute.milligram protein.

Oxidative Stress Parameters

Striatal Preparation

To measure the oxidative stress parameters (except for DCFH oxidation), striatum was homogenized in 9 volumes (1:10, *w*/*v*) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl and centrifuged at 750×*g* for 10 min at 4 °C to discard nuclei and cell debris [49]. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and used to quantify these parameters. Striatum slices were also prepared (400 μm) for DCFH oxidation measurement.

Malondialdehyde (MDA) Levels

We evaluated lipid oxidative damage by measuring malondialdehyde (MDA) levels through the thiobarbituric acid-reactive substances (TBA-RS) method. TBA-RS levels were measured according to the method described by Yagi [50] with slight modifications. Briefly, 200 μL of 10 % trichloroacetic acid and 300 μL of 0.67 % TBA in 7.1 % sodium sulfate were added to 100 μL of tissue supernatants containing 0.3 mg of protein and incubated for 2 h in a boiling water bath. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained complex was extracted with 400 μL of butanol. Fluorescence of the organic phase was read at 515 and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. TBA-RS levels were calculated as nanomole MDA/milligram protein.

Sulfhydryl Content

Protein oxidative damage was tested by measuring the sulfhydryl content, which is based on the reduction of 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm [51]. Briefly, 30 μL of 10 mM DTNB and 980 μL of PBS were added to 50 μL of tissue supernatants containing 0.3 mg of protein. This was followed by 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. Sulfhydryl content is inversely correlated to oxidative damage (sulfhydryl oxidation). Results were calculated and expressed as nanomole thiol/milligram protein.

2′,7′-Dihydrodichlorofluorescein (DCFH) Oxidation

Reactive oxygen species production (ROS) was assessed according to LeBel et al. [52] by using 2′,7′-dihydrodichlorofluorescein diacetate (DCF-DA). DCF-DA was prepared in 20 mM sodium phosphate buffer pH 7.4, also containing 140 mM KCl and incubated together with tissue slices (30 mg) during 30 min at 37 °C. DCF-DA is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity parallels the amount of reactive species present. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. A calibration curve was prepared with DCF (0.25–10 mM). The levels of ROS were calculated as picomole DCF formed/milligram protein.

Nitrate and Nitrite Content

Nitrate and nitrite concentrations were evaluated according to Navarro-Gonzálvez et al. [53] with some modifications. One hundred and fifty microliters of tissue supernatants (containing approximately 1.2 mg of protein) was deproteinized by adding 125 μL of 75 mM $ZnSO_4$ solution, followed by centrifugation at 9000×*g* for 2 min at 25 °C. The supernatant obtained was neutralized with 55 mM NaOH solution and diluted in 5 volumes of glycine buffer solution, pH 9.7. Copper-coated cadmium granules (600–1000 mg) were added to the supernatants to convert all nitrates into nitrite in the biological samples. Aliquots of 200 μL were then treated with 200 μL of Griess reagent (2 % sulfanilamide in 5 % HCl and 0.1 % N-1-(naphtyl)ethylenediamine in H_2O) and incubated at room temperature by 10 min. The absorbance was read at 505 nm. A calibration curve was prepared with $NaNO_2$ at concentrations ranging from 1 to 125 μM. The final results were expressed as micromole nitrate and nitrite/milligram of protein.

Antioxidant Defenses

Reduced Glutathione (GSH) Concentrations

GSH concentrations were measured according to Browne and Armstrong [54]. A hundred and fifty microliters of metaphosphoric acid was added to an equal volume of tissue supernatants containing 30 μg protein and centrifuged for 10 min at 7000×*g*. Then 30 μL from the supernatants were diluted with 70 μL of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA. This preparation was incubated with o-phthaldialdehyde (1 mg/mL in methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. Concentrations were calculated from a calibration curve of a GSH standard (0.001–1 mM) and expressed as nanomole GSH/milligram protein.

Enzymatic Antioxidant Enzymes

Glutathione Peroxidase (GPx) Activity

GPx activity was measured according to Wendel [55] using tert-butyl hydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM ethylenediaminetetraacetic acid, pH 7.7, 2 mM GSH, 0.1 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl hydroperoxide, 0.1 mM NADPH, and tissue supernatants (approximately 3 μg of protein). One GPx unit (U) is defined as 1 μmol of NADPH consumed per

minute. The specific activity was calculated and expressed as unit/milligram protein.

Glutathione Reductase (GR) Activity

GR activity was measured according to Carlberg and Mannervik [56] using oxidized glutathione (GSSG) and NADPH as substrates. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium with 200 mM sodium phosphate buffer, pH 7.5, containing 6.3 mM EDTA, 1 mM GSSG, 0.1 mM NADPH, and tissue supernatants (approximately 0.065 mg of protein). The results were calculated and expressed as unit/milligram protein.

Superoxide Dismutase (SOD) Activity

Total SOD activity was assayed according to Marklund [57] and is based on the capacity of pyrogallol to autooxidize, a process highly dependent on superoxide, which is a substrate for SOD. The inhibition of autoxidation of pyrogallol occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The reaction medium contained 50 mM Tris buffer/1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U/mL catalase, 0.38 mM pyrogallol, and tissue supernatant (approximately 1 μg of protein).

We also determined SOD1 (cytosolic isoform) and SOD2 (mitochondrial isoform) activities in the presence or absence of KCN (1.0 mM). SOD2 corresponds to the activity obtained in the presence of KCN, whereas SOD1 activity was calculated by subtracting the CN-sensitive activity from total SOD activity. Corrections were made for direct effects of CN^- on pyrogallol autoxidation [58]. Calibration curves were performed with purified SOD as standard to calculate SOD activities present in the samples. The results were expressed as unit/milligram protein.

Catalase (CAT) Activity

CAT activity was assayed according to Aebi [59] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1 % Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and tissue supernatants (approximately 1 μg of protein). One unit (U) of the enzyme is defined as 1 μmol of H_2O_2 consumed per minute. The specific activity was calculated and expressed as unit/milligram protein.

Glucose-6-Phosphate Dehydrogenase (G6PDH) Activity

G6PDH activity was measured by the method of Leong and Clark [60] in a reaction mixture containing 100 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$, and tissue supernatants (approximately 0.035 mg of protein). The reaction was started by the addition of 1 mM glucose-6-phosphate and was followed in a spectrophotometer at 340 nm. The results were calculated and expressed as unit/milligram protein.

Glutathione S-Transferase (GST) Activity

The activity of GST was measured according to Guthenberg and Mannervik [61] with slight modifications. GST activity was measured by the rate of formation of dinitrophenyl-S-glutathione at 340 nm in a medium containing 50 mM potassium phosphate, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, and tissue supernatants (approximately 0.045 mg of protein). The results were calculated and expressed as unit/milligram protein.

Western Blot Assays

Nuclear and Cytosolic Fraction Preparation for the Measurement of Transcription Factors and Inhibitors

Striatum was washed with cold PBS and lysed with a pre-cooled homogenizer in 300 μL cold buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 1 mM KCl, and 1 mM DTT) plus 1 μg/μL protease, phosphatase inhibitor cocktail, 1 mM PMSF, and 0.5 % Nonidet P-40, and incubated on ice for 15 min. The homogenates were centrifuged at 850×*g* for 10 min at 4 °C, and the supernatants (cytoplasmic extracts, SN1) were collected and stored at −80 °C. The pellets were resuspended in 200 μL of cold buffer, transferred to pre-cooled microcentrifuge tubes, and incubated on ice for 15 min. Then, 0.5 % Nonidet P-40 was added and the samples were incubated on ice for 5 min and mixed for 10 s. The suspensions were centrifuged at 14,000×*g* for 30 s at 4 °C, and the supernatants were collected in SN1. Then, the pellets were resuspended in 50 μL of complete lysis buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 % glycerol, 420 mM NaCl, and 1 mM DTT), plus 1 μg/mL protease, phosphatase inhibitor cocktail, and 1 mM PMSF, mixed for 10 s, and incubated on ice for 40 min (mixed for 10 s each 5 min). Finally, the suspensions were mixed for 30 s and centrifuged at 14,000×*g* for 10 min at 4 °C. The supernatants (nuclear extracts, SN2) were collected and stored at −80 °C. Inmunodetection was performed using the following primary antibodies, according to datasheet specifications: anti-Nrf2 (1:500, Abcam® ab31163), anti-Keap1 (1:500, Cell Signaling® D6B12), anti-NF-κB p-65 (1:500, Cell Signaling® D14E12), and anti-IκBα (1:500, Cell Signaling® L35A5).

Striatal Preparation for the Measurement of Akt and Erk 1/2 Protein Levels

Striatum was homogenized in Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 1 % SDS (*w*/*v*), 10 % glycerol (*v*/*v*)) and normalized by quantifying protein content. Inmunodetection was performed using the anti-Akt antibody (1:1,000, R&D Systems® MAB2055) and anti-Erk 1/2 and anti-phospho-Erk 1/2 (1:1000; Cell Signaling® p44/42 MAPK and Phospho-p44/42 MAPK), according to datasheet specifications.

Immunoblotting

To perform immunoblot experiments, equal amounts of protein (30 μg/well) were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes. After verifying protein loading and electroblotting efficiency through Ponceau S staining, the membrane was blocked in Tween-Tris buffered saline (TTBS: 100 mM Tris–HCl, pH 7.5, 0.9 % NaCl, and 0.1 % Tween-20) containing 5 % albumin. Membranes were then incubated overnight at 4 °C with each antibody separately in TTBS, at different working dilutions as suggested by the manufacturers, and afterwards washed with TTBS. Anti-rabbit or anti-mouse IgG peroxidase-linked secondary antibody (1:10,000; Santa Cruz®, sc-2030 and sc-2031, respectively) was incubated with the membranes for an additional 2 h, washed again, and the immunoreactivity was detected by enhanced chemiluminescence. Densitometric analysis was performed with ImageJ software. Blots were developed to be linear in the range used for densitometry. All results were expressed as a ratio relative to the β-actin (1:1000, Sigma-Aldrich® A1978) or lamin B1 (1:1000, Abcam® ab133741) internal control.

Protein measurement

Protein content in samples was quantified for data normalization according to Lowry et al. [62].

Statistical Analysis

Results are presented as mean ± standard deviation. Assays were performed in triplicate and the mean was used for statistical calculations. Duplicate or triplicate experiments were always carried out and the mean used for the calculations. Statistical analysis was performed with GraphPad 5.0 software. Student's *t* test (independent) was applied for simple comparisons between groups. Differences were considered significant when $P < 0.05$. Only significant results are presented.

Results

We first observed that $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice receiving 4.7 % dietary Lys for 48 h and injected intrastriatally with NaCl did not have apparent motor or behavioral alterations. In contrast, generalized convulsions were observed in all animals injected with QUIN.

Intrastriatal QUIN Administration Increases Lactate Release in the Striatum of $Gcdh^{+/+}$ and $Gcdh^{-/-}$ Mice

We evaluated the influence of a single intrastriatal injection of QUIN to $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice submitted to Lys overload (4.7 %) for 48 h on lactate release in the striatum, in an attempt to evaluate mitochondrial oxidative metabolism. Our results demonstrated that QUIN provoked a significant increase of lactate production in both $Gcdh^{+/+}$ [$t_{(7)} = 4.941$; $P < 0.01$] and $Gcdh^{-/-}$ mice [$t_{(6)} = 3.303$; $P < 0.05$] (Fig. 1), indicating a disruption of oxidative metabolism.

Intrastriatal QUIN Administration Decreases Complex IV Activity in the Striatum of $Gcdh^{-/-}$ Mice

Since QUIN impaired oxidative mitochondrial respiration, we next investigated the effects of in vivo QUIN administration on the activities of complexes II, II-III, and IV of the respiratory chain in striatum of $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice. We found that complex IV activity was significantly reduced in $Gcdh^{-/-}$ but not in $Gcdh^{+/+}$ mice injected with QUIN [$t_{(9)} = 2.150$; $P < 0.05$] (Table 1). It can be also seen in the table that no significant differences were found in the activities of complexes II and II-III in striatum of both genotypes. The data indicate a failure of electron transfer through the respiratory chain at the COX activity step.

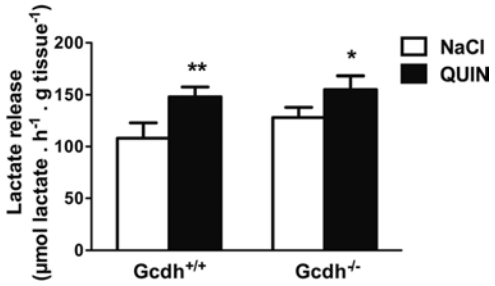

Fig. 1 Lactate release in the striatum of $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice on a high Lys (4.7 %) diet. Lactate concentrations were measured 24 h after a single intrastriatal injection of NaCl or QUIN (50 nmol). Results are represented as mean ± standard deviation for five independent experiments (animals) per group. *$P < 0.05$, **$P < 0.01$, compared to $Gcdh^{-/-}$ injected with NaCl (Student's *t* test for unpaired samples)

Intrastriatal QUIN Administration Decreases CK Activity in the Striatum of *Gcdh*$^{-/-}$ Mice

A significant reduction of CK activity in the striatum of *Gcdh*$^{-/-}$ mice injected with QUIN and submitted to a high Lys diet (4.7 %) was also observed as compared to NaCl-injected *Gcdh*$^{-/-}$ mice [$t_{(9)} = 8.781$; $P < 0.001$] (Fig. 2).

Intrastriatal QUIN Administration Induces Lipid Peroxidation in the Striatum of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ Mice

QUIN intrastriatal infusion caused a significant increase of MDA levels in the striatum of *Gcdh*$^{+/+}$ [$t_{(7)} = 4.493$; $P < 0.01$] and *Gcdh*$^{-/-}$ mice [$t_{(8)} = 4.265$; $P < 0.01$] as compared to animals injected with NaCl (Fig. 3). Furthermore, it can be observed that the increase of MDA concentrations was higher in knockout animals when compared to wild-type (WT) animals injected with QUIN [$t_{(8)} = 3.351$; $P < 0.05$].

Intrastriatal QUIN Administration Reduces Sulfhydryl Content in the Striatum of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ Mice

Figure 4 shows that sulfhydryl content was markedly reduced in the striatum of *Gcdh*$^{+/+}$ [$t_{(6)} = 3.164$; $P < 0.05$] and *Gcdh*$^{-/-}$ [$t_{(8)} = 3.837$; $P < 0.01$] mice after QUIN injection.

Intrastriatal QUIN Administration Increases DCFH Oxidation in the Striatum of *Gcdh*$^{-/-}$ Mice

Next, we assessed DCFH oxidation in the striatum of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice injected with QUIN. We observed that DCFH oxidation was significantly increased only in *Gcdh*$^{-/-}$ animals [$t_{(10)} = 4.989$; $P < 0.001$] (Fig. 5), indicating increased ROS generation in the mutant mice.

Table 1 Respiratory chain complex II, II-III, and IV activities in striatum from *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice under a high Lys (4.7%) diet and measured 24 h after a single intrastriatal injection of NaCl or QUIN (50 nmol)

	Gcdh$^{+/+}$ plus NaCl	*Gcdh*$^{+/+}$ plus QUIN	*Gcdh*$^{-/-}$ plus NaCl	*Gcdh*$^{-/-}$ plus QUIN
Complex II	2.06 ± 0.51	2.10 ± 0.40	1.92 ± 0.16	2.06 ± 0.21
Complex II-III	10.1 ± 1.51	9.50 ± 0.98	9.62 ± 0.74	9.12 ± 0.76
Complex IV	45.6 ± 6.40	39.1 ± 6.39	48.6 ± 12.5	34.0 ± 9.95*

Results are represented as mean ± standard deviation for five independent experiments (animals) per group

*$P < 0.05$, compared to *Gcdh*$^{-/-}$ injected with NaCl (Student's *t* test for unpaired samples). Results are expressed as nanomole per minute per milligram protein

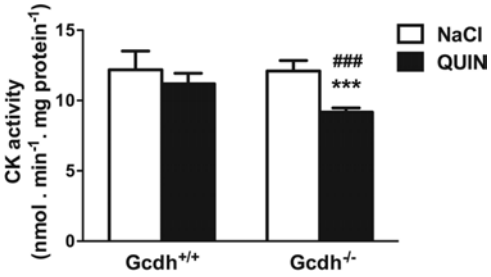

Fig. 2 Creatine kinase (CK) activity in the striatum of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice on a high Lys (4.7 %) diet. CK activity was measured 24 h after a single intrastriatal injection of NaCl or QUIN (50 nmol). Results are represented as mean ± standard deviation for five independent experiments (animals) per group. ***$P < 0.001$, compared to *Gcdh*$^{-/-}$ injected with NaCl; $^{###}P < 0.001$, compared to *Gcdh*$^{+/+}$ injected with QUIN (Student's *t* test for unpaired samples)

Intrastriatal QUIN Administration Increases Nitrate and Nitrite Production in the Striatum of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ Mice

We next verified that QUIN in vivo administration provoked a significant increase of nitrate and nitrite levels in the striatum of *Gcdh*$^{+/+}$ [$t_{(7)} = 3.55$; $P < 0.01$] and *Gcdh*$^{-/-}$ [$t_{(5)} = 4.071$; $P < 0.01$] mice as compared to animals injected with NaCl (Fig. 6). It can be also seen in the figure that nitrate and nitrite production was slightly higher in *Gcdh*$^{-/-}$ mice relative to *Gcdh*$^{+/+}$ mice.

GSH Concentrations Are Decreased in the Striatum of *Gcdh*$^{-/-}$ Mice Fed a High Lys Diet

Figure 7 shows that GSH concentrations were significantly lower in *Gcdh*$^{-/-}$ mice receiving Lys overload, as compared to *Gcdh*$^{+/+}$ mice [NaCl: $t_{(8)} = 4.262$; $P < 0.01$; QUIN: $t_{(8)} = 2.448$; $P < 0.05$]. In contrast, QUIN injection had no effect on this parameter.

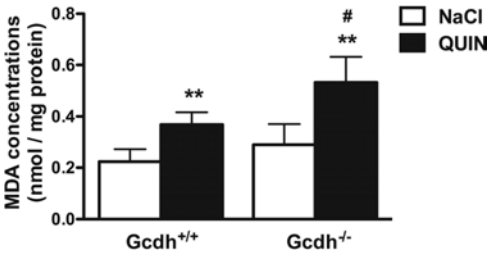

Fig. 3 Malondialdehyde (MDA) concentrations in the striatum of *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ mice on a high Lys (4.7 %) diet. MDA concentrations were measured 24 h after a single intrastriatal injection of NaCl or QUIN (50 nmol). Results are represented as mean ± standard deviation for five independent experiments (animals) per group. **$P < 0.01$, compared to *Gcdh*$^{-/-}$ injected with NaCl; $^{#}P < 0.05$, compared to *Gcdh*$^{+/+}$ injected with QUIN (Student's *t* test for unpaired samples)

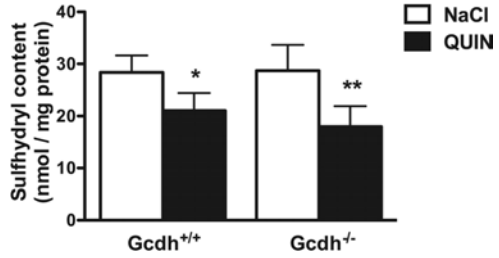

Fig. 4 Sulfhydryl content in the striatum of $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice on a high Lys (4.7 %) diet. Thiol oxidation was measured 24 h after a single intrastriatal injection of NaCl or QUIN (50 nmol). Results are represented as mean ± standard deviation for five independent experiments (animals) per group. $^{**}P < 0.01$, compared to $Gcdh^{-/-}$ injected with NaCl (Student's *t* test for unpaired samples)

Intrastriatal QUIN Administration Alters the Antioxidant Enzyme Activities in the Striatum of $Gcdh^{-/-}$ Mice

As regards to the enzymatic antioxidant defenses, we observed a reduction of GST activity in $Gcdh^{-/-}$ animals (Fig. 8f) [$t_{(7)} = 3.75$; $P < 0.01$]. Moreover, QUIN injection increased GPx (Fig. 8a) and SOD2 (Fig. 8d) activities in both $Gcdh^{+/+}$ [GPx: $t_{(8)} = 13.34$; $P < 0.001$; SOD2: $t_{(8)} = 2.824$; $P < 0.05$] and $Gcdh^{-/-}$ mice [GPx: $t_{(8)} = 6.298$; $P < 0.001$; SOD2: $t_{(8)} = 3.108$; $P < 0.05$], whereas GST activity was increased in the knockout mice [$t_{(7)} = 3.512$; $P < 0.01$] (Fig. 8a), with no changes of GR, SOD1, CAT, and G6PDH activities (Fig. 8b, c, e, g). It is also important to emphasize that the increase of GPx activity was more pronounced in the knockout animals relative to the WT animals [$t_{(8)} = 4.079$; $P < 0.01$] (Fig. 8a).

Intrastriatal QUIN Administration Induces Early Nrf2, Akt, and Erk 1/2 Expression in the Striatum of $Gcdh^{-/-}$ Mice

Figure 9 depicts the effects of QUIN injection into the striatum on Nrf2, Keap1, Akt, and Erk 1/2 protein levels of mouse striatum 30 min after its infusion. QUIN administration induced an increase of Nrf2 expression in both $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice [$Gcdh^{+/+}$: $t_{(4)} = 5.519$; $P < 0.01$; $Gcdh^{-/-}$: $t_{(4)} = 3.988$; $P < 0.05$] (Fig. 9a). QUIN also provoked a significant decrease of the Nrf2 inhibitor protein Keap1 in $Gcdh^{-/-}$ mice [$t_{(4)} = 3.127$; $P < 0.05$] (Fig. 9b). Furthermore, QUIN-induced increase of Nrf2 and decrease of Keap 1 were more pronounced in $Gcdh^{-/-}$ mice [$t_{(4)} = 5.635$; $P < 0.05$]. We also found that QUIN injection associated with high Lys dietary intake significantly increased Akt [$t_{(4)} = 3.518$; $P < 0.05$] (Fig. 9c) and phospho-Erk 1/2 [$t_{(4)} = 7.627$; $P < 0.01$] (Fig. 9d) expression in $Gcdh^{-/-}$ mice.

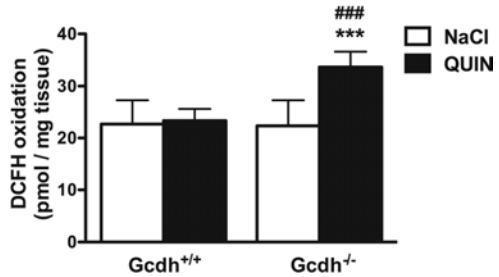

Fig. 5 2′,7′-Dihydrodichlorofluorescein (DCFH) oxidation in the striatum of $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice under a high Lys (4.7 %) diet. DCFH oxidation was measured 24 h after a single intrastriatal injection of NaCl or QUIN (50 nmol). Results are represented as mean ± standard deviation for five independent experiments (animals) per group. $^{***}P < 0.001$, compared to $Gcdh^{-/-}$ injected with NaCl; $^{###}P < 0.001$, compared to $Gcdh^{+/+}$ injected with QUIN (Student's *t* test for unpaired samples)

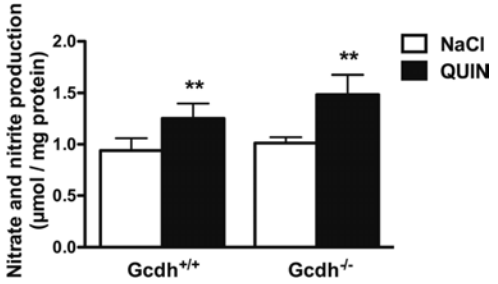

Fig. 6 Nitrate and nitrite production in the striatum of $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice on a high Lys (4.7 %) diet. Nitrate and nitrite concentrations were measured 24 h after a single intrastriatal injection of NaCl or QUIN (50 nmol). Results are represented as mean ± standard deviation for five independent experiments (animals) per group. $^{**}P < 0.01$, compared to $Gcdh^{-/-}$ injected with NaCl (Student's *t* test for unpaired samples)

Intrastriatal QUIN Administration Induces Early NF-κB Expression in the Striatum of $Gcdh^{-/-}$ Mice

Figure 10a shows a moderate increase of NF-κB-p65 expression in the nuclear fraction caused by QUIN in $Gcdh^{-/-}$ mice fed a high Lys diet [$t_{(4)} = 3.255$; $P < 0.05$]. QUIN injection also augmented the content of cytosolic NF-κB-p65 in $Gcdh^{-/-}$ mice relatively to knockout mice injected with NaCl [$t_{(4)} = 5.694$; $P < 0.05$] and to WT mice that received QUIN [$t_{(4)} = 3.224$; $P < 0.05$] (Fig. 10b). Furthermore, it can be observed that QUIN caused a strong decrease of the cytosolic IκBα

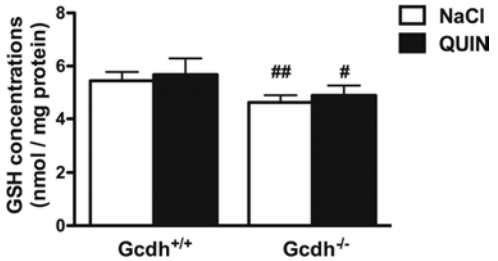

Fig. 7 Reduced glutathione (GSH) concentrations in the striatum of $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice on a high Lys (4.7 %) diet. GSH concentrations were measured 24 h after a single intrastriatal injection of NaCl or QUIN (50 nmol). Results are represented as mean ± standard deviation for five independent experiments (animals) per group. $^{#}P < 0.05$, $^{##}P < 0.01$, compared to $Gcdh^{+/+}$ injected with QUIN (Student's *t* test for unpaired samples)

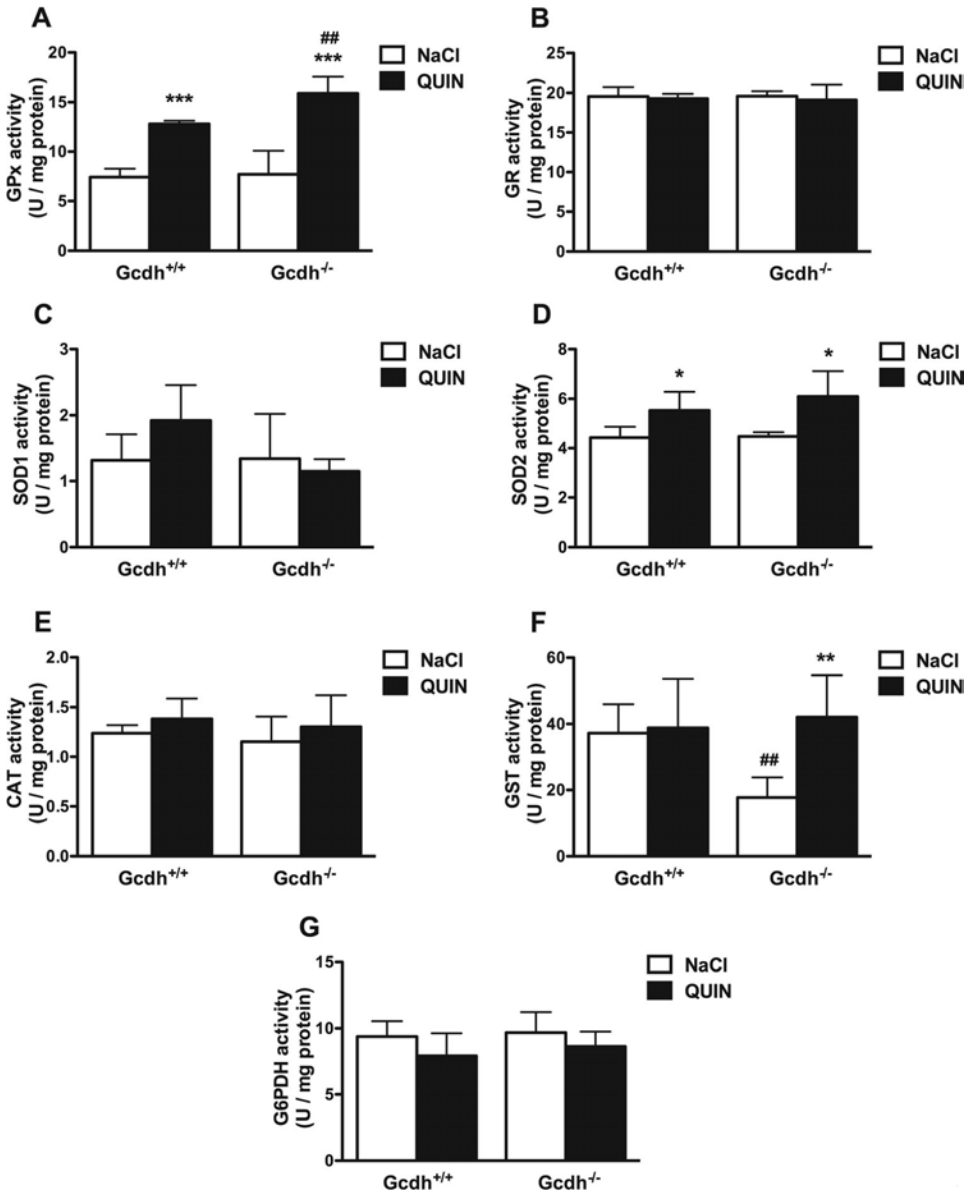

Fig. 8 Glutathione peroxidase (GPx, **a**), glutathione reductase (GR, **b**), superoxide dismutase 1 (SOD1, **c**), superoxide dismutase 2 (SOD2, **d**), catalase (CAT, **e**), glucose-6-phosphate dehydrogenase (G6PDH, **f**), and glutathione-S-transferase (GST, **g**) activities in the striatum of $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice on a high Lys (4.7 %) diet. The enzyme activities were measured 24 h after a single intrastriatal injection of NaCl or QUIN (50 nmol). Results are represented as mean ± standard deviation for five independent experiments (animals) per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to $Gcdh^{-/-}$ injected with NaCl; $^{\#\#}P < 0.01$, compared to $Gcdh^{+/+}$ injected with QUIN (Student's *t* test for unpaired samples)

content in both $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice [$Gcdh^{+/+}$: $t_{(4)} = 3.667$; $P < 0.05$; $Gcdh^{-/-}$: $t_{(4)} = 3.238$; $P < 0.05$] (Fig. 10c).

Discussion

Although much work has been performed investigating the molecular mechanisms implicated in the neuropathology of GA I, the exact processes responsible for the acute striatal degeneration characteristic of this disorder are still poorly established. It is of note that destruction of the basal ganglia follows catabolic events during encephalopathic crises that are triggered by infections or immunizations associated with inflammatory processes [2, 22].

In a previous report, it was postulated that the KP pathway, and more particularly QUIN, is activated during infections contributing to the neuropathology of GA I [15], although to the best of our knowledge so far no work investigated this hypothesis. QUIN is a neurotoxin associated to neurodegeneration through activation of distinct signaling pathways and transcription factors [17]. The most important mechanism for QUIN deleterious effects towards the CNS is excitotoxicity because this metabolite is a glutamatergic agonist of NMDA receptors, preferentially activating the subunits NR2A and NR2B. Through this mechanism, QUIN causes an augment of Ca^{2+} influx that leads to a pathological cascade resulting in neuronal death [17].

Considering that chronic and acute Lys overload to GA I knockout mice give rise to increased GA and 3HGA

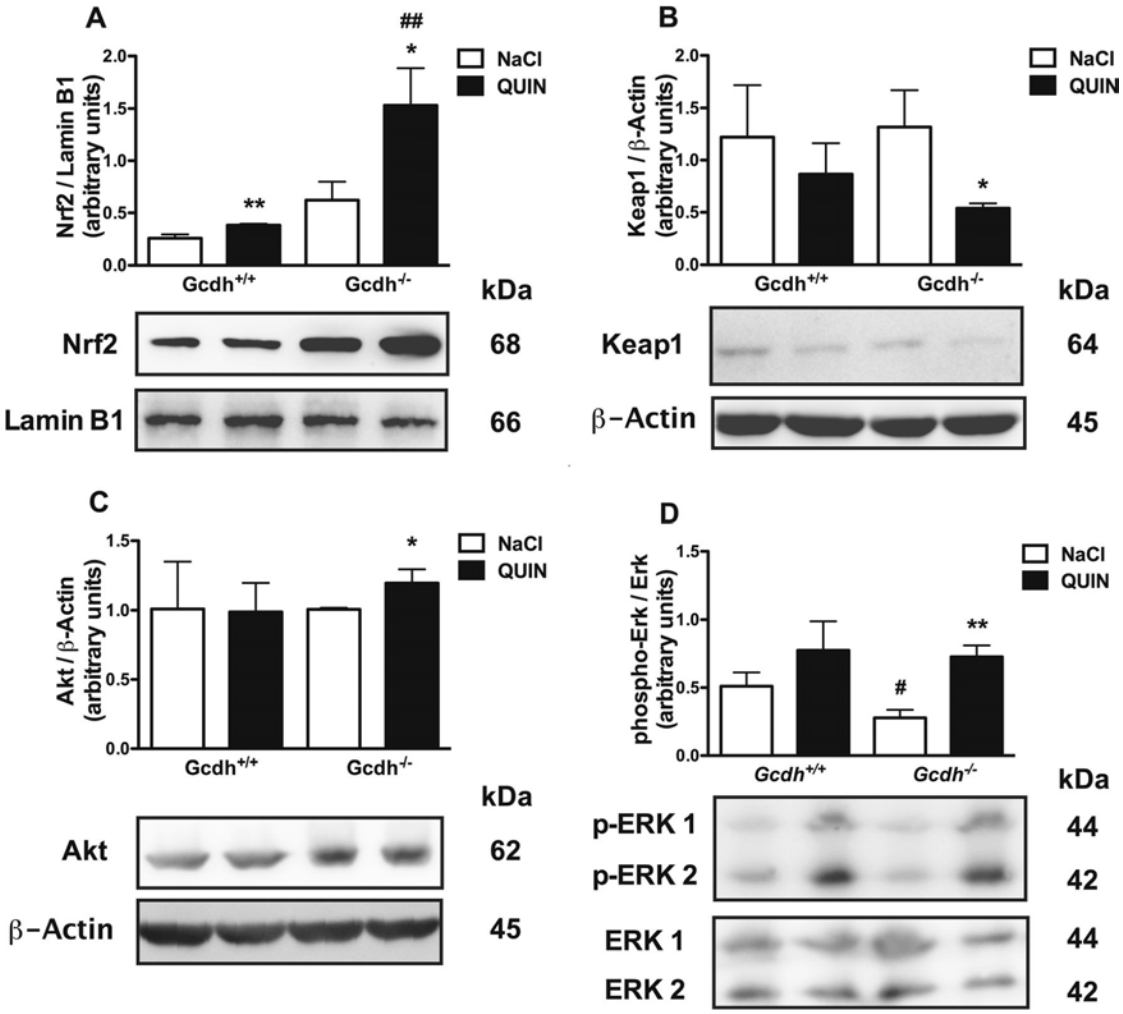

Fig. 9 Immunoblot and densitometric analysis for nuclear Nrf2 (**a**), cytosolic Keap1 (**b**), Akt (**c**), and phospho-Erk 1/2 (**d**) protein content in the striatum of $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice on a high Lys (4.7 %) diet. Protein levels were measured 30 min after a single intrastriatal injection of NaCl or QUIN (50 nmol). Lamin B1 and β-actin were used as endogenous controls. Results are represented as mean ± standard deviation for three independent experiments (animals) per group. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to $Gcdh^{-/-}$ injected with NaCl; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared to $Gcdh^{+/+}$ injected with QUIN (Student's *t* test for unpaired samples). Protein levels are expressed as arbitrary units (AU)

concentrations in tissues and body fluids, as well as striatal degeneration [63–65], in the present work we submitted $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice to a high Lys dietary intake for 48 h followed by a single intrastriatal injection of QUIN to simulate an acute neuroinflammatory process. We thereafter evaluated bioenergetics and oxidative stress parameters, as well as the content of proteins involved in critical cellular signaling pathways.

QUIN significantly increased lactate production in both $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice, implying an impairment of oxidative metabolism. Elevated lactate concentrations were probably secondary to a blockage of the respiratory chain since COX activity was decreased by this treatment in $Gcdh^{-/-}$ mice. We cannot however exclude that a disturbance in the glycolytic pathway and/or citric acid cycle functioning was also involved in lactate production increase.

QUIN also significantly reduced CK activity in the striatum of $Gcdh^{-/-}$ mice, indicating that intracellular energy transfer is impaired in the knockout mice. This is in line with previous data showing that the activity of CK is markedly diminished in brain and skeletal muscle from $Gcdh^{-/-}$ mice that received intraperitoneal Lys administration [66]. Consistent with this observation, Zinnanti and colleagues found markedly decreased concentrations of phosphocreatine in the brain of $Gcdh^{-/-}$ mice on a high Lys diet [63, 64]. Our findings are also in accordance with a previous study showing that QUIN intrastriatal injection induced a decrease of CK activity in rat striatum [18].

Taken together, our data strongly indicate that oxidative phosphorylation and intracellular energy transfer are compromised by QUIN in vivo in the striatum of $Gcdh^{-/-}$ mice. We also verified that QUIN-induced disruption of energy

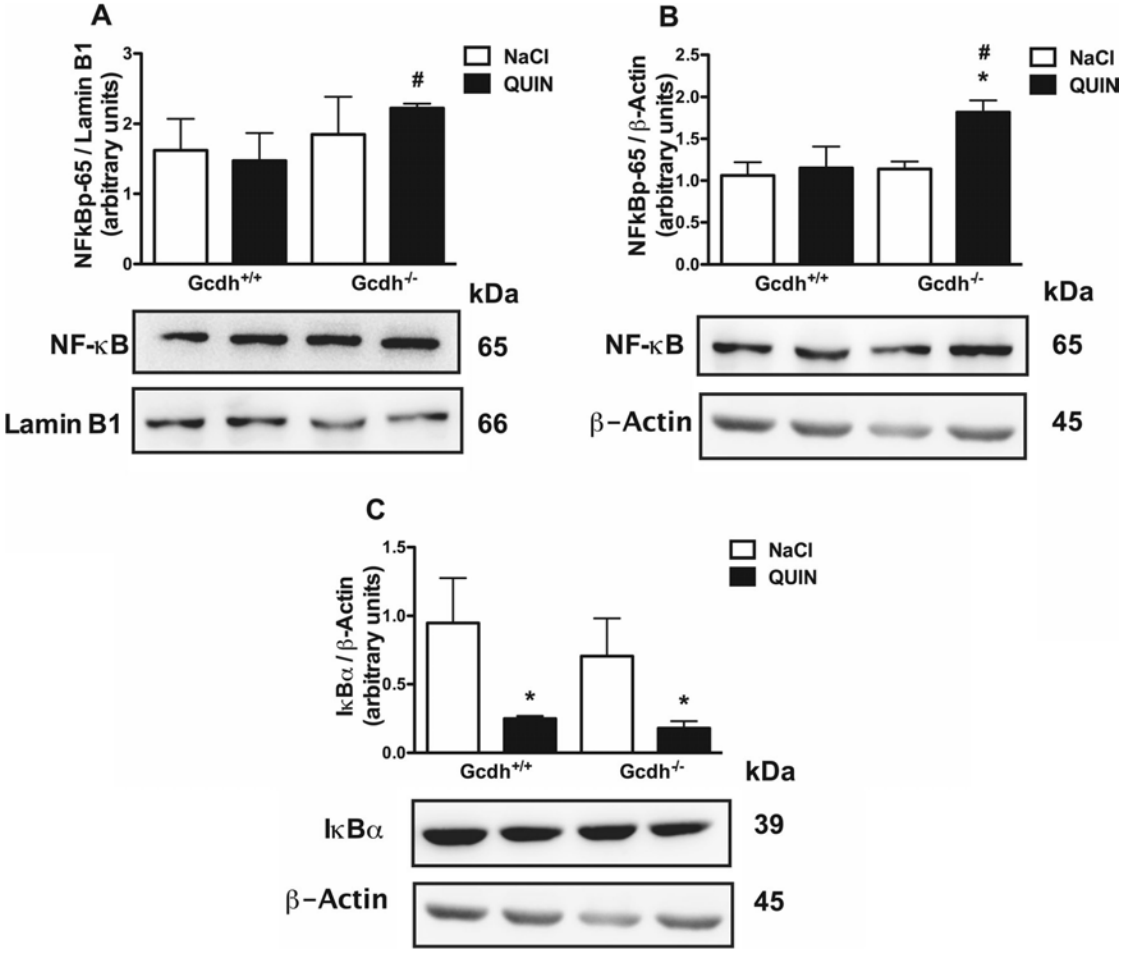

Fig. 10 Immunoblot and densitometric analysis for nuclear (**a**) and cytosolic (**b**) NF-κB, and cytosolic IκBα (**c**) protein content in the striatum of $Gcdh^{+/+}$ and $Gcdh^{-/-}$ mice on a high Lys (4.7 %) diet. Protein levels were measured 30 min after a single intrastriatal injection of NaCl or QUIN (50 nmol). Lamin B1 and β-actin were used as endogenous controls. Results are represented as mean ± standard deviation for three independent experiments (animals) per group. $^{*}P < 0.05$, compared to $Gcdh^{-/-}$ injected with NaCl; $^{\#}P < 0.05$, compared to $Gcdh^{+/+}$ injected with QUIN (Student's *t* test for unpaired samples). Protein levels are expressed as arbitrary units (AU)

metabolism was more evident in $Gcdh^{-/-}$ mice under a high Lys dietary intake and this may have happened because of the additive effects of QUIN, GA, and 3HGA [35, 66, 67]. Regarding to the mechanisms by which QUIN disturbs bioenergetics, it was previously shown that activation of glutamate receptors by QUIN leads to impairment of energy production via free radical formation [18, 68–71]. Thus, it is conceivable that the inhibition of CK activity observed in the present work could be due to oxidative attack to the enzyme protein structure [72–77].

Therefore, we evaluated important redox homeostasis parameters after QUIN injection and observed that this neurotoxin increased MDA levels reflecting lipid peroxidation, in both $Gcdh^{-/-}$ and $Gcdh^{+/+}$ mice, although knockout mice was more susceptible to this oxidative damage. Noteworthy, previous results showed that GCDH knockout mice submitted to Lys overload present enhanced MDA levels that was attributed to the increased production of GA and 3HGA [63, 78]. Our results are not surprising because QUIN was previously demonstrated to induce lipid peroxidation in animal tissues [79]. It was suggested that QUIN is able to combine with Fe^{2+}, inducing the formation of the highly reactive hydroxyl radical through the Fenton reaction. Therefore, although we cannot at the present establish the mechanisms by which QUIN caused lipid peroxidation, it is feasible that this compound could induce reactive species formation by stimulating the Fenton reaction or secondarily via other mechanisms including overstimulation of NMDA receptors.

QUIN instrastriatal administration also reduced sulfhydryl content that implies increased sulfhydryl oxidation. Considering that two thirds of thiol groups are associated with protein cysteine residues, it is assumed that QUIN induced protein oxidative damage. We cannot however rule out the possibility that part of these thiol groups belong to unbound cysteine or cysteine derivative molecules, including GSH, so that decreased sulfhydryl content could be alternatively attributed

to a reduction of antioxidant defenses. However, since QUIN was not able to change GSH concentrations in both *Gcdh*$^{-/-}$ and WT mice, the reduction of thiol groups caused by QUIN was more likely due to protein oxidation. On the other hand, although QUIN did not change GSH levels, it was observed that *Gcdh*$^{-/-}$ mice had lower concentrations of this antioxidant in the striatum relatively to WT animals. Similar findings were demonstrated previously in GA I knockout mice on an enriched Lys chow for 60 h or after an acute Lys injection [63, 78].

We also found a reduction of GST activity in *Gcdh*$^{-/-}$ mice. The mechanisms by which GST activity was reduced in our work is so far unknown; however, it is feasible that this decrease could be due to reactive species attack causing modifications in the enzyme protein structure as previously shown for GST and other antioxidant enzymes [80–82]. In fact, this was probably the case since QUIN also induced reactive oxygen and nitrogen species in our model in *Gcdh*$^{-/-}$ mice. As regards to the consequences of the decreased GST activity, it may compromise detoxification of xenobiotics, as well as conjugates of hydrogen peroxide and lipid peroxidation products with GSH, helping their excretion [83, 84]. It was also observed that QUIN increased GPx and SOD2 activities in the striatum of both *Gcdh*$^{+/+}$ and *Gcdh*$^{-/-}$ animals treated with dietary supplementation of Lys, but did not alter GR, SOD1, CAT, and G6PDH activities. The increase of SOD2 and GPx activities provoked by QUIN suggests that superoxide and hydrogen peroxide are probably involved in its pro-oxidant effects because these reactive species are scavenged by SOD and GPx, respectively. Furthermore, the fact that GPx also detoxifies lipid peroxides and that GST metabolizes lipid peroxides, 4-hydroxynonenal, and isoprostanes reinforces the view that lipid peroxidation occurs in brain of *Gcdh*$^{-/-}$ mice, especially during inflammation.

Our results showing lipid and protein oxidative damage as well as reduction of GSH are likely due to QUIN-induced reactive nitrogen species generation that could be secondary to NMDA receptor overstimulation leading to high calcium influx and activation of nitric oxide synthase [85]. Furthermore, since COX is markedly inhibited by nitric oxide, the QUIN-induced decrease of this activity could be similarly due to the production of nitric oxide, a classical COX inhibitor. Otherwise, ROS may also contribute to the effects caused by QUIN especially in *Gcdh*$^{-/-}$ mice, since this neurotoxin provoked DCFH oxidation, a probe that is mainly oxidized by ROS. ROS generation in striatum of *Gcdh*$^{-/-}$ mice may also explain the higher effects detected in the knock out animals.

Considering that QUIN increased the activities of SOD2 and GPx that may result from a compensatory mechanism triggered by cellular signaling pathways, we investigated the effect of this metabolite on the Nrf2/antioxidant response element (ARE) pathway. Nrf2 is a transcription factor behaving as a primary sensor of oxidative stress and a master regulator of the antioxidant system due to its ability to modulate the expression of numerous antioxidant and detoxifying genes [86–92]. We found that QUIN administration increased nuclear Nrf2 protein levels in *Gcdh*$^{+/+}$ and more markedly in *Gcdh*$^{-/-}$ mice. Furthermore, the content of cytosolic Keap1, an Nrf2-binding protein that hampers the translocation of this transcription factor to the nucleus and promotes Nrf2 ubiquitination-proteasomal degradation (Gan and Johnson, 2014), was significantly decreased by QUIN only in *Gcdh*$^{-/-}$ mice, and this may explain the augmented content of nuclear Nrf2.

We cannot at the present establish the mechanisms by which QUIN induced prominent alterations of Nrf2 and Keap1 in *Gcdh*$^{-/-}$ mice. However, since oxidative stress was induced in *Gcdh*$^{-/-}$ mice submitted to high Lys overload leading to increased brain concentrations of GA and 3HGA [63, 64, 78], it may be presumed that QUIN acted synergistically with GA and 3HGA inducing these oxidative stress-sensitive signaling proteins in striatum of GCDH-deficient mice. Furthermore, Nrf2 translocation into the nucleus where it binds to ARE to further transactivate cytoprotective enzymes may represent an early attempt to protect cells against the ongoing oxidative damage. Our present results are in accordance with previous findings showing that QUIN provokes an early up-regulation of Nrf2 in rodent striatal slices [79].

A mechanism of Nrf2 activation is disruption of the interaction between Keap1 and Nrf2 by oxidation of critical cysteine thiols in Keap1 [93–95] induced by oxidants and/or reactive species. Thus, once QUIN is able to induce RNS and ROS production, as well as sulfhydryl oxidation in striatum of *Gcdh*$^{-/-}$, we presume that QUIN activated Nrf2 translocation by oxidation of thiol groups releasing this transcription factor from its inhibitor. Nrf2 can be also regulated by other signal transduction pathways, such as Akt (also called PKB) that mediates Nrf2 phosphorylation. Our results also showed that QUIN increased Akt protein content in the striatum of GA I knockout mice, implying that this kinase was also involved in Nrf2 activation. Phospho-Erk 1/2 protein levels were also found to be augmented by QUIN treatment. Since Nrf2 can also be regulated by this mitogen-activated protein kinase (MAPK) favoring the release of Nrf2 from its inhibitory protein Keap1 [90, 96], our results indicate the participation of this pathway in QUIN-induced Nrf2 activation.

QUIN also increased the protein content of NF-κB in striatal nuclear and cytosolic fractions of *Gcdh*$^{-/-}$ and *Gcdh*$^{+/+}$ mice and decreased the NF-κB inhibitor IkBa in the cytosol, implying that NF-κB was translocated into the nucleus. This signaling pathway was previously shown to be activated under pathological conditions associated with intracellular redox state disturbances and inflammatory processes [97, 98]. Therefore, we cannot rule out this additional

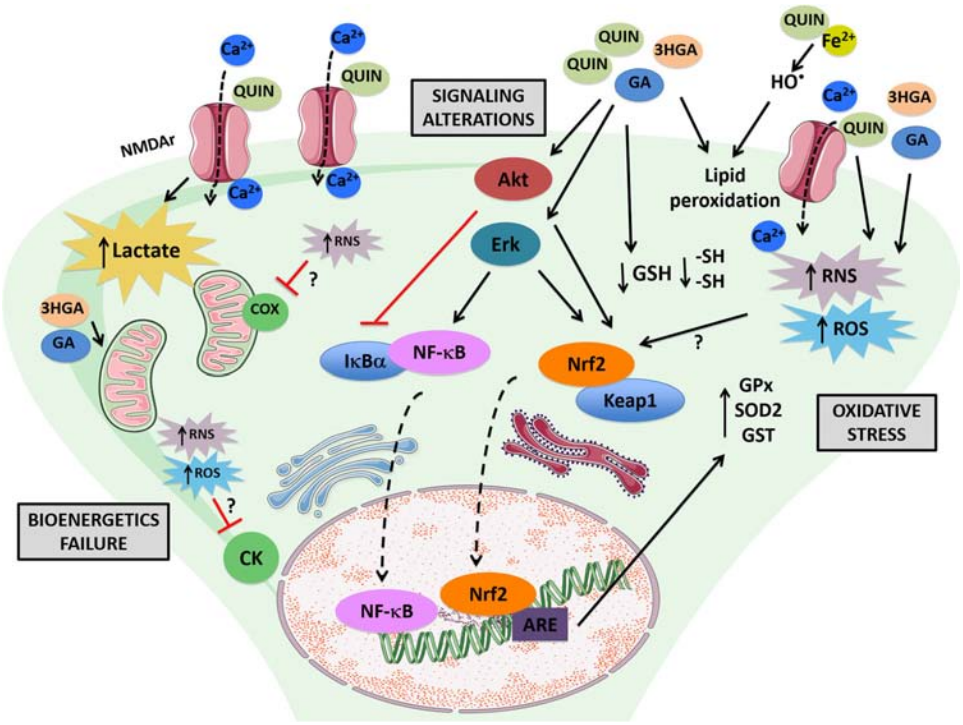

Fig. 11 Schematic representation of underling mechanisms involved in the toxicity of quinolinic acid (QUIN) in striatum of *Gcdh*$^{-/-}$ mice. QUIN activates N-methyl-D-aspartate (NMDA) receptors, provoking cytotoxic mechanisms, including oxidative stress and bioenergetics dysfunction. Nrf2 and NF-kB signaling pathways are stimulated as a compensatory response to the pro-inflammatory/oxidative stress elicited by QUIN

mechanism elicited to overcome the pro-oxidant cellular status in our animal model.

On the other hand, Akt- and Erk 1/2-mediated signaling pathways that were stimulated by QUIN may also be involved in the transactivation of NF-κB in *Gcdh*$^{-/-}$ mice since these pathways are interconnected [99, 100]. As regards to Erk, this MAPK protein induces dissociation of IκBα from NF-κB, allowing nuclear translocation and DNA-binding of NF-κB [101].

Taken together, we demonstrate that multiple signaling pathways participate in QUIN toxic effects leading to redox homeostasis alterations in striatum of *Gcdh*$^{-/-}$ mice submitted to high Lys overload. It is also emphasized that QUIN stimulates NMDA receptors, particularly the NR2A and NR2B subunits [17], that may secondarily result in RNS generation and Erk signaling pathway activation [102, 103]. Noteworthy, a recent report described that NR2A and NR2B receptor subunits are highly expressed in *Gcdh*$^{-/-}$ animals receiving Lys overload [104] so that these observations may signalize a mechanism of QUIN neurotoxicity in our *Gcdh*$^{-/-}$ mouse model of GA I.

Conclusions

The present work shows for the first time that QUIN impairs energy and redox homeostasis in striatum of *Gcdh*$^{-/-}$ mice submitted to Lys overload. Furthermore, QUIN activated Nrf2 and NF-kB that may possibly represent compensatory mechanisms that take place during oxidative stress/inflammation induced by QUIN as an attempt to protect the striatum from these deleterious pathological mechanisms (Fig. 11). Our findings support the hypothesis that activation of KP that occur during inflammatory processes, resulting in increased concentrations of QUIN, may play a role in the acute striatal degeneration that follows infections in patients affected by GA I as previously hypothesized [15]. It is also conceivable that QUIN may act synergistically with the accumulating organic acids GA and 3HGA, whose concentrations dramatically increase during these crises of metabolic decompensation, leading to destruction of the striatum.

Acknowledgments We are grateful to the financial support of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, # 404883/2013-3), Pró-Reitoria de Pesquisa/Universidade Federal do Rio Grande do Sul (PROPESQ/UFRGS, # PIBITI 18489), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, # 10/0031-1), PRONEX, FINEP Rede Instituto Brasileiro de Neurociência (IBN-Net, # 01.06.0842-00), and Instituto Nacional de Ciência e Tecnologia-Excitotoxicidade e Neuroproteção (INCT-EN, # 573677/2008-5).

Compliance with the Ethical Standard

Conflict of Interest The authors declare that there are no conflicts of interest.

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10. **DISCUSIÓN**

En este trabajo se exploraron los efectos ejercidos por la 3-HK en ensayos *in vitro* e *in vivo*. En un principio la hipótesis se orientaba a declarar al metabolito como una molécula pro-oxidante y neurotóxica. Sin embargo, los resultados colectados demuestran que es una molécula con actividad dual (antioxidante y pro-oxidante), dependiendo de las condiciones experimentales, que no debe ser considerada como tóxica, la cual denotó un posible perfil neuroprotector que requiere ser explorado con más detalle. Los efectos pro-oxidantes de la molécula evaluados no fueron capaces de producir daño tisular o celular alguno en ninguno de los dos modelos estudiados, mientras que sus efectos antioxidantes *in vitro* previnieron la falla mitocondrial y el estrés oxidante producidos por el QUIN, el 3-NP y $FeSO_4$. La activación compensatoria de enzimas antioxidantes y la activación del Nrf2 parecen ser los mecanismos mediante los cuales la 3-HK previene los efectos de estas toxinas en una estrategia que se enfoca a disminuir las ERO, ERN y el estrés oxidante general.

Varios reportes indican que la 3-HK induce muerte celular en diferentes cultivos neuronales de cerebelo, estriado e hipocampo (revisado por Colín-González et al., 2013). La ausencia de toxicidad observada en este trabajo puede deberse a que se utilizó un sistema más complejo compuesto de neuronas, astrocitos y microglia. La KMO, la enzima encargada de producir la 3-HK, se encuentra preferentemente en células gliales, por lo que las neuronas al estar aisladas y al no tener la maquinaria necesaria para degradarla podrían sufrir en su presencia (Guidetti et al., 1995), pero nuestros resultados sugieren que éste no es el caso.

Por su otro lado, la inyección intraestriatal de la 3-HK produjo un aumento en los marcadores de estrés en tiempos tempranos (6 y 24 h) y estimuló las actividades de las enzimas glutatión reductasa y glutatión-S-tranferasa, sin mostrar efectos conductuales ni morfológicos en los animales tratados. De acuerdo con lo anterior, Guidetti y Schwarcz (1999) no encontraron diferencia significativa en los volúmenes de lesión de animales inyectados intraestriatalmente

con la 3-HK (10 nmol). Por lo tanto, se puede concluir que niveles incrementados de esta molécula (10 - 80 nmol) en el estriado no están relacionados con procesos de neurodegeneración.

La falta de toxicidad de la 3-HK en el estriado puede deberse a su permanencia limitada o transitoria en el tejido. La 3-HK es metabolizada rápidamente y su presencia en el cerebro sano induce la activación de mecanismos de sobrevivencia mediante una respuesta adaptativa que parece ser suficiente para neutralizar la generación de los radicales formados (Guidetti y Schwarcz, 1999).

Dado que la toxicidad de la 3-HK no logró ser comprobada podría asumirse que sus efectos deletéreos comienzan cuando es transformada en el QUIN. En concentraciones que exceden el rango fisiológico, este metabolito se relaciona con procesos patológicos tanto a nivel periférico como central (Pérez De la Cruz et al., 2012). El estudio de esta hipótesis requiere de métodos con los que no contamos actualmente. Por tal razón, se consideró incluir los estudios realizados en paralelo con el QUIN y así proveer de más información sobre el papel de la VK en procesos neurodegenerativos.

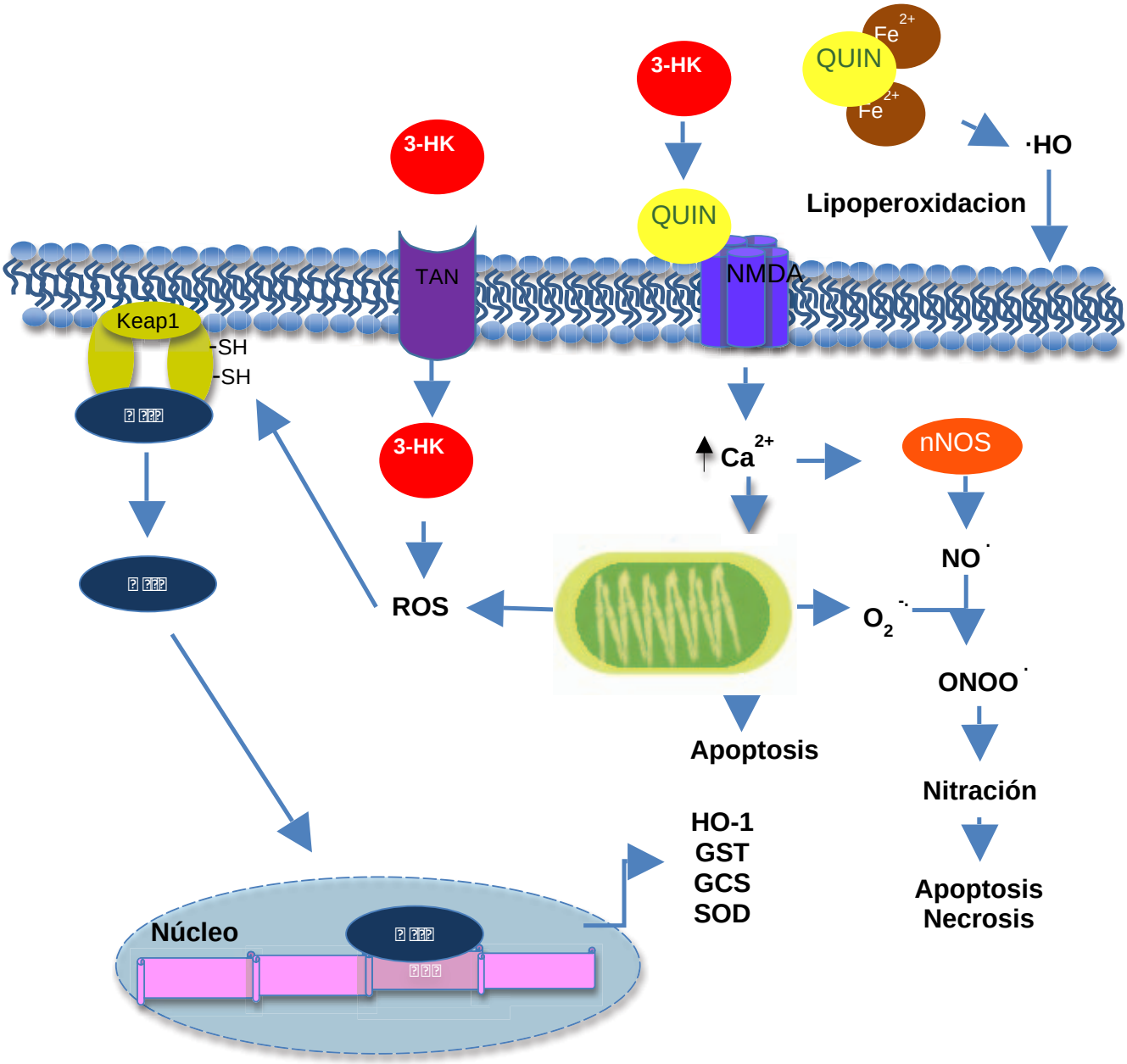
El primer punto a investigar fue el papel del Nrf2, un factor de transcripción que regula funciones de protección antioxidantes, en el modelo tóxico producido por el QUIN. El ensayo permitió establecer un patrón de regulación temprana sobre este factor, al favorecer su sobre-regulación en tejido neuronal como una respuesta adaptativa de protección ante el inminente efecto de este metabolito. Desde este punto de vista, el estrés oxidante es una respuesta importante que contribuye al patrón de daño ejercido por el QUIN, sugiriendo así que la estimulación temprana del Nrf2 podría ser una estrategia terapéutica para prevenir el desarrollo de procesos neurodegenerativos (**Figura 6**).

El aumento de la cantidad proteica de la hemo-oxigenasa-1, en presencia del QUIN, demuestra la activación del Nrf2. Este evento, a diferencia de lo visto con la 3-HK, representa una acción compensatoria del sistema biológico que no es suficiente para evitar el daño que está en progreso. Entonces, el nivel de protección deseado para el modelo solo podría ser alcanzado a través de un estímulo externo provisto por un inductor de la activación de Nrf2 y/o un

antioxidante. A este respecto, y confirmando lo anterior, el ácido cafeico previno el daño excitotóxico inducido por QUIN en el estriado de rata. Este efecto claramente está relacionado con sus propiedades antioxidantes demostradas y su capacidad para inducir al Nrf2 (Kalonia et al., 2009; Pang et al., 2016).

Una vez caracterizados estos mecanismos se investigó mediante que otros procesos el QUIN amplifica su toxicidad en el SNC. El cerebro de personas afectadas con acidemias orgánicas desarrolla una degeneración aguda relacionada con la acumulación de metabolitos tóxicos tales como AG, 3-OHGA, AMM, AP, AMG, entre otros; sin embargo, su acción aislada no justifica el grado de daño que se observa en estas patologías. Varadkar y Surtees (2004) propusieron que la VK tiene un papel activo en la neurodegeneración observada en estos desordenes. En este trabajo se obtuvo evidencia no solo *in vitro,* sino también *in vivo* del efecto sinérgico entre el QUIN y los ácidos orgánicos: Los ratones *gcdh* $^{-/-}$ cuando son alimentados con una dieta alta en lisina tienen niveles aumentados de las subunidades NR2A y NR2B (Lagranka et al., 2014). Estos animales cuando son administrados intraestriatalmente con el QUIN, el cual interactúa también con los rNMDA a través de las mismas subunidades, exhibieron efectos tóxicos más intensos en comparación con aquellos que solo acarrearon la mutación. Por consiguiente, la interacción del QUIN con los ácidos orgánicos en puede contribuir a entender el mecanismo tóxico que ocurre en estos y en otros desórdenes neurológicos.

Finalmente, el descubrimiento de que la combinación de ácidos orgánicos con QUIN puede agravar la pérdida de células en el SNC sugiere que una disminución del flujo de la VK enfocada a inhibir la producción de QUIN podría ser utilizada como estrategia terapéutica en éste y otro tipo de enfermedades.


3-HK
3-HK
QUIN
Fe2+
Fe2+
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3-HK
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-SH
-SH
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nNOS
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ROS
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ONOO·
Apoptosis
Nitración
HO-1
GST
GCS
SOD
Apoptosis
Necrosis
Núcleo

11. **CONCLUSIÓN**

Los efectos de la 3-HK en el estriado de roedores dependen de las condiciones experimentales empleadas. La presencia de la 3-HK en concentraciones elevadas en el cerebro normal podría constituir una señal de defensa para contrarrestar el estrés oxidante por activación de señales de sobrevivencia. Las especies reactivas producidas por la 3-HK activan las defensas antioxidantes, y de esta forma la célula es capaz de contrarrestar los efectos deletéreos de otros agentes tóxicos. Por lo tanto, la 3-HK podría ser una molécula que regula del ambiente redox en condiciones fisiológicas con acciones neuroprotectoras u homeostáticas.

De la misma forma que la 3-HK, el QUIN modula el ambiente redox mediante el factor Nrf2. La activación del factor depende del tiempo de exposición, de las concentraciones probadas y del daño oxidante generado. Sin embargo, esta modulación es solo un evento compensatorio en un intento de la célula para disminuir el daño que está en progreso. La protección observada con antioxidantes sugiere que el daño causado por el QUIN pude ser prevenido o disminuido.

El sinergismo del QUIN con otros metabolitos tóxicos y otras de sus acciones caracterizadas en este estudio ayudan a comprender los mecanismos por los cuales la neurodegeneración ocurre. En consecuencia, la identificación de mecanismos tóxicos producidos por neurotoxinas endógenas con diferentes perfiles podría ser útil para el diseño de estrategias terapéuticas más efectivas.

12. **PERSPECTIVAS**

Elaborar un diseño experimental que permita identificar si las acciones protectoras de la 3-HK son suficientes para prevenir el daño *in vivo* de toxinas conocidas.

Investigar mediante marcadores bioquímicos, de falla energética, conductuales y morfológicos si el uso de inhibidores de la VK (con especial atención en moléculas que eviten la producción de QUIN) puede conferir protección en ratones *knockout* para la glutaríl-CoA deshidrogenasa alimentados con una dieta alta de lisina.

13. **ANEXO**

NeuroToxicology 34 (2013) 189–204

Contents lists available at SciVerse ScienceDirect

NeuroToxicology

Review

3-Hydroxykynurenine: An intriguing molecule exerting dual actions in the Central Nervous System

Ana Laura Colín-González [a,b,1,*], Perla D. Maldonado [b], Abel Santamaría [a,*]

[a] Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, Insurgentes Sur 3877, Mexico City 14269, Mexico
[b] Laboratorio de Patología Vascular Cerebral, Instituto Nacional de Neurología y Neurocirugía, Mexico City 14269, Mexico

ARTICLE INFO

Article history:
Received 20 September 2012
Accepted 27 November 2012
Available online 3 December 2012

Keywords:
3-Hydroxykynurenine
Kynurenine pathway
Neurological diseases
Oxidative stress
Brain damage
Redox modulation

ABSTRACT

Kynurenine pathway is gaining attention due to the many metabolic processes in which it has been involved. The tryptophan conversion into several other metabolites through this pathway provides neuronal and redox modulators useful for maintenance of major functions in the brain. However, when physiopathological conditions prevail – i.e. oxidative stress, excitotoxicity, and inflammation – preferential formation and accumulation of toxic metabolites could trigger factors for degeneration in neurological disorders. 3-Hydroxykynurenine has been largely described as one of these toxic metabolites capable of inducing oxidative damage and cell death; consequently, this metabolite has been hypothesized to play a pivotal role in different neurological and psychiatric disorders. Supporting evidence has shown altered 3-hydroxykynurenine levels in samples of patients from several disorders. In contrast, some experimental studies have provided evidence of antioxidant and scavenging properties inherent to this molecule. In this review, we explored most of literature favoring one or the other concept, in order to provide an accurate vision on the real participation of this tryptophan metabolite in both experimental paradigms and human brain pathologies. Through this collected evidence, we provide an integrative hypothesis on how 3-hydroxykynurenine is exerting its dual actions in the Central Nervous System and what will be the course of investigations in this field for the next years.

Contents

E-mail address: absada@yahoo.com.
* Corresponding authors at: Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, Insurgentes Sur 3877, Mexico City 14269, Mexico. Tel.: +52 55 5606 3822x2013.
E-mail address: absada@yahoo.com (A. Santamaría).
[1] Doctoral Program in Biomedical Sciences, Universidad Nacional Autónoma de México.

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http://dx.doi.org/10.1016/j.neuro.2012.11.007

1. Oxidative stress in neurodegenerative disorders

Neurodegeneration is the result of pathological processes producing severe and specific patterns of brain cell damage in a concerted manner (Coyle and Puttfarcken, 1993; Santamaria and Jimenez, 2005). Neurodegenerative events constitute a major cause for the development of neurological disorders. Human diseases coursing with neurodegeneration involve excitotoxicity as a triggering event for deadly cascades (Halliwell, 2006; Brouillet et al., 1999; Cowan and Raymond, 2006). In turn, excitotoxicity is currently defined as a toxic process characterized by a sustained stimulation of excitatory amino acids receptors (Schwarcz et al., 1978; Nicholls et al., 2007), mainly involving N-methyl-D-aspartate receptors (NMDAR). Different toxic events derived from excitotoxicity have been characterized in experimental models, including upregulation of detrimental signaling pathways, disrupted Ca^{2+} homeostasis, and recruitment of reactive oxygen/nitrogen species (ROS/RNS), with further oxidative/nitrosative stress (Santamaria and Jimenez, 2005; Halliwell, 2006; Nicholls et al., 2007; Beal, 2004; Lin and Beal, 2006; Sas et al., 2007), ultimately leading to cell death (see below).

Although oxidative damage to proteins, DNA and lipids has been characterized in different experimental models (reviewed by Halliwell, 2006; Jimenez-Del-Rio and Velez-Pardo, 2012) and in *post mortem* brain tissue of patients with neurodegenerative diseases (reviewed by Ischiropoulos and Beckman, 2003), it still remains difficult to determine whether oxidative stress is a primary causal event or merely a secondary end-stage epiphenomenon.

Actually, it is recognized that oxidative stress is one of the most important mechanisms involved in deadly events observed in neuronal cells in different neurodegenerative disorders. It is also accepted that the brain is particularly sensitive to oxidative stress as 20% of the total oxygen consumed by the body is employed by this organ, which constitutes only 2% of the total body weight. This feature makes the brain the major generator of ROS/RNS when compared with other organs. Moreover, the brain is rich in numerous conditions favoring ROS/RNS production, including: (1) high content of unsaturated lipids, (2) chemical reactions involving dopamine oxidation, (3) high concentrations of iron in various regions, and (4) lower activity of antioxidant systems as compared with other organs (including kidney and liver) (Halliwell, 2006).

Oxidative stress in the CNS recruits the following events: interrupted mitochondrial functions in neuronal cells induced by toxins, together with a failure to supply O_2 or substrates for energy production that will generate an impairment of ATP production and a rapid brain damage due to oxidative and excitotoxic components. The decrease in ATP synthesis disrupts Na^+/K^+-ATPase and Ca^{2+}/H^+-ATPase pumps and reverses the Na^+/Ca^{2+}-transporter. Upon these conditions, cells are unable to maintain membrane potential and then, voltage-gated Ca^{2+} channels are activated, leading to depolarization of cellular membranes. Once depolarized, membranes are more vulnerable to the action of excitatory amino acids – mostly glutamate – even at normal concentrations. Glutamate then activates NMDAR, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, and metabotropic glutamate receptors, thereby increasing intracellular Ca^{2+} and Na^+ levels. In turn, voltage-gated Ca^{2+} channels, together with reverse operation of the Na^+/Ca^{2+} exchanger, are responsible for the increased levels of intracellular Ca^{2+}. Once in the cytoplasmic domain at high concentrations, Ca^{2+} activates a variety of Ca^{2+}-dependent enzymes, including protein kinase C, phospholipase A2, phospholipase C, proteases, endonucleases, and a variety of pro-oxidant enzymes that trigger protein phosphorylation, proteolysis, mitochondrial damage, and oxidative stress (Hardingham and Bading, 2001).

In cells from different tissues, the predominant ROS/RNS produced are superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($^{\bullet}NO$), peroxynitrite anion ($ONOO^-$) and nitrogen dioxide ($^{\bullet}NO_2$). Under "normal" physiological conditions, natural defense against ROS/RNS is provided by endogenous antioxidant molecules such as glutathione (GSH), ascorbic acid, α-tocopherol, and a number of antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). SOD converts $O_2^{\bullet-}$ into H_2O_2, whereas GPx and CAT convert H_2O_2 into H_2O. However, an imbalance in the formation and clearance of ROS/RNS leads to oxidative stress and subsequent changes affecting the cell dynamics (Halliwell, 2006). Main sources of ROS/RNS are: (a) mitochondrial respiratory chain that generates $O_2^{\bullet-}$ (Schon and Area-Gomez, 2012); (b) xanthine oxidase that produces $O_2^{\bullet-}$ when it catalyzes oxidation of hypoxhantine to uric acid (Fatokun et al., 2007); (c) cyclooxygenase 2 that produces $O_2^{\bullet-}$ during oxidative metabolism of arachidonic acid (Teismann, 2012); (d) NADPH oxidase that produces $O_2^{\bullet-}$ during NADPH oxidation (Maldonado et al., 2010); and (e) Ca^{2+}-dependent nitric oxide synthase (NOS) that, under normal conditions, produces $^{\bullet}NO$, which in turn can react with $O_2^{\bullet-}$ to generate the strong oxidant $ONOO^-$ (Aguilera et al., 2007). Tetrahydrobiopterin (BH4) is an important regulator of NOS function because it is required to maintain enzymatic coupling. Loss or oxidation of BH4 to 7,8-dihydrobiopterin (BH2) is associated with NOS uncoupling, resulting in the production of $O_2^{\bullet-}$ rather than $^{\bullet}NO$ (Crabtree and Channon, 2011). ROS/RNS produce cellular damage through lipid peroxidation, membrane injury, nucleic acid alteration and enzyme inactivation. These species also modify cellular signaling and gene regulation, contributing to breakdown of the blood-brain barrier, and allowing the infiltration of neutrophils and other cells that activate multiple neuroinflammatory cascades (Mollica et al., 2012). Ultimately, oxidative stress induces cell damage, leading to neuronal death by apoptosis or necrosis (Loh et al., 2006).

2. The Kynurenine pathway (KP)

2.1. Overview

Kynurenine pathway (KP) is the most prominent metabolic pathway for degradation of tryptophan into a series of metabolites with relevance for the modulation of different redox functions in different physiological systems. It has been largely assumed that the main goal of KP is to endorse the formation of coenzyme NAD^+, which is relevant for the modulation of major physiological processes (Massudi et al., 2012). In particular, in the CNS, some

metabolites formed toward this pathway are relevant for the functioning of nerve tissue, as they exhibit neuromodulatory properties. Recently, KP has also gain attention due to two additional characteristics: (1) it metabolizes more than 90% of the peripheral tryptophan in mammals, and (2) many of its metabolites could play a causative role in disorders of the CNS (recently reviewed by Schwarcz et al., 2012). KP is known to be mostly active in glial cells, and the concentration range of its metabolites varies from nanomolar to low micromolar concentrations. Key limiting enzymes in KP are indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), and pivotal metabolite for triggering KP is kynurenine (KYN) (reviewed by Schwarcz et al., 2012).

In regard to tryptophan metabolism and 3-hydroxykynurenine (3-HK), it is well-known that KP activation yields the formation of KYN from tryptophan with the enzymatic participation of IDO or TDO and formamidase. On one hand, KYN can form kynurenic acid (KYNA) in a side terminal arm of the pathway through an irreversible transamination catalyzed by kynurenine aminotransferases (KATs). On the other hand, KYN is converted into 3-HK through the catalytic intervention of kynurenine 3-monooxygenase (KMO), which similarly to 3-hydroxyanthranilic acid (3-HANA), is more related to some functions on redox modulatory activity than to neuronal activity (recently reviewed by Schwarcz et al., 2012). 3-HK is then converted into 3-HANA by kynureninase. Picolinic acid formation corresponds to another side arm of KP and involves the participation of 2-amino-3-carboxymuconic-6-semialdehyde decarboxylase to convert 3-HK into this metabolite. The neuroactive metabolite quinolinic acid (QUIN) is also formed from 3-HK with the enzymatic participation of 3-hydroxyanthranilate oxygenase (3-HAO). Finally, transiently formed QUIN yields the formation of NAD^+ in a step involving the enzyme quinolinate phosphorobosyltransferase. A simplified representation of KP is presented in Fig. 1, giving relevance to 3-HK formation.

Despite KP is responsible for the formation of a number of neuroactive and redox active metabolites, in this review we will focus our attention on 3-HK, one of the most relevant metabolites for redox modulatory activity in the tryptophan metabolism. Therefore, neuroactive metabolites will be just briefly mentioned as follows.

2.2. KP neuroactive metabolites

KYNA was the first characterized kynurenine derived from tryptophan. KYNA, an endogenous competitive antagonist of ionotropic glutamate receptors, acts at micromolar concentrations and is synthesized as a terminal metabolite of the short arm of KP after KYN is irreversibly transaminated by KATs (Yu et al., 2004). In NMDAR, this metabolite binds to the glycine co-agonist site,

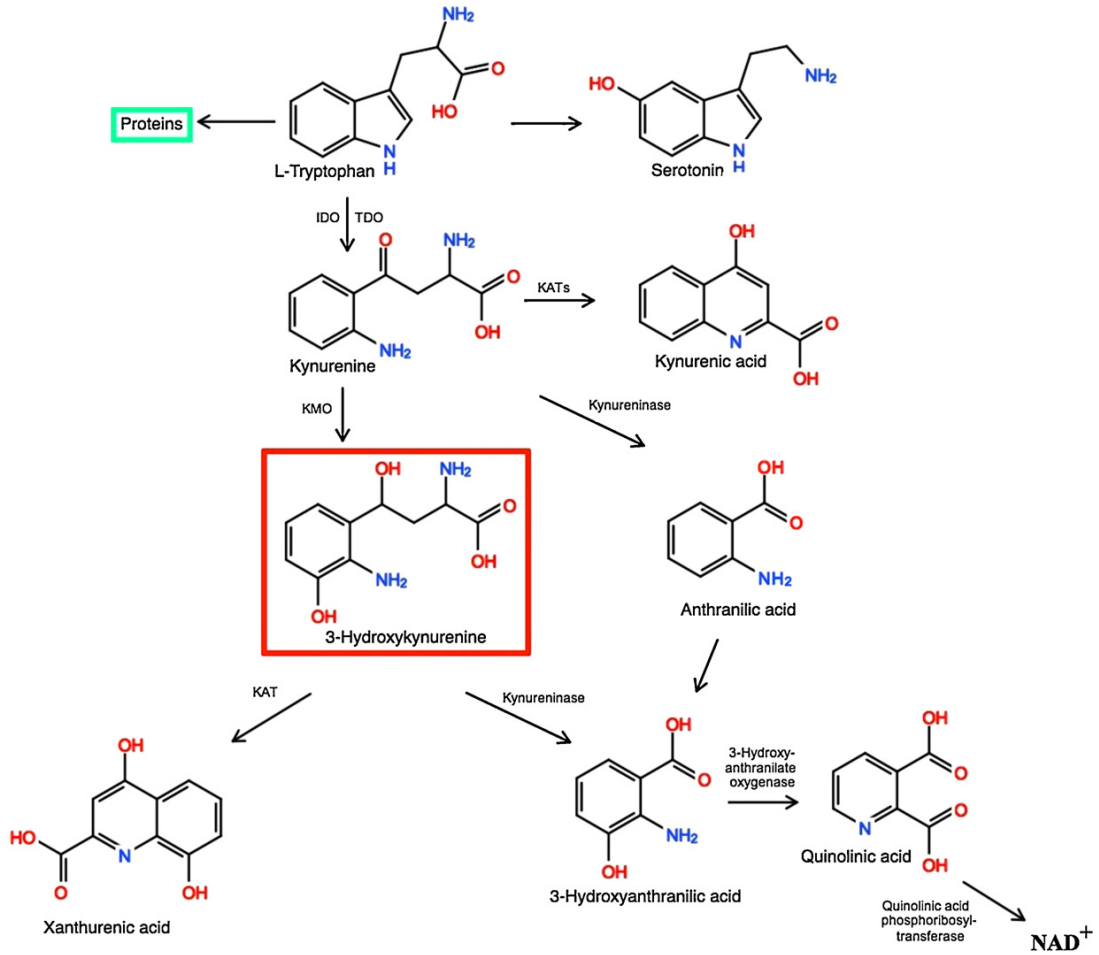

Fig. 1. Simplified schematic representation of the tryptophan metabolism pathway known as the kynurenine pathway (KP). Scheme shows 3-hydroxykynurenine (3-HK) formation and location within KP. Some intermediate metabolites were omitted. Abbreviations: IDO, indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; KATs, kynurenine aminotransferases.

blocking the receptor. It has also been reported that KYNA to inhibit α7 nicotinic acetylcholine receptors (α7nAChR) at central level (reviewed by Schwarcz et al., 2012). In addition, recent evidence supports a role for KYNA as an antioxidant agent and free radical scavenger (Lugo-Huitrón et al., 2011).

QUIN is another KP metabolite exerting activity at central level. QUIN is synthesized from 3-HANA through the activation of 3-HAO (Stachowski and Schwarcz, 2012). The primary mechanism of action reported for this metabolite is through direct activation of NMDAR, preferentially those receptors expressing the NR2B subunits (reviewed by Schwarcz et al., 2012). QUIN may also exert its effects through the stimulation of oxidative stress (Rios and Santamaria, 1991).

As aforementioned, several studies have reveal that other metabolites of KP – including kynurenine, 3-HK, 3-HANA and anthranilic acid – seem to lack of direct effects on neuronal activity (Stone, 1993). For the purposes of this review, these metabolites will not occupy our attention.

2.3. KP in neurodegenerative diseases

The concept that KP metabolites could be involved in neurodegenerative disorders came in first place from studies describing the toxic effects of QUIN in animals, resembling the pathological characteristics of Huntington's disease (HD) (Schwarcz et al., 1983). Later on, evidence on the protective and antagonistic actions exerted by KYNA served to hypothesize that changes in the QUIN:KYNA ratio might be responsible for excitotoxic brain damage (Schwarcz et al., 1984). Increased levels of QUIN in *post mortem* brains of HD patients at the initial stages of the disease helped to establish a role for this metabolite in neurodegeneration (Guidetti et al., 2004). In further support for this concept, reduced KYNA levels were seen in HD, suggesting that the QUIN:KYNA ratio could be relevant for this disorder.

Further evidence for a role of KP in neurodegenerative disorders emerged from studies demonstrating decreased levels of KYN and KYNA in the basal ganglia of patients with Parkinson's disease (PD) (Ogawa et al., 1992). In addition, QUIN seems to be increased in those glial cells surrounding amyloid plaques and neurofibrillary tangles in Alzheimer's disease (AD) (Guillemin et al., 2005). Abnormal KP metabolism is also present in animal models of hypoglycaemia, ischemia, perinatal hypoxia and traumatic spinal cord injury (reviewed by Schwarcz et al., 2012), supporting a role of this metabolic pathway as a physiological and physiopathological modulator in the CNS. Altogether, this evidence supports an active role of an altered KP metabolism in different pathological conditions in the CNS; however, more detailed studies are needed to demonstrate this hypothesis.

3. 3-Hydroxykynurenine (3-HK)

3-HK is an intriguing and puzzling compound found at increased levels in pathological conditions; thus, characterizing and identifying the basic and clinical relevance of this tryptophan metabolite in brain disorders may help to elucidate toxic mechanisms involved in cell dysfunction and death. A summary of the chemical properties of 3-HK is presented in Table 1.

Table 1
Chemical properties of 3-HK.

Average molecular weight	224.213
Predicted water solubility	3.33 mg/mL [Predicted by ALOGPS]
log *P*	−2.09 [Predicted by ALOGPS]
log *S*	−1.771
H-bond donor	4
H-bond acceptor	6
Molar extinction coefficient	$3650\,M^{-1}\,cm^{-1}$ at 368 nm (Bando et al., 1981)

3.1. Sources for 3-HK in the brain

Brain levels of 3-HK can be enriched by two manners: either from systemic origin, or through local production. Interesting circumstances create contrasting scenarios for both of these conditions. For instance, it seems that under normal conditions, the most important contributor to enhance the extracellular striatal 3-HK levels (50 nM) comes from the circulating kynurenine (KYN) when this precursor is systemically administered to rats (Notarangelo et al., 2012). However, it must be taken into consideration that 3-HK detection specifically corresponded to extracellular levels, which do not reflects the total content of this metabolite, therefore allowing no major inferences on this phenomenon. In contrast, in another study carried out by Bellac et al. (2006), these authors compared the plasma, cortical and hippocampal levels of 3-HK in animals subjected to Pneumococcal meningitis, and found that this metabolite was significantly increased locally under pathological conditions in both brain regions, and this increase was accompanied by an augmented mRNA and activity of kynurenine 3-hydroxylase, supporting the concept that glial cells responsive to infectious/inflammatory processes could be responsible for these effects. This topic points out to important differences in 3-HK sources when either normal or pathological conditions prevail. Although it is likely that local formation of 3-HK is more prominent in toxic conditions, these differences deserve further confirmation.

3.2. 3-HK chemical properties

3-HK is a hydrophilic yellow compound commonly found in lens of primates (man and baboon), sciuridae, sheep, and in some fish (Van Heyningen, 1971; Bando et al., 1981; Truscott et al., 1992; Truscott and Wood, 1994). In primates, it is present as 3-hydroxy-L-kynurenine *O*-β-D-glucoside (3-HKG) in a micro molar range, it decreases linearly with age over the period from birth to the age of 30–40, and subsequently it remains at a constant level (Bando et al., 1981; Wood and Truscott, 1994). 3-HK has also been found in squirrel and insect eyes as xanthomatin (3-HK dimer), N-acetyl and sulphate derivatives, and glucoside conjugates (Van Heyningen, 1971; Han et al., 2007). 3-HK and its glucoside interact with lysyl residues of lens proteins and may function as a shortwave ultraviolet light filter absorbing maximally at approximately 365 nm (Goldstein et al., 2000). A summarized list of the basal levels of 3-HK in different tissues and fluids of different species is depicted in Table 2.

In a neutral pH solution, in presence of oxygen, 3-HK easily oxidizes (autooxidation). The oxidation of 3-HK forms an *o*-semiaminoquinone which reacts with oxygen to yield an *o*-aminoquinone and a large production of $O_2^{\bullet-}$ and H_2O_2; consequently, there is $^{\bullet}OH$ formation via Fenton reaction (Vazquez et al., 2000; Eastman and Guilarte, 1990; Okuda et al., 1996; Ishii et al., 1992; Hiraku et al., 1995) (Fig. 2). Trace metals, such as Cu^{2+} and Fe^{3+}, strongly catalyze the oxidation of 3-HK (Goldstein et al., 2000). Moreover, iron chelators like potassium ferricyanide, hematin, and haemoglobin are effective oxidizing agents of 3-HK. The heme enzyme horseradish peroxidase, in the presence of H_2O_2, acts in the same manner, suggesting that $^{\bullet}OH$ may be involved in its oxidation (Ishii et al., 1992). A change in the UV–vis spectrum of the autoxidized solutions indicates that some autoxidation products of 3-HK, which are formed initially, may be susceptible to further oxidation (Stutchbury and Truscott, 1993).

The characterization of the major autooxidation products of 3-HK under physiological conditions shows that 3-HK dimerizes to hydroxyxanthommatin and xanthommatin, which in turn generate at least four different compounds – including the *p*-quinone 4,6-dihydroxyquinolinequinonecarboxylic acid – and at least two minor species that were unable to be identified (Vazquez et al.,

Table 2
Levels of 3-HK in different tissues.

Tissues and fluids	Concentrations	References
Iris/ciliary body (bovine)	0.07 μg/g	Malina and Martin (1995)
Retina (bovine)	0.19 μg/g	Malina and Martin (1995)
Transparent lenses (bovine)	1.14 μg/g	Malina and Martin (1995)
Urine (human)	2.2 (1.0–3.4) μmol/mmol creatinine	http://www.hmdb.ca/metabolites/HMDB11631
Serum (human)	0.001–0.4 μM	Pearson and Reynolds (1992), Heyes et al. (1994), Chiarugi et al. (1996), Hervé et al. (1996)
Human lens	70,000 μM (3-HKG)	Luthra and Balasubramanian (1992)
Basal brain extracellular levels (rat)	<0.002 μM	Notarangelo et al. (2012)
Whole brain (rat)	0.000008 μM	Gál and Sherman (1978, 1980)

2000; Ishii et al., 1992) (Fig. 2). Moreover, 3-HK oxidation is effectively inhibited by the inclusion of thiols such as glutathione, cysteine and ascorbate.

3-HK is also a reducing agent that participates in redox reactions by donating electrons; thus, it can be an antioxidant and restore the oxidative balance in the cellular environment (Christen et al., 1990; Luthra and Balasubramanian, 1992; Goshima et al., 1986). However, under oxidative conditions, 3-HK can bind to protein amino groups to further yield cross-linked polypeptide chains (Aquilina et al., 1999). Furthermore, during the oxidation of 3-HK: (1) ROS ($O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$) are produced which promote modification of proteins, oxidation of lipids, DNA strand breaks and modification of nucleic acids, modulation of gene expression through activation of redox-sensitive transcription factors, and modulation of inflammatory responses through signal transduction, to name a few events (Goldstein et al., 2000; Ishii et al., 1992; Hiraku et al., 1995); (2) transition metal ions (Cu^{2+}, Fe^{3+}) are reduced, turning themselves into pro-oxidants (Cu^{+}, Fe^{2+}), to further participate in the formation of more radicals toward a Fenton reaction-like mechanism (Goldstein et al., 2000); (3) the quinone-imine formed is highly reactive, favoring further oxidative reactions; and (4) reactivity of oxidized 3-HK toward amino acid functional groups is similar to DOPA, catecholamines, catechol, and other aminophenols (Foye, 1979; Lévay et al., 1997; Bindoli et al., 1992).

3.3. Effects of 3-HK in biological systems: anti-oxidative activity vs. oxidative damage

ROS production and their scavenging are both processes with particular relevance to maintain the cellular homeostasis; thereby, small molecules such as certain vitamins and metabolites show this double behavior. Among these, 3-HK may be physiologically and pathologically relevant. 3-HK may function as a modulator of synaptic neurotransmission in the prenatal brain. During the last week of gestation, 3-HK levels are remarkably high, to further decrease dramatically around the time of birth. In adulthood, 3-HK concentrations gradually change, exhibiting alterations during the occurrence of pathological conditions; these effects appear to be determined by the timing and mature of the pathogenic challenge

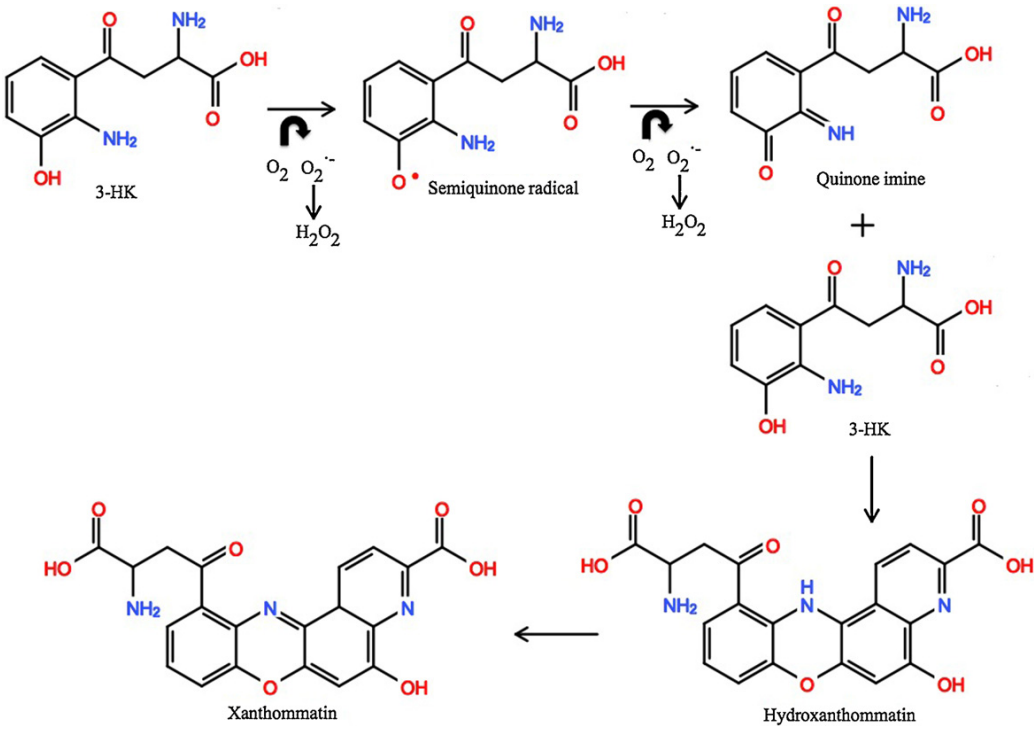

Fig. 2. 3-HK is oxidized at lower potentials. The oxidation of 3-HK under physiological conditions, generates several compounds such as the unstable xanthommatin formed by the oxidative dimerization of 3-HK in the presence of H_2O_2. This compound is degraded into other products: p-quinone (not shown), 4,6-dihydroxyquinolinequinonecarboxylic acid (not shown) and hydroxyxanthommatin. Superoxide anion and hydrogen peroxide are also formed rapidly upon oxidation of 3-HK.

(Ceresoli-Borroni and Schwarcz, 2000). However, 3-HK could play a dual role at higher levels, showing a beneficial role in the initial stages of development and shifting to a detrimental phase under prolonged exposure. The molecular mechanisms underlying these activities remain to be determined.

Another role suggested for 3-HK is related with its preventive properties on oxidative damage through scavenging activity. Goshima et al. (1986) demonstrated that 3-HK (1 μg) is able to scavenge $O_2^{\bullet-}$ in Malpighian tubules of blowfly (*Aldrichia graham*). In addition, this metabolite (1–100 μM) scavenged $^{\bullet}OH$, exhibiting scavenging capacity similar to 500 μM melatonine, a classical $^{\bullet}OH$ scavenger. Furthermore, 3-HK significantly decreased the peroxyl radicals induced by the thermolysis of 2,2′-azo-bis-(2-aminopropane)-derived peroxyl radicals in a higher degree than trolox, a well-known peroxyl scavenger, as well as ascorbate (Christen et al., 1990; Leipnitz et al., 2007; Goda et al., 1999). Interestingly, compelling evidence has shown that 3-HK can easily undergo reactions with $^{\bullet}NO$ and may be classified as a potent $^{\bullet}NO$ scavenger. Intensely colored yellow products are rapidly formed from 3-HK in presence of two different donors of $^{\bullet}NO$: nitrite at pH 5, and $NO^{+}SbF_6^{-}$ at pH 2.0. Nitrosation of 3-HK leads, presumably via a nitrosamine intermediate, to a diazonium ion, which forms an oxadiazole, tautomerizing to a yellow *o*-quinone diazide and other additional substances of different color, depending on the $^{\bullet}NO$ source (Backhaus et al., 2008). Hence, 3-HK is able to scavenge ROS such as $O_2^{\bullet-}$, $^{\bullet}HO$, and peroxyl radical and $^{\bullet}NO$; consequently, 3-HK can prevent lipid oxidation and protein oxidation and nitration.

Leipnitz et al. (2007) showed that 3-HK inhibits spontaneous lipid peroxidation in supernatants from cerebral cortex of rats. 3-HK (1 h, 100 μM) reduced the thiobarbituric acid-reactive substances (TBARS) formation in the absence and presence of Fe^{3+} and Fe^{2+}, which in turn stimulate 3-HK auto-oxidation and $^{\bullet}OH$ formation, respectively. The same authors also demonstrated that 3-HK (1 h, 100 μM) increased up to 400% the total antioxidant reactivity of C6 glioma cells, and these values gradually decreased as incubation time advanced. Treatment with 3-HK in these cells also decreased the basal TBARS when incubated for 1 and 6 h, but not at longer incubation times (Leipnitz et al., 2007). Additionally, it has been demonstrated that 3-HK (3 mM) inhibits the oxidative covalent cross-linking of crystallins, using riboflavin, a compound known to produce a variety of oxyradicals when used as a photocrosslinking agent of proteins (Luthra and Balasubramanian, 1992).

3-HK avoided the increased free radical production induced by glutaric acid in supernatants from cerebral cortex of rats, the major metabolite accumulating in glutaric acidemia type 1, an autosomal recessive disorder of lysine, hydroxylisine, and tryptophan metabolism. Patients with this disorder exhibit brain atrophy, macrocephaly, and acute dystonia secondary to striatal degeneration (Viau et al., 2012). 3-HK fully prevented and attenuated the increased levels of TBARS and 2′,7′-dihydrodichlorofluorescein oxidation produced by glutaric acid (1 mM) administration (Leipnitz et al., 2007) respectively.

Altogether, the aforementioned evidence serves to suggest that 3-HK could act as an endogenous natural antioxidant in a variety of diseases. However, in other studies, 3-HK has also shown to be a powerful generator of reactive species and a potential endogenous neurotoxin. A large body of literature has characterized its *in vitro* neurotoxicity (Table 3). Nonetheless, as the same table shows, most of the concentrations tested correspond to supraphysiological (pharmacologic) concentrations.

Data collected and described in Table 2 clearly show that concentrations used in most of *in vitro* studies are far higher than those described as normal brain concentrations (~0.08–0.3 μM, cited by Okuda et al., 1996), reaching considerably higher concentrations upon diverse pathological conditions (0.3–1.2 μM), including Huntington's disease, AIDS/dementia and hepatic encephalopathy. The issue of pharmacological vs. normal brain functions of 3-HK raises a relevant consideration. Fundamentally, use of high concentrations of this molecule to evoke cell toxicity suggest that most of these studies could be describing pharmacological (non-physiological) effects, thereby strengthening the concept that, under normal conditions, 3-HK could be more a modulatory metabolite mostly responsible for maintaining cellular redox homeostasis than a prooxidant, a feature that could be shared with other KP metabolites. Despite this key consideration, it is pertinent to describe neurotoxic actions exerted by 3-HK, since dual effects for this metabolite still remain in literature.

In primary cultured striatal neurons, 3-HK (10 μM) induced cell death with apoptotic features. 3-HK-induced apoptosis involved generation of ROS that were suppressed by antioxidants (catalase, allopurinol, α-tocopherol, trolox, N-acetylcysteine, ascorbic acid and 3,3,5,5-tetramethyl-1-pyrroline-1-oxide, xanthine oxidase inhibitor, and deferoxamine, a chelating agent). Furthermore, it was demonstrated that 3-HK toxicity in these cells depends on both its cellular uptake via transporters for large neutral amino acids in a sodium-dependent process, and the intracellular increase of ROS (Okuda et al., 1996, 1998).

Cerebellar granule neurons are likely to be more resistant to 3-HK-induced cell death than striatal cells, requiring much higher concentrations of the toxin (250 μM) to provoke the same degree of toxicity. The co-application of catalase caused a significant reduction in its neurotoxic effect, whereas SOD, allopurinol and the neutral amino acid transporter blockade failed to provide protection; likewise, 3-HK did not induce caspase-3 activation but produced p38 MAPK activation (Smith et al., 2009; Wei et al., 2000). Similar to

Table 3
Concentrations of 3-HK tested in *in vitro* studies.

Cells	3-HK tested concentration	Periods	% Cellular death	Reference
Cerebellar granule neurons	10 μM–1 mM	1–9 h	20–80%	Smith et al. (2009)
	250–500 μM	7 days	50–90%	Jeong et al. (2004)
Striatal neurons	1– 10 μM	24–48 h	50–65%	Jeong et al. (2004)
	50 μM	48 h	75%	
Neuronal hybrid cell line	200 μM	24 h	80%	Eastman and Guilarte (1989)
Hippocampal neurons	10 μM	48 h	50%	Okuda et al. (1998)
Cortical neurons	10 μM	48–72 h	–	Chiarugi et al. (2001a,b)
	100 μM	24 h		
Human neuroblastoma SH-SY5Y	240 μM	24 h	40%	Jeong et al. (2004)
PC-12 pheochromocytoma cells	500–600 μM	24 h	53–23%	Jeong et al. (2004)
GT1-7 hypothalamic neurosecretory cells	400 μM	24 h	38%	Jeong et al. (2004)
C6 glioma	100 μM	1–48 h	0%[a]	Leipnitz et al. (2007)

[a] C6 cell morphology did not change in the presence of 3-HK at all incubation times.

cerebellar granule cells, immortalized cells, PC-12 pheochromocytoma cells and GT1-7 hypothalamic neurosecretory cells required pharmacological concentrations of 3-HK to show apoptotic features, whereas 3-HK-induced toxicity was unaffected by the neutral amino acid transporter. Of note, dantrolene, an inhibitor of calcium release from the endoplasmic reticulum, protected these cells. The protection exerted by dantrolene was associated with a marked increase in the protein levels of a prominent anti-apoptotic gene product, Bcl-2, suggesting that other mechanisms are likely involved in 3-HK toxicity (Wei et al., 2000). Neuronal hybrid cell line and human neuroblastoma SH-SY5Y cells treated with 3-HK showed an increase in ROS concentration, alteration in the catalase activity and enhanced apoptotic features (Eastman and Guilarte, 1990, 1989; Jeong et al., 2004).

Noteworthy, treatment of human astrocytes with 3-HK at concentrations below 100 nM significantly augmented intracellular NAD^+ levels compared to non-treated cells. However, higher doses (>100 nM) decreased intracellular NAD^+ levels and increased extracellular LDH activity (Brandy et al., 2009). NAD^+ is an important co-factor of many enzymes and, as a molecule, is involved in several metabolic processes. For instance, NAD^+ is a substrate for mono- and poly-ADP-ribosylation of proteins, and a precursor for calcium mobilizing agents that regulates the functions of NAD^+-dependent enzymes such as the protein deacetylase SIRT1, which in turn modulates target gene transcription through the modification of chromatin-associated proteins (Zhang et al., 2009; Berger et al., 2004) Hence, changes in 3-HK concentrations may indirectly alter: (i) DNA repair mechanisms, (ii) intracellular free Ca^{2+} levels, and (iii) general patterns of gene expression.

On the other hand, it has been reported that sodium-dependent 3-HK uptake in slices from separate brain regions was in the following order: cortex > striatum = hippocampus > cerebellum (Eastman et al., 1992). In a similar manner, sodium-dependent KYN uptake activity reported by Speciale and Schwarcz (1990) was as follows: cortex > striatum > hippocampus > cerebellum, suggesting that 3-HK toxicity may be dependent on cellular uptake (Okuda et al., 1998).

Remarkably, there are only a few studies with 3-HK in *in vivo* models in the CNS; neither of them have characterized in a detailed manner its mechanisms of toxicity. For instance, intraventricular administration of 3-HK (634.21 μg/rat) caused convulsive attacks in rats (Pinelli et al., 1984). Moreover, the intrastriatal injection of 3-HK (50 nmol) induced only tissue damage around the injected site, without abnormal behavior in rats (Nakagami et al., 1996). Later on, it was suggested that 3-HK could potentiate QUIN toxicity through a possible synergic interaction with a combination of direct NMDA receptor activation and free radical production. Guidetti and Schwarcz (1999) evaluated this effect in the rat brain *in vivo* using an intrastriatal co-injection of 5 nmol 3-HK plus 15 nmol QUIN, doses which, when injected separately, caused no or minimal neurodegeneration. Co-injection of 3-HK and QUIN caused substantial increases in the lesion volumes and altered rotation behavior. However, these authors were unable to find *de novo* production of QUIN, suggesting that potentiation of QUIN toxicity by 3-HK was not due to the *in vivo* conversion of 3-HK to QUIN (Guidetti and Schwarcz, 1999). The authors proposed then that the failure of 3-HK to cause cellular damage is the result of the radical scavenging capacity of the normal brain, which may be sufficient to neutralize 3-HK-induced free radical generation and to prevent cell death (Schwarcz et al., 2010). Nonetheless, it is also pertinent to consider that the experimental design employed in this work did not assume possible chemical interactions between these two molecules, a process that could ultimately modify the reactive identity of each agent. Therefore, optimal conditions to test these agents should imply a separate administration. In the meantime, these findings might indicate that the mild effects exerted by 3-HK upon *in vivo* conditions could recruit free radicals generated from the autoxidation process, with no deleterious consequences to nerve tissue, as it can be assumed that defense systems are capable to counteract 3-HK toxic signals.

3.4. Other effects linked to 3-HK

Neurodegenerative diseases such as AD, PD, and HD, are likely to share common cellular and molecular deleterious mechanisms, including protein aggregation and mitochondrial dysfunction (Ross and Poirier, 2004; Coyle and Puttfarcken, 1993; Santamaria and Jimenez, 2005). Several reports suggest that high concentrations of 3-HK might promote these events.

As previously mentioned, IDO catalyzes the transformation of tryptophan into N-formylkynurenine; the hydrolysis of N-formylkynurenine produces KYN, which is hydroxylated and glycosylated, yielding 3-HK and 3-HKG (Snytnikova et al., 2008). At physiological conditions, 3-HK and 3-HKG undergo spontaneous deamination, and the resulting deaminated kynurenines-carboxyketoalkenes are highly reactive species susceptible to nucleophilic attack via Michael addition, forming an unstable adduct with amino acid residues such as cysteine, histidine, and lysine (Garner et al., 1999, 2000) (Fig. 3).

In the presence of oxygen, 3-HK attaches to several sites on the crystallins, especially in cysteine residues (γS crystallin and βB1 crystallin) (Aquilina and Truscott, 2002). 3-HK binding is accompanied by crystalline aggregation, and its inclusion with GSH results in a delayed onset of crystalline modification (Stutchbury and Truscott, 1993; Garner et al., 2000; Aquilina and Truscott, 2002). Another protein target for 3-HK is bovine serum albumin (Goldstein et al., 2000). 3-HK also interacts with zinc/thiolate coordination environments such as metallothionein (Giles et al., 2003).

The formation of cysteine adduct with 3-HK was confirmed using a preparation of bovine calf lens protein at pH 7.2, whereas His and Lys residues only became modified when the incubation was performed at pH 9.5, conditions under which the polypeptides are likely to be unfolded (Korlimbinis and Truscott, 2006). On the contrary, Staniszewska and Nagaraj (2005) showed that lysine adducts can be generated in human lens proteins treated with 3-HK using a monoclonal antibody.

Appropriate GSH levels are crucial for minimizing polypeptide modification by 3-HK. GSH acts as an oxidant scavenger; thus, GSH possesses the ability to prevent covalent modification of proteins (Aquilina et al., 1997). Binding of 3-HK to crystallins in the lens may be a dynamic process, with binding and release, both taking place. If there is sufficient GSH, the reactive deamination product would most likely react with GSH. In contrast, if there is a decrease in GSH levels, the deaminated 3-HK may oxidize and bind irreversibly to proteins (Korlimbinis and Truscott, 2006).

The deposition of proteins also takes place when proteins and lipids are exposed to sugars, forming advanced glycation-end products (AGEs). Sugars, ascorbate, and dicarbonyl compounds react with amino groups of lysine and arginine residues of proteins through the formation of ketoamine adducts; these adducts further produce ROS. Several proteins implicated in neurodegenerative diseases, such as amyloid β, tau, α-synuclein, and prions are glycated and the extent of glycation is correlated with the pathologies seen in patients, contributing to the development of neurodegenerative diseases. Nagaraj et al. (2010) demonstrated that 3-HK may exert a stimulatory effect on pentoside synthesis from ascorbate and ribose, two AGEs that have been detected in the human lens, suggesting that 3-HK may modulate AGE formation. Therefore, 3-HK could play a pivotal role in protein damage due to the following considerations: (1) this molecule is highly susceptible to oxidize and produce formation of extremely toxic reactive species (o-aminoquinone and ROS) (Okuda et al., 1996; Eastman and Guilarte, 1990; Vazquez et al., 2000; Aquilina et al., 1997, 1999; Aquilina and Truscott, 2002); (2) the formation of these

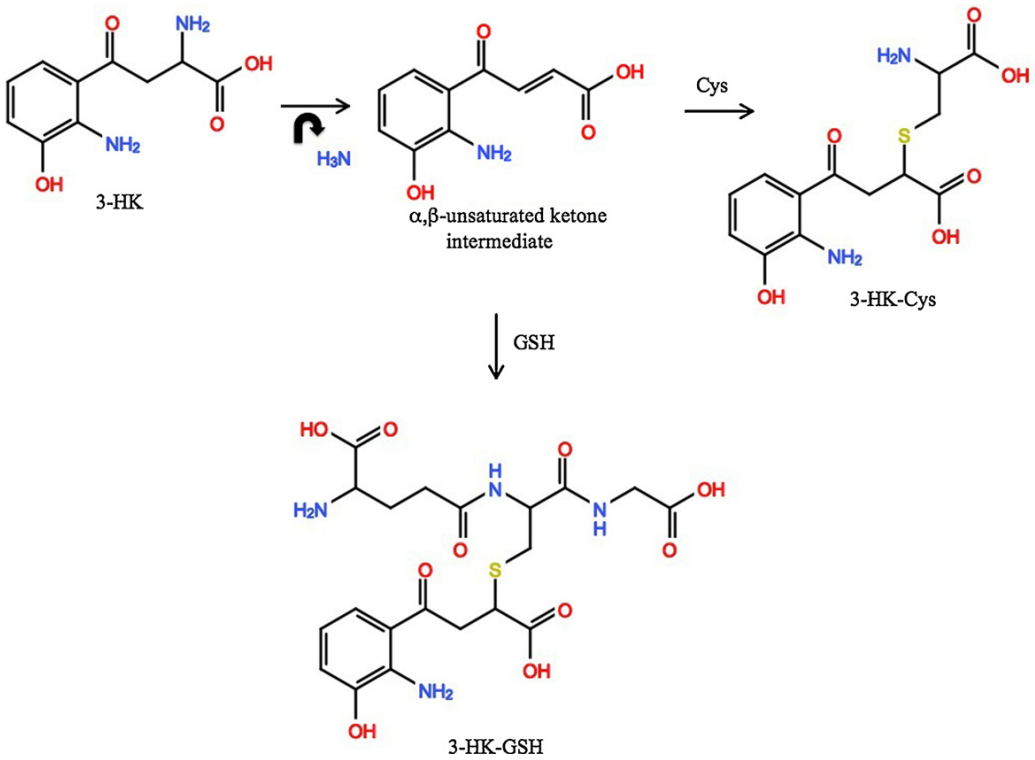

Fig. 3. Deamination of 3-HK and its covalent attachment to amino acid residues. Under physiological conditions, the carbonyl group acidifies the adjacent H atom, resulting in the elimination of ammonia from the β-carbon and bound rearrangement to form an α,β-unsaturated carbonyl. The β-carbon atom is highly susceptible to non-stereoselective nucleophilic attack, resulting in the formation of an enolate intermediate that becomes protonated to form the 3-HK-protein adduct.

species could decrease the concentration of GSH and other antioxidants (Berry and Truscott, 2001); (3) protein radicalization, adduct formation, cross-linking and fragmentation occur when 3-HK concentrations increase because this metabolite can react with polypeptides at neutral pH via modification of amino acid residues (Stutchbury and Truscott, 1993; Goldstein et al., 2000); (4) the interaction of 3-HK with proteins may affect the cellular redox status, causing the release of metal ions (Giles et al., 2003; Aquilina et al., 1999; Backhaus et al., 2008; Viau et al., 2012); and (5) it is also possible that the *o*-aminophenol moiety of 3-HK may act as a site for chelation of redox-active metals such as Fe^{2+} or Cu^{+}, metals that could contribute to $^{\bullet}OH$-induced damage to protein (Goldstein et al., 2000). Altogether, this evidence suggests that these processes could modify the enzymatic activity of several proteins, as occurs with the creatinine kinase activity, which is inhibited by 3-HK in a time- and dose-dependent manner. This inhibition was prevented, but not reversed, by incubation with reduced glutathione, dithiothreitol, ascorbic acid and trolox. Nevertheless, under nitrogen atmosphere, the enzyme was not inhibited by 3-HK, indicating that the inhibition was caused by products of autoxidation of 3-HK, and not by 3-HK itself (Cornelio et al., 2006). Thus, protein modification exerted by 3-HK could be carried out by multiple mechanisms, including a direct interaction of 3-HK with proteins, together with further reactions of the 3-HK autoxidation-derived reactive species with a variety of macromolecules.

In addition, 3-HK can modify mitochondrial function and trigger DNA damage. Mitochondria have several important functions in the cell. Mitochondrial dysfunction causes abatement in ATP production, oxidative damage, excitotoxicity, and the induction of apoptosis, all of which are involved in the pathogenesis of numerous disorders (Lin and Beal, 2006; Beal, 2004). Lee et al. (2004) showed that 3-HK causes neuronal cell death by inducing mitochondrial dysfunction. 3-HK induced the collapse of mitochondrial membrane potential and cytochrome c release. Interestingly, inhibition of the Extracellular Signal-Regulated Kinase (ERK) dramatically increased mitochondrial malfunction and enhanced caspase activity under conditions of cellular stress. 3-HK (≥2.5 mM) also affected the respiratory parameters of heart mitochondria in a dose-dependent manner. This metabolite significantly decreased respiratory control index and the ADP:oxygen ratio of glutamate/malate respiring rat heart mitochondria. However, the high concentrations of 3-HK used in this work are difficult to achieve under physiological conditions (Baran et al., 2003). Finally, DNA damage by 3-HK has also been studied. 3-HK induced DNA double-strand breaks in cultured human cells. Hiraku et al. (1995) suggested that this damage is mediated by an increase in the production of H_2O_2 and the presence of Mn^{2+} or Cu^{2+}. Therefore, high concentrations of 3-HK observed in pathological events could be related to protein, mitochondria and DNA modifications.

Of consideration, Krause et al. (2011) have recently described anti-inflammatory and neuroprotective effects for 3-HK and 3-hydroxyanthranilic acid (3-HAA), and these effects involved the suppression of cytokine-induced neuronal death and associate antioxidant responses in primary human fetal central nervous system cultures treated with cytokines (IL-1 with or without interferon-γ) or with Toll-like receptor ligands mimicking the proinflammatory environment of the CNS. These findings raise the controversy on the real role exerted by 3-HK and related metabolites in the brain, as described in the following section.

3.5. Effects of 3-HK-related compounds

3.5.1. Xanthurenic acid

Interestingly, 3-HK metabolism proffers complementary concepts to the effects already reported for this metabolite in the CNS. Xanthurenic acid (XA), another metabolite of KP, is formed after

transamination of 3-HK (Fig. 1). Since 3-HK autooxidation is assumed to be involved in oxidative damage, XA formation is believed to be part of a detoxification process (Gobaille et al., 2008) as it prevents 3-HK accumulation. In turn, XA synthesis from 3-HK seems to involve the same kynurenine transmaninase isoenzymes that catalyze the transamination of KYN to KYNA (Urenjak and Obrenovitch, 2000). Besides of its sedative actions at high concentrations (Heyliger et al., 1998), XA has been demonstrated to be present in different brain regions at concentrations about 1 μM; therefore, this molecule has been proposed to exert physiological actions in the brain (Gobaille et al., 2008). Moreover, XA can penetrate de blood-brain barrier and its distribution in the brain is heterogeneous, being particularly accumulated in the frontal cortex. Indeed, some physiological actions exerted by XA have been attributed to its potential role as a neurotransmitter-like molecule (Gobaille et al., 2008), an issue that still deserves investigation. In the meantime, being the major catabolic product of 3-HK, XA requires special attention. While some reports describing antioxidant properties for this metabolite might serve to confirm its formation in the brain as a major detoxification route for 3-HK production (Murakami et al., 2001; López-Burillo et al., 2003), only a few of them describe pro-oxidant actions (Murakami et al., 2006); hence, whether the role of this molecule on the effects of 3-HK is oriented to detoxify or to exert synergic toxic actions through its redox modulatory actions is a question needing experimental approaches to be elucidated, and so, no scenario should be ruled out so far for the actions of XA and 3-HK.

3.5.2. Anthranilic acid:3-hydroxyanthranilic acid ratio

An alternative arm of the KP converts KYN into anthranilic acid (AA). The enzyme responsible for this alternative metabolic step is kynureninase. In turn, AA is responsible for 3-HAA formation, without involving 3-HK in the process (Fig. 1). This part of KP metabolism is relevant when trying to understand those physiological and physiopathological processes linked to 3-HK (Darlington et al., 2010). In fact, since 3-HAA can be formed either directly from 3-HK or from AA, only part of the toxic actions of 3-HAA could be related with 3-HK. Moreover, 3-HAA yields the formation of a more toxic metabolite, QUIN. It has been demonstrated that QUIN levels are increased after 3-HAA is topically administered into the rat striatum (Notarangelo et al., 2012; reviewed by Schwarcz et al., 2010). This effect was assessed by biochemical and microscopic assays; thereby, enhanced levels of 3-HAA – and probably 3-HK too – would lead to the formation of a potent excitotoxic/pro-oxidant KP metabolite (QUIN) that might contribute to a more complex toxic pattern in the brain.

Noteworthy, the chemical structures of 3-HAA and 3-HK are quite similar, as they have hydroxyl and amino groups in ortho positions to each other, both are good electron donors and their oxidation lead to the formation of a highly reactive quinoneimine. Nonetheless, 3-HAA, similar to 3-HK and other KP redox metabolites, carries out the same duality, since on one hand it has been shown to induce ROS-mediated cell damage in different cell cultures (Goldstein et al., 2000; Brandy et al., 2009), and on the other hand, this metabolite is responsible for antioxidant responses by diminishing TBARS in cerebral cortex supernatants (Leipnitz et al., 2007), scavenging peroxyl radicals, and protecting β-phycoerythrin from these species (Christen et al., 1990). Furthermore, 3-HAA might exert a relevant role during inflammatory conditions since it has shown to suppress the activation of the proinflammatory transcription factor NFκB while inhibiting nitric oxide synthase (reviewed by Darlington et al., 2010). Altogether, this contrasting evidence encourages the study of 3-HAA and the 3-HAA:AA ratio since these two elements could be relevant to understand the actions of 3-HK at central level.

3.6. 3-HK in brain disorders

3-HK is known to be a neuroactive metabolite that may produce damage by overproduction of reactive species, direct protein interaction and mitochondrial dysfunction, although its extracellular levels, determined by *in vivo* microdialysis in the rat brain, are below 2 nM (Notarangelo et al., 2012). Nevertheless, in a number of different pathological conditions – possibly as a consequence of enzyme induction –, the amounts of 3-HK in the CNS are increased in astrocytes, macrophages, microglia and other cells of the immune system. Thus, this tryptophan metabolite may play an important role in neurodegenerative diseases, psychiatric disorders, and seizures (Chiarugi et al., 2001a,b), as shown in Table 4.

Excitotoxic mechanisms are believed to play a central role in the pathogenesis of a broad spectrum of neurological diseases. Ceresoli et al. (1997) showed that both the developing and adult brains respond to an acute excitotoxic insult by shifting brain KP metabolism, and provided evidence that an excitotoxic brain lesion in rats of 7 days of age causes a preferential increase in KYNA formation, while an increase in 3-HK levels was more pronounced

Table 4
3-HK levels (mean ± se.mean) in normal and pathological conditions.

Pathological conditions; Disorder/experimental model	Mean 3-HK levels (controls)	Mean 3-HK levels (pathological conditions)	References
PD			
CFS	6.1 ± 2.8 nM	1.1 ± 0.1 nM	Tohgi et al. (1993a,b)
HD			Pearson and Reynolds (1992)
Temporal cortex	0.29 ± 0.22 nmol/g	0.49 ± 0.21 nmol/g	
Frontal cortex	0.15 ± 0.12 nmol/g	0.41 ± 0.27 nmol/g	
Putamen	0.08 ± 0.01 nmol/g	0.29 ± 0.21 nmol/g	
Infant pneumococcal meningitis model in rats			
Cortex	42 ± 15 nM	590 ± 237 nM	Bellac et al. (2006)
Experimental encephalomyelitis in rats			Chiarugi et al. (2001a,b)
Spinal cord	0.14 ± 0.03 nmol/g	1.2 ± 0.2 nmol/g	
Forebrain	0.12 ± 0.02 nmol/g	0.2 ± 0.02 nmol/g	
Infantile spasms			
CSF	0.57–85 nM	1.56–3.26 nM	Yamamoto (1991), Yamamoto et al. (1994)
Rats with experimental allergic encephalomyelitis			
Spinal cord	0.14 nM	1.21 nM	Chiarugi et al. (2001a,b)
Forebrain	0.12 nM	0.20 nM	
Blood	0.08 nM	0.11 nM	

in adult animals, demonstrating that age-dependent changes in cerebral KP metabolism occur in response to an excitotoxic insult (Ceresoli et al., 1997). In another work, the basal levels of 3-HK in rat brains were unchanged after receiving intrastriatal injections of NMDA a week earlier, while tryptophan, kynurenine and QUIN showed increased levels. Animals receiving intrastriatal injections of NMDA (8 μg/2 μl) plus systemic administration of kynurenine (100 mg/kg, i.p.) reached a peak in 3-HK of approximately 50 nM 2 h following kynurenine injection (100 mg/kg, i.p.). Notably, 3-HK levels decreased 7 h later. These authors suggested that 3-HK enters the brain from the circulation and once there, it is rapidly sequestered and degraded into QUIN (Notarangelo et al., 2012).

Inflammation processes contribute to the progression and manifestation of a broad spectrum of disorders in the CNS. Excessive release of IFN-γ has been associated with the pathogenesis of chronic inflammatory and autoimmune diseases (Mühl and Pfeilschifter, 2003). IFN-γ induces the expression of IDO and so, it enhances the production of kynurenines from tryptophan, including 3-HK. The induction of IDO by IFN-γ triggers an increase in intracellular ROS, cytosolic cytochrome c and caspase-3 activity, along with a decrease in protein-free thiol content and apoptotic cell death. IDO (1-methyl-DL-tryptophan) and kynurenine 3-hydroxylase inhibitors (Ro61-8048) effectively inhibited IFN-γ-mediated apoptosis (Mailankot and Nagaraj, 2010). These data suggest that increased levels of 3-HK could play a role in diseases related to excitotoxic and inflammatory processes such as HD, AD, PD, convulsive and depressive disorders, to name a few.

3.6.1. Huntington's disease

HD is a fatal inherited neurodegenerative disorder characterized by abnormal motor movements, personality changes and dementia. Cellular toxicity, which initially affects the striatum – and progressively the cortex –, has been attributed to a polyglutamine expansion in the huntingtin protein. Oxidative stress, protein oxidation, and consecutively, aberrant inactivation/or degradation of proteins, can aggravate molecular damage (Sorolla et al., 2012). New studies carried out in HD brains, genetic model organisms, and mouse models suggest that this disease may in fact be causally related to early abnormalities in KP (Schwarcz et al., 2010). In support to this concept, it has been demonstrated that neurolopathological features in HD can be reproduced in experimental animals by an intrastriatal injection of QUIN. Further evidence indicates that expression of a mutant huntingtin fragment induces transcription of KP in yeast, which is abrogated by impairing the activity of the histone deacetylase Rpd3. Histone deacetylase-dependent regulation of the KP was also observed in a mouse model of HD, in which the treatment with a neuroprotective histone deacetylase inhibitor, suberoylanilide hydroxamic acid, blocked KP activation in microglia expressing a mutant huntingtin fragment *in vitro* and *in vivo*, thereby suggesting that this pathway plays a critical downstream role in mediating mutant huntingtin toxicity (Giorgini et al., 2008).

Previous reports have indicated abnormalities in the concentration of tryptophan metabolites in the HD brain, including an increase in 3-HK levels (Schwarcz et al., 1984; Reynolds and Pearson, 1989; Pearson et al., 1995; Pearson and Reynolds, 1992; Guidetti and Schwarcz, 2003). Concentrations of 3-HK were determined in brain tissues taken from *post mortem* brains from patients with HD in the early stages (Guidetti and Schwarcz, 2003; Schwarcz et al., 2012). 3-HK was significantly increased in frontal and temporal cortex and putamen of these patients (Pearson and Reynolds, 1992), while others at very advanced stages, and in a state of severe disability, showed lower levels (Stoy et al., 2005).

Several models have been developed in order to elucidate the toxic mechanisms leading to neuronal damage, their possible interactions in HD, and potential therapies. Among these, genetic models using invertebrates, such as fruit flies and yeast, as well as genetic mice, have been the most commonly used (Ramaswamy et al., 2007). Using lower model organisms, it has been demonstrated that the toxic effects of mutant huntingtin could be related to an increase in KP metabolites. In a transgenic *Drosophila melanogaster* model of HD, genetic or pharmacological KMO inhibition ameliorated neurodegeneration. Importantly, this effect was reduced by feeding flies with 3-HK (Campesan et al., 2011). In a similar manner, 3-HK levels increased around 2.2-fold in yeast cells that expressed a mutant hutingtin. The genetic deletion and pharmacologic inhibition of KMO in these cells suppressed the toxicity caused by a mutant huntingtin fragment, suggesting that 3-HK levels could play a role in the observed toxic effects. In addition, pharmacologic inhibition of KMO reduced ROS levels (Giorgini et al., 2005); thereby, the polyQ toxicity mechanisms in *Drosophila melanogaster*, yeasts and HD patients might involve upregulation of KP to form 3-HK, and consequently, overproduction of ROS.

Transgenic models have also been used to determine the 3-HK role in HD. The brain levels of this compound are increased in several mouse models. R6/2 mice are the most commonly used transgenic murine model of HD; these animals possess a 1.9 kB fragment derived from the 5′ end of the human huntingtin gene inserted into the mouse genome, and they express approximately 144 CAG repeats. R6/2 mice received an intrastriatal injection of tryptophan in order to evaluate the production of 3-HK *in vivo*. No effect of genotype was observed between 4 and 12 weeks of age. In contrast, intrastriatally applied kynurenine resulted in an increased neosynthesis of 3-HK, but no in other KP metabolites. Subsequent *ex vivo* studies in striatal, cortical and cerebellar tissue revealed substantial increases in the activity KMO and significant reductions in the activity of the 3-HK degradative enzyme, kynureninase, in HD mice, this changes starting at 4 weeks of age. Decreased kynureninase activity was most evident in the cortex and preceded the increase in KMO activity. The activities of other KP enzymes showed no consistent brain abnormalities in the mutant mice. These findings suggest that impairment in KP metabolic enzymes jointly account for the abnormally high brain levels of 3-HK in the R6/2 model of HD, and are related to symptoms developed in these animals (Sathyasaikumar et al., 2010; Guidetti et al., 2006).

YAC 128 and the chimeric HdhQ lines (HdhQ92 and HdhQ111) are models that express the entire human huntingting gene and contain a mutated exon 1 with 111 or 92 CAG repeats, respectively. These models, together with heterozygous transgenic mice containing 89 CAG repeats, have also shown a substantial elevation of 3-HK content in both the striatum and cortex (Guidetti et al., 2000). No changes were seen in 13-month-old shortstop mutant mice for HTT (Guidetti et al., 2006).

Altogether, this information supports the concept that cerebral KP is affected in the HD brain, showing differences between early and late stages of the disease. Increased 3-HK levels might contribute to cell damage in early phases of illness. Perhaps, in response to mitochondrial impairment and/or a plethora of other abnormalities, dysfunctional cells then create a pro-inflammatory environment, leading to the activation of surrounding microglia. This, in turn, would lead to an up-regulation of microglial KP metabolism, enhanced release of 3-HK and QUIN, and, finally, the demise of already weakened neurons by a combination of excessive ROS production and NMDA receptor activation (Schwarcz et al., 2010).

3.6.2. Alzheimer's disease

AD is a complex neurodegenerative disease characterized by hippocampal neuronal loss and severe dementia in later stages, resulting in a gradual and irreversible loss of memory and cognitive functions. The severity of AD depends on the dysfunction of two molecules: Amyloid protein precursor and Tau protein.

Protein aggregation results in senile plaque formation and neurofibrillary degeneration, which together trigger a cascade of toxic events, including oxidative stress, glutamate-induced excitotoxicity and inflammation.

Through its neuroactive products – especially QUIN –, KP is considered to be involved in the neuropathogenesis of AD. However, in regard to 3-HK, different results have been found. Using circulating antibodies directed against tryptophan derivatives conjugated to proteins in serum samples from AD patients, it was demonstrated that the overproduction of 3-HK is associated with hyperactivation of IDO-1 (Duleu et al., 2010). In support to this concept, in another assay with *post mortem* hippocampal sections of AD patients using immunocytochemical methods, elevated 3-HK levels were demonstrated. Western blot analysis showed 3-HK to be slightly elevated, although statistical significance was not reached (Bonda et al., 2010). Similar to this, in *post mortem* brains of AD patients, 3-HK was slightly increased in temporal cortex, although once again, this was not significantly changed from matched controls (Pearson and Reynolds, 1992). In the cerebrospinal fluid and plasma from patients with Alzheimer-type dementia, 3-HK levels decreased 81% when compared with controls, with no marked increases in 3-HK levels in plasma concentrations (Bonda et al., 2010; Tohgi et al., 1992).

Although there is no experimental evidence demonstrating an increase of 3-HK levels in AD phenotype, the elevated expression and activity of IDO-1 and the presence of increased levels of QUIN indicate that there is an increase in 3-HK production that is rapidly catabolized. Moreover, in *Drosophila* mutants it was also observed that cytotoxicity of 3-HK is relevant to produce memory alterations similar to AD (Savvateeva et al., 2000), whereas chronic administration of a KMO inhibitor in a transgenic mouse model of AD prevented spatial memory deficits, anxiety-related behavior, and synaptic loss (Zwilling et al., 2012), suggesting that increased levels of 3-HK may participate in the cellular damage observed in AD. Therefore, it is likely that amyloid-β activates microglial and astrocytic cells in the brain following accumulation and aggregation; consequently, it also may induce enhanced levels of the toxic intermediates of KP, whereby reducing the formation of toxic KP metabolites may emerge as a new therapeutic strategy against AD.

3.6.3. Parkinson's disease

PD is a disabling neurodegenerative disease which involves several interacting toxic mechanisms: mitochondrial dysfunction, oxidative stress and cell energy insufficiency (Beal, 2004). Alterations in KP have been demonstrated in this disorder. Serum and cerebral spinal fluid samples of PD patients have shown increased levels in the KYN:tryptophan ratio in comparison with controls. Furthermore, KATI and KATII activities in serum of PD patients are reduced (Szabó et al., 2011). The cerebrospinal fluid of parkinsonian patients shows a reduction in 3-HK concentrations (Tohgi et al., 1993a,b). In contrast, 3-HK levels were increased in putamen of PD brains of patients without L-DOPA therapy, as well as in the frontal cortex, putamen, and substantia nigra of patients treated with L-DOPA (Ogawa et al., 1992). Therefore, it can be assumed that a shift in the tryptophan metabolism towards 3-HK and QUIN, and consequently, a fall in KYNA concentrations, are related to the alterations observed in PD.

3.6.4. Microbial chronic infections

3-HK levels are elevated in viral and microbial infections such as VIH and Pneumococcal meningitis. VIH-associated dementia is a frequent consequence of HIV infection and relates to neuronal damage. IDO activity is increased in *post mortem* brain tissues (frontal cortex) from AIDS patients; as a result, 3-HK concentrations were increased (over three-fold) (Sardar and Reynolds, 1995; Sardar et al., 1995; Heyes et al., 1989). Thus, elevated 3-HK levels may contribute to the neuronal deficits underlying HIV-associated dementia.

On the other hand, Pneumococcal meningitis is characterized by an intense inflammatory host reaction that contributes to the development of cortical necrosis and hippocampal apoptosis. Inflammatory conditions in the brain are known to induce tryptophan degradation along KP, resulting in accumulation of neurotoxic metabolites. In the late phase of acute Pneumococcal meningitis, it was found a transcriptional upregulation of kynurenine-3-hydroxylase and a 3-HK accumulation in cortex (590 nM) and hippocampus (313 nM), while in plasma (115 nM), no increases were observed. In line with this concept, enhanced 3-HK levels were associated with KMO upregulation (Bellac et al., 2006). Therefore, under inflammatory conditions, it is likely that KP could be metabolically oriented to form 3-HK at central level, instead of other metabolites.

3.6.5. Cerebral ischemia

Cerebrovascular disease is the second cause of death and the most frequent cause of non-traumatic disability in adults worldwide, according to the World Health Organization. Nowadays, there is no effective treatment to reverse the morphological and behavioral alterations induced by stroke. Moreover, inflammatory cytokines and protease secretion by microglia, leukocytes and resident cells of the neurovascular unit, are pivotal factors for cerebral injury, which in turn might induce upregulation of the KP, as that found in animal models of focal cerebral ischemia and patients with this disorder (Saito et al., 1993); however, the role of 3-HK in acute ischemic stroke remains uncertain.

Carpenedo et al. (2002) used *m*NBA and Ro 61-8048, two different KMO inhibitors, in organotypic hippocampal slice cultures exposed to 30 min of oxygen and glucose deprivation. 3-HK and QUIN neo-synthesis decreased, hence facilitating kynurenine metabolism towards KYNA formation, and reducing tissue damage. The addition of 3-HK and QUIN to oxygen and glucose-deprived hippocampal slices prevented the neuroprotective activity of these KMO inhibitors. However, 3-HK administration (300 μM) was non-toxic since it did not exacerbated oxygen and glucose deprivation-induced damage. Thus, the reduced tissue damage observed under these conditions may be the consequence of KYNA overproduction.

3.6.6. Convulsive disorders

Seizures are characterized by sudden and unusually unprovoked attacks as a result of abnormal brain electric activity. Not all seizures imply epilepsy; they can be the consequence of hepatic encephalopathy and Vitamin B6 deficiency. KP metabolites have been hypothetically linked to the occurrence of seizure phenomena because 3-HK and QUIN administration into the lateral ventricle caused convulsive attacks in rats (Pinelli et al., 1984; Yokoi et al., 1998).

Cortical 3-HK concentrations were substantially increased in *post mortem* brain tissues of patients with hepatic encephalopathy. On the other hand, accumulation of 3-HK was observed in urine and plasma of rats and mice with induced vitamin B6 deficiency (Kimoto et al., 1991; Pearson and Reynolds, 1991; Bender et al., 1990). Moreover, increased concentrations of this metabolite were found in different brain regions of vitamin B6 deficient neonatal rats: cerebellum, striatum, frontal cortex, and pons/medulla at 14 and 18 days of age (Guilarte and Wagner, 1987a,b). The elevation of brain 3-HK levels is likely occurring simultaneously with the onset of neurological signs, suggesting that the accumulation of this metabolite in the brain may be a putative endogenous convulsant in hepatic encephalopathy and vitamin B6 deficiency.

Another cause of seizures is cerebral infection. Intracerebral injection of hamster neurotropic measles virus to weanling Balb/C mice leads to encephalitis, which is characterized by seizures, selective neurodegeneration, and, after approximately 7 days, death. To provide a better understanding of the underlying molecular pathology, the brain content of KP metabolites was evaluated. 3-HK and QUIN levels increased in CA1 and CA3 areas, and these changes preceded the onset of electroencephalographic seizures; thus, excess formation of 3-HK and QUIN caused seizures and eventually neurodegeneration, whereby a decrease in the 3-HK:QUIN ratio might be of benefit in preventing or arresting seizure-induced neuronal damage (Lehrmann et al., 2008).

Infantile spasms are a form of age-dependent intractable epilepsy in childhood and are often associated with mental retardation. Cerebrospinal fluid from patients with symptomatic infantile spasms showed increased 3-HK levels (Yamamoto, 1991; Yamamoto et al., 1994). Moreover, infants and children with high fevers can also produce febrile seizures. Yamamoto et al. (1994) found an imbalance in the tryptophan metabolites during convulsions in this disorder. These findings suggest that seizures in hepatic encephalopathy, vitamin B6 deficiency, cerebral infection, infantile spasms, and febrile seizures could be attributable to an altered 3-HK production. Additionally, 3-HK could act as a competitive inhibitor at benzodiazepine receptor in GABA receptor complex; thus, the putative endogenous convulsant activity of 3-HK may be exerted by modulation of the GABA/BDZ receptor complex (Guilarte et al., 1988). Therefore, 3-HK might contribute to the pathogenesis of convulsive disorders.

3.6.7. Depression

Depressed patients show peripheral changes of circulating levels of proinflammatory cytokines and altered neurotransmission, resulting in a KP up-regulation. This phenomenon has been reported in patients suffering from various types of depressive disorders, including major depression, bipolar disorders, major/minor depressive states related to immunotherapeutic treatments, pre- and post-partum depression, and cardiovascular disease-related depression. Indeed, this phenomenon is positively correlated with the intensity of depressive symptoms (Zunszain et al., 2011; Leonard and Myint, 2006; Miura et al., 2008).

Several attempts have been assayed to determine the role of tryptophan metabolites in brain abnormalities taking place in depression. Firstly, the Unpredictable Chronic Mild Stress model is an informative paradigm to study depression in animals. Laugeray et al. (2010) demonstrated that 3-HK levels are increased preferentially at the subcortical level (amygdala/striatum) in this model, whereas it is decreased at a cortical level (cingulate cortex). Interestingly, no KP changes were observed in hippocampus (Laugeray et al., 2010). Secondly, fractalkine receptor (CX(3)CR1)-deficient mice following lipopolysaccharide (LPS) challenge showed prolonged depressive-like behavior associated with exaggerated microglial activation and induction of the tryptophan degrading enzyme IDO. In these animals, the levels of 3-HK were increased in the brain 72 h after LPS injection. The depressive-like behavior evidenced in CX(3)CR1(−/−) mice 72 h after LPS injection was abrogated by inhibition of IDO, suggesting that IDO activation and subsequent generation of neuroactive kynurenine metabolites may exert a pivotal role in the development of depression (Corona et al., 2012). Thirdly, physiological stressors are consistently associated to depression; thus, a mild electrical shock administered to feet of laboratory animals can be used as a model. In these conditions, 3-HK is increased in plasma and several cerebral regions (cerebellum, medulla, hypothalamus, striatum, midbrain, hippocampus and cortex) (Pawlak et al., 2000). Finally, it is possible that antidepressants may change the KYNA:3-HK ratio. In primary astroglial cultures, fluoxetine, citalopram, amitriptyline and imipramine (1–10 μM) increased the *de novo* production of KYNA and diminished 3-HK synthesis. At 24 and 48 h the expression of KAT1 and KAT2 was enhanced, whereas KMO was diminished by all these drugs. These findings indicate that tricyclic antidepressants could correct the behavioral depression through the re-establishment of the beneficial ratio KYNA:3-HK (Kocki et al., 2012).

3.6.8. Multiple sclerosis

Experimental allergic encephalomyelitis in rats represents an autoimmune inflammatory disorder of the CNS characterized by demyelination, paralysis and histopathology similar to multiple sclerosis. 3-HK levels in the spinal cord of these animals increased 10-fold over control. IDO and KMO largely increased in the spinal cord, with not changes in the forebrain, but a decrease in the spleen (Chiarugi et al., 2001a,b).

4. Concluding remarks

Increased levels of 3-HK in a variety of neurological disorders would initially suggest an active role of this KP metabolite in the development and progression of degenerative events; however, we cannot discard that its enhanced levels could also correspond to a mere increase in metabolic activation of KP as an epiphenomenon related with inflammatory processes or other toxic mechanisms. Thus, despite a large body of evidence points out to a neurotoxic/pro-oxidative profile for this molecule, antioxidant properties are not discarded at all. Indeed, it is likely that the very nature of the effects evoked by 3-HK will depend on several circumstances and/or experimental conditions. While in some specific paradigms 3-HK acts as a pro-oxidant throughout direct or indirect reactions – when high doses or concentrations are tested –, under other conditions this metabolite has been shown to scavenge free radicals and prevent oxidative damage. This dual evidence leads us to consider that there are more than one possible scenario for the actions of this agent: (1) If the signals recruited by 3-HK are noxious enough to trigger toxic cascades, if its concentrations are in a supraphysiopathological rank, or if the redox conditions for the formation of toxic derivatives (i.e., toxic quinones or other KP metabolites) prevail in the environment, then cells and tissues will suffer from injury and degeneration via oxidative stress and inflammation; in contrast, if 3-HK exerts moderate oxidative stress, then cells will be able to sense and counteract the deleterious effects of ROS/RNS by activating antioxidant systems, and so, they will be able to initiate concerted antioxidant and anti-inflammatory responses, thereby leading to survival signals. In this point, and in consideration to the collected and discussed evidence, we propose the following hypothesis: objectively, as a transitory metabolite of the KP, 3-HK seems to be a molecule which, under physiological conditions represents a candidate to be claimed as a redox modulatory agent, and its actions in this regard – whether scavenging ROS or even inducing moderate oxidative stress by toxic derivatives – would be accounting for a sensed response of the brain tissue to counteract any further major attack, expressed as chronic or intense acute damage. In favor of this concept, basic experimental evidence for a toxic role of this molecule considers so far the use of extremely high concentrations of 3-HK, whereas clinical evidence on the increased levels of this metabolite in different pathologies could be reflecting secondary and late scenarios of the real causal components of these disorders. Evidently, this is only a speculative statement aimed at objectively direct the efforts of different groups interested in this topic for further experimental considerations. So, to be fair, we suggest a series of possible experiments to be carried out in order to discard or accept neurotoxic or

neuroprotective actions of 3-HK in the brain. To our concept, in an ideal experimental scenario, comparisons between *in vivo* an *in vitro* effects of 3-HK should be explored using different solvents, concentrations, and volumes of infusion to animals, as well as routes of administration. In addition to these controlled conditions, metabolically handling and modifying KP function to generate a more "natural" manner to induce 3-HK accumulation would represent a more reliable model to explore its actions. Either through pharmacological agents oriented to selectively block KATs and kynureninases, and the unequivocal determination of 3-HK levels, or by mean of selective mutations in cells and animals on these key enzymes, our knowledge on the role of 3-HK in the brain will be enhanced. Moreover, specific and more detailed determination of the roles exerted by the 3-HK derivatives (toxic quinones and the metabolites 3-HAA and QUIN) under different experimental conditions, require more attention. In the meantime, the dual actions of 3-HK in the CNS resemble the dual behavior of KP. A schematic representation of the mechanisms by which 3-HK may exert its effects in the brain is summarized in Fig. 4.

Further considerations must be taken in regard to the possible involvement of signaling pathways and their role in the integral pattern of toxicity evoked by this metabolite, in order to elucidate in a more detailed manner all those mechanisms involved in the effects of 3-HK. Of further consideration is the proven interaction of 3-HK with proteins and other biological molecules. Particularly, the cost of these interactions is high as it comprises protein cross-linking, as well as depletion of thiol pools, mostly GSH. Furthermore, a possible role of 3-HK as a neurotransmitter-like molecule, as it has been suggested for XA, should be explored in a near future. One question emerges at this specific point: is 3-HK directly responsible for damage in the CNS, or are its derivatives, such as *o*-aminoquinone, the inducers of cell demise? Trying to answer this question through the reviewed evidence is difficult. One clue could be related with the valuable report of Guidetti and Schwarcz (1999) in which 3-HK was tested under *in vivo* conditions. Once again, when administered alone, this metabolite was unable to extend the lesion area of the striatum. Because authors declared that 3-HK did not yield QUIN synthesis, then the metabolite itself is assumed to be unable of inducing toxicity. The question now is whether oxidative damage, as mild as it can be, could recruit survival responses. In this regard, it is also known that some antioxidants and electrophilic compounds act through the induction of integrative antioxidant responses, comprising the activation of signaling pathways, such as the Nrf2/Keap1/Are axis, a master modulator of antioxidant responses (Lee and Johnson, 2004). Either as a Michael acceptor or as an electrophilic compound, 3-HK could recruit this resource. In addition, since one of the most important components of neurodegenerative events involves

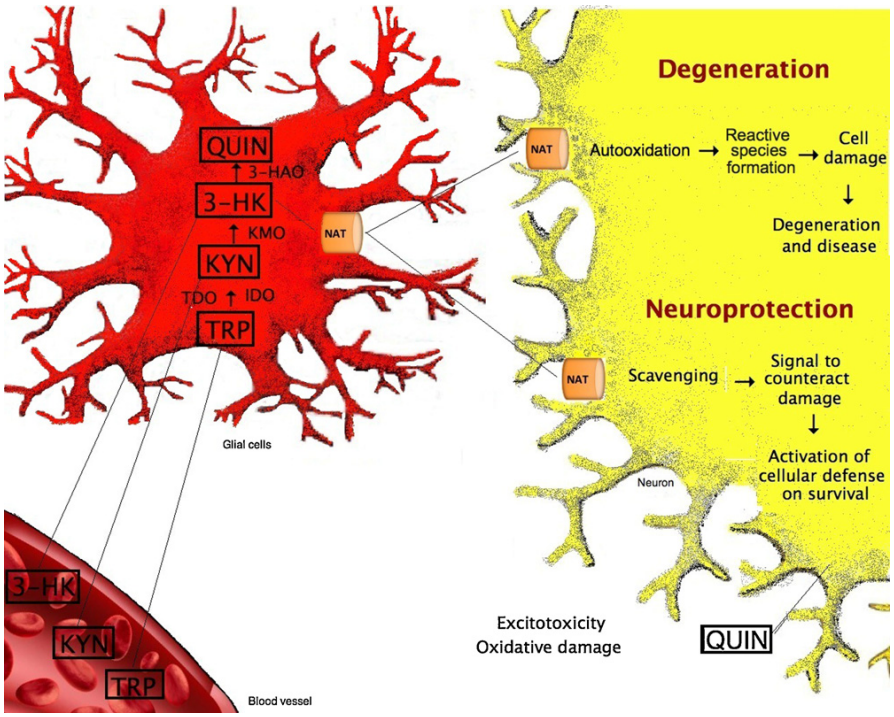

Fig. 4. Schematic representation of the mechanisms underlying 3-HK formation and its effects in the brain. Tryptophan (TRP), kynurenine (KYN), and 3-HK can directly cross the blood-brain barrier to reach glial cells, where they will be inserted into KP. Under physiological conditions, KP is activated to form the metabolic precursor NAD^+; however, under inflammatory, oxidative or neurotoxic attack, there is an increased activation of indoleamine dioxygenase (IDO), tryptophan dioxygenase (TDO) and kynurenine monooxygenase (KMO), yielding a preferential formation of putative toxic metabolites, including 3-HK and quinolinic acid (QUIN). Whether 3-HK is then toxic, is the topic discussed in this review. Both metabolites can be released to the extracellular cleft to reach near targets. Particularly, 3-HK can cross neuronal cell membranes through the Neutral Amino Acid Transporter (NAT), and once inside, it can follow two routes: (1) if this molecule displays scavenging/antioxidant properties, then it would be able to counteract the deleterious actions of preformed ROS/RNS either through a direct scavenging effect or by means of triggering integral antioxidant responses evoked by transcription signaling pathways, such as the Nrf2/Keap1/ARE axis, a master modulator of antioxidant responses in the brain. (2) If 3-HK undergoes autooxidation, then it would yield the formation of highly reactive molecules, such as *o*-aminoquinone, which in turn could be the real responsible for exacerbated oxidative damage, activation of deadly cascades, and massive cell death. An alternative explanation for the toxic pattern exerted by this molecule comprises the formation of other toxic metabolites from 3-HK metabolism. Being a direct precursor of 3-hydroxyanthranilic acid, 3-HK might eventually account for QUIN formation. The latter is responsible not only for excitotoxicity, but also for oxidative stress. Therefore, in a scenario where both 3-HK and QUIN could be present, neurodegeneration is expected to be potentiated. For further consideration in this figure, Guillemin et al. (2007) have demonstrate that primary cultured neurons and SK-N-SH cells can directly produce enhanced levels of 3-HK and other KP metabolites in response to pro-inflammatory stimuli.

inflammatory processes, the precise role of 3-HK in the induction/modulation of the NFκB pro-inflammatory signaling needs detailed characterization. This is particularly relevant as a cross-talk between these two pathways cannot be ruled out in physiological and physiopathological events in the brain. These key considerations shall be explored in a near future in order to provide 3-HK with its real position in the KP modulatory actions on the CNS: a redox sensitive modulator or a toxic intermediate metabolite.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

Ana Laura Colín-González is a CONACyT scholarship holder (239954). She belongs to the Doctoral Program in Biomedical Sciences from the Universidad Nacional Autónoma de México.

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