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Lista de abreviaturas

Ado-Met	S-adenosil-L-metionina
Akt	Proteína cinasa B
APAP	Acetaminofén (N-acetil-p-amino-fenol)
ARE	Elementos de respuesta antioxidante
ASK1	Cinasa que regula la señal de apoptosis
As3MT	Arsénico (III) metiltransferasa
ATF-2	Factor de transcripción activador 2
BHE	Barrera hematoencefálica
BDNF	Factor neurotrófico derivado de cerebro
BSO	L-buthionin-*S-R*-sulfoximina
cAMP	AMP cíclico
CCl_4	Tetracloruro de carbono
CEC	Células endoteliales de cerebro
cGMP	GMP cíclico
CREB	Factor de transcripción dependiente de cAMP
DAG	Diacilglicerol
ERK2	MAPK cinasa regulada extracelularmente 2
ERK5	MAPK cinasa regulada extracelularmente 5
ERO	Especies reactivas de oxígeno
GCL	γ-glutamil-cisteín ligasa
GCLC	GCL subunidad catalítica
GCLM	GCL subunidad modulatorio
Grx	Glutarredoxina
GS	GSH sintetasa
GSH	Glutatión reducido
GSSG	Glutatión oxidado
GSSG-Rd	Glutatión reductasa
G6PDH	Glucosa 6 fosfato deshidrogenasa
HEK 293	Línea celular de riñón de embrión humano 293

iAs	Arsénico inorgánico
IP$_3$	Inositol 1,4,5-triofosfato
JNK	Cinasa de c-jun N-terminal
MAPK	Proteína cinasas activadas por mitógenos
mRNA	RNA mensajero
mTORC2	Blanco mecanístico de rapamicina en células de mamífero
NAC	N-acetil-cisteína
NAPQI	N-acetil-p-enzoquinona imina
NF-κB	Factor nuclear Rel - κB
NGF	Factor de crecimiento nervioso
NIH-3T3	Línea celular de fibroblastos de embrión de ratón 3T3
Nrf2	Factor nuclear 2 (asociado al factor eritroide 2)
NT	Neurotrofina
NT-3	Neurotrofina 3
NT-4/5	Neurotrofina 4/5
PC12	Línea celular de feocromocitoma de ratón 12
PDK1	Cinasa dependediente de 3-fosfoinositol 1
PI3K	Fosfoinositol 3-cinasa
PLC-γ	Fosfolipasa C gamma
p75^{NTR}	Receptor de neurotrofinas de baja afinidad p75
TNF	Factor de necrosis tumoral
Tx	Tiorredoxina
TR	Tiorredoxin reductasas
TrkA	Receptor tipo tirosina cinasa de alta afinidad A
RTK	Receptores tirosina cinasa
SNC	Sistema nervioso central

Abstract

Nerve growth factor (NGF) is one of several structurally related proteins, named neurotrophins (NTs), that regulate neuronal survival, development, function, and plasticity. Moreover, NGF is an important activator of antioxidant mechanisms. These NGF functions are mediated by tropomyosin-related kinase receptor A (TrkA). Although NTs and their receptors are expressed in visceral tissues, the extent to which NTs are involved in their physiology is less clear. NGF is one of the most expressed NTs in adult mouse livers. Although NGF is an important modulator of antioxidant mechanisms in neural tissues, few studies describe the relationship between oxidative stress and NGF expression in the liver. In the present study, we demonstrate that *ngfb* mRNA is positively modulated in mouse liver after oxidative injury induced by the intraperitoneal injection of 14 mg/kg of sodium arsenite, 6 mmol/kg of L-buthionine-S-R-sulfoximine or 300 mg/kg of acetaminophen. In addition to the up regulation of *ngfb*, we observed the phosphorylation of the NGF high-affinity receptor TrkA in the liver as well as downstream phosphorylation of Akt, NF-kB nuclear migration and *Iκbα* and *Tx-1* mRNA up regulation. These effects were abolished when a neutralizing anti-NGF antibody was used. Furthermore, the anti-NGF antibody alone induced oxidative stress in the liver observed as a decrease of reduced glutathione, increase oxidized glutathione and down regulation of *Tx-1* mRNA. Thus, NGF plays a critical role in liver protection against oxidative stress and xenobiotic injury as well as maintaining a reduced thiol state.

Resumen

El factor de crecimiento nervioso (NGF) forma parte de una familia de proteínas estructuralmente relacionadas y denominadas neurotrofinas (NT), que están asociadas a la regulación del desarrollo, migración, función, plasticidad, sobrevivencia y muerte neuronal. Además, se ha reportado que NGF es capaz de modular mecanismos de respuesta antioxidante. Estas funciones son mediadas por la unión a su receptor tipo tirosina cinasa de alta afinidad A (TrkA). A pesar de que se ha reportado que las NT's y sus receptores se expresan en tejidos viscerales, no se sabe con certeza el papel que juegan en la fisiología de estos tejidos. En este sentido, NGF es la NT con mayor expresión en el hígado de ratones adultos. A pesar de que NGF es un importante inductor de respuestas antioxidante en tejidos nerviosos, pocos estudios describen la relación entre estrés oxidante y la expresión de NGF en el hígado. En el presente estudio se demuestra en un modelo murino, que el tratamiento via intraperitoneal con agentes químicos que inducen estrés oxidante sistémico como lo son 14 mg/kg de arsenito de sodio, 6 mmol/kg de L-butionina-S-R-sulfoximina o 300 mg/kg acetaminofén, son capaces de inducir la transcripción de *ngfb* en hígado. Aunado a ello, se observa la activación de su receptor TrkA así como cascada abajo la migración nuclear de la proteína cinasa Akt y del factor de transcripción NF-kB, junto como un incremento en los niveles de mRNA de *Iκbα* y *Tx-1*. Dichos efectos fueron eliminados cuando se empleó un pre-tratamiento con el anticuerpo neutralizante anti-NGF. Aún más, el tratamiento con el anticuerpo anti-NGF por sí solo disminuye los niveles de glutatión reducido, incrementa los niveles de glutatión oxidado y disminuye la transcripción de *Tx-1*. Los resultados muestran evidencia que sugiere que NGF juega un papel crítico en

la regulación del estado redox en hígado, tanto de manera basal como ante un estrés oxidante y el daño por xenobióticos.

Introducción

Estrés Oxidante y respuesta antioxidante

Las especies reactivas de oxígeno (ERO) son consecuencia del metabolismo aeróbico. Aún en estado de reposo, cerca del 2 % del oxígeno consumido es convertido en ERO (Bridges et al., 2012). Algunos de estas ERO activan señales intracelulares involucradas en la protección contra el estrés oxidante, como la síntesis de glutatión (GSH), tiorredoxina (Tx) y enzimas antioxidantes (Limon-Pacheco et al., 2007). En un organismo, los tejidos y órganos, existen diferencias en sus tasas metabólicas, lo cual se observa en los niveles de flujo sanguíneo, consumo de oxígeno, y reservorios específicos de enzimas y proteínas antioxidantes (Griffith & Meister, 1979a, Limon-Pacheco & Gonsebatt, 2009), de tal manera que algunos órganos, como el cerebro, son más susceptibles al daño por estrés oxidante.

El GSH, γ-glutamil-cisteinilglicina, es el tiol intracelular más abundante, cuyas principales funciones son: servir como antioxidante, participar en el metabolismo de xenobióticos, ser un reservorio y transportador de cisteínas, modular la proliferación celular, ayudar a mantener el estado redox celular y participar en la síntesis de ribonucleótidos (Meister & Anderson, 1983, Dringen, 2000). La oxidación del grupo sulfhidrilo (-SH), presente en el residuo de cisteína del glutatión, le permite existir en forma reducida (GSH) u oxidada, como un complejo disulfuro (GSSG) (DeLeve & Kaplowitz, 1991), el cual se reduce a GSH por la flavoenzima NAPDH dependiente, glutatión reductasa (GSSG-Rd). En estado basal existe una relación entre GSH y GSSG de 10:1 (Meister, 1988b), modificándose de acuerdo al consumo de GSH o bien por el incremento de GSSG. El cociente GSSG/2GSH proporciona un buen estimado del potencial reductor

(ΔE), que es una medida de la energía potencial para la reducción u oxidación: ΔE (GSSG + 2H$^+$ → 2GSH) = ΔE^0-RT/nF ln([GSH]^2/[GSSG]) . Cuando más negativo es este potencial mayor es la capacidad reductora del GSH y menor la capacidad oxidante del GSSG (Dalton et al., 2004). Cambios en este potencial se asocian a proliferación celular (-260 a -230 mV) en células diferenciadas o que no están proliferando a menores potenciales (-230 a -190 mV), mientras que mecanismos de muerte celular como necrosis y/o apoptosis se observan a potenciales celulares entre los -170 a -150 mV (Schafer & Buettner, 2001).

La sensibilidad de cada órgano para responder a estímulos oxidantes depende de sus niveles de GSH, estos niveles son específicos del órgano, tejido y/o tipo celular (Griffith & Meister, 1979a) y no son estáticos, sino que muestran variaciones que incluyen desde cambios por ritmos circadianos (Neuschwander-Tetri & Rozin, 1996), cambios por la fase del ciclo celular en el que se encuentren las células que componen el tejido (Schafer & Buettner, 2001), así como la capacidad de inducir su síntesis en momentos de alta demanda, lo cual nos habla de mecanismos altamente especializados que permitan una regulación fina y plástica de su concentración.

El hígado es el órgano con la mayor concentración de GSH, alcanzando valores de hasta 10 mM (Meister & Anderson, 1983), y es producido a partir de sus precursores al igual que en prácticamente todas las células (DeLeve & Kaplowitz, 1991). La síntesis, a partir de los aminoácidos L-glu, L-cys y L-gly, se lleva a cabo en el citoplasma de todas las células animales e implica dos pasos enzimáticos dependientes de ATP (Shih et al., 2006, Meister & Anderson, 1983). El primer paso es el limitante por la disponibilidad reducida de L-cys, y es catalizado por la γ-glutamil-cisteína ligasa (GCL), que media la unión entre L-

glu y L-cys para formar el dipéptido γ-glutamyl-cysteina, el cual a su vez es unido con L-gly mediante la GSH sintetasa (GS) para producir GSH (Meister & Anderson, 1983).

La GCL es un heterodímero dependiente de Mg^{2+} o Mn^{2+}, compuesto por una subunidad catalítica (GCLC, 73 kDa) y otra moduladora ligera (GCLM, 31 kDa), que se encuentran unidas por un enlace disulfuro, que puede ser modulado por las concentraciones de GSH, generando una regulación no alostérica (Meister & Anderson, 1983, Wu et al., 2004). Por otra parte, se ha observado que la concentración de la enzima correlaciona bastante bien con la concentración de su RNA mensajero (mRNA), ya sea por el aumento en su transcripción o bien por el cambio en su estabilidad (Wild & Mulcahy, 2000). Su actividad o transcripción es inducida por una gran variedad de factores, dentro de los que se encuentran: el estrés oxidante, las citocinas, la radiación ionizante, el estrés calórico, la inhibición de la GCL, el consumo de GSH o su conjugación, la prostaglandina A2, la presencia de metales pesados, algunos antioxidantes y la insulina (Lu, 2000, Townsend et al., 2003).

Debido a las múltiples funciones que cumple el GSH en los procesos celulares, como proteger contra el estrés oxidante, inactivar compuestos tóxicos electrofílicos, así como también participar en la proliferación, la diferenciación y la apoptosis celular (Dringen, 2000, Zhang & Forman, 2012), la alteración de su homeostasis, por una disminución en su síntesis o reciclamiento o un incremento en su consumo, está asociada a cambios en el estado redox que pueden devenir en enfermedades. Algunos ejemplos de condiciones patológicas asociadas a la deficiencia de GSH son la anemia hemolítica, anormalidades neurológicas, como el autismo y enfermedades del hígado (Ballatori et al., 2009). Además, en pacientes con enfermedades asociadas al envejecimiento, como las de

Parkinson y de Huntington se han reportado bajas concentraciones de GSH (Ballatori et al., 2009).

Otro sistema antioxidante es el sistema Tiorredoxina (Tx). Estas sulfoproteínas catalizan reacciones mediadas por NADPH e incluye a las Tx's (Tx1, citosólica y nuclear, y Tx2 mitocondrial) y a las tiorredoxina reductasas (TR1 y TR2) (Jurado et al., 2003). Las Tx son proteínas de bajo peso molecular (12 kDa) que se encuentran en todos los organismos vivos (Stefankova et al., 2005). Se caracterizan por poseer el motivo CysXXCys en su sitio activo (Holmgren, 1989). Este sistema antioxidante juega un papel relevante en la síntesis y reparación del DNA (Powis & Montfort, 2001), ya que regula factores de transcripción como al factor nuclear Rel - κB (NF-κB) (Hanschmann et al., 2013), además de modular proteína cinasas activadas por mitógenos (MAPK), como a la cinasa que regula la señal de apoptosis (ASK1) (Lu & Holmgren, 2012).

Aunque el GSH se encuentra en concentraciones de mmoles y Tx en μmolares, aunado a que tienen moléculas y reacciones blanco que comparten, existe evidencia que sugiere que sus estados redox son controlados de manera independiente (Trotter & Grant, 2003) y como sistemas antioxidantes dependientes de L-cys, son parte de la primera línea de defensa ante cambios en el estado redox (Go & Jones, 2013); además de jugar un papel importante en el metabolismo de xenobióticos y de fármacos (Hanschmann et al., 2013).

Fármacos y xenobióticos que consumen GSH

El acetaminofén (N-acetil-p-amino-fenol; APAP) es un fármaco analgésico y antipirético cuya sobredosis es una de las principales causas de hepatotoxicidad por estrés oxidante (Lee, 2004). El APAP es biotransformado por los citocromos P450 CYP1A2, 2E1 y 3A4 en su metabolito reactivo N-acetil-p-enzoquinona imina (NAPQI), el cual en dosis

elevadas es capaz de disminuir los niveles de GSH intracelular (Martin-Murphy et al., 2010). El estrés oxidante generado por dicha intoxicación está asociado con alteraciones en la homeostasis del calcio así como a daño en mitocondria que impide la síntesis de ATP, lo que da lugar a procesos necróticos (Hinson et al., 2010). La sobredosis de APAP también está asociada a toxicidad renal, aún en ausencia de daño hepático (Stern et al., 2005), lo cual puede conllevar a un daño en múltiples órganos (Fontana, 2008).

En un modelo murino el consumo de GSH debido a la sobre dosis de APAP se ha asociado con la activación del factor nuclear 2 (asociado al factor eritroide 2) (Nrf2) así como genes que inducen enzimas antioxidantes dependientes de este factor de transcripción, como la epóxido hidrolasa microsomal, la hemo-oxigenasa 1 y la *GCLc* (Goldring et al., 2004). Además, se ha observado que parte del daño inducido por el APAP en dicho órgano es mediado por la oxidación de *Tx-1* (Nakagawa et al., 2008). En este sentido, como parte de esta tesis investigamos el efecto del APAP en un modelo murino y observamos que 300 mg/kg de APAP vía intraperitoneal disminuye los niveles de GSH en hígado y riñón (Resultados I, Figura 1), asociado con un incremento en la transcripción de *Nrf2* y glutarredoxina-1 (*Grx-1*) en hígado (Resultados I, Figura 3), mientras que en cerebro la inducción del mRNA de *Tx-1* y *Grx-1* se asocia con la translocación nuclear del factor de transcripción NF-κB (Resultados I, Figura 4). Finalmente, en riñón no se observó inducción en la transcripción de *Grx-1* ni *Tx-1* lo cual concuerda con un incremento en la lipoperoxidación (Resultados I, Figura 2 y 5).

Por otra parte, el arsénico (iAs) es un metaloide ampliamente distribuido en la corteza terrestre. Es un carcinógeno, teratógeno y co-mutágeno (IARC, 1987, Albert, 1997) cuya principal ruta de exposición humana es a través del consumo de agua contaminada con dicho metaloide ya sea de manera natural o antrópica (Tapio & Grosche, 2006). El principal

órgano que lleva a cabo la biotransformación del iAs es el hígado, aunque no es el único con la capacidad para hacerlo (Hughes & Kitchin, 2006, Rodriguez et al., 2005). Su biotransformación se lleva a cabo por la enzima arsénico (III) metiltransferasa (As3MT), la cual emplea como sustrato la forma trivalente del arsénico (As^{3+}) y como donador de grupos metilo a la S-adenosil-L-metionina (Ado-Met) (Challenger et al., 1933). Este proceso se conoce como metilación oxidativa, porque en el proceso se oxida el arsénico trivalente a pentavalente y da como productos secuenciales especies mono-, di- y trimetiladas de arsénico (Thomas et al., 2001). Las formas trivalentes de estos compuestos metilados presentan una mayor toxicidad que las especies inorgánicas, por lo que la metilación de arsénico es considerada como un proceso de bioactivación (Thomas et al., 2001).

Existen varios modelos que explican la biometilación del iAs. Estos involucran al GSH o el sistema Tx como agentes reductores de las formas pentavalentes (Thomas, 2009, Hayakawa et al., 2005). Se encuentra ampliamente documentado la generación de estrés oxidante por la exposición a iAs, el cual está implicado en la etiología de su toxicidad (Hughes & Kitchin, 2006, Hei & Filipic, 2004, Kessel et al., 2002). En consecuencia, se ha reportado que la exposición a este metaloide activa factores de transcripción relacionados con la inducción de enzimas antioxidantes como Nrf2 y NF-κB (Pi et al., 2003, Hu et al., 2002).

Finalmente, en estudios farmacológicos se emplea al L-buthionin-*S*-*R*-sulfoximina (BSO) como un análogo del γ-glutamil fosfato por lo que se une al sitio catalítico de la GCLc, inhibiéndola de manera irreversible, impidiendo así la síntesis de GSH (Griffith, 1982, Meister, 1995, Limon-Pacheco et al., 2007).

Como se mencionó anteriormente, los tejidos y órganos en un mamífero presentan reservorios específicos de enzimas y proteínas antioxidantes (Griffith & Meister, 1979a, Limon-Pacheco & Gonsebatt, 2009), lo que les confiere una capacidad órgano-específica ante estímulos oxidantes. Sin embargo, estos órganos y tejidos no se encuentran funcionando de manera aislada, de tal manera que los efectos de cada órgano pueden a su vez repercutir en los otros o bien mediante mecanismos de comunicación inter-órgano, lo que da paso a procesos de protección (Plant, 2004, Droujinine & Perrimon, 2013).

En ese sentido, se tiene evidencia en un modelo murino de una respuesta órgano-específica y de un probable evento de comunicación entre tejidos periféricos y el sistema nervioso central (SNC). La inhibición en la síntesis de GSH por BSO, vía intraperitoneal, disminuyó el contenido de GSH en hígado y en riñón. Ante este estímulo en hígado se incrementó la fosforilación de la MAPK p38, la traslocación nuclear del factor de transcripción activador 2 (ATF-2) y de NF-κB, así como la fosforilación de c-Jun y el incremento de la transcripción de *Tx-1*. En riñón se incrementó la fosforilación de la MAPK p38 y la transcripción de *Tx-1* pero solo se observó la fosforilación de c-Jun. Sin embargo, en cerebro se observó un incremento en la síntesis de GSH asociado con un aumento en la transcripción de *Nrf2*, *GCLc* y la subunidad responsable de la incorporación de cistina, *xCT,* todo ello mediado, al menos en parte, por la MAPK cinasa regulada extracelularmente 2 (ERK2) y el factor de transcripción Nrf2 (Limon-Pacheco et al., 2007).

Hasta ahora poco se sabe acerca de la manera a través de la cual se percibe el estado de estrés oxidante en plasma así como el ó los mecanismos por medio de los cuales se induce esta respuesta en el SNC.

Vías de señalización

Si bien el mantenimiento de la homeostasis redox intracelular es esencial para las funciones y sobrevivencia celular, se sabe que cambios en el estado redox intra y extra celular son capaces de inducir alteraciones en el transcriptoma, lo cual indica la presencia de mecanismos que permiten sensar el estado redox y responder de manera específica y coordinada, desde el nivel celular hasta entre órganos y tejidos (Plant, 2004, Droujinine & Perrimon, 2013, Winterbourn & Hampton, 2008). Los mecanismos por medio de los cuales las células se comunican entre sí son químicos, eléctricos o electroquímicos. Los mecanismos que implican señales químicas son capaces de difundir por el torrente sanguíneo hasta encontrar sus células blanco. El proceso por medio del cual las señales extracelulares son convertidas en intracelulares es denominado transducción de señales.

Las cascadas de señalización son un circuito molecular que detecta, amplifica e integra diversas señales externas para generar respuestas que pueden incluir cambios en la expresión génica así como en la actividad enzimática o de canales iónicos (Berg et al., 2002). Así, una señal del medio ambiente, como una hormona, se recibe por la interacción de un componente celular, un receptor de membrana por ejemplo, la cual lo transduce a otra forma química de señal que es amplificada antes de inducir una respuesta, siendo en cada paso sujeta a regulación por retroalimentación (Berg et al., 2002).

Para los casos en que la molécula señaladora (ligando) es no polar, es posible que difunda a través de la membrana, se una a proteínas e interactué directamente con el DNA modulando la transcripción de genes, como es el caso de las hormonas esteroideas. Sin embargo, para la mayoría de las moléculas señalizadoras, denominadas mensajeros primarios, es necesaria la presencia de proteínas receptoras asociadas a la membrana celular (Berg et al., 2002).

Existen cuatro tipo de receptores de membrana que interactúan con ligandos específicos: 1) los receptores acoplados a proteínas G; 2) los receptores acoplados a canales iónicos; 3) Los receptores unidos a tirosina cinasas y; 4) Los receptores con actividad enzimática intrínseca (Lodish, 2000).

La unión del ligando con su receptor puede conllevar a cambios en las concentraciones de moléculas denominadas segundos mensajeros, como lo son el AMP y el GMP cíclico (cAMP y cGMP respectivamente), calcio, inositol 1,4,5-triofosfato (IP$_3$) y diacilglicerol (DAG). Estos segundos mensajeros amplifican la señal pues se generan en altas concentraciones y son capaces de difundir lejos de su lugar de origen (Alberts et al., 2008).

Estos segundos mensajeros, o bien los receptores mismos en el caso de los receptores con actividad enzimática intrínseca, activan proteínas denominadas proteínas cinasas, las cuales son capaces transducir la señal a cambios en la estructura de otras proteínas al transferir un grupo fosfato del ATP a residuos de L-ser, L-thr y/o L-tyr en otras proteínas (Alberts et al., 2008). La actividad de estas proteínas cinasas es contrarrestada por fosfatasas, las cuales remueven el grupo fosfato (Lodish, 2000).

Uno de los grupos más importantes de este tipo de proteínas cinasas son las MAPK, las cuales se pueden activar por estímulos internos, como los factores de crecimiento, o hasta por estímulos ambientales como la radiación, la exposición a xenobioticos, etc. En los mamíferos existen 4 tipos principales de MAPK: 1) las cinasas reguladas por estímulos extracelulares (ERKs); 2) las cinasas de c-jun N-terminal (JNK); 3) las p38^{MAPK} y; 4) las cinasas reguladas por estímulos extracelulares 5 (ERK 5) (Seger, 2004).

Otra proteína cinasa es la proteína cinasa B (Akt) que es una proteína cinasa específica de serina/treonina la cual es activada por fosforilación en la L-thr 308 por la vía

fosfoinositol 3-cinasa (PI3K)/ la cinasa dependediente de 3-fosfoinositol 1 (PDK1) y en la L-ser 473 por el complejo 2 del blanco mecanístico de rapamicina en células de mamífero (mTORC2) (Sarbassov et al., 2005). Cabe señalar que su fosforilación puede ser regulada por la fosfatasa supresora de tumores PTEN que desfosforila al PIP_3 (Lee et al., 2002).

Así mediante estas y otras proteína cinasas se transducen señales de diversos estímulos, desde la proliferación celular, mitosis, diferenciación, respuestas al estrés, sobrevivencia celular y la apoptosis.

Estás cascadas de señalización pueden emplear como segundos mensajeros ERO o bien ser moduladas por estas, tal que son sensibles a cambios en el estado redox. Así, Tx se encuentra constitutivamente unida a la MAPKKK ASK1 inhibiendo su actividad cinasa, separándose de ésta cuando Tx se oxida en las L-cys 32 y 35 ante un incremento en las ERO, lo cual conlleva a la activación de ASK1 que puede inducir procesos de muerte celular vía las MAPK, JNK y p38 (Tobiume et al., 2001) así como procesos de diferenciación (Choi et al., 2011) o respuesta inmune (Matsuzawa et al., 2005). Por otra parte, las ERO son capaces de inhibir las fosfatasas que inactivan a Akt y a las MAPK, lo cual potencia el efecto de las cascadas de señalización iniciadas por receptores, citocinas y estresores (Ray et al., 2012).

Factores neurotróficos. El factor de crecimiento nervioso

Las neurotrofinas (NT) son una familia de proteínas homodiméricas estructural y funcionalmente relacionadas que comparten aproximadamente el 50% de identidad que incluyen al factor de crecimiento nervioso (NGF), al factor neurotrófico derivado de cerebro (BDNF), la neurotrofina-3 (NT-3) y la neurotrofina 4/5 (NT-4/5). (Lessmann et al., 2003).

El NGF regula el desarrollo neuronal, la diferenciación, la plasticidad, la actividad sináptica, la muerte y la sobrevivencia celular (Lu et al., 2005, Reichardt, 2006); además NGF está implicado en la respuesta antioxidante en el SNC (Satoh et al., 2013).

El NGF se sintetiza, al igual que todas las NT's, como una pre-pro-neurotrofina de 241 aminoácidos que consiste en un pre-prodominio, un pro-dominio y el dominio carboxilo terminal de la neurotrofina madura. La pre-pro-proteína contiene el péptido señal para su secreción. Cuando el extremo hidrofóbico del péptido señal es removido en el retículo endoplásmatico da lugar al precursor de la proteína (pro-proteína) el cual es glicosilado en el amino terminal y sulfatado en dicho oligosacárido. Posteriormente, se puede ser fragmentar el pro dominio por furinas y pro-convertasas intracelulares o por plasmina y metaloproteasas extracelularmente a su forma madura de 120 aminoácidos denominada NGFβ (Seidah et al., 1996, Wiesmann & de Vos, 2001, Lessmann et al., 2003, Ibanez, 2002). Debido a ello, es posible observar en plasma la forma que contiene el prodominio, denominado proNGF, el cual se ha observado presenta actividad biológica (Ibanez, 2002, Al-Shawi et al., 2007).

La forma biológicamente activa de las NT's maduras es de dos monómeros dispuestos en forma paralela para formar un homodímero unido por tres enlaces disulfuro generado por el motivo estructural nudo de cisteína (Wiesmann & de Vos, 2001). La actividad autócrina y parácrina del NGF depende de activar cascadas de señalización al unirse a dos tipos de receptores transmembranales: el receptor de neurotrofinas de baja afinidad p75 (p75^{NTR}) y el receptor tirosina cinasa de alta afinidad A (TrkA) (Lu et al., 2005).

El receptor p75^{NTR} es una glicoproteína que pertenece a la superfamilia de receptores del factor de necrosis tumoral (TNF), el cual es capaz de unirse de manera

similar con todas las neurotrofinas a concentraciones nanomolares (Sofroniew et al., 2001). El dominio extracelular de p75^{NTR} presenta cuatro dominios ricos en cisteína dispuestos en tándem necesario para la unión de las NT's (Nykjaer et al., 2005). Cabe señalar que la unión de las NT's con p75^{NTR} forma un complejo asimétrico de un homodímero de NT con un solo receptor p75^{NTR} (He & Garcia, 2004), lo cual, aunado con la capacidad de este receptor de interactuar con los dominios citoplasmáticos y transmembranales de los receptores Trk's, permite al receptor p75^{NTR} fungir como un correceptor que modula la afinidad y especificidad de los Trk's a las NT's (Huang & Reichardt, 2003), así como de alterar la cascada de señalización mediada por cada receptor por separado (Kaplan & Miller, 1997, Yoon et al., 1998). La activación del receptor p75^{NTR}, de manera similar a todos los receptores de la superfamilia TNF, está asociada con el inicio de procesos de muerte celular mediados por la MAPK JNK, ceramida y NF-κB (Gentry et al., 2004). Además, p75^{NTR} puede ser sustrato de la α- y γ-secretasa y de la TNF-α convertasa separando el dominio extracelular de la proteína, lo cual evita la señalización mediada por ligando (Weskamp et al., 2004). Sin embargo, el fragmento carboxilo terminal intracelular aún es capaz de modular la actividad de los receptores Trk (Jung et al., 2003). Aún más, se ha reportado que el dominio intracelular puede translocarse al núcleo (Parkhurst et al., 2010) y en la línea celular de feocromocitoma de ratón (PC12) el dominio intracelular de p75^{NTR} es el responsable de iniciar una respuesta antioxidante, sin necesidad de mediar la unión con alguna NT (Tyurina et al., 2005). También se ha reportado en estudios *in vivo* e *in vitro* que el proNGF se une preferentemente a este receptor induciendo procesos pro-apoptóticos (Beattie et al., 2002, Harrington et al., 2004).

El receptor TrkA es una proteína glicosilada de 140 kDa que pertenece a la familia de receptores tirosina cinasa (RTK) los cuales son proteínas transmembranales de un solo

paso que incluyen al receptor de BDNF y NT-4, denominado TrkB, y al receptor de la NT-3, TrkC (Wiesmann & de Vos, 2001). Los receptores RTK presentan tres regiones estructurales: el dominio extracelular, que se caracteriza por dos clusters ricos en cisteínas, uno de los cuales es seguido por tres repeticiones ricas en leucina y dos dominios parecidos a inmunoglobulinas; la región transmembranal y la región citoplasmática (Bartkowska et al., 2010). El receptor TrkA presenta dos isoformas biológicamente activas, TrkA-I y TrkA-II que presenta una inserción de 6 aminoácidos entre el segundo dominio parecido a inmunoglobulina y la región transmembranal del dominio extracelular (Barker et al., 1993). Por estudios de hibridación *in situ* TrkA-I se ha encontrado que se expresa en tejidos periféricos mientras que TrkA-II en SNC (Barker et al., 1993).

La activación de TrkA se da una vez que se ha unido el homodímero de NT al segundo dominio parecido a inmunoglobulina del TrkA, induciendo la homodímerización del receptor, que activa al dominio catalítico tirosina cinasa de la región citoplasmática mediante autofosforilación con lo que se inicia la cascada de señalización intracelular (Figura 1) (Wiesmann & de Vos, 2001, Huang & Reichardt, 2003). Estos sitios de autofosforilación sirven como sitios de unión para proteínas adaptadoras o señaladoras como la proteína adaptadora que contiene el dominio SHC 2 (Shc) y la fosfolipasa C gamma (PLC-γ) (Sofroniew et al., 2001). Así, la autofosforilación de la tirosina 490 del receptor TrkA permite la unión de Shc, que a su vez se une al complejo de proteínas adaptadoras Grb2-Sos, el cual activa a la proteína G monomérica Ras, iniciando la activación de la cascada de señalización MEK1/ERK1/2 (Sofroniew et al., 2001).
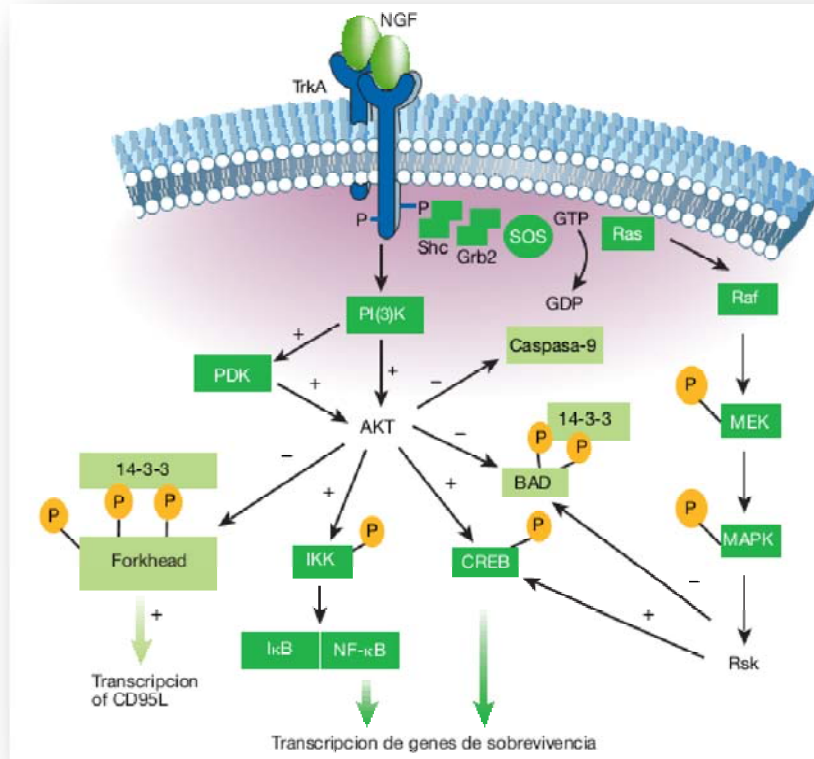
Figura 1. La vía inducida por la unión de NGF a TrkA. NGF induce la autofosforilación intracelular de TrkA que a su vez permite el acoplamiento de proteínas señalizadoras cómo PLC-γ, PI3K y la proteínas adaptadora Shc. La activación de PI3K induce la activación de Akt de manera directa o bien a través de PDK1. La fosforilación de CREB así como del inhibidor de NF-κB, IKK, estimulan la transcripción de factores de sobrevivencia, mientras que la fosforilación de las proteínas Bad, Forkhead y caspasa-9 inhiben la vía pro-apoptótica. De manera paralela, la interacción de las proteína adaptadora Shc con Grb2 y SOS pueden activar la vía Ras–Raf–MEK–ERK que a su vez activan a Rsk. Esto podría inducir respuestas mediante Bad y CREB que en sinergia con la vía de Akt induzcan respuesta de sobrevivencia. Traducido de (Yuan & Yankner, 2000).

Además, el factor de transcripción dependiente de cAMP (CREB) es activado por fosforilación en la serina 133 por la vía de Ras y la proteína serina treonina cinasa Rsk, vía que también se ha visto es posible de activar al factor de transcripción NF-κB y c-fos (Sofroniew et al., 2001). Aunado a ello, se ha observado que el receptor TrkA media la activación de la cascada de señalización de PI3K/PDK1/Akt que activa al factor de transcripción NF-κB y la inducción de proteínas antiapoptóticas como Bcl-2 y Bcl- X$_L$ (Wooten et al., 2000). Finalmente, se ha observado que en todos los casos la activación del receptor TrkA conlleva a la inducción de mecanismos de sobrevivencia, diferenciación celular y en el SNC y periférico a migración celular y el crecimiento de neuritas (Sofroniew et al., 2001).

NGF y respuesta antioxidante

Aunado a estas respuestas, de manera importante, se ha documentado que la cascada de señalización NGF/TrkA es capaz de inducir una respuesta antioxidante, tanto en estudios *in vivo* como *in vitro* (Satoh et al., 2013). Por ejemplo, en la línea celular PC12 y en cultivo primario de neuronas simpáticas se demostró que el tratamiento o pre condicionamiento con NGF es capaz de disminuir la muerte celular por H$_2$O$_2$ (Jackson et al., 1990, Kirkland et al., 2007). En la línea celular PC12, el tratamiento con NGF incrementa los niveles de GSH además de inducir la incorporación del aminoácido limitante para su síntesis, L-cisteína y/o L-cistina, promoviendo la sobrevivencia celular contra el estrés oxidante (Pan & Perez-Polo, 1996). En modelos similares, se demostró que el tratamiento con NGF induce la actividad de enzimas antioxidantes como la glutatión peroxidasa y la catalasa,

incrementa la actividad de enzimas relacionadas con el ciclo del GSH, como la GCL y la glucosa 6 fosfato deshidrogenasa (G6PDH), así como de los niveles de GSH reducido (Pan & Perez-Polo, 1993, Sampath et al., 1994). Además, se evaluó si algún otro factor trófico cómo BDNF o NT3 (20 ng/ml) o EGF, IGF o bFGF (40 ng/ml) es capaz de inducir un incremento en los niveles de GSH, encontrando que sólo el NGF y el EGF lo hacen, aunque fue mayor el incremento observado con NGF (Pan & Perez-Polo, 1993). Por otra parte, en modelos *in vivo* se ha demostrado que una inyección intracerebroventricular de NGF o la implantación de fibroblastos que sobre-expresan NGF tiene efectos neuroprotectores ante episodios de hipoxia-reoxigenación (Shigeno et al., 1991, Pechan et al., 1995). Aún más, modelos murinos que sobre-expresan NGF presentan un incremento en la actividad de enzimas antioxidantes como la superóxido dismutasa y la glutatión transferasa, un incremento de la proteína antiapoptótica Bcl-2, así como mayores concentraciones de GSH en cerebro total, plasma e hígado (Guegan et al., 1999, Arsenijevic et al., 2007).

Aunque el NGF fue inicialmente caracterizado como factor trófico específico para ciertos tipos neuronales del SNC y periférico, se ha observado que su expresión así como la de sus receptores no se limita a ellos sino que presentan una amplia distribución incluyendo tejidos periféricos como corazón, hígado, músculo, riñón, vejiga, pulmón, bazo, en células del sistema inmune, en tejidos endocrinos como ovario, testículo, glándula tiroidea, páncreas, glándula salivar etc., en donde se ha relacionado con eventos de sobrevivencia y respuesta antioxidante (Yamamoto et al., 1996, Sofroniew et al., 2001, Vega et al., 2003, Navarro-Tableros et al., 2004). De hecho, se ha reportado la inducción de NGF y/o su receptor en varios tipos celulares ante un estímulo o después de daño tisular. Por ejemplo, en un modelo murino de asma alérgica, la vía de señalización mediada por NGF/TrkA es

capaz de incrementar la viabilidad de células plasmáticas pulmonares mediante la inducción de NF-κB y Bcl-2 (Abram et al., 2009). Estudios *in vitro* mostraron que la vía NGF/TrkA induce un efecto pro-fibrogénico en fibroblastos de piel y pulmón indicando su rol en la reparación del tejido (Micera et al., 2001). En corazón se ha demostrado tanto en modelos *in vivo* como *in vitro* que los cardiomiocitos expresan NGF y sus receptores, y que se inducen ante episodios de hipoxia reoxigenación, previniendo la apoptosis por medio de la vía de señalización NGF/TrkA/PI3K/PDK1/Akt (Caporali et al., 2008, Meloni et al., 2010). En un modelo murino, la exposición a una mezcla de compuestos orgánicos volátiles conlleva a estrés oxidante en pulmón que incrementa los niveles de NGF en plasma (Wang et al., 2013). Además, se ha observado en modelos *in vitro* que la glucosa es capaz de inducir la expresión de TrkA en páncreas, además de que NGF juega un rol importante en la sobrevivencia de las células β en modelos de trasplantes *in vivo* (Raile et al., 2006, Navarro-Tableros et al., 2004, Miao et al., 2006).

Por otra parte, en un modelo murino se encontró que el hígado expresa las NT's NGF, BDNF y NT-3, aunque NGF es la única que incrementa su expresión en dicho órgano a lo largo de la vida, de hecho es la NT más abundante en él (Lommatzsch et al., 2005). Además se reportó que en el modelo murino de inducción de cirrosis hepática por tetracloruro de carbono (CCl_4), que al ser metabolizado por los citocromos P450 da como producto el radical triclorometil que es un compuesto altamente oxidante, se induce dramáticamente la expresión de NGF, asociado a la activación del receptor p75[NTR] y el inicio de procesos apoptóticos en células estelares, sin encontrarse expresión del receptor TrkA (Oakley et al., 2003). Sin embargo, estudios más recientes demostraron por inmunotinción en hígado humano la expresión del receptor en hepatocitos, las células

localizadas en los sinusoides hepáticos, y en estructuras localizadas en el tracto portal, incluyendo células epiteliales de los ductos biliares y en las células localizadas en la pared de la ramas de la arteria hepática en tejido hepático normal y fibrótico; además de demostrar *in vitro* la activación de la cascada de señalización mediada por NGF/TrkA (Bonacchi et al., 2008). De igual forma, se ha reportado que en un modelo murino de diabetes se encuentran inducidos NGF y su receptor TrkA en hígado un 36 y 17 % respectivamente, asociados a la proliferación celular en hepatocitos (Gezginci-Oktayoglu et al., 2011). De igual manera, en un modelo murino de hiperplasia en hígado por 0.1 mmol/kg de nitrato de plata, se observó un incremento en la expresión del mRNA de NGF y TrkA (Nemoto et al., 2000). Además, en pacientes con hepatocarcinoma se observa la expresión de NGF y TrkA por inmunohistoquímica en hepatocitos, células endoteliales y células de Kupffer, aunado a un incremento de NGF en plasma 25 veces mayor al nivel de los controles (con un rango de 73 - 520 pg/mL, comparado a la media de 20 pg/mL en controles sanos) (Rasi et al., 2007). Finalmente, como se mencionó anteriormente, en un modelo murino que sobreexpresa NGF, se observa en hígado un incremento en la concentración de GSH (Arsenijevic et al., 2007).

Planteamiento del problema

Estos datos sugieren que: a) la síntesis de NGF puede ser modulada ante cambios en el estado redox a nivel sistémico; b) la cascada de señalización mediada por NGF/TrkA probablemente participe en la activación de una respuesta antioxidante y/o de sobrevivencia

en el SNC y tejidos periféricos; c) Fallas en la cascada de señalización de NGF podrían estar asociadas a un incremento en la vulnerabilidad ante daño oxidante.

Hipótesis

Con base en lo anterior, hipotetizamos que los cambios en el estado redox sistémico serán capaces de inducir en hígado la síntesis de NGF y activar de manera autócrina la cascada de señalización NGF/TrkA iniciando así una respuesta antioxidante y/o de sobrevivencia.

Objetivos

Determinar si la exposición a agentes químicos capaces de generar un daño oxidante sistémico son capaces de modular la expresión de NGF en hígado de ratón.

Determinar la activación de la cascada de señalización NGF/TrkA en hígado de ratón y su relación con una respuesta antioxidante ante un daño oxidante sistémico

Determinar el papel de NGF en la regulación y mantenimiento del estado redox en hígado de ratón.

Resultados

Los resultados de esta tesis se presentan en tres partes:

Resultados 1.

Se observa que la exposición aguda a APAP ejerce un efecto órgano específico. Disminuye los niveles de GSH en hígado y riñón. Esto induce una respuesta antioxidante en hígado, observado cómo un incremento en la transcripción de *Nrf2 y Grx-1*. Por otra parte en cerebro no cambian los niveles de GSH pero se observa una inducción de los niveles transcripcionales de *Grx-1* y *Trx-1* asociada a la translocación nuclear del factor de transcripción NF-κB.

Resultados II.

Se presenta un artículo publicado que describe el rol que juega en hígado el NGF y su cascada de señalización mediada por TrkA/PI3K/PDK1/Akt/NF-κB ante estrés oxidante sistémico así como también el papel que tiene en el mantenimiento del estado redox basal.

Resultados III

Se presenta una revisión publicada que brinda una aproximación sobre la relevancia y modulación de los transportadores de cisteína/cistina en el sistema nervioso central, que permiten el incremento en el contenido de GSH.

Resultados I

Artículo enviado para su publicación

Acetaminophen induces the transcription of the antioxidant proteins thioredoxin 1 and glutaredoxin 1 in the brain and liver of balb/c mice

ACETAMINOPHEN INDUCES THE TRANSCRIPTION OF THE ANTIOXIDANT PROTEINS THIOREDOXIN 1 AND GLUTAREDOXIN 1 IN THE BRAIN AND LIVER OF BALB/c MICE

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Abstract

Tissues and organs express different levels of antioxidants, such as glutathione (GSH). Exposure to environmental toxins and/or pharmaceutical drugs induces differential tissue specific responses and/or damage. Acetaminophen (APAP) is an analgesic and an antipyretic drug that can cause severe liver damage when high doses are administered. The damage is caused by the depletion of intracellular GSH and an excess of the N-acetyl-p-benzoquinone imine (NAPQI) metabolite that reacts with cellular and mitochondrial proteins producing cell damage and tissue necrosis by mechanisms involving oxidative stress. In this study, we analyzed the response to an intraperitoneal (i.p.) injection (300 mg/kg) of APAP at 1, 2 and 3 h after administration in the livers, brains, and kidneys of BALB/c male mice. We observed a significant decrease in the levels of GSH in the livers and kidneys, and an increase in the transcription of *Nrf2* and *Grx-1* in the liver. In the brain, we found an increase in the transcription of *Grx-1* and *Trx-1* associated with NF-κB nuclear migration. In the kidney, the up-regulation of these antioxidant proteins was not observed, which coincides with the significant increase in lipid peroxidation.

Key words: acetaminophen, glutathione, thioredoxin, glutaredoxin, mice, liver, brain and kidneys response.

Abbreviations: APAP, acetaminophen; AP-1, activating protein 1; BSO, L-buthionine-S-R sulfoximine; GAPDH, glyceraldehyde phosphate dehydrogenase; γ-GCS, gamma-glutamylcysteine synthetase; GSH-Rd, glutathione reductase; GSH, reduced glutathione; Grx, glutaredoxins; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; NF-κB, nuclear factor kappa B; Nrf2, nuclear factor E2 p45-related factor

1. Introduction

Tissues and organs have different rates of metabolism, blood flow, and oxygen consumption, and they express different levels of antioxidant enzymes and proteins. In mammals, the liver is the organ with the highest metabolic capacity, and proportional to its weight, the central nervous system has the highest oxygen requirement. Exposure to environmental toxins and/or pharmaceutical drugs, then, induces differential tissue specific responses and/or damage (Limón Pacheco and Gonsebatt, 2009).

Glutathione (GSH, L-γ-glutamyl-L-cysteinyl glycine) is the most abundant antioxidant in the cells and mediates the metabolism of xenobiotics through conjugantion reactions catalyzed by glutatathione s-transferases. GSH levels are quite different among organs. The mammalian liver is considered to be the organ with the highest concentration of GSH (~7-10 mM), while the kidneys, spleen, small intestine, muscles and brain contain moderate amounts of the antioxidant. (~1-3 mM) (Dringen R, 2000). Moreover, GSH together with glutaredoxins (Grx), glutathione reductase (GSH-Rd) and NADPH constitute the GSH-Grx system which participates in catalysis of thiol disulfide oxidoreductions in proteins or small molecules (Lillig et al., 2008).

Another important antioxidant system is the thioredoxin (Trx) system. This group of thiol-specific proteins catalyzes reactions mediated by NADPH and includes thioredoxins (Trx-1, -2) and thioredoxin reductases (Trx-R1, -R2) (Jurado et al., 2003). In adult mice, the highest levels of *Trx-1* mRNA have been observed in the kidney, with intermediate levels present in the lungs and the lowest levels present in the brain, heart, and testis. Both GSH (through the modulation of γ-GCS) and *Trx-1* are modulated by the Nuclear related factor-erythroid 2 transcription factor (Nrf2) (Jurado et al., 2003; Limón-Pacheco and Gonsebatt, 2009).

Modulation of GSH content induces organ-specific responses in male mice by systemic administration of L-buthionine-S-R-sulfoximine (BSO), a specific inhibitor of the GSH synthesis. The activation of the transcription factor NF-κB and an increase in the transcription of *Trx-1* were observed in the liver. In the brain, an increase in the transcription of *Trx-1* was associated with Nrf2 activation, but in contrast, in kidneys, the up-regulation of *Trx-1* was not associated with these transcription factors (Limón-Pacheco et al., 2007).

Acetaminophen (APAP) is an analgesic and antipyretic drug and overdose is one of the most common causes of drug-induced hepatotoxicity in developed and undeveloped countries (Lee, 2004; Montoya, 1996). APAP is biotransformed by the cytochromes CYP1A2, 2E1, and 3A4 to its reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). NAPQI is eliminated by its conjugation with GSH, but it can also bind to proteins. APAP overdose depletes intracellular GSH. The resulting oxidative stress is associated with alterations in calcium homeostasis ande the loss of the ability of mitochondria to synthesize ATP leading to cell necrosis (Park et al., 2005; Hinson et al., 2010; Martin-Murphy et al., 2010). APAP overdose not only caused liver damage but also renal toxicity even in the absence of hepatic toxicity (Curry et al., 1982; Jones and Vale, 1993; Proudfoot and Wright, 1970; Stern et al., 2005; Zao et al., 2011) that might progress to multiorgan failure (Fontana, 2008).

In CD-1 mice, the depletion of GSH by APAP has been associated with the activation of Nrf2 and of the *Nrf2*-dependent genes, such as *mEH* (microsomal epoxide hydrolase), GCL_C (glutamate cysteine ligase catalytic subunit) and *HO-1* (heme oxygenase-1) (Goldring et al., 2004). Interestingly, when the authors of this study treated mice with BSO (L-buthionine-S-R-sulfoximine), a specific inhibitor of γ-GCS that also depletes liver GSH, the activation of Nrf2 was not observed (Goldring et al., 2004).

To analyze the organ-specific early responses to APAP, we treated mice with an intraperitoneal (i.p.) injection of APAP (300 mg/kg). The animals were sacrificed at 1, 2 and 3 h after the administration of the drug. Diminished levels of GSH were found in the livers and kidneys but not in the brains. The changes in GSH levels in the kidneys were associated with an increase in lipid peroxidation. In the livers, an up-regulation of the transcription of *Nrf2*, as well as of the *Grx*, was observed. In the brain, we found an increase in the transcription of *Grx-1* and *Trx-1* associated with NF-κB nuclear migration.

2. Materials and Methods

2.1 Chemicals

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

2.2 Animals and treatments

Male BALB/c mice 6 weeks of age were obtained from the animal care facility of the Instituto de Investigaciones Biomédicas at Universidad Nacional Autónoma de México. Animals were maintained in 12-h light/dark cycles. Mice were injected i.p. with APAP dissolved in saline solution at a dose of 300 mg/kg of body weight. Control mice received saline solution only. Animals were killed by cervical dislocation at 1, 2, and 3 h following the administration of APAP.

2.3 Determination of GSH

The content of reduced glutathione was determined by a fluorometric method (Senft et al., 2000). Briefly, a small portion of fresh tissue was washed in an ice cold isotonic saline solution to remove debris and blood. Each sample tissue was homogenized at 1:10 in buffer A (154 mM KCl, 5 mM diethylene-triamine-penta-acetate (DTPA), and 0.1 M phosphate buffer) and then in an equal volume of buffer B (40 mM HCl, 10 mM DTPA and 20 mM ascorbic acid), and 10% trichloroacetic acid was added. The samples were centrifuged at 14,000 x g for 20 min at 4 °C, and the supernatant was passed through a 0.45 µm filter. The final homogenate remained stable for four weeks at -70 °C. The samples were analyzed in duplicate. The tube labeled A and classified as background contained 5 µl of homogenate, 145 µl of a solution of 5% trichloroacetic acid in a redox quenching buffer (20 mM HCl, 5 mM DTPA and 10 mM ascorbic acid), 20 µl of 7.5 mM N-ethyl maleimide in a redox quenching buffer and 250 µl of a 1 M phosphate buffer. Tube B contained 5 µl of homogenate, 165 µl of 5% trichloroacetic acid in a redox quenching buffer and 150 µl of a 1 M phosphate buffer. Tubes A and B were incubated for 5 min at room temperature. The samples were then incubated for 30 min in the dark in 1 ml of a 0.1 M phosphate buffer and 150 µl of *O*-phthaldialdehyde (5 mg per ml of absolute methanol; J. T Baker, NJ, USA). Fluorescence readings were then taken at 365 nm of excitation and 430 nm of emission in a Labsen Fluorometer 450 (Labsen Scientific, ILL, USA). The final values were calculated as the fluorescence of unit A minus the fluorescence of unit B [$UF_A - UF_B = UF_F$]. The

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standard curves of GSH at 0.1 mM of concentration were used to calculate the GSH content.

2.4 Lipid peroxidation assay

Lipid peroxidation in the fresh tissue homogenates was determined based on the formation of thiobarbituric acid-reactive substances (TBARS), as described elsewhere (Buege and Aust 1978). Briefly, fresh tissue was homogenized in 30% (w/v) 0.1 M butylated hydroxytoluene (0.1M PB:methanol, 1:1) and centrifuged for 15 min at 9800 x *g*. A total of 100 μl of supernatant fraction was then added to the 0.5 ml of TBA solution (26 mM thiobarbituric acid, 0.2 M HCl, 6.66% trichloroacetic acid and 1 mM deferoxamine mesylate) followed by 30 min of heating at 95°C. After cooling, 0.5 ml of butanol:pyridine 15:1 was added, and the sample was shaken vigorously. The mixture was then centrifuged for 10 min at 800 x *g* at 4 °C. The supernatant was read spectrophotometrically at 532 nm against a "blank" reaction mixture that did not contain the homogenate, although the mixture had been subjected to the entire procedure. The concentration of TBARS was expressed in nmol per g of wet tissue weight.

2.5 cDNA synthesis and semiquantitative PCR

Total RNA was isolated by the TRIzol method (Invitrogen, Carlsbad, CA, USA). RNA concentration was determined by absorbance at 260 nm. The integrity of RNA was verified by electrophoresis in 1.1% denaturing agarose gels in the presence of formaldehyde at 2.2 M. The RNA samples were transcribed into first strand cDNA using MMLV retrotranscriptase (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The cDNA was amplified by PCR with primers from the sequences of the following proteins: thioredoxin 1 (*Trx-1*), glutaredoxin 1 *(Grx-1)*, glyceraldehyde phosphate dehydrogenase (*GAPDH*), and the nuclear related factor-erythroid 2 transcription factor (*Nrf2*) (Limón-Pacheco et al., 2007). The primers used were as follows: *Trx-1*, 5' CGT GGT GGA CTT CTC TGC TAC GTG GTG 3' (sense) and 5' GGT CGG CA GCA TTT GAC TTC ACA GTC 3' (antisense); *GAPDH*, 5' TGA AGG TCG GTG TGA ACG GAT T 3' (sense) and 5' CAT GTA GGC CAT GAG GTC CAC C 3' (antisense); *Grx-1,* 5' TGC AGA AAG ACC CAA GAA ATC CTC AGT CA 3' (sense) and 5' TGG AGA TTA GAT CAC TGC ATC CGC CTA TG 3' (antisense); and *Nrf2*, 5'

TAC TCC CAG GTT GCC CAC ATT 3' (sense) and 5' TAT CCA GGG CAA GCG ACT CA 3'. The PCR reaction consisted of 30 cycles for *Nrf2*, *Trx-1*, *Grx-1* and *GAPDH*. The cycle profiles for the PCR reactions were 95 °C, 30 sec; 55 °C, 30 sec; 72 °C, 30 sec for *Nrf2*; 95 °C, 30 sec; 60 °C, 30 sec; 72 °C, 30 sec for *Trx-1 and Grx-1;* and 95 °C, 30 sec; 60 °C, 30 sec; 72 °C, 60 sec for *GAPDH*. The PCR products were separated by electrophoresis on a 2% agarose gel. The gel was visualized on a UV transilluminator (BioRad), and the images were captured with the Kodak ID version 3.6 Image analyzer (Kodak, NY, USA). Images were analyzed by densitometry and data of *Trx-1*, *Grx-1* and *Nrf2* were normalized against the housekeeping gene *GAPDH*. Changes in expression were presented as percent of change with respect to control group.

2.5 NF-κB western blot analysis

NF-κB transcription factor nuclear migration was analyzed in the cytosolic and nuclear fractions. Fresh tissue samples were homogenized, as previously described (Kaneko *et al*., 2004), at 30% (w/v) in kinase extraction buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 10 mM EDTA, 1 mM DTT, 0.4% v/v IGEPAL, 1 mM Na_3VO_4, 1 mM PMSF, and 10 mg/ml of aprotinin and leupeptin), incubated on ice for 10 min and then centrifuged for 15 min at 8000 x g 4 °C. The supernatant fractions (cytoplasmic soluble proteins) were collected. The nuclear pellet was then washed and lysed in buffer C (20 mM HEPES, pH7.9, 200 mM NaCl, 1 mM EDTA, 5% v/v glycerol, 1 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF and 10 mg/ml of aprotinin and leupeptin). The lysates were incubated on ice for 2 h and then centrifuged at maximum speed for 15 min. Protein concentrations were determined using the micro method by Bradford (Bradford, 1976; Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The lysates were separated by SDS–PAGE on 10% gels and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences). The membranes were blocked overnight at 4 °C in Tris-buffered saline containing 5% nonfat milk. The membranes were then incubated with the primary antibody against the native form, which was used to detect the total protein content. Actin protein levels were used as the loading control. The proteins were visualized by chemiluminescence using the ECL Advance Western Blotting Detection Kit (Amersham

Biosciences). The images were captured and analyzed by densitometry with the Kodak ID version 3.6 Image Analyzer (Kodak, NY).

2.7 Data analysis

Each assay was performed in triplicate, unless otherwise indicated. Data are expressed as the means ± SE. Statistical significance was assessed by a one-way ANOVA, followed by a Tukey's multiple mean comparison test, Dunnet's multiple comparison test or by Student's *t*-test, as indicated in each case. A *p* value of <0.05 was considered significant in all cases.

2.8 Ethics

The experiments reported in this article were performed following the guidelines stated in "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985) and the "Norma Oficial Mexicana de la Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación" (SAGARPA) entitled "Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio" (Clave NOM-062-ZOO-1999, published on August 2001).

3. Results and Discussion

APAP significantly reduced the levels of GSH in the livers and kidneys of BALB/c mice (Fig. 1 A, B; P<0.05). The reduction of GSH in the liver cells was initially between 20-30% in the first 2 h and approximately 50% 3 h after APAP administration. In the kidneys, the reduction of GSH was less pronounced, between 27-30%. We did not observe a reduction of GSH levels in the brain (Fig. 1 C, P>0.05). This finding is of interest because it has been shown that, in mice and in humans, APAP traverses the blood brain barrier and is detected in the cerebrospinal fluid 20 min after intravenous administration (Bannawarth *et al.*, 1992; Godgrey *et al.*, 2005; Kumpulainen *et al.*, 2007). In rats [^{3}H]-acetaminophen is detected in brain structures such as anterior and posterior cortex, striatum, hippocampus, hypothalamus, brain stem, ventral and dorsal spinal cord, 45 min after i.v. administration (Courad et al., 2001). Studies have also shown the expression of cytochrome P450 enzymes capable of metabolizing APAP in the brain (Dutheil *et al.*, 2008). Although intact APAP and lack of conjugates has been also reported (Fisher et al., 1981)

The depletion of GSH in the kidneys was associated with a significant increase in the formation of TBARS in these organs (Fig. 2). This suggests that among the three organs studied, in kidneys oxidative stress is rapidly generated after APAP administration which might contribute to nephrotoxicity. We did not observe changes in the generation of TBARS between the treated and control animals when we examined the liver and brain homogenates.

Specific early responses were observed in the assayed organs. In the liver, GSH depletion was associated with increased levels of *Nrf2* and *Grx-1* mRNA at 2 and 3 h after APAP injection, while the transcription of *Trx-1* was not up-regulated (Fig. 3; $P<0.05$). Our results were similar to the observations made by Goldring et al. (2004) who reported that the depletion of GSH in the liver following the administration of APAP was associated with liver Nrf2 activation and nuclear translocation in CD-1 mice. Moreover, in a previous work it has been described that APAP induces the expression of mRNA and protein of GSH related enzymes (i.e. γ-GCS) in livers of CD-1 mice (Kitteringham et al., 2000). These observations and our results suggest that the GSH-Grx system might be the main mechanism to confront APAP toxicity in the liver, however further investigation is needed to corroborate this hypothesis.

We did not observe changes in GSH levels or in the transcription of *Nrf2* in the brain, but the levels of *Grx-1* and *Trx-1* mRNA were significantly higher at 2 and 3 h after APAP administration (Fig. 4), which suggests the participation of different signaling pathways related to their transcriptional regulation. There is evidence that NF-κB and AP-1 activity mediate cellular responses to stress (Limón-Pacheco and Gonsebatt, 2009), and we observed an increase in the nuclear translocation of NF-κB in brain homogenates at 2 h after APAP administration (Fig. 4).

The depletion of GSH in the kidneys was not associated with an antioxidant response. This is probably due to the smaller reduction of GSH levels in the kidneys than in liver. The lack of response may also be due to the limited antioxidant capacity of the kidneys as was shown by the increase in TBAR production. This finding suggests a higher susceptibility to oxidative stress (Figs. 2 and 5) and could help explain the renal damage due to APAP overdose which occurs sometimes in the absence of liver injury (Manzer and Perrone, 2008; Ozkaya et al., 2010).

An interesting observation was the induction of the transcription of antioxidant proteins in the brain tissue, although the levels of GSH were not significantly altered. This up-regulation of *Trx-1* and *Grx-1* could explain the neuroprotection that was induced by APAP against lipid peroxidation and against cellular damage induced by 1-methyl-4-phenyl pyridinium or quinolinic acid in rat brains (Tanito et al., 2007; Malek et al., 2010).

We have previously observed a similar antioxidant response in the brain when the GSH content is decreased by inhibition of its synthesis by BSO, which diminished the levels of GSH in the liver and kidneys (Limón-Pacheco et al., 2007). This supports our hypothesis that "systemic oxidative stress" signals, such as those induced by BSO or APAP treatments, initiate a protective response in the CNS through the transcription of antioxidant proteins. Taken together these observations make evident the organ-specific responsed to confront toxic insults, where a limited or exhausted antioxidant capacity could be associated with an increased susceptibility to cell damage.

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FIGURE LEGENDS

Fig. 1. GSH levels (nmol/g of wet tissue) in mice organs in control (n=3) and treated BALB/c mice (n=3) at 1, 2 and 3 h after 300 mg kg−1 of APAP i.p. injection in A) liver B) kidneys and C) brain. Determinations were performed in triplicates, bars represent mean levels ± SE. Data were analyzed using a one way ANOVA test followed by Dunnett´s multiple comparison test. Differences were considered significant when P<0.05. ** P<0.001; * P<0.05.

Fig 2. Thiobarbituric acid-reactive substance (TBARS) production as a measure of oxidative damage at 2 h APAP treatment in brain, kidneys and liver of control (n=3) and treated (n=3) BALB/c mice. Determinations were performed in triplicates, bars represent mean levels ± SE. Data between control and treated animals were analyzed using Student's *t* test. Differences were considered significant when P<0.05. * P<0.05.

Fig 3. The effect of APAP treatments in the transcription of *Grx-1, Trx-1* and *Nrf2* in the liver of control (n=3) and treated (n=3) BALB/c mice. Upper panels show a representative agarose gels; lower panel show densitometric values of mRNA levels of *Grx-1, Trx-1* (A) and *Nrf2* (B), GAPDH was used as a house keeping gene. Bars represent means ± SE of triplicate experiments. Data were analyzed using ANOVA and Dunnett's multiple comparison test. Differences were considered significant when P<0.05. * P<0.05.

Fig 4. The effect of APAP treatment in the transcription of *Grx-1, Trx-1* in the brain of control (n=3) and treated (n=3) BALB/c mice. Upper panels show a representative agarose gels; lower panel show densitometric values of mRNA levels of *Grx-1, Trx-1* (A) and *Nrf2*

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(B) GAPDH was used as a housekeeping gene. NF-κB protein was measured by western blot in in the cytosolic and nuclear fractions (C). Actin was used as a housekeeping gene. Bars represent means ± SE of triplicate experiments. Data were analyzed using ANOVA and Dunnett's multiple comparison test. NF-κB data were analyzed using Student's *t* test. Differences were considered significant when P<0.05. * P<0.05.

Fig 5. The effect of APAP treatment in the transcription of *Grx-1, Trx-1* and *Nrf2* in the kidneys of control (n=3) and treated (n=3) BALB/c mice. Upper panels show a representative agarose gels; lower panels show densitometric values of mRNA levels of *Grx-1, Trx-1 (*A) and *Nrf2* (B) GAPDH was used as a housekeeping gene. Bars represent means ± SE of triplicate experiments. Data were analyzed using ANOVA and Dunnett's multiple comparison test. Differences were considered significant when P<0.05. * P<0.05.
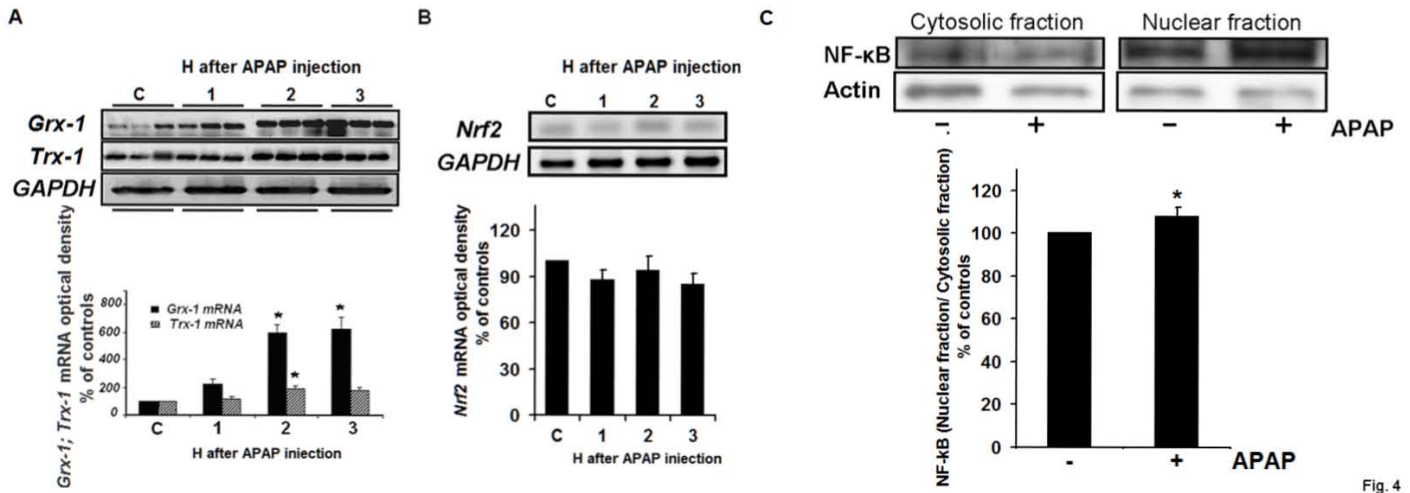
Fig. 1

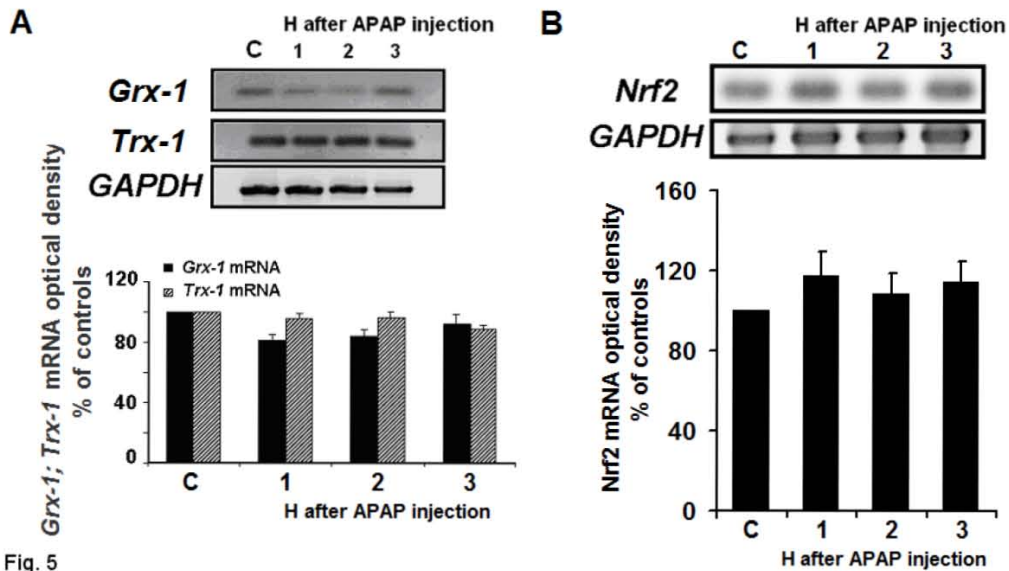Fig. 2

Fig. 3

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Fig. 4

Fig. 5

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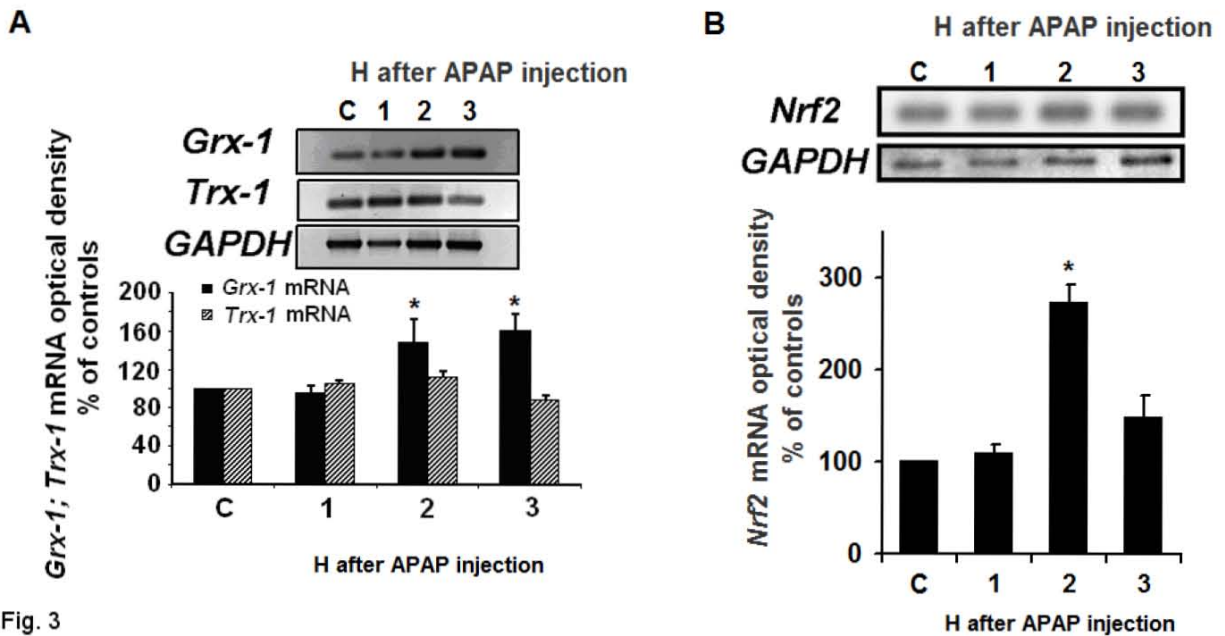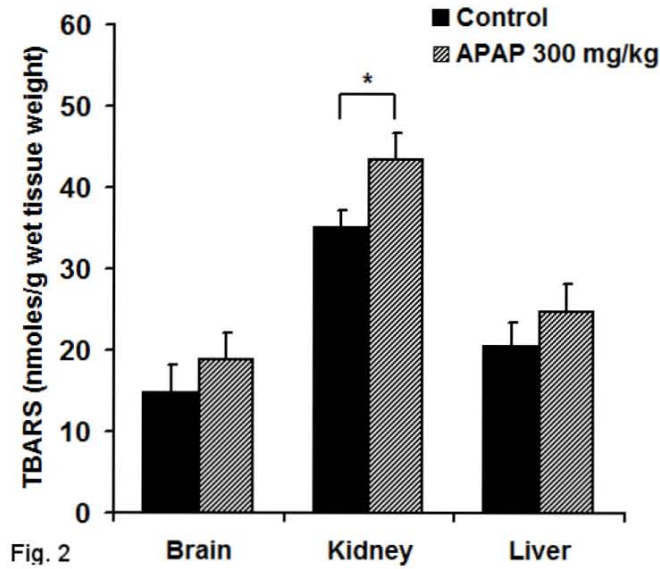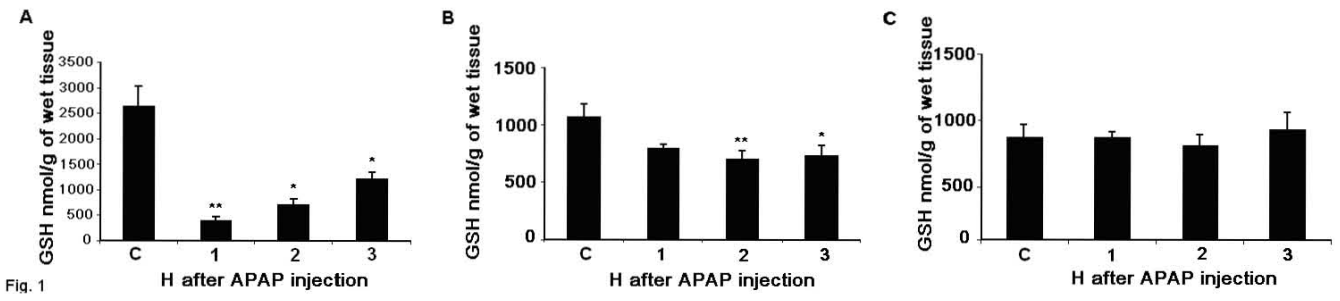
Resultados II

Nerve growth factor exhibits an antioxidant and an autocrine activity in mouse

liver that is modulated by buthionine sulfoximine, arsenic, and acetaminophen

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ORIGINAL ARTICLE

Nerve growth factor exhibits an antioxidant and an autocrine activity in mouse liver that is modulated by buthionine sulfoximine, arsenic, and acetaminophen

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Abstract

Nerve growth factor (NGF) is one of the several structurally related proteins, named neurotrophins (NTs), that regulate neuronal survival, development, function, and plasticity. Moreover, NGF is an important activator of antioxidant mechanisms. These NGF functions are mediated by tropomyosin-related kinase receptor A (TrkA). Although NTs and their receptors have been shown to be expressed in visceral tissues, the extent to which NTs are involved in the physiology of visceral tissues is less clear. NGF is the most expressed NT in adult mouse livers. Although NGF is an important modulator of antioxidant mechanisms in neural tissues, few studies describe the relationship between oxidative stress and NGF expression in the liver. In this study, we demonstrate that *ngfb* mRNA is positively modulated in mouse livers after oxidative injury via intraperitoneal injection of 14 mg/kg sodium arsenite, 6 mmol/kg L-buthionine-S-R-sulfoximine (BSO), or 300 mg/kg acetaminophen (APAP). In addition to the upregulation of *ngfb*, we observed the phosphorylation of the NGF high-affinity receptor TrkA in the liver as well as the downstream phosphorylation of Akt, NF-kB nuclear migration and *iκbα* and *tx-1* mRNA upregulation. These effects were abolished when a neutralizing anti-NGF antibody was used. Furthermore, this anti-NGF antibody alone induced oxidative stress in the liver by decreasing the reduced glutathione, increasing the oxidized glutathione, and downregulating *tx-1* mRNA. Thus, NGF plays a critical role in liver protection against oxidative stress and xenobiotic injury as well as maintains a reduced thiol state.

Keywords: liver, oxidative stress, NGF, L-buthionine-S-R-sulfoximine, arsenic, acetaminophen

Introduction

Nerve growth factor (NGF), a member of a family of structurally related proteins called neurotrophins (NTs), regulates neuronal survival, development, differentiation, function, and plasticity [1,2]. Moreover, NGF is an important activator of antioxidant mechanisms in the central nervous system [3–5].

NGF binds to two distinct classes of transmembrane receptors, the low-affinity p75 NT receptor (p75^{NTR}), a member of the tumor necrosis receptor superfamily, and the high-affinity tropomyosin-related kinase receptor A (TrkA) [1]. The activation of p75^{NTR} is associated with cell death [6], whereas TrkA activation leads to cell survival [1]. The major pathways activated by the TrkA receptor include Ras, Rac, PI3-kinase, and PLC-γ1, and their downstream effectors, mitogen-activated protein (MAP) kinase cascades, protein kinase B (Akt), and the generation of IP3 and diacylglycerol [2].

NTs and their receptors have been shown to be expressed in non-neural tissues [7–9]. For example, NGF is one of the several factors that induce and maintain allergic airway inflammation and fibrosis [10–11] as well as lung and skin tissue repair [12]. Furthermore, the addition of NGF prevents cardiomyocyte apoptosis following hypoxia/reoxygenation or stimulation with angiotensin II through the TrkA/PI3K/Akt pathway [13].

In the adult mouse liver, NGF is the most abundant NT [14]. NGF has been shown to be modulated positively in mouse liver after CCl$_4$ injection [15] as well as after lead nitrate injection, which induces liver hyperplasia [16]. These data suggest that NGF responds to agents that induce oxidative stress. Additionally, transgenic mice overexpressing NGF display elevated glutathione (GSH) concentrations in the liver [17].

The peptide GSH (L-γ-glutamyl-L-cysteinyl-glycine) critically protects cells against oxidative stress and xenobiotic injury as well as maintains the cellular thiol redox state by preventing the oxidation of protein-SH groups [18]. The liver is the most active organ for the *the novo* synthesis of GSH which can be inhibited by L-buthionine-*S-R*-sulfoximine (BSO) and is widely used to deplete cellular GSH [19]. On the other hand, the metabolism of xenobiotics and drugs such as inorganic arsenic and acetaminophen (N-acetyl-p-amino-phenol (APAP) consume liver GSH [18,20,21], decreasing the cellular antioxidant levels.

We observed an upregulation of *ngfb* transcription, associated with phosphorylation of the TrkA receptor and downstream phosphorylation of Akt, after mice were treated with an intraperitoneal (i.p.) injection of BSO, sodium arsenite (iAs), or APAP. Results suggest that this NT participates in the maintenance of redox state in liver and initiates a protective response when GSH or reduced

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thiol levels are compromised. Our results also indicate that this effect shows an autocrine function for NGF.

Materials and methods

Chemicals and antibodies

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. A primary antibody against native TrkA was purchased from Millipore (Bedford, MA, USA). Antibodies against phospho-TrkA (Tyr 490), Akt, phospho-Akt (Thr 308), Rel/nuclear factor-κB (NF-κB) p65, and the secondary anti-rabbit antibody were obtained from Cell Signaling Technology (Danvers, MA, USA), whereas the antibody against β-actin was from Santa Cruz (CA, USA). The neutralizing anti-NGF antibody was purchased from Abcam (Cambridge, MA, USA).

Animal use and treatments

Six-week-old male BALB/c mice were obtained from the animal care facility at the Instituto de Investigaciones Biomédicas at Universidad Nacional Autónoma de México. Animals were maintained in 12-h light/dark cycles. Mice were injected i.p. with 6 mmol/kg BSO, 14 mg/kg iAs, or 300 mg/kg APAP dissolved in a 0.9% saline solution. Control and treated mice were killed by cervical dislocation 0.5, 2, 6, and 24 h following BSO and iAs administration. For APAP treatment, control and treated mice were killed 1, 2, and 3 h after dosage. To determine the effects of thiol levels, mice were treated with 5 mmol/kg NAC dissolved in a saline solution 30 min prior to BSO [18] or APAP administration. Control mice received the saline solution or NAC only. The mice treated with NAC were killed 2 h following compound administration. To confirm the autocrine signaling of NGF in the liver, the animals were pre-treated with 1 mg/kg neutralizing anti-NGF antibody dissolved in a saline solution 1 h prior to BSO or APAP administration [22,23]. The livers were harvested and washed with ice-cold PBS to remove blood and tissue debris, immediately frozen in liquid nitrogen and stored at −70°C.

Western blotting

For kinase assays, fresh tissue was homogenized, as previously described [24], at 30% (w/v) in kinase extraction buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 10 mM EDTA, 1 mM DTT, 0.4% v/v IGEPAL, 1 mM Na_3VO_4, 1 mM PMSF, and 10 mg/ml aprotinin and leupeptin), incubated on ice for 10 min and centrifuged for 15 min at 4000 g at 4°C. The supernatant fractions (cytoplasmic-soluble proteins) were collected. The nuclear pellet was washed and lysed in buffer C (20 mM HEPES, pH 7.9, 200 mM NaCl, 1 mM EDTA, 5% v/v glycerol, 1 mM DTT, 1 mM Na3VO4, 1 mM PMSF and 10 mg/ml aprotinin and leupeptin). The lysates were incubated on ice for 2 h and then

centrifuged at maximum speed for 15 min. Protein concentrations were determined using the micro-method by Bradford (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The lysates were separated using SDS–PAGE in 10% gels and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences; Piscataway, NJ, USA). The membranes were blocked overnight at 4°C in Tris-buffered saline containing 5% non-fat milk and incubated with the primary antibody against the phosphorylated kinase. Antibodies against the native form were used to detect the total protein content. For analysis of transcription factor translocation, both nuclear and cytoplasmic extracts were employed, in which β-actin was used as a loading control. Proteins were visualized by chemiluminescence using the ECL Advance Western blotting detection kit (Amersham Biosciences), and images were captured and analyzed densitometrically by Kodak ID version 3.6 Image Analyzer (Kodak, NY, USA).

Semiquantitative RT-PCR analysis

The expression of the *ngfb* gene was quantified using semi-quantitative RT-PCR. Total RNA was isolated using TRI-zol (Invitrogen). RNA concentrations were determined by an absorbance at 260 nm. RNA integrity was verified using electrophoresis in 1% agarose gels. One microgram of total RNA was reverse-transcribed using the M-MLV reverse transcriptase (Invitrogen). cDNA was amplified using the following primer pairs: *ngfb* (412-bp product): 5′ TGT GCC TCA AGC CAG TGA AAT T 3′; 5′ TCC ACA GTG ATG TTG CGG GTC T 3′ [25], 33 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; *gapdh* (940-bp product), 5′ TGA AGG TCG GTG TGA ACG GAT T 3′ and, 5′ CAT GTA GGC CAT GAG GTC CAC C 3′ [18], 26 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 30 s. *gapdh* mRNA levels were used as a loading control. The PCR products were separated using electrophoresis in a 2% agarose gel and visualized on a UV transilluminator (Bio-Rad). Images were captured and analyzed densitometrically using Kodak ID version 3.6 Image Analyzer.

Glutathione determination

GSH and GSH disulfide (GSSG) contents were assayed using the fluorometric o-phthalaldehyde (OPA) method [26], which was previously proven in our laboratory by HPLC [18], using 96-well black microplates. Fluorescence readings were taken at 365-nm excitation and 430-nm emission with a Beckman Coulter DTX 800/880 Multimode Detector (Beckman Coulter, Fullerton, CA, USA). The final values were calculated as the fluorescence of unit B minus the fluorescence of unit A (UF_B-UF_A = UF_F).

Real-time RT-PCR analysis

For real-time RT-PCR analysis, the Rotor-Gene Q (Qiagen GmbH, Hilden, Germany) was used. Previously prepared cDNA was diluted 1:125 and used as a template for

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real-time PCR. The PCR products were detected using the Kapa SYBR FAST qPCR kit (Kapa Biosystems, Woburn, MA). Each reaction included 8 μL of diluted cDNA, 10 μL of Kapa SYBR FAST qPCR kit, and 1 μl of each 5 μM forward and reverse primers in a total reaction volume of 20 μL. The following reaction conditions were used: a first cycle at 94°C for 3 m; 30 cycles of 94°C for 5 s and 60°C for 20 s; and a final melt curve from 73°C to 93°C to ensure that only one product was amplified. The following primers were used for *gapdh*: F: 5′ ACC ACC AAC TGC TTA GCC CC 3′ and R: 5′ CAG CTC TGG GAT GAC CTT GC 3′; for *tx-1*: F: 5′ CGT GGT GGA CTT CTC TGC TAC GTG GTG 3′ and R: 5′ GGT CGG CAT GCA TTT GAC TTC ACA GTC 3′ [18]; and for *iκba*: F: 5′ AAA TCT CCA GAT GCT ACC CGA GAG 3′ and R: 5′ ATA ATG TCA GAC GCT GGC CTC CAA 3′ [27]. Mean ± standard deviation (SD) of amplification efficiency was 1 ± 0.029, and mean ± SD correlation coefficient was 0.994 ± 0.008. The results were analyzed by the $2^{-\Delta\Delta CT}$ method using *gapdh* as a reference gene [28].

Data analysis

Each assay was performed in triplicate unless otherwise indicated. The data are expressed as the means ± SE, and the significance was assessed using Student's t-test comparison or a two-way ANOVA with a Tukey's *post hoc* as indicated in each case. A p value ≤ 0.05 was considered significant in all cases.

Ethics

The experiments reported in this manuscript were performed in accordance with the guidelines stated in the Principles of Laboratory Animal Care (NIH publication No. 85–23, revised 1985) and the Norma Oficial Mexicana de la Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA, México) Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio (NOM-062-ZOO-1999, published in August 2001).

Results

Agents that modulate redox status induce ngfb expression in the liver

We initially investigated *ngfb* mRNA expression levels in the liver at 0.5, 2, 6, and 24 h after injection with BSO [18] to assess when the response began and how persistent it was. Significant increases in *ngfb* mRNA levels were observed at 0.5 and 2 h after BSO injection (Figure 1A). We also treated mice with iAs and APAP, which have been reported to consume liver GSH [20,21]. *ngfb* mRNA levels were also significantly upregulated at 2, 6, and 24 h after iAs i.p. injection (Figure 1B), and 2 h after APAP injection (Figure 1C). BSO, iAs, and APAP activated the TrkA/PI3K/Akt pathway.

Liver cells are known to express the high-affinity NGF receptor TrkA [8,9]; thus, we investigated whether increased *ngfb* transcription in the liver induced an autocrine response, namely if NGF released by liver cells activated TrkA. To evaluate this, we determined the Tyr 490 phosphorylation status of the TrkA receptor in liver homogenates of BSO-, iAs-, and APAP-treated mice at 2 h (Figure 2A). Furthermore, to test the downstream signaling initiated by NGF, the phosphorylation status of nuclear Akt (at Thr 308) was also evaluated (Figure 2B). Significant increases in both phosphorylated forms, TrkA and Akt, for all the treatments were observed.

TrkA activation observed in the liver after BSO and APAP treatments depends on the thiol redox status

To confirm that changes in the thiol levels modulated TrkA phosphorylation, NAC was administered 30 min before BSO or APAP treatments as an exogenous thiol source [18]. In both cases, TrkA activation was abrogated (Figure 3A). Moreover, NAC inhibited the increased transcription of *ngfb* induced by BSO (Figure 3B).

The NGF signaling pathway modulates GSH and GSSG levels in the mouse liver and is associated with the upregulation of tx-1 mRNA levels through NF-κB after BSO treatment

To confirm the autocrine effect of NGF in the liver, a neutralizing anti-NGF antibody was injected i.p. 1 h prior to BSO or APAP treatment to abrogate TrkA phosphorylation (Figure 4A and B). Concurrently, changes in GSH and GSSG levels were evaluated (Figure 5A and B). Although we did not observe significant differences in GSH or GSSG levels when animals were treated with either BSO or BSO in the presence of the neutralizing anti-NGF antibody, the anti-NGF antibody alone diminished GSH levels and increased GSSG levels (P ≤ 0.05).

Subsequently, the nuclear translocation of the transcription factor NF-κB after BSO, neutralizing anti-NGF antibody or BSO plus anti-NGF antibody treatment was evaluated. BSO treatment significantly reduced nuclear NF-κB levels, but anti-NGF antibody pretreatment plus BSO significantly reduced nuclear NF-κB levels ever further in comparison with BSO treatment alone and controls (Figure 6A).

To clarify the activity of NF-κB observed in Figure 6A, the transcription of *iκba* was evaluated. Whereas BSO treatment alone significantly increased *iκba* mRNA levels, pretreatment with the neutralizing anti-NGF antibody abrogated this transcription (Figure 6B). Finally, as observed previously, BSO treatment increased the mRNA levels of *tx-1* through NF-κB (Figure 6C) [18]. In agreement with the observed modulation of *iκba* mRNA, the BSO-mediated increase in liver *tx-1* mRNA was abrogated when mice were pretreated with the neutralizing anti-NGF antibody. Moreover, neutralizing anti-NGF alone reduced *tx-1* transcript levels (Figure 6C).
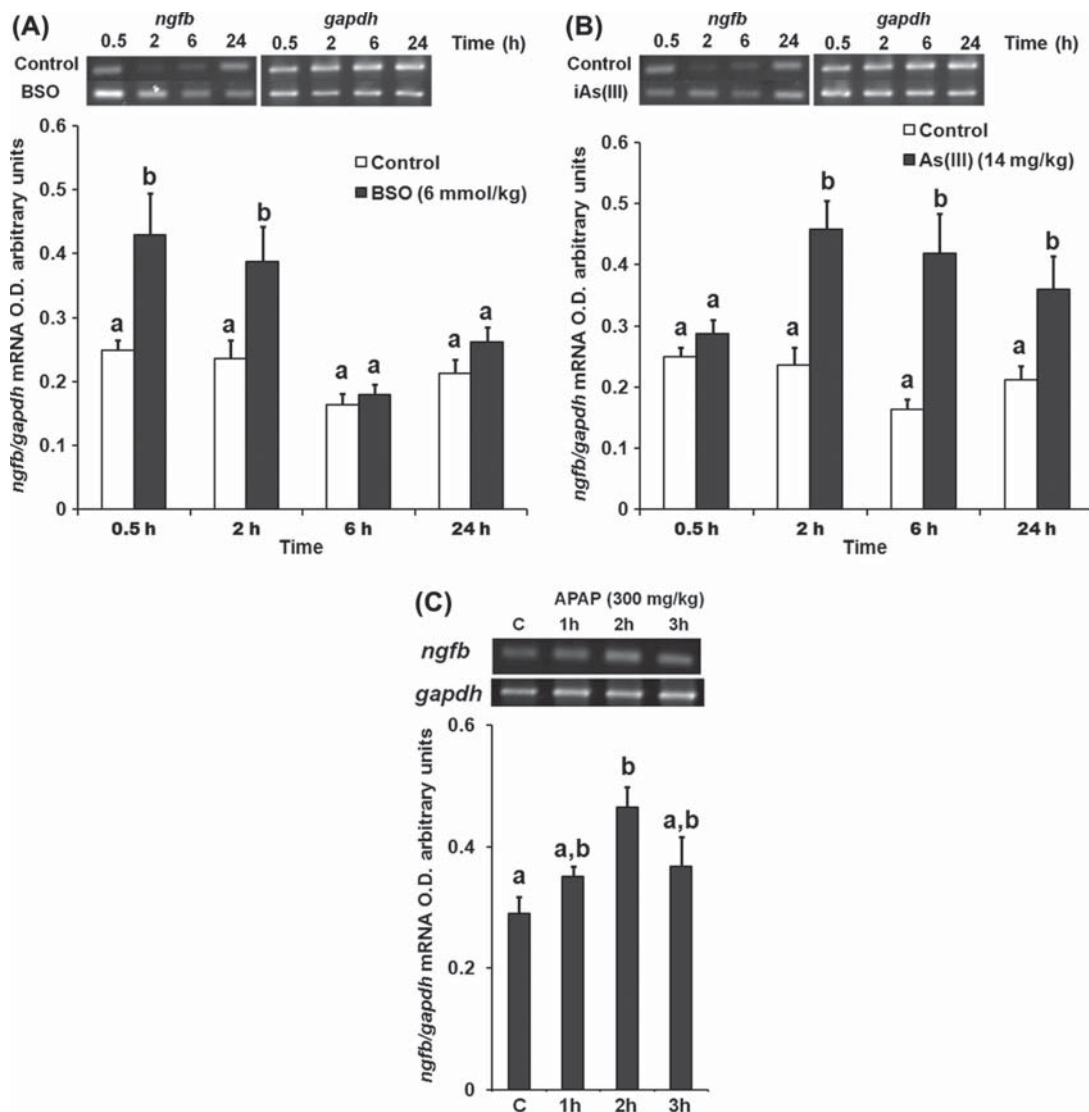
Figure 1. *ngfb* mRNA levels in the liver after treatment with different agents known to induce oxidative injury. Total RNA from mouse livers treated i.p. with (A) 6 mmol/kg BSO, (B) 14 mg/kg iAs, or (C) 300 mg/kg APAP was extracted as indicated in the methods. Upper panels present a representative agarose gel of the *ngfb* transcript, and the lower panel shows the densitometric values of the mRNA levels of the genes analyzed. *gapdh* was used as a loading control. The bars represent the mean ± SE of triplicate experiments and were calculated from densitometry data (n = 16). The data were analyzed using Student's t-test. Different letters in superscript above each column indicate statistical significance difference according to t-test; P ≤ 0.05.

Discussion

In vitro and *in vivo* studies have shown that NGF is an important activator of the antioxidant response in the central nervous system [3–5] as well as an anti-apoptotic/prosurvival factor in cardiac myocytes [13]. NGF has also been shown to be expressed in the adult mouse liver [14] and has been demonstrated to be upregulated after liver injury [15,16,29–31]. Also, transgenic mice overexpressing NGF displayed a positive correlation between GSH concentrations and NGF levels in the liver [17].

Our results suggest that GSH depletion upon exposure to BSO and other toxicologically relevant redox state modulators, such as iAs and APAP, upregulated *ngfb* transcription in mouse liver. This induction was maintained from 0.5 to 2 h after BSO administration, from 2 to 24 h upon iAs injection and 2 h after APAP treatment (Figure 1), suggesting that the response depends on the

toxico-dynamic and toxico-kinetic features of each chemical.

The increased *ngfb* transcription was associated with the activation of the TrkA/Akt/NFκB signaling pathway (Figures 2A, 2B, 6A and 6B) and *tx-1* increase transcription (Figure 6C). These results suggest that NGF is synthesized in liver exercising an autocrine effect, similar to what has been previously reported in other peripheral tissues after injury [13,15]. Moreover, the ability of the circulating neutralizing NGF antibody to abolish TrkA activation (Figure 2A) suggests that NGF is present in plasma and in the extracellular fluid after oxidative stress.

NAC has been used as an exogenous thiol source when GSH synthesis is inhibited [18]. In our study, the administration of NAC prevented TrkA phosphorylation induced by BSO, iAs, or APAP, suggesting that TrkA activation depends on the levels of reduced thiols (Figure 3A). These findings agree with those of a previous report, wherein
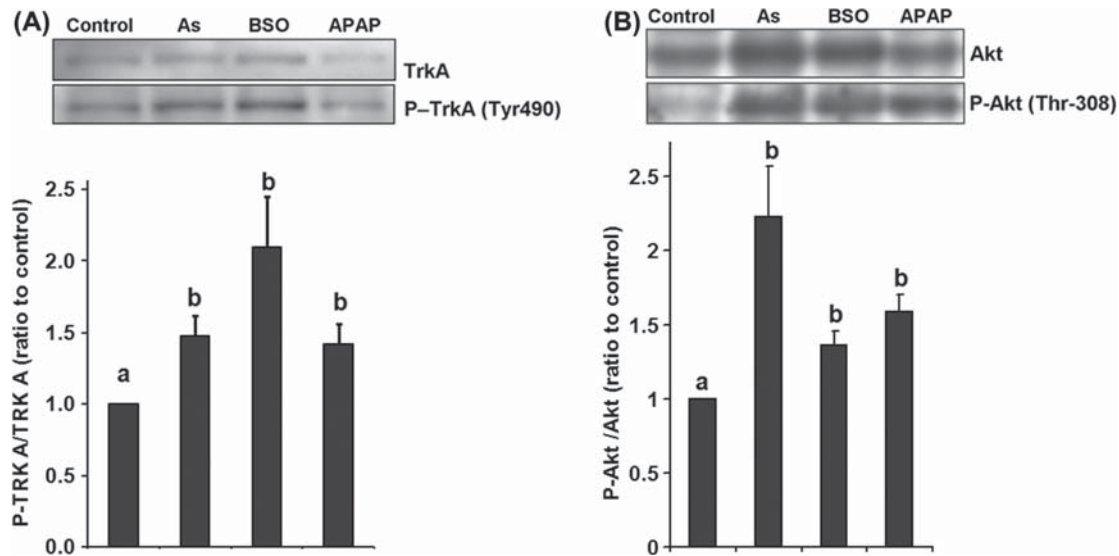
Figure 2. The TrkA/Akt signaling pathway is activated by agents that modulate the thiol redox status. (A) Upper panels present representative Western blots of TrkA Tyr 490 phosphorylation after iAs, BSO and APAP at 2 h. Densitometric evaluation of the blot images was performed using total TrkA as the loading control. (B) Upper panels show representative Western blots of nuclear Akt Thr 308 phosphorylation after treatments. Bars represent the mean ± SE of triplicate experiments (n = 16). Different letters in superscript above each column indicate statistical significance difference according to t-test; $P \leq 0.05$.

NAC was found to inhibit TrkA activation in a PC-12 cell line [32]. Moreover, our data suggest that changes in the cellular thiol redox state are responsible for the *ngfb* mRNA modulation by BSO (Figure 3B).
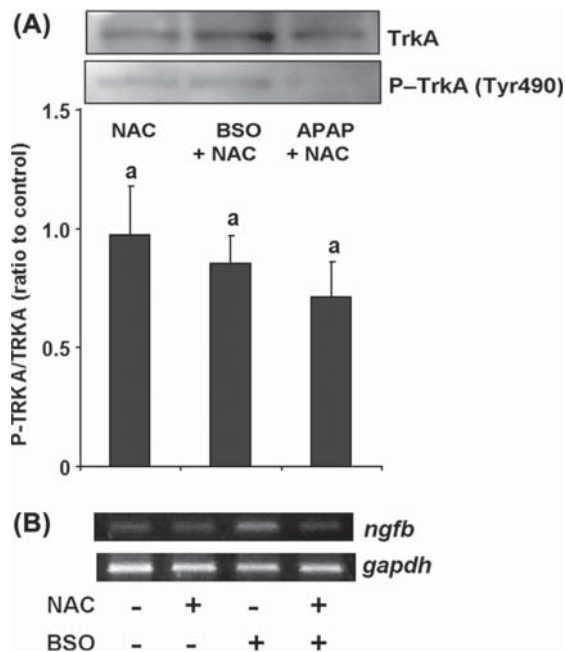

Figure 3. The effect of NAC on TrkA activation by BSO and APAP exposures in the liver. Five mmol/kg of NAC was injected i.p. 30 min before treatment. (A) Western blots of TrkA Tyr 490 phosphorylation after BSO, and APAP at 2 h. Densitometric evaluation of the blot images was performed using total TrkA as the loading control. (B) *ngfb* mRNA modulation after 2 h BSO i.p. injection in the mouse liver is abrogated by NAC. *gapdh* was used as a loading control. Bars represent the mean ± SE of triplicate experiments and were calculated from densitometry data (n = 16). The data were analyzed using Student's t-test. Different letters in superscript above each column indicate statistical significance difference according to t-test; $P \leq 0.05$.

Hepatocytes constitute 60% of the total cells of liver, whereas that sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, and biliary epithelium add each to 20% of the remaining biologically relevant cells [33]. Therefore, even though we did not determine the type of liver cells that comprise this pathway activation, we believe that in our experimental conditions, the hepatocytes are the cells responding to NGF through the TrkA/PI3K/Akt/NF-κB/*trx-1* signaling pathway. Whereas TrkA activation leads to cell survival [1,6], NGF has been also reported to illicit proapoptotic effects via the p75[NTR]. Hepatic stellate cells express p75[NTR], and its activation is related to cell differentiation and apoptosis [15,34,35]. Hyperthyroidism-induced fibrosis and apoptosis in rat liver involve the activation of death receptor-mediated pathways, including p75[NTR] [36,37]. On the other hand, TrkA activation has been reported to be expressed and is related to stimulate proliferation in injured hepatocytes [9]. Moreover, we have observed using immunofluorescence staining that only hepatocytes express TrkA receptor (Supplementary Methods and Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2013.783210) as previously reported by Gezginci-Oktayoglu and collaborators [9].

Although BSO exposure significantly decreased the nuclear levels of NF-κB (Figure 6A), the transcriptional levels of *iκba* were increased (Figure 6B). Because *iκba* is one of the several proteins that inhibit NF-κB, activation of this transcription factor results in *iκba* degradation followed by an increased *iκba* transcription levels. Thus, upregulation of *iκba* mRNA levels indicates NF-κB activity in cells [38]. BSO treatment induced NF-κB activation in mouse liver which is in accordance with a previous report by Chia and collaborators [39] using a murine hepatoma Hepa-1c1c7 cell line. The use of the anti-NGF antibody abrogated the increase in *iκba* mRNA levels (Figure 6B),
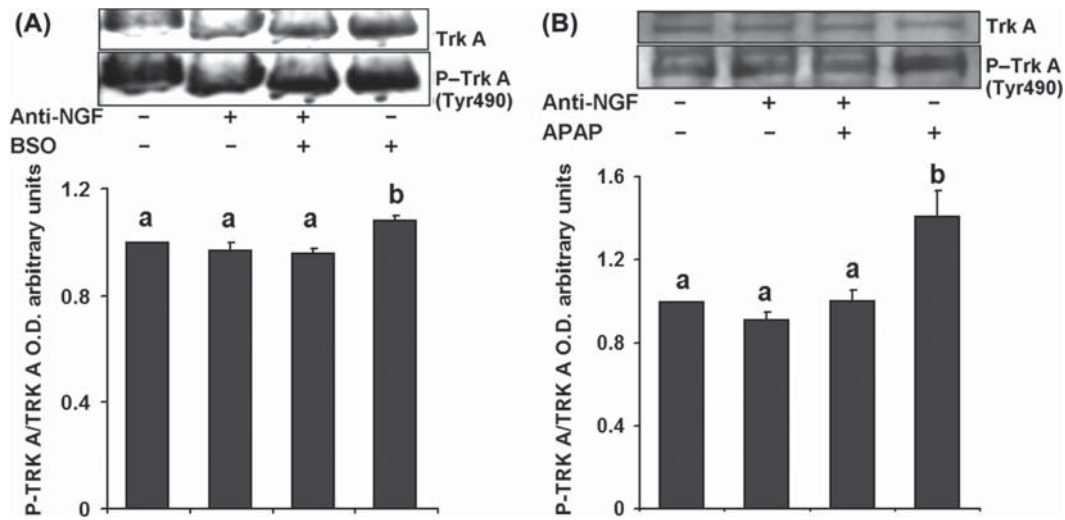
Figure 4. The neutralizing anti-NGF antibody abrogates TrkA activation by BSO and APAP; 1 mg/kg of anti-NGF was injected i.p. 1 h before treatment. Upper panels demonstrate representative Western blots of TrkA Tyr 490 phosphorylation after (A) BSO and (B) APAP at 2 h. Densitometric evaluation of the blot images was performed using total TrkA as a loading control. Bars represent the mean $\pm$ SE of triplicate experiments (n = 16). The data were analyzed using a two-way ANOVA with a Tukey *post hoc* test. Different letters in superscript above each column indicate statistical significance difference; $P \leq 0.05$.

suggesting that NGF modulates the NF-κB transcription factor through the TrkA/PI3K/Akt autocrine pathway in mouse liver treated with BSO.

NF-κB is a transcription factor that modulates early response genes, including transcription factors, cytokines, inflammatory mediators, and processes such as stress responses and cell growth [40,41]. NF-kB has been associated with both beneficial and damaging effects in liver [42–44], and is a critical participant in the regulation of antioxidants and survival responses to oxidative stress [45].

As was mentioned earlier, NGF has been reported to bind to two distinct classes of transmembrane receptors p75[NTR] and TrkA both modulating gene expression through NF-κB [1,6]. The activation of NF-κB through the NGF/TrkA signaling pathway has been demonstrated in rat pheochromocytoma PC12 cell line [46]. Moreover,

in human embryonic kidney 293 (HEK 293) and mouse embryo fibroblast (NIH-3T3) cell lines, the atypical protein kinase C-interacting protein p62 serves as scaffold for the activation of NF-κB, which in turns mediates the NGF/TrkA survival pathway [47], whereas that the NGF/p75[NTR] pathway has been shown to inhibit NF-κB DNA binding in hepatic stellate cells in rats treated with CCl$_4$ [15]. Some reports show that although p75[NTR] and TrkA activate differential pathways, simultaneous expression of both modified certain transduction events mediated by either receptor expressed alone [48,49]. Our experimental results suggest the participation of NGF/TrkA/Akt/NF-κB signaling pathway in hepatocytes, but whether NF-κB activation in liver is mediated by TrkA, or TrkA in addition with p75[NTR] remains to be determined.
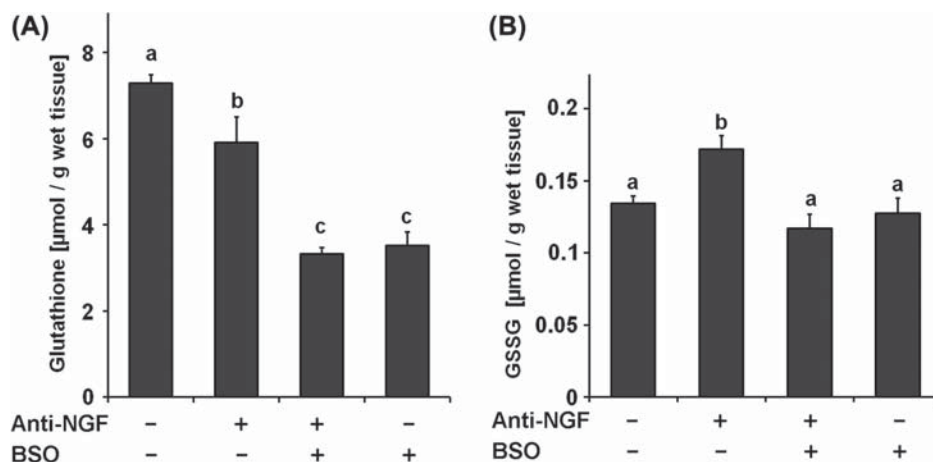

Figure 5. The neutralizing anti-NGF antibody modulates the redox state in the liver. Assessment, as described in the methods, of both (A) GSH and (B) GSSG levels in the liver after 2 h of 6 mmol/kg of BSO i.p., 1 mg/kg of anti-NGF injected i.p. 1 h before BSO treatment or 1 mg/kg of anti-NGF injected alone. Control mice were injected with a saline solution. Bars represent the mean $\pm$ S.E. of triplicate experiments (n = 16). The data were analyzed using a two-way ANOVA with a Tukey *post hoc* test. Different letters in superscript above each column indicate statistical significance difference; $P \leq 0.05$.
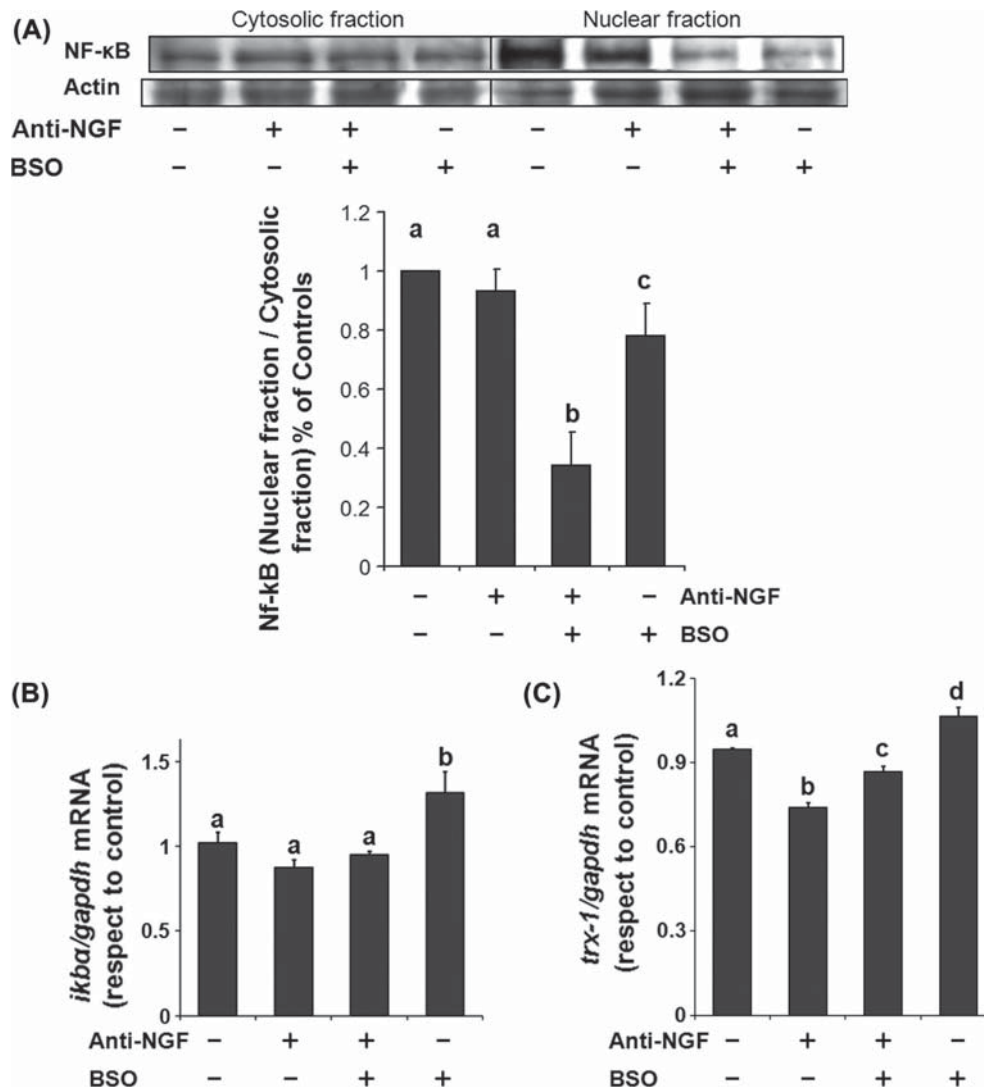
Figure 6. The neutralizing anti-NGF antibody abrogates the NGF-induced pathway by BSO. (A) The neutralizing anti-NGF antibody reduces the translocation of the transcription factor NF-κB induced by BSO. 1 mg/kg of anti-NGF was injected i.p. 1 h before treatment. Upper panels display representative Western blots of NF-κB from cytoplasmic and nuclear fractions after BSO and APAP at 2 h. The densitometric evaluation of the NF-κB nuclear/cytoplasmatic ratio of the blot images was performed using β-actin as the loading control. Bars represent the mean ± S.E. of triplicate experiments, (n = 16). Neutralizing anti-NGF antibody pretreatments abrogate the transcriptional up regulation of (B) *iκba* and (C) *tx-1* genes induced by BSO in the mouse liver. Total RNA from mouse livers was extracted, and cDNA was synthesized as described in the methods. The data were normalized with *gapdh*. The vertical bars indicate the mRNA levels relative to the control group. Bars represent the mean ± S.E. of triplicate experiments (n = 16). For both (A) and (B), the data were analyzed using a two-way ANOVA with a Tukey *post hoc* test. Different letters in superscript above each column indicate statistical significance difference P ≤ 0.05.

BSO treatment induced the transcription of thioredoxin-1 (*tx-1*) in the mouse liver as a compensatory or protective mechanism [18]. Tx-1 is a small cytosolic protein that exhibits many biological functions similar to GSH as an antioxidant, both of these compounds modulate cellular responses to oxidative stress at different levels [18,50]. The promoter region of *tx-1* harbors many potential regulatory binding motifs compatible with inducible expression through NF-κB [51]. In this work, we observed that *tx-1* transcription after BSO treatment was mediated through the NGF-pathway in the mouse liver (Figure 6). Additionally, we have evidence suggesting that this NT plays a significant role in *tx-1* mRNA modulation, given that only the neutralizing anti-NGF antibody treatment reduced its transcriptional levels.

The neutralizing anti-NGF antibody treatment induced oxidative stress in the liver, which was observed as decreased GSH levels, increased GSSG levels (Figure 5), and decreased *tx-1* mRNA levels (Figure 6). This suggests a relevant role for NGF in maintaining the redox homeostasis in the liver, contingent on GSH, GSSG, and *tx-1* levels. Furthermore, this role of NGF is compatible with its elevated levels in the liver [14] and with the increased GSH levels in the liver of transgenic mice overexpressing NGF [17].

In conclusion, we found evidence suggesting that NGF activates the TrkA/PI3K/Akt/NF-κB pathway in the mouse liver, which in turn aids to maintain liver redox state. Furthermore, NGF is upregulated in the liver by different agents known to induce oxidative injury and modulates, at least in part, the antioxidant response (Figure 7).
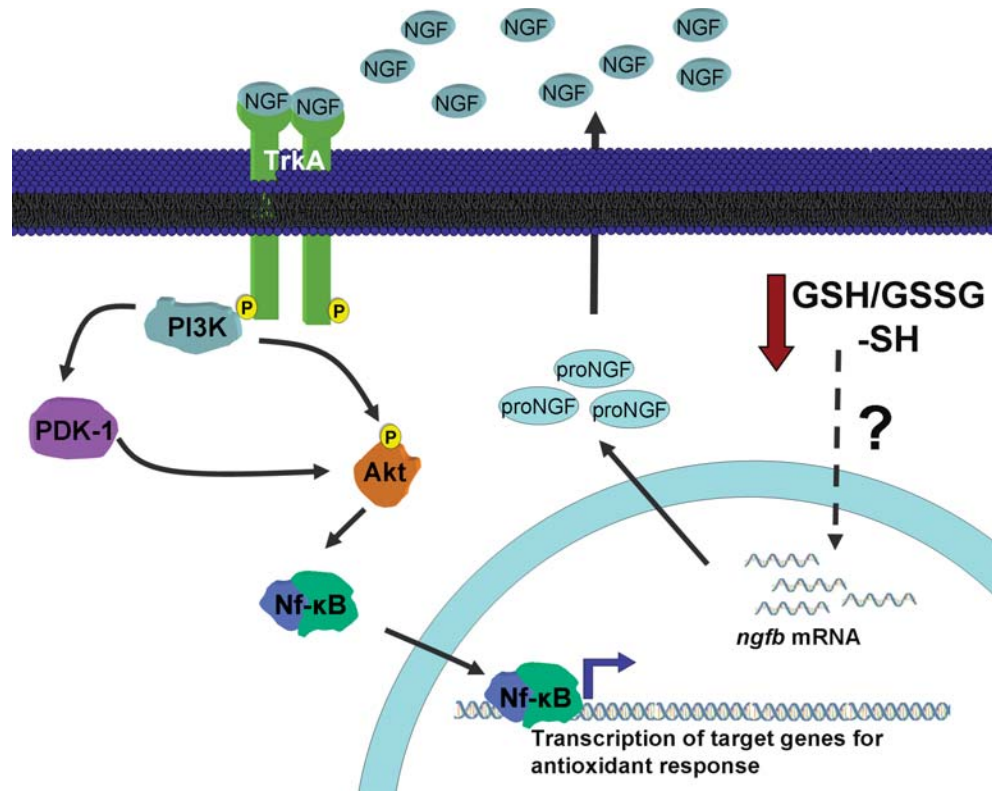
Figure 7. A hypothetical scenario for the activity of NGF after oxidative injury in the mouse liver. NGF levels increase, and NGF is secreted by the liver, resulting in an autocrine/paracrine activation of the TrkA/PI3K/Akt/NF-κB pathway in the liver, which induces the transcription of antioxidant genes.

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Supplementary material available online

Supplementary methods

Supplementary methods

Control mice were perfused through the heart with cold saline solution followed by buffered paraformaldehyde (4%). Their livers were removed, cut in small cubes, postfixed in the same fixative during 24 h at 4°C, and then cryoprotected through graded buffered sucrose solutions (20% and 30%). Sections (50 μm thick) were cut in a cryostat and individually collected in 96-well culture plates filled with 0.1 M phosphate buffer (PB). After 3 washes with PB supplemented with 0.3% Triton X-100 (PBT), sections were blocked 2 h in PBT containing 0.3% BSA (Santa Cruz) and 2% normal horse serum (Vector Laboratories, Burlingame, CA, USA) and incubated overnight at 4°C with primary antibodies against TrkA (Millipore) and CD-31 (BD PharMingen, San Diego, CA, USA) raised in rabbit and rat respectively (1:100 in both cases). After 3 washes with PBT, sections were incubated with secondary antibodies Alexa Fluor 546 anti-Rabbit (Invitrogen) and Alexa Fluor 647 donkey anti-rat (Jackson ImmunoResearch, West Grove, PA; USA; 1:400 in both cases) for 2 h at room temperature protected from light. Then, sections were washed in PB 0.1 M and nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole, Invitrogen) according to manufacturer's protocol. Finally, liver sections were mounted in Fluorescent Mounting Medium (DAKO, Glostrup, Denmark). Photomicrographs were taken on Olympus BX51WI DSU confocal microscope (Olympus, Center Valley, PA, USA) coupled with a Hamamatsu EM-CCD C9100 camera (Hamamatsu, Hamamatsu, Japan). For the final images, Z-stack optical slices (3 μm spacing each) of the sections were taken and combined to maximum intensity projections with ImageJ version 1.46r software (U. S. National Institutes of Health, Bethesda, Maryland, USA).
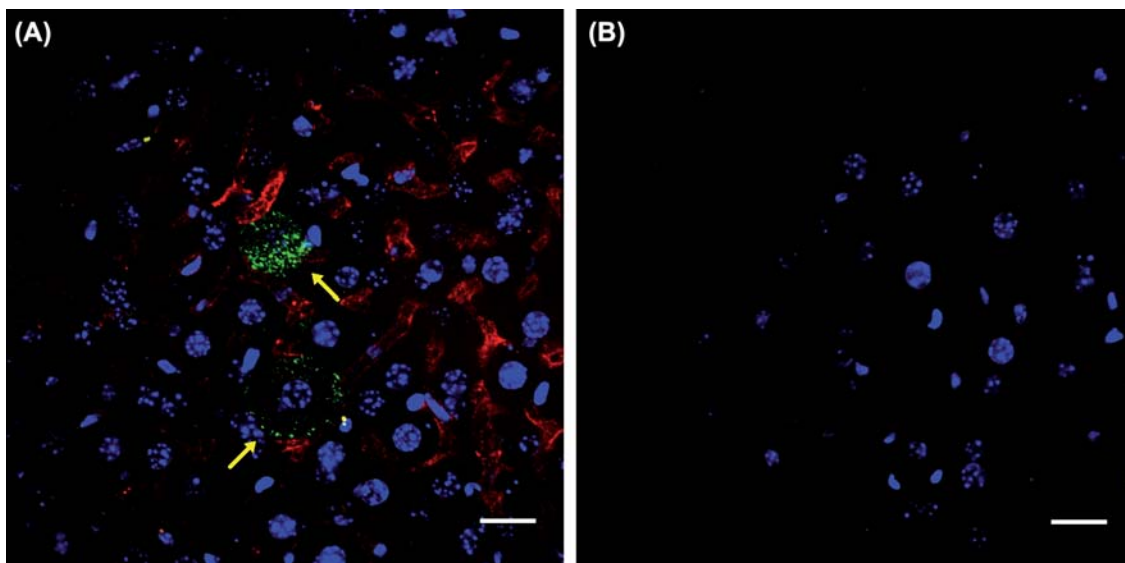
Supplementary Figure 1. TrkA is expressed on mice hepatocytes. Confocal image of liver control mice sections immunostained for TrkA and CD-31. (A) shows a positive cell for TrkA receptor (green, arrow), endothelial cells (CD-31, red) and nuclei (DAPI, blue) staining. (B). Control image where primary antibodies were omitted. Scale bar: 20 microns.

Resultados III

The role of amino acid transporters in GSH synthesis in the blood–brain barrier

and central nervous system

Cesar Valdovinos-Flores, María E. Gonsebatt

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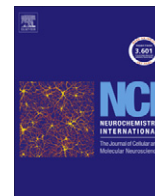
Review

The role of amino acid transporters in GSH synthesis in the blood–brain barrier and central nervous system

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ABSTRACT

Glutathione (GSH) plays a critical role in protecting cells from oxidative stress and xenobiotics, as well as maintaining the thiol redox state, most notably in the central nervous system (CNS). GSH concentration and synthesis are highly regulated within the CNS and are limited by availability of the sulfhydryl amino acid (AA) L-cys, which is mainly transported from the blood, through the blood–brain barrier (BBB), and into neurons. Several antiporter transport systems (e.g., x_c^-, x_{AG}^-, and L) with clearly different luminal and abluminal distribution, Na^+, and pH dependency have been described in brain endothelial cells (BEC) of the BBB, as well as in neurons, astrocytes, microglia and oligodendrocytes from different brain structures. The purpose of this review is to summarize information regarding the different AA transport systems for L-cys and its oxidized form L-cys$_2$ in the CNS, such as expression and activity in blood–brain barrier endothelial cells, astrocytes and neurons and environmental factors that modulate transport kinetics.

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1. Introduction

Glutathione (GSH) plays a critical role in protecting cells from oxidative stress and xenobiotics, as well as maintaining the thiol redox state, most notably in the central nervous system (CNS) (Dringen, 2000). Its CNS concentration is approximately 2–3 mM, which is much higher than that in blood or cerebrospinal fluid (CSF) (Cooper and Kristal, 1997; Limón-Pacheco et al., 2007). However, brain levels of GSH are lower than those reported for other organs (Commandeur et al., 1995) and decline with aging (Maher,

2005). This is of concern because, compared with other organs, the brain has lower antioxidant enzyme activities, such as SOD, catalase, and GPx. The abundance of lipids with unsaturated fatty acids, which are targets of lipid peroxidation, make the brain especially vulnerable to oxidative stress (Dringen, 2000).

GSH is synthesized in the cytoplasm of all animal cells from the aminoacids (AAs) L-glu, L-cys and L-gly in two enzymatic steps that consume ATP. γ-Glutamylcysteine synthetase (GCL) catalyzes the first rate-limiting enzymatic step in GSH synthesis (Meister and Anderson, 1983). GCL mediates the first reaction between L-glu and L-cys to form a dipeptide, γ-glutamyl-cysteine (γGluCys), which in turn reacts with L-gly catalyzed by GSH synthetase (GS) to produce GSH. GCL is a heterodimer composed of a catalytic heavy (73 kD) subunit, GCLC, and a modulatory light (31 kD) subunit, GCLM. GCLC, but not GCLM, has enzymatic activity and is subject to feedback inhibition by GSH competitively with glutamate and has not been shown to be substrate-saturated at normal cellular L-cys levels, thus increased L-cys levels can promote GSH synthesis (Meister and Anderson, 1983; Wu et al., 2004).

2. Amino acid (AA) transport in astrocytes and neuronal GSH synthesis

After intracarotid injection, only 0.5% of radiolabeled GSH can be detected in brain extracts, indicating that GSH poorly penetrates the blood–brain barrier (BBB) (Cornford et al., 1978; Anderson

et al., 1989). Moreover, *in vitro* studies have shown that basal levels of GSH are different among neurons, astrocytes, microglia and oligodendrocytes (Makar et al., 1994; Thornburne and Juurlink, 1996; Hirrlinger et al., 2002), suggesting different mechanisms for maintaining GSH homeostasis. Nevertheless, because the different types of brain cells are in close contact to each other *in vivo*, information obtained from *in vitro* experiments does not necessarily reflect the *in vivo* situation (Dringen, 2000).

GSH synthesis is limited by the availability of the sulfhydryl AA L-cys, especially in neurons (Shih et al., 2006). However, because L-cys autoxidizes to its disulfide form cystine (L-cys$_2$) under aerobic conditions (Wang and Cynader 2000), the plasma concentration of L-cys (10–20 μM) is 10-fold lower than that of L-cys$_2$ (100–200 μM) (Dröge et al., 1991; Wang and Cynader, 2000).

In the CNS, and most notably in astrocytes, an exchanger referred to as system x$_c^-$ is responsible for the Na$^+$-independent, chloride-dependent, pH-dependent and electroneutral uptake of L-cys$_2$ into cells in exchange for the excitatory AA L-glu in a 1:1 ratio (Makowske and Christensen, 1982; Bannai, 1986; Cho and Bannai, 1990; Lim and Donaldson, 2011). System x$_c^-$ consists of a specificity-conferring light chain, xCT (also known as SLC7A11) and a single membrane spanning type II membrane glycoprotein heavy chain 4F2hc (also known as SLC3A2 or CD98) (Sato et al., 2000). The subunits are linked by a disulfide bridge (Lewerenz et al., 2011). xCT has been identified in a wide variety of CNS cells, including astrocytes (Cho and Bannai, 1990), microglia (Piani and Fontana, 1994), retinal Müller cells (Kato et al., 1993), immature cortical neurons (Murphy et al., 1990), glioma cells (Cho and Bannai, 1990), and the BBB (Sato et al., 2002). Importantly, xCT is also expressed in cerebral cortex neurons (Pow, 2001; Burdo et al., 2006). xCT protein (Shih et al., 2006) and mRNA expression (Sato et al., 2002) have been studied *in vivo,* and it was found to be widely expressed in mature and immature brain structures, including cortex, hippocampus, striatum and cerebellum, although at much lower levels than in BBB endothelial cells (EC) and meningeal tissue. Burdo and colleagues (2006) localized xCT in neurons and astrocytes in both mouse and human brain, in addition to border areas between the BBB and the blood-CSF barrier, including vascular EC, ependymal cells, choroid plexus, and leptomeninges. Protein levels of xCT in brain and spinal cord increase during development, reaching highest expression in adulthood (La Bella et al., 2007). The relevance of system x$_c^-$ in the CNS is due to the range of processes linked to this transport system. For example, Baker and collaborators (2002) reported that in rat striatum and nucleus accumbens, system x$_c^-$ is the major source of extracellular glutamate. Moreover, x$_c^-$ is related to oxidative protection (Shih et al., 2006), BBB physiology (Hosoya et al., 2002), brain tumor growth (Chung et al., 2005), and synaptic organization (Augustin et al., 2007).

However, because system x$_c^-$ mediates L-cys$_2$ uptake into cells in exchange for the excitatory AA L-glu in a 1:1 ratio, exacerbated action of this AA transporter system could be deleterious by increasing extracellular L-glu to excitotoxic levels (Baker et al., 2002; Lewerenz et al., 2006; Lau and Tymianski, 2010; Tan et al., 2001).

The intracellular glutamate concentration is >12,000 μmol/g, whereas its extracellular levels have been reported to be in the 1–3 μM range. This pronounced difference (4000–12,000 times) is maintained through energy-dependent transport (Hawkins et al., 2002). System x$^-_{AG}$ is a subgroup of the family of SLC1 Na$^+$-dependent transporters that is important for the clearance of L-glu and includes neuronal EAAC1 (EAAT3, SLC1A1) (Kanai and Hediger, 1992), cerebellum excitatory AA transporter 4 (EAAT4, SLC1A6) (Fairman et al., 1995), glial transporter Glu-Asp (GLAST, EAAT1, SLC1A3) (Storck et al., 1992), L-glu transporter-1 (GLT-1, EAAT2, and SLC1A2) (Pines et al., 1992), and the retinal transporter EAAT5 (SLC1A7) (Arriza et al., 1997). These transporters share

approximately 50–60% AA sequence homology (Chao et al., 2010). The transport of L-glu is coupled with the inward co-transport of three Na$^+$ and one H$^+$ and the counter transport of one K$^+$, enabling the maintenance of a concentration gradient across the cell membrane (Zerangue and Kavanaugh, 1996a). GLAST plays a major role in extracellular glutamate clearance (Amara and Fontana, 2002), whereas GLT-1 represents almost 1% of brain protein and performs over 90% of cortical L-glu uptake (Lehre and Danbolt, 1998; Sheldon and Robinson, 2007). The relationship between systems x$_c^-$ and x$^-_{AG}$ was demonstrated in glioma cells by Patel and collaborators (2004); L-glu released by system x$_c^-$ is instantly taken up again by system x$^-_{AG}$.

While high levels of system x$_c^-$ protein expression have been reported in neurons (Pow, 2001; Burdo et al., 2006) and L-cys$_2$ is the major form in plasma, L-cys concentrations in CSF (0.68–1.75 μM) are much higher than that of L-cys$_2$ (Wang and Cynader, 2000; Lewerenz et al., 2011). For this reason, AA transporters that incorporate L-cys should be more important in neurons, although it has been reported that high extracellular concentrations of L-cys may be neurotoxic (Gazit et al., 2004; Janáky et al., 2000).

In this sense, the AA transporter EAAC1 was first identified as a high-affinity neuronal L-glu transporter (Kanai and Hediger, 1992), but it was later shown to also transport L-cys in neuronal cultures (Chen and Swanson, 2003; Himi et al., 2003). EAAC1 is ubiquitously expressed in the perisynaptic areas and cell bodies of neurons and some white matter cells, particularly developing oligodendroglia, but not astrocytes. It is highly expressed in both glutamatergic and γ-aminobutyric acid (GABA)-ergic neurons (Rothstein et al., 1994; Conti et al., 1998; He et al., 2000). EAAC1 immunoreactivity is found in the cell cytoplasm, implying that there is a pool of transporters available for trafficking into the plasma membranes (Robinson, 2006).

EAAC1 did not appear to play a major role in the clearance of glutamate from the extracellular space (Rothstein et al., 1996; Peghini et al., 1997); EAAC1-deficient mice have normal brain histology, do not exhibit spontaneous seizures, and have normal motor coordination with a decrease in spontaneous locomotor activity (Peghini et al., 1997). Conversely, GLAST or GLT-1 (Tanaka et al., 1997; Watase et al., 1998; Rao et al., 2001) knock-out mice have elevated extracellular glutamate levels and spontaneous seizures. It was also later shown that EAAC1 knock-out mice have increased neuronal oxidative stress markers, decreased neuronal GSH contents (∼40%) and develop brain atrophy and severe spatial reference memory deficits with aging (Aoyama et al., 2006). *In vitro* studies have shown that inhibition of EAAC1 transport reduces both neuronal GSH and neuronal survival after oxidative stress (Nafia et al., 2008; Watabe et al., 2008). Furthermore, an *in vitro* study by Himi and collaborators (2003) has demonstrated that EAAC1-knockdown neurons show a 30% reduction of L-cys uptake and a 25% reduction of intracellular GSH, providing evidence that EAAC1 is an important route for neuronal uptake of L-cys. Indeed, L-cys was shown to be transported by EAAC1 with a maximal flux rate 10- to 20-fold higher than that of GLAST or GLT-1, which is comparable to the flux rates for L-glu (Zerangue and Kavanaugh, 1996b). Recently, an *in vivo* study showed that reduced expression of EAAC1 exacerbates ischemic injury by reducing thiol content, increasing oxidative stress and impairing neuronal zinc homeostasis after ischemia–reperfusion (Won et al., 2010).

However, as mature cultured neurons preferentially utilize L-cys, but not L-cys$_2$, for GSH synthesis, it is thought that the rate of L-cys$_2$ uptake into the brain is especially important for maintaining GSH levels in glial cells (Cho and Bannai 1990; Kranich et al., 1996). *In vitro* studies suggest that mature neurons utilize L-cys for GSH synthesis, while glial cells utilize both L-cys and L-cys$_2$ (Dringen and Hirrlinger 2003; Sagara et al., 1993). For example, the addition of L-cys can increase GSH levels in neuronal cultures

temporally deprived of AA, whereas L-cys$_2$ cannot (Kranich et al., 1996). According to Wang and collaborators (2000), murine astroglial cultures directly release GSH (first demonstrated by Sagara et al., 1996), and L-cys is generated by an extracellular thiol/disulfide exchange reaction that can occur nonenzymatically (Jocelyn, 1967) as follows:

$$GSH + L\text{-}cys_2 \rightleftarrows L\text{-}cys + CSSG \qquad (1)$$

However, although astrocytes release approximately 10% of their intracellular GSH within one hour, it is consumed by the ecto-enzyme γ-glutamyl transpeptidase (γGT) (Dringen et al., 1997). This enzyme catalyzes the transfer of the γ-glutamyl moiety from GSH or its glutathione conjugate onto an acceptor L-gly molecule, thereby generating the dipeptide L-cysgly or L-cysgly conjugate, respectively (Meister and Anderson, 1983). The dipeptide L-cysgly functions like GSH in reaction [1] to reduce L-cys$_2$ (Jones et al., 2000). Neurons can use L-cysgly dipeptide to synthesize GSH after having hydrolyzed it by the ectopeptidase ApN, generating L-cys and L-gly, which are subsequently taken up as precursors for GSH synthesis (Dringen et al., 2001).

Thus, for GSH synthesis in astrocytes, L-cys$_2$ uptake is mediated by system x_c^- (xCT with 2F2hc) in close relationship with system x^-_{AG} (specifically GLT-1 and GLAST) to prevent excitotoxic damage. Once GSH is synthesized by GCL and GS is exported from astrocytes and catabolized by γGT to L-cysgly, both peptides, GSH and L-cysgly, reduce extracellular L-cys$_2$ to L-cys and L-cys$_2$-mono conjugated, respectively. L-Cysgly can also be cleaved by the neuronal peptidase ApN to L-cys and L-gly. These mechanisms provide the L-cys that is required for neuronal GSH synthesis and is taken into neurons by EAAC1 (Fig. 1). Danbolt (2001) suggests the existence of several different transporters for L-cys$_2$ in brain due to observation by Flynn and Mac Bean (2000) who described in brain synaptosomes the uptake of L-cys$_2$ by three different mechanisms. Thus, the existence of other L-cys/L-cys$_2$ transporters might explain the mild phenotypic effects observed in the EAAC1 knock-out mouse as well as the decreased extracellular glutamate and lack of oxidative stress in the system x_c^- knock-out mouse.

There are numerous studies that have demonstrated GSH synthesis mechanisms and the uptake of substrates in neurons and astrocytes, as well as neuronal dependence on astrocytic GSH

synthesis. However, the uptake of rate-limiting substrates through the BBB has been less studied.

3. BBB structural components and function

There are three main cell layers that act as barriers in the CNS: the EC of brain or retinal capillaries (BBB and the blood retinal barrier), the choroid plexus epithelium (blood–CSF), and the arachnoid epithelium of the meninges (Abbott et al., 2010). These barriers differ in localization, size, morphology, and function. Because the BBB has a surface area 1000 times that of the choroid plexus, the extent to which a given molecule enters the brain is determined almost exclusively by BBB permeability characteristics (Boado et al., 1999; Pardridge et al., 1990).

The concept of the BBB was suggested by Lewandowsky (1900) to explain the absence of pharmacological actions of intravenously administered bile acids or ferrocyanide in the CNS. Studies using electron microscopy revealed that the BBB is an EC barrier present in brain capillaries (Reese and Karnovsky 1967). These brain endothelial cells (BECs) show a typical continuous cobblestone appearance, with high mitochondria content (8–11% of the total BEC volume; Oldendorf et al., 1977), high electrical impedance of ∼1900 Ω cm^2 (Crone and Olesen, 1982; Butt et al., 1990) and a lack of fenestrations (Abbott et al., 2010). BEC surface area within the CNS is, depending on the anatomical region, between 150 and 200 cm^2 g^{-1} tissue, giving a total area for exchange in the brain between 12 and 18 m^2 for the average adult (Nag and Begley, 2005). It has been estimated that almost every neuron in the human brain has its own capillary (Zlokovic, 2005).

BECs preclude the free exchange of solutes between the blood and the brain, except for small (<400 Da), lipid-soluble molecules with fewer than nine hydrogen bonds, which cross the BBB via lipid-mediated diffusion (Pardridge, 2007). If plasma components (red blood cells and leukocytes) cross the BBB, they generally generate neurotoxic products that can compromise synaptic and neuronal functions (Zlokovic, 2005; Abbott et al., 2006).

BBB impermeability results from the physical barrier between adjacent BECs that line the microvessel wall (Lee et al., 2009); they are connected by junctional integral membrane protein complexes, such as tight junctions (TJ) (occludin, claudins and adaptor mole-
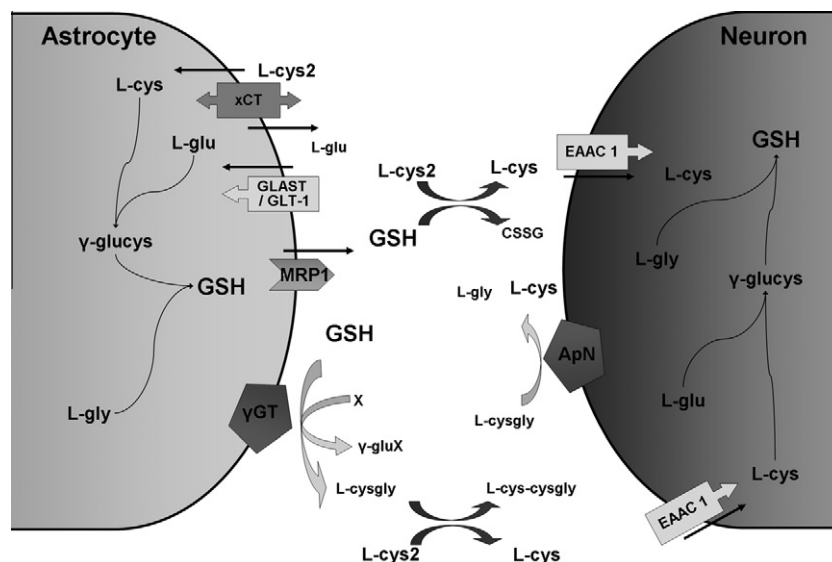
Fig. 1. Model showing how astrocytes provide L-cys to neurons. Astrocytes uptake L-cys$_2$ by system x_c^- and synthesize GSH that can be released to the extracellular fluid, where GSH or its metabolites react with L-cys$_2$ to generate L-cys and L-cys- conjugated, respectively. L-cys is then taken up by neurons through EAAC1 for GSH synthesis.

cules (MAGUK)), adherens junctions (AJ) and junctional adhesion molecules (JAM) (for review see Abbott et al., 2006).

4. The BBB as a neurovascular unit

Although functional events that define the BBB occur at the level of BECs, the BBB also consists of pericytes, astrocytes, microglia and neurons. All of them are in very close proximity to BECs through the basement membrane, which is composed of collagen type IV, laminin, fibronectin, and heparin sulfate proteoglycan (Farkas and Luiten, 2001; Prat et al., 2001). All these elements are part of the functional neurovascular unit (Park et al., 2003; Hawkins and Davis, 2005; McCarty, 2005). The relationships between different cell types allow for effective paracrine modulations that are critical for CNS homeostasis (Dermietzel and Krause, 1991; Lok et al., 2007) such as the coordination of neurovascular hemodynamic and microvascular permeability, neurotrophic, angiogenic and neurogenic signals, as well as neurotransmitter inactivation and matrix interactions (Engelhardt, 2003; Zlokovic, 2008).

Pericytes cover between 22% and 70% of the abluminal BEC abluminal surface at a 1:3 ratio (Allt and Lawrenson, 2001; von Tell et al., 2006). Pericytes have many cytoplasmic processes that encircle BECs (Dehouck et al., 1997). It has been suggested that pericytes regulate multiple cell functions such as contractile, immune and phagocytic activities, as well as BECs migration, survival, cell proliferation, differentiation and angiogenesis (Minakawa et al., 1991; for review see Lai and Kuo 2005; Fisher, 2009).

Neurons and BECs are generally physically distant, although neural stem cells and perivascular nerves are close to BECs and even make direct contact in specialized regions (Tavazoie et al., 2008). Despite the distance between neurons and BECs, it has been suggested that neuron–BECs and astrocyte–BECs communication can modulate BBB permeability and blood flow (Attwell et al., 2010; Paemeleire, 2002). BECs and/or associated astrocytic processes are contacted by different kinds of neurons (Persidsky et al., 2006). Moreover, the neuronal environment seems to induce BBB features in the BECs during embryonic development, through the secretion of neurogenic factors such as neurotrophins and vascular endothelial growth factor (VEGF) (for review see Bauer and Bauer, 2000).

Astrocytes end-foot processes surround more than 99% of BECs and attached pericytes (Risau and Wolburg, 1990; Hawkins and Davis, 2005). Astrocyte differentiation occurs in the late embryonic and postnatal periods, whereas BECs sprouting is completed before birth. Although astrocytes do not modulate angiogenesis, they play a major role in barrier maintenance and breakdown through astrocyte-derived factors and astrocyte–BEC physical interactions (Garcia et al., 2004; Lee et al., 2009). Astrocyte–BEC interactions have been demonstrated to regulate brain water levels and electrolyte exchange by modulating the presence and abundance of transporter proteins in the luminal and abluminal BEC membranes (Abbott et al., 2006). Moreover, it has been demonstrated that constant interaction between BECs and astrocytes is necessary to maintain BBB features. Finally, as an example of the relevant BEC–astrocytes interactions in GSH synthesis, DeBault (1981) demonstrated that glial cell-conditioned media did not induced γGT expression in γGT-negative BECs, whereas a single astrocyte foot process did.

5. Nutrients cross the BBB

Transport across the BBB is the limiting step for nutrient and metabolite movement from blood to intracellular CNS spaces (Boado et al., 1999). In order to ensure a constant optimal chemical environment adequate for CNS functions, BECs have a complex network of specific carrier-mediated transport systems in the respective luminal and abluminal membranes that allow the transfer of metabolites and nutrients between blood and CNS (Prat et al., 2001; Ohtsuki, 2004). Apart from inhibiting paracellular movement, TJ protein complexes also divide the BEC membrane into abluminal (brain) and luminal (blood) sides. This prevents the movement of membrane proteins (e.g., transporters) and lipids (van Meer et al., 1986), so that there is a different content of both lipids and intrinsic proteins on the luminal and abluminal sides (Tewes and Galla, 2001; Betz et al., 1980). For this reason, all the solutes must cross both membranes. It is the combined characteristics of both membranes that determine which molecules cross the barrier and how fast transport takes place (Pappenheimer and Setchell, 1973; Oyler et al., 1992; Hawkins et al., 2002).

As an example, the BBB plays a crucial role in supplying glucose to the brain, which consumes 20% of total body energy since the amount of glycogen stored in brain is small (Obel et al., 2012). There is a highly expressed Na^+-independent glucose transporter GLUT1 (SLC2A1) constituting more than 90% of BECs glucose transporters (Pardridge et al., 1990), with a 1:3 ratio of distribution between the luminal and abluminal surface (Abbott 2005).

As previously mentioned, the AA content in the extracellular fluid of the brain is much lower than that in plasma, and the individual protein composition is markedly different. In fact, the concentrations of AAs in CSF, with the exception of L-gln, are ~6–30 times lower than their concentrations in plasma (Table 1), indicating that AAs leave the brain against a concentration gradient (Hawkins et al., 2006; O'Kane and Hawkins, 2003).

More than 20 specific transport systems have been described in BBB BECs. They are classified in five main categories: carrier-mediated transport, ion transport, active efflux transport, caveolae-mediated transport and receptor-mediated transport (for review see: Smith, 2000; Ohtsuki, 2004; Hawkins et al., 2006; Zlokovic, 2008).

6. Systems AA transporter in the BECs of the BBB

Ten AA transport systems with clearly different luminal and abluminal distributions have been reported in BBB BECs (Fig. 2). All AAs are incorporated into BECs, and from there are moved into the CNS by the Na^+-independent system L and system y$^+$, which are expressed on both luminal and abluminal BEC membranes, and system x$_c^-$, which is expressed only on the luminal membrane (Hawkins et al., 2006; Shih et al., 2006). The facilitative transporters on the luminal side, x$_G^-$ and n (that transport L-asp, L-glu and L-gln), mediate efflux from the endothelium to blood (Zlokovic, 2008). Five Na^+-dependent transport systems present only on the abluminal side of BECs are responsible for AA efflux from CNS to BECs to the circulation. These ASC systems transport L-ala, L-ser, L-asn and L-cys, A (L-ala, L-pro and N-methyl AA), B$^{0,+}$ (neutral and basic AA), x$^-_{AG}$ (L-glu, L/D-asp) and N (L-gln, L-asn and L-his) (O'Kane and Hawkins 2003; Zlokovic, 2008). To maintain Na^+-dependent transport, the sodium pump (Na^+, K^+-ATPase) is highly expressed on the abluminal BEC membranes (Sánchez del Pino et al., 1995a).

7. L-Cys transport across the BBB

As mentioned at the beginning of this review, GSH poorly crosses the BBB. Therefore, L-cys/L-cys$_2$ transport is the limiting step of GSH synthesis in the CNS, and their flux across BBB BECs is crucial (Bannai and Tateishi, 1986).

System x$_c^-$ is responsible for L-cys$_2$ influx in the luminal membrane of BECs, as well as in astrocytes (Hosoya et al., 2002). xCT is expressed in all brain areas with particularly high mRNA

Table 1
AA concentrations in plasma and brain (from: O'Kane and Hawkins, 2003; Abbott et al., 2010). CSF concentrations are presumably similar to those in the extracellular fluid of brain. Units are in μM.

Solute	Plasma	CSF	Ratio CSF/plasma
Total AA	2890	890	0.31
Neutral AA			
L-Gly	249	4.7–8.5	0.012–0.034
L-Ala	330	23.2–32.7	0.07–0.1
L-Ser	149	23.5–37.8	0.16–0.25
L-Pro	139	5	0.035
L-Thr	77	13	0.016
L-Cys	64	3	0.046
L-Met	20	1	0.05
L-Asn	53	8	0.15
L-Gln	669	547	0.82
L-His	40	4	0.1
L-Val	174	12	0.068
L-Leu	109	10.1–14.9	0.10–0.14
L-Ile	46	3	0.06
L-Phe	45	5	0.11
L-Tyr	61	7	0.11
L-Trp	30	1	0.03
Basic-positively AAs			
L-Arg	80	14.2–21.6	0.18–0.27
L-Orn	44	2	0.04
L-Lys	147	9	0.06
Acidic-negatively AAs			
L-Glu	83	1.79–14.7	0.02–0.18
L-Asp	4	0.02	0.005
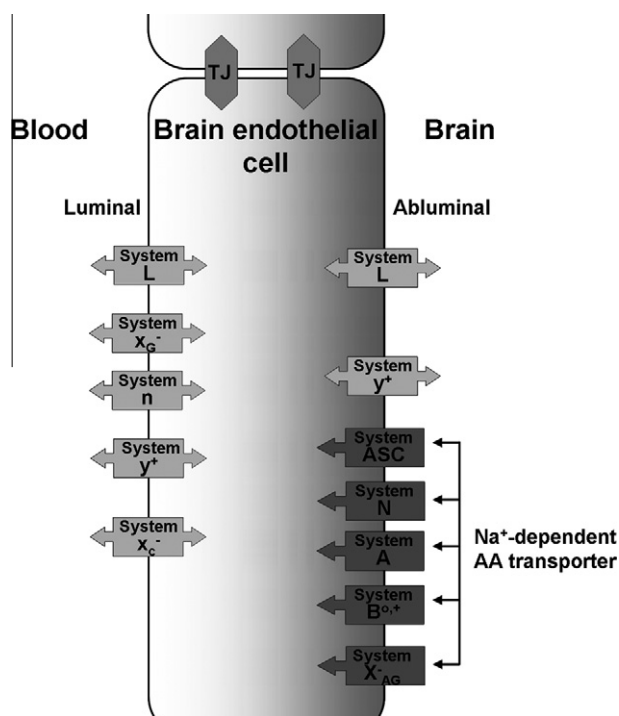
Fig. 2. AA transporter systems in the BBB. (A) AAs are transported by systems L and y^+ from blood to BECs and then into the brain. Five Na⁺-dependent transport systems on abluminal BEC membranes mediate AA efflux from the brain. Facilitative transporters x_G^- and n on the luminal membrane mediate L-glu, L-asp and L-gln efflux to blood.

expression levels in the meninges, and high protein levels have been located at border areas between the brain and the periphery, including BECs, ependymal cells, choroid plexus, and leptomenin-

ges (Sato et al., 2002; Burdo et al., 2006). System x_c^- expression in choroid plexus is relevant because it also contains high levels of GCL, GS and GGT (Tate et al., 1973; Okonkwo et al., 1974; and Orte et al., 1999) that could indicate high GSH consumption of GSH in this region, supporting its metabolic relevance.

Once L-cys$_2$ is incorporated into cells, it is rapidly reduced to L-cys (Ishii et al., 1991). Then, L-cys is taken up for protein and GSH synthesis inside BECs. However, because there does not seem to be a system to transport GSH to the CNS in BECs, L-cys must be transported as a free AA.

Fig. 2 shows that there are only two AA transporter systems on both the abluminal and luminal BEC membranes that provide essential gatekeeping functions by controlling AA uptake and efflux from the blood to the brain parenchyma.

System L-mediates L-cys efflux from BECs to CNS. System L is part of the SLC7 family that consists of Na⁺-independent, heterodimeric AA transporters with wide substrate selectivity (also systems x_c^-, y^+L, $b^{0,+}$, and asc). They consist of a light chain, which confers specificity, and a single heavy chain membrane-spanning type II membrane glycoprotein (Chillarón et al., 2001; Kanai and Endou, 2001). System L is the major route by which mammalian cells take up nutritionally essential AAs from extracellular fluids (Liu et al., 2004). System L operates as an obligatory 1:1 heteroexchanger (Meier et al., 2002). It consists of two catalytic subunits referred to as LAT1 (SLC7A5) and LAT2 (SLC7A8), which are linked by a disulfide bridge to the heavy chain 4F2hc (Kanai and Endou, 2001).

Although LAT2 is not found in the BBB (Killian and Chikhale, 2001; Kageyama et al., 2000), LAT1 is highly expressed in the BECs, the subfornical organ, the subcommissural organ, the ventromedial nucleus of the hypothalamus, the subgranular zone of the dentate gyrus, the ependymal layer of the lateral ventricles, and the olfactory bulb. Preston et al. (1989) determined their activity in choroid plexus. Measurements in membranes indicate that LAT1 is present in a luminal:abluminal ratio of 2:1 in the BBB (Sánchez del Pino

et al., 1995b; O'Kane, 2000). LAT1 mediates transport of essential zwitterionic AAs with large and small neutral side chains (e.g., L-leu, L-ile, L-val, L-trp, L-tyr, L-phe, L-thr, and L-met), with affinity constants similar to their plasma concentrations (Kanai et al., 1998; Verrey, 2003). It has been reported that the Km is 10- to 100-fold lower in the BBB as compared to system L in peripheral tissues (Pardridge, 1983). Because system L is a Na^+-independent AA exchanger, the net direction of transport depends on unidirectional transporters that are co-expressed in the cells (Del Amo et al., 2008).

In vitro studies have shown that LAT2, but not LAT1, accepts L-cys as substrate (Segawa et al., 1999; Meier et al., 2002). Nevertheless, *in vivo* experiments have suggested that LAT1 is able to transport L-cys in the BBB. [^{35}S]-Cysteine uptake was readily inhibited by the specific system L inhibitor 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH) in the luminal membrane of Ehrlich ascites tumor cells (Wade and Brady, 1981). *In situ* application of a brain perfusion technique to measure the rate of [^{14}C] L-ala passage between blood and brain demonstrated that L-cys produced similar uptake inhibition as the preferred AAs for system L, L-leu and L-phe (Benrabh and Lefauconnier, 1996). Killian and collaborators (2001) demonstrated that the relative *in vivo* affinities of LAT1 in the BBB are as follows: L-leu > L-phe > L-cys > L-ser > L-asn ⩾ L-ala ⩾ L-gly. Moreover, both LAT1 and LAT2 transport cysteine conjugates (Li and Whorton, 2005; Mokrzan et al., 1995). This discrepancy in substrate specificity between models of LAT1 expression has been also reported in human bladder carcinoma cells by Kim and colleagues (2002).

Before L-cys is oxidized to L-cys$_2$ in the extracellular space, it is transported against a concentration gradient to BECs by system ASC, which maintains low concentrations of L-cys/L-cys$_2$ at the CNS parenchyma (Zerangue and Kavanaugh, 1996b; Bridges, 2011; Kasai et al., 2011). System ASC is a Na^+-dependent, L-ala, L-ser and L-cys transporter that consists of two members (ASCT1 and ASCT2), although ASCT2 (SLC1A5) is expressed on BEC abluminal membranes (Tayarani et al., 1987; Kasai et al., 2011).

8. Modulation of L-cys/L-cys$_2$ transport systems in the BBB

To maintain appropriate GSH concentrations in the CNS under both normal and pathologic conditions, L-cys/L-cys$_2$ needs to be moved from circulating blood into the CNS through the BBB. Little is known about the modulation of L-cys/L-cys$_2$ incorporation through the BBB, which would require signals originating from both the CNS and blood (Limón-Pacheco et al., 2007).

BEC permeability and remodeling, as well as angiogenesis, can be modulated by conditioned medium containing soluble factors or co-culture with pericytes, astrocytes and neurons (Rist et al., 1997; for review see: Kim et al., 2006; Abbott et al., 2006; Shimizu et al., 2011). Moreover, the modulation of AA transporter systems by soluble factors or intracellular secondary messengers has been demonstrated. As mentioned above, it has been reported that astrocytes up-regulate luminal γGT expression in BECs (DeBault, 1981).

Chishty et al. (2002) used human umbilical vein endothelial cells to show that acute exposure to modulators of protein kinase A (PKA)- and protein kinase C (PKC)-dependent signal transduction pathways significantly reduced the maximal transport capacity and diffusion constants of system L, but the transport affinity remained unchanged. An *in vitro* study in the b.End3 mouse brain capillary endothelial cell line suggests that astrocytes positively modulate the transcription, expression and uptake functionalities of system L but do not affect BEC transport activity (Omidi et al., 2008). In vascular smooth muscle cells, treatment with platelet-derived growth factor (PDGF) stimulates LAT1 mRNA and protein

expression in a time-dependent manner *via* mammalian target of rapamycin (mTOR) kinase activation (Liu et al., 2004).

EAAC1 is also modulated by PDGF in primary neuronal cultures; it induces a nearly twofold increase in surface expression mediated by phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB) (Fournier et al., 2004). Cell surface levels of EAAC1 protein are regulated by different stimuli, such as PKC subtypes, growth factor receptor activation, NMDA receptor activation, and the glutamate transporter-associated protein 3-18 (GTRAP) (for reviews see Robinson, 2006 and Aoyama et al., 2012). An *in vitro* study in C6 glioma cells showed that PKCα activation is related to the redistribution of EAAC1 to the plasma membrane, whereas PKCε increases transport activity without affecting EAAC1 surface expression (González et al., 2002). Some GTRAPs modulate glutamate transporters activity increasing transport V_{max} (Hyde et al., 2003) whereas GTRAP3-18 inhibits glutamate transport activity (Lin et al., 2001). GTRAP3-18-deficient mice showed increased plasma membrane EAAC1 expression, increased neuronal GSH content and better performance on motor/spatial learning and memory tests than wild-type mice, indicating that EAAC1 is important for learning processes (Aoyama et al., 2011).

System x$_c$$^-$ is upregulated in primary astrocyte cultures following treatment with dibutyryl-cAMP (Gochenauer and Robinson, 2001). *In vitro* studies have demonstrated that activation of PKA and PKC inhibits system x$_c$$^-$ activity in rat cortical astrocytes (Tang and Kalivas, 2003). However, PKA activation was also associated with system x$_c$$^-$ induction, not inhibition, in astrocytes and HT22 cells (Lewerenz et al., 2003).

9. Transcriptional regulation of AA transporter systems

xCT expression is reportedly modulated by numerous stimuli (for review see Lewerenz et al. 2011), including electrophilic agents (Kim et al., 2001; Hosoya et al., 2002), oxygen (Bannai et al., 1989), bacterial lipopolysaccharide, nitric oxide and nuclear factor erythroid-2-related factor (Nrf2) overexpression. Four antioxidant response elements (ARE) motifs (Sasaki et al., 2002), as well as a tandem of amino acids response elements (AARE's) (Sato et al., 2004), which are putative binding sites for NFκB, and multiple putative AP-1 (activator protein 1) binding sites (Sato et al., 2001) are found in the proximal promoter sequences of both human and mouse xCT. These observations are in accordance with postulated models that imply modulation of xCT expression by these effectors.

The rat LAT1 proximal promoter was cloned, and numerous potential cis-acting elements were predicted by database analysis (MatInspector and TRANSFAC; Campbell and Thompson, 2001) for Sp1 (specificity protein 1), AP1, and glucocorticoid receptor, as well as two ARE consensus sequences and several sites which have 78–100% identity with the AARE consensus sequence (Diah et al., 2001). In this sense, *in vitro* models that involve ROS generation due to intoxication by TCDD, CCl$_4$ or the pesticide piperonyl butoxide has been demonstrated to upregulate LAT1 mRNA (Shultz et al., 1999; Muguruma et al., 2007) and protein levels (Sarkar et al., 1999). Boado and colleagues (2004) observed a down-regulation of LAT1 at the mRNA level but not at the protein level. They demonstrated that LAT1 mRNA is bound to several polysome proteins in rabbit BECs, and that it is regulated posttranscriptionally during development. There are some studies suggesting LAT1 modulation at mRNA level, but the mechanisms have not been elucidated. In a hepatic epithelial cell line, arginine depletion resulted in an approximately 10-fold elevation of steady-state levels of LAT1 mRNA, which is surprising, as arginine is not a substrate for this transporter (Shultz et al., 1999; Campbell et al., 2000). It has been suggested that aldosterone (Mastroberardino et al., 1998),

arginine-vasopressin and adrenergic agents (Duelli et al., 2000) are involved in regulating LAT1 expression. Pathologic conditions, such as hepatic encephalopathy or aminoaciduria, can also alter BBB LAT1 expression (Duelli et al., 2000). An *in vitro* study showed that LAT1 is initially down-regulated due to mRNA de-stabilization during hypoxia in cultured bovine brain capillary endothelial cells (Boado et al., 2003).

The promoter region sequence of EAAC1 has at least one ARE motif in various mammalian species, even though Nrf2-ARE signaling is not necessary for basal EAAC1 expression (Escartin et al., 2011). Hypoxic-ischemic episodes also decrease the EAAC1 expression (Martin et al., 1997; Dallas et al., 2007; Montori et al., 2010). Both mGluR1 and mGluR5 activation contribute to increased EAAC1 translation, which is mediated by mTOR and the extracellular signal-regulated kinase (ERK) signaling pathways (Ross et al., 2011).

The expression of ARE-containing genes is upregulated by the binding of Nrf2, which is translocated into the nucleus in response to redox status changes (Osburn and Kensler, 2008; Giudice et al., 2010). Nrf2 can also be translocated if it is phosphorylated by a number of kinase cascades (e.g., PKC, PI3K, JNK, p38, and ERK) (Kandil et al., 2010; Lima et al., 2008; Limón-Pacheco et al., 2007).

It has been reported that neurotrophic factors prevent the toxic effects of a variety of ROS-generating compounds *via* GSH upregulation in both *in vivo* and *in vitro* neural models (Mena et al., 1997; Gong et al., 1999; Pan and Perez-Polo, 1993; Spina et al., 1992; Jackson et al., 1994; Guégan et al., 1999). It is possible that these neurotrophic factors also modulate AA transporter systems related to L-cys/L-cys$_2$ in the BBB.

10. System L as modulator of signaling pathways

The role of the system L in modulating signaling pathways such as mTOR (Hyde et al., 2003; Fuchs and Bode, 2005; Nicklin et al., 2009) and the subsequent up-regulation of the system L transporter has recently been demonstrated (Edinger and Thompson, 2002). mTOR is a serine/threonine protein kinase that regulates tightly controlled processes of cell growth, such as increased cell mass/size and proliferation, by controlling mRNA translation in response to nutrient availability (Wullschleger et al., 2006; Avruch et al., 2008). For this reason, it is not surprising that LAT1 is over-expressed in cultured cells and malignant tumors; it likely supports their continuous growth and proliferation (Yanagida et al., 2001; Kim et al., 2002). Thus, regulating LAT1 at both luminal and abluminal BEC membranes could play an important role in the brain's adaptive response to systemic and localized abnormal redox status. In fact, the brain is selectively protected from hypoaminoacidemia due to malnutrition (Freedman et al., 1980) and systemic GSH synthesis inhibition, at least in the first hours (Limón-Pacheco et al., 2007). This protection may be due to an up-regulation of BBB LAT1 gene expression or activity.

11. Conclusion remarks

GSH synthesis is highly dependent on L-cys$_2$/L-cys levels, mainly in CNS. Thus, it becomes relevant to identify the transport systems that incorporate these AAs and the mechanisms that regulate their expression and function. Neurons require EAAC1 transport system to incorporate L-cys, however extracellular L-cys levels are astrocyte-GSH dependent, which in turn depends on L-cys$_2$ incorporation by system x$_c^-$. EAAC1 and xCT are modulated by stress and nutritional conditions that could be pharmacologically manipulated to enhance GSH levels in CNS. *In vivo* models demonstrate that the incorporation of L-cys$_2$/L-cys into the CNS requires the expression of transport system x$_c^-$ and L at the BBB, where LAT1

regulation seems to play an important role in the brain adaptive response to systemic and localized abnormal redox status. We have nowadays a better understanding of the transcription factors and kinases responsible for the modulation of L-cys$_2$/L-cys transport. The use of knock-out mice or inhibitors/enhancers of these modulating factors could become important strategies to deal with CNS damage due to GSH down-regulation.

Acknowledgements

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Discusión

Nuestros resultados concuerdan con trabajos previamente reportados donde se observó la expresión de *ngfb* de manera basal en hígado (Gezginci-Oktayoglu et al., 2011, Bonacchi et al., 2008), además de que cambios en el estado redox sistémico inducen su expresión en modelos murinos (Oakley et al., 2003). Dado que la disminución en los niveles de GSH se logró mediante agentes químicos con mecanismos de acción particulares, la inhibición en su síntesis, por BSO (Griffith, 1982), así como por un incremento en su consumo, mediante la exposición con iAs (Tapio & Grosche, 2006) y APAP (Martin-Murphy et al., 2010); se observó que, aunque en todos los casos se incrementaron los niveles de mRNA de *ngfb*, la rapidez así como la duración de dicho incremento depende de las características toxicodinámicas y toxicocinéticas de cada agente químico (Resultados II, Figura 1). Esto sugiere que la síntesis de *ngfb* es dependiente del estado redox, sin importar el mecanismo por medio del cual sea alterado. Con lo cual, si esto se traduce a cambios en los niveles de proteína, dado que NGF es secretado al espacio extracelular y finalmente se distribuye por torrente sanguíneo, sugeriría que NGF juega un rol como sensor redox, el cual dada la información sobre su acción en otros tejidos (Yamamoto et al., 1996, Sofroniew et al., 2001, Vega et al., 2003, Navarro-Tableros et al., 2004), sería capaz de integrar respuestas antioxidantes sistémicas homeostáticas.

Debido a problemas metodológicos, no fue posible determinar si los cambios en los niveles de mRNA de *ngfb* reflejaban un incremento a nivel de proteína. Sin embargo, dado que los cambios en la transcripción de *ngfb* se asociaron con la activación de la cascada de

señalización NGF/TrkA/PI3K/PDK1/Akt/NFκB (Resultados II, Figura 2A, 2B, 6A y 6B) así como con el incremento en la expresión de *Tx-1* (Resultados II, Figura 6C) se sugiere un aumento en los niveles de proteína de NGF, además de que dicho incremento es capaz de ejercer un efecto autócrino, similar al reportado para otros tejidos ante daño tisular (Oakley et al., 2003, Caporali et al., 2008). Aún más, dado que la activación de TrkA fue evitada por el pre-tratamiento con el anticuerpo neutralizante de NGF (Resultados II, Figura 2A) sugiere que los cambios observados en la transcripción de *ngfb* ante estrés oxidante en tejido hepático se asocian con cambios en los niveles de NGF en plasma y fluido extracelular, además de que permite hipotetizar que el hígado no es el único tejido que responde ante el estrés oxidante sistémico incrementando la síntesis de *ngfb*; sobre todo, si se toma en cuenta que si bien en el presente trabajo solo se evaluó el efecto en hígado, ya que ha sido demostrado que los agentes químicos empleados son capaces de ejercer un daño oxidante en varios tejidos (Griffith & Meister, 1979b, Stern et al., 2005, Hughes & Kitchin, 2006)

Puesto que se ha observado en modelos humanos que ante eventos de estrés psicológico se incrementan los niveles de NGF en plasma (Aloe et al., 1994) fue necesario el demostrar que los cambios observados en la transcripción de *ngfb* y la activación de la vía TrkA/Akt/NFκB son debidos a cambios en el estado redox. Para ello se realizó un pre-tratamiento con N-acetil-cisteína (NAC) como fuente exógena de grupos tiol (Limon-Pacheco et al., 2007), el cual previno la fosforilación de TrkA inducida por BSO, iAs o APAP (Resultados II, Figura 3A). Esto concuerda con el reporte previo donde se observa que la dosificación de NAC es capaz de inhibir la activación de TrkA en la línea celular PC12 (Kamata et al., 2005). Además, nuestros datos sugieren que cambios en el estado

redox son los responsables de la modulación a nivel de mensajero de *ngfb* por el tratamiento con BSO (Resultados II, Figura 3B).

El hígado es un órgano heterogéneamente complejo, ya que se debe tomar en cuenta su estructura tridimensional, su fisiología y sus componentes celulares para la comprensión de los fenómenos que ocurren en la patogénesis de una enfermedad o bien en el planteamiento de mecanismos de prevención. En el hígado sano es posible encontrar al menos 15 tipos celulares. Los hepatocitos comprenden cerca del 60 % del total de las células y el 80 % del volumen del órgano, convirtiéndolos en el tipo celular más numeroso, mientras que las células endoteliales sinusoidales, las células de Kupffer, las células estelares y las células del epitelio biliar comprenden del 3 al 20 % cada una del total de células biológicamente importantes (Malarkey et al., 2005).

Los hepatocitos son quienes contienen toda la maquinaria necesaria para llevar a cabo las funciones vitales del órgano. Cerca del 15 % del volumen de cada hepatocito lo conforma el retículo endoplasmático, que es donde se lleva a cabo el metabolismo de fármacos, además de contener cerca de 1,000 mitocondrias, 30 lisosomas y 500 peroxisomas. Son células morfológicamente hexagonales, cuyas superficies contactan con células del parénquima adyacente, canalículos biliares frontera, o están expuestos al espacio perisinusoidal. Su disposición espacial es como cordones en placa de una célula de espesor, denominado *muralium*, que se ramifican y anastomosan limitados por las regiones portales (Malarkey et al., 2005).

Las células de Kupffer son derivadas de monocitos circulantes y representan el 15 % de las células del hígado, cumplen funciones fagocíticas y son las mayores productoras

de citocinas (Bykov et al., 2004). Finalmente, las células estelares, comprenden cerca del 5 % de las células del hígado, son el principal tipo celular responsable en la regeneración, fibrogénesis y cirrosis hepática (Mabuchi et al., 2004). De manera basal producen la matriz extracelular, controlan el tono microvascular, metabolizan y son sitio de almacenamiento de la vitamina A y lípidos. Cuando el hígado sufre un daño, las células estelares reciben señales secretadas por hepatocitos dañados y células inmunes, lo que conlleva a que se diferencien en miofibroblastos activados con capacidad proliferativa, fibrogénica y secretan citocinas y factores de crecimiento que promueven la regeneración hepática; sin embargo, en procesos inflamatorios crónicos son capaces de inducir procesos de muerte celular (Yin et al., 2013, Mabuchi et al., 2004).

El presente trabajo se realizó en homogenado de tejido hepático de ratón, lo cual imposibilitó determinar el tipo celular responsable del incremento en la expresión de *ngfb* y de la activación de la vía de señalización NGF/TrkA/PI3K/PDK1/Akt/NFκB. Sin embargo, en cortes de hígado de ratón observamos por inmunofluorescencia la expresión de TrkA en hepatocitos (Resultados II, métodos suplementarios, Figura 1S) como había sido previamente reportado (Gezginci-Oktayoglu et al., 2011). Además, existe evidencia que sugiere que dentro del hígado, las células estelares y los hepatocitos son responsables del incremento en la síntesis de NGF ante daño por cambios en el estado redox (Oakley et al., 2003, Lin et al., 2009, Yin et al., 2013). Sin embargo, aunque las células estelares expresan el receptor $p75^{NTR}$, la cascada de señalización asociada a $p75^{NTR}$ es la activación de mecanismos proapoptóticos y de diferenciación (Passino et al., 2007, Lin et al., 2009, Oakley et al., 2003), así como de inducción de fibrosis hepática en modelos murinos de hipertiroidismo (Kumar et al., 2007, Zvibel et al., 2010), mientras que la activación de

TrkA en hepatocitos está asociada con la estimulación de la proliferación celular (Gezginci-Oktayoglu et al., 2011). Por lo que la evidencia sugiere que en nuestras condiciones experimentales son los hepatocitos quienes responden a NGF a través de la vía TrkA/PI3K/PDK1/Akt/NFκB *Tx-1*.

A pesar de que en nuestro modelo experimental la exposición a BSO decreció significativamente la concentración de NF-κB en núcleo (Resultados II, Figura 6A), la expresión transcripcional de *iκbα* se incrementó (Resultados II, Figura 6B). En condiciones basales NF-κB se encuentra secuestrado en citoplasma por una familia de inhibidores denominados IκB (inhibidores de κB), la activación del factor de transcripción trae consigo la degradación de dichos inhibidores y la inducción de su transcripción por el mismo NF-κB en un clásico modelo de retroalimentación negativa (Quan et al., 1997). En ese sentido, *iκbα* es el gen que codifica para el principal inhibidor de NF-κB, por lo que la determinación de cambios en los niveles de mRNA mensajero de *iκbα* es uno de los mejores marcadores de la actividad de NF-κB. Por lo tanto, en nuestro modelo, el tratamiento con BSO indujo la activación de NF-κB en el hígado de ratón, lo cual concuerda con reportes previos en modelos *in vitro* e *in vivo* (Chia et al., 2010, Limon-Pacheco et al., 2007). Aún más, dado que el pre-tratamiento con el anticuerpo neutralizante anti-NGF fue capaz de evitar el incremento transcripcional de *iκbα* (Resultados II, Figura 6B), refuerza la hipótesis de que la cascada de señalización activada por NGF es la responsable de la activación del factor de transcripción NF-κB mediante la vía TrkA/PI3K/PDK1/Akt en el hígado de ratones tratados con BSO, o bien juega un papel clave en su activación y sugiere que el dominio intracelular de p75^{NTR} no participa en la respuesta observada.

La activación de NF-kB es relevante ya que es un factor de transcripción que regula genes de respuesta temprana, incluyendo a otros factores de transcripción, citocinas, mediadores inflamatorios y procesos celulares como la respuesta a estrés y crecimiento (Schreck et al., 1991, Baeuerle & Henkel, 1994, Song et al., 2008). Parte de sus funciones es prevenir o mediar procesos apoptóticos en el hígado (Montiel-Duarte et al., 2004), y recientemente se ha observado que juega un papel relevante en la inducción de respuestas antioxidantes y de sobreviencia en modelos de estrés oxidante (Denk et al., 2000, Morante et al., 2005, Li & Karin, 1999)

En ese sentido, la activación de NF-κB mediante la vía de señalización NGF/TrkA/PI3K/PDK1/Akt ha sido demostrada en modelos *in vitro*, en líneas celulares como la PC12 (Bui et al., 2001), la línea de riñón de embrión humano 293 (HEK 293) y en fibroblastos de embrión de ratón 3T3 (NIH-3T3), donde se ha observado que la proteína cinasa atípica C, p62, funge como proteína de andamiaje para inducir la respuesta de sobrevivencia y diferenciación de la vía NGF/TrkA/PI3K/PDK1/Akt mediante la activación de NF-κB (Wooten et al., 2001, Wooten et al., 2000). Por otra parte, la activación de la vía de NGF/p75^{NTR} en ratas tratadas con CCl$_4$ inhibió la unión de NF-κB con el DNA en células estelares (Oakley et al., 2003). Y si bien, como ya se ha mencionado, el dominio intracelular de p75^{NTR} es capaz de iniciar una respuesta antioxidante, no requiere de la unión a ligando para que se lleve a cabo (Tyurina et al., 2005). Finalmente, como se mencionó anteriormente p75^{NTR} tiene la capacidad de interactuar con los dominios citoplasmáticos y transmembranales de TrkA lo cual modula la afinidad y especificidad de los Trk's a las NT's (Huang & Reichardt, 2003), así como de alterar la cascada de señalización mediada por cada receptor por separado (Kaplan & Miller, 1997, Yoon et al.,

1998). Por lo cual, nuestros resultados experimentales sugieren la participación de la cascada de señalización NGF/TrkA/PI3K/PDK1/Akt/NFκB pero aún queda por determinar si la respuesta observada está mediada solo por TrkA, o TrkA con p75[NTR].

Conforme lo reportado por nuestro grupo de trabajo, el tratamiento con BSO incrementó la expresión de mRNA de *Tx-1* en el hígado de ratón como mecanismo de compensación o protector ante el cambio en el estado redox (Resultados II, Figura 6C) (Limon-Pacheco et al., 2007). Cómo se mencionó anteriormente, esta sulfoproteína cataliza reacciones de oxidorreducción (Jurado et al., 2003) y es capaz de regular factores de transcripción como NF-κB (Hanschmann et al., 2013), además de modular MAPK, como a ASK1 (Lu & Holmgren, 2012). Además dentro de su secuencia regulatoria *Tx-1* presenta varios motivos potenciales de unión a NF-κB (Kaghad et al., 1994) así como motivos de unión a CREB (Masutani et al., 2004), los cuales forman parte de la cascada de señalización mediada por TrkA (Sofroniew et al., 2001). De hecho, trabajos previos en modelos *in vitro* en la línea celular PC12 han reportado que dentro de los genes que induce la vía NGF/TrkA se encuentra *Tx-1* participando en mecanismos de sobrevivencia celular (Masutani et al., 2004), lo cual confirma el por qué el pre-tratamiento con el anticuerpo neutralizante anti-NGF es capaz de evitar el incremento en su transcripción ante BSO y, mucho más interesante, que el tratamiento con sólo el anticuerpo anti-NGF reduce su expresión, lo cual sugiere que la vía de señalización activada por NGF se encuentra activada de manera basal y que juega un papel significativo en la regulación basal de la expresión de esta proteína (Resultados II, Figura 6C).

Si bien el efecto en la disminución de GSH en hígado por BSO bajo el mismo modelo experimental ya ha sido documentado (Limon-Pacheco et al., 2007), de manera no

esperada, aunque el cotratamiento de BSO con el anticuerpo neutralizante anti-NGF no afectó la disminución en los niveles hepáticos de GSH, el tratamiento por sí solo del anticuerpo decreció los niveles de GSH y aún más interesante, incrementó los de GSSG (Resultados II, Figura 5A y 5B), lo cual sugiere que, en conjunto con la modulación de *Tx-1*, la cascada de señalización mediada por NGF cumple un papel regulador de las enzimas y proteínas que mantienen la homeostasis redox, al menos en hígado, y que probablemente dentro de las enzimas que modula se encuentra la GSSG-Rd y el ciclo de las pentosas. Lo cual está en concordancia con el incremento en los niveles de GSH en el hígado de organismos transgénicos que sobre expresan NGF (Arsenijevic et al., 2007).

En conclusión en el presente trabajo encontramos evidencia que sugiere que NGF activa la vía TrkA/PI3K/PDK1/Akt/NFκB en hígado de ratón, la cual ayuda a mantener de manera basal el estado redox en este órgano. Además, la transcripción y síntesis de NGF se incrementa en hígado por diferentes agentes cuyo mecanismo de acción es la generación de daño por estrés oxidante, activando, al menos en parte, una respuesta antioxidante (Resultados II, Figura 7).

Perspectivas

La modulación y el papel que juega NGF en hígado en el presente modelo se enmarca en el trabajo previo de nuestro grupo de trabajo dónde se demostró una respuesta

órgano específica así como un probable evento de comunicación inter órgano. La inhibición en la síntesis de GSH por BSO, disminuyó el contenido de GSH en hígado y en riñón, mas en cerebro se observó un incremento en la síntesis de GSH asociado con un aumento en la transcripción de *Nrf2*, *GCLc* y la subunidad responsable de la incorporación de cistina, *xCT,* todo ello mediado, al menos en parte, por ERK2 y el factor de transcripción Nrf2 (Limon-Pacheco et al., 2007).

Estas evidencias, sumado a los trabajos dónde se demuestra la expresión y modulación de NGF y su cascada de señalización vía TrkA bajo modelos de estrés en tejidos periféricos (Wooten et al., 2001, Wooten et al., 2000, Guegan et al., 1999, Arsenijevic et al., 2007, Vega et al., 2003, Navarro-Tableros et al., 2004, Caporali et al., 2008, Abram et al., 2009, Micera et al., 2001) así como su incremento ante estrés físico (Aloe et al., 1994), sugiere un papel sistémico de NGF como un modulador neuro-inmuno-endocrino con funciones fundamentales en la regulación de procesos homeostáticos, como fue sugerido previamente (Levi-Montalcini et al., 1990).

De tal manera que, el incremento observado en la transcripción de NGF en hígado es probable que suceda en otros órganos y tejidos ante cambios en el estado redox, lo cual permitiría un incremento en plasma de NGF, tal como se ha observado en diversos modelos de estrés (Guegan et al., 1999, Arsenijevic et al., 2007, Wang et al., 2013, Rasi et al., 2007, Aloe et al., 1994), con lo cual el NGF circulante podría orquestar una respuesta de neuro-protección. Dado, que las NT son poco permeables en la barrera hematoencefálica (BHE) (Pan et al., 1998) el mecanismo por medio del cual se daría la comunicación entre SNC y tejidos periféricos es posible que involucre la participación de las células endoteliales de

cerebro (CEC), las cuales expresan el receptor TrkA y son capaces de secretar NGF ante

condiciones que modifican el estado redox (Tanaka et al., 2004, Moser et al., 2004).

Bajo este modelo hipotético, una vez que se han incrementado los niveles de NGF

en el SNC, se une a su receptor TrkA activa la cascada de señalización que activa una

respuesta antioxidante y/o de sobrevivencia, mediado, al menos en parte, por Nrf2 (Satoh et

al., 2013, Limon-Pacheco et al., 2007, Kosaka et al., 2010). Dentro de esta respuesta, en el

trabajo previo, en el cerebro se observó un incremento en el contenido de GSH, el cual

dado que el GSH no atraviesa la BHE (Anderson et al., 1989), debe depender de la síntesis

de novo, el cual es dependiente de la concentración de los aminoácidos L-cys y $L-cys_2$

(Meister & Anderson, 1983, Shih et al., 2006). Estos aminoácidos dependen de

transportadores para atravesar la BHE, como lo son xCT, LAT1 y EAAC1. xCT participa

en la incorporación de L-cys2 por astrocitos y CEC de la BHE, mientras que LAT1 es una

subunidad del systema L, el cual es el único transportador capaz de translocar L-cys de las

CEC hacía el parénquima neuronal, y EAAC1 forma parte del sistema x-AG el cual

incorpora en las neuronas L-cys para la síntesis de GSH (Resultados III, Figura 1 y 2). Cabe

señalar que estos transportadores contienen posibles elementos de respuesta antioxidante

(ARE) en su secuencia promora.

Tomando en cuenta lo antes mencionado, es posible hipotetizar que los cambios en

el estado redox sistémico inducen respuestas órgano específicas que incluyen la síntesis de

NGF, lo cual incrementa los niveles de NGF circulante en plasma. Este NGF periférico

activaría la cascada de señalización en las CEC que inducirían la incorporación de L-cys y

$L-cys_2$ en el parenquima cerebral, además de secretar NGF en el SNC dónde jugaría un

papel importante en la repuesta antioxidante neuroprotectora en la que intervendría la cascada de señalización NGF/TrkA.

Cabe señalar que en atletas de alto rendimiento se ha documentado la presencia de elevados niveles de NGF en suero. En el mismo trabajo los autores sugieren una asociación con la respuesta alérgica documentado mediantes pruebas clínicas (Bonini et al., 2013), sin embargo los autores sugieren que el incremento de NGF en suero puede ser producto del ejercicio físico solamente sumada y/o una consecuencia del ejercicio extremo e inflamación muscular. Por otro lado varios trabajos han reportado cambios en los niveles tisulares de NGF en pacientes diagnosticados o tratados por disfunción en la vegija urinaria (Ochodnicky et al., 2011).

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