UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS - BIOQUÍMICA

Investigação dos efeitos da ornitina e da homocitrulina sobre as homeostases energética e redox em cerebelo e cultura de astrócitos de córtex cerebral de ratos

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ÂNGELA ZANATTA

ORIENTADOR: PROF. DR. MOACIR WAJNER

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“Às vezes você consegue o que deseja e às vezes você consegue o que precisa e, às vezes, você consegue o que você consegue”

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INTRODUÇÃO E OBJETIVOS

RESUMO

A síndrome hiperornitinemia-hiperamonemia-homocitrulinúria (HHH) é um erro inato do metabolismo do ciclo da ureia, de caráter autossômico recessivo e caracterizada por um defeito no transporte mitocondrial de ornitina (Orn), levando a um acúmulo citosólico desse aminoácido, além do acúmulo de homocitrulina (Hcit) e amônia. Os sinais e sintomas clínicos são variáveis, dentre eles podemos citar coagulopatia, coma, letargia, retardo mental, atrofia cortical e ataxia cerebelar. Embora os sintomas neurológicos sejam frequentes, a fisiopatogenia dessa doença ainda é desconhecida. Nesse trabalho investigamos os efeitos *in vitro* e *ex vivo* da Orn e Hcit sobre importantes parâmetros de homeostase redox e energética em cerebelo de ratos de 30 dias de vida. Além disso, também investigamos os efeitos desses metabólitos sobre alguns parâmetros em astrócitos cultivados de córtex cerebral. Primeiramente investigamos os efeitos *in vitro* da Orn e Hcit sobre importantes parâmetros de homeostase redox e energética em cerebelo de ratos jovens. Ambos metabólitos aumentaram significativamente os níveis de substâncias reativas ao ácido tiobarbitúrico (TBA-RS). Além disso, esses níveis foram totalmente prevenidos pela melatonina e pela glutationa reduzida (GSH). Observamos também que os níveis da produção de nitratos e nitritos não foi alterada por nenhum dos metabólitos utilizados, por outro lado, a Hcit aumentou a produção de peróxido de hidrogênio. Ao investigarmos os níves de GSH observamos que tanto a Orn quanto a Hcit reduziram esse parâmetro e que a Orn reduziu o conteúdo de grupamentos sulfidrila, indicando uma dimunioção nas defeas antioxidantes não enzimáticas. Com relação ao metabolism energetic, a Orn e Hcit diminuíram a atividade da enzima aconitase, porém não alteraram outros parâmetros estudados. Além disso, a redução causada pela Orn foi prevenida pela GSH, indicando que essa enzima é suscetível a oxidação causada por exxe aminoácido. Também investigamos os efeitos *ex vivo* da injeção intracerebelar de Orn e Hcit sobre importantes parâmetros de estresse oxidativo e sobre a ativdade da Na^+, K^+ ATPase. A Orn aumentou significativamente os níveis de TBA-RS e atividade da superóxido dismutase, catalase, glutationa peroxidase, glutationa redutase e glucose 6-fosfato desidrogenase e diminuiu a ativdade da Na^+,K^+-ATPase. Por outro lado, as concentrações de GSH não foram alteradas pelo tratamento com ornitina. Por fim, a Hcit não foi capaz de alterar nenhum dos parâmetros avaliados. Os efeitos da Orn e Hcit foram também investigados em astrócitos cultivados a partir de córtex cerebral de ratos neonatos. Observamos que Orn e Hcit diminuíram a viabilidade celular, a função mitocondrial e os níveis de GSH, porém não alteraram a produção de citocinas. Nossos resultados demonstram um prejuízo moderado na homeostase energética, porém, quando observamos a homeostase redox, observamos um dano mais pronunciado. Dessa forma, concluímos que alterações na homeostase energética, mas principalmente na homeostase redox podem estar envolvidos na fisiopatogenia do dano neurológico observado nos pacientes com a síndrome HHH.

ABSTRACT

Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome is an autosomal recessive disorder of urea cycle characterized by a defect in the transport of ornithine (Orn) into mitochondrial matrix leading to accumulation of Orn, homocitrulline (Hcit) and ammonia. The clinical signs and symptoms are variable, ranging from coagulopathy, coma, lethargy, mental retardation, cortical atrophy and cerebellar ataxia. Although neurological symptoms are frequent, the pathophysyology of this disorder is poorly known. In the present study, we investigated the *in vitro* and *ex vivo* effects of Orn and Hcit on important parameters of redox and energy homeostasis in cerebellum of 30-day-old rats. Besides that, we also investigated the effects of these metabolites on some parameters in cultured cortical astrocytes. We first studied the *in vitro* effects of Orn and Hcit on important parameters of redox and energy homeostasis in cerebellum of young rats. Orn and Hcit significantly increased thiobarbituric acid reative species (TBA-RS) levels that was totally prevented by melatonin and reduced glutathione (GSH). We also found that nitrate and nitrite production was not altered by any of the metabolites, in contrast to hydrogen peroxide production, which was significantly enhanced by Hcit. These results suggest that probably reactive nitrogen species were not involved on the observed results. Furthermore, GSH concentrations were significantly reduced by Orn and Hcit and sulfhydryl content by Orn, implying an impairment of antioxidant defenses. As regards to energy metabolism, Orn and Hcit provoked a significant reduction of aconitase activity, without altering other parameters of energy metabolism. Furthermore, Orn-elicited reduction of aconitase activity was totally prevented by GSH, indicating that critical groups of this enzyme were susceptible to oxidation caused by this amino acid. We also investigated the *ex vivo* effects of intracerebellar administration of Orn and Hcit on important parameters of oxidative stress and on Na^+, K^+ ATPase activity. Similarly with our *in vitro* results, Orn significantly increased TBA-RS levels and the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glucose 6-phosphate dehydrogenase (G6PDH), and reduced Na^+,K^+-ATPase activity. In contrast, GSH concentrations were not changed by Orn treatment. Furthermore, intracerebellar administration of Hcit was not able to alter any of these parameters. The effects of Orn and Hcit were also investigated in cultured cortical astrocytes. We observed that Orn and Hcit diminished cellular viability, mitochondrial function, GSH levels, without altering cytokine production. Our results demonstrate a mild impairment in energy metabolism, but mostly in redox homeostasis in cerebellum and cortical cultured astrocytes. Therefore, we conclude that alterations of energy metabolism and especially redox homeostasis may be involved in the pathophysiology of the neurological damage observed in patients with HHH syndrome.

LISTA DE ABREVIATURAS

8-OHdGA – 8-hidróxi-2'-deoxiguanosina

AL – argininosuccinato liase

AS – argininosuccinato sintetase

BB-CK – creatina quinase cerebral

CAT – catalase

ci-CK – creatina quinase citosólica

CK – creatina quinase

CPS – carbamoil fosfato sintetase

EIM – erro inato do metabolismo

ER – espécie reativa

ERN – espécie reativa de nitrogênio

ERO – espécie reativa de oxigênio

G6PDH – glicose 6-fosfato desidrogenase

GPx – glutationa peroxidase

GR – glutationa redutase

GSH – glutationa reduzida

GSSG – glutationa oxidada

Hcit – homocitrulina

HNE – 4-hidroxinonenal

LA – ácido lipóico

LDL – lipoproteínas de baixa densidade

L-NAME – L-NG-nitroarginina metil Ester

MB-CK – creatina quinase específica de músculo cardíaco

MDA – malondialdeído

MiaCK – creatina quinase ubíqua

MibCK – creatina quinase sarcomérica músculo específica

MiCK – creatina quinase mitocondrial

MM-CK – creatina quinase específica de músculo esquelético

MTT – 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio

Nrf-2 – fator eritróide nuclear 2 relacionado ao fator 2

OAT – ornitina amino transferase

ORC – carreador de ornitina

Orn – ornitina

ORNT – ornitina translocase

OTC – ornitina transcarbamilase

Pi – fosfato inorgânico

PUFA – ácido graxo de cadeia poliinsaturada

Síndrome HHH – Síndrome hiperornitinemia – hiperamonemia – homocitrulinúria

SNC – sistema nervoso central

SOD – superóxido dismutase

TBA-RS – espécies reativas ao ácido tiobarbitúrico

TRO – trolox

I.1 INTRODUÇÃO

I.1.1 Erros inatos do metabolismo

O termo erros inatos do metabolismo (EIM) foi utilizado pela primeira vez por Archibald Garrod em 1908 durante estudos realizados em pacientes com alcaptonúria, doença em que os pacientes afetados excretam grandes quantidades de ácido homogentísico na urina. O pesquisador observou que um ou mais indivíduos da mesma família eram afetados sem que seus pais apresentassem a doença. Baseado também na observação da maior incidência de consanguinidade entre os pais dos pacientes e nas leis de Mendel, Garrod propôs um modelo de herança autossômica recessiva para este distúrbio. Através da determinação do ácido homogentísico na urina de pacientes com alcaptonúria e da observação de que esta substância era um metabólito normal da degradação da tirosina, Garrod relacionou este acúmulo a um bloqueio no metabolismo do ácido homogentísico. Verificou-se mais tarde que tais alterações resultavam da síntese qualitativa ou quantitativamente anormal de uma proteína, usualmente uma enzima (Scriver et al, 2001). Presumiu-se então que, em consequência deste bloqueio metabólico, pode ocorrer o acúmulo de precursores tóxicos da reação catalisada pela enzima envolvida, com a formação de rotas metabólicas alternativas e a deficiência de produtos essenciais ao organismo (Bickel, 1987).

Até o momento se conhece o defeito bioquímico em mais de 600 EIM, a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Scriver et al, 2001). Embora

individualmente raras, essas doenças em seu conjunto afetam aproximadamente 1 a cada 500 a 1.000 nascidos vivos (Baric et al, 2001).

I.1.2 Doenças do metabolismo da ornitina

A ornitina (Orn) é um aminoácido não proteico que exerce um papel fundamental no metabolismo da ureia, da creatinina e das poliaminas. As vias metabólicas da Orn interagem com o ciclo da ureia e o ciclo dos ácidos tricarboxílicos. A Orn é substrato ou produto de cinco enzimas e é transportada por um proteína carreadora mitocondrial. O metabolismo deste aminoácido ocorre no ciclo da ureia, na biossíntese de poliaminas e creatina e na reação da ornitina amino transferase (OAT) (Valle e Simell, 2001).

No que se refere ao ciclo da ureia, três enzimas são citosólicas (argininosuccinato sintetase, argininosuccinato liase e arginase), enquanto duas estão presentes na matriz mitocondrial (ornitina transcarbamilase e carbamoil fosfato sintetase). Portanto, o funcionamento do ciclo da ureia requer o transporte de Orn para a matriz mitocondrial e citrulina para fora da mitocôndria (Klingenberg, 1970). O transporte de Orn para dentro da mitocôndria no tecido hepático (principal tecido ureogênico) é mediado por um carreador específico, ornitina translocase 1 (ORNT1) e, em menor extensão, pelo ORNT2 (Gamble e Lehninger, 1973; Fiermonte et al, 2003). A captação de Orn para dentro da mitocôndria em células extra-hepáticas parece envolver diferentes transportadores, incluindo o ORNT2 (Passarella et al, 1990; Fiermonte et al, 2003). Estas observações são consistentes com a ideia de que o transporte de Orn para dentro da mitocôndria tem diferentes propósitos em diferentes tecidos; a captação de Orn nas células hepáticas serve,

primariamente, para a síntese de ureia, enquanto que em outros tecidos serve para a degradação da Orn e a biossíntese de arginina e creatina (Valle e Simell, 2001).

As doenças do metabolismo da Orn são caracterizadas pelo acúmulo de Orn, o que leva à hiperornitinemia. Até o momento foram descritos dois erros inatos do metabolismo que causam hiperornitinemia: a atrofia girata do coróide e da retina (OMIM 258870) e a síndrome hiperornitinemia-hiperamonemia-homocitrulinúria (HHH), com sintomas e sinais predominantemente neurológicos (OMIM 238970). Trataremos aqui da síndrome HHH.

I.1.3 Síndrome hiperornitinemia-hiperamonemia-homocitrulinúria (HHH)

Em 1969, Shih e colaboradores descreveram o primeiro paciente com aumento no plasma de Orn (hiperornitinemia) e amônia (hiperamonemia), bem como elevada excreção de homocitrulina na urina (homocitrulinúria) (Shih et al, 1969).

A síndrome HHH é um EIM de caráter autossômico recessivo (Palmieri, 2008; Tessa et al, 2009; Martinelli et al, 2015) na qual o aumento da concentração de Orn plasmática diferencia esta síndrome de outras doenças do ciclo da ureia, e a hiperamonemia e a homocitrulinúria, também característicos dessa condição, a distingue da atrofia girata, em que os pacientes afetados também apresentam hiperornitinemia (Valle e Simell, 2001).

Essa doença do ciclo da ureia é caracterizada por uma mutação no gene ORNT1 (SLC25A15), o qual codifica um transportador mitocondrial de ornitina (ORC1) (Figura 1) (Fell et al, 1974; Camacho et al, 1999; Palmieri, 2008; Tessa et al, 2009; Martinelli et al, 2015). A inabilidade de transportar Orn do citosol

para a mitocôndria resulta em uma deficiência intramitocondrial de Orn e consequentemente da função do ciclo da ureia ao nível da enzima ornitina trascarbamilase, levando à hiperamonemia. Como a rota normal do catabolismo da Orn ocorre através da enzima intramitocondrial ornitina aminotrasferase, a Orn citosólica se acumula, sendo secretada pela célula, resultando em hiperornitinemia. Na ausência de Orn dentro da mitocôndria, o carbamoil fosfato acumulado condensa com lisina e forma homocitrulina (Hcit), através de um mecanismo ainda não esclarecido, levando a homocitrulinúria. Alternativamente, o carbamoil fosfato pode ser direcionado para a biossíntese citosólica das pirimidinas, levando ao aumento da excreção de ácido orótico (Oro) e uracila na urina (Valle e Simell, 2001) (Figura 1).

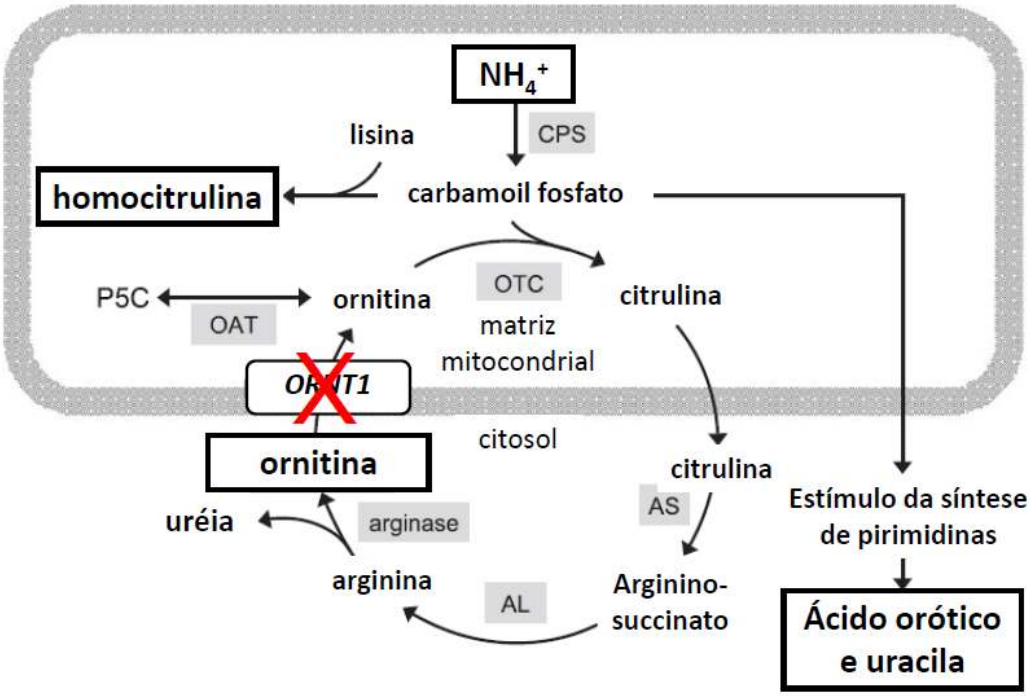

Figura 1. Rotas metabólicas envolvidas na síndrome HHH devido à deficiência do transportador mitocondrial de ornitina ORNT1 (SLC25A15), levando ao acúmulo dos metabólitos marcados nos retângulos pretos. AL= argininosuccinato liase; AS = argininosuccinato sintetase; CPS = carbamoil fosfato sintetase; OAT = ornitina aminotransferase; OTC = ornitine transcarbamilase; P5C = D1 pirrolina-5-carboxilato (adaptada de Korman et al, 2004)

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I.1.3.1 Achados clínicos

Os sintomas da síndrome HHH normalmente aparecem durante o período neonatal ou na infância. No entanto, alguns casos são diagnosticados na idade adulta (Gatfield et al, 1975). A doença é clinicamente variável, onde alguns pacientes podem apresentar sintomas mais brandos, como por exemplo

dificuldades de aprendizado e sintomas neurológicos leves, até uma forma mais severa com distúrbios de coagulação, encefalopatia hepática, coma, letargia, ataxia cerebelar e convulsões. Exceto pela forma neonatal, mais severa, não há evidência direta de uma correlação entre a idade e a severidade da doença (Häberle et al, 2012; Martinelli et al, 2015).

I.1.3.2 Achados bioquímicos e diagnóstico

Os pacientes acometidos pela síndrome HHH apresentam um aumento plasmático de Orn e amônia, juntamente com elevada excreção urinária de Hcit, sendo esses achados bioquímicos as características principais deste distúrbio e que dão o nome e levam ao diagnóstico deste EIM (Valle e Simell, 2001). A concentração plasmática de Orn varia de 200 a 1080 μM (valores normais: 20 a 100 μM) e a excreção urinária de Orn livre de 20 a 8160 μmol/g de creatinina (valores normais: 9 a 120 μmol/g de creatinina) (Korman et al, 2004; Debray et al, 2008; Tessa et al, 2009). Em relação à Hcit, a concentração urinária varia de 20 a 2380 μmol/g de creatinina (valores normais: 0 a 30 μmol/g de creatinina), excedendo em muito os valores normais. Os níveis plasmáticos de amônia durante o período de jejum estão elevados nos pacientes relativamente aos indivíduos normais. Os valores de amônia posprandial aumentam com a ingestão aumentada de proteínas e pacientes com dieta hiperproteica possuem hiperamonemia crônica (Valle e Simell, 2001).

Em relação a outros aminoácidos, a concentração plasmática de arginina é normal, enquanto a lisina está mais baixa e a glutamina e a alanina estão elevadas. Outro metabólito que aparece aumentado no plasma dos

pacientes com a síndrome HHH é o lactato, bem como a razão lactato/aspartato (Korman et al, 2004).

A síndrome HHH pode ser diferenciada através dos achados laboratoriais de outras síndromes que levam à hiperamonemia, incluindo as doenças do ciclo da ureia. A tríade hiperornitinemia, hiperamonemia e homocitrulinúria são achados patognomônicos desta doença.

I.1.3.3 Tratamento

O objetivo principal do tratamento é prevenir a toxicidade da amônia seguindo os princípios delineados para as doenças do ciclo da ureia. De maneira geral, os pacientes devem evitar situações de jejum e ingestão excessiva de proteínas. Restrição protéica para menos do que 1,2 g/kg/dia previne a hiperamonemia e resulta também em uma diminuição nas concentrações de Orn plasmática (Valle e Simell, 2001). Além disso, a suplementação com arginina e citrulina também tem sido instituída para diminuir a hiperamonemia e em alguns estudos mostrou-se efetiva para tanto. A utilização de citrulina parece ser mais efetiva, pois é capaz de incorporar dois grupamentos amino na molécula (Gordon et al, 1987).

I.1.3.4 Fisiopatogenia

Até o momento os mecanismos que levam aos achados clínicos, especialmente ao dano neurológico, nos pacientes com a síndrome HHH não estão bem esclarecidos. A hiperamonemia tem sido associada com o aumento na produção de espécies reativas de oxigênio (ERO) na mitocôndria e no

citosol, assim como com a diminuição na atividade de enzimas antioxidantes no cérebro (Kosenko et al, 2003). Altas concentrações de amônia também estão relacionadas com disfunção neurológica, provocada por uma diminuição no metabolismo cerebral (Norenberg et al, 2004). No entanto, alguns pacientes afetados pela síndrome HHH apresentam níveis normais de amônia sanguínea que está associada à disfunção neurológica (Valle e Simell, 2001). Sendo assim, é possível que o acúmulo de Orn e Hcit possa exercer um papel importante na fisiopatologia desta síndrome. Nesse particular, um estudo demonstrou que a Orn e a Hcit induzem *in vitro* estresse oxidativo em córtex cerebral de ratos jovens (Amaral et al, 2009). Além disso, também foi demonstrado *in vivo* que ambos metabólitos interferem na homeostase redox em córtex cerebral de ratos jovens (Viegas et al, 2011). Os pacientes afetados pela síndrome HHH frequentemente apresentam aumento dos níveis plasmáticos de ácido lático e da razão lactato/piruvato, sugerindo a possibilidade de possuírem alterações na homeostase mitocondrial. Nesse contexto, Viegas e colaboradores demonstraram que tanto a Orn quanto a Hcit podem induzir um desequilíbrio na homeostase energética em córtex cerebral de ratos jovens (Viegas et al, 2009).

Uma lacuna no estudo da patogênese da síndrome HHH é que, apesar dos pacientes frequentemente apresentarem sinais clínicos e sintomas envolvendo o córtex cerebral e o cerebelo, não existem estudos sobre o efeito dos metabólitos acumulados nessa doença sobre o cerebelo.

I.1.4 Espécies reativas e radicais livres

"Espécies reativas" (ER) é um termo usado coletivamente para designar tanto espécies "radicais" quanto "não radicais", agentes oxidantes que facilmente são convertidos a radicais, tais como o ácido hipocloroso (HOCl), o ácido hipobromoso (HOBr), o ozônio (O_3), o peroxinitrito ($ONOO^-$), o oxigênio *singlet* ($^1O_2$) e o peróxido de hidrogênio (H_2O_2). Sendo assim pode-se dizer que todo radical livre é uma espécie reativa, entretanto, nem toda espécie reativa é considerada um radical livre. E dentro dessa definição enquadram-se uma ampla gama de espécies e algumas delas são listadas na Tabela I (Halliwell, 2006).

Radicais livres são moléculas ou átomos que contenham um ou mais elétrons desemparelhados e possuam a capacidade de existir de forma independente (Southorn e Powis, 1988). O desemparelhamento de elétrons, situação energeticamente instável, é o que confere alta reatividade a essas espécies. Os radicais livres podem ser formados pela perda de um elétron de um não-radical ou pelo ganho de um elétron por um não-radical. Radicais podem também ser formados em um processo de fissão homolítica, no qual uma ligação covalente é quebrada e cada elétron do par compartilhado permanece com cada um dos átomos envolvidos (Halliwell e Gutteridge, 1996). Quando um radical livre reage com um composto não-radical, outro radical livre pode ser formado; assim, a presença de um único radical pode iniciar uma seqüência de reações em cadeia de transferência de elétrons (redox) (Maxwell, 1995). Nas reações em cadeia induzidas pelos radicais livres, um radical reativo leva à formação de um produto que também é um radical livre e que, por sua vez, reage produzindo um terceiro radical.

Tabela 1 – Espécies reativas mais frequentes

Radicais Livres	Não radicais
Espécies reativas de oxigênio (ERO)	
Superóxido, $O_2^{\bullet -}$	Peróxido de hidrogênio, H_2O_2
Hidroxila, $OH^{\bullet}$	Ozônio, O_3
Hidroperoxila, $OH^{\bullet}_2$ (superóxido protonado)	*Singlet*, $^1O_2$
Carbonato, $CO^{\bullet -}_3$	Peróxidos orgânicos, ROOH
Peroxila, $RO^{\bullet}_2$	Peroxomonocarbonato, $HOOCO_2^-$
Alcoxila, $RO^{\bullet}$	Peróxicarbonato nitroso, $ONOOCO_2^-$
Radical dióxido de carbono, $CO^{\bullet -}_2$	
Espécies reativas de nitrogênio (ERN)	
Óxido nítrico, $NO^{\bullet}$	Ácido nitroso, HNO_2
Dióxido de nitrogênio, $NO^{\bullet}_2$	Cátion nitrosil, NO^+
Nitrato, $NO^{\bullet}_3$	Ânion nitroxila, NO^-
	Tetróxido dinitrogenado, N_2O_4
	Trióxido nitrogenado, N_2O_3
	Peroxinitrito, $ONOO^-$
	Peroxinitrato, O_2NOO^-
	Ácido peroxinitroso, ONOOH
	Cátion nitronico, NO_2^+
	Peroxinitritos alquila, ROONO
	Peroxinitratos de alquila, RO_2ONO
	Cloreto de nitrila, NO_2Cl
	Peróxiacetil nitrato, $CH_3C(O)OONO_2$

Fonte: adaptado de Halliwell (2006)

I.1.4.1 Mecanismos de dano celular induzido por espécies reativas

ERO e espécies reativas de nitrogênio (ERN) ocorrem tanto em processos fisiológicos quanto patológicos do organismo. Fisiologicamente, essas espécies reativas apresentam diversas funções (Bergendi et al, 1999). Assim, um aumento da liberação local de radicais livres usualmente é benéfico, como é o caso da liberação de espécies tóxicas oxidantes pelos neutrófilos, que podem atuar na defesa do hospedeiro contra uma infecção (Delanty e Dichter, 1998). Participam ainda de processos de sinalização celular e também estão envolvidos na síntese e regulação de algumas proteínas (Ward e Peters, 1995). Por outro lado, quando formadas em excesso, essas espécies altamente reativas têm o potencial de oxidar moléculas biológicas incluindo proteínas, lipídios e DNA (Maxwell, 1995). Com relação aos efeitos prejudiciais das reações oxidantes ao organismo, os radicais livres podem promover lipoperoxidação; podem causar a oxidação de lipoproteínas de baixa densidade (LDL); podem reagir com proteínas, levando à sua inativação e consequente alteração de sua função; e podem também reagir com o DNA e RNA, levando a mutações somáticas e a distúrbios de transcrição (Delanty e Dichter, 1998), entre outros efeitos.

I.1.5 Defesas antioxidantes

Antioxidantes são substâncias endógenas ou exógenas que reduzem a formação de radicais livres ou reagem com os mesmo, neutralizando-os. A célula pode se proteger contra o dano oxidativo através de antioxidantes não enzimáticos e enzimáticos.

Embora diferindo na composição, as defesas antioxidantes estão amplamente distribuídas no organismo e compreendem agentes que removem cataliticamente os radicais livres, como as enzimas superóxido dismutase, catalase, glutationa peroxidase, entre outras; proteínas que minimizam a disponibilidade de pró-oxidantes (íons de ferro e cobre, por exemplo), ao se ligarem aos mesmos como as transferrinas; proteínas que protegem biomoléculas de danos (incluindo dano oxidativo) por outros mecanismos; agentes de baixo peso molecular que aprisionam espécies reativas de oxigênio e nitrogênio, como glutationa, α-tocoferol, ácido ascórbico e a bilirrubina.

I.1.5.1 Defesas antioxidantes não enzimáticas

As defesas antioxidantes não enzimáticas podem ser separadas em dois grandes grupos: agentes hidrossolúveis e lipossolúveis. O primeiro grupo inclui glutationa reduzida (GSH), ácido ascórbico, ácido úrico, melatonina, os cofatores selênio e coenzima Q10 e proteínas plasmáticas. O tripeptídeo GSH é um dos mais efetivos e abundantes antioxidantes contra ERO, particularmente no cérebro onde as concentrações alcançam 1-10 mmol/L (Bast, 1993). A GSH mantém o equilíbrio redox na célula e inativa ERO. O ácido ascórbico também parece ser muito importante no cérebro porque células neurais têm um sistema de captação altamente eficiente e os níveis de ácido ascórbico no líquor são muito mais altos do que no plasma (Lonnrot et al, 1996).

I.1.5.2 Defesas antioxidantes enzimáticas

A atividade de antioxidantes enzimáticos também é importante para a detoxificação de radicais livres. Exemplos desses são as izoenzimas da superóxido dismutase contendo cobre e zinco (Cu, Zn-SOD) ou magnésio (Mn-SOD), a catalase (CAT), a glutationa peroxidase (GPx) e a glutationa redutase (GR). Qualquer desequilíbrio nesse sistema pode resultar em um aumento de radicais livres derivados do oxigênio. As isoformas Cu, Zn-SOD e Mn-SOD estão presentes no citosol e mitocôndria cerebrais, e juntos com a GSH, representam a linha de defesa mais importante contra a toxicidade do $O_2^{\bullet-}$. Isoformas da SOD geram H_2O_2, o qual é removido pela atividade da GPx combinada com GSH ou GR, ou pela CAT (Chance et al, 1979; Halliwell e Gutteridge, 1996), ou adicionalmente por outras peroxidases (Chae et al, 1999).

I.1.6 Estresse oxidativo

Espécies reativas são necessárias para a função nomal da célula, conforme acima referido, servindo como moléculas de sinalização para importantes papéis fisiológicos. Elas são continuamente produzidas e neutralizadas por sistemas de defesa antioxidante. No entanto, quando produzidos em altas concentrações ou quando defesas antioxidantes estão deficientes, elas podem causar dano celular. Se o aumento de espécies reativas é relativamente pequeno, a resposta antioxidante será suficiente para compensar esse aumento. No entanto, sob certas condições patológicas, a produção de espécies reativas está muito mais aumentada, e as defesas

antioxidantes podem ser insuficientes para restabelecer a homeostase redox (Halliwell, 2006).

O rompimento entre o equilíbrio pró-oxidante e antioxidante em favor do pró-oxidante é descrito como estresse oxidativo e pode representar um mecanismo fundamental de doenças humanas (Halliwell e Gutteridge, 2007; Sies, 1985). Assim, o termo “Estresse Oxidativo” é usado para se referir à situação na qual a geração de espécies reativas ultrapassa a capacidade das defesas antioxidantes disponíveis. Pode resultar tanto de uma diminuição das defesas antioxidantes quanto de uma produção aumentada de oxidantes, bem como da liberação de metais de transição ou a combinação de quaisquer desses fatores (Halliwell, 2001).

O estresse oxidativo pode promover adaptação, dano ou morte celular.

- Adaptação: as células podem tolerar um estresse oxidativo moderado, que geralmente resulta em um aumento da síntese de sistemas de defesa antioxidante a fim de restaurar o balanço pró-oxidante / antioxidante.
- Dano celular: o estresse oxidativo pode provocar dano a alvos moleculares (DNA, proteínas, carboidratos e lipídios) (Halliwell e Gutteridge, 2007b). Nesses casos, a resposta à injúria tecidual pode ser reversível: a célula entra em um estado de homeostase alterado temporário ou prolongado, que não leva à morte celular.
- Morte celular: pode ocorrer tanto por necrose quanto por apoptose. Na morte celular por necrose, a célula incha e se rompe, liberando seu conteúdo para o meio extracelular. Pode haver a liberação de

antioxidantes, como CAT e GSH, e também de pró-oxidantes, como os íons cobre e ferro e proteínas do grupo heme, agentes esses que podem afetar as células adjacentes, podendo até mesmo induzi-las a um estresse oxidativo. Já na apoptose, o mecanismo intrínseco de morte celular programada é ativado e não há a liberação do conteúdo celular. A apoptose pode estar acelerada em certas doenças, tais como as desordens neurodegenerativas, havendo envolvimento do estresse oxidativo (Halliwell e Gutteridge, 2007c).

Além da indução de necrose e apoptose, o estresse oxidativo pode levar a um aumento da lipoperoxidação, cujos produtos (malondialdeído (MDA) e 4-hidroxinonenal (HNE), entre outros) são altamente neurotóxicos, e a um dano oxidativo tanto às proteínas, inibindo a atividade de diversas enzimas e alterando a função celular, quanto ao DNA, causando alteração de bases púricas e pirimídicas (Halliwell e Gutteridge, 2007).

O cérebro é altamente dependente de energia para o seu funcionamento normal e a mitocôndria é a estrutura intracelular responsável pela produção dessa energia. Para a produção eficiente de energia na forma de ATP, a mitocôndria possui uma alta demanda por oxigênio, já que utiliza uma grande quantidade de O_2 em uma massa de tecido relativamente pequena, o que torna esse tecido altamente susceptível à ação de espécies reativas. Além disso, a presença de membranas celulares ricas em ácidos graxos de cadeia poliinsaturada (PUFA), os quais são especialmente sensíveis ao ataque de radicais livres e conseqüente oxidação de lipídeos pode levar ao dano por radicais livres no tecido cerebral. O conteúdo aumentado de ferro, o qual favorece a lipoperoxidação e a autooxidação de neurotransmissores através da

$OH^{\bullet}$ formado na reação de Fenton (Zaleska e Floyd, 1985), a considerável quantidade de microglia, macrófagos residentes do sistema nervoso que podem produzir $O_2^{\bullet-}$ e H_2O_2, a modesta defesa antioxidante, sendo os níveis de catalase particularmente baixos em muitas regiões cerebrais (Halliwell e Gutteridge 1996 e 2007), aumentam a suscetibilidade do cérebro ao dano causado por radicais livres. Além disso, uma disfunção mitocondrial pode ocorrer por diminuição da atividade dos complexos da cadeia respiratória com um conseqüente prejuízo no transporte de elétrons, o que leva a uma dispersão dos elétrons na forma de radicais livres potencialmente danosos à célula. Numerosas evidências sugerem que os radicais livres e o estresse oxidativo possam estar envolvidos na patogênese dos danos neurológicos de várias doenças neurodegenerativas, como doença de Alzheimer, doença de Parkinson e esclerose lateral amiotrófica (Perry et al, 2003; Aoyama e Nakaki, 2015). Como medida de parâmetros de estresse oxidativo, existe um considerável número de casos onde se relatou aumento nos níveis de MDA e HNE no cérebro de pacientes, além de produtos da oxidação protéica, como grupos carbonil e 3-nitrotirosina, e também produtos que resultam da oxidação de DNA, bem como concentrações reduzidas dos antioxidantes não enzimáticos GSH e ácido ascórbico e diminuição da atividade das enzimas antioxidantes CAT e GPx (Jenner e Olanow 1996; Liu et al, 1999; Perry et al, 2003).

Estudos demonstraram uma diminuição na atividade do complexo I da cadeia respiratória em cérebros *postmortem* de pacientes portadores de doença de Parkinson. Essa inibição do complexo I pode acarretar na geração de espécies reativas, tais como ânion superóxido, radicais hidroxila e

peroxinitrito, as quais poderiam causar um prejuízo ainda maior na cadeia transportadora de elétrons. Dessa forma, é possível que o estresse oxidativo e as disfunções mitocondriais formem um ciclo vicioso na doença de Parkinson (Gu et al, 1996; Janetsky et al, 1994; Schapira et al, 1989, 1990 a, b). Na doença de Alzheimer, é possível que o estresse oxidativo tenha um papel chave na morte neuronal. Tem sido proposto que o peptídeo β-amilóide, o formador das chamadas placas senis, tenha a capacidade de gerar radicais livres espontaneamente. Estudos *in vivo* também evidenciaram um dano oxidativo em cérebros humanos *postmortem* com doença de Alzheimer através da observação de aumento de 8-hidroxi-2'-deoxiguanosina (8-OHdGA), produtos de oxidação de outras bases e de RNA, carbonilas de proteínas, nitrotirosina e marcadores de peroxidação lipídica (Smith et al, 1991; Marksbery et al, 1999; Nourooz-Zadeh et al, 1999; Lovell et al, 2000).

I.1.7 Metabolismo energético no cérebro de mamíferos

O cérebro possui uma intensa atividade metabólica, porém suas reservas energéticas são extremamente pequenas em relação à sua demanda. Assim, há necessidade contínua de substratos energéticos para o cérebro de mamíferos, sendo a glicose o principal deles onde, em contraste com outros tecidos, não necessita de insulina para ser captada e oxidada (Dickinson, 1996). O padrão de utilização de glicose varia conforme a etapa de desenvolvimento do sistema nervoso central (SNC), o estado nutricional do indivíduo e o destino de sua cadeia de átomos de carbono (Erecinska et al, 2004; Lieberman e Marks, 2008). Situações de jejum prolongado fazem com que o SNC passe a utilizar corpos cetônicos para a obtenção de energia, a fim

de poupar o organismo de um catabolismo protéico exacerbado resultante da necessidade da manutenção da glicemia via gliconeogênese (Lieberman e Marks, 2008). A glicose captada pelo cérebro é, também, fonte de carbono para a síntese de diversas outras biomoléculas (por exemplo, neurotransmissores), o que reforça a idéia de que a utilização de glicose não está atrelada somente à produção de energia.

Mitocôndrias de mamíferos são organelas intracelulares ubíquas, responsáveis pela produção de ATP pelo metabolismo aeróbico, mas também desempenham outras funções intracelulares, tendo um papel crítico no processo de apoptose e servindo como um tampão de cálcio. Tecidos com alta atividade oxidativa tais como cérebro, músculos esquelético e cardíaco, apresentam altas concentrações de mitocôndrias (Orth e Schapira, 2001).

I.1.8 Ciclo dos ácidos tricarboxílicos

O ciclo dos ácidos tricarboxílicos que ocorre nas mitocôndrias é a via comum de oxidação dos carboidratos, aminoácidos e ácidos graxos. O metabolismo energético cerebral se mostra essencialmente oxidativo, sendo a glicose o principal substrato utilizado (Clark et al, 1993), entrando no ciclo sob a forma de acetil-CoA, que é então oxidada completamente a CO_2 e a energia é conservada na forma de coenzimas reduzidas (NADH e $FADH_2$) e GTP (Nelson e Cox, 2008).

I.1.9 Fosforilação oxidativa

A fosforilação oxidativa é o processo principal da produção de energia celular. Todos os passos oxidativos na degradação de carboidratos, lipídios e aminoácidos convergem a esse estágio final da respiração celular, em que a energia da oxidação, provida pelo fluxo de elétrons através das enzimas da cadeia transportadora de elétrons, promove a síntese de ATP (Nelson e Cox, 2008). Quando não há hipóxia, a fosforilação oxidativa é dependente da concentração de ATP, ADP e fosfato inorgânico (Pi) e da razão mitocondrial de $NADH/NAD^{+,}$ que é determinada pela atividade da cadeia transportadora de elétrons e pela transferência de elétrons provenientes de reações catalisadas por enzimas mitocondriais (Erecinska e Silver, 1994).

A cadeia transportadora de elétrons, composta por quatro complexos enzimáticos (complexos I-IV) além da ATPase (complexo V), recebe elétrons das coenzimas NADH e $FADH_2$, e os transfere através de uma série de reações de oxidação-redução até o oxigênio molecular e simultaneamente acopla essa reação exergônica à translocação de prótons através da membrana mitocondrial interna (Wallace, 1999). O fluxo de prótons (gradiente eletroquímico de prótons) gerado durante o transporte de elétrons na cadeia transportadora de elétrons leva à formação de ATP a partir de ADP e Pi pelo complexo V (ATP sintase) (Babcock e Wikstrom, 1992; Wallace, 1999).

Os complexos da cadeia transportadora de elétrons são formados por várias cadeias polipeptídicas associadas a grupos transportadores de elétrons: NADH desidrogenase (complexo I), succinato: ubiquininona oxirredutase (complexo II), complexo citocromo b-c_1 (complexo III) e citocromo c oxidase (complexo IV), além de elementos móveis que se localizam entre os

complexos, são eles a coenzima Q, um componente não protéico lipossolúvel que carreia elétrons entre os complexos I e III, e o citocromo *c*, uma proteína localizada na face externa da membrana que transfere os elétrons do complexo III para o complexo IV (Lieberman e Marks, 2008).

I.1.10 Creatina quinase

A CK consiste de um grupo de isoenzimas com um papel central no metabolismo energético, principalmente para tecidos com alta demanda energética, como cérebro, músculo cardíaco e esquelético, onde funciona como um efetivo sistema de tampão e transferência intracelular de energia. A CK catalisa a transfosforilação reversível entre ATP e creatina a ADP e fosfocreatina [$MgATP^-$ + creatina $\leftrightarrow$ $(fosfocreatina)^-$ + $MgADP^-$ + H^+], ajudando a manter os níveis dos substratos fosforilados. Sabe-se que durante a excitação nervosa e neuromuscular ocorre um aumento de dez vezes no *turnover* celular de ATP, e que durante essas mudanças rápidas, o sistema creatina/fosfocreatina é necessário tanto como um tampão energético quanto como um sistema de transporte entre os locais de produção e consumo de ATP pelas ATPases para evitar grandes flutuações nos níveis de ATP/ADP celulares nesses tecidos excitáveis (Bessman e Carpenter, 1985; Schnyder et al, 1991; Wallimann et al, 1992).

As isoformas da CK estão localizadas em sítios de demanda e produção energética. A isoforma citosólica (Ci-CK) consiste de dímeros e é expressa de uma maneira tecido-específica, isto é, cérebro específica (BB-CK), músculo esquelético específica (MM-CK) e um heterodímero músculo cardíaco-específico (MB-CK) (O'Gorman et al., 1996; Schnyder et al, 1991; Wallimann et

al, 1992). As formas mitocondriais da CK (Mi-CK) são dispostas em octâmeros e são compostas da isoforma sarcomérica músculo específica Mib-CK e da forma ubíqua Mia-CK, que é encontrada nas mitocôndrias do tecido cerebral (Gross et al, 1996; Saks et al, 1985; Schlegel et al, 1988; Wallimann et al, 1992).

Devido à sua localização próxima a sítios onde ocorrem a geração de energia e o transporte de íons através de membranas, o sistema CK/fosfocreatina desempenha um papel fundamental na homeostase energética neuromuscular. Assim, é presumível que alterações na função da CK levem ao desenvolvimento de vários estados patológicos envolvendo o cérebro, músculo esquelético e músculo cardíaco (Aksenov et al, 2000; Aksenov et al, 1997; Aksenova et al, 1999; David et al, 1998; Hamman et al, 1995).

I.1.11 Na^+,K^+ ATPase

A Na^+,K^+-ATPase é uma proteína transmembrana constituída por dois tipos de subunidades, a subunidade α, não-glicosilada que contém os sítios de atividade catalítica da enzima e de ligação de íons, e a subunidade β, que é uma glicoproteína de função desconhecida, formando uma estrutura dimérica $(\alpha\beta)_2$.

A função dessa enzima é translocar Na^+ (muito mais concentrado fora do que dentro da célula) e K^+(muito mais concentrado dentro do que fora da célula), através da membrana plasmática, contra seus gradientes de concentração utilizando energia (ATP). A enzima transporta simultaneamente 2 Na^+ para fora e 3 K^+ para dentro da célula. A saída de Na^+ capacita as células

animais a controlar osmoticamente seu conteúdo de água. Como três cargas positivas são transportadas para o meio extracelular e apenas duas para o meio intracelular, o fluxo de íons Na^+ e K^+ produz um gradiente eletroquímico através da membrana celular (Lingrel & Kuntzweiler, 1994). Esse gradiente é usado como fonte de energia para a despolarização e repolarização do potencial de membrana, para a manutenção e regulação do volume celular, para transporte ativo, transporte dependente de íon Na^+, de glicose, de aminoácidos e de neurotransmissores e para cotransporte/antiporte de outros íons (Geering, 1990). Enfatiza-se que todas as células eucarióticas superiores consomem o ATP por elas produzido para a manutenção das concentrações citosólicas de Na^+ e K^+, sendo que esse consumo chega a ser de 40 a 60% nas células neuronais (Whittan, 1962).

A reação catalisada pela Na^+,K^+-ATPase é a seguinte:

$$3Na^+_{(intracelular)} + 2K^+_{(extracelular)} + ATP \rightleftharpoons 3Na^+_{(extracelular)} + 2K^+_{(intracelular)} + ADP + Pi$$

I.1.12 Metabolismo energético e doenças neurodegenerativas

Numerosas evidências relacionam doenças neurodegenerativas a uma alteração no metabolismo energético. Estudos demonstraram uma diminuição na atividade do complexo I da CTE em cérebros *postmortem* de pacientes portadores de doença de Parkinson (Schapira et al, 1989; Janetzky et al, 1994). Também há relatos de defeitos nos complexos II e III da CTE e na enzima α-cetoglutarato desidrogenase, importante enzima do ciclo do ácido cítrico, nessa doença (Mizuno et al, 1990).

Na doença de Alzheimer, a mais comum dentre as doenças neurodegenerativas, é encontrada principalmente uma redução na atividade do complexo IV da CTE (Maurer et al, 2000). Estudos em cérebros postmortem de pacientes demonstraram uma diminuição na atividade do complexo enzimático da piruvato desidrogenase e na atividade da enzima α-cetoglutarato desidrogenase (Perry et al, 1980).

Além disso, a fisiopatologia de diversas doenças, como a isquemia cerebral (Wyse et al, 2000), epilepsia (Grisar 1984), processos neurodegenerativas (Yu 2003) e doença de Alzheimer (Hattori et al, 1998) tem sido associada à uma redução na atividade da Na^+-K^+ -ATPase, a qual possui fundamental importância para o funcionamento normal do organismo e do SNC, particularmente na manutenção do gradiente elétrico da membrana celular.

I. 1.13 Astrócitos

Os astrócitos são importantes moduladores da função normal cerebral, promovendo estabilidade e suporte aos neurônios (Kahlert and Reiser, 2004; Takuma et al, 2004). Além disso, tais células parecem ter importância em processos neurodegenerativos, especialmente em respostas ao estresse, quando secretam citocinas e quimiocinas (Sofroniew and Vinters, 2010). Esse processo, conhecido como astrogliose, pode ser benéfico ou deletério, uma vez que astrócitos reativos podem induzir respostas tanto respostas anti inflamatórias como pró inflamatórias, sendo essa última um dos possíveis mecanismos envolvidos na patogênese de processos neurodegenerativos

(Zhang et al, 2010). Por outro lado, astrócitos podem modular a biossíntese e liberação de defesas antioxidantes, como por exemplo GSH (Hertz and Zielke, 2004).

I.2 OBJETIVOS

I.2.1 Objetivos gerais

O objetivo deste trabalho foi o de investigar os efeitos *in vitro* e *ex vivo* dos principais metabólitos acumulados na síndrome HHH (ornitina e homocitrulina) sobre a homeostase redox e energética em cerebelo de ratos jovens. Também objetivamos avaliar os efeitos da Orn e Hcit sobre a viabilidade, função mitocondrial e homeostase redox em astrócitos cultivados de córtex cerebral de ratos neonatos. A ênfase de nosso trabalho foi investigar os mecanismos patogênicos que pudessem explicar as alterações nas principais estruturas cerebrais (cerebelo e córtex cerebral) alteradas em pacientes afetados por essa doença.

I.2.2 Objetivos específicos

- Investigar os efeitos *in vitro* da ornitina e homicitrulina sobre o dano oxidativo lipídico (níveis de substâncias reativas ao ácido tiobarbitúrico), dano oxidativo proteico (conteúdo de grupamento sulfidrila e formação de carbonilas) e defesa antioxidantes não-enzimáticas (concentrações de glutationa reduzida), bem como sobre a homeostase energética (atividades enzimáticas do ciclo do ácido

cítrico, dos complexos da cadeia transportadora de elétrons, da creatina quinase e da Na^+,K^+-ATPase em cerebelo de ratos de 30 dias de vida.

- Investigar os efeitos *ex vivo* da administração da ornitina e homicitrulina sobre o dano oxidativo lipídico (níveis de substâncias reativas ao ácido tiobarbitúrico), defesa antioxidantes não-enzimáticas (concentrações de glutationa reduzida) e enzimáticas (atividade das enzimas superóxido dismutase, catalase, glutationa peroxidase, glutationa redutase e glicose 6-fosfato desidrogenase) e sobre a atividade da enzima Na^+,K^+-ATPase em cerebelo de ratos de 30 dias de vida.
- Investigar os efeitos *in vitro* da ornitina e homocitrulina sobre a viabilidade celular (incorporação do iodeto de propídio), função mitocondrial (formação do formazan e potencial de membrana mitocondrial - JC-1), as defesas antioxidantes não-enzimáticas (concentrações de glutationa reduzida), bem como sobre a resposta inflamatória (concentrações de IL-1β, IL-6, TNFα e NFκB) em astrócitos de córtex cerebral de ratos neonatos não estimulados e estimulados por uma dose subtóxica de menadiona.

PARTE II

ARTIGOS CIENTÍFICOS

Capítulo I

Disturbance of redox homeostasis by ornithine and homocitrulline in rat cerebellum: A possible mechanism of cerebellar dysfunction in HHH syndrome

Ângela Zanatta, Carolina Maso Viegas, Anelise Miotti Tonin, Estela Natacha Brandt Busanello, Mateus Grings, Alana Pimentel Moura, Guilhian Leipnitz, Moacir Wajner

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Disturbance of redox homeostasis by ornithine and homocitrulline in rat cerebellum: A possible mechanism of cerebellar dysfunction in HHH syndrome

Ângela Zanatta [a], Carolina Maso Viegas [a], Anelise Miotti Tonin [a], Estela Natacha Brandt Busanello [a], Mateus Grings [a], Alana Pimentel Moura [a], Guilhian Leipnitz [a], Moacir Wajner [a,b,*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

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ABSTRACT

Aims: Cerebellar ataxia is commonly observed in hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome, an inherited metabolic disorder biochemically characterized by ornithine (Orn), homocitrulline (Hcit) and ammonia accumulation. Since the pathophysiology of cerebellum damage in this disorder is still unknown, we investigated the effects of Hcit and Orn on important parameters of redox and energy homeostasis in cerebellum of young rats.
Material and methods: We determined thiobarbituric acid-reactive substance (TBA-RS) levels, carbonyl content, nitrate and nitrite production, hydrogen peroxide production, GSH concentrations, sulfhydryl content, as well as activities of respiratory chain complexes I–IV, creatine kinase, Na^+,K^+-ATPase, aconitase and α-ketoglutarate dehydrogenase.
Key findings: Orn and Hcit significantly increased TBA-RS levels (lipid oxidation), that was totally prevented by melatonin and reduced glutathione (GSH). We also found that nitrate and nitrite production was not altered by any of the metabolites, in contrast to hydrogen peroxide production which was significantly enhanced by Hcit. Furthermore, GSH concentrations were significantly reduced by Orn and Hcit and sulfhydryl content by Orn, implying an impairment of antioxidant defenses. As regards energy metabolism, Orn and Hcit provoked a significant reduction of aconitase activity, without altering the other parameters. Furthermore, Orn-elicited reduction of aconitase activity was totally prevented by GSH, indicating that the critical groups of this enzyme were susceptible to oxidation caused by this amino acid.
Significance: Taken together, our data indicate that redox homeostasis is disturbed by the major metabolites accumulating in HHH syndrome and that this mechanism may be implicated in the ataxia and cerebellar abnormalities observed in this disorder.

Introduction

The hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (MIM #238970) is an inherited disorder caused by a defect in the transport of ornithine (Orn) into the mitochondrial matrix due to mutations in the SLC25A15 gene, encoding the ornithine translocase 1 transporter previously termed ORNT1 also named ORC1 (Camacho et al., 1999; Fiermonte et al., 2003; Kim et al., 2012). The inability to import Orn from the cytosol into the mitochondria results in intramitochondrial Orn deficiency and a functional impairment of the urea cycle at the level of ornithine transcarbamoylase, with consequent hyperammonemia. Because the normal pathway for Orn catabolism proceeds via the intramitochondrial enzyme ornithine aminotransferase, cytosolic Orn accumulates resulting in hyperornithinemia. In the absence of intramitochondrial Orn, accumulating carbamoyl phosphate either condenses with lysine to form homocitrulline (Hcit), leading to homocitrullinuria, or is shunted through the cytosolic pyrimidine biosynthetic pathway leading to increased excretion of orotic acid and uracil in the urine (Korman et al., 2004).

HHH syndrome is clinically characterized by acute intermittent episodes of hyperammonemia accompanied by ataxia, vomiting, lethargy, and confusion. Besides cerebellar signs, patients also present movement disorders, dystonia, epilepsy, liver dysfunction and aversion to protein (Palmieri, 2008; Valle and Simell, 2001; Miyamoto et al., 2002; Kim et al., 2012; Filosto et al., 2012).

The central nervous system (CNS) pathology consists of demyelinization, atrophy and stroke-like lesions in the cerebral cortex and cerebellum, which have been ascribed to the toxic effects of ammonia and glutamine on the astrocyte, including changes in cellular bioenergetics, mitochondrial dysfunction, osmotic swelling, and alterations in glutamine–glutamate cycling (Gropman and Batshaw, 2004; Gropman, 2010;

* Corresponding author at: Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul. Rua Ramiro Barcelos No 2600 — Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 5571; fax: +55 51 3308 5535.
E-mail address: mwajner@ufrgs.br (M. Wajner).

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Tuchman et al., 2008; Braissant, 2010; Sofroniew and Vinters, 2010). However, it is unlikely that hyperammonemia is solely responsible for the pathophysiology of HHH syndrome since affected individuals who are diagnosed early and maintain good metabolic control (normal plasma ammonia levels) nonetheless develop progressive neurologic dysfunction years after their initial diagnosis. Therefore, chronic accumulation of Orn, HCit, orotic acid (Oro) and other metabolic factors cannot be ruled out as the contributing causes of the neurological symptoms seen in patients affected by HHH syndrome and investigation of the role of these accumulating metabolites on the CNS function will eventually lead to a better understanding of the relationship between the clinical features and the biochemical abnormalities of this disorder. In this context, recent studies revealed that Orn and Hcit disrupt mitochondrial homeostasis in vitro and in vivo in rat cerebral cortex (Amaral et al., 2009; Viegas et al., 2009, 2011).

It is of note that cerebellar ataxia and abnormalities are common findings in HHH syndrome, although the pathogenesis of the cerebellar damage in patients affected by this disorder is practically unknown. Therefore, in the present study we investigated the effects of Hcit and Orn on important biochemical parameters of oxidative stress and energy metabolism in cerebellum of young rats. We determined thiobarbituric acid-reactive substances (TBA-RS) (lipid peroxidation), carbonyl formation (protein oxidative damage), nitrate and nitrite formation (reactive nitrogen species) and hydrogen peroxide production (reactive oxygen species), reduced glutathione (GSH) concentrations and sulfhydryl content (non-enzymatic antioxidant defenses), as well as the activities of complexes I to IV (oxidative phosphorylation), aconitase and α-ketoglutarate dehydrogenase (citric acid cycle functioning), creatine kinase (intracellular energy transfer) and Na^+,K^+-ATPase (neurotransmission).

Experimental procedures

Animal and reagents

We used 30-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, UFRGS. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80–23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA. Hcit and Orn were dissolved on the day of the experiments in the buffer used for each assay to final concentrations in the incubation medium ranging from 0.1 to 5 mM, and the pH adjusted to 7.4.

Ethical statement

This study was performed in strict accordance with the EU Directive 2010/63/EU for Animal Experiments and approved by the Ethical Committee for the Care and Use of Laboratory Animals of HCPA. All efforts were also made to use the minimal number of animals necessary to produce reliable scientific data and to minimize the animal discomfort.

Preparation of cerebellum samples and incubation

Rats were sacrificed by decapitation without anesthesia, and the cerebellum was dissected, weighed and homogenized in 10 volumes (w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and pre-incubated for 1 h at 37 °C with Hcit or Orn. Controls did not contain these metabolites in the incubation medium. Immediately after pre-incubation, aliquots were taken to measure the oxidative stress parameters TBA-RS, carbonyl formation, sulfhydryl content, GSH levels, nitrate and nitrite production. In some experiments, antioxidants were co-incubated with supernatants at the following final concentrations: 1000 μM reduced glutathione (GSH), 1000 μM melatonin (MEL), 7.5 μM Trolox (TRO, soluble α-tocoferol), 500 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) and 100 μM lipoic acid (LA). The doses of antioxidants used in the present study were selected according to the literature and to previous experiments demonstrating that these doses are capable of preventing oxidative damage and do not alter per se on the biochemical parameters analyzed (Leipnitz et al., 2008; Ribeiro et al., 2011; Moura et al., 2012; Tonin et al., 2012).

For the determination of the activities of the respiratory chain complexes I–III, II, II–III and IV, the cerebellum was homogenized (1:20, w/v) in SETH buffer (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base and 50 $UI \cdot mL^{-1}$ heparin), pH 7.4. The homogenates were centrifuged at 800 *g* for 10 min and the supernatants were kept at −70 °C until being used for enzyme activity determination. For the measurement of hydrogen peroxide (H_2O_2) release and the activities of aconitase and α-ketoglutarate dehydrogenase, mitochondrial fractions from cerebellum were prepared according to Rosenthal et al. (1987). For creatine kinase activity determination, the cerebellum was homogenized (1:10 w/v) in isosmotic saline solution and he homogenates used in the assay. For Na^+,K^+-ATPase activity, synaptic plasma membranes were prepared according to the method of Jones and Matus (1974). The cerebellum was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. Then, the homogenates were added to a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 *g* for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the membrane enzyme preparation. These samples were pre-incubated for 30 min at 37 °C with Hcit or Orn. Controls did not contain these metabolites in the incubation medium.

We always performed the experiments using blanks in order to detect artifacts and validate our methodology. Whereas controls contained tissue preparations but not Orn and Hcit, some blanks were devoid of cerebellum preparations in the incubation medium supplemented by Orn or Hcit and served to detect interferences of the tested metabolites on the techniques utilized to measure the oxidative stress and bioenergetics parameters (results not shown).

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS was determined according to the method of Esterbauer and Cheeseman (1990). Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL of pre-incubated cerebellum supernatants and centrifuged at 3000 *g* for 10 min. Three hundred microliters of the pre-incubated supernatants (containing approximately 0.3 mg of protein) were transferred to a pyrex tube and incubated with 300 μL of 0.67% TBA in 7.1% sodium sulphate on a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool on running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. TBA-RS values were calculated as nmol/ mg protein.

Protein carbonyl content

Protein carbonyl formation, a marker of protein oxidative damage, was measured spectrophotometrically according to Reznick and Packer (1994). Two hundred microliters of the aliquots from the pre-treated supernatants (containing approximately 0.3 mg of protein) were treated with 400 μL of 10 mM 2,4-dinitrophenylhidrazine

(DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 1 h. Samples were then precipitated with 600 μL 20% trichloroacetic acid and centrifuged for 5 min at 9000 *g*. The pellet was then washed with 1 mL ethanol:ethyl acetate (1:1, v/v) and dissolved in 550 μL 6 M guanidine prepared in 2.5 N HCl at 37 °C for 5 min. The difference between the DNPH-treated and HCl-treated samples (blank) was used to calculate the carbonyl content determined at 365 nm. The results were calculated as nmol of carbonyl groups/mg of protein, using the extinction coefficient of 22,000 × 10^6 nmol/mL for aliphatic hydrazones.

Nitrate and nitrite formation

Nitrate and nitrite production was measured according to Miranda et al. (2001). Two hundred microliters of vanadium chloride was added to the tube containing 200 μL of pre-treated supernatants (0.3 mg of protein) for complete reduction of nitrate to nitrite. Then, 200 μL of Griess reagent (a mixture of N-1-naphtylethylenediamine dihydrochloride and sulfanilamide) were added, and the tube was incubated for 30 min at 37 °C in a water bath in a dark room. The resulting pink-stained pigment was determined in a spectrophotometer at 540 nm. A calibration curve was performed using sodium nitrate (2.5–100 μM), and each curve point was subjected to the same treatment as supernatants. Nitrate and nitrite formation values were calculated as nmol/mg protein.

Mitochondrial hydrogen peroxide (H_2O_2) release

Mitochondrial preparations (0.2 mg protein·mL^{-1}) supported by 5 mM glutamate and 5 mM malate as substrates were incubated in standard reaction medium in the presence of 10 μM Amplex red and 1 U/mL horseradish peroxidase. The fluorescence was monitored during 500 s on a Hitachi F-4500 spectrofluorometer operated at excitation and emission wavelengths of 563 and 587 nm, respectively, and slit widths of 5 nm. Antimycin A (0.1 μg/mL^{-1}) was added at the end of the measurements (Mohanty et al., 1997). Data were expressed as FAU.

Reduced glutathione (GSH) concentrations

GSH concentrations were measured according to Browne and Armstrong (1998). One volume of metaphosphoric acid was added to the pre-treated samples, which were centrifuged for 10 min at 7000 *g*. Then, 185 μL of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and 15 μL of o-phthaldialdehyde (1 mg/mL) were added to 30 μL of cerebellum supernatants (0.3–0.5 mg of protein). This mixture was incubated at room temperature in a dark room for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.001–1 mM) and the concentrations were calculated as nmol/mg protein.

Sulfhydryl content

This assay is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). Briefly, 30 μL of 10 mM DTNB was added to an aliquot of 50 μL of pre-treated supernatants. This was followed by 30 min incubation at room temperature in a dark room. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were calculated as nmol TNB/mg protein.

Determination of the respiratory chain complexes activities

Mitochondrial respiratory chain enzyme activities (complexes I–III, II, II–III and IV) were measured in the supernatants with a protein concentration varying from 1.5 to 4.0 mg protein/mL. The activity of NADH:cytochrome c oxidoreductase (complexes I–III) was assessed as described by Schapira et al. (1990). The activities of succinate: DCIP-oxidoreductase (complex II) and succinate:cytochrome c oxidoreductase (complexes II–III) were determined according to the method of Fischer et al. (1985) and of cytochrome c oxidase (complex IV) according to Rustin et al. (1994). The methods described to measure these activities were slightly modified, as described in details in a previous report (da Silva et al., 2002). The activities of the respiratory chain complexes were calculated as nmol/ min/ mg protein.

Activities of aconitase and α-ketoglutarate dehydrogenase complex

The activity of the enzyme aconitase was measured according to Morrison (1954), following the reduction of $NADP^+$ at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activity of α-ketoglutarate dehydrogenase complex was evaluated according to Lai and Cooper (1986) and Tretter and Adam-Vizi (2004), with modifications. The incubation medium contained 50 mM K_2PO_4, 1 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 0.3 mM DTT, 100 μM EGTA, 50 μM coenzyme A-SH, 250 μM α-ketoglutarate and 2 mM NAD^+, pH adjusted to 7.35. The reduction of NAD^+ was recorded in a Hitachi F-4500 spectrofluorometer at wavelengths of excitation and emission of 340 e 466 nm, respectively.

Determination of creatine kinase activity

Creatine kinase activity was measured in the supernatants containing 0.4–1.2 μg of protein in a reaction mixture consisting of 60 mM Tris–HCl, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$ and 0.625 mM lauryl maltoside in a final volume of 100 μL. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of 0.3 μmol of ADP. The reaction was stopped after 10 min by the addition of 1 μmol of p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962) with slight modifications (Schuck et al., 2002). The color was developed by the addition of 100 μL of 2% α-naphthol and 100 μL of 0.05% diacetyl in a final volume of 1 mL and read spectrophotometrically at 540 nm after 20 min. Results were calculated as μmol creatine/min/mg protein.

Determination of Na^+,K^+-ATPase activity

The reaction mixture for the Na^+,K^+-ATPase assay consisted of 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes containing approximately 3 μg of protein in a final volume of 200 μL. The enzymatic assay occurred at 37 °C during 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. The reaction was stopped by the addition of 200 μL of 10% trichloroacetic acid. Mg^{2+}-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos, 1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Enzyme specific activity was calculated as nmol Pi released/min/mg protein.

Protein determination

Protein content was determined in cerebral cortex supernatants by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Statistical analysis

Unless otherwise stated, results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data was analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. Only significant F values are shown in the text. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

Results

Orn and Hcit induce lipid peroxidation in rat cerebellum probably through reactive oxygen species formation

First, we investigated the in vitro effects of Orn and Hcit on TBA-RS levels in cerebellum of young rats. Fig. 1 shows that Orn [A: $F_{(4,24)} = 30.998$; $P < 0.001$] and Hcit [B:$F_{(4,29)} = 8.897$; $P < 0.001$] significantly increased TBA-RS levels in a dose-dependent manner [Orn: $\beta = 0.919$; $P < 0.001$; Hcit: $\beta = 0.761$; $P < 0.001$], indicating that these metabolites induce lipid peroxidation. We then evaluated the role of antioxidants on Orn-induced lipid oxidative damage. Cerebellar supernatants were co-incubated with 5.0 mM Orn and each of the antioxidants GSH (1500 μM), MEL (1000 μM), TRO (7.5 μM), L-NAME (500 μM) or LA (100 μM). Our results show that MEL [$F_{(7,47)} = 33.249$; $P < 0.001$] and GSH [$F_{(5,35)} = 83.773$; $P < 0.001$] were able to fully prevent Orn-induced TBA-RS levels increase, in contrast to TRO, L-NAME and LA, which did not alter Orn-induced lipid peroxidation (Fig. 4).

Orn and Hcit do not alter carbonyl formation in rat cerebellum

The next set of experiments was carried out to evaluate the in vitro effects of Orn and Hcit on carbonyl formation. We observed that neither Orn (control: 2.47 ± 0.89; 5 mM Orn: 2.69 ± 0.57; n = 6) nor Hcit (control: 4.24 ± 0.50; 5 mM Hcit: 4.65 ± 0.79; n = 6) significantly altered this parameter.

Orn and Hcit do not induce nitrate and nitrite production in rat cerebellum

We also assessed the effect of Orn and Hcit on nitrate and nitrite formation. These metabolites did not induce reactive nitrogen species generation in cerebellum (control: 1.51 ± 0.65; 5 mM Orn: 1.44 ± 0.51; 5 mM Hcit: 1.45 ± 0.58; n = 6).

Orn and Hcit provoke a reduction of non-enzymatic antioxidant defenses in rat cerebellum

The non-enzymatic antioxidant defenses were also assessed by investigating the effects of Orn and Hcit on GSH concentrations and sulfhydryl content in the cerebellum. We observed that Orn [$F_{(4,28)} = 42.691$; $P < 0.001$] and Hcit [$F_{(4,28)} = 4.932$; $P < 0.05$] significantly decreased GSH concentrations in a dose-dependent fashion [Orn: $\beta = -0.662$, $P < 0.001$; Hcit: $\beta = -0.662$; $P < 0.001$] (Fig. 2A). Furthermore, Orn decreased sulfhydryl content [$F_{(4,29)} = 3.013$; $P < 0.05$] in a dose-dependent manner [$\beta = -0.552$; $P < 0.01$], in contrast to Hcit that did not alter this parameter (Fig. 2B).

Hcit increases hydrogen peroxide production in rat cerebellum

Next, we evaluated whether Orn or Hcit were able to alter the hydrogen peroxide (H_2O_2) production in the presence of glutamate and malate as substrates. We found that only Hcit significantly increased H_2O_2 production (Fig. 3).

The respiratory chain complexes I–IV activities are not altered by Orn and Hcit in rat cerebellum

We also tested the influence of Orn and Hcit on the activities of complexes I–III, II, II–III and IV of the respiratory chain. We verified that these activities were not altered by these metabolites (Table 1).

Orn and Hcit significantly inhibit aconitase activity in rat cerebellum

The effects of Orn and Hcit on α-ketoglutarate dehydrogenase and aconitase activities were also tested in mitochondrial preparations from cerebellum. We observed that these compounds did not change α-ketoglutarate dehydrogenase activity (control: 23.9 ± 5.27; 5 mM Orn: 26 ± 9.23; 5 mM Hcit: 21.2 ± 8.87; n = 6). However, Orn [$F_{(4,15)} = 5.667$; $P < 0.01$] and Hcit [$F_{(4,16)} = 5.667$; $P < 0.05$] markedly diminished aconitase activity in a dose-dependent manner [Orn: $\beta = -0.819$; $P < 0.001$; Hcit: $\beta = -0.697$; $P < 0.01$] (Fig. 5A). We then evaluated the role of antioxidants on Orn-induced aconitase activity inhibition. Cerebellar mitochondria were co-incubated with 5.0 mM Orn and GSH (1500 μM), MEL (1000 μM) or TRO (7.5 μM). Our results show that GSH [$F_{(4,27)} = 3.279$; $P < 0.05$] was able to fully prevent Orn-induced aconitase activity decrease, whereas MEL and TRO did not alter this effect (Fig. 5B).

Orn and Hcit do not change creatine kinase and synaptic membrane Na^+, K^+-ATPase activities in rat cerebellum

We also verified that Orn and Hcit did not altered the activities of creatine kinase (control: 6.13 ± 1.11; 5 mM Orn: 7.7 ± 0.22; n = 6)

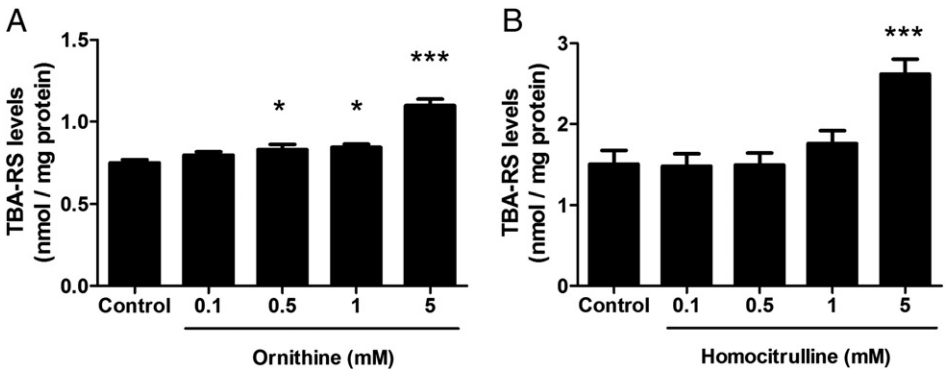

Fig. 1. Effect of ornithine (Orn) (A) and homocitrulline (Hcit) (B) on thiobarbituric acid-reactive substances (TBA-RS) in rat cerebellum. Values are means ± standard deviation for six independent experiments performed in triplicate and expressed as nmol/mg protein. *$P < 0.05$, ***$P < 0.001$, compared to controls (Duncan multiple range test).

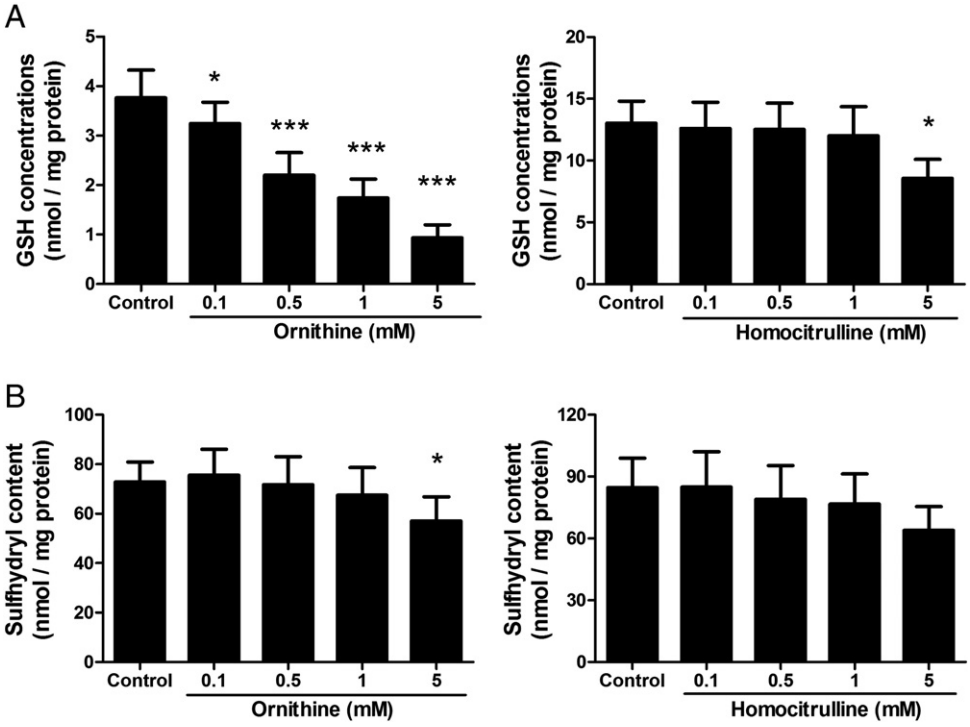

Fig. 2. Effect of ornithine (Orn) and homocitrulline (Hcit) on reduced glutathione (GSH) concentrations (A) and sulfhydryl content (B) in rat cerebellum. Values are means ± standard deviation for six independent experiments performed in triplicate and expressed as nmol/mg protein. $^{*}P < 0.05$, $^{***}P < 0.001$, compared to controls (Duncan multiple range test).

(control: 5.67 ± 2.22; 5 mM Hcit: 5.08 ± 1.46; n = 6) and synaptic membrane Na^+, K^+-ATPase (control: 804 ± 127; 5 mM Orn: 816 ± 57; n = 6) (control: 892 ± 225; 5 mM Hcit: 648 ± 374; n = 6).

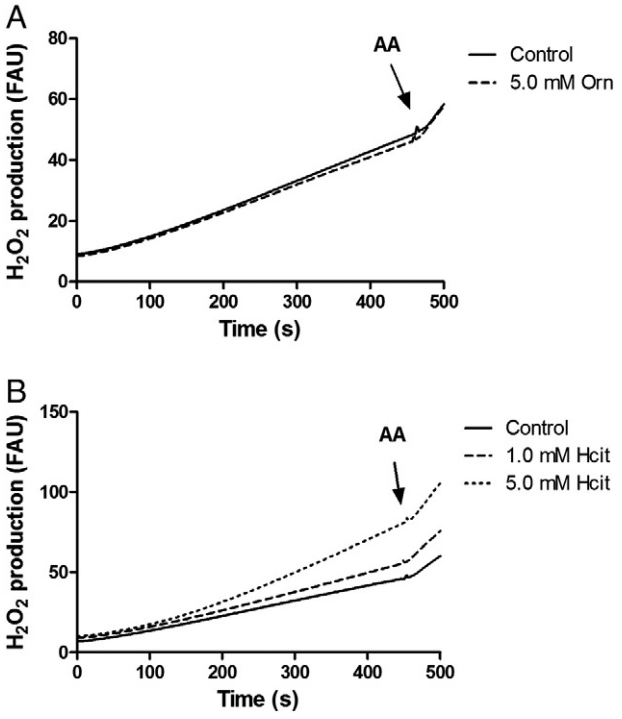

Fig. 3. Effect of ornithine (Orn) (A) and homocitrulline (Hcit) (B) on hydrogen peroxide production using 2.5 mM glutamate plus 2.5 mM malate as substrates in mitochondrial preparations from rat cerebellum. Antimycin (AA) (0.1 μg/mL) was added at the end of assays, as indicated. Traces are representative of four independent experiments and are expressed as fluorescence arbitrary units (FAU).

Discussion

Ataxia and cerebellar atrophy are observed in HHH syndrome (Tsujino et al., 2000; Miyamoto et al., 2002; Korman et al., 2004; Filosto et al., 2012). Although the pathogenesis of the cerebellum damage is practically unknown, the CNS cellular pathophysiology in this disorder has been generally attributed to the toxic effects of ammonia (Braissant, 2010; Sofroniew and Vinters, 2010). However, it is unlikely that hyperammonemia is solely responsible for the pathophysiology of HHH syndrome since affected individuals who are diagnosed early and maintain good metabolic control (normal plasma ammonia levels) nonetheless develop progressive neurologic dysfunction. Therefore, it is conceivable that the other metabolites accumulating in HHH syndrome, i.e., Orn and Hcit, may be neurotoxic.

Therefore, we investigated the role of Orn and Hcit on important parameters of oxidative stress and energy homeostasis in rat cerebellum. We initially verified that Orn and Hcit significantly increased TBA-RS levels, reflecting an induction of malondialdehyde generation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge, 2007). Thus, it may be presumed that Orn and Hcit cause lipid peroxidation in cerebellum.

The next step of our investigation was to evaluate the effects of the classic antioxidants GSH, MEL, TRO, LA, SOD plus CAT and L-NAME on the lipid peroxidation induced by Orn. In this context, it is well known that GSH, MEL, TRO and LA are scavengers of the free radicals superoxide, hydroxyl and peroxyl, as well as of peroxynitrite. Besides its scavenging properties, GSH also protects protein bound sulfhydryl groups and eliminates hydrogen peroxide and lipid peroxide as a cofactor for the antioxidant enzyme glutathione peroxidase. The effect of the nitric oxide synthase inhibitor L-NAME was finally evaluated in order to specifically determine the involvement of reactive nitrogen species.

We observed that Orn-induced lipid oxidative damage was totally prevented by the free radical scavengers GSH and MEL, suggesting that Orn provokes a prooxidant effect on membrane lipids from cerebellum probably by the hydroxyl and other radicals that are scavenged by

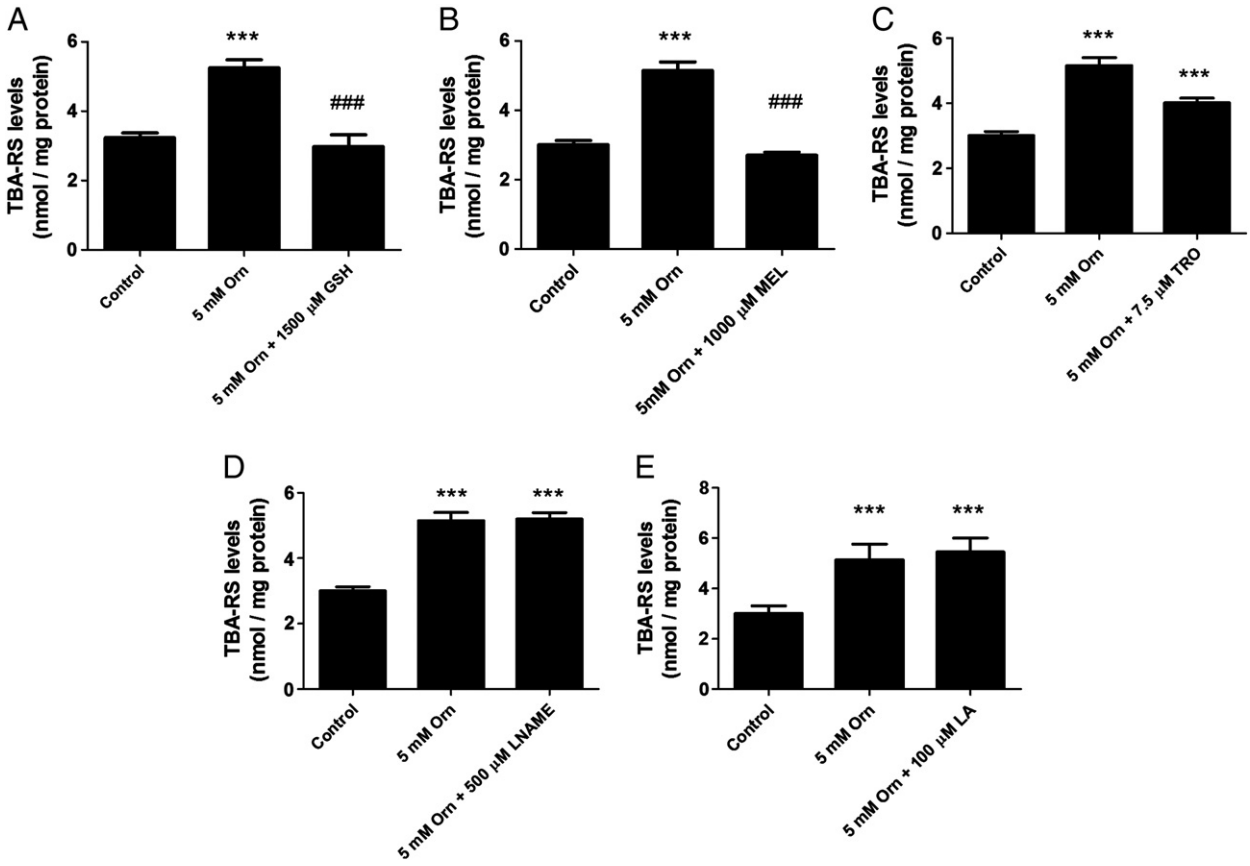

Fig. 4. Effect of antioxidants on ornithine (Orn)-induced lipid peroxidation (thiobarbituric acid-reactive species increase) in rat cerebellum. Cerebellum supernatants were co-incubated for 1 h at 37 °C with 5 mM Orn and either 1500 μM reduced glutathione (GSH) (A), 1000 μM melatonin (MEL) (B), 7.5 μM trolox (TRO, soluble α-tocopherol) (C), 500 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) (D) or 100 μM lipoic acid (LA) (E). Values are means ± standard deviation for six independent (animals) experiments performed in triplicate and are expressed as nmol/mg protein. $^{***}P < 0.001$, compared to control; $^{###}P < 0.001$, compared to 5 mM Orn (Duncan multiple range test).

these antioxidants. Since nitrate and nitrite production was not altered by any of these metabolites, in contrast to hydrogen peroxide production, which was significantly enhanced by Hcit, it may be presumed that Orn- and Hcit-elicited oxidative damage through induction of reactive oxygen species generation. Interestingly, hydrogen peroxide gives rise to the hydroxyl radical by the Fenton reaction (Halliwell and Gutteridge, 2007).

We also found that GSH concentrations were decreased by Orn and Hcit, whereas sulfhydryl content was diminished by Orn. It should be emphasized here that GSH is the major naturally-occurring brain antioxidant (Halliwell and Gutteridge, 2007) and that sulfhydryl groups, which are found as protein-bound groups and as components of small molecules, represent an important redox antioxidant pool in the cellular defenses against oxidative stress (Hansen and Winther, 2009; Thomas et al., 1995; Requejo et al., 2010). Taken together, these findings indicate that the non-enzymatic antioxidant defenses are compromised by these metabolites in the cerebellum.

Table 1
Effect of ornithine (Orn) and homocitrulline (Hcit) on the activities of respiratory chain complexes in rat cerebellum.

	Control	0.1 mM	0.5 mM	1.0 mM	5.0 mM
I–III					
Orn	6.72 ± 1.82	7.23 ± 1.6	6.97 ± 2.04	7.33 ± 1.6	7.25 ± 1.42
Hcit	7.95 ± 1.06	6.96 ± 2.91	5.81 ± 1.58	5.61 ± 1.49	7.1 ± 2.01
II					
Orn	4.82 ± 1.19	6.16 ± 0.75	6.18 ± 2.01	6.57 ± 1.73	6.21 ± 1.69
Hcit	6.59 ± 1.22	5.99 ± 1.39	6.23 ± 1.48	6.11 ± 1.47	6.11 ± 1.05
II–III					
Orn	22.3 ± 4.8	23.4 ± 5.14	22.7 ± 5.43	23.4 ± 5.69	23.5 ± 4.54
Hcit	21.6 ± 3.85	21.9 ± 4.42	22.6 ± 5.34	23.2 ± 7.35	21.7 ± 4.52
IV					
Orn	10.9 ± 1.19	10 ± 2.84	10.3 ± 2.62	10.8 ± 2.91	12 ± 2.67
Hcit	14.5 ± 3.43	11.1 ± 2.78	12.4 ± 1.01	10.5 ± 2.63	13.8 ± 2.35

Values are mean ± standard deviation for four to six independent (animals) experiments per group. The activities of complexes I–IV are expressed as nmol/min/mg protein. No significant differences between groups were detected (One-way ANOVA).

Since oxidative stress results from an imbalance between the total antioxidant defenses and the reactive species generated in a tissue (Halliwell and Gutteridge, 2007), our present data strongly indicate that Orn and Hcit, the major amino acids accumulating in HHH syndrome, induce this deleterious cell condition in rat cerebellum. It should be also emphasized that the CNS has low cerebral antioxidant defenses compared with other tissues (Halliwell and Gutteridge, 2007), a fact that makes this tissue more vulnerable to increased reactive species.

With respect to the parameters of energy metabolism, Orn and Hcit did not change the activities of the respiratory chain complexes I–IV, α-ketoglutarate dehydrogenase complex, creatine kinase and Na^{+},K^{+}-ATPase. In contrast, we observed that Orn and Hcit decreased aconitase activity. In this particular, it is well reported that aconitase is a sensitive target of reactive oxygen species representing a parameter of oxidative stress (Liang et al., 2000; Patel et al., 1996; Tretter and Adam-Vizi, 2000; Tretter et al., 2005; Myers et al., 2010). In fact, Orn-induced decrease of aconitase activity was totally prevented by the antioxidant GSH, reinforcing the other findings demonstrating that the effects caused by Orn are mediated by reactive oxygen species. Furthermore, considering that aconitase is susceptible to oxidative inactivation, particularly by superoxide, resulting in the release of a labile Fe from its catalytic 4Fe–4S center and hydrogen peroxide

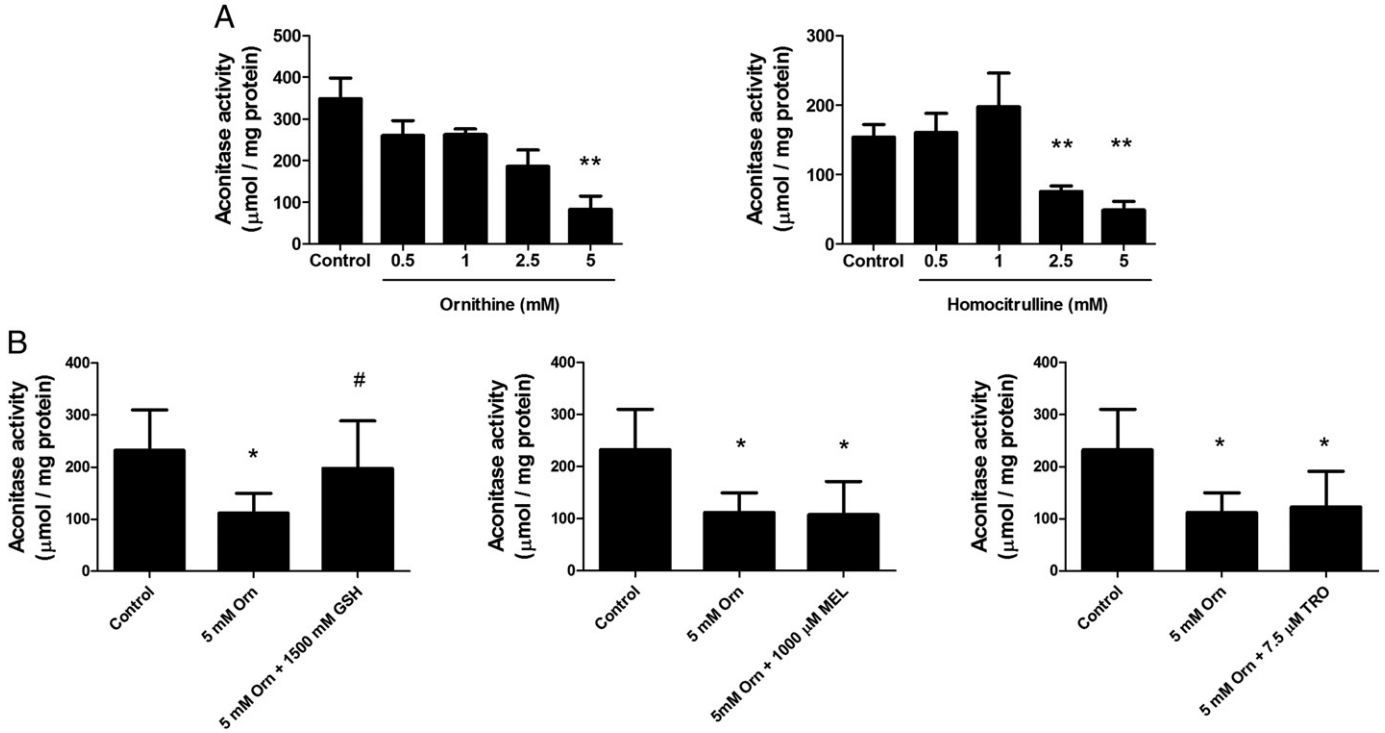

Fig. 5. Effect of ornithine (Orn) and homocitrulline (Hcit) on aconitase activity in rat cerebellum. Mitochondrial fractions were incubated for 30 min at 37 °C with Orn or Hcit (0.5–5 mM) (A). In some experiments, 5 mM Orn was co-incubated with 1500 µM reduced glutathione (GSH), 7.5 µM trolox (TRO, soluble α-tocopherol) or 1000 µM melatonin (MEL) (B). Values are means ± standard deviation for six independent experiments performed in triplicate and expressed as µmol/ min/ mg protein. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to controls; $^{\#}P < 0.05$, compared to 5 mM Orn (Duncan multiple range test).

formation (Vasquez-Vivar et al., 2000; Myers et al., 2010), it is presumed that Hcit-induced inhibition of aconitase may be related to the increase of hydrogen peroxide. However, it should be noted that other reactive species are capable of inactivating aconitase (Gardner et al., 1997). Therefore, our findings demonstrating that Orn inhibited aconitase activity, but did not alter hydrogen peroxide levels, indicate that other reactive species are involved in Orn-induced aconitase inhibition.

Previous results obtained in cerebral cortex of rats showed a dual mechanism of brain damage caused by Orn and Hcit. It was revealed that these metabolites disturb energy homeostasis, by inhibiting the activities of respiratory chain complexes, CK and α-ketoglutarate dehydrogenase, as well as redox homeostasis, by causing protein and lipid oxidative damage and decreasing the antioxidant defenses (Amaral et al., 2009; Viegas et al., 2009, 2011). Our present results, demonstrating that oxidative stress is induced by Orn and Hcit in cerebellum with no disruption of energy dysfunction, indicate that oxidative damage and disturbance of antioxidant defenses probably represent the major mechanism of injury in this cerebral structure.

At the present we cannot determine the pathophysiological relevance of the present data since to our knowledge concentrations of Orn and Hcit in cerebellum or other brain structures were never measured in HHH syndrome. On the other hand, it should be considered that blood Orn concentrations may achieve 1 mM in affected patients and that some of our positive significant results were obtained at these doses (Palmieri, 2008; Valle and Simell 2001). Although our present results better mimic an acute effect of the major metabolites accumulating in HHH syndrome, we cannot rule out that a persistent effect of elevated concentrations of Orn and Hcit may also induce a disruption of redox homeostasis in this disorder. Therefore, in case the present in vitro results are confirmed in vivo in animal studies and in affected patients, it may be presumed that this pathomechanism may contribute to the cerebellar atrophy characteristic of HHH syndrome. It should be also noted that during episodes of metabolic decompensation characterized by encephalopathy and intense proteolysis, much higher concentrations of these amino acids take place therefore facilitating CNS injury (Camacho et al., 1999).

Conclusion

In conclusion, this is the first report showing that oxidative stress is elicited by Orn and Hcit in cerebellum of young rats. It is therefore presumed that, besides hyperammonemia, the major metabolites accumulating in HHH syndrome may contribute to the cerebellum abnormalities and symptoms found in patients affected by this disease possibly through reactive oxygen species attack. Furthermore, it is conceivable that a protein restricted diet with high caloric intake that reduce the risk of increased elevation of brain Orn and Hcit during catabolic states allied to the administration of antioxidants may represent a potential adjuvant therapy for patients affected by HHH syndrome.

Conflict of interest statement

The authors declare that there are no potential conflicts of interest.

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Capítulo II

Ornithine In Vivo Administration Disrupts Redox Homeostasis and Decreases Synaptic Na+, K+-ATPase Activity in Cerebellum of Adolescent Rats: Implications for the Pathogenesis of Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) Syndrome

Ângela Zanatta, Carolina Maso Viegas, Fernanda Hermes Hickmann, Wagner de Oliveira Monteiro, Angela Sitta, Daniela de Moura Coelho, Carmen Regla Vargas, Guilhian Leipnitz, Moacir Wajner

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ORIGINAL RESEARCH

Ornithine In Vivo Administration Disrupts Redox Homeostasis and Decreases Synaptic Na^+, K^+-ATPase Activity in Cerebellum of Adolescent Rats: Implications for the Pathogenesis of Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) Syndrome

Ângela Zanatta[1] · Carolina Maso Viegas[1] · Fernanda Hermes Hickmann[1] · Wagner de Oliveira Monteiro[1] · Angela Sitta[2] · Daniela de Moura Coelho[2] · Carmen Regla Vargas[3] · Guilhian Leipnitz[1] · Moacir Wajner[1,2]

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Abstract Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome is an inborn error of metabolism caused by a defect in the transport of ornithine (Orn) into mitochondrial matrix leading to accumulation of Orn, homocitrulline (Hcit), and ammonia. Affected patients present a variable clinical symptomatology, frequently associated with cerebellar symptoms whose pathogenesis is poorly known. Although in vitro studies reported induction of oxidative stress by the metabolites accumulating in HHH syndrome, so far no report evaluated the in vivo effects of these compounds on redox homeostasis in cerebellum. Therefore, the present work was carried out to investigate the in vivo effects of intracerebellar administration of Orn and Hcit on antioxidant defenses (reduced glutathione concentrations and the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase), lipid oxidation (malondialdehyde concentrations), as well as on the activity of synaptic Na^+, K^+-ATPase, an enzyme highly vulnerable to free radical attack, in the cerebellum of adolescent rats. Orn significantly increased malondialdehyde levels and the activities of all antioxidant enzymes, and reduced Na^+, K^+-ATPase activity. In contrast, glutathione concentrations were not changed by Orn treatment. Furthermore, intracerebellar administration of Hcit was not able to alter any of these parameters. The present data show for the first time that Orn provokes in vivo lipid oxidative damage, activation of the enzymatic antioxidant defense system, and reduction of the activity of a crucial enzyme involved in neurotransmission. It is presumed that these pathomechanisms may contribute at least partly to explain the neuropathology of cerebellum abnormalities and the ataxia observed in patients with HHH syndrome.

Keywords Ornithine · Homocitrulline · Redox homeostasis · Na^+, K^+-ATPase activity · Cerebellum

✉ Moacir Wajner
mwajner@ufrgs.br

[1] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos No 2600 – Anexo, Porto Alegre, RS CEP 90035-003, Brazil

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

[3] Programa de Pós Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

Introduction

Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (OMIM 238970) is an autosomal recessive inborn error of the urea cycle and ornithine (Orn) degradation pathway caused by a defect in the transport of Orn into the mitochondrial matrix due to mutations in the *SLC25A15* gene, encoding ornithine translocase 1 (ORNT1) (Camacho et al. 1999; Camacho and Rioseco-Camacho 2012; Filosto et al. 2013). This leads to cytoplasmic accumulation of Orn and impairment of the urea cycle resulting in hyperammonemia, as well as in the accumulation of carbamoyl phosphate, which condenses with lysine to form homocitrulline (Hcit) (Korman et al. 2004; Al-Hassnan et al. 2008).

The clinical phenotype of HHH syndrome varies widely in its severity and age of onset. The infantile variants are clinically characterized by acute intermittent episodes of hyperammonemia, vomiting, confusion, lethargy, and cerebellar ataxia. Besides cerebellar signs, patients also present developmental delay, cognitive impairment, movement disorders, dystonia, epilepsy, and liver dysfunction. Infants may refuse to eat or have poorly controlled breathing rate and body temperature. Late-onset forms of HHH syndrome are usually less severe than the infantile phenotypes and clinical manifestations may include pyramidal–cerebellar syndrome with ataxic gait, nystagmus and poor fine motor coordination (Tsujino et al. 2000; Miyamoto et al. 2002; Palmieri 2008; Wang and Chou 2012; Tezcan et al. 2012; Filosto et al. 2013). Chronic neurocognitive deficits, such as developmental delay, learning disabilities, and unexplained seizures, are also common.

Neuropathological findings consist of demyelinization, accompanied by atrophy and stroke-like lesions in the cerebral cortex and cerebellum, which have been ascribed to the toxic effects of ammonia and glutamine on the central nervous system (CNS) and impaired bioenergetics (Gropman and Batshaw 2004; Gropman 2010; Tuchman et al. 2008; Braissant 2010; Sofroniew and Vinters 2010). In this scenario, hyperammonemia has been associated to increased production of toxic reactive oxygen species (ROS) in the mitochondria and in the cytosol by xanthine and aldehyde oxidases reactions, as well as to decreased activities of free radical scavenging enzymes in the brain (Kosenko et al. 2003). High tissue ammonia levels were also reported to cause neurological dysfunction by decreasing the brain metabolic rate and the concentrations of high-energy phosphates (Norenberg et al. 2004).

It is unlikely that hyperammonemia is mainly or solely responsible for the pathophysiology of HHH syndrome since affected individuals who are diagnosed early and maintain good metabolic control and normal plasma ammonia levels nonetheless develop progressive neurologic dysfunction years after the initial diagnosis (Valle and Simell 2001). Therefore, it is conceivable that other metabolic factors including chronic or acute accumulation of Orn and Hcit may contribute to the neurological symptoms characteristic of patients affected by this disorder.

It was previously reported that some patients with HHH syndrome present mitochondrial abnormalities in liver, muscle, leukocytes, and fibroblasts, as well as excrete high amounts of lactate, glutarate, and Krebs cycle intermediates, suggesting an impairment of mitochondrial function (Haust et al. 1981; Korman et al. 2004; Fecarotta et al. 2006). Accordingly, recent in vitro studies revealed that Orn and Hcit disrupt mitochondrial energy and redox homeostasis in rat cerebral cortex and cerebellum (Amaral et al. 2009; Viegas et al. 2009, 2011; Zanatta et al. 2013). However, so far no investigation evaluated the in vivo effects of these compounds on redox homeostasis and more particularly on the enzymatic antioxidant defenses in cerebellum, as well as on Na^+, K^+-ATPase activity, that is crucial for neurotransmission (de Lores Arnaiz and López Ordieres 2014).

Thus, in the present work, we determined whether in vivo intracerebellar administration of Orn and Hcit to adolescent rats could induce lipid oxidative damage (malondialdehyde levels) and compromise the antioxidant defenses that include reduced glutathione concentrations and the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glucose-6-phospate dehydrogenase in rat cerebellum. The in vivo effects of Orn and Hcit on the activity of Na^+, K^+-ATPase, an important enzyme necessary for neurotransmission and highly susceptible to free radical attack (Satoh and Nakazato 1992; Lees 1993; Erecinska and Silver 1994; Kurella et al. 1997; Muriel and Sandoval 2000; Yousef et al. 2002; Erecinska et al. 2004), was also investigated. We hope that the evaluation of the in vivo role of Orn and Hcit on the cerebellum function may lead to a better understanding of the brain abnormalities and clinical features in HHH syndrome.

Materials and Methods

Reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for Hcit, which was obtained from MP Biomedicals, LLC Solon, Ohio, USA. Orn and Hcit were dissolved in saline solution (0.9 % NaCl) on the day of the experiments.

Animals

A total number of 72 thirty-day-old male Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, UFRGS were used in the experiments. We used 18 animals (six for controls and for each experimental group) to determine malondialdehyde levels, GSH concentrations, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glucose-6-posphate dehydrogenase activities. We also used 18 rats to measure the effects of Orn and Hcit on malondialdehyde levels 30 min after their administration, following the suggestion of the reviewer. The same number of animals was used to evaluate Na^+, K^+ ATPase activity and another set of 18 rats for Orn measurements.

The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) were followed in all experiments. The experimental protocol was also approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

Orn and Hcit Intracerebellar Administration

Rats were deeply anesthetized with ketamine plus xilazine (75 and 10 mg/kg i.p., respectively) and placed in a stereotaxic apparatus. Two small holes were drilled in the skull for microinjection. One microliter of a 2.5 M Orn solution (2.5 μmol), 0.8 M Hcit solution (0.8 μmol), or NaCl solution (controls) containing the same amount of sodium was administered into the cerebellum bilaterally over 3 min via needles connected by a polyethylene tube to a 10-μL Hamilton syringe coupled to a pump with controlled speed. The needle was left in place for another 1 min before being softly removed, so that the total procedure lasted 4 min. The coordinates for injections were as follows: 9.5 mm posterior to bregma, 1.4 mm lateral to midline, and 2.8 mm ventral from dura (Paxinos and Watson 1986; Lekic et al. 2008). The correct position of the needle was tested by injecting 0.5 μL of methylene blue injection (4 % in saline solution). The chosen doses of Orn and Hcit were based on a previous protocol showing that they provoked significant effects in the cerebral cortex (Viegas et al. 2011).

Orn concentrations were measured 30, 60, and 120 min after its injection by high-performance liquid chromatography (HPLC), using homocysteic acid as the internal standard (Joseph and Marsden 1986) with slight modifications (Viegas et al. 2012). Briefly, cerebellum was dissected on an inverted Petri dish placed on ice and homogenized in 5 volumes (1:5, w/v) of saline solution (0.9 % NaCl) in a ground-glass type Potter–Elvehjem for 1 min with 15 up and down strokes. Cerebellum homogenates were centrifuged at 500×*g* for 10 min. The obtained supernatant was carefully removed for Orn determination.

Cerebellum Preparation

Animals were sacrificed by decapitation without anesthesia 4 h after intracerebellar injection of Orn, Hcit, or NaCl. In some experiments, we killed the animals 30 min after drug injection. The brain was rapidly excised on a Petri dish placed on ice. The olfactory bulb, pons, medulla, forebrain, and cerebral cortex were discarded, and the cerebellum was dissected, weighed, and kept chilled until homogenization with a ground-glass type Potter–Elvehjem homogenizer in the specific buffer.

The cerebellum was homogenized in a similar way in 10 volumes (1:10, w/v) of cold 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 750×*g* for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al. 2001). The supernatants containing a suspension of mixed and preserved organelles, including mitochondria were used for the determination of oxidative stress parameters.

For the determination of Na^+, K^+-ATPase activity, synaptic plasma membranes were prepared from rat cerebellum according to Jones and Matus (1974). Cerebellum was initially homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA and centrifuged at 800×*g* for 10 min. The supernatants were removed and centrifuged at 9000×*g* for 20 min. The pellet was then resuspended in hypotonic buffer (5 mM Tris–HCl buffer, pH 8.1). This preparation was submitted to 6 strokes with vigorous shaking in vortex during a total period of 30 min, which are known to disrupt intact synaptosomes, releasing various components including synaptic membranes. The synaptic plasma membranes were prepared afterward using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8, and 1.0 M. After centrifugation at 69,000×*g* for 2 h, the fraction at the 0.8–1.0 M sucrose interface was taken as the membrane enzyme preparation.

Determination of Biochemical Parameters

Malondialdehyde (MDA) Levels

MDA levels were assayed according to the method described by Yagi (1998) with slight modifications. Briefly, 200 μL of 10 % trichloroacetic acid and 300 μL of 0.67 % thiobarbituric acid in 7.1 % sodium sulfate were added to 100 μL of tissue supernatants and incubated for 2 h in a boiling water bath. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained complex was extracted with 400 μL of butanol. Fluorescence of the organic phase was read at 515 and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. MDA levels were calculated as nmol MDA/mg protein and expressed as percentage of control.

Reduced Glutathione (GSH) Concentrations

GSH concentrations were measured according to Browne and Armstrong (1998). One volume of metaphosphoric acid was added to the pre-treated samples, which were

centrifuged for 10 min at 7000 × *g*. Then, 185 µL of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and 15 µL of *o*-phthaldialdehyde (1 mg/mL) were added to 30 µL of cerebellum supernatants (0.3–0.5 mg of protein). This mixture was incubated at room temperature in a dark room for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. Calibration curve was prepared with standard GSH curve (0.001–1 mM), and the cerebellum concentrations were calculated as nmol GSH/mg protein.

Superoxide Dismutase (SOD) Activity

SOD activity was assayed according to Marklund (1985) and is based on the capacity of pyrogallol to autooxidize, a process highly dependent on O_2^-, which is a substrate for SOD. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm. The reaction medium contained 50 mM Tris buffer/1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U/mL catalase, 0.38 mM pyrogallol, and approximately 1 µg of protein. A calibration curve was performed with purified SOD as standard to calculate the activity of SOD present in the samples. The specific activity was calculated as U/mg protein.

Catalase (CAT) Activity

CAT activity was assayed according to Aebi (1984) by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1 % Triton X-100, and 10 mM potassium phosphate buffer, pH 7.0, and approximately 1 µg of protein. One unit (U) of the enzyme is defined as 1 µmol of H_2O_2 consumed per minute. The specific activity was calculated as U/mg protein.

Glutathione Peroxidase (GPx) Activity

GPx activity was measured according to Wendel (1981) using tert-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM ethylenediaminetetraacetic acid, pH 7.7, 2 mM GSH, 0.1 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH, and approximately 3 µg of protein. One GPx unit (U) is defined as 1 µmol of NADPH consumed per minute. The specific activity was calculated as U/mg protein.

Glutathione Reductase (GR) Activity

GR activity was measured according to Calberg and Manervik (1985) using oxidized glutathione (GSSG) and NADPH as substrates. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium with 200 mM sodium phosphate buffer, pH 7.5, containing 6.3 mM ethylenediaminetetraacetic acid, 1 mM GSSG, 0.1 mM NADPH, and approximately 3 µg of protein. One GR unit (U) is defined as 1 µmol of GSSG reduced per minute. The specific activity was calculated as U/mg protein.

Glucose-6-Phosphate Dehydrogenase (G6PD) Activity

G6PD activity was measured by the method of Leong and Clark (1984), in which the reaction mixture (1 mL) contained 100 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, and 0.5 mM $NADP^+$. The reaction was started by the addition of 1 mM glucose-6-phosphate and was followed in a spectrophotometer at 340 nm by NADPH formation. One G6PD unit corresponds to 1 µmol of substrate transformed per minute and the specific activity was calculated as U/mg protein.

Na^+, K^+-ATPase Activity

The reaction mixture for the Na^+, K^+-ATPase assay consisted of 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes containing approximately 3 µg of protein in a final volume of 200 µL. The enzymatic assay occurred at 37 °C during 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. The reaction was stopped by the addition of 200 µL of 10 % trichloroacetic acid. The ouabain-insensitive Mg^{2+}-ATPase activity was assayed under the same conditions with the addition of 1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos 1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Enzyme-specific activity was calculated as nmol Pi released/min/mg protein.

Protein Determination

Protein content was determined in cerebellum supernatants by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Statistical Analysis

Results are presented as mean ± SD. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc Duncan's multiple range test when *F* was

significant. Only significant F values are shown in the text. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

Results

Orn Concentrations in Cerebellum

Table 1 displays the concentrations of Orn in the cerebellum 30, 60, and 120 min after its injection (5 μmol/g body weight). It can be seen in the table that Orn concentrations were up to 60-fold higher (2.29 μmol/g) at 30 min after administration, decreasing afterward to levels of 1.18 μmol/g (threefold higher) 120 min after injection as compared to the normal values in the cerebellum (0.036 μmol/g).

In vivo Orn Administration Induces Lipid Peroxidation in Rat Cerebellum

Figure 1 shows that Orn significantly increased MDA levels at 30 min (25 %) (A) [$F_{(2,16)} = 5.507$; $P < 0.05$] and 4 h (83 %) (B) [$F_{(2,16)} = 25.878$; $P < 0.001$] after its administration, implying that this amino acid induces lipid peroxidation in cerebellum of adolescent rats. In contrast, Hcit did not change MDA levels.

Table 1 Time course of cerebellum ornithine concentrations after intracerebellar ornithine injection (5 μmol)

	Ornithine concentrations (μmol/g)
Control	0.036
30 min after injection	2.29
60 min after injection	2.14
120 min after injection	1.18

Data represent the median of three independent experiments (animals)

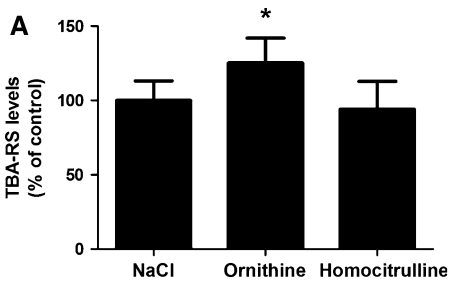


Fig. 1 Effect of intracerebellar administration of ornithine (2.5 μmol) or homocitrulline (0.8 μmol) on malondialdehyde (MDA) levels in rat cerebellum 30 min (**a**) and 4 h (**b**) after injection. Data are expressed as mean ± SD of 5 or 6 independent experiments

In vivo Orn and Hcit Administration Does Not Change GSH Concentrations in Rat Cerebellum

We then investigated the effects of intracerebellar administration of Orn and Hcit on GSH concentrations. We observed that GSH levels were not changed either by Orn or Hcit (controls 6.56 ± 0.76; Orn: 8.98 ± 0.75; Hcit: 6.68 ± 1.81, as nmol GSH/mg protein, $n = 6$).

In vivo Orn Administration Increases Enzymatic Antioxidant Defenses in Rat Cerebellum

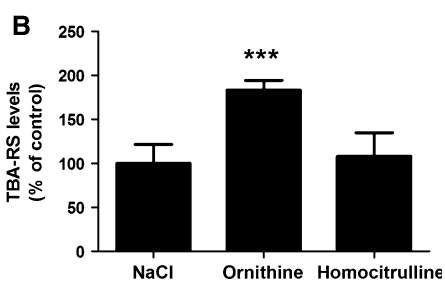
Next, we measured various antioxidant enzyme activities after injection of Orn and Hcit into the cerebellum. It was observed that Orn significantly increased the activities of SOD (56 %) [$F_{(2,16)} = 15.862$; $P < 0.001$], CAT (41 %) [$F_{(2,16)} = 5.575$; $P < 0.01$], GPx (54 %) [$F_{(2,16)} = 37.831$; $P < 0.001$], GR (64 %) [$F_{(2,16)} = 17.910$; $P < 0.001$], and G6PD (65 %) [$F_{(2,16)} = 27.425$; $P < 0.001$]. In contrast, Hcit did not change the activity of any of these enzymes (Fig. 2).

In vivo Orn Administration Reduces Na^+, K^+-ATPase Activity in Rat Cerebellum

We also tested the influence of Orn and Hcit on the activity of synaptic Na^+, K^+-ATPase. We found that this activity was significantly reduced (37 %) by intracerebellar administration of Orn [$F_{(2,16)} = 8.152$; $P < 0.01$], while Hcit had no effect on this parameter (Fig. 3). In contrast, Mg^{2+}-dependent ATPase activity was not changed by these treatments (controls 1991 ± 498; Orn: 2336 ± 440; 2151 ± 278, as nmol Pi released/min/mg protein, $n = 6$).

Discussion

Patients affected by HHH syndrome commonly present neurological symptoms including cerebellar abnormalities and ataxia, whose pathogenesis is not yet well established.



(animals) performed in triplicate and are expressed as percentage of controls [in Controls (nmol/mg protein), 30 min:0.55 ± 0.07; 4 h:0.28 ± 0.06]. *$P < 0.05$; ***$P < 0.001$, compared to rats that received NaCl injection (Duncan's multiple range test)

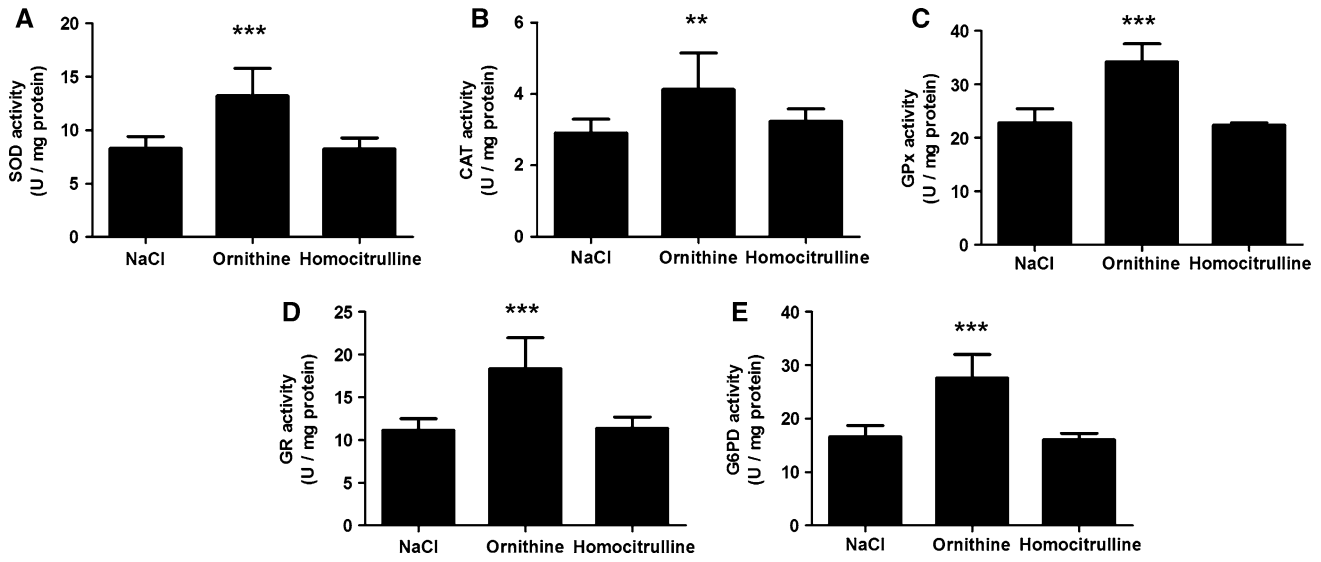


Fig. 2 Effect of intracerebellar administration of ornithine (2.5 μmol) or homocitrulline (0.8 μmol) on the activities of superoxide dismutase (SOD) (**a**), catalase (CAT) (**b**), glutathione peroxidase (GPx) (**c**), glutathione reductase (GR) (**d**), and glucose-6-phosphate dehydrogenase (G6PD) (**e**) in rat cerebellum 4 h after injection. Data are expressed as mean ± SD of five or six independent experiments (animals) performed in triplicate and are expressed as U/mg protein: SOD is expressed as μmol pyrogallol oxidized/min/mg protein; CAT as μmol H_2O_2 consumed/min/mg protein; GPx as μmol NAPDH consumed/min/mg protein; GR as μmol GSSG reduced/min/mg protein; G6PD as μmol glucose-6-phosphate consumed/min/mg protein. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to rats that received NaCl injection (Duncan's multiple range test)

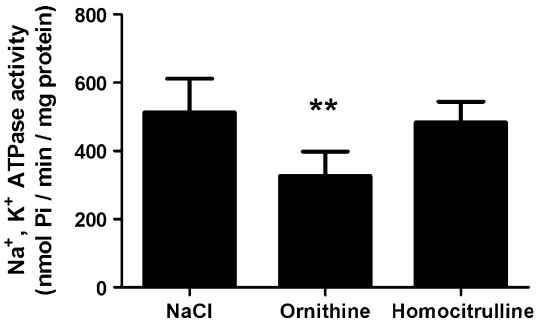


Fig. 3 Effect of intracerebellar administration of ornithine (2.5 μmol) or homocitrulline (0.8 μmol) on the activity of Na^+, K^+-ATPase in rat cerebellum 4 h after injection. Data are expressed as mean ± SD of five or six independent experiments (animals) performed in triplicate and are expressed as nmol Pi released/min/mg protein. $^{**}P < 0.01$, compared to rats that received NaCl injection (Duncan's multiple range test)

However, since hyperammonemia can induce ROS formation and cause ATP depletion (Kosenko et al. 2003; Norenberg et al. 2004), the brain damage in this disorder has been tentatively attributed to the toxic effects of ammonia and glutamine on the CNS impairing bioenergetics and redox homeostasis (Gropman and Batshaw 2004; Gropman 2010; Tuchman et al. 2008; Braissant 2010; Sofroniew and Vinters 2010). In line with these reports, mitochondrial dysfunction has been found in tissues from patients with HHH syndrome and in some cases the severity of the neurologic presentation of this disorder may be indistinguishable from primary mitochondrial diseases (Haust et al. 1981; Smith et al. 1992; Korman et al. 2004). On the other hand, some individuals with HHH syndrome continue to worsen neurologically with progressive pyramidal tract disease and cognitive deterioration in the absence of chronic or acute hyperammonemia and encephalopathic crises (Shih and Ficicioglu 2000; Debray et al. 2008), making unlikely that high ammonia levels are mainly or solely responsible for the brain pathogenesis in this disease. This is supported by experimental data from our group demonstrating that Orn and Hcit provoke in vitro and in vivo disruption of energy and redox homeostasis in cerebral cortex of rats (Viegas et al. 2009, 2011; Amaral et al. 2009; Zanatta et al. 2013). However, to the best of our knowledge, no study has reported the in vivo role of Orn and Hcit on cellular redox status in the cerebellum.

We showed here that a single intracerebellar administration of Orn to adolescent rats disrupts redox homeostasis by causing lipid peroxidation (high MDA levels) and activating the enzymatic antioxidant defense system in cerebellum. Furthermore, Orn injection markedly decreases Na^+, K^+-ATPase activity, which is critical for neurotransmission. However, Orn did not alter GSH levels and Hcit administration was unable to change any of these parameters in the cerebellum, indicating an in vivo selective effect for Orn.

Regarding the elevated MDA concentrations observed in Orn-injected animals, since this compound is an end

product of membrane fatty acid oxidation, it is presumed that Orn induced in vivo lipid oxidation in cerebellum. We have previously found that Orn provoked lipid oxidative damage in vitro in cerebellum at doses as low as 0.5 mM, whereas Hcit caused significant effects only at 5 mM concentration (tenfold higher). We found here that Orn concentrations in the cerebellum ranged from 1.18 to 2.29 μmol/g after a single injection, indicating that these concentrations are able to disrupt redox homeostasis in the cerebellum. Unfortunately, we were not able to measure Hcit concentrations, but our results indicate that cerebellum Hcit concentrations did not reach levels capable of inducing lipid oxidation. Another fact that probably makes our present results relevant to the pathophysiological condition is that blood Orn levels in this disorder may reach over 1.0 mM (normal: 30–110 μmol/L), whereas Hcit concentrations are much lower (Palmieri 2008; Valle and Simell 2001; Camacho and Rioseco-Camacho 2012).

To our mind, this is the first study comprehensively investigating the ex vivo effects of the major compounds accumulating in HHH syndrome on the antioxidant enzymatic system in the cerebellum. We found that important enzyme activities of this system, namely SOD, CAT, GPx, GR, and G6PD, were significantly increased, probably as a response to the higher reactive species formation that were previously shown to be increased by Orn (Amaral et al. 2009; Viegas et al. 2009, 2011; Zanatta et al. 2013). In this context, it is widely known that reactive species may induce up-regulation of antioxidant enzyme genes via distinct signaling mechanisms (Rushmore et al. 1991; Lakshminarayanan et al. 1998; Halliwell and Gutteridge 2007; Seminotti et al. 2013) in an effort to establish a more effective antioxidant defense and tissue adaptation to augmented production of free radicals (Nelson et al. 2006).

Thus, the increased activities of SOD and CAT possibly represent a compensatory response to Orn-induced overproduction of superoxide and hydrogen peroxide, respectively. Furthermore, the increased activity of GPx supports an important role for peroxides in Orn prooxidant effects since this enzyme decomposes hydrogen peroxide and lipid peroxides. The presumption of Orn-induced increase of peroxides is strengthened by its effect causing lipid oxidative damage in cerebellum. Regarding GR and G6PD, that are necessary to catalyze the NADPH-dependent conversion of GSSG to GSH, their augmented activities may reflect a cellular response in order to maintain a normal GSH/GSSG ratio. This may indeed be the case since Orn administration increased GPx activity without significantly altering GSH concentrations.

The present observation that Orn administration did not alter GSH concentrations in vivo in cerebellum is in disagreement with the previous in vitro results showing a diminution of this antioxidant in cerebellum supernatants exposed to Orn (Zanatta et al. 2013). These apparently controversial findings may be explained by the fact that in our present in vivo study the cerebellum had the full cellular machinery to up-regulate the genes encoding GR and G6PD, thus compensating GSH consumption due to Orn-induced reactive species generation. In contrast, the previous in vitro experiments were carried out in cerebellum supernatants devoid of nuclei and consequently of transcriptional and translational machinery, so that GSH cannot be recycled (Zanatta et al. 2013).

Previous findings showed that cerebellum is particularly vulnerable to oxidative stress because of its high oxygen consumption and ROS production rate, as well as low mitochondrial GSH levels compared to other brain structures (Mori et al. 2007). This is in line with our results showing that Orn induced a strong lipid peroxidation and marked alterations on the activity of various antioxidant enzymes in rat cerebellum. Accordingly, we have found that other metabolites accumulating in various inherited metabolic disorders disturb redox homeostasis in this cerebral structure (Leipnitz et al. 2010; Busanello et al. 2014; da Rosa et al. 2015).

Our work also demonstrated that Na^+, K^+-ATPase activity, but not Mg^{2+}-dependent ATPase activity, was markedly decreased by intracerebellar injection of Orn, indicating a selective effect for this activity. Although we did not determine the exact mechanism involved in Orn-induced decrease of Na^+, K^+-ATPase activity, it is conceivable that this reduction may be a consequence of free radical attack on the enzyme that contains critical groups that are highly vulnerable to oxidative damage (Lees 1993; Hitschke et al. 1994; Kurella et al. 1997; Muriel and Sandoval 2000; Yousef et al. 2002). This hypothesis is supported by previous data demonstrating that Orn induces oxidative stress in rat brain (Amaral et al. 2009; Viegas et al. 2011; Zanatta et al. 2013). However, we cannot rule out that Orn could decrease Na^+, K^+-ATPase activity by interfering with membrane lipids anchoring this enzyme, based on the observations that alterations of these lipids and membrane fluidity by other compounds are able to compromise various membrane enzyme activities (Wheeler et al. 1975; Busanello et al. 2013).

It is emphasized that this enzyme is present at high concentrations in the brain and consumes about 40–50 % of the ATP generated in this tissue, highlighting its importance for normal brain functioning. Indeed, Na^+, K^+-ATPase plays a crucial role in maintaining the resting membrane potential and excitable properties of neurons by controlling Na^+ and K^+ gradient across the plasma membrane. Since reduction of Na^+, K^+-ATPase activity is observed in patients and animal models of neurodegenerative disorders (Lees 1993; Cousin et al. 1995; Lees and Leong 1995; Ellis et al. 2003; Dickey et al. 2005; Vignini et al. 2007; Bagh et al. 2008; Busanello

et al. 2010, 2011, 2013; Seminotti et al. 2011; Moura et al. 2012; Amaral et al. 2012; de Lores Arnaiz and López Ordieres 2014), it may be presumed that the decrease of this enzyme activity caused by Orn, as here verified, may impair neurotransmission in cerebellum.

In conclusion, our present ex vivo data allied to previous in vitro and in vivo animal and human experimental findings (Korman et al. 2004; Amaral et al. 2009; Viegas et al. 2009, 2011; Zanatta et al. 2013) support a role for the major accumulating metabolites provoking disruption of redox homeostasis and mitochondrial dysfunction in the pathogenesis of HHH syndrome. Furthermore, we show for the first time that Orn decreases Na^+, K^+-ATPase activity, representing a possible new pathomechanism contributing to the cerebellar symptoms of this disease.

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

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Capítulo III

Ornithine and homocitrulline impair mitochondrial function, decrease antioxidant defenses and induce cell death in menadione-stressed rat cortical astrocytes: potential mechanisms of neurological dysfunction in HHH syndrome

Ângela Zanatta, Marília Danyelle Nunes Rodrigues, Alexandre Umpierrez Amaral, Débora Guerini Souza, André Quincozes-Santos, Moacir Wajner

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Corresponding Author:	Moacir Wajner, PhD, MD Universidade Federal do Rio Grande do Sul Porto Alegre, Rio Grande do Sul BRAZIL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universidade Federal do Rio Grande do Sul
Corresponding Author's Secondary Institution:	
First Author:	Ângela Zanatta
First Author Secondary Information:	
Order of Authors:	Ângela Zanatta
	Marília Danyelle Nunes Rodrigues
	Alexandre Umpierrez Amaral
	Débora Guerini Souza
	André Quincozes-Santos
	Moacir Wajner, PhD, MD
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Suggested Reviewers:	Jose A Camacho University of California Irvine School of Medicine jcamacho@uci.edu
	Diego Martinelli Children Research Hospital Bambino Gésu diego_martinelli@libero.it
	Ferdinando Palmieri Universita degli Studi di Bari Aldo Moro ferdinando.palmieri@uniba.it

Departamento de Bioquímica
Instituto de Ciências Básicas da Saúde, UFRGS
Rua Ramiro Barcelos 2600 - Anexo
CEP: 90035-003 Porto Alegre - RS - Brasil
Tel: +55 51 3308-5571
Fax: +55 51 3308-5540/5535
E-mail: mwajner@ufrgs.br

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Prof. Arne Schousboe
Senior Editor,
Neurochemical Research

Dear Prof. Schousboe,

I am sending you our manuscript entitled "Ornithine and homocitrulline impair mitochondrial function, decrease antioxidant defenses and induce cell death in menadione-stressed rat cortical astrocytes: potential mechanisms of neurological dysfunction in HHH syndrome", which we would like to submit for publication in Neurochemical Research.

The Principles of Laboratory Animal Care, National Institute of Health of United States of America, NIH, publication n° 85-23, revised in 2011 were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

The paper demonstrates for the first time the vulnerability of unstimulated and particularly menadione-stressed astrocytes to the toxic effects of the major metabolites accumulating in HHH syndrome, Orn and Hcit, disrupting essential mitochondrial functions (redox and energy homeostasis) and viability.

I also inform you that all authors have contributed significantly to the manuscript and agree with the submission of the paper at its present version. Moreover, the whole manuscript, or parts of it, will not be submitted elsewhere for publication. Finally, there is no possible conflict of interest in the conduct and reporting of research.

I look forward to hear from you in the near future.

Yours sincerely,

M Wajner, MD, PhD

Ornithine and homocitrulline impair mitochondrial function, decrease antioxidant defenses and induce cell death in menadione-stressed rat cortical astrocytes: potential mechanisms of neurological dysfunction in HHH syndrome

Ângela Zanatta[1], Marília Danyelle Nunes Rodrigues[1], Alexandre Umpierrez Amaral[1], Débora Guerini Souza[1], André Quincozes-Santos[1], Moacir Wajner[1,2]

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

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

* Corresponding Author: Moacir Wajner

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal de Rio Grande do Sul. Rua Ramiro Barcelos N° 2600 – Anexo, CEP 90035-003, Porto Alegre, RS – Brasil. Phone: +55 51 3308-5571, fax: +55 51 3308-5535, e-mail: mwajner@ufrgs.br

Abstract

Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome is caused by deficiency of ornithine translocase leading to predominant tissue accumulation and urinary excretion of ornithine (Orn), homocitrulline (Hcit) and ammonia. Patients commonly present neurological dysfunction manifested by cognitive deficit, spastic paraplegia, pyramidal and extrapyramidal signs, stroke-like episodes, hypotonia and ataxia, whose pathogenesis is poorly known. Although astrocytes are necessary for neuronal protection, they are susceptible to damage by neurotoxins. Therefore, in the present study we investigated the effects of Orn and Hcit on cell viability (propidium iodide incorporation), mitochondrial function (thiazolyl blue tetrazolium bromide – MTT - reduction and mitochondrial membrane potential - $\Delta\Psi_m$), antioxidant defenses (GSH) and pro-inflammatory response (NFkB, IL-1β, IL-6 and TNF-α) in unstimulated and menadione-stressed cortical astrocytes. As regards to unstimulated astrocytes, Orn impaired MTT reduction and both amino acids decreased GSH levels, but did not alter cell viability and the pro-inflammatory factors. When astrocytes were challenged by menadione, there was a decrease of cell viability associated with a reduction of $\Delta\Psi_m$ that occurred in synergism with Orn or Hcit. The present data indicate that the major compounds accumulating in HHH syndrome impair mitochondrial function and reduce cell viability and the antioxidant defenses in cultured astrocytes especially when stressed by menadione. It is presumed that these mechanisms may be involved in the neuropathology of this disease.

Key words: HHH syndrome, ornithine, homocitrulline, mitochondrial function, oxidative stress, astrocytes

Introduction

Hyperornithinemia-hyperammonemia-homocitrullinuria syndrome (HHH syndrome; MIM#238970) is an autosomal recessive disorder caused by a defect in ornithine translocase (*SLC25A15* or *ORNT1*, MIM*603861). The disorder is rare, with approximately 100 patients having been so far reported worldwide, although its incidence is higher in Canada, Italy and Japan [1]. The biochemical phenotype includes hyperornithinemia, hyperammonemia and homocitrulinuria. Hyperammonemia occurs due to the inability to import ornithine (Orn) from the cytosol into the mitochondria resulting in a functional impairment of the urea cycle at the level of ornithine transcarbamoylase. Furthermore, because the normal pathway for Orn catabolism proceeds via the intramitochondrial enzyme ornithine aminotransferase, cytosolic Orn accumulates resulting in hyperornithinemia. In the absence of intramitochondrial Orn, accumulating carbamoyl phosphate either condenses with lysine to form homocitrulline (Hcit), leading to homocitrullinuria, or is shunted through the cytosolic pyrimidine biosynthetic pathway leading to increased excretion of orotic acid and uracil in the urine [2].

The clinical features of HHH syndrome are highly variable, ranging from a mild form with learning difficulties and slight neurological involvement to a more severe form with coma, lethargy, hepatic signs and seizures. Patients commonly have neurological dysfunction manifested by mental retardation, spastic paraplegia, pyramidal and extrapyramidal signs, stroke-like episodes, hypotonia, ataxia, failure to thrive and hepatic failure [3-8]. Demyelinization associated with atrophy and stroke-like lesions in the cerebral cortex and cerebellum are commonly found in HHH syndrome [5]. Although neurological dysfunction and brain abnormalities are observed in HHH syndrome, the pathogenesis of this disorder is not yet well established. However, bizarre-looking mitochondria on microscopy in liver, muscle, leukocytes and fibroblasts, as well as high urinary excretion of lactate, glutarate, and Krebs cycle intermediates, are observed in patients with HHH syndrome [2, 9-11], strongly suggesting that impairment of mitochondrial function may play a major role in HHH syndrome pathophysiology.

In what concerns to hyperammonemia, it has been related to increased production of reactive oxygen species (ROS) and decreased activities of free radical scavenging enzymes in the brain [12], as well as with neurodegeneration in common CNS disorders and some inborn errors [13, 14]. Therefore, it

may be presumed that hyperammonemia found in many patients with HHH syndrome may be involved in its neuropathology. High tissue ammonia levels was also shown to decrease the brain metabolic rate and the concentrations of high-energy phosphates [15], that in turn may be related to the mitochondrial morphological alterations found in the tissues from the affected patients [16].

However, it is unlikely that hyperammonemia is solely responsible for the pathophysiology of this disorder since affected individuals who are diagnosed early and maintain good metabolic control and normal plasma ammonia levels, nonetheless develop progressive neurologic dysfunction years after the initial diagnosis [17]. Therefore, other metabolic factors including persistent or acute accumulation of Orn and Hcit probably contribute to the neurological symptoms characteristic of patients affected by this disorder. In this scenario, recent *in vitro* studies performed in brain cellular fractions (homogenates) revealed that Orn and Hcit disrupt mitochondrial energy and redox homeostasis in rat cerebral cortex and cerebellum, thus supporting neurotoxic effects of the major compounds that accumulate in this syndrome [18-20].

Astrocytes are important modulators of normal brain functioning providing neuronal support and stability [21,22]. They are also involved in neurodegeneration, especially in damaging and stress responses when they become reactive and synthesize cytokines and chemokines [23]. Thus, astrogliosis may be beneficial or deleterious because reactive astrocytes can induce pro-inflammatory or anti-inflammatory responses, being the pro-inflammatory implicated in the pathogenesis of neurodegenerative processes [24]. In contrast, astrocytes also modulate the biosynthesis and release of antioxidant defenses including reduced glutathione (GSH) in the CNS [25]. Taken together, astrocytes may represent an interesting and promising biological tool to study neurodegeneration associated with inflammation.

Therefore, since astrocytes have critical roles in the neuronal–astrocytic interactions [26, 27] and nothing has been reported on the influence of the major metabolites accumulating in HHH syndrome on an intact cell system and more particularly on astrocyte functions, in the present work we evaluated the effects of Orn and Hcit on cell viability (propidium iodide (PI) incorporation) and mitochondrial function (MTT reduction and JC1 levels), as well as on the antioxidant defense system (GSH levels) and inflammatory parameters (interleukin 1β (IL- 1β), interleukin 6 (IL-6), tumor necrosis factor α (α -TNF)

and NFKβ levels) in primary cortical astrocyte cultures from cerebral cortex of neonatal rats. We also stressed astrocytes with a subtoxic dose of menadione, an inducer of free radical production [28-31].

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM) and other materials for cell cultures were purchased from Gibco. DNase, MTT, PI, and ELISA for NFκB, TNF α), IL-1β and IL-6 were purchased from Gibco/Invitrogen (Carlsbad, CA, USA), PeproTech, and eBioscience. All other chemicals were purchased from Sigma-Aldrich.

Animals

Neonatal male Wistar rats were obtained from our breeding colony (Department of Biochemistry, UFRGS, Brazil) and maintained in a controlled environment (12-h light/ 12-h dark cycle; 22±1 °C; ad libitum access to food and water).

Ethical statement

This study was performed in strict accordance with the Principles of Laboratory Animal Care, National Institute of Health of United States of America, NIH, publication n° 85-23, revised in 2011, and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

Primary Astrocyte Cultures

Animals had their cerebral cortex aseptically removed from cerebral hemispheres. All meninges were removed. During the dissection, the cerebral cortex was kept in Hank's balanced salt solution (HBSS) containing 0.05 % trypsin and 0.003 % DNase at 37 °C for 8 min. The tissue was then mechanically dissociated for 7 min using a Pasteur pipette and centrifuged at 100 × *g* for 5 min. The cells were resuspended in HBSS containing DNase (0.003 %) and left for decantation during 20 min. The

supernatant was collected and centrifuged for 400 × *g* for 7 min. The cells from the supernatant were resuspended in DMEM (10 % fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM NaHCO3, 1 % fungizone, and 0.04 % gentamicin) and plated in 24-well plates pre-coated with poly-L-lysine at a density of $3–5\times10^5$ cells/cm^2. The cells were cultured at 37 °C in a 95 % air / 5 % CO_2 incubator. The first medium exchange occurred 24 h after culture begins.

Orn, Hcit and menadione treatment

After astrocytes reached confluence, the culture medium was removed and the cells were incubated in the absence (controls) or presence of Orn or Hcit (1 to 5 mM) for 6 h at 37 °C in a 95 % air/5 % CO_2 incubator in DMEM with 1 % FBS. The ROS stimulator menadione was used in some experiments at a subtoxic dose in order to stress astrocytes. So, after 4 h of pre-incubation with Orn or Hcit, some cultures were exposed to 25 μM menadione for 2 h, after which the biochemical parameters were measured. This dose was lower than those used in other studies [30-31] and was shown in preliminary experiments not to induce alterations of pro-inflammatory factors that would otherwise mask our results on oxidative stress parameters, mitochondrial function and cell viability (results not shown).

Astrocyte viability

Astrocyte viability was determined by the PI incorporation assay using phase contrast optics. The optical density of fluorescent nuclei (labeled with PI), indicative of cell death, was determined with Optiquant software (Packard Instrument Company). Density values obtained were expressed as a percentage of the control condition. Cells were treated simultaneously with 7.5 μM PI for 10 min. Density values obtained are expressed as percentage relative to the control values [32].

Mitochondrial function

Mitochondrial function was assessed by the MTT assay in which formazan is produced (activity of mitochondrial dehydrogenases) and by $\Delta\Psi_m$ determined by JC-1.

MTT was added to the medium at a concentration of 50 μg/mL and cells were incubated for 30 min at 37 °C in an atmosphere with 5 % of CO_2. Subsequently, the medium was removed and the MTT crystals were dissolved in DMSO. Absorbance values were measured at 560 and 650 nm. The results

were calculated as arbitrary unit (AU) / absorbance and expressed as percentages relative to the control conditions. Only functional cells are able to reduce MTT.

For $\Delta\Psi_m$ determination, astrocytes were incubated for 30 min with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, 2 µg/mL) [33]. Cells were then homogenized and centrifuged, washed once with HBSS, and transferred to a 96-well plate. Fluorescence was measured using an excitation wavelength of 485 nm and emission wavelengths of 540 and 590 nm. The $\Delta\Psi_m$ was calculated using the ratio of 590 nm (red fluorescent J-aggregates) to 540 nm (green monomers). The results were calculated as AU / $\Delta\Psi_m$ and expressed as percentages relative to the control conditions.

Antioxidant defenses

GSH levels

GSH levels were assessed as previously described by Souza and colleagues [34]. Astrocyte homogenates (50 mg) were diluted in 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM EDTA, and the protein was precipitated with 1.7 % meta-phosphoric acid. The supernatant was assayed with *o*-phthaldialdehyde (1 mg/ml methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions from 0 to 500 µM. GSH concentrations were calculated as nmol/mg protein. The results are expressed as percentages relative to the control conditions.

Pro-inflammatory factors

IL-1β, IL-6 and TNF- α measurements

The levels of IL-1β, IL-6 and TNF- α were measured in the extracellular medium of astrocytes cultured, using ELISA kits from eBioscience (USA). The results were calculated as ng/mL and expressed as the percentage of the control levels.

Nuclear Factor-KB Levels

The levels of NF-KB p65, were measured in the nuclear fraction from lysed astrocytes using an ELISA commercial kit from Invitrogen (USA). The results are expressed as percentages relative to the control levels.

Protein Assay

Protein content was measured trough the Lowry's method, using bovine serum albumin as a standard [35].

Statistical Analyses

Results were expressed as mean ± standard deviation (SD), unless otherwise stated. All measures were done in triplicates and the mean used for the statistical calculations. Differences among groups were calculated using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. Only significant F values are shown. All analyses were performed using the Statistical Package for Social Sciences software, version 18.0 (SPSS, Inc., Chicago, IL, USA).

Results

Orn and Hcit do not induce cytokine production in cultured astrocytes

We first verified that Orn and Hcit did not change the levels of the interleukins IL-1β, IL-6 and TNF-α, neither of NFKβ levels (Table 1). These results indicate that these accumulating amino acids in HHH syndrome do not induce *per se* an inflammatory response in cultured astrocytes.

Ornithine (Orn) and homocitruline (Hcit) decrease viability of rat cortical astrocytes stimulated by menadione

Next, we studied the effects of Orn and Hcit on astrocyte viability. Figure 1 shows that PI incorporation was significantly increased by Orn and Hcit in menadione-treated astrocytes [$F_{(4,16)} = 19.5$; $P<0.001$]. However, Orn and Hcit *per se* were not able to alter cell viability.

Ornithine (Orn) and homocitruline (Hcit) impair mitochondrial function in rat cortical astrocytes

We also verified that formazan formation was significantly decreased in unstimulated astrocytes exposed to Orn at 1 mM and higher concentrations (Fig. 2A [$F_{(3,23)}$ = 17.081; P<0.001]), in contrast to Hcit which did not alter this parameter (Fig. 1B).

Furthermore, Orn and Hcit were not able to alter $\Delta\Psi_m$ in unstimulated astrocytes but both significantly reduced $\Delta\Psi_m$ in menadione-treated cells [$F_{(4,29)}$ = 159.48; P<0.001] (Fig. 3).

Orn and Hcit reduce the antioxidant defenses in rat cortical astrocytes

We also evaluated the effect of Orn and Hcit on GSH levels, the major naturally-occurring brain antioxidant. Figure 4 shows that Orn ($F_{(3,21)}$ = 9.494; P<0.01) and Hcit ($F_{(3,21)}$ = 6.063; P<0.01) significantly decreased GSH concentrations in unstressed astrocytes, indicating a decrease of antioxidant defenses.

Discussion

Patients affected by HHH syndrome present blood accumulation of Orn and ammonia, as well as high urinary excretion of Hcit. Although progressive cortical brain atrophy with leukodystrophy of the white matter is common in patients with this disorder, the pathomechanisms of brain injury in this disease are poorly known. It is therefore conceivable that Orn and/or Hcit that mainly accumulate in HHH syndrome may potentially behave as neurotoxins, contributing to its pathogenesis. This hypothesis is supported by previous studies reporting mitochondrial dysfunction and induction of oxidative stress in cerebral cortex and cerebellum of rats provoked by these accumulating amino acids [18-20, 36, 37]. It is however emphasized that these in vitro and in vivo data showing alterations of mitochondrial energy and redox homeostasis caused by Orn and Hcit were obtained with subcellular fractions from whole brain that contain all neural cells.

Astrocytes modulate energy metabolism, as well as regulate the antioxidant defenses and anti-inflammatory responses, among other important CNS functions [38 – 43]. Since the utilization of primary astrocyte cultures has contributed to the understanding of the role of astrocytes in physiological and pathological conditions [27], in the present study we exposed unstressed and menadione-stressed

astrocytes derived from cerebral cortex of neonatal rats to Orn and Hcit to mimic the biochemical phenotype of HHH syndrome and evaluated cell viability, mitochondrial function, antioxidant defenses and cytokine production. It is here emphasized that to the best of our knowledge no study investigated whether Orn and Hcit could disturb important cellular functions in astrocytes.

We first observed that menadione *per se* did not alter any of the parameters evaluated, but when added together with Orn or Hcit provoked synergistic effects in some measurements, implying that menadione-stressed astrocytes are more vulnerable to Orn and Hcit deleterious effects.

We also verified that Orn and Hcit significantly reduced cell viability (PI incorporation increase) in menadione-stressed astrocytes, with no effects on unstimulated astrocytes. Furthermore, mitochondrial function was significantly reduced by Orn in unstimulated cells as measured by MTT reduction, whereas $\Delta\Psi_m$ (JC-1) was markedly compromised by both Orn and Hcit in stressed but not in unstimulated astrocytes. These data are in accordance with previous findings demonstrating that mitochondrial energy homeostasis is compromised by Orn and Hcit [19] and reinforce the deleterious effects of these metabolites compromising important astrocyte mitochondrial functions.

With regard to the antioxidant defense system, we found that these metabolites markedly decreased GSH concentrations in an intact cell system (astrocytes), corroborating with previous findings obtained with subcellular fractions from total brain [18, 20]. So, we demonstrated that astrocytes are involved in the reduction of GSH levels induced by the major metabolites accumulating in HHH syndrome. It is emphasized that astrocytes are considered to be a major source of GSH by synthesizing and secreting this antioxidant, helping the maintenance of the CNS redox state [23, 44-49].

GSH is the most abundant antioxidant in the brain, exerting a critical role to scavenge ROS, protecting protein sulfhydryl groups, and also regulating cell death and survival pathways [50]. Since adequate levels of antioxidants are essential to protect cells against oxidative damage and an imbalance in the pro-oxidant/antioxidant homeostasis induces oxidative stress [51], it is possible that the significant reduction of GSH provoked by the major metabolites accumulating in HHH syndrome may cause loss of functionality of astrocytes under Orn and Hcit exposure. Indeed, the significant reduction of MTT and cell viability (PI incorporation) in cultured astrocytes exposed to Orn and Hcit under the same conditions

may reflect low GSH availability. However, we cannot rule out that loss of viability may also be reflected by less number of astrocytes to synthesize GSH that may enter a vicious circle. Alternatively, the reduction of GSH levels may have occurred due to its oxidation by reactive species leading to GSH consumption and a pro-oxidant status. In this context, previous reports demonstrated that Orn and Hcit elicit free radical generation and lipid and protein oxidative damage [20], implying increase of reactive species generation. It should be also emphasized that depletion of GSH takes place during stress conditions giving rise to an oxidative stress response that plays a critical role in astrocytic functionality and viability [52]. Noteworthy, astrocytes have been demonstrated to be vulnerable to various toxins and in particular to 1- methyl-4-phenylpyridinium (MPP+), and this susceptibility seems to be dependent on a pro-oxidant condition induced by MPP+, leading astrocytes to lose functionality and viability [53,54].

The pathophysiological importance of our present data is still uncertain because brain concentrations of Orn and Hcit are not yet established in HHH-syndrome. However, since neurological symptoms appear or become worse during these catabolic crises, in which the concentrations of the accumulating metabolites dramatically increase because of increased proteolysis, we may presume that worsening of clinical features in the affected patients may be at least in part due to the toxic effects of the accumulating amino acids.

In summary, we describe for the first time a high susceptibility of neonatal rat cortical astrocytes, especially when stressed by menadione, to the toxic insults of Orn and Hcit that caused a decrease of cell viability, mitochondrial dysfunction and impaired antioxidant defenses. Our findings obtained in intact neural cells confirm previous results carried out in subcellular fractions and homogenates from whole brain and indicate that disruption of astrocyte redox and energy homeostasis caused by the major organic acids accumulating in HHH syndrome may contribute to the neuropathology of patients affected by this disease. We also demonstrated here for the first time that these critical systems necessary for normal brain functioning are more vulnerable in neural cells stressed by a pro-inflammatory agent (menadione) leading them to loose viability. The present findings provide insight into possible mechanisms of brain damage in HHH syndrome caused by Hcit and Orn and suggest that the pathogenesis of this disorder cannot be exclusively attributed to hyperammonemia. Furthermore, our present data indicate that cultured astrocytes represent an interesting and promising biological tool to study toxicity of compounds accumulating in

inherited disorders, although their main limitation lies on the fact that astrocyte cultures do not involve the intricate relationships between neurons and glial cells. It is therefore presumed that disruption of energy and redox homeostasis contributes to the neurological dysfunction observed in this disorder, particularly during metabolic crises, when metabolite production and accumulation are even higher.

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Legends to figures

Fig 1. Effects of ornithine (Orn) and homocitrulline (Hcit) on propidium iodide (PI) incorporation in rat cortical astrocytes. Orn or Hcit were incubated with astrocytes for 6 h. In some experiments, astrocytes were also treated with 25 uM menadione for 2 h following Orn or Hcit 4 h pre-incubation. Data are represented as mean ± SD of six independent experiments (wells) performed in triplicate and expressed as percentage of controls (Control: [dead cells] 192 ± 28.3, ***P*<0.01, ****P*<0.001, compared to control (Duncan multiple range test).

Fig 2. Effects of ornithine (A) and homocitrulline (B) on thiazolyl blue tetrazolium bromide (MTT) reduction in rat cortical astrocytes. Orn or Hcit were incubated with astrocytes for 6 h. Data are represented as mean ± SD of six independent experiments (wells) performed in triplicate and expressed as percentage of controls (controls: [AU / Absorbance] Orn: 0.67 ± 0.14), ***P*<0.01, compared to control (Duncan multiple range test).

Fig 3. Effects of ornithine (Orn) and homocitrulline (Hcit) on mitochondrial membrane potential ($\Delta\Psi_m$) in rat cortical astrocytes. Orn or Hcit were incubated with astrocytes for 6 h. In some experiments, astrocytes were also treated with 25 uM menadione for 2 h following Orn or Hcit 4 h pre-incubation. Data are represented as mean ± SD of six independent experiments (wells) performed in triplicate and expressed as percentage of controls ([AU / $\Delta\Psi_m$] 2.99 ± 0.18),****P*<0.001, compared to control (Duncan multiple range test).

Fig 4. Effects of ornithine (Orn) and homocitrulline (Hcit) on reduced glutathione (GSH) levels in rat cortical astrocyte cultures. Orn or Hcit were incubated with astrocytes for 6 h. Data are represented as mean ± SD of five to six independent experiments (wells) performed in triplicate and expressed as percentage of controls (Controls [nmol / mg protein] Orn: 22.1 ± 1.51; Hcit: 43.6 ± 5.82). * *P*<0.05, ***P*<0.01, compared to control (Duncan multiple range test).

Figure 1

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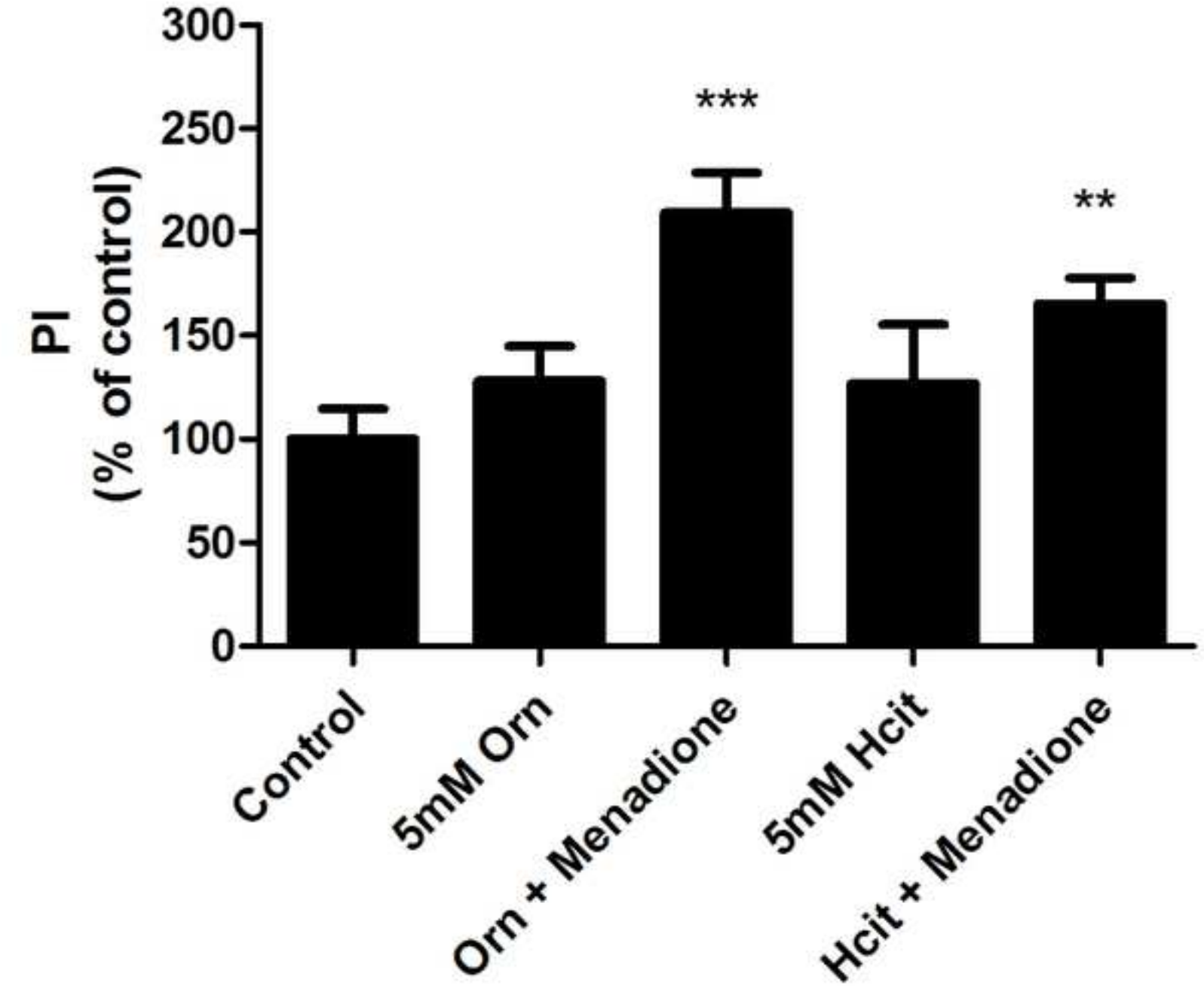

Figure 2

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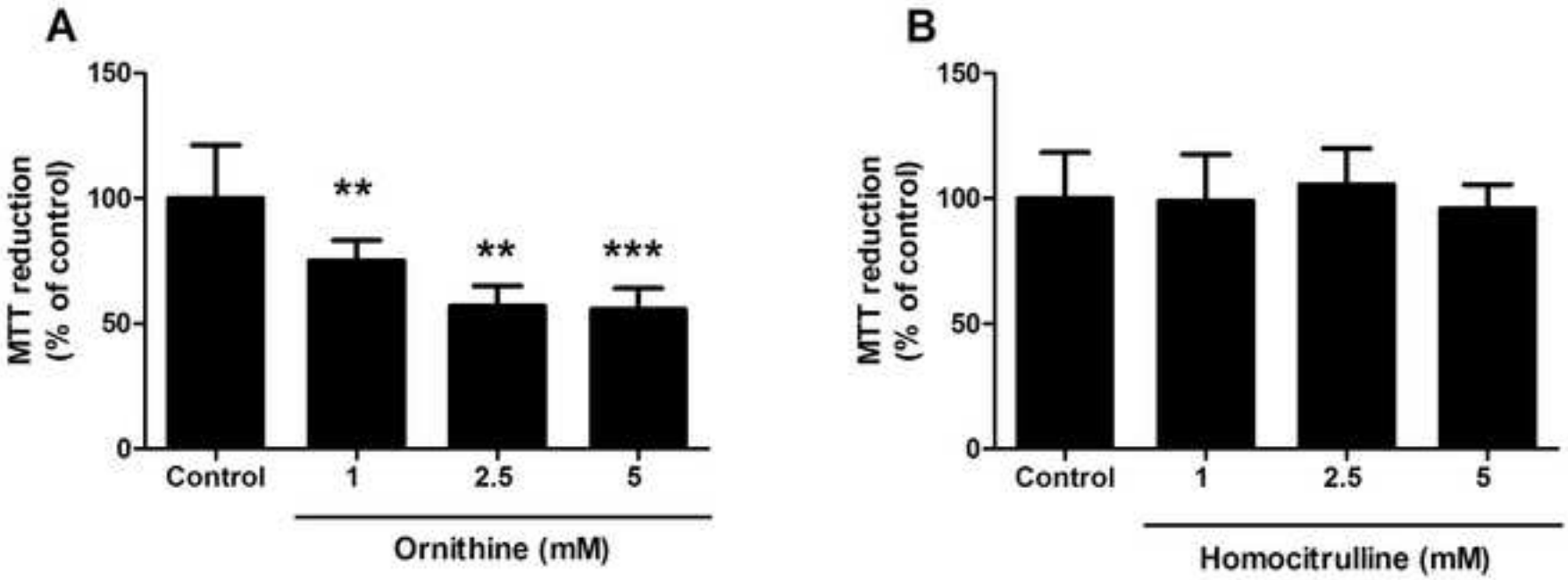

Figure 3

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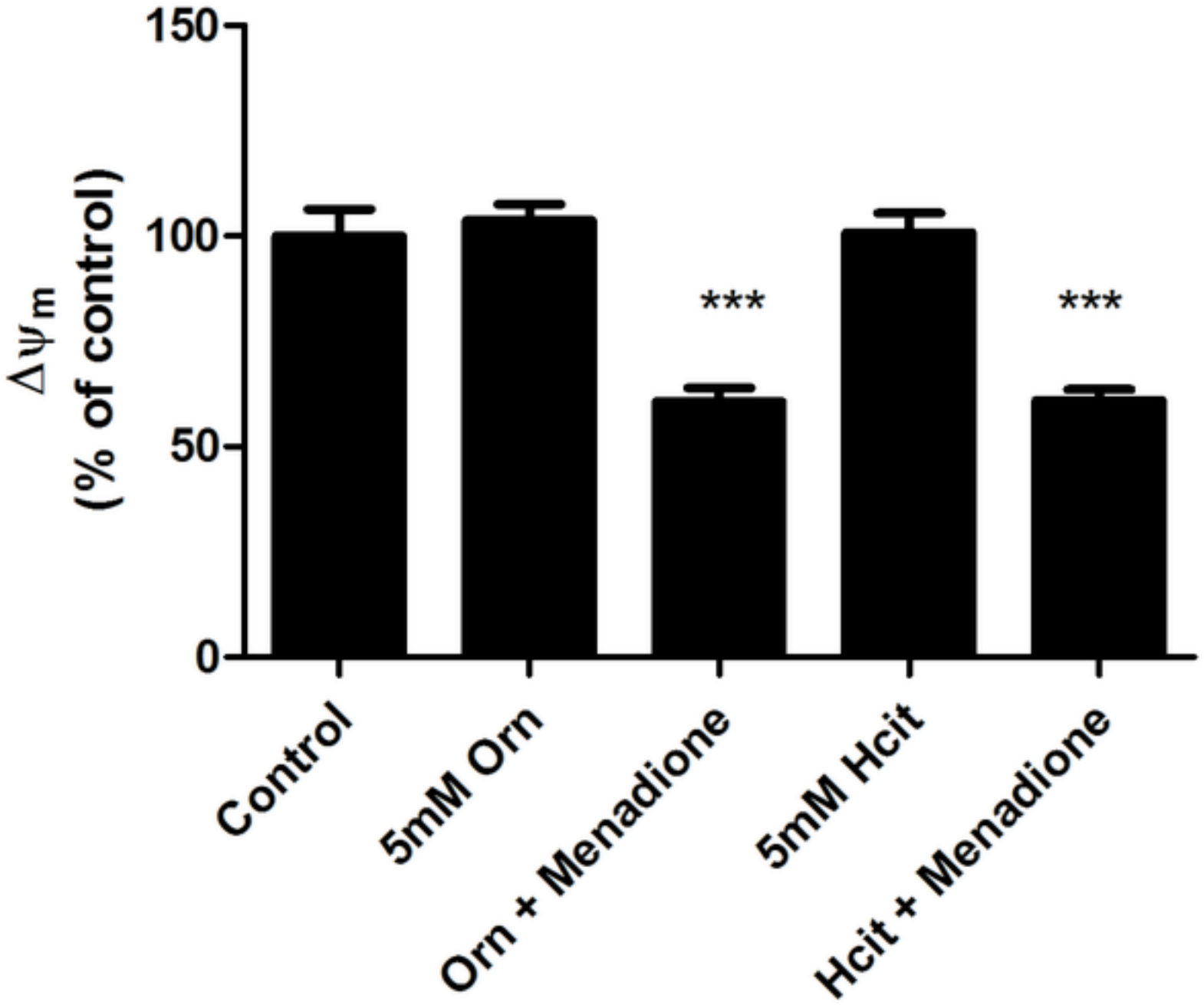

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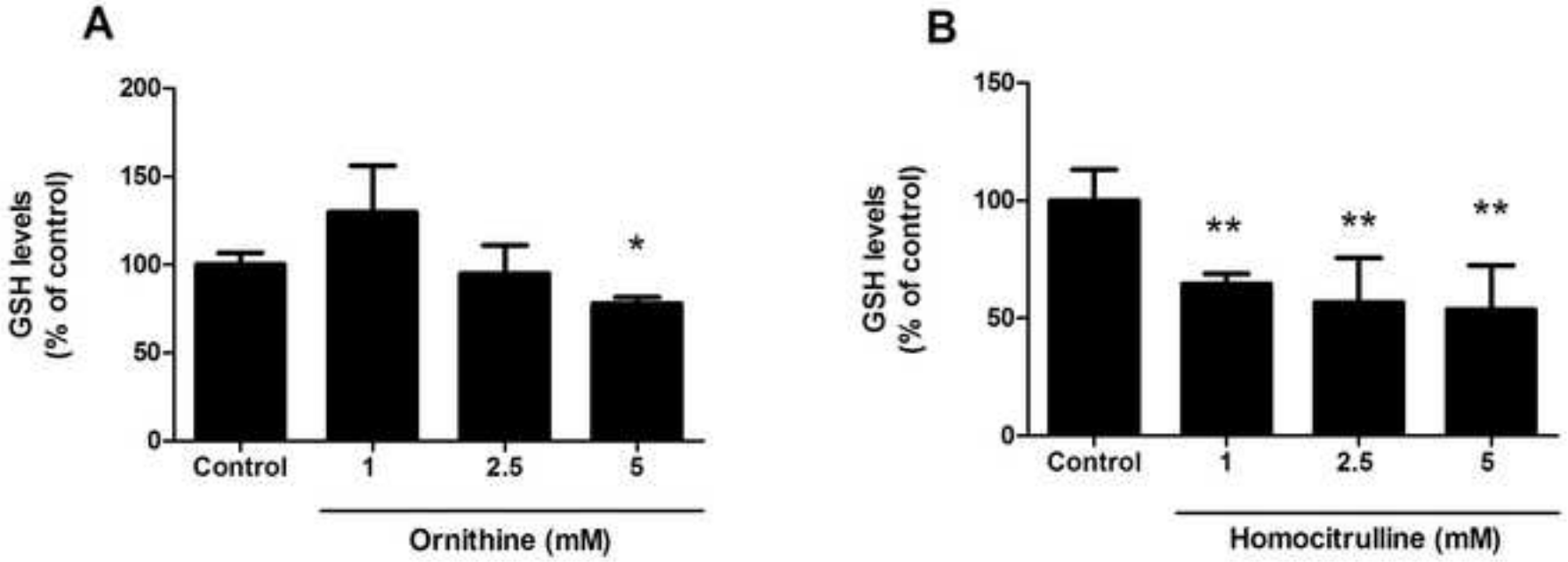

Table 1. Effects of ornithine (Orn) and homocitrulline (Hcit) on interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and NFKβ levels in astrocyte cultures from cerebral cortex of rats.

	IL-1β	IL-6	TNF-α	NFKβ
1 mM Orn	99.5 ± 3.31	112.3 ± 3.51	102 ± 2.08	99.2 ± 3.77
1 mM Hcit	94.5 ± 9.81	110 ± 10.2	99.7 ± 4.03	100 ± 2.98

Values are mean ± standard deviation for three to four independent experiments (wells) performed in triplicate and expressed as percentage of controls. IL- 1β, IL-6 and TNF-α were calculated as ng/mL and expressed as percentage of controls. No differences between groups were detected (one-way ANOVA).

PARTE III

DISCUSSÃO E CONCLUSÕES

III.1 DISCUSSÃO

Pacientes afetados pela síndrome HHH apresentam frequentemente sinais clínicos e sintomas neurológicos, tais como letargia, coma, atraso no desenvolvimento, encefalopatia, atrofia cortical, ataxia, dificuldades na coordenação motora fina, disdiadococinesia e nistagmo (Martinelli et al, 2015). Exames de imagem do SNC dos pacientes revelam alterações no córtex cerebral e cerebelo que podem estar relacionados à sintomatologia neurológica. No entanto, pouco se sabe sobre a patogenia da doença no que se refere às alterações neuropatológicas. Embora de uma forma geral hiperamonemia esteja associada à encefalopatia, alguns pacientes com dano grave no SNC apresentam níveis plasmáticos de amônia normais, de tal forma que os sintomas neurológicos podem não estar associados à hiperamonemia (Salvi et al, 2001; Korman et al, 2004). Sendo assim, é possível que os metabólitos acumulados na doença, tais como a ornitina (Orn) e homocitrulina (Hcit), possam ser potencialmente tóxicos e estarem envolvidos na patogênese da síndrome HHH. Neste particular, trabalhos prévios têm demonstrando um envolvimento desses metabólitos na alteração da homeostase redox e energética *in vitro* e *ex vivo* em córtex cerebral de ratos jovens (Amaral et al, 2009; Viegas et al, 2009; 2011). Considerando que o cerebelo também está comprometido em muitos pacientes com a síndrome HHH, iniciamos nosso trabalho estudando os efeitos *in vitro* da Orn e Hcit sobre a homeostase redox e energética em cerebelo de ratos jovens.

Inicialmente, observamos que tanto a Orn, quanto a Hcit foram capazes de aumentar significativamente os níveis de espécies reativas ao ácido

tiobarbitúrico (TBA-RS) refletindo um aumento nos níveis de malondialdeído (MDA), o qual é um produto final da lipoperoxidação particularmente de ácidos graxos presentes nas membranas celulares (Halliwell and Gutteridge, 2007). Dessa forma, pode-se sugerir que a Orn e a Hcit causam dano oxidativo lipídico em cerebelo. Para elucidar quais espécies reativas estavam envolvidas com o dano lipídico observado, co-incubamos a Orn com alguns antioxidantes clássicos, como GSH, melatonina (MEL), trolox (TRO), ácido lipóico (LA), SOD mais CAT e L-NAME (L-NG-nitroarginina metil Ester). Sabe-se que GSH, MEL, TRO e LA são sequestradores de espécies reativas como o superóxido, hidroxila, peroxila e peroxinitrito. Além de ser sequestrador, o GSH também atua como um cofator da enzima glutationa peroxidase, auxiliando na eliminação de peróxido de hidrogênio. Para determinarmos o possível envolvimento de espécies reativas de nitrogênio com o dano oxidativo observado, avaliamos também a ação do L-NAME, o qual é um inibidor da enzima óxido nítrico sintase. Pudemos observar que o dano oxidativo lipídico foi completamente revertido pelo GSH e MEL, sugerindo que o provavelmente tenha o envolvimento do radical hidroxila, uma vez que tanto o GSH quanto a MEL são sequestradores dessa espécie reativa de oxigênio. Por outro lado, o L-NAME não foi capaz de reverter o efeito pró-oxidante causado pela Orn, o que está de acordo com outros experimentos realizados que mostraram que a produção de nitratos e nitritos não foi alterada por nenhum desses metabólitos. Assim, concluímos que as espécies reativas induzidas pela Orn são fundamentalmente de oxigênio (ERO). Corroborando com isso, observamos também que a Hcit, quando incubada em uma preparação enriquecida de mitocôndria, foi capaz de induzir a produção de peróxido de hidrogênio, o qual,

através da reação de Fenton, é capaz de formar radical hidroxila, ambos ERO (Halliwell and Gutteridge, 2007).

Verificamos também que ao incubarmos o homogeneizado de cerebelo com Orn ou Hcit, ambos metabólitos foram capazes de diminuir as concentrações de GSH, sendo que a Orn também diminuiu o conteúdo de grupamentos sulfidrila. Deve-se salientar que o GSH é o antioxidante encontrado em maior quantidade no cérebro (Halliwell and Gutteridge, 2007). Por outro lado, dois terços dos grupamentos sulfidrila são ligados a proteínas, enquanto um terço está livre na forma de pequenas moléculas, tais como a cisteína e a glutationa reduzida, entre outros, que representam um importante *pool* antioxidante na defesa celular contra o estresse oxidativo (Hansen and Winther, 2009; Thomas et al, 1995; Requejo et al, 2010). A diminuição nos níveis de GSH e do conteúdo de grupamentos sulfidrila indica que as defesas antioxidantes não enzimáticas estão comprometidas por esses aminoácidos.

Avaliamos também a formação de grupamentos carbonila (aldeídos e cetonas) que são principalmente produzidos por oxidação da cadeia lateral das proteínas (especialmente Pro, Arg, Lys e Thr) por uma clivagem oxidativa, bem como pela reação da redução de açúcares ou pela oxidação de seus produtos com resíduos de lisina (Dalle-Done et al, 2003). Geralmente, a detecção de elevados níveis de grupamentos carbonila (dano oxidativo lipídico) leva a perda de função protéica (Dalle-Donne et al, 2009). Embora a formação de grupamentos carbonila é principalmente causada por um dano protéico mediado por ERO, não se pode excluir a possibilidade de que aldeídos resultantes da lipoperoxidação podem induzir a geração desses grupamentos (Dalle-Done et al, 2003). Porém, não observamos nenhuma alteração desse

parâmetro causada pela Orn ou pela Hcit, sugerindo que não houve um dano oxidativo proteico provocado por esses metabólitos no cerebelo.

Uma vez que o estresse oxidativo resulta de um desequilíbrio entre as defesas antioxidantes totais e a produção de espécies reativas (Halliwell and Gutteridge, 2007), nossos dados sugerem que tanto a Orn, quanto a Hcit, são capazes de alterar a homeostase redox em cerebelo de ratos jovens, causando estresse oxidativo. Cabe ainda ressaltar que o SNC é particularmente vulnerável ao aumento de espécies reativas, uma vez que, comparativamente a outros sistemas, ele apresenta menos defesas antioxidantes. Além disso, o cérebro é altamente dependente de energia para o seu funcionamento normal, o que implica em um fluxo aumentado de elétrons pela cadeia respiratória com a consequente formação primária do radical superóxido pelo escapamento de elétrons pela cadeia, e de outros radicais derivados do mesmo como o peróxido de hidrogênio e mesmo o radical hidroxila. Assim, para a produção eficiente de energia na forma de ATP, a mitocôndria possui uma alta demanda por oxigênio, já que utiliza uma grande quantidade de O_2 em uma massa de tecido relativamente pequena, o que torna esse tecido altamente susceptível à ação de espécies reativas (Halliwell and Gutteridge, 2007).

Considerando que o SNC necessita de uma alta demanda de energia para seu funcionamento normal, avaliamos também parâmetros de metabolismo energético em homogeneizado de cerebelo de ratos jovens. Pudemos observar que a Orn ou a Hcit não alteraram as atividades dos complexos da cadeia transportadora de elétrons, do complexo enzimático α-cetoglutarato desidrogenase, da creatina quinase e da enzima Na^+,K^+-ATPase. Além disso, constatamos que tanto a Orn quanto a Hcit foram capazes de

diminuir a atividade da enzima aconitase. Ao tratar especificamente da enzima aconitase, pode-se observar na literatura que a mesma é sensível ao ataque de espécies reativas de oxigênio, podendo representar um parâmetro de estresse oxidativo (Liang et al, 2000; Patel et al, 1996; Tretter e Adam-Vizi, 2000; Tretter et al, 2005; Myers et al, 2010). Para elucidar se a diminuição dessa atividade enzimática foi devido ao ataque oxidativo e, se tal for o caso, investigar quais espécies reativas estavam envolvidas na diminuição da atividade da aconitase causada pela Orn, utilizamos alguns antioxidantes durante os ensaios de medida dessa atividade. Observamos que a GSH foi capaz de reverter completamente a diminuição da atividade da aconitase causada pela Orn, corroborando com os dados anteriores, de um aumento na produção de espécies reativas de oxigênio causando os danos oxidativos observados.

Além disso, sabe-se que a aconitase é suscetível a inativação por ataque oxidativo, principalmente pelo superóxido, resultando na liberação de uma molécula de ferro do centro de seu sítio catalítico 4Fe–4S e na formação de peróxido de hidrogênio (Vasquez-Vivar et al, 2000; Myers et al, 2010). Dessa forma, sugere-se que a inibição induzida pela Hcit pode estar relacionada ao aumento de peróxido de hidrogênio. Por outro lado, deve-se observar que outras espécies reativas são capazes de inativar a aconitase (Gardner et al, 1997). Sendo assim, nossos achados demonstram uma inibição dessa enzima, provocada também pela Orn, a qual não alterou a formação de peróxido de hidrogênio, indicando que outras espécies reativas estão envolvidas na inibição causada pela Orn.

O próximo passo da nossa investigação foi avaliar os efeitos *ex vivo* da Orn e da Hcit através da administração intracerebelar *in vivo* desses

metabólitos em cerebelo de ratos jovens sobre parâmetros de estresse oxidativo e sobre a atividade da enzima Na^+, K^+ ATPase.

Ao avaliarmos os níveis de MDA, pudemos observar que a Orn foi capaz de aumentar significativamente esse parâmetro em dois tempos diferentes de injeção, após 30 minutos e 4 horas de injeção, porém o mesmo efeito não foi observado nos animais que receberam Hcit. Esses resultados sugerem que a Orn induz a oxidação de lipídios *in vivo*. Ao compararmos esses dados com os apresentados nos experimentos *in vitro,* observamos que a Orn foi capaz de aumentar os níveis de MDA em concentrações baixas (0,5 mM), enquanto que a Hcit aumentou esse parâmetro apenas com concentrações mais altas (5 mM). Sendo assim, podemos sugerir que a concentração de Hcit no cerebelo dos animais após a injeção intracerebelar não foi capaz de induzir lipoperoxidação.

Avaliamos também as defesas antioxidantes. Primeiramente, testamos a atividade de importantes enzimas antioxidantes (SOD, CAT, GPx, GR e G6PDH) e observamos que a Orn foi capaz de aumentar a atividade de todas as enzimas testadas. Esse aumento pode ser atribuído a uma resposta rebote com maior síntese na produção da proteína/enzima com a finalidade de compensar e controlar um provável aumento da produção de espécies reativas. Trabalhos anteriores demonstraram que a Orn está envolvida com o aumento de espécies reativas (Amaral et al, 2009; Viegas et al, 2009, 2011). Nesse particular, sabe-se que as espécies reativas podem regular a expressão de enzimas antioxidantes através de diferentes cascatas de sinalização (Halliwell and Gutteridge 2007; Olsen et al, 2014), provocando, dessa forma, o aumento das defesas antioxidantes. Uma das vias de sinalização mais conhecidas é a

do fator eritroide nuclear 2 relacionado com o fator 2 (Nrf-2) que regula o estado redox celular, além de desempenhar outras funções como modular o metabolismo intermediário e auxiliar na degradação de macromoléculas (Hayes e Dinkova-Kostova, 2014).

Por outro lado, um aumento na atividade da SOD pode ocorrer em resposta a um aumento na formação de superóxido, o qual, através de uma dismutação proporcionada pela enzima, irá gerar um aumento na formação de peróxido de hidrogênio, o que poderia explicar o aumento na atividade da CAT. Por sua vez o aumento na atividade da GPx pode estar relacionado a uma indução na formação de peróxido de hidrogênio pela Orn, uma vez que a GPx degrada essa espécie reativa que é utilizada na oxidação do GSH. Por fim, um aumento na GR e na G6PDH que são responsáveis pelo *pool* de NADPH utilizado pela GR para realizar a conversão de glutationa oxidada (GSSG) a GSH, podem refletir uma resposta celular para garantir a manutenção da razão GSH/GSSG. Além disso, o aumento na atividade da GR pode também explicar o fato de a administração de Orn não ter alterado os níveis de GSH. Enfatize-se que nos experimentos *in vitro* pudemos observar que a Orn alterou os níveis de GSH, porém esse mesmo efeito não foi observado ao injetarmos esse metabólito no cerebelo. Esses resultados controversos podem ser explicados pelo fato de que nos experimentos *in vivo* existe um sistema integro, onde todos os componentes celulares são preservados, podendo ocorrer a regulação dos genes da GR e G6PDH.

Outro parâmetro avaliado foi a atividade da enzima Na^+,K^+-ATPase que é muito importante para a manutenção do gradiente eletroquímico da membrana celular e, portanto, para a neurotransmissão (Lingrel & Kuntzweiler

1994; Geering, 1990). Observamos que a Orn foi capaz de reduzir significativamente a atividade dessa enzima. Tal redução pode ser atribuída ao ataque oxidativo por espécies reativas, uma vez que a interação entre as subunidades α (catalítica) e β (regulatória) da enzima é vulnerável ao dano oxidativo (Kurella et al, 1997; Muriel e Sandoval 2000; Figtree et al, 2006). Neste sentido, ao injetarmos a Orn, observamos um aumento em torno de 87% dos níveis de espécies reativas ao ácido tiobarbitúrico (TBA-RS) que indica dano oxidativo lipídico devido à espécies reativas. Além disso, oxidação lipídica na membrana celular íntegra pode diminuir a atividade da Na^+,K^+-ATPase, uma vez que ela está integrada à membrana plasmática e depende da composição de lipídios próximos. Outra hipótese é a de que a Orn pode potencialmente interferir na fluidez de membrana, local onde a enzima está ancorada, alterando dessa forma a sua atividade (Wheeler et al, 1975; Busanello et al, 2013). Devemos, entretanto esclarecer que esses mecanismos não foram investigados no presente trabalho.

Enfatize-se que diminuição na atividade dessa enzima são muito importantes, uma vez que a mesma consome em torno de 40 a 50% do ATP cerebral para poder desempenhar sua função crucial na manutenção do gradiente eletroquímico celular e consequentemente na manutenção do potencial de repouso, o qual é de suma importância na neurotransmissão. Nesse sentido, outros trabalhos demonstraram uma diminuição da atividade da Na^+,K^+-ATPase em doenças neurodegenerativas (Ellis et al. 2003; Dickey et al. 2005; Vignini et al. 2007; Bagh et al. 2008; Busanello et al. 2010, 2011, 2013; Seminotti et al. 2011; Moura et al. 2012; Amaral et al. 2012; de Lores Arnaiz

and Ordieres 2014). Sendo assim, uma diminuição na atividade dessa enzima, provocada pela Orn, pode prejudicar a neurotransmissão no cerebelo.

Considerando em seu conjunto os dados *in vitro* e *ex vivo*, pudemos observar que a Orn apresentou resultados mais proeminentes em relação a Hcit. Esses achados podem ser relevantes para a fisiopatologia da doença, uma vez que os níveis de Orn plasmática podem ser superiores a 1,0 mM (valores normais 30-110 µmol/L). Além disso, a excreção urinária de Orn (8100 µmol/g de creatinina – valores normais: 9 - 120 µmol/g de creatinina) é superior a de Hcit (2380 µmol/g de creatinina – valores normais: 0 - 30 µmol/g de creatinina) (Valle e Simell 2001; Palmieri 2008)

Nossos resultados demonstram um envolvimento da Orn e da Hcit na indução *in vitro* e *ex vivo* de estresse oxidativo em cerebelo. No entanto, não observamos um dano significativo na homeostase energética provocado por esses metabólitos. Dessa forma, podemos concluir que o dano oxidativo e o desequilíbrio nas defesas antioxidantes (distúrbio na homeostase redox) estejam potencialmente envolvidos no dano cerebelar observado nos pacientes afetados pela síndrome HHH.

Devemos ainda enfatizar que estudamos os efeitos da Orn e Hcit em um modelo subcelular (homogeneizado celular) e também em tecido íntegro (injeção intracerebelar) em cerebelo. Os resultados que obtivemos com esses modelos de frações celulares e células íntegras foram muito semelhantes, indicando alteração preponderante dos metabólitos afetando o estado redox celular. Tendo em vista que resultados prévios realizados com córtex cerebral foram realizados em frações celulares (homogeneizados de córtex cerebral) e pelo fato de que nessas preparações todas as células neurais estão

envolvidas, decidimos investigar os efeitos da Orn e Hcit sobre algumas parâmetros em astrócitos (células intactas) cultivados de córtex cerebral de ratos neonatos.

Enfatize-se que os astrócitos são células neurais fundamentais nas interações neuronais-astrocitárias e nada foi relatado sobre a influência dos principais metabolitos que se acumulam na síndrome HHH sobre funções importantes em sistemas celulares com células intactas e mais particularmente sobre astrócitos.

Determinamos então os efeitos da Orn e Hcit sobre a viabilidade celular (medida pela incorporação de iodeto de propídio), a função mitocondrial (medida pela redução do 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT) que representa atividade de desidrogenases mitocondriais e medição de JC1 que indica potencial de membrana mitocondrial), bem como sobre as defesas antioxidantes e parâmetros inflamatórios (citocinas) em culturas primárias de astrócitos de córtex cerebral de ratos neonatos. Também expusemos os astrócitos a uma dose subtóxica de menadiona, um indutor da produção de radicais livres para mimetizar o estado do cérebro durante crises metabólicas devidas a infecções que estão associadas a processo inflamatório. Verificamos que a dose utilizada de menadiona não alterou per se os parâmetros medidos e também não foi capaz de induzir uma produção aumentada de citocinas, o que era nosso propósito. Por outro lado, observamos que os astrócitos estimulados por menadiona foram mais sensíveis aos efeitos deletérios da Orn e da Hcit.

Assim, verificamos que a Orn e a Hcit diminuíram a viabilidade, a função mitocondrial, as defesas antioxidantes (GSH), sem alterar a produção de citocinas principalmente em astrócitos estimulados por menadiona. Tais

resultados indicam a participação de astrócitos nos efeitos provocados pelos principais metabólitos acumulados na síndrome HHH.

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III. 2 CONCLUSÕES

O presente trabalho indica que a Orn e a Hcit alteram pouco a homeostase energética, mas comprometem a homeostase redox em cerebelo e em cultura de astrócitos de córtex cerebral. Presumimos que essas alterações possam representar mecanismos fisiopatogênicos do dano cerebral característico da síndrome HHH.

III.3 PERSPECTIVAS

- Injetar Orn e Hcit no 4º ventrículo de ratos neonatos (1 dia de vida) e investigar parâmetros de estresse oxidativo e metabolismo energético após diferentes tempos de exposição aos metabólitos
- Analisar parâmetros de imunohistoquímica (GFAP, S100B, FluoroJade, BrdU) após injeção de Orn e Hcit no 4º ventrículo em ratos neonatos
- Analisar parâmetros do metabolismo energético e da homeostase redox em tecidos de pacientes com a síndrome HHH

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