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Efeito do ácido lipoico sobre parâmetros de estresse oxidativo em modelo animal de fenilcetonúria

Tese de Doutorado

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"Ao meu irmão, mãe e pai (*in memoriam*), que sempre me inspiram e me apoiam!"

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PARTE III

LISTA DE ABREVIATURAS

ADHL – ácido diidrolipoico
AL – ácido lipoico
BH_{2q} – diidrobiopterina (forma quinóide)
BH_4 – tetra-hidrobiopterina
BHE – barreira hematoencefálica
CAT – catalase
DHPR – diidropterina redutase
EIM – erros inatos do metabolismo
ER – espécies reativas
ERO – espécies reativas de oxigênio
ERN – espécies reativas de nitrogênio
EO – estresse oxidativo
G6PD – glicose 6-fosfato desidrogenase
GCL – glutamato cisteína ligase
GR – glutationa redutase
GS – glutationa sintetase
GSH – glutationa reduzida
GSH-Px – glutationa peroxidase
GSSG – glutationa oxidada
GST – glutationa S-transferase
HPA – hiperfenilalaninemia
LPO – lipoperoxidação ou peroxidação lipídica
PAA - fenilacetato
PAH – fenilalanina hidroxilase
Phe – L-fenilalanina
PKU – fenilcetonúria
PLA – fenilactato
PPA – fenilpiruvato
SC – subcutânea
SNC – sistema nervoso central
SOD – superóxido dismutase
TBA-RS – substâncias reativas ao ácido tiobarbitúrico
Trp – triptofano
Tyr – tirosina

RESUMO

A fenilcetonúria (PKU) é causada pela deficiência severa da atividade da fenilalanina hidroxilase (PAH), enzima responsável pela conversão de fenilalanina (Phe) em tirosina (Tyr), levando ao aumento dos níveis sanguíneos e teciduais de Phe, bem como de seus metabólitos fenilpiruvato (PPA), fenilactato (PLA) e fenilacetato (PAA). Os pacientes com PKU apresentam disfunção neurológica severa, manifestando convulsões, retardo mental e psicomotor, sintomas que estão associados ao acúmulo desse aminoácido e seus metabólitos. A restrição dietética, que faz parte do tratamento da PKU, nem sempre é mantida pelos pacientes e pode afetar o *status* antioxidante devido à restrição de nutrientes. Estudos recentes em ratos e com pacientes fenilcetonúricos mostram que o estresse oxidativo (EO) pode estar envolvido na neurofisiopatologia dessa doença, possivelmente devido ao aumento na produção de espécies reativas, e diminuição das defesas antioxidantes. O cérebro, órgão afetado na doença, é extremamente sensível ao EO devido a baixas defesas antioxidantes, alta concentração de ferro e lipídeos insaturados. Aparentemente o fígado não é afetado na PKU, mas é um importante órgão de detoxificação e reservatório de GSH (tripeptídeo antioxidante). O ácido lipoico (AL) é um potente antioxidante facilmente adquirido da dieta, absorvido pelo organismo e tem sido sugerido em estudos para o tratamento e prevenção de EO em modelos de doenças neurodegenerativas. Um estudo recente demonstrou um efeito protetor do AL contra o EO gerado por uma concentração tóxica de Phe em cérebro de ratos jovens. O objetivo do trabalho foi avaliar o efeito do AL na prevenção do EO gerado em um modelo crônico de PKU induzido por injeções diárias de Phe e α-metilfenilalanina (inibidor da PAH). No presente trabalho, o modelo de hiperfenilalaninemia (HPA) causou, no cérebro, aumento de dano a lipídeos, proteínas e DNA; aumento da atividade da superóxido dismutase e diminuição da atividade da catalase, além de mostrar que essas enzimas podem ter um papel importante neste processo, uma vez que suas atividades são afetadas diretamente pela presença de Phe e seus metabólitos. Também foi possível descrever algumas espécies reativas específicas geradas no processo oxidativo envolvido na HPA, como de H_2O_2, $NO^{\bullet}$ e $O_2^{\bullet-}$. E ainda, identificou-se que o EO não está restrito ao cérebro e que o fígado pode ter um papel importante em defesa ao EO encontrado na doença. As enzimas glutationa peroxidase, glutationa redutase, glicose-6-fosfato desidrogenase e o conteúdo total de GSH foram diminuídos pela HPA em cérebro dos animais e a atividade da glutamato cisteína ligase foi aumentada. Já no fígado, todas as enzimas relacionadas ao metabolismo da GSH foram aumentadas pela HPA. O tratamento com AL foi capaz de prevenir as alterações enzimáticas além de impedir o dano a biomoléculas. O AL também preveniu o aumento da produção de H_2O_2 e $NO^{\bullet}$ no cérebro dos animais submetidos ao tratamento de HPA. Quanto ao metabolismo da GSH, o AL foi capaz de manter as atividades enzimáticas aos níveis do controle além de restaurar a produção de GSH no cérebro dos animais afetados pela HPA. De acordo com os resultados, é possível que um tratamento com antioxidantes seja eficaz na manutenção da homeostasia *redox* nos pacientes servindo como uma abordagem terapêutica inovadora e adicional ao tratamento dietético já aplicado aos pacientes de PKU.

ABSTRACT

Phenylketonuria (PKU) is caused by a severe deficiency of phenylalanine hydroxylase (PAH), the enzyme responsible for the conversion of phenylalanine (Phe) to tyrosine (Tyr), leading to increased blood and tissue levels of Phe and its metabolites phenylpyruvate (PPA), phenylactate (PLA) and phenylacetate (PAA). Patients with PKU have severe neurological dysfunction, characterized by seizures, mental retardation and psychomotor symptoms that are associated with the accumulation of this amino acid and its metabolites. A restrict diet important for PKU treatment, is not always maintained by patients and this can affect the antioxidant status due to nutrient limitation. Recent studies in rats and patients with PKU show that oxidative stress may be involved in the neuropathophysiology of this disease, possibly due to increased production of reactive oxygen species and decreased antioxidant defenses. The brain, organ affected in the disease, is extremely sensitive to oxidative stress due to low antioxidant defenses and high concentrations of Fe and unsaturated lipids. Apparently, the liver is not affected in PKU, but it is an important organ of detoxification and GSH reservoir (tripeptide antioxidant). Lipoic acid (LA) is a potent antioxidant easily acquired from the diet, absorbed by the body and has been suggested for the treatment and prevention of oxidative stress in different neurodegenerative diseases in many studies. A recent study showed a protective effect of LA against the oxidative stress generated by a toxic concentration of Phe in the brain of young rats. The aim of this study was to evaluate the effect of LA in preventing the oxidative stress generated in a chronic model of PKU induced by daily injections of Phe and α-methylphenylalanine (PAH inhibitor) for 7 days. The hyperphenylalaninemia (HPA) model caused in brain an increase of damage to lipids, proteins and DNA; increased superoxide dismutase activity and decreased catalase activity, showing that these enzymes may play an important role in this process, since their activities are directly affected by the presence of Phe and its metabolites. It was also reported some specific reactive species generated in the oxidation process involved in HPA as H_2O_2, $NO^\bullet$ and $O_2^{\bullet-}$. In addition, it was found that oxidative stress is not restricted to the brain, and the liver may play an important role in defense to oxidative stress found in the disease. Activities of glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and total content of GSH were decreased by the HPA in the brain of animals and the activity of GCL was increased. In the liver, all enzymes related to GSH metabolism were increased by the HPA. Treatment with LA was able to prevent the enzymatic changes in addition to preventing damage to biomolecules. The overproduction of H_2O_2 and $NO^\bullet$ by HPA model was inhibited by LA treatment. Regarding to GSH metabolism, LA was able to maintain enzyme activities and GSH production at control levels in the brain of animals affected by HPA. According to our results, it is possible that a treatment with antioxidants is effective in maintaining redox homeostasis in patients and may be a novel therapeutic approach additional to dietary treatment already applied to PKU patients.

PARTE I

INTRODUÇÃO

1.1. Erros Inatos do Metabolismo (EIM)

Archibald Garrod propôs em 1908, a partir de observações em pacientes com alcaptonúria, que algumas doenças ocorriam devido à deficiência de uma enzima específica responsável por uma rota metabólica. Fundamentado nos conceitos das leis de Mendel, Garrod pôde explicar os casos observados como defeitos hereditários com caráter de herança autossômica e recessiva. Porém, somente em 1941 George Beadle e Edward Tatum elaboraram a teoria "um gene – uma enzima" na qual um gene é responsável pela síntese de uma única enzima, esclarecendo as causas dos EIM (Beadle e Tatum, 1941). Sendo assim, os EIM são considerados doenças hereditárias causadas por alguma deficiência (parcial ou severa) em uma enzima, causando o bloqueio de uma determinada rota metabólica e consequente acúmulo tóxico de substratos e/ou escassez de produtos essenciais ao metabolismo (Scriver et al., 2001).

Desde então, muitos estudos têm descrito e classificado os erros inatos em todas as áreas do metabolismo, sendo atualmente mais de 500 doenças (Scriver et al., 2001). Apesar de serem doenças raras, como grupo a frequencia é relativamente alta, 1 para cada 2.000 nascimentos (Baric et al., 2001). Os EIM foram classificados em três grandes grupos por Saudubray e Charpentier (2001): doenças com defeito na síntese ou catabolismo de moléculas complexas, que incluem distúrbios de depósito lisossômico e doenças peroxissomais; doenças com defeito no metabolismo intermediário, como as aminoacidopatias e as acidemias orgânicas; e doenças com defeito na produção ou utilização de energia, que incluem os distúrbios de depósito de glicogênio, defeitos na

gliconeogênese e oxidação de ácidos graxos. As aminoacidopatias, que incluem a PKU, e as acidemias orgânicas, são os EIM mais frequentes (Hoffmann, 1984).

1.2. Fenilcetonúria e hiperfenilalaninemia

A L-fenilalanina (Phe) é um aminoácido essencial cujo primeiro passo de seu metabolismo requer a ação de uma enzima hepática denominada fenilalanina hidroxilase (PAH – EC 1.14.16.1), responsável pela conversão de Phe em L-tirosina (Tyr), com a redução do cofator tetra-hidrobiopterina (BH_4). O BH_4 é regenerado pela enzima diidropteridina redutase (DHPR – EC 1.6.99.7) que é dependente de NADH (Figura 1). Já por descarboxilação e transaminação, a Phe é convertida em seus metabólitos fenilpiruvato (PPA), fenilacetato (PAA) e fenilactato (PLA), os quais são livremente excretados em indivíduos normais (Nyhan, 1984) (Figura 2). Qualquer impedimento da hidroxilação da Phe (por deficiência na atividade da PAH, da DHPR ou na síntese de BH_4) gera uma condição de hiperfenilalaninemia (HPA) com aumento plasmático de Phe (acima de 120 µM). O gene que codifica a PAH está localizado no cromossomo 12 e pode apresentar mais de 500 mutações (Woo et al., 1983). Cada mutação ou combinação de mutações causa variações na atividade residual da PAH como característica fenotípica. A principal causa de HPA é a deficiência severa na atividade da PAH conhecida como fenilcetonúria (PKU) (Scriver e Kaufman, 2001). O bloqueio da rota principal do catabolismo da Phe provoca o acúmulo deste aminoácido e de seus metabólitos no plasma e tecidos dos pacientes afetados, e a consequente presença de altos níveis dessas substâncias na urina dos pacientes (Knox, 1972; Nyhan, 1984).
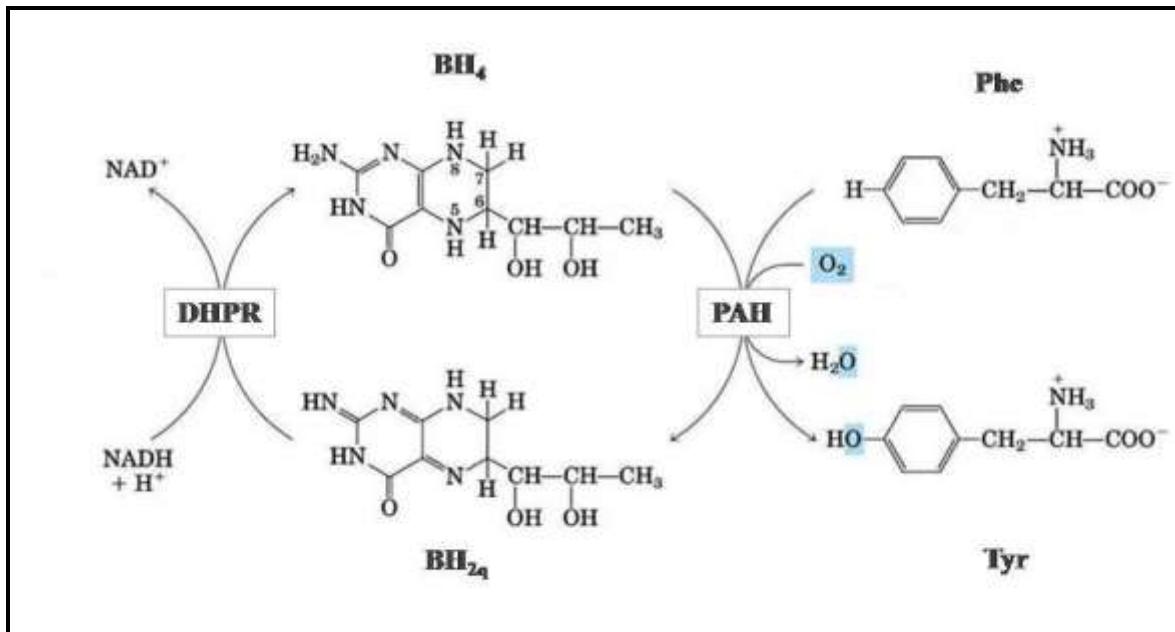
Figura 1. Reação de hidroxilação da fenilalanina. Adaptado de Lehninger et al. (1993).

A PKU tem uma incidência mundial de aproximadamente 1:10.000 nascimentos (Scriver e Kaufman, 2001), sendo que no Brasil há, atualmente, mais de 1.200 pacientes em tratamento (Monteiro e Candido, 2006). Os pacientes não tratados podem apresentar sinais e sintomas que se manifestam em maior ou menor intensidade: alterações cutâneas, distúrbios de pigmentação, hipoplasia dentária, descalcificação de ossos longos, atraso no desenvolvimento psicomotor, falha no sistema cognitivo, microcefalia, epilepsia, retardo no crescimento e convulsões (Williams et al., 2008).

1.3. Diagnóstico e tratamento

O diagnóstico já nos primeiros dias de vida é importante devido ao dano neurológico irreversível causado se os pacientes não forem tratados precocemente (Scriver e Kaufman, 2001; Kohli et al., 2005). Na triagem neonatal, essa doença pode ser identificada precocemente por diversos testes, incluindo o teste de inibição bacteriana em

uma amostra de sangue coletada em papel filtro e métodos cromatográficos, fluorimétricos e enzimáticos (Clague e Thomas, 2002). Alguns países já utilizam espectrometria de massa em Tandem para a identificação de EIM (Simon et al., 2006).

O tratamento para a PKU consiste principalmente de uma dieta hipoproteica, restrita no aminoácido acumulado, a fim de reduzir ou normalizar os níveis deste aminoácido, já que o grau de retardo mental está diretamente relacionado com os níveis elevados no plasma e tecidos dos pacientes afetados (Scriver e Kaufman, 2001). Para os pacientes, alimentos como ovos, leite, pães, queijos e carne são proibidos. Entretanto reposições proteicas, necessárias para o crescimento da criança, são feitas a partir de suplementos comerciais sem Phe (Williams et al., 2008). Os benefícios do tratamento dietético são a melhora neuropsiquiátrica dos pacientes afetados e a prevenção do dano neurológico (Zeman et al., 1996; Hanley, 2004).

1.4. Fisiopatologia da PKU

As principais alterações da PKU ocorrem no desenvolvimento e funções neurológicas. Muitas são as hipóteses de neurofisiopatologia nesse EIM, como o acúmulo do aminoácido ou metabólito não degradado pela enzima deficiente gerando toxicidade. Em pacientes a neurotoxicidade está relacionada ao acúmulo de Phe. Já em modelos experimentais com animais tanto a Phe quanto os metabólitos causam toxicidade, sendo o PAA considerado o mais tóxico (Kaufman, 1989).

Por outro lado, outra hipótese de mecanismo de neuropatologia neste EIM está relacionada ao transporte dos aminoácidos. O acúmulo de Phe no caso da PKU pode dificultar a passagem de outros aminoácidos neutros de cadeia longa pela BHE, visto que utilizam o mesmo tipo de transportador (LNAAt). Isso resulta em menor concentração

destes outros aminoácidos no cérebro e consequentemente a diminuição de síntese proteica e de neurotransmissores (Killian e Chikhale, 2001; Le Masurier et al., 2006; Hoeksma et al., 2009). A redução na síntese de serotonina, de dopamina e de noradrenalina está relacionada à deficiência relativa de Tyr e triptofano (Trp) (Tam e Roth, 1997). Na PKU a Tyr passa a ser um aminoácido essencial, uma vez que a rota de síntese está bloqueada na doença (van Spronsen et al., 1996).

Os mecanismos de neuropatologia neste tipo de EIM permanecem pouco esclarecidos, porém estudos indicam envolvimento de outras enzimas que podem estar relacionadas ao dano neurológico encontrado na PKU, como a Na^+K^+-ATPase de membranas sinápticas de ratos submetidos a um modelo animal de HPA, a qual está inibida (Wyse et al., 1995). Além disso, mostrou-se que o metabolismo energético é prejudicado em preparados de cérebro de ratos em presença de Phe (Rech et al., 2002), indicando que alterações no metabolismo energético também podem estar relacionadas ao mecanismo de neuropatologia na PKU.

Recentemente o estresse oxidativo (EO) tem sido sugerido como um participante na fisiopatologia de alguns EIM devido ao acúmulo de metabólitos tóxicos que podem levar à excessiva produção de radicais livres e/ou à diminuição das defesas antioxidantes (Wajner et al., 2004). Diversos estudos relatam o aumento de marcadores de EO tanto em modelos animais quanto em pacientes com PKU (Sitta et al., 2009 a,b; Vargas et al., 2011; Ribas et al., 2011). E ainda, o dano morfológico cerebral ou ao DNA relacionado ao EO em modelos animais de PKU pode ser revertido pelo uso de antioxidantes (Martinez-Cruz et al., 2002). Além disso, a terapia de dietas restritivas também pode alterar o *status* antioxidante dos pacientes contribuindo para a indução de EO nestes pacientes (Artuch et

al., 2004; Schulpis et al., 2005). Este trabalho tem como foco de estudo o EO na PKU em animais tratados com antioxidante.
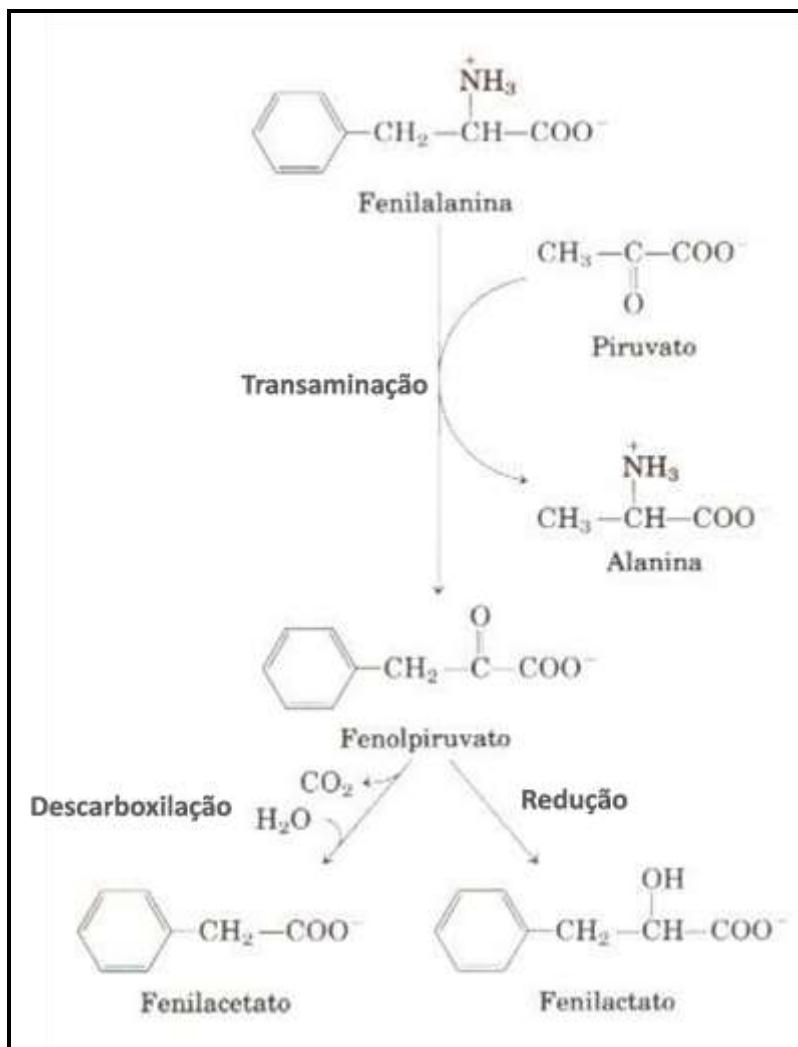
Figura 2. Rota alternativa para o catabolismo da fenilalanina em pacientes. Adaptado de Lehninger et al. (1993).

1.5. Radicais livres e espécies reativas de oxigênio e nitrogênio

O termo radical livre refere-se a moléculas ou átomos de existência independente que possuem um ou mais elétrons desemparelhados, ou seja, ocupam um orbital atômico ou molecular sozinhos. Essa característica confere uma reatividade e instabilidade energética a essas moléculas que tenderão a adquirir um segundo elétron para emparelhar o orbital

(Halliwell e Gutteridge, 2007). Assim, além da alta reatividade dos radicais livres, a presença de um único radical pode iniciar uma sequência de reações em cadeia de transferência de elétrons (Maxwell, 1995). Nessas reações em cadeia, um único radical pode levar à formação de um produto que também é um radical livre e que, por sua vez, reage produzindo um terceiro radical e assim sucessivamente.

Nos sistemas biológicos são produzidos diversos tipos de radicais livres. No metabolismo celular aeróbico o próprio oxigênio molecular é um birradical estável que pode sofrer redução tetravalente, resultando na formação de água (H_2O). Ao longo deste processo parte do oxigênio pode ser convertido a intermediários reativos como o radical ânion superóxido ($O_2^{\bullet-}$) e o radical hidroxila ($^{\bullet}OH$). No entanto, existem compostos reativos como os radicais livres, mas que não possuem elétrons desemparelhados, sendo classificados como espécies reativas (ER). Assim, o termo espécies reativas de oxigênio (ERO) refere-se não somente aos radicais livres, mas também a alguns derivados não radicalares do oxigênio como o peróxido de hidrogênio (H_2O_2). Dessa forma, todo radical livre é uma ERO, mas nem toda ERO é um radical livre (Halliwell e Gutteridge, 2007). Além dessas, existem as espécies reativas de nitrogênio (ERN), sendo as principais o óxido nítrico ($NO^{\bullet}$) e o peroxinitrito ($ONOO^{-}$).

A formação de ERO e ERN ocorre tanto em processos fisiológicos quanto patológicos do organismo, apresentando diversas funções fisiológicas. O $O_2^{\bullet-}$ é formado no organismo principalmente através da cadeia transportadora de elétrons mitocondrial, mas também pela ação de células fagocitárias durante o processo de defesa contra uma infecção (Delanty e Dichter, 1998). Além desse, o $^{\bullet}OH$ é o radical de oxigênio mais reativo e uma vez formado reage rapidamente, podendo atacar qualquer biomolécula. O $^{\bullet}OH$ é formado pela reação entre o $O_2^{\bullet-}$ e o H_2O_2 (Reação de Haber-Weiss) ou pela reação entre o H_2O_2 e

metais de transição como o ferro e o cobre (Reação de Fenton), a partir de ONOO⁻, ou irradiação de alta energia (Halliwell e Gutteridge, 2007). O H_2O_2 é formado em praticamente todos os tecidos do organismo e apesar de fracamente reativo sua importância está relacionada à sua capacidade de formar o $^{\bullet}OH$ (Halliwell, 2001). Já o $NO^{\bullet}$ é um radical pouco reativo que apresenta grande importância biológica atuando na vasorregulação e neurotransmissão, mas em excesso pode ser tóxico. A reação entre os radicais $NO^{\bullet}$ e $O_2^{\bullet-}$ leva a formação de ONOO⁻, o qual apresenta maior reatividade podendo oxidar lipídios, DNA e aminoácidos (Halliwell, 2001).

A produção deliberada de ER tóxicos pode ocorrer em resposta inflamatória do organismo ou condições patológicas (Boveris e Chance, 1973), porém outros fatores externos como dieta inadequada, consumo exagerado de álcool e drogas, radiação, poluentes, etc. Também podem aumentar a produção de radicais livres nas células (Aseervatham et al., 2013).

1.6. Defesas antioxidantes

As células possuem um sistema de defesa contra os danos produzidos pelas ER, transformando-as em compostos inativos, o chamado sistema antioxidante. Os antioxidantes são definidos como qualquer substância endógena ou exógena que, quando presente em baixas concentrações, previne ou retarda a oxidação de um substrato oxidável. As defesas antioxidantes podem ser enzimáticas ou não enzimáticas, podendo remover as ER cataliticamente (catalase, superóxido dismutase); diminuir a disponibilidade de substâncias pró-oxidantes (íons de Fe e Cu) como as transferinas, proteger biomoléculas contra dano (chaperonas), ou sequestrar diretamente ER como glutationa (GSH), ácido ascórbico e α-tocoferol (Halliwell e Gutteridge, 2007).

Agentes hidrossolúveis e lipossolúveis compõem o sistema antioxidante não-enzimático, incluindo vitaminas (A, C e E), selênio, GSH, ácido úrico, melatonina e polifenóis. O modo de ação dos antioxidantes não-enzimáticos abrange desde a remoção direta do oxigênio e o sequestro das ER ou de seus precursores já presentes no organismo, como a prevenção da formação de novas ER (Halliwell, 1994).

O sistema antioxidante enzimático também é importante na eliminação de ER. As enzimas mais estudadas são a superóxido dismutase (SOD) que catalisa a dismutação de $O_2^{\bullet-}$ reduzindo-o a H_2O_2. A catalase (CAT), presente principalmente nos peroxissomos, reduz esse H_2O_2 a H_2O prevenindo a formação de $^{\bullet}OH$. As glutationas-peroxidases, encontradas no citosol e mitocôndrias, eliminam o H_2O_2 utilizando a glutationa (GSH) como substrato, gerando ao final da reação H_2O e glutationa oxidada (GSSG). A GSSG consiste de duas GSH ligadas por ponte dissulfeto e pode ser convertida novamente a GSH pela enzima glutationa redutase (GR) com a utilização de NADPH que, por sua vez, é fornecido pela ação de enzimas do metabolismo da glicose-6-fosfato, como a glicose-6-fosfato desidrogenase (Halliwell, 2006).

O sistema de defesa antioxidante não é totalmente efetivo, uma vez que em sistemas biológicos aeróbicos há presença de dano a biomoléculas. Portanto, alguns autores classificam como defesa antioxidante os sistemas de reparo necessários para combater moléculas danificadas, como reparo ao DNA, lipídios e proteínas danificados (Halliwell e Gutteridge, 2007).

1.7. Metabolismo da glutationa

A GSH é um tripeptídeo (γ-glutamilcisteinilglicina), e um dos mais importantes e abundantes antioxidantes contra ER, principalmente no cérebro onde as concentrações

alcançam 1-10 mmol/L (Bast, 1993). Esse tripeptídeo está amplamente distribuído nos organismos e é consequentemente o composto tiol mais prevalente sendo, portanto, fundamental na manutenção do equilíbrio redox na célula.

A GSH é sintetizada em praticamente todas as células, podendo ser armazenada em altas concentrações ou mesmo exportada para fora das células. A síntese da GSH é catalisada por duas enzimas, γ-glutamilcisteína ligase (GCL) e GSH sintetase (GS). A GCL é a enzima-chave limitante da síntese de GSH e é conhecida por regular todo *turnover* de controle de GSH intracelular, ou seja, quando a concentração intracelular de GSH está baixa, a GCL é estimulada por retroalimentação negativa e mais GSH é produzida. Assim, a atividade da GCL é regulada não-alostericamente por retroalimentação inibitória pela GSH (Lu, 2009). A GSH pode ser armazenada em organelas como mitocôndrias e retículo endoplasmático, podendo ser exportada para o espaço extracelular como plasma sanguíneo e fluido cérebro-espinhal através de transportadores específicos (MRP1 e MRP2), porém esse mecanismo ainda é pouco esclarecido (Forman et al., 2009). Já a degradação deste tripeptídeo é iniciada pela enzima γ-glutamil transpeptidase, uma enzima conjugada à superfície externa de certas membranas celulares que exporta o composto para o meio extracelular (Bertini et al., 2003). Posteriormente, pode haver a utilização dos produtos de degradação ou conjugados de cisteína (Cys-SR) (Bonnefoy et al., 2002).

A GSH participa de reações de conjugação com vários tipos de compostos, como xenobióticos, sendo importante para a detoxificação do organismo. O metabolismo dessas substâncias é realizado por reações de fase I e II. A conjugação da GSH a xenobióticos, caracterizada como reação de fase II, é realizada pelas enzimas da família glutationa S-transferases (GST) que estão presentes na maioria dos tecidos, embora em concentrações elevadas em órgãos como rim e fígado (Dringen, 1999). Além da detoxificação de

compostos tóxicos, como os resultantes de lipoperoxidação através de conjugação, a GSH é a primeira linha de defesa do organismo contra ERO neutralizando a ação de radicais livres e do H_2O_2, podendo reagir diretamente com esses compostos ou servindo de substrato para enzimas antioxidantes (Franklin, 2010). A expressão de enzimas envolvidas no ciclo da GSH é induzida em resposta ao EO, por uma sequência denominada ARE (do inglês *antioxidante responsive element*) (Satoh et al., 2013).

Alterações da GSH celular para GSSG levam a um desequilíbrio do balanço redox favorecendo a permanência e ação de ERO e ERN. Essas alterações no metabolismo de GSH em associação com o aumento do EO têm sido relacionadas com a patogênese de diversas doenças. Contudo, é ainda desconhecido se estratégias que tenham como objetivo restaurar a concentração de GSH e a homeostasia redox são eficientes na melhoria ou modificação destes estados.

1.8. Estresse oxidativo (EO)

Tanto as ERO e as ERN são indispensáveis fisiologicamente em baixas concentrações em processos como sinalização celular e apoptose, além de estarem envolvidas na síntese e regulação de algumas proteínas (Veal e Day, 2011). Nos organismos saudáveis há um equilíbrio entre a produção de ER e as defesas antioxidantes. As defesas antioxidantes controlam os níveis de ER, permitindo que estas desempenhem seu papel dentro do metabolismo normal. O rompimento do equilíbrio entre a produção de ER e da ação dos antioxidantes é descrito como EO e pode representar um mecanismo fundamental de doenças humanas (Halliwell e Gutteridge, 2007).

O EO refere-se à situação de desequilíbrio entre a capacidade antioxidante e as ER formadas, em favor destas, na qual a geração de ER ultrapassa as defesas antioxidantes

disponíveis, seja através da diminuição das defesas antioxidantes, seja pelo aumento na produção de ER ou ambos, resultando em lesão a componentes celulares (Halliwell e Gutteridge, 2007; Halliwell, 2001). O estado redox pró-oxidante favorece a ocorrência de lesões oxidativas em macromoléculas como proteínas, lipídios e DNA, causando lipoperoxidação, oxidação de proteínas e sua inativação e podem também reagir com DNA e RNA causando mutações ou distúrbios de transcrição. Segundo Halliwell e Gutteridge (2007), a célula pode responder de várias maneiras frente ao EO dependendo do tipo celular e da severidade do EO: 1) proliferação aumentada; 2) adaptação por regulação dos sistemas de reparo onde a proteção pode ser completa ou não, ou as células se tornam resistentes aos altos níveis de EO; 3) injúria celular, envolvendo dano a marcadores moleculares (lipídeos, proteínas, carboidratos, etc.); 4) senescência quando as células sobrevivem, mas não podem mais se dividir; 5) morte celular.

Assim, as principais consequências secundárias do EO nos organismos biológicos são a lipoperoxidação das membranas celulares, podendo alterar sua fluidez e permeabilidade; a oxidação de proteínas, que leva à alteração da atividade enzimática ou mesmo à desnaturação; e lesão ao DNA/RNA celular, podendo causar mutações (Halliwell e Gutteridge, 2007). O EO está envolvido na fisiopatologia de diversas doenças neurodegenerativas e também em EIM, como a PKU (Wajner et al., 2004), principalmente devido à suscetibilidade do cérebro ao ambiente oxidante.

1.9. Ácido lipoico

O ácido lipoico (AL), também conhecido como ácido tioico e lipoamida, é encontrado naturalmente na mitocôndria, participando do ciclo de Krebs como cofator para as enzimas piruvato desidrogenase e α-cetoglutarato desidrogenase (Hagen et al., 1999;

Mclain et al., 2011). Nas células, o AL é reduzido à ácido diidrolipoico (ADHL) no citoplasma pela GR e na mitocôndria pela enzima diidrolipoamida desidrogenase, podendo ser re-oxidado pela lipoamida desidrogenase (Packer et al., 1997; Gorąca et al., 2011).

Como o ácido tioico é uma molécula pequena, lipo e hidrossolúvel, pode atravessar facilmente as membranas celulares, incluindo a BHE (Samuel et al., 2005). Sua importância metabólica já é conhecida há muitos anos, mas somente recentemente esta substância está sendo reconhecida como um poderoso antioxidante. O AL aproxima-se muito do ideal como antioxidante, apresentando especificidade na eliminação de radicais livres, induzindo a expressão de genes importantes na defesa antioxidante, quelando metais e interagindo com outros antioxidantes, incluindo a GSH (Packer et al., 1995; Gorąca et al., 2011). O AL vem sendo utilizado em diversos modelos animais no tratamento e prevenção de EO em doenças, como a doença de Alzheimer e de Parkinson (Packer et al., 1997), isquemia-reperfusão (Ghibu et al., 2009), diabetes (Minjhout et al., 2010), SIDA (Síndrome da Imuno Deficiência Adquirida) (Fuchs et al., 1993) e até para o envelhecimento (Matsugo et al., 2011).

O uso de AL como agente terapêutico em EIM não é muito descrito na literatura científica, mas alguns trabalhos mostram uma melhora intelectual e neuropsicomotora em pacientes diariamente suplementados com AL para doenças do metabolismo do piruvato (Hommes et al., 1968; Blass, 1983), já que o AL é cofator para as enzimas envolvidas nessas doenças. Em estudos em animais, o AL mostrou prevenção ao EO cerebral em ratos submetidos ao modelo de doença de Canavan (Pederzolli et al., 2010). Além disso, um trabalho recente do nosso grupo de estudo mostrou que o AL foi capaz de prevenir o EO

induzido por uma concentração tóxica de Phe e em um modelo agudo (um dia de injeções) de HPA em cérebro de ratos (Moraes et al., 2010).

Assim, a avaliação do efeito do AL na HPA poderá contribuir para o estudo da neuropatologia envolvendo EO nessa doença além de poder contribuir para o estudo da uma abordagem terapêutica adicional ao tratamento dietético.

OBJETIVO GERAL

Avaliar o efeito antioxidante do AL sobre parâmetros de estresse oxidativo em ratos submetidos a um modelo de HPA induzido quimicamente a partir de sete dias de injeções de Phe e α-MePhe (inibidor da PAH).

2.1. Objetivos específicos

- Avaliar o efeito de sete dias de injeções subcutâneas de Phe (duas vezes ao dia) e α-MePhe (uma vez ao dia), como modelo de HPA, sobre parâmetros gerais de estresse oxidativo e o efeito do tratamento com o antioxidante AL em cérebro dos animais;

- Identificar algumas possíveis espécies reativas envolvidas no estresse oxidativo em cérebro de ratos submetidos ao modelo animal de HPA com sucessivas injeções de Phe e α-MePhe e o efeito do tratamento antioxidante com AL;

- Avaliar o conteúdo de GSH e atividade das enzimas envolvidas no seu metabolismo em cérebro e fígado de ratos submetidos ao modelo animal de HPA com sucessivas injeções de Phe e α-MePhe e o efeito do tratamento antioxidante com AL;

PARTE II

RESULTADOS

Capítulo I – Artigo 1

ROLE OF CATALASE AND SUPEROXIDE DISMUTASE ON OXIDATIVE STRESS

IN THE BRAIN OF A PHENYLKETONURIA ANIMAL MODEL AND THE EFFECT

OF LIPOIC ACID

Tarsila Barros Moraes, Carlos Eduardo Diaz Jacques, Andrea Pereira Rosa, Giovana Reche Dalazen, Melaine Terra, Juliana Gonzalez Coelho, Carlos Severo Dutra-Filho

ORIGINAL RESEARCH

Role of Catalase and Superoxide Dismutase Activities on Oxidative Stress in the Brain of a Phenylketonuria Animal Model and the Effect of Lipoic Acid

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Andrea Pereira Rosa · Giovana Reche Dalazen · Melaine Terra ·
Juliana Gonzalez Coelho · Carlos Severo Dutra-Filho

Abstract Phenylketonuria (PKU) is an inherited metabolic disorder caused by deficiency of phenylalanine hydroxylase which leads to accumulation of phenylalanine and its metabolites in tissues of patients with severe neurological involvement. Recently, many studies in animal models or patients have reported the role of oxidative stress in PKU. In the present work we studied the effect of lipoic acid against oxidative stress in rat brain provoked by an animal model of hyperphenylalaninemia (HPA), induced by repetitive injections of phenylalanine and α-methyl-phenylalanine (a phenylalanine hydroxylase inhibitor) for 7 days, on some oxidative stress parameters. Lipoic acid prevented alterations on catalase (CAT) and superoxide dismutase (SOD), and the oxidative damage of lipids, proteins, and DNA observed in HPA rats. In addition, lipoic acid diminished reactive species generation compared to HPA group which was positively correlated to SOD/CAT ratio. We also observed that in vitro Phe inhibited CAT activity while phenyllactic and phenylacetic acids stimulated superoxide dismutase activity. These results demonstrate the efficacy of lipoic acid to prevent oxidative stress induced by HPA model in rats. The possible benefits of lipoic acid administration to PKU patients should be considered.

Introduction

Phenylketonuria (PKU) is an inborn error of amino acid metabolism caused by mutations in the gene of phenylalanine hydroxylase (PAH) which converts phenylalanine (Phe) to tyrosine (Tyr). Consequently, the deficiency on PAH activity causes hyperphenylalaninemia (HPA) and accumulation of Phe and its metabolites phenylpyruvic acid (PPA), phenyllactic acid (PLA) and phenylacetic acid (PAA) in tissues of patients (Scriver and Kaufman 2001). The main neurological involvement found in disease includes demyelization, delayed psychomotor development, mental retardation, and behavioral changes (Kayaalp et al. 1997; Dyer 1999).

In the last years many studies have reported alterations on oxidative stress parameters in many inborn errors of intermediary metabolism, including PKU, either in animal models or patients (Sierra et al. 1998; Wajner et al. 2004). In addition, some important brain features such as the low antioxidant defenses, the high polyunsaturated lipids content and oxygen consumption, which award to brain vulnerability to oxidative processes, indicate the possibility of an association of the oxidative stress presented in PKU with neuropathophysiology found in disease (Ribas et al. 2011; Sanayama et al. 2011). Sirtori et al. (2005) and Sitta et al. (2009a) demonstrated that PKU patients have shown oxidative damage to biomolecules, such as lipid, protein, and DNA, concomitant with a decrease of enzymatic and

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non-enzymatic antioxidant defenses in plasma and blood cells, which can lead to reactive species generation. In this context, data from in vitro and in vivo studies have shown that Phe accumulation and its metabolites lead to generation of reactive species and increased lipid peroxidation in rat brain, which can be related to the decrease of antioxidant defenses and increase of oxidative damage (Hagen et al. 2002; Fernandes et al. 2010). All these findings regarding to oxidative damage indicate that oxidative stress may contribute to neurological symptoms found in PKU patients (Vargas et al. 2011).

On the other hand, deprivation of essential micronutrients due to the restrict diet treatment to these patients may lead to a poor antioxidant intake (Acosta et al. 1999; van Backel et al. 2000). A recent study has demonstrated that selenium and L-carnitine supplementation alleviates the oxidative damage generated by the disease (Sitta et al. 2011). In this way our research group has proposed the use of lipoic acid (LA) as a treatment to attenuate the oxidative stress found in PKU (Moraes et al. 2010). LA is a natural antioxidant that is absorbed from diet, transported to cells, and capable to cross the blood–brain barrier (Packer et al. 1997; Samuel et al. 2005). LA and its reduced form, dihydrolipoic acid (DHLA), are considered as potent antioxidants and have been shown to prevent oxidative stress in experimental animal models of many disorders of the neurological system (Packer et al. 1997). Moraes et al. 2010 demonstrated that LA was effective to prevent oxidative stress in rat brain provoked by in vitro and in vivo Phe high concentrations exposure; e.g., LA prevented the inhibition of antioxidants enzymes and the increase of lipid peroxidation and reactive species production.

In the present work we studied the effect of LA against oxidative stress in rat brain provoked by an animal model of HPA induced by repetitive injections of Phe for 7 days, and some oxidative stress parameters were evaluated to assess enzymatic antioxidant defenses, reactive species generation, lipid peroxidation, and protein and DNA oxidative damage in the brain of young rats.

Materials and Methods

Animals, Reagents, and Equipment

Litters of seven Wistar rats of both sex of approximately 6-day-old obtained from the Department of Biochemistry, ICBS, UFRGS were maintained with dams and bred in a room with 12:12 h light/dark cycle (lights on 07:00–19:00 h), with controlled temperature (22 ± 1 °C) and dams had free access to water and a 20 % (w/w) protein commercial chow (Nuvilab, Porto Alegre, RS, Brazil). The NIH Guide for the Care and Use of Laboratory Animals (NIH publication # 80–23, revised 1996) was

followed in all experiments, and the research project was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul. The chemicals reagents were purchased from Sigma (St. Louis, MO, USA), and a SpectraMax M5 plate spectrophotometer of absorbance/fluorescence were used for the measurements. Eppendorf 5417R (refrigerated version) were used for the centrifugation procedures.

PKU Animal Model and LA Treatment

It was demonstrated by Hagen et al. (2002) that the present model of HPA is capable to reflect the Phe levels found in patients (up to 3.5 mM in plasma and 1 mmol/kg wet weight tissue in the brain) and it is induced by successive administrations of Phe twice a day and the PAH inhibitor α-methylphenylalanine (α-MePhe) once a day, while LA was also administered once a day. The proposed model of HPA was produced by successive administrations of Phe twice a day and the PAH inhibitor α-MePhe once a day, while LA was also administered once a day. The treatments were performed for 8 days, beginning at 6th day of life. Animals were divided into four groups: Control group (saline solution); LA group (40 mg/kg LA—chosen after a dose curve); Phe group (1.6 μmol/g α-MePhe and 2.1 μmol/g L-phenylalanine); and Phe+LA group (40 mg/kg LA, 1.6 μmol/g α-MePhe, and 2.1 μmol/g Phe). Solutions were prepared each day and were administered by subcutaneous injections according to Hagen et al. (2002) and Podda et al. (1996).

Tissue Preparation and Protein Content

Animals were killed by decapitation 14 h after the last injection and the brain was immediately removed and kept on an ice plate. The olfactory bulb, pons, and medulla were discarded and the forebrain was cleaned, weighed, and homogenized. These procedures were done with controlled cold temperature. Samples were homogenized (1:10, w/v) in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at 800 g for 10 min at 4 °C to separate nuclei and cell debris (Llesuy et al. 1985; Lissi et al. 1986). The pellet was freezed to DNA damage technique and the supernatant was immediately used for the other measurements. All experiments were repeated with different animals. Protein concentration was determined in brain supernatants using bovine serum albumin as a standard (Lowry et al. 1951).

Antioxidants Enzymes Activity

Measurement of catalase (CAT) activity was based on the disappearance of H_2O_2 at 240 nm in a specific 96-wells

plate with 240 μL of a reaction medium containing 20 mM H_2O_2 and 10 mM potassium phosphate buffer pH 7.0, and 10 μL of samples treated with 0.1 % Triton X-100 according to Aebi (1984). One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute and the specific activity is represented as CAT units/mg protein. To evaluate superoxide dismutase (SOD) activity, the method described by Marklund (1985) was used. This method is based on capacity of pyrogallol to autoxidize, a process highly dependent on superoxide radical. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be indirectly assayed spectro-photometrically at 420 nm. A calibration curve was performed with purified SOD as standard. A 50 % inhibition of pyrogallol autoxidation is defined as one unit of SOD and the specific activity is represented as units per mg protein. Furthermore, we evaluated the direct effect of Phe (5 mM) and its metabolites (PPA, PLA, and PAA—1.2 mM) on specific activity of commercial purified preparations of CAT (from bovine liver—EC 1.11.1.6) and SOD (from bovine erythrocytes—EC 232-943-0) without pre-incubation.

2′7′-Dichlorofluorescein Oxidation Assay (DCF)

The production of unspecific reactive species was measured following Lebel et al. (1992) method based on 2′7′-dichlorofluorescein (DCFH) oxidation by ROS and RNS presenting in samples formatting DCF•. Samples (30 μL) were incubated for 30 min at 37 °C in the dark with 30 μL of 20 mM sodium phosphate buffer pH 7.4 with 140 mM KCl and 240 μL of 100 μM 2′7′-dichlorofluorescein diac-etate (DCF-DA) solution in a 96-wells plate. DCF• was measured at 488 nm excitation and 525 nm emission, and the results were represented by nmol DCF•/mg protein.

Thiobarbituric Acid-reactive Substances (TBA-RS)

TBA-RS was measured according to Ohkawa et al. (1979); the following were added to eppendorffs: 200 μL of tissue supernatant; 50 μL of SDS 8.1 %; 375 μL of 20 % acetic acid in aqueous solution (v/v) pH 3.5 and 375 μL of 0.8 % thiobarbituric acid. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 h. The mixture was cooled at room temperature for 5 min and centrifuged at 850 g for 10 min. The resulting pink stained TBA-RS obtained were determined spectrophotometrically at 532 nm. TBA-RS were represented as nmol/mg protein.

Protein Carbonyl Content

Oxidative protein damage produces an enhancement of carbonyl content (Stadtman 1990; Stadtman and Levine 2003). The protein carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer 1994). In brief, 200 μl of homogenate was added to plastic tubes containing 400 μl of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark for 1 h, and vortexed every 15 min. After that, 500 μl of 20 % trichloroacetic acid was added to each tube. The mixture was vortexed and centri-fuged at $20,000 \times g$ for 3 min. The supernatant obtained was discarded and pellet was washed with 1 ml ethanol: ethyl acetate (1:1, v/v), vortexed, and centrifuged at $20,000 \times g$ for 3 min. The supernatant was discarded, and the pellet re-suspended in 600 μl of 6 M guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at 60 °C for 15 min. Subsequently, it was centrifuged at $20,000 \times g$ for 3 min, and the absorbance was measured at 370 nm (UV). The results were represented as protein carbonyl content (nmol/mg protein).

DNA–Protein Cross-links Assay (DNA-PC)

DNA damage was measured by DNA–protein cross-links (DNA-PC) formed as described previously by Zhitkovich and Costa (1992) with minor modifications. The method is based on selective precipitation of protein-cross-linked DNA in the presence of KCl–SDS leaving protein-free DNA fragments in the supernatant. Briefly, 500 μl of 5 % SDS were added to the pellet and this mixture was vortexed and frozen at −20 °C overnight. After thawing at 37 °C, the mixture was vigorously vortexed for 10 s, warmed for 10 min at 65 °C, and 0.5 ml of 100 mM KCl in 20 mM Tris–HCl pH 7.5 was added, followed by passing the mixture through a 1 ml pipette tip for five times. The SDS-K precipitate was formed by cooling the samples on ice for 10 min and collected by centrifugation at $3,000 \times g$ for 3 min at 4 °C. The supernatant obtained was separated (DNA free) and the pellet was resuspended in 1 ml 100 mM KCl, 20 mM Tris–HCl pH 7.5. Samples were heated for 10 min at 65 °C, chilled on ice, and centrifuged at $3,000 \times g$ for 3 min. The washing step was repeated three more times. Proteinase K (15 units/mg protein) was added to the pellet and the suspension was placed for 3 h at 50 °C. The samples were centrifuged at $6,000 \times g$ for 5 min. An aliquot of 250 μl of this supernatant and of the DNA-free previously obtained were used to determine the amount of DNA using 30 μl of the fluorescent dye Sybr Gold (diluted 10,000 times). Fluorescent measurements were performed in a fluorometer at 365 nm excitation and 460 nm emission. Samples incubated with formaldehyde 40 % and bovine serum albumin were used as positive

control and blank, respectively. Results were reported as % DNA-PC.

Statistical Analysis

The results obtained were represented as mean ± S.D. and were analyzed by one-way ANOVA followed by the Tukey post hoc test for multiple comparisons. Analysis of the normality and variance homogeneity was previously verified ($\alpha = 0.05$). The correlation between some parameters was estimated using Pearson's correlation test when necessary. All data were processed using the GraphPad Prism software (version 5.0) in a PC-compatible computer. A value of $p < 0.05$ was considered to be statistically significant.

Results

In order to study the status of some antioxidants enzymes, we analyzed CAT and superoxide dismutase activities from rat brain. Figure 1a, b present the activities of these enzymes in hyperphenylalaninemic rats and the treatment with LA. It can be seen in the figure that LA prevented the inhibition caused by HPA condition on the activity of CAT [$F(3,37) = 16.07$, $p < 0.001$] (Fig. 1a). LA treatment increased SOD activity but was also able to restore to control levels the increase of SOD activity in HPA rats [$F(3,22) = 9.01$, $p < 0.01$] (Fig. 1b).

We also evaluated the oxidative damage to biomolecules including proteins, lipids, and DNA by measuring protein carbonyl content, TBA-RS levels, and generation of DNA–protein cross-links, respectively. As shown in Fig. 1c, TBA-RS were increased in the HPA model [$F(3,40) = 6.14$, $p < 0.01$] and LA treatment inhibited this increase. Phe accumulation promoted a significant increase on protein carbonyl content [$F(3,60) = 16.83$, $p < 0.001$] and LA treatment returns these levels to control ones (Fig. 1d). Figure 1e demonstrates that DNA damage generated by HPA condition was inhibited when rats were treated with LA [$F(3,22) = 7.80$, $p < 0.05$].

Figure 2a shows that generation of unspecific reactive species, assessed by 2'7'-dichlorofluorscein oxidation assay (DCF), was also increased in HPA group [$F(3,54) = 7.37$, $p < 0.001$] and LA was able to efficiently reduce the formation of reactive species to control levels. So, to confirm if the imbalance observed between SOD and CAT activities could be really correlated with reactive species accumulation, Fig. 2b interestingly shows that there is a positive correlation between SOD/CAT ratio and DCF production in rat brain ($r = 0.5694$; $p < 0.01$).

Finally, to investigate the alterations on enzymes activity found in this study we next evaluate the direct in vitro effect of Phe and metabolites (PPA, PLA, and PAA) on commercial purified preparations of CAT and SOD. Figure 3a shows that PLA and PAA were able to increase SOD activity [$F(4,14) = 13.36$, $p < 0.01$] and in
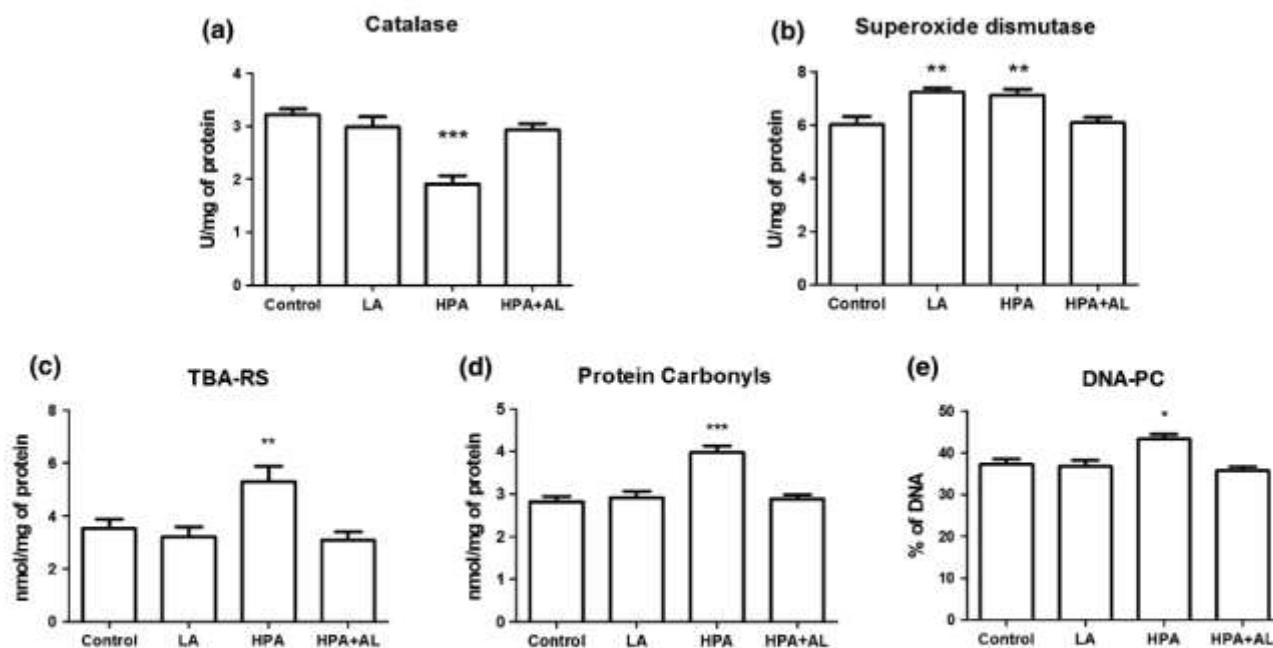

Fig. 1 Effect of lipoic acid treatment on CAT (a) and SOD (b) activities; on lipoperoxidation (c); protein oxidation (d) and DNA damage (e) in brain from rats subjected to daily administration of Phe and PAH inhibitor. Results are mean ± SD ($n = 6$–17). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to control (Tukey test)

Fig. 2 Effect of lipoic acid treatment in brain from rats subjected to daily administration of Phe and PAH inhibitor on reactive species production (a) and correlation between SOD/CAT ratio and reactive species production (b). Results are mean ± SD ($n = 6$–16). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared to control (Tukey test)
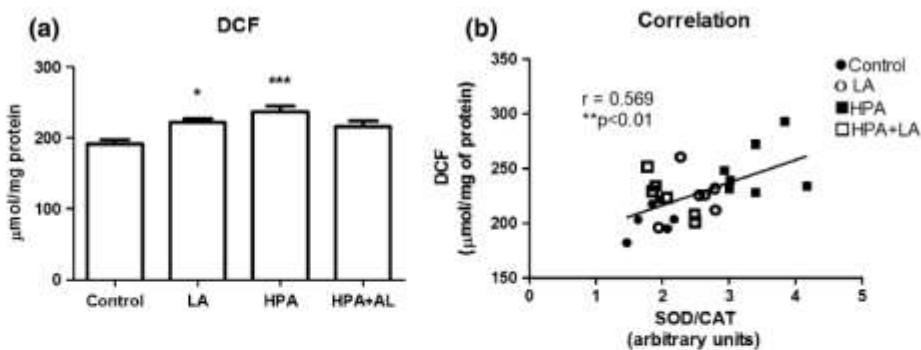
Fig. 3b it can be seen that Phe (5 mM) inhibited the activity of CAT [$F(5, 16) = 3.35$, $p < 0.05$].

Discussion

Several recent research papers on literature have been describing the involvement of oxidative stress in some inborn errors of metabolism especially PKU (Vanzin et al. 2011; Ribas et al. 2011, 2012; Rocha and Martins 2012; Sanayama et al. 2011). Moreover, studies in vitro/in vivo in animals and not-treated PKU patients are altered at short and long time of Phe exposition (Hagen et al. 2002; Sitta et al. 2009b) probably indicating that parameters of oxidative stress altered in these HPA conditions seems not to be an adaptive condition (Ristow and Zarse 2010). In this way, as study of our research group described the prevention by LA against oxidative stress observed in vitro by a toxic concentration of Phe and in an acute HPA model (Moraes et al. 2010), the present data show the effect of LA in a HPA model with successive daily administrations of Phe and PAH inhibitor mimicking a repetitive exposition to high Phe levels.

Initially, the activities of CAT and superoxide dismutase were analyzed. A decrease of CAT and an increase of SOD activities induced by the model of HPA were found. This corroborates with the alterations in antioxidant enzymes (CAT and SOD) already observed in rat brain using the same animal model of HPA described above (Hagen et al. 2002; Mazzola et al. 2011) and also findings in erythrocytes of PKU patients demonstrating the same enzymatic profile of CAT and superoxide dismutase (Gassió et al. 2008; Sanayama et al. 2011). In the present study, LA was able to prevent the inhibition of CAT and also returned SOD activity to the levels found in the control group, showing to be an effective scavenger of reactive species such as hydrogen peroxide and superoxide anion in this specific situation (Packer et al. 1995; Li et al. 2004; Moraes et al. 2010). Moreover, increased SOD activity by LA alone (LA group) is in accordance to other findings in some studies with a similar LA treatment in rats to that used in this study (Kim et al. 2011; Perera et al. 2011). Interestingly, LA can counteract oxidative stress by inducing the expression of the SOD gene, thus up regulating the nerve growth factor (NGF) (Garrett et al. 1997; Nistico et al. 1992).

After that, we evaluated the oxidative status of biomolecules as lipids, protein, and DNA, analyzing their oxidative biomarkers. As shown in Fig. 1c–e, HPA group increased all these parameters, indicating oxidation of lipids by the generation of thiobarbituric reactive substances (Fig. 1c), protein oxidation by the formation of carbonyls group in proteins of brain (Fig. 1d), and DNA damage by the increase of DNA–protein cross-links

Fig. 3 In vitro direct effects of Phe, PPA, PLA, and PAA on enzyme activity of commercial purified preparations SOD (a) and CAT (b). Substances were added to incubation medium immediately before measuring the enzyme activities (without pre-incubation). Results are mean ± SD ($n = 3$–4); $*p < 0.05$; $**p < 0.01$ compared to control (Tukey test)
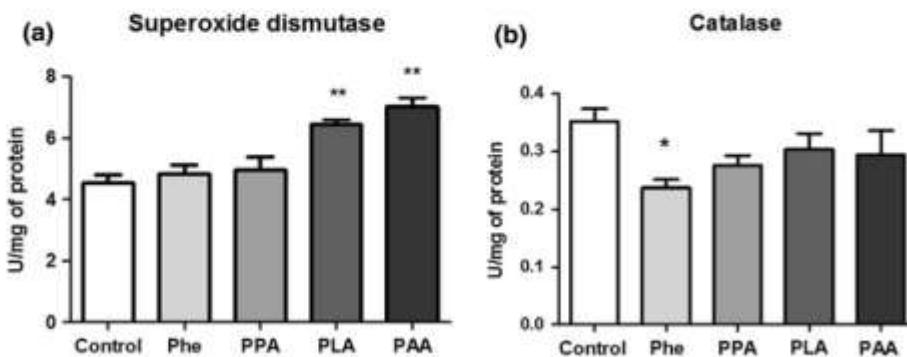
(Fig. 1e). Many recent studies have demonstrated significant changes in these parameters of oxidative damage to biomolecules in phenylketonuric patients (Sitta et al. 2009a; Sanayama et al. 2011; Vanzin et al. 2011). Furthermore, LA treatment was able to inhibit the oxidative damage found in the present work. These results were in agreement with previous studies showing that LA were effective to prevent lipid peroxidation provoked by HPA (Moraes et al. 2010) and protein oxidative damage induced by acute toxicity of N-acetylaspartic acid (Pederzolli et al. 2010).

Assuming that the overproduction of reactive species, including free radicals, can produce oxidative damage to biomolecules (Halliwell and Gutteridge 2007), we evaluated the content of reactive nonspecific of samples by DCF assay. As observed in Fig. 2a, LA was efficient to restore DCF to control levels in HPA+LA group, but LA and HPA groups present a significant increase in the content of DCF compared with the control group. These results indicate an imbalance in redox status of the brain of rats in favor of pro-oxidants, which can be due to increase in SOD activity without compensation by CAT observed in LA and HPA groups (Fig. 1a, b). Moreover, a positive correlation between DCF content and SOD/CAT ratio was observed, indicating that the increase in the production of reactive species may be due to the influence of the changes found in the activity of these two antioxidant enzymes. Since SOD metabolizes the superoxide anion ($^{\bullet}O_2^-$) to hydrogen peroxide (H_2O_2) and CAT converts H_2O_2 to water, the consequence of the increase in the ratio SOD/CAT might be the more availability of H_2O_2 which can form the most potent pro-oxidant molecule, hydroxyl radical, by Fenton reaction as displayed in Fig. 4a (Halliwell and Gutteridge 2007). Another H_2O_2 detoxification pathway is by glutathione peroxidase activity (GPx), which is a selenium dependent enzyme. Studies with PKU patients have

reported a decreased GPx activity probably due to selenium deficiency of such patients (van Backel et al. 2000; Ercal et al. 2002; Gassió et al. 2008). In addition, as unpublished data of our group using the same HPA and LA treatments of this work showed inhibition of GPx activity in HPA group, it is feasible to speculate that HPA condition leads to H_2O_2 accumulation and that the antioxidant enzymes play an important role on redox status in the disease.

Although the technique to measure the generation of reactive species used here is a nonspecific assay that uses diclofluorescein (DCFH), this fluorochrome is more readily oxidized by $^{\bullet}OH$ and peroxyl radicals than by hydrogen peroxide and superoxide anion radical (Halliwell and Whiteman 2004). In the present work we find even a positive correlation between DCF and TBA-RS, indicating that lipoperoxidation induced by the model of HPA is associated with increased reactive species. According to this, an interesting result was observed by Fernandes et al. (2010) with in vitro incubation of cerebral cortex homogenates of rats showing that Phe causes lipid damage by increasing TBA-RS. In these experiments, the co-incubation with SOD and CAT did not alter the effect of Phe on TBA-RS, but the lipid damage was reversed by the addition of trolox or melatonin, which may indicate that lipid peroxidation caused by accumulation of Phe is possibly by hydroxyl ($^{\bullet}OH$) and peroxyl ($LOO^{\bullet}$) radicals than by a direct action of H_2O_2 or $^{\bullet}O_2^-$ (Fig. 4a).

In order to further investigate the role of SOD and CAT in a situation of HPA, we evaluated the in vitro influence of Phe and its metabolites on the activities of commercial purified preparations of SOD and CAT. Fig. 3 shows that Phe inhibits purified CAT (Fig. 3b), and that PLA and PAA increase the activity of purified SOD (Fig. 3a). These set of experiments were performed without pre-incubation, so the effects were obtained during the measurement of the enzyme activities. Thus, the observed increase in SOD

Fig. 4 Initiation and propagation processes of lipid peroxidation indirectly formed from hydrogen peroxide (H_2O_2) (a): *SOD* superoxide dismutase, *CAT* catalase, *GSH-Px* glutathione peroxidase, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *LH* unsaturated lipids, $L^{\bullet}$ lipid radical, $LOO^{\bullet}$ lipid peroxyl radical. Alternative rote of phenylalanine (Phe) metabolism (b): *PPA* phenylpyruvic acid, *PLA* phenyllactic acid, *PAA* phenylacetic acid, *1* transamination reaction, *2* descarboxylation reaction, *3* reduction reaction
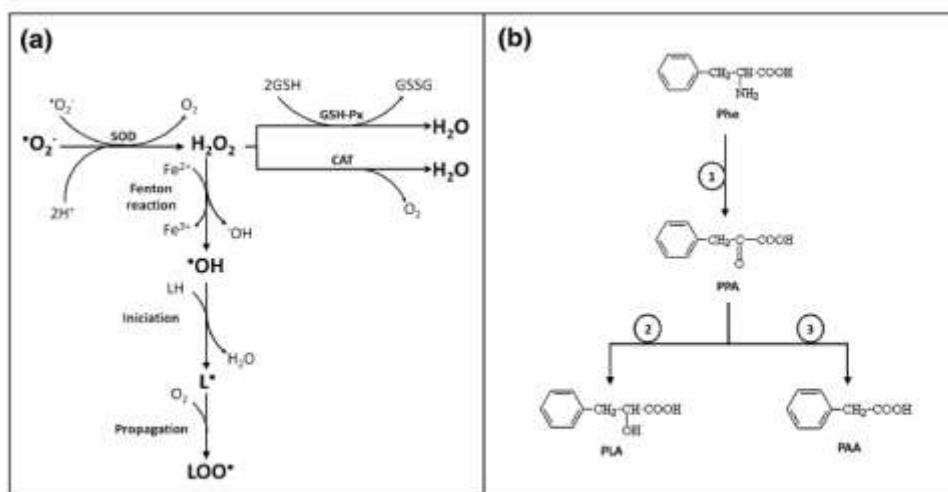
activity in the group of animals submitted to the HPA model is probably due to the metabolites (PLA and PAA) formed from the accumulation of Phe by an alternative route as shown in Fig. 4b (Scriver and Kaufman 2001). However, the hypothesis of adaptation against oxidative stress cannot be totally ruled out since Moraes et al. (2010) showed a decrease in SOD activity in rats submitted to acute treatment of HPA (single Phe administration). Thereby, further studies are needed to evaluate the expression and quantity of these enzymes in the brain of rats subjected to HPA models. In addition, we evaluated the in vitro influence of LA on commercial purified preparations of SOD and CAT (data not shown). LA did not alter the activity of these enzymes. Considering that LA did not show a direct effect on CAT activity in vitro, a possible mechanism whereby LA may protect CAT activity in the HPA rat by preventing oxidative inactivation of the enzyme.

The results shown are consistent with oxidative profile found in patients, an increase of damage to biomolecules and concomitant decrease in antioxidant defenses (Sirtori et al. 2005; Sitta et al. 2009a). Furthermore, we show that the increase in reactive species correlated with the profile of antioxidant enzymes CAT and SOD. Therefore, considering the data presented now, we can conclude that the treatment with daily injections of Phe (and PAH inhibitor) to induce HPA, with plasmatic and cerebral levels of Phe equivalent to those found in patients, altered oxidative stress biomarkers in rat brain and that the role of antioxidant enzymes (SOD and CAT) in this model is possibly essential for the maintenance of redox homeostasis, since the substances accumulated in PKU are capable of directly altering the activity of these enzymes. Moreover, continuous treatment with LA is effective to normalize these parameters indicating that this antioxidant may be effective not only in acute models of HPA (Moraes et al. 2010), but also in a daily treatment. Since some patients have repeated increases on Phe levels due to lack of adherence to the dietary treatment, it is possible that oxidative stress can be mitigated with the use of antioxidants, such as LA, as an additional therapy treatment in PKU.

References

Acosta PB, Yannicelli S, Marriage B, Steiner R, Gaffield B, Arnold G, Lewis V, Cho S, Berstein L, Parton P, Leslie N, Korson M (1999) Protein status of infants with phenylketonuria undergoing nutrition management. J Am Coll Nutr 18:102–107

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126

Dyer CA (1999) Pathophysiology of phenylketonuria. Ment Retard Dev Disabil Res Rev 5:104–112

Ercal N, Aykin-Burns N, Gurer-Orhan H, McDonald JD (2002) Oxidative stress in a phenylketonuria animal model. Free Radic Biol Med 32:906–911

Fernandes CG, Leipnitz G, Seminotti B, Amaral AU, Zanatta A, Vargas CR, Dutra Filho CS, Wajner M (2010) Experimental evidence that phenylalanine provokes oxidative stress in hippocampus and cerebral cortex of developing rats. Cell Mol Neurobiol 30:317–326

Garrett NE, Malcangio M, Dewhurst M, Tomlinson DR (1997) α-Lipoic acid corrects neuropeptide deficits in diabetic rats via induction of trophic support. Neurosci Lett 222:191–194

Gassió R, Artuch R, Vilaseca MA, Fusté E, Colome R, Campistol J (2008) Cognitive functions and the antioxidant system in phenylketonuric patients. Neuropsychology 22(4):426–431

Hagen MEK, Pederzolli CD, Sgaravatti AM, Bridi R, Wajner M, Wannmacher CM, Wyse AT, Dutra-Filho CS (2002) Experimental hyperphenylalaninemia provokes oxidative stress in rat brain. Biochim Biophys Acta 1586:344–352

Halliwell B, Gutteridge JMC (2007) Free radicals in biology and medicine, 4th edn. Oxford University Press, New York

Halliwell B, Whiteman M (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: How should you do it and what do the results mean? Br J Pharmacol 142:231–255

Kayaalp E, Treacy E, Waters PJ, Byck S, Nowacki P, Scriver CR (1997) Human phenylalanine hydroxylase mutations and hyperphenylalaninemia phenotypes: a meta-analysis of genotypephenotype correlations. Am J Hum Genet 61:1309–1317

Kim MY, Kim EJ, Kim Y, Choi C, Lee B (2011) Effects of α-lipoic acid and L-carnosine supplementation on antioxidant activities and lipid profiles in rats. Nutr Res Pract 5(5):421–428

Lebel CP, Ischiropoulos H, Bondy SC (1992) Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Rés Toxicol 5:227–231

Li Y, Zhao Y, Yu W, Jiang S (2004) Scavenging ability on ROS of alpha-lipoic acid (ALA). Food Chem 84:563–567

Lissi E, Caceres T, Videla LA (1986) Visible chemiluminescence from rat brain homogenates undergoing autoxidation. I. Effect of additives and products accumulation. Free Radic Biol Med 2:63–69

Llesuy SF, Milei J, Molina H, Boveris A, Milei S (1985) Comparison of lipid peroxidation and myocardial damage induced by adriamycin and 4′-epiadriamycin in mice. Tumori 71:241–249

Lowry OH, Rosebrough NJ, Lewis-Farr A, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Marklund SL (1985) Pyrogallol autoxidation. In: Greenwald RA (ed) Handbook of methods for oxygen radical research. CRC Press, Boca Raton, pp 243–247

Mazzola PN, Terra M, Rosa AP, Mescka CP, Moraes TB, Piccoli B, Jacques CE, Dalazen GR, Cortes MX, Coelho JC, Dutra-Filho CS (2011) Regular exercise prevents oxidative stress in the brain of hyperphenylalaninemic rats. Metab Brain Dis 26:291–297

Moraes TB, Zanin F, da Rosa A, de Oliveira A, Coelho J, Petrillo F, Wajner M, Dutra-Filho CS (2010) Lipoic acid prevents oxidative stress in vitro and in vivo by an acute hyperphenylalaninemia chemically-induced in rat brain. J Neurol Sci 292:89–95

Nistico G, Ciriolo MR, Fiskin K, Iannone M, De Martino A, Rotilio G (1992) NGF restores decrease in catalase activity and increases superoxide dismutase and glutathione peroxidase activity in the brain of aged rats. Free Radic Biol Med 12:177–181

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358

Packer L, Eric HW, Hans JT (1995) Alpha lipoic acid as a biological antioxidant. Free Rad Biol Med 19:227–250

Packer L, Tritschler HJ, Wessel K (1997) Neuroprotection by the metabolic antioxidant α-lipoic acid. Free Radic Biol Med 22:359–378

Pederzolli CD, Rosa AP, Oliveira AS, Coelho JG, Becker DL, Dalazen GR, Moraes TB, Dutra-Filho CS (2010) Neuroprotective role of lipoic acid against acute toxicity of N-acetylaspartic acid. Mol Cell Biochem 344:231–239

Perera J, Tan JH, Jeevathayaparan S, Chakravarthi S, Haleagrahara N (2011) Neuroprotective effects of alpha lipoic acid on haloperidol-induced oxidative stress in the rat brain. Cell Biosci 1:1–12

Podda M, Rallis M, Cuber MG, Packer L, Maibachl H (1996) Kinetic study of cutaneous and subcutaneous distribution following topical application of lipoic acid onto hairless mice. Biochem Pharmacol 52:627–633

Potocnik U, Widhalm K (1994) Long-term follow-up of children with classical phenylketonuria after diet discontinuation: a review. J Am Coll Nutr 3:232–236

Reznick AZ, Packer L (1994) Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol 233:357–363

Ribas GS, Sitta A, Wajner M, Vargas CR (2011) Oxidative stress in phenylketonuria: What is the evidence? Cell Mol Neurobiol 31(5):653–662

Ribas GS, Biancini GB, Mescka CP, Wayhs CY, Sitta A, Wajner M, Vargas CR (2012) Oxidative stress parameters in urine from patients with disorders of propionate metabolism: a beneficial effect of L-carnitine supplementation. Cell Mol Neurobiol 32:77–82

Ristow M, Zarse K (2010) How increased oxidative stress promotes longevity and metabolic health: the concept of mitochondrial hormesis (mitohormesis). Exp Gerontol 45:410–418

Rocha JC, Martins MJ (2012) Oxidative stress in Phenylketonuria: future directions. J Inherit Metab Dis 35:381–398

Samuel S, Kathirvel R, Jayavelu T, Chinnakkannu P (2005) Protein oxidativee damage in arsenic induced rat brain: influence of DL-α-lipoic acid. Toxicol Lett 155:27–34

Sanayama Y, Nagasaka H, Takayanagi M, Ohura T, Sakamoto O, Ito T, Ishige-Wada M, Usui H, Yoshino M, Ohtake A, Yorifuji T, Tsukahara H, Hirayama S, Miida T, Fukui M, Okano Y (2011) Experimental evidence that phenylalanine is strongly associated to oxidative stress in adolescents and adults with phenylketonuria. Mol Genet Metab 103(3):220–225

Scriver CR, Kaufman S (2001) Hyperphenylalaninemia: phenylalanine hydroxylase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 1667–1724

Sierra C, Vilaseca MA, Moyano D, Brandi N, Campistol J, Lambruschini N, Cambra FJ, Deulofeu R, Mira A (1998) Antioxidant status in hyperphenylalaninemia. Clin Chim Acta 276:1–9

Sirtori LR, Dutra-Filho CS, Fitarelli D, Sitta A, Haeser A, Barschak AG, Wajner M, Coelho DM, Llesuy S, Bello'-Klein A, Giugliani R, Deon M, Vargas CR (2005) Oxidative stress in patients with phenylketonuria. Biochim Biophys Acta 1740:68–73

Sitta A, Barschak AG, Deon M, Terroso T, Pires R, Giugliani R, Dutra-Filho CS, Wajner M, Vargas CR (2006) Investigation of oxidative stress parameters in treated phenylketonuric patients. Metab Brain Dis 4:287–296

Sitta A, Manfredini V, Biasi L, Treme'a R, Schwartz IV, Wajner M, Vargas CR (2009a) Evidence that DNA damage is associated to phenylalanine blood levels in leukocytes from phenylketonuric patients. Mutat Res 679:13–16

Sitta A, Barschak AG, Deon M, Barden AT, Biancini GB, Vargas PR, de Souza CF, Netto C, Wajner M, Vargas CR (2009b) Effect of short- and long-term exposition to high phenylalanine blood levels on oxidative damage in phenylketonuric patients. Int J Dev Neurosci 27:243–247

Sitta A, Vanzin CS, Biancini GB, Manfredini V, de Oliveira AB, Wayhs CA, Ribas GO, Giugliani L, Schwartz IV, Bohrer D, Garcia SC, Wajner M, Vargas CR (2011) Evidence that L-carnitine and selenium supplementation reduces oxidative stress in phenylketonuric patients. Cell Mol Neurobiol 31:429–436

Stadtman ER (1990) Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. Free Radic Biol Med 9:315–325

Stadtman ER, Levine RL (2003) Free-radical mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids 25:207–218

van Backel MME, Printzen G, Wermuth B, Wiesmann UN (2000) Antioxidant and thyroid hormone status in selenium-deficient phenylketonuric and hyperphenylalaninemic patients. Am J Clin Nutr 72:976–981

Vanzin CS, Biancini GB, Sitta A, Wayhs CAY, Pereira YN, Rockenbach F, Garcia SC, Wyse ATS, Schwartz IVD, Wajner M, Vargas CR (2011) Experimental evidence of oxidative stress in plasma of homocystinuric patients: a possible role for homocysteine. Mol Genet Metab 104:112–117

Vargas CR, Wajner M, Sitta A (2011) Oxidative stress in phenylketonuric patients. Mol Genet Metab 104:S97–S99

Wajner M, Latini A, Wyse AT, Dutra-Filho CS (2004) The role of oxidative damage in the neuropathology of organic acidurias: insights from animal studies. J Inherit Metab Dis 27:427–448

Zhitkovich A, Costa M (1992) A simple, sensitive assay to detect DNA protein-crosslinks in intact cells and in vivo. Carcinogenesis 13:1485–1489

Capítulo II – Artigo 2

EVALUATION OF SPECIFIC REACTIVE SPECIES PRODUCTION IN A

HYPERPHENYLALANINEMIA ANIMAL MODEL AND THE EFFECT OF LIPOIC

ACID ADMINISTRATION

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ABSTRACT

Recent *in vitro* and *in vivo* studies indicate the involvement of oxidative stress in the neuropathology of hyperphenylalaninemia (HPA), by diminishing antioxidant defenses and causing damage to biomolecules. This work is part of an investigation that has shown an increase in the ratio SOD/CAT activities, suggesting a hypothesis for brain reactive species accumulation in an HPA model. The aim of this study was to identify some specific reactive species in the brain of young hyperphenylalaninemic rats, with or without treatment of lipoic acid (LA). The results confirmed our previous hypothesis, showing that brain accumulation of phenylalanine led to an increased generation of H_2O_2, $O_2^{\bullet-}$ and $NO^\bullet$ in the brain. In addition, LA totally inhibited the H_2O_2 overproduction and partially prevented the $NO^\bullet$ elevation induced by HPA in the brain. This is the first identification of reactive species which may be involved in the oxidative stress found in HPA and that could be associated with biomolecules damage implicated in this disease.

INTRODUCTION

Phenylketonuria (PKU) is the most frequent inborn error of amino acid metabolism. The blockage of phenylalanine catabolism and the consequent hyperphenylalaninemia (HPA) causes several neurological and behavioral dysfunctions in patients *(1)*. In the last decade, several studies have reported the possible involvement of oxidative stress in the neuropathophysiology of inborn errors of metabolism and PKU *(2)*. In addition, it was showed that the high phenylalanine (Phe) plasma levels are associated with oxidative stress *(3)* which were supported by alterations in oxidative stress biomarkers in PKU patients *(4)*. On the other hand, it is known that the treatment with restricted diet alters antioxidant status and may contribute to oxidative stress in PKU patients *(5)*. In accordance with this finding,

studies have indicated that the damage to biomolecules could be associated with decrease in antioxidant defenses and/or increase in reactive species *(6,7,8)*. The initial hypothesis of this work was that mainly hydrogen peroxide (H_2O_2), but possibly other reactive species could be accumulated in brain of hyperphenylalaninemic rats since antioxidant enzymes activities (SOD/CAT ratio) were altered in a previous study *(9)*, and this suggested idea was confirmed as following described.

Hydrogen peroxide, hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$) are examples of reactive species (RS) including radical and non-radical molecules. These RS are normally produced in physiological conditions and oxidative stress occurs in consequence of a serious imbalance between RS overproduction and antioxidant system deficient causing damage. Importantly, $^{\bullet}OH$ has the higher rate for hydrogen atom abstraction from methylene group of lipids, and it may be formed from H_2O_2 and ferrous ion (Fe^{2+}) (Fenton reaction), from peroxynitrite ($ONOO^-$), or high energy irradiation *(10)*. In relation to H_2O_2 it can be normally produced from $O_2^{\bullet-}$ by superoxide dismutase (SOD – E.C. 1.15.1.1) and its elimination is performed by catalase (CAT – E.C. 1.11.1.6) and glutathione peroxidase (GSH-Px – E.C. 1.11.1.9) activities. Although changes on the activities of important antioxidant enzymes have been reported in PKU neuropathology *(3,11)*, it still remains to be determined the RS involved in oxidative stress in PKU causing biomolecular damage.

Previous studies have been shown lipoic acid (LA) capacity against oxidative stress induced by Phe accumulation. LA was able to protect lipid, protein and DNA damage and prevent antioxidant inhibition *(9,12)*. LA is considered an available antioxidant for

therapeutic uses *(13)* as well it can attenuate deleterious effects of oxidative stress in neurons cells *(14)*. In addition, according to Packer and colleagues LA and its reduced form (DHLA) are considered ideal antioxidants mainly to scavenger most oxygen and nitrogen reactive species *(15)*. Considering the failed antioxidant system by HPA condition and biomolecular damage possibly caused by RS accumulation. In the present report, the aim was to identify some RS present in the brain of rats subjected to a chemically-induced chronic HPA model and the LA effect.

MATERIALS AND METHODS

Animals, reagents and equipment

Six-day-old female and male litters (n=72) from seven Wistar rats obtained from the Department of Biochemistry, ICBS, UFRGS were maintained with dams and bred and kept in appropriate cages in air-conditioned rooms at constant temperature (22 ± 1 ℃), and cycles of light / dark 12 hours, with access to a standard commercial food and water *ad libitum*. The animal care were followed according to official government official guidelines as the Federation of Brazilian Societies for Experimental Biology, and the project was approved by Ethics Committee on Animal Use (CEUA) of the Universidade Federal do Rio Grande do Sul. All reagent used were purchased from Sigma (St. Louis, MO, USA). A Liquid Scintillation and Luminescence Counter from Perkin Elmer (MicroBeta TriLux) and a SpectraMax® M5 plate spectrophotometer were used for the measurements.

Experimental design

Litters were divided in four groups: control (saline); LA group (40mg/Kg body weight of lipoic acid in alkaline solution); HPA group and HPA+LA group. At first day injection, it

was administrated lipoic acid in animals of LA groups. And from second to eight day treatment, all groups received LA and HPA model administration except control group. The HPA model was established by the administration of L-phenylalanine (Phe - 2.1 ml/g) twice daily and α-methylphenylalanine (α-MePhe - 1.6 mmol/g), an inhibitor of phenylalanine hydroxylase, once a day injection. Both administrations were subcutaneous and all solutions were fresh daily prepared with saline and pH 7.4 adjusted. LA dose was chosen after a previous pilot curve and antioxidant effect found by Moraes and collaborators *(9)*. HPA chemically model was defined by Kienzle-Hagen and collaborators *(6)* which demonstrated that this proposed model of HPA is capable to reflect the plasma Phe levels found in patients (3.6±0.2 mM).

Tissue preparation

Twelve hours after the last injection the animals were killed by decapitation, the brain (forebrain) was immediately removed and cleaned, weighed and processed according to each assay. To evaluate H_2O_2 content prisms samples were used, and to measure $O_2^{\bullet-}$, $^{\bullet}OH$ and NO content it was used homogenates. The samples were kept on ice and were freshly used and homogenized (1:10, w/v) in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl and centrifuged at 800 g for 10 min at 4°C to separate nuclei and cell debris. Protein content was measured with bovine albumin *(16)*.

Reactive Species analyses

The $O_2^{\bullet-}$ content was determined according to Gupte and collaborators *(17)* with adaptations. The homogenates (100 μL) were added in medium reaction (200 μL) with Krebs HEPES buffer (pH 8.6) in the presence of lucigenin (20 μmol/L). The samples

luminescences were counting for 10 minutes and background (buffer and lucigenin without samples) discounted. The data expressed in RLU (relative luminescence unit)/min/mg tissue.

Nitric oxide was assessed by Griess reaction method, 50 µL of supernatant were mixed with 50 µL of Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% dihydrochloride naphthylethylenediamine in water) and incubated in 96-well plates for 10 min at room temperature and protected from light. The absorbance was measured in at 543 nm. Nitrite concentration was represented as mol of nitrite / mg of protein *(18)*.

The method used to measure the H_2O_2 was based on the oxidation of phenol red by H_2O_2 mediated by horseradish peroxidase, followed by the increase of absorbance at 610 nm *(19)*. Briefly, 400 mg of the brain were cut in two perpendicular directions, to produce prisms and was added to a glass vial containing 5.5 mM buffer dextrose, pH 7.0, and the mixture is kept at room temperature for 1 hour. After, 60 µL of supernatant was softly mixed with 235 µL of a medium containing 50 mM sodium phosphate buffer pH 7.4, 85 µL of peroxidase and 1 mg / mL of red phenol. The reaction occurred in the dark for 10 minutes. Thereafter, 5 µL of 1N NaOH was added to each tube and the absorbance was read at 610 nm. The calibration curve was performed with commercial solution of H_2O_2. Results were expressed as nmol of H_2O_2 /mg of tissue.

As previously described by Gutteridge *(20)*, the deoxyribose assay was used to evaluate the $^•OH$ production from the auto-reduction of ferric citrate complex. The pentose sugar is attacked by $^•OH$ with releasing thiobarbituric acid (TBA) reactive substances. Firstly,

samples were incubated at 37°C for 1 h in dark room with a medium reaction containing 3 mM 2-deoxy-D-ribose; 20 µM $FeCl_3$; 100 µM EDTA; 500 µM H_2O_2 and 100 µM ascorbate. After, 10% TCA and 0.67% TBA were added, vortexed and incubated for 1 h in a boiling water bath. The absorbance determined at 532 nm and 1,1,3,3-tetra-methoxypropane (TMP) was used as standard. Results expressed as µmol TMP/mg of protein.

Statistical analysis

All data were processed using the GraphPad Prism software (version 5.0) in a PC-compatible computer. The results obtained were represented as mean±S.D. and analyzed by one-way ANOVA followed by the Tukey post-hoc test for multiple comparisons. Analysis the normality and variance homogeneity was previously verified ($\alpha=0.05$) and a value of $p<0.05$ was considered to be statistically significant.

RESULTS

Oxidative stress markers are altered in PKU patients and animal models and possibly the neuropathology of PKU requires redox homeostasis alterations. However it remains unknown which reactive species could be accumulated in HPA condition. Our results indicated that Phe accumulation in brain of HPA rats induced an overproduction of $O_2^{\bullet-}$ showed in fig.1.a [$F_{(3, 11)} = 6.37$; $p <0.01$; n = 3-4], the LA treatment was unable to inhibit this $O_2^{\bullet-}$ generation. Figure 1.b shows H_2O_2 data, and HPA model increased brain concentration of H_2O_2 [$F_{(3, 37)} = 21.69$; $p <0.001$; n = 10-11] and treatment with LA totally inhibited this effect. Nitric oxide content is presented in fig.2.a and HPA model significantly increased $NO^{\bullet}$ concentration in the brain [$F_{(3, 25)} = 21.24$; $p <0.001$; n = 6-

10]. LA treatment partially prevented this effect, since $NO^{\bullet}$ levels of HPA+LA group are not similar to control. On the other hand, it was not detected changes on $^{\bullet}OH$ concentration in groups of this experiment [$F_{(3, 47)} = 0.3569$; $p > 0.05$; $n = 11$-14], indicating that $^{\bullet}OH$ is not accumulated in brain of HPA rats (Fig.2.b).

DISCUSSION

The present study contributes to a further elucidation of specific RS involved in the oxidative stress associated with HPA, since previous investigations with the same experimental design showed increased SOD activity and inhibition of CAT and GSH-Px activity *(9 and data not published)*. These enzymatic changes indicated a possible accumulation of H_2O_2 in brain tissue and this effect was successfully confirmed in the present study. In addition, knowing which RS are involved in brain Phe accumulation could support investigations with antioxidant therapy that have been proposed to PKU *(21)*. Importantly, we observed an increase in $O_2^{\bullet-}$ in HPA group. $O_2^{\bullet-}$ is considered the first RS formed and can be a precursor of H_2O_2, $^{\bullet}OH$ and lipoperoxyl radicals. The overproduction of $O_2^{\bullet-}$ and H_2O_2 observed in fig. 1. can possibly be derived from mitochondrial disruption due to accumulation of Phe as described by Rech and collaborators *(22)*. However, studies have showed that NADPH oxidase also plays an essential role in the generation of $O_2^{\bullet-}$ and hence oxidative stress observed in PKU patients *(23, 24)*. Although the mechanism of activation of NADPH oxidase in PKU is unclear, this increased activity could support the accumulation of $O_2^{\bullet-}$ observed in our study.

In consequence of brain overproduction of $O_2^{\bullet-}$ HPA animals may have presented elevation in nitrite content, given that on inflammatory condition or oxidative stress induces $NO^{\bullet}$

release by the stimulation of the activity of nitric oxide synthase (NOS) *(25)*. In addition, LA treatment increased $NO^\bullet$ in brain and it could indicate a possible effect of LA inducing eNOS activity by activation of AMP-activated protein kinase *(26)* or mitogen-activated protein kinase *(27)*. $NO^\bullet$ presents crucial role in the regulation of neuronal function and synaptic transmission and as an important radical and second messenger *(28)*. Furthermore, it is known that low $NO^\bullet$ concentrations have some antioxidant feature directly scavenger RS *(29)*. On the other hand, the $NO^\bullet$ reacting with $O_2^{\bullet-}$ can generate $ONOO^-$, which is a highly reactive nitrogen species and causes nitrosylation of amino acids and consequent inhibition of protein leading to enzymatic deficiency *(30)*. Thereby, in terms of effects on inhibition of enzymes observed in PKU could be related to the increase of $O_2^{\bullet-}$ and $NO^\bullet$.

Another effect widely reported in PKU is lipid peroxidation (LPO) that can be initiated by three generation mechanisms: (1) free radical-mediated oxidation, (2) free radical-independent, non-enzymatic oxidation, and (3) enzymatic oxidation *(31)*. First cited mechanism proceeds with an initiating free radical which oxidize lipid molecules by chain reactions e.g. by $^\bullet OH$. In addition, it has been shown that the TBA-RS (a lipid peroxidation marker) generated directly by the accumulation of Phe (5 mM) may be the action of $^\bullet OH$ and peroxyl radicals ($ROO^\bullet$), as verified *in vitro* experimental conditions *(32)*. Furthermore, an interesting result observed was the concentration of $^\bullet OH$ which remained the same as control. Even considering that $^\bullet OH$ is a rapidly and reactive oxygen specie to be measured by an indirect methodological protocol, the results lead us to suggest that HPA did not increased $^\bullet OH$ in rat brain and that lipid damage observed in PKU may be due to a possible action of $ROO^\bullet$ formed by other chain-initiating RS. Although we did not observe a significant difference in animal groups, regarding $^\bullet OH$ we could not discard the possibility

of a previous increase of $^{\bullet}OH$ generation during treatments. On the other hand, according to our results $O_2^{\bullet-}$ and $NO^{\bullet}$ are increased in HPA group. Neither of them is active enough to initiate lipid peroxidation directly, but they release $ONOO^-$, which may initiate LPO chain reactions, from react quite rapidly at a diffusion-controlled rate *(33)*. Therefore, both $O_2^{\bullet-}$ and $NO^{\bullet}$ increased by Phe accumulation in this study may be an indicative these RS are involved on biomolecules damage reported in disease.

Finally, the figure 3 compiles all data discussed in this section and suggestions about possible pathways of RS overproduction involved in PKU. Mitochondrial metabolism disrupted and NADPH oxidase induced by Phe accumulation can increase $O_2^{\bullet-}$. Increased SOD activity simultaneously with CAT and GSH-Px inhibited enables H_2O_2 accumulation. Recently, studies have been introduced an additional mechanism for the rapid upregulation of Nrf2 (nuclear factor erythroid2-related factor 2) by H_2O_2, which governs basal and inducible antioxidant enzymes expression *(34)*. However, studies are necessary to clarify this concept it is possible that one of the mechanisms of antioxidant response against oxidative stress observed in PKU is by Nrf2 activation system and H_2O_2. In addition, data suggest a role of inflammation and immune activation in the Phe accumulation *(35)*. Cytokines induce the release of reactive $O_2^{\bullet-}$ and $NO^{\bullet}$ by NOS enzymes, and the availability of BH_4 regulates the direction of this RS production *(36)*. Consequently, under oxidative stress condition BH_4 is oxidized and BH4-dependent enzymes lose their activity further increasing Phe concentration *(37, 38)*. It becomes a vicious cycle with low production of catecholamine neurotransmitters followed by neurological and psychiatric symptoms.

Antioxidant therapy has been postulated as an additional approach to the restricted dietary treatment for PKU patients. Regarding the action of LA, it was possible to identify a consistent elimination of RS overproduction in brain from HPA rats, especially H_2O_2 but also $NO^\bullet$. However, under a lesser extent, animals only administered with LA also presented increase H_2O_2 and $NO^\bullet$. This result indicated a pro-oxidant action of LA which it is a common effect performed on excessive use of antioxidants *(39)*. Thus, it is necessary future investigations on dose adjustment of LA in order to remove this effect. To our knowledge, this study originally demonstrates the brain production of specific RS accumulation a HPA rat model injected with Phe and PAH inhibitor. If these results are confirmed in humans, we can suggest that H_2O_2, $NO^\bullet$ and $O_2^{\bullet-}$ present an important a role on mechanisms involved in PKU pathophysiology. In addition, it is also possible that accumulation of these RS can vary in among brain regions, but this study showed that HPA is able to increase some specific RS in total brain.

CONFLICT OF INTEREST

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

Scriver CR, Kaufman S (2001) Hyperphenylalaninemia: phenylalanine hydroxylase deficiency, in The metabolic and molecular bases of inherited disease. (Scriver CR, Beaudet AL, Sly WS, Valle D, eds), McGraw-Hill, New York, pp. 1667–1724.

Wajner M, Latini A, Wyse AT, Dutra-Filho CS (2004) The role of oxidative damage in the neuropathology of organic acidurias: insights from animal studies. J Inherit Metab Dis. 2004. 27, 427–448.

Sanayama Y, Nagasaka H, Takayanagi M, Ohura T, Sakamoto O, Ito T, Ishige-Wada M, Usui H, Yoshino M, Ohtake A, Yorifuji T, Tsukahara H, Hirayama S, Miida T, Fukui M, Okano Y (2011) Experimental evidence that phenylalanine is strongly associated to oxidative stress in adolescents and adults with phenylketonuria. Molecular Genetics and Metabolism. 2011. 103(3), 220-225.

Vargas CR, Wajner M, Sitta A (2011) Oxidative stress in phenylketonuric patients. Molecular Genetics and Metabolism. 2011. 104, S97–S99.

Rocha JC, Martins MJ (2011) Oxidative stress in phenylketonuria: futures directions. J Inherit Metab Dis. 2011. 35, 381–398.

Kienzle-Hagen ME, Pederzolli CD, Sgaravatti AM, Bridi R, Wajner M, Wannmacher CM, Wyse AT, Dutra-Filho CS (2002) Experimental hyperphenylalaninemia provokes oxidative stress in rat brain. Biochem Biophys Acta. 2002. 1586, 344-352.

Martinez-Cruz F, Pozo D, Osuna C, Espinar A, Marchante C, Guerrero JM (2002) Oxidative stress inuced by phenylketonuria in the rat: Prevention by melatonin, vitamin E and vitamin C. J Neurosci Res. 2002. 69(4), 550-558.

Berti SL, Nasi GM, Garcia C, Castro FL, Nunes ML, Rojas DB, Moraes TB, Dutra-Filho CS, Wannmacher CM (2012) Pyruvate and creatine prevent otxidative stress and behavioral alterations causaed by phenylalanine administratios into hippocampus of rats. Metab Brain Dis. 2012. 27(1), 79-89.

Moraes TB, Jacques CED, Rosa AP, Dalazen GR, Terra M, Coelho JG, Dutra-Filho CS (2013) Role of catalase and superoxide dismutase activities on oxidative stress in the brain of a phenylketonuria animal model and the effect of lipoic acid. Cell Mol Neurobiol. 2013. 33(2), 253-260.

Halliwell B and Gutteridge JMC (2007) Free Radicals in Biology and Medicine, Oxford University Press, NY.

Sitta A, Barschak Ag, Deon M, Terroso T, Pires R, Giugliani R, Dutra-Filho CS, Wajner M, Vargas CR (2006) Investigation of oxidative stress parameters in treated phenylketonuric patients. Metabolic Brain Dis. 2006. 21(4), 287-296.

Moraes TB, Zanin F, da Rosa A, de Oliveira A, Coelho J, Petrillo F, Wajner M, Dutra-Filho CS (2010) Lipoic acid prevents oxidative stress in vitro and in vivo by an acute hyperphenylalaninemia chemically-induced in rat brain. J Neurol Sci. 2010. 292, 89-95.

Halliwell B (2011) Free radical and antioxidants – quo vadis? (Opinion) Cell Press – Trends in Pharmacological Science. 2011. 32, 3.

Ji D, Majid ASA, Yin ZQ (2013) α-Lipoic Acid Attenuates Light Insults to Neurones. Biol Pharm Bull. 2013. 36(7), 1060–1067.

Packer L, Tritschler HJ, Wessel K (1997) Neuroprotection by the metabolic antioxidant α-lipoic acid. Free Rad Biol and Med. 1997. 22, 359-378.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951. 193, 265–275.

Gupte SA, Levine RJ, Gupte RS, Young ME, Lionetti V, Labinskyy V, Floyd BC, Ojaimi C, Bellomo M, Wolin MS, Recchia FA (2006) Glucose-6-phosphate dehydrogenase-derived NADPH fuels superoxide production in the failing heart. J Moll Cell Cardiol. 2006. 41, 340-349.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite and nitrate in biological fluids. Anal Biochem. 1982. 126, 131–138.

Pick E, Keisari Y (1980) A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Methods. 1980. 38, 161-170.

Gutteridge JMC (1991) Hydroxyl radical formation from the auto-reduction of a ferric citrate complex. Free Rad Biol Med. 1991. 11, 401-406.

Mazzola PN, Karikas GA, Schulpis KH, Dutra-Filho CS (2013) Antioxidant treatment strategies for hyperphenylalaninemia. Metab Brain Dis. 2013. DOI 10.1007/s11011-013-9414-2.

Rech VC, Feksa LR, Dutra-Filho CS, Wyse AT, Wajner M, Wannmacher CM (2002) Inhibition of the mitochondrial respiratory chain by phenylalanine in rat cerebral cortex. Neurochem Res. 2002. 27(5), 353-357.

Lu L, Gu X, Li D, Liang L, Zhao Z, Gao J (2011) Mechanisms regulating superoxide generation in experimental models of phenylketonuria: An essential role of NADPH oxidase. Mol Gent and Metabol. 2011. 104, 241-248.

He Y, Gu X. Lu L, Liagn L, Gao J, Zhang X (2013) NOX, the main regulator in oxidative stress in experimental models of phenylketonuria? J Pediatr Endocrinol Metab. doi: 10.1515/jpem-2012-0387.

Manning P, Cookson MR, McNeil CJ, Figlewicz D, Shaw PJ (2001) Superoxide-induced nitric oxide release from cultured glial cells. Brain Res. 2001. 911, 203-210.

Lee WJ, Lee IK, Kim HS, Kim YM, Koh EH, Won JC, Han SM, Kim MS, Jo I, Oh GT, Park IS, Youn JH, Park SW, Lee KU, Park JY (2005) Alpha-Lipoic acid prevents endothelial dysfunction in obese rats via activation of AMP-activated protein kinase. Arterioscler Thromb Vasc Biol. 2005. 25,2488–2494.

Anter E, Thomas SR, Schulz E, Shapira OM, Vita JA, Keaney JF Jr (2004) Activation of endothelial nitric-oxide synthase by the p38 MAPK in response to black tea polyphenols. J Biol Chem. 2004. 279, 46637–46643.

Prast H, Philippu A (2001) Nitric oxide as a modulator of neuronal function. Prog Neurobiol. 2001. 64, 51-68.

Wink DA, Miranda KM, Espey MG, Pluta RM, Hewett SJ, Colton C, Vitek M, Feelisch M, Grisham MB (2001) Mechanisms of the antioxidant effects of nitric oxide. Antiox Redox Signal. 2001. 3, 203-213.

Zielonka J, Sikora A, Joseph J, and Kalyanaraman B (2010) Peroxynitrite is the major species formed from different flux ratios of co-generated nitric oxide and superoxide: direct reaction with boronate-based fluorescent probe. J Biol Chem. 2010. 285, 14210–14216.

Niki E, Yoshida Y, Saito Y, Noguchi N (2005) Lipid peroxidation: mechanisms, inhibition, and biological effects. Biochem Biophys Res Commun. 2005. 338, 668–676.

Fernandes CG, Leipnitz G, Seminotti B, Amaral AU, Zanatta A, Vargas CR, Dutra Filho CS, Wajner M (2010) Experimental evidence that phenylalanine provokes oxidative stress in hippocampus and cerebral cortex of developing rats. Cell Mol Neurobiol. 2010. 30, 317–326.

Pacher P, Beckman J S, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007. 87, 315–424.
Covas G, Marinho HS, Cyrne L, Antunes F (2013) Chapter Nine – Activation of Nrf2 by H2O2: De novo synthesis versus nuclear translocation. Methods in Enzymology. 2013. 528, 157-171.

Sperner-Unterweger B, Kohl C, Fuchs D (2014) Immune changes and neurotransmitters: Possible interactions in depression? Progress in Neuro-Psychopharmacology & Biological Psychiatry. 2014. 48, 268–276.

Crabtree MJ, Channon KM (2011) Synthesis and recycling of tetrahydrobiopterin in endothelial function and vascular disease. Nitric Oxide. 2011. 25, 81–8.

Fuchs D, Jäger M, Widner B, Wirleitner B, Artner-Dworzak E, Leblhuber F (2001) Is hyperhomocysteinemia due to the oxidative depletion of folate rather than to insufficient dietary intake? Clin Chem Lab Med. 2001. 39, 691–694.

Widner B, Fuchs D, Leblhuber F, Sperner-Unterweger B (2001) Does disturbed homocysteine and folate metabolism in depression result from enhanced oxidative stress? J Neurol Neurosurg Psychiatry. 2001. 70, 419.

Moini H, Tirosh O, Park Y C, Cho K J, Packer L (2002) R-a-lipoic acid action on cell redox status, the insulin receptor, and glucose uptake in 3T3–-L1 adipocytes. Arch. Biochem. Biophys. 397: 384–391.

Figure captions

Fig.1. Effect of lipoic acid treatment on anion superoxide (a) and hydrogen peroxide (b) production in brain from young rats subjected to hyperphenylalaninemia model. Results are expressed by mean±SD (n=4-10). * $p < 0.05$; **$p<0.001$ compared to control (Tukey test).

Fig.2. Effect of lipoic acid treatment on oxide nitric (a) and hydroxyl radical (b) content in brain from young rats subjected to hyperphenylalaninemia model. Results are expressed by mean±SD (n=6-14). * $p < 0.05$; **$p<0.001$ compared to control (Tukey test).

Fig.3. Possible pathways of reactive species production in hyperphenylalaninemia based on literature review. In red represents specific reactive species increased in this hyperphenylalaninemia model investigated.

Figure 1

O$_2^{\cdot-}$ content   (a)

H$_2$O$_2$ production   (b)

46
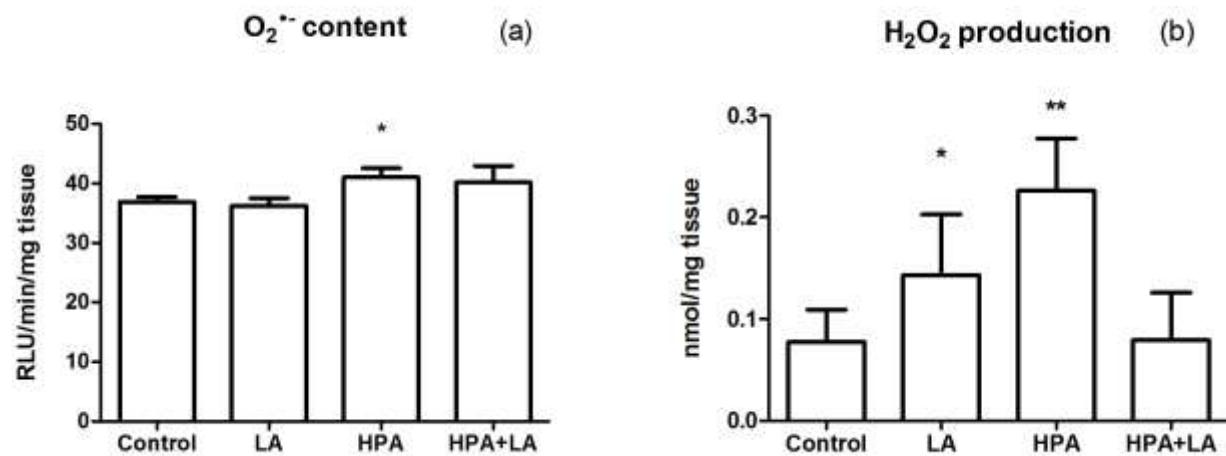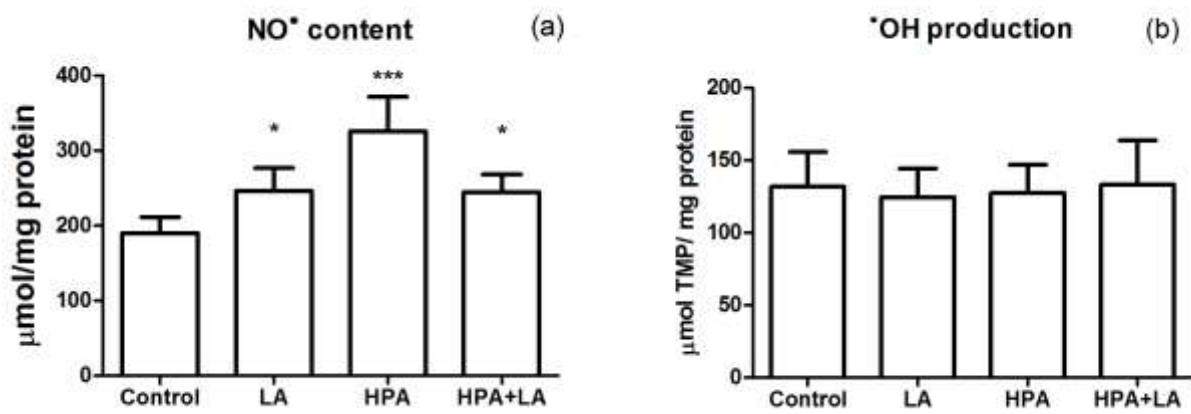
Figure 2
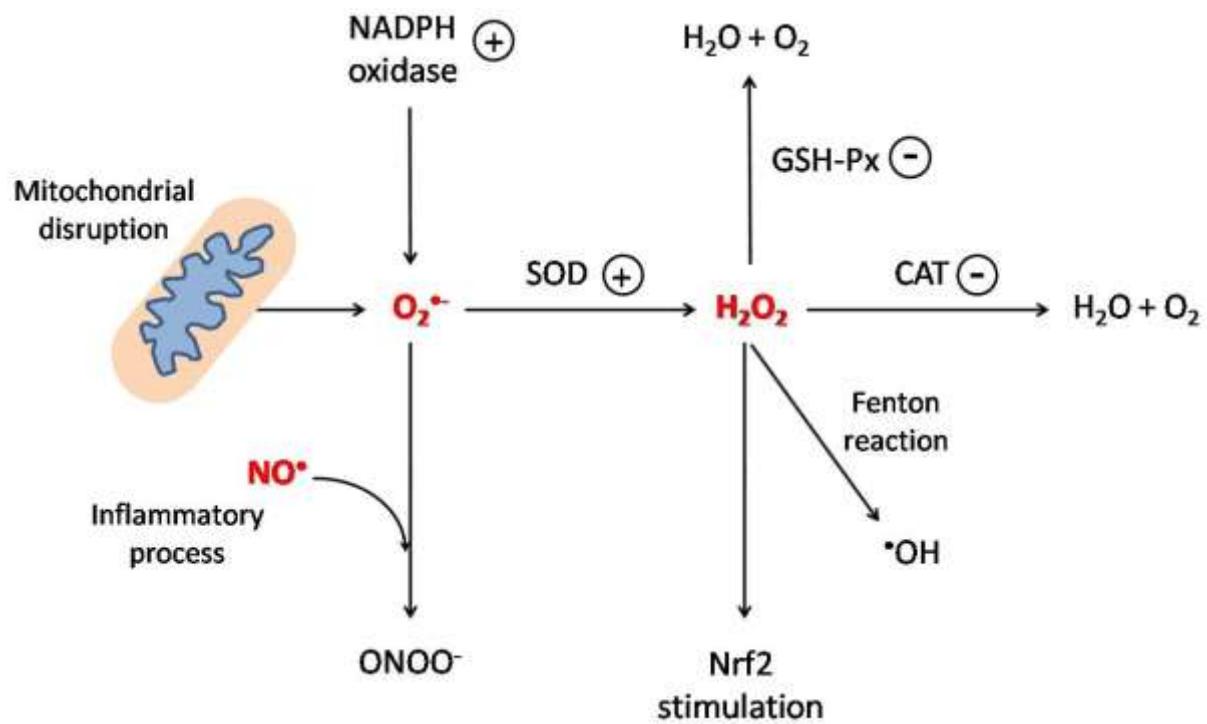
Figure 3

GLUTATHIONE METABOLISM ENZYMES IN BRAIN AND LIVER OF HYPERPHENYLALANINEMIC RATS AND THE EFFECT OF LIPOIC ACID TREATMENT

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ABSTRACT

Phenylketonuria (PKU) is a disorder caused by a deficiency in phenylalanine hydroxylase, which converts phenylalanine (Phe) to tyrosine, leading to hyperphenylalaninemia (HPA) with accumulation of Phe in tissues of patients. The neuropathophysiology mechanism of this disease remains unknown. However, the involvement of oxidative stress with decrease glutathione in PKU has been reported recently. Intracellular glutathione (GSH) levels may be maintained by the antioxidant action of lipoic acid (LA). The aim of this study was to evaluate the activity of the enzymes involved in the metabolism and function of GSH, such as glutathione peroxidase (GSH-Px), glucose-6-phosphate dehydrogenase (G6PD), glutathione reductase (GR), glutamate-cysteine ligase (GCL), glutathione-S-transferase (GST) and GSH content in brain and liver of young rats subjected to a chemically induced model of HPA and the effect of LA for a week. In brain, the administration of Phe reduced the activity of the GSH-Px, GR and G6PD and LA prevented GSH-Px and G6PD inhibition totally or partially. GCL activity was increased by HPA and was not affect by LA antioxidant treatment. GST activity did not differ between groups. GSH content was increased by LA and decreased by HPA treatment in brain samples. Considering the liver, all parameters analyzed were increased in studied HPA animals and LA was able to hinder some effects except for the GCL and GST enzymes, and GSH content. These results suggested that HPA model alters the metabolism of GSH in rat brain and liver, which may have an important role in the maintenance of GSH function in PKU although liver is not a directly affected organ in this disease. So, an antioxidant therapy with LA may be useful in the treatment of oxidative stress in HPA.

INTRODUCTION

The deficiency of phenylalanine - 4 - hydroxylase (PAH) causes phenylketonuria (PKU), a disorder characterized by HPA with accumulation of phenylalanine (Phe) and its metabolites in biological fluids and tissues of patients and scarcity of L-tyrosine (Tyr) (Scriver and Kaufman 2001). Typical neurological features are growth retardation, delayed psychomotor development, microcephaly, seizures, epilepsy and mental retardation (Pietz 1998; Scriver and Kaufman 2001). PKU treatment consists mainly of a Phe-restricted and low-protein diet supplemented with essential micronutrients such as vitamins, minerals and trace elements (Vilaseca et al 2010). The mechanisms of toxicity of Phe (and its metabolites) in the central nervous system (CNS) are not well understood. However, an increasing number of reports has proposed that increased Phe in plasma associated with oxidative stress is involved in the neuropathology PKU (Sanayama et al 2011; Vargas et al 2011; Sitta et al 2009).

Reduced glutathione (GSH) is the most abundant non-protein thiol present in cells at millimolar concentrations, participating in important cellular processes including cell proliferation and regulation of gene expression (Dickinson and Forman 2002). However, GSH is one of the most important mechanisms of protection against oxidative injuries in cell being a scavenger of reactive species or a cofactor for antioxidant enzymes (Dickinson et al 2003). Glutathione peroxidase (GSH-Px – EC 1.11.1.9) reduces peroxides oxidizing GSH to oxidized glutathione (GSSG), and GSSG is reduced by glutathione reductase (GR – EC 1.8.1.7) with NADPH as a coenzyme, the main product of glucose-6-phosphate dehydrogenase (G6PD – EC 1.1.1.49) (Järvinen et al 2003). Additionally, GSH participates in detoxification reactions of phase II with a group of GSTs (glutathione S-transferases –

EC 2.5.1.18) isoenzymes, protecting the cell against oxidative stress through metabolism byproducts (Fonseca et al 2010; Huerta-Olvera et al 2010).

GSH intracellular levels might be maintained by LA reduction potential action; it is synthesized naturally in tissues and participates as a cofactor for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase enzymes and is capable to cross the blood-brain barrier (Hagen et al 1999; Samuel et al 2005). Both LA and its reduced form dihydrolipoic acid (DHLA) are considered potent antioxidants (Packer et al 1997) and LA has been used as experimental therapy of models for neurological dysfunction such as seizures, schizophrenia and Parkinson's disease (de Souza et al 2010; Seybolt 2010; Abdin and Sarhan 2011). Considering that PKU neuropathology is not completely understood and that oxidative stress may be involved in the mechanism of action of the disease, we investigated the effect of a chronic model of chemically-induced HPA on parameters of GSH metabolism in homogenates of brain and liver of young rats and the effect of LA treatment.

MATERIALS AND METHODS

Six-day-old Wistar rats were obtained from the Department of Biochemistry, ICBS, UFRGS and were maintained with dams in a room with 12:12 h light/dark cycle with controlled temperature (22°C±1°C) and dams had water and a protein commercial chow (Nuvilab, Porto Alegre, RS, Brazil) *ad libitum*. The NIH Guide for the Care and Use of Laboratory Animals (NIH publication # 80–23, revised 1996) was followed in all experiments. All chemicals were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Eppendorf 5417R (refrigerated version) were used for the

centrifugation procedures. SpectraMax M5 plate spectrophotometer of absorbance / fluorescence with temperature control was used for all measurements.

HPA animal model and LA treatment

A chronic chemically-induced model of HPA was produced by the administration of L-phenylalanine (Phe) twice a day (2.1 µmol/g) and PAH inhibitor α-methylphenylalanine (α-MePhe) once a day (1.6 µmol/g), while LA was administered once a day (40mg/Kg). Injections were performed for eight days, beginning at post-natal day 6. Animals were divided into four groups: Control group (saline solution all administrations); LA group; HPA group (α-MePhe + Phe) and HPA+LA group (α-MePhe + Phe + LA). Solutions were prepared each day and were administered by subcutaneous injections according to Kienzle-Hagen et al (2002) which demonstrated that this proposed model of HPA is capable to reflect the plasma Phe levels found in patients (3.6±0.2 mM). In addition, many experimental models chemically inducing HPA by Phe administration in animals promote behavioral, neurochemical and neuromorphological alterations similar to those found in PKU patients (Luttges and Gerren 1979; Diamond et al 1994; Berti et al 2012; dos Reis et al 2013).

Tissue preparation

Litters with 14 days of life (after 8 days of treatment) were killed by decapitation 12 hours after the last injection and the brain and liver were immediately removed and kept on an ice-plate. The olfactory bulb, cerebellum, pons and medulla were discarded and the forebrain and liver were cleaned, weighed and maintained on ice. Samples were homogenized (1:10, w/v) in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM

KCl. Homogenates were centrifuged at 800 g for 10 min at 4°C to separate nuclei and cell debris (Lissi et al 1986). Protein tissue concentration was determined in brain and liver supernatants using bovine serum albumin as a standard (Lowry et al 1951).

Glutathione peroxidase (GSH-Px) activity

GSH-Px activity was determined according to the method of Wendel (1981) from the monitoring of disappearance of NADPH at 340 nm. The medium contained 2 mM GSH, 0.15 U / mL GR, 0.4 mM sodium azide, 10 mM tert-butyl hydroperoxide, 0.5 mM NADPH 0.1 mM and 26 µL of sample (0.08 – 0.16 mg of protein). One unit of GSH-Px was defined as µmol of NADPH consumed per minute and specific activity is represented as units/mg protein.

Glucose 6-phosphate dehydrogenase (G6PD) activity

The method to measure G6PD activity is based on the formation of NADPH at 340 nm in a reaction medium containing 100 mM Tris-hydrochloryde buffer pH 7.5, 10 mM magnesium chloryde, 0.1% triton X-100, 0.5 mM $NADP^+$, 1 mM glucose 6-phosphate and 30 µL of sample (0.09 – 0.18 mg of protein) according to Leong and Clark (1984). One G6PD unit is defined as µmol of NADPH produced per minute and the specific activity is represented as G6PD units/mg protein.

Glutathione reductase (GR) activity

GR activity was determined by the method of Calberg and Manervik (1985) which is based on the reduction of GSSG using NADPH as a coenzyme. The reaction medium contained 200 mM sodium phosphate buffer, 6.3 mM EDTA pH 7.5, 10 mM GSSG, 0.4 mM NADPH

and 30 µL of sample (0.09 − 0.18 mg of protein). The consumption of NADPH was measured in a spectrophotometer at 340 nm. One unit of GR is defined as µmol of GSSG reduced per minute and specific activity is represented as GR units/mg protein.

Activity of glutamate-cysteine ligase (GCL)

GCL activity was determined based on the reaction of naphthalene-2,3-dicarboxialdehyde (NDA) with the γ-glutamylcysteine produced in a medium containing 40 mM ATP, 20 mM glutamic acid, 2 mM cysteine and 25 µL of sample (0.075 − 0.15 mg protein). The product formed is measured in the spectrofluorometer at 472 nm excitation and 528 nm emission according to White et al (2003). One unit of GCL is defined as µmol of GSH formed per minute and specific activity is represented as GCL units/mg protein.

Glutathione S-transferase (GST) activity

Activity of GST was determined by the method of Habig et al (1974), based on the conjugation of CDNB (1-chloro-2,4-dinitrobenzene) to GSH. The product formed is measured in a spectrophotometer at 340 nm in a reaction medium containing 100 mM potassium phosphate buffer, 50 mM CDNB, 25 mM GSH and 30 µL of sample (0.09 − 0.18 mg of protein). One unit of GST is defined as µmol of CDNB conjugated to GSH per minute and the specific activity is represented as GST units/mg protein.

Reduced glutathione (GSH) content

GSH levels were evaluated according to Browne and Armstrong (1998) with slight modifications. Samples were deproteinized with 1.85% metaphosphoric acid and centrifuged at 6000g for 10 minutes and 15 µL of supernatant was incubated with a

medium containing 0.38 mM of *o*-phthaldialdehyde (1mg/mL methanol) at room temperature for 15min. Fluorescence was measured using wavelengths of excitation and emission of 350 and 420 nm, respectively. Calibration curve was performed with a commercial standard GSH (0.0025–0.1mM), and GSH concentrations were calculated as µmol/g tissue.

Statistical analysis

Results are expressed by mean ± standard deviation (SD) and statistical analysis was performed by one-way (ANOVA) analysis of variance followed by the Tukey test for multiple comparisons when the F value was significant. All analyzes were performed by GraphPad version 5.0 software. Significant level was considered as 95% ($\alpha=0.05$).

RESULTS

To evaluate the metabolic profile of GSH in HPA condition and LA treatment, we analyzed the activity of several important enzymes involved in processes of use, regeneration and production of GSH in brain (fig.1 and 2) and liver (fig. 3 and 4) of animals. First, we observed that in the brain, HPA inhibited the activity of GSH-Px [$F_{(3,24)}$ = 36.98; p <0.001; n = 7] and the treatment with LA was able to maintain the activity of this enzyme to the control values (fig.1). The activity of GR, the enzyme responsible for the conversion of GSSG to GSH, was inhibited by the accumulation of Phe, and this effect was not altered by treatment with the antioxidant LA [$F_{(3,21)}$ = 6.99; p <0.01; n = 6-7] as shown in fig. 1. The activity of G6PD, responsible for the production of NADPH, was also inhibited in HPA rats when compared to controls [$F_{(3,32)}$ = 16.24; p <0.001; n = 8-10], and the treatment with LA was able to partially prevent this effect. The activity of GCL, the

limiting enzyme in the route for synthesis of GSH, was significantly increased in the HPA group [F (3,25) = 4.256; p <0.05; n = 6 -9] (fig. 2) and the treatment with LA did not change this effect. Fig. 2 also presents the results on the activity of the GST, responsible for the conjugation of GSH to harmful molecules like metals and xenobiotics. It can be observed that the activity of this enzyme was not changed by any treatment in the brain of these animals [F (3,18) = 1.917; p> 0.05; n = 5-6].

In liver, fig. 3 presents a significant increase of the GSH-Px activity in LA and HPA groups [F (3,22) = 19.60; p <0.001; n = 6-7] and HPA+LA group showed that this activity was preserve to the level of control. Moreover, in liver samples, the HPA model increased the activity of enzyme GR [F (3,24) = 10.77; p <0.001; n = 5-9], and LA treatment also normalized after this change. Fig. 3 shows that the model of HPA significantly increased the activity of G6PD, which was also and subsequently normalized by treatment with LA [F (3,27) = 19.56; p <0.001; n = 7-8]. GCL activity was increased in the HPA group but was not maintained to control levels by LA (fig. 4) [F (3,21) = 15.12; p <0.001; n = 5-8] . Moreover, GST activity was also significantly increased in the groups that received HPA and treatment with LA [F (3,27) = 14.97; p <0.001; n = 7-9].

Finally, GSH content of brain and liver samples was evaluated and was shown in table 1. HPA significantly diminished GSH content in brain of young rats [F(3,28) = 112.8; p<0.001; n = 6-9]. LA alone increased GSH in brain and this antioxidant treatment was able to preserve the levels of GSH diminished by HPA. On the other hand, HPA induced model significantly increased GSH content in liver and LA treatment did not alter this effect [F(3,19) = 19.43; p<0.001; n = 5-6].

DICUSSION

GSH is important in protecting brain against oxidative stress and its regulation and synthesis is limited by thiol amino acids transport in blood brain barrier (Valdovinos-Flores and Gosenbatt 2012). In addition, liver plays a critical role in GSH homeostasis between different organs (Ookhtens and Kaplowitz 1998). Since there is evidence of change in GSH-Px activity and reduced GSH content in PKU patients and that oxidative stress is possibly associated in PKU neuropathology (Sanayama et al 2011; Sitta et al 2009). The present study evaluated the main enzymes involved in GSH metabolism in brain and liver. Initially, we observed that the activities of GSH-Px, GR and G6PD were inhibited in the brain of HPA animals. Recent studies in erythrocytes of PKU patients and HPA animal model have demonstrated an inhibition of GSH-Px activity (Kienzle-Hagen et al 2002; Sirtori et al 2005; Sitta et al 2006), and this inhibition is correlated with low selenium plasma concentrations in PKU patients (Sitta et al 2009). A NADPH source used as electron acceptor necessary to reduce GSSG to GSH by GR activity is provided from G6PD (Brigelius-Flohé 1999). Consequently, according to data exhibited the inhibition of GR and G6PD activities by HPA administration may hinder the recycling of GSH from GSSG shifting the cerebral tissue to a more oxidant status. On the other hand, Ercal et al (2002) showed an increase of G6PD activity in red blood cells (RBC) of *BTBR-Pah* [(enu2)] animal model for PKU and a concomitant decrease in GSH levels in RBC and brain of mices. Although, in physiological conditions the levels of GSH are higher than GSSG (Griffith 1999). Administration of LA is capable to increase intracellular GSH levels up to approximately 70% (Packer et al 1997), and it was observed in animals of LA group. Therefore, high levels of GSH found in LA group can promote an inhibition of GR activity

and eventually accumulating NADPH in cells which in turn can also down regulate the activity of G6PD (Chung et al 1991; Singh et al 2012).

GCL activity was also evaluated in the brain and a significant increase was observed in the HPA group. It possibly suggests that there may be some feedback mechanism trying to maintain the levels of intracellular GSH, possibly through induction of GCL which may represent the main means of keeping brain levels of GSH, since GR was inhibited in brain samples (Aoyama et al 2008; Lu 2009). GST catalyzes the conjugation of compounds containing carbon and nitrogen electrophilic with GSH, contributing to cellular detoxification (Wojtkowiak-Giera et al 2011). However, there was no change in the activity of GST in any group comparing to controls and possibly the mechanism whereby oxidative stress is involved in brain of HPA animals is not through phase II reactions detoxification (Townsend et al 2008). In addition, Artuch et al (2001) described no differences in GST activity in erythrocytes of PKU patients.

Liver is an important organ of detoxification with high levels of antioxidants, especially GSH (Reed et al 2008). In order to evaluate the effect of HPA in GSH metabolism, we also analyzed the activity of enzymes involved in the metabolism of GSH in liver. The results observed in samples from liver of HPA animals showed an increase of the activity of all enzymes analyzed which are involved in GSH metabolism and an elevation of GSH concentration. The results possible indicate an effective participation of this organ in the detoxification of reactive species and redox homeostasis maintenance from the use of GSH as an antioxidant against oxidative stress in HPA experimental condition. Indeed, the liver plays an important role in body homeostasis of GSH it is the main organ that releases GSH

into the blood (Kretzschmar 1996; Ookhtens and Kaplowitz 1998; Lu 1999). Besides, alterations of GSH homeostasis is involved in neurodegenerative disease and it is known that brain uptakes GSH through specific transporters in the blood brain barrier (BBB) (Favilli et al 1997; Johnson et al 2012; Valdovinos-Flores and Gosenbatt 2012), although the specific mechanisms are not completely understood. In addition, liver GSH reservoir is a source of cysteine, which is a limiting amino acid in the synthesis of new GSH molecules and can cross BBB by amino acids transporters (Tateishi et al 1977). Therefore, it is possible that the liver is contributing for the maintenance of GSH levels in the brain of rats subjected to chronic treatment of HPA. More studies are necessary to better understand this hypothesis. A possible mechanism of GSH enzymes induction could be the transcription factor Nrf2 (nuclear factor erythroid2-related factor 2) which governs basal and inducible antioxidant enzymes expression by means of the antioxidant response element (ARE). Increase of GSH concentration was accompanied by increased Nrf2 protein levels, regulating GCL and GST gene expression (Satoh et al 2013). Although it was not measured Nrf2 in this work, it has been proposed that LA induces Nrf2 from ARE-specific DNA-binding and, consequently, increases GCL activity and GSH synthesis (Suh et al 2004; Wagner et al 2012).

Recently, antioxidant treatment strategies have been proposed to HPA (Mazzola et al 2013). In addition, L-carnitine and selenium supplementation reduced oxidative stress in PKU patients (Sitta et al 2011). In this work LA administration was effective to normalize the redox state altered by HPA, though LA per se increased GSH levels and it should be considered in future studies. However, if these results were confirmed in patients, antioxidants like LA could be a potential adjunctive therapy in the treatment of PKU.

REFERENCES

Abdin AA, Sarhan NI (2011) Intervention of mitochondrial dysfunction-oxidative stress-dependent apoptosis as a possible neuroprotective mechanism of α-lipoic acid against rotenone-induced parkinsonism and L-dopa toxicity. Neuroscience Research. 71(4): 387-395

Artuch R, Colome C, Vilaseca MA et al (2001) Plasma phenylalanine is associated with decreased serum ubiquinone-10 concentrations in phenylketonuria. J Inherit Metab Dis 24:359–366

Aoyama K, Watabe M, Nakaki T (2008) Regulation of Neuronal Glutathione Synthesis. J Pharmacol Sci 108, 227 – 238

Berti SL, Nasi GM, Garcia C, de Castro FL, Nunes ML, Rojas DB, Moraes TB, Dutra-Filho CS, Wannmacher CMD (2012) Pyruvate and creatine prevent oxidative stress and behavioral alterations caused by phenylalanine administration into hippocampus of rats. Metab Brain Dis 27:79–89

Browne RW, Armstrong D (1998) Reduced glutathione and glutathione disulfide. Methods Mol Biol 108: 347–352

Brigelius-Flohe´ R (1999) Tissue-specific functions of individual glutathione peroxidases. Free Radic Biol Med 27:951–965

Calberg I, Manervik B (1985) Glutathione Reductase. Methods Enzymol 113: 484-490

Chung PM, Cappel RE, Gilbert HF (1991) Inhibition of glutathione disulfide reductase by glutathione. Arch Biochem Biophys 288(1): 48-53

de Souza GF; Saldanha GB; de Freitas RM (2010) Lipoic acid increases glutathione peroxidase, Na+, K+-atpase and acetylcholinesterase activities in rat hippocampus after pilocarpine-induced seizures? Arq Neuropsiquiatr 68(4): 586-591

Diamond A, Ciaramitaro V, Donner E, Djali S, Robinson MB (1994) An Animal Model of Early-treated PKU. The Journal of Neuroscience 14(5): 3072-3082

Dickinson DA, Forman HJ (2002) Cellular glutathione and thiols metabolism. Biochem Pharmacol 64: 1019–1026

Dickinson DA, Moellering DR, Iles KE et al (2003) Cytoprotection against oxidative stress and the regulation of glutathione synthesis. Biol Chem 384: 527–537

dos Reis EA, Rieger E, de Souza SS, Rasia-Filho AA, Wannmacher CMD (2013) Effects of a co-treatment with pyruvate and creatine on dendritic spines in rat hippocampus and posterodorsal medial amygdala in a phenylketonuria animal model. Metab Brain Dis 28:509–517

Ercal N, Aykin-Burns N, Gurer-Orhan H, Mcdonald JD (2002) Oxidative stress in a phenylketonuria animal model. Free Radic Biol Med 32:906–911

Favilli F, Marraccini P, Iantomasi T, Vincenzini MT (1997). Effect of orally administered glutathione on glutathione levels in some organs of rats: role of specific transporters. Br. J. Nutr. 78: 293-300

Fonseca RR, Johnson WE, O'Brien SJ et al (2010) Molecular evolution and the role of oxidative stress in the expansion and functional diversification of cytosolic glutathione transferases. BMC Evol Biol 10: 281

Habig WH, Pabst MJ, Jacoby WB (1974) Glutathione S-Transferase: the first enzymatic step in mercapturic acid formation. J Biol Chem 249: 7130-7139

Hagen TM, Ingersoll RT, Lykkesfeldt J et al (1999) (R)-α-lipoic acid-supplemented old rats have improved mitochondrial function, decreased oxidative damage, and increased metabolic rate. *Faseb J* 13: 411–418

Huerta-Olvera SG, Macías-Barragán J, Ramos-Márquez ME et al (2010) Alpha-lipoic acid regulates heme oxygenase gene expression and nuclear Nrf2 activation as a mechanism of protection against arsenic exposure in HepG2 cells. Envir Toxicol Pharmacol 29: 144-149

Järvinen K, Soini Y, Kinnula VL (2003) γ-Glutamylcysteine Synthetase in Lung Cancer. Effect on Cell Viability. Methods Mol Med 74: 211-222

Johnson WM, Wilson-Delfosse AL, Mieyal JJ (2012) Dysregulation of Glutathione Homeostasis in Neurodegenerative Diseases. Nutrients 4: 1399-1440

Kienzle-Hagen ME, Pederzolli CD, Sgaravatti AM et al (2002) Experimental hyperphenylalaninemia provokes oxidative stress in rat brain. Biochim Biophys Acta 1586(3):344-352

Kretzschmar M (1996). Regulation of hepatic glutathione metabolism and its role in hepatotoxicity. Exp Toxic Pathol 48: 439-446

Leong SF Clark JB (1984) Regional enzyme development in rat brain. Enzymes associated with glucose utilization. Biochem J 218:131-138

Lissi EA, Cáceres T, Videla LA (1986) Visible chemiluminescence from rat brain homogenates undergoing autoxidation. I. Effect of additives and products accumulation. Free Radic Biol Med 2(1): 63-69

Lowry OH, Rosebrough NJ, Farr AL et al (1951) Protein measurement with the folin phenol. J Biol Chem 193: 265-275

Lu SC (1999) Regulation of hepatic glutathione synthesis: current concepts and controversies. The FASEB Journal 13(10): 1169-1183

Lu SC (2009) Regulation of glutathione synthesis. Mol Aspects Med. 30(1-2): 42–59

Luttges MW, Gerren RA (1979) Postnatal alpha-methylphenylalanine treatment effects on adult mouse locomotor activity and avoidance learning. Pharmacol Biochem Behav 11(5):493-498

Mazzola P.N., Karikas G.A., Schulpis K.H., Dutra-Filho C.S. (2013). Antioxidant treatment strategies for hyperphenylalaninemia. Metab Brain Dis. DOI 10.1007/s11011-013-9414-2

Ookhtens O and Kaplowitz N (1998) Role of the liver in intergorgan homeostasis of glutathione and cyst(e)ine. Semin Liver Dis 18: 313-329

Packer L, Tritschler HJ, Wessel K (1997) Neuroprotection by the metabolic antioxidant α-lipoic acid. Free Rad Biol and Med 22: 359-378

Pietz J (1998) Neurological aspects of adult phenylketonuria. Curr Opin Neurol 11: 679-688

Reed MC, Thomas RL, Pavisic J, James SJ, Ulrich CM, Nijhon HF (2008) A mathematical model of glutathione metabolism. Theoretical Biology and Medical Modelling 5:8 doi:10.1186/1742-4682-5-8

Samuel S, Kathirvel R, Jayavelu T, Chinnakkannu P (2005) Protein oxidative damage in arsenic induced rat brain: influence of DL-α-lipoic acid. Toxicol Lett 155: 27-34

Sanayama Y, Nagasaka H, Takayanagi M et al (2011) Experimental evidence that phenylalanine is strongly associated to oxidative stress in adolescents and adults with phenylketonuria. Molecular Genetics and Metabolism 103(3): 220-225

Satoh T, McKercher SR, Lipton SA (2013) NrF2/ARE-mediated antioxidant actions of pro-electrophilic drugs. Free Radic Biol Med doi: 10.1016/j.freeradbiomed.2013.07.022

Scriver CR, Kaufman S (2001) Hyperphenylalaninemia: phenylalanine hydroxylase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 1667–1724

Seybolt SEJ (2010) Is it time to reassess alpha lipoic acid and niacinamide therapy in schizophrenia? Medical Hypotheses 75(6): 572-575

Singh S, Anand A, Srivastava PK (2012) Regulation and properties of glucose-6-phosphate dehydrogenase: A review. International Journal of Plant Physiology and Biochemistry Vol. 4(1): 1-19

Sirtori LR, Dutra-Filho CS, Fitarelli D et al (2005). Oxidative stress in patients with phenylketonuria. Biochim Biophys Acta 1740(1): 68-73

Sitta A, Barschak AG, Deon M et al (2006) Investigation of oxidative stress parameters in treated phenylketonuric patients. Metab Brain Dis 21(4): 287-296

Sitta A, Barschak AG, Deon M et al (2009) Effect of short- and long-term exposition to high phenylalanine blood levels on oxidative damage in phenylketonuric patients. Int J Dev Neurosci 27:243–247

Sitta A, Vanzin CS, Biancini GB, Manfredini V, de Oliveira AB, Wayhs CA, Ribas GO, Giugliani L, Schwartz IV, Bohrer D, Garcia SC, Wajner M, Vargas CR (2011) Evidence that Lcarnitine and selenium supplementation reduces oxidative stress in phenylketonuric patients. Cell Mol Neurobiol 31(3):429–436

Suh JH, Shenvi SV, Dixon BM et al (2004) Decline in transcriptional activity of Nrf2 causes age related loss of glutathione synthesis, which is reversible with lipoic acid. Proc Natl Acad Sci 101:3381–3386

Tateishi N, Higashi T, Naruse A, Nakashima K, Shiozaki H (1977). Rat liver glutathione: possible role as a reservoir of cysteine. J Nutr. 107:51–60

Townsend DM, Manevich Y, He L, Hutchens S, Pazoles CJ, Tew KD (2008) Novel Role for Glutathione S-Transferase: Regulator of protein S-glutathionylation following oxidative and nitrosative stress Journal of Biological Chemistry 284(1): 436-445

Valdovinos-Flores C, Gosenbatt ME (2012) The role of amino acids transporters in GSH synthesis in the blood brain barrier and central nervous system. Neurochem Intern 61: 405-414

Vargas CR, Wajner M, Sitta A (2011) Oxidative stress in phenylketonuric patients. Molecular Genetics and Metabolism 104: S97–S99

Vilaseca MA, Lambruschini N, Gómez-López L et al (2010) Quality of dietary control in phenylketonuric patients and its relationship with general intelligence. Nutr Hosp 25: 60-66

Wagner AE, Ernst IMA, Birringer M, Sancak Ö, Barella L, Rimbach G (2012) A Combination of Lipoic Acid Plus Coenzyme Q10 Induces PGC1α, a Master Switch of Energy Metabolism, Improves Stress Response, and Increases Cellular Glutathione Levels in Cultured C2C12 Skeletal Muscle Cells. Oxidative Medicine and Cellular Longevity doi:10.1155/2012/835970

Wendel A (1981) Glutathione peroxidase. Meth Enzymol 77: 325-332

White CC, Viernes H, Krejsa CM et al (2003) Fluorescence-based microtiter plate assay for glutamate–cysteine ligase activity. Anal Biochem 318: 175–180

Wojtkowiak-Giera A, Wandurska-Nowak E, Michalak M (2011) Changes in the activity and kinetics of mouse intestinal glutathione transferase during experimental trichinellosis. Parasitol Res 108: 1309-1313

Figure Captions

Fig.1 Effect of lipoic acid treatment on glutathione peroxidase (GSH-Px), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) activities in brain of rats subject to chronic hyperphenylalaninemia model. Results are expressed by mean±SD (n=6-10). * $p<0.05$ and ** $p<0.01$ compared to control group (Tukey test).

Fig.2 Effect of lipoic acid treatment on glutamate-cysteine ligase (GCL) and glutathione S-transferase (GST) activities in brain of rats subject to chronic hyperphenylalaninemia model. Results are expressed by mean±SD (n=5-9). * $p<0.05$ compared to control group (Tukey test).

Fig.3 Effect of lipoic acid treatment on glutathione peroxidase (GSH-Px), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) activities in liver of rats subject to chronic hyperphenylalaninemia model. Results are expressed by mean±SD (n=5-9). *** $p<0.001$ compared to control group (Tukey test).

Fig.4 Effect of lipoic acid treatment on glutamate-cysteine ligase (GCL) and glutathione S-transferase (GST) activities in liver of rats subject to chronic hyperphenylalaninemia model. Results are expressed by mean±SD (n=5-9). ** $p<0.01$; ***$p<0.001$ compared to control group (Tukey test).

Figure 1
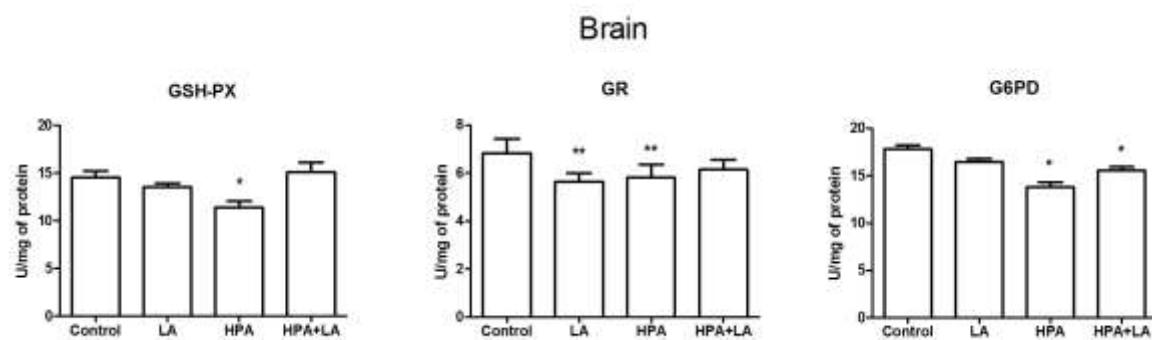
Figure 2
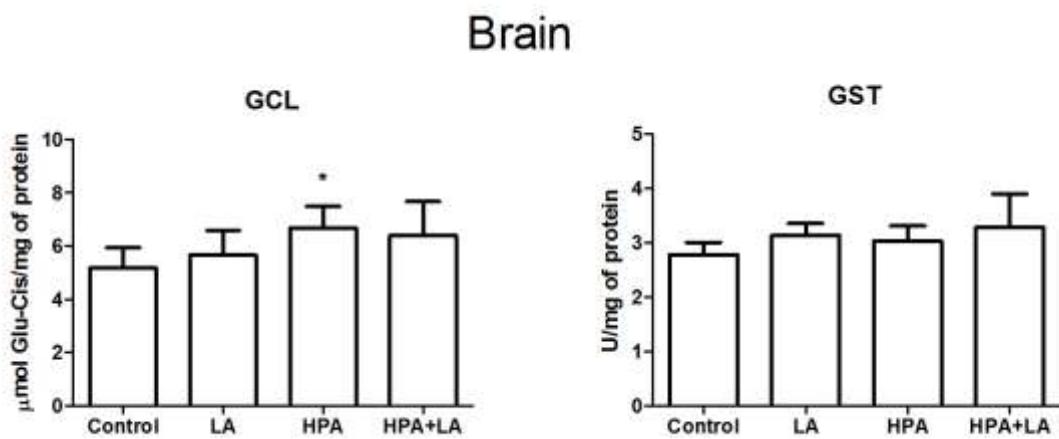
Figure 3
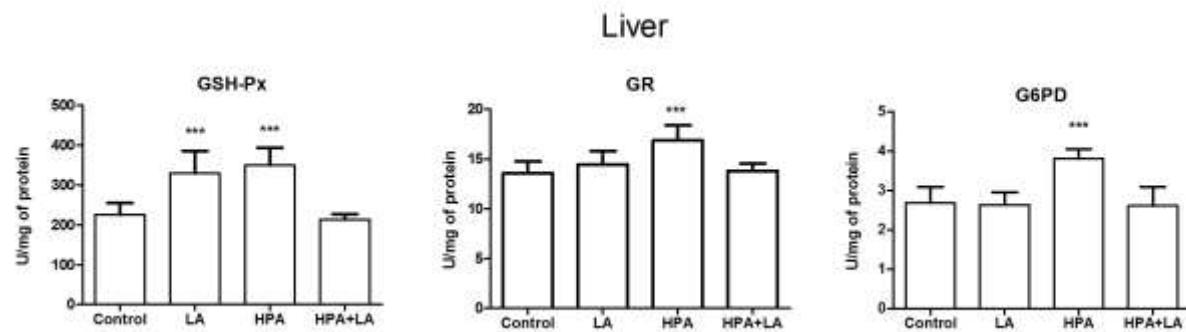
Liver

Figure 4
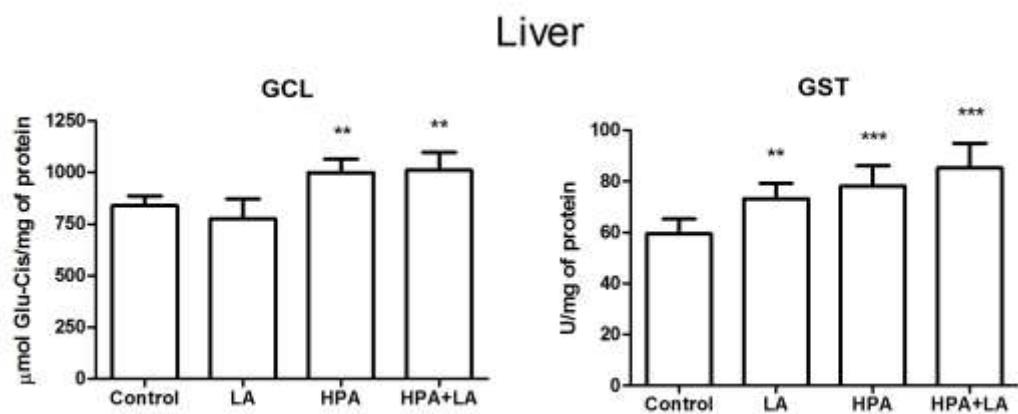
Table 1. Effect of LA treatment on GSH content in brain and liver of rats subject to chronic HPA.

		Control	LA	HPA	HPA+LA
GSH	Brain	264.1 ± 8.5	356.7 ± 14.2***	233.9 ± 18.0***	277.2 ± 8.3
	Liver	772.4 ± 133.4	$942.8 \pm 78,2$	1214.4 ± 31.9***	1301.9 ± 208.7***

Content of GSH is expressed as µmol/g of tissue. Data are means $\pm$ SD for n = 6-9 independent experiments performed. ***$p<0.001$, compared to control (Tukey).

PARTE III

DISCUSSÃO GERAL

Na PKU, o metabolismo de Phe encontra-se prejudicado devido a uma deficiência total ou parcial na atividade da enzima hepática PAH. Consequentemente, há um excesso de Phe no plasma dos pacientes, principalmente, naqueles que não estão em tratamento. As altas concentrações de Phe nos tecidos estão relacionadas ao retardo mental nos pacientes afetados (Scriver et al., 2001). O diagnóstico e tratamento precoce com uma dieta sem Phe atenuam as alterações neurofisiológicas e comportamentais (Channon et al., 2004, Huijbregts et al., 2002). Porém, mesmo após muitos anos de pesquisa e investigações com pacientes ou em modelos animais, os mecanismos envolvidos na neurofisiopatologia da doença ainda não estão completamente elucidados (Sierra et al., 1998; Kienzle-Hagen et al., 2002; Ercal et al., 2002). É possível, no entanto, que as alterações no SNC se tratem de um somatório de mecanismos neurotóxicos incluindo alterações do estado redox celular.

Diversos trabalhos de pesquisa, em pacientes fenilcetonúricos ou animais hiperfenilalaninêmicos, induzidos quimicamente ou em modelos *knockout*, têm descrito o envolvimento do EO na neurofisiopatologia da PKU (Vanzin et al., 2011; Ribas et al., 2011; Ribas et al., 2012; Rocha e Martins 2011; Sanayama et al., 2011). Muitos dos pacientes com PKU apresentam, ao longo da vida, aumentos reincidentes nos níveis plasmáticos de Phe, devido à dificuldade de adesão ao tratamento dietético principalmente a partir da adolescência (Vilaseca et al., 2010) e, consequentemente, mesmo pacientes diagnosticados e tratados precocemente podem apresentar níveis plasmáticos de Phe acima do normal, o que está correlacionado com a piora nas funções cognitivas desses pacientes (Schulpis et al., 2004; Gassió et al., 2008; Lindegren et al., 2012). Por outro lado, a dieta restritiva para o tratamento da PKU, que também altera o perfil antioxidante dos pacientes (Artuch et al., 2004; Schulpis et al., 2005), se cumprida rigorosamente, pode impedir os

prejuízos neurológicos que são reversíveis (Schulpis et al., 2011). Estratégias antioxidantes como tratamento para a HPA vêm sendo estudadas (Mazzola et al., 2013). Além disso, trabalhos mostraram que alguns biomarcadores de EO estão alterados tanto a curto quanto a um longo período de exposição à Phe no plasma (Kienzle-Hagen et al., 2002; Sitta et al., 2009c), possivelmente indicando que o EO nestas condições de HPA não parece ter uma característica adaptativa (Ristow e Zarse 2010).

Em 2010, um estudo do nosso grupo de pesquisa indicou o efeito preventivo do AL contra o EO observado em cérebro de ratos submetidos a elevadas concentrações *in vitro* de Phe, ou HPA induzido em experimento agudo a partir de injeção do inibidor da PAH (Moraes et al., 2010). Com base no trabalho citado acima, foi feita a avaliação de um possível efeito crônico do AL no tratamento do EO em cérebros de ratos jovens induzidos à HPA a partir de sucessivas administrações diárias de Phe e do inibidor da PAH imitando uma exposição repetitiva a altos níveis de Phe. Sendo assim, investigações contemplando o estudo do EO em modelos de HPA utilizando substâncias antioxidantes podem ser ferramentas importantes para elucidar melhores formas de tratamento adicionais na doença. Neste sentido, o presente trabalho pode contribuir para avaliar como o EO em condições de HPA se comporta frente a um antioxidante com amplo espectro de ação. Na sequência um resumo dos resultados obtidos está apresentado na forma de duas figuras nesta discussão (Figuras 3 e 4).

Os resultados apresentados, no primeiro capítulo desta tese e obtidos com o modelo de experimentação animal induzido quimicamente a partir de sucessivas injeções diárias de Phe e α-MePhe, demonstraram alterações na atividade de duas importantes enzimas antioxidantes: CAT e SOD. Os dados encontrados de diminuição na atividade da CAT e aumento da SOD induzidas pelo modelo de HPA corroboram outros trabalhos nos quais foi

observado o mesmo padrão de resultado para essas enzimas em cérebro de ratos com modelo crônico de HPA (Kienzle-Hagen et al., 2002; Mazzola et al., 2011). Além disso, esse perfil de atividade enzimática da CAT e SOD também foi descrito em pacientes com PKU (Gassió et al., 2008; Sanayama et al., 2011).
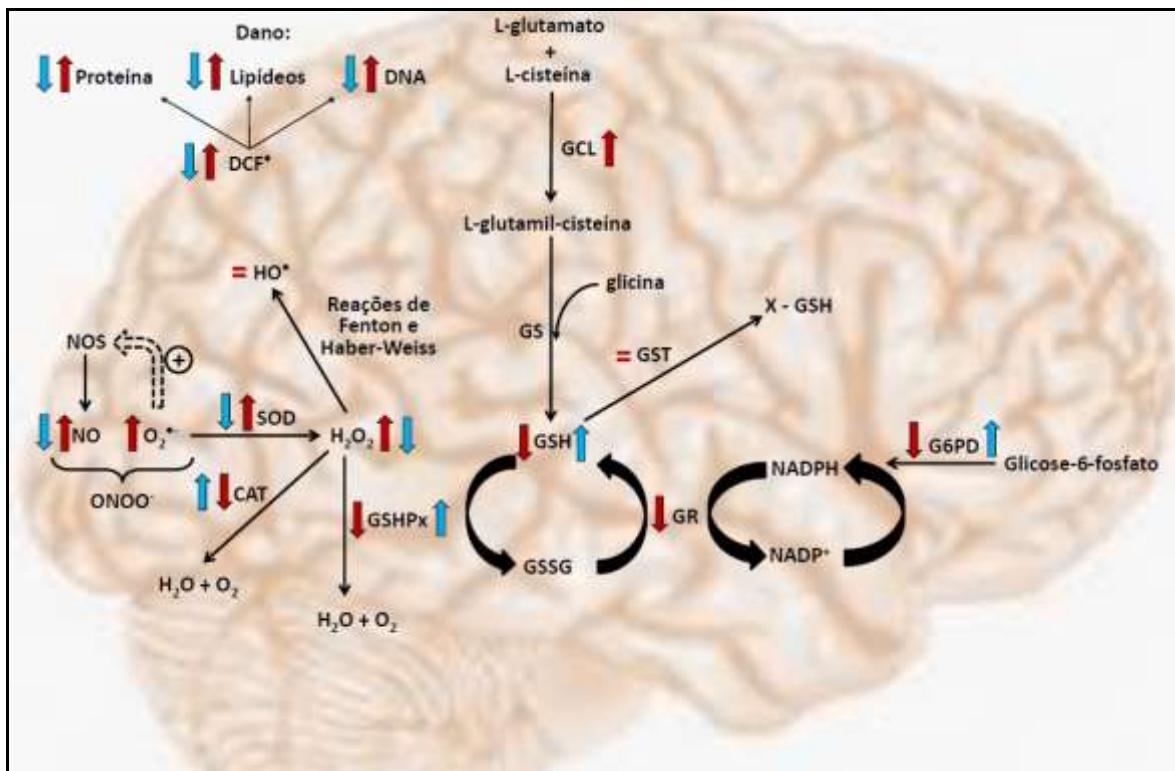
Figura 3. Resumo dos resultados observados no cérebro dos animais em relação aos tratamentos de hiperfenilalaninemia com ou sem ácido lipoico. Setas indicam aumento ou diminuição. = indica que não houve diferença dos grupos experimentais em relação ao controle. Em vermelho (efeito do HPA) e em azul (efeito do HPA+AL quando houve prevenção antioxidante).

A CAT e a SOD são enzimas responsáveis pela dismutação do H_2O_2 e do $O_2^{\bullet-}$, respectivamente, no meio celular. A CAT está presente no interior dos peroxissomos (exceto em eritrócitos) e a atividade de CAT no cérebro é de aproximadamente 15 U/mg de proteína. Já a SOD apresenta variações como CuZnSOD, que está localizada em sua maior

parte no citosol, e a MnSOD, maior parte presente na mitocôndria. O nível de SOD (total) cerebral é em torno de 4 µg/mg de proteína. Uma vez que a SOD degrada o $O_2^{\cdot-}$ em H_2O_2 e a CAT converte H_2O_2 à água, a consequência do aumento da taxa de SOD / CAT pode ser a maior disponibilidade de H_2O_2 no meio. Isso pode acarretar na formação de outras moléculas oxidantes ainda mais potentes, como o radical hidroxil ($^{\cdot}OH$), através da reação Fenton e de Haber-Weiss exibida na figura 3 desta discussão (Halliwell e Gutteridge 2007). Esse acúmulo de H_2O_2 foi confirmado nos achados do segundo capítulo, nos quais os resultados demonstraram maior produção de H_2O_2 justamente nos grupos que apresentaram aumento da razão SOD/CAT.

Outra via de eliminação do H_2O_2 é pela ação das peroxidases usando o H_2O_2 para oxidar outros substratos (SH_2), como a GSH-Px, que é uma enzima dependente de selênio, cuja atividade encontra-se diminuída em pacientes com PKU, provavelmente devido à deficiência de selênio desses indivíduos (van Backel et al., 2000; Ercal et al., 2002; Gassió et al., 2008). No último capítulo deste trabalho foi mostrado que os animais com HPA também apresentaram uma inibição na atividade da GSH-Px. Sendo assim, é possível especular que a HPA conduz a uma acumulação de H_2O_2 no cérebro dos animais e que as enzimas antioxidantes desempenham um importante papel do estado redox na doença, sendo que esse desequilíbrio no estado redox no cérebro de ratos em favor de pró-oxidantes pode ser devido ao aumento da atividade da SOD sem a compensação necessária pela CAT e GSH-Px observada nos grupos AL e HPA.

Contudo, em homogeneizados de cérebro de ratos jovens a Phe alterou somente a atividade da CAT, com inibição já em baixas concentrações (0,5 mM) (Kienzle-Hagen et al., 2002). Nosso trabalho mostrou que a Phe inibe a CAT purificada a partir de preparados comerciais extraídos de fígado bovino, e que os metabólitos PLA e PAA aumentam a

atividade da SOD purificada a partir de preparados comerciais extraídos de eritrócitos bovinos. Estes resultados em conjunto sugerem que o aumento observado na atividade da SOD no grupo de animais submetidos ao modelo HPA é provavelmente devido à ação direta dos metabólitos (PLA e PAA) formados a partir do acúmulo de Phe, em ratos, por uma via alternativa de metabolização (Scriver e Kaufman, 2001). No entanto, a hipótese de adaptação contra o EO, e consequente aumento na atividade da SOD, não pode ser totalmente descartada já que Moraes et al. (2010) mostraram uma diminuição na atividade desta enzima em ratos submetidos a tratamento HPA agudo (uma única administração de Phe e α-MePhe). Portanto, mais estudos são necessários para avaliar tanto a expressão quanto a quantidade dessas enzimas no cérebro de ratos submetidos a modelos de HPA.

Segundo Sies (1991), o EO é definido como um desequilíbrio entre pró-oxidantes e antioxidantes em favor dos pró-oxidantes, podendo levar a um dano. Este prejuízo é considerado como o dano a biomoléculas causado pela ação de ER em organismos vivos, não sendo necessariamente um dano oxidativo (Halliwell e Gutteridge 2007). Para avaliar a lesão celular usamos marcadores em biomoléculas como lipídeos (TBA-RS), proteína (Carbonilas proteicas) e DNA (DNA-*Protein crosslink*). Esses parâmetros se apresentaram aumentados no grupo HPA e o efeito foi prevenido pela a ação antioxidante do AL. Esses dados estão de acordo com estudos em pacientes com PKU que demonstrou alterações significativas em marcadores de dano a biomoléculas como lipídeos, proteína e DNA (Sitta et al., 2009a; Sanayama et al., 2011; Vargas et al., 2011).

Sabendo que o excesso na produção de ER, incluindo radicais livres, pode produzir dano a biomoléculas, e que o método DCF utilizado no primeiro detecta muitas ER, tais como H_2O_2, $^{\bullet}OH$, $ONOO^-$ e os radicais peroxil ($ROO^{\bullet}$), sem distingui-los (Halliwell e

Whiteman, 2004), o segundo trabalho realizado para identificar quais as ER específicas estariam envolvidos no EO relacionado com HPA.

Os resultados discutidos anteriormente nos levaram a esperar que o H_2O_2 estaria aumentado nos grupos HPA e AL, o que de fato foi confirmado, porém, observamos também um aumento de $O_2^{\bullet-}$ e de $NO^{\bullet}$ no grupo HPA. O $O_2^{\bullet-}$ é considerado a primeira ER formada na célula sendo um precursor do H_2O_2 e, de $^{\bullet}OH$ e lipoperoxil ($LOO^{\bullet}$) (Halliwell e Gutteridge, 2007). Esta produção de $O_2^{\bullet-}$ também pode ser proveniente da alteração do metabolismo mitocondrial já observado em modelos com acúmulo de Phe (Rech et al., 2002). Segundo Lu et al. (2011), a NADPH oxidase possui um papel essencial na geração de $O_2^{\bullet-}$ e, consequentemente, do EO observado em pacientes com PKU. Apesar de não estar claro o mecanismo de ativação da NADPH oxidase na PKU, esse aumento de sua atividade poderia levar ao acúmulo de $O_2^{\bullet-}$ observado neste estudo.

Sob condições inflamatórias ou de EO o $O_2^{\bullet-}$ induz a liberação de $NO^{\bullet}$ a partir da estimulação da atividade da enzima óxido nítrico sintase (NOS) (Manning et al., 2001). Nossos experimentos mostraram um aumento de $NO^{\bullet}$ no grupo HPA e nos grupos com tratamento antioxidante. O $NO^{\bullet}$ é um radical e segundo mensageiro, tendo um importante papel na regulação da função neuronal e transmissão sináptica (Prast e Philippu, 2001). Além disso, o $NO^{\bullet}$, quando em baixas concentrações, tem ação antioxidante removendo diretamente algumas ER (Wink et al., 2001). Por outro lado, o $NO^{\bullet}$ em contato com o $O_2^{\bullet-}$ pode gerar $ONOO^-$, que é uma ERN altamente reativa que causa nitrosilação de aminoácidos e consequente inibição de proteínas causando, por exemplo, deficiência enzimática (Zielonka et al., 2010).

No primeiro trabalho também encontramos uma correlação positiva entre a geração de ER (DCF) e lipoperoxidação (TBA-RS), indicando que a lipoperoxidação (LPO)

induzida pelo modelo de HPA está associada com o aumento de ER. A lipoperoxidação pode ser iniciada a partir de três mecanismos: (1) a oxidação mediada por radicais livres; (2) radical livre - independente, a oxidação não enzimática, e (3) a oxidação enzimática (Niki et al., 2005). O primeiro mecanismo prossegue com iniciação da oxidação de moléculas lipídicas por radicais livres em reações em cadeia. Os radicais $^\bullet$OH têm a taxa mais elevada de abstração para um átomo de hidrogênio do grupo metileno dos lipídeos. Além disso, experimentos com homogeneizados cerebrais demonstraram o TBA-RS gerado diretamente pela acumulação de Phe (5 mM), pode ser pela ação dos radicais $^\bullet$OH e ROO$^\bullet$ (Fernandes et al., 2010).

Porém, um resultado interessante observado foi com relação à concentração de $^\bullet$OH no cérebro dos animais HPA, a qual se manteve igual aos animais do grupo controle. Mesmo considerando que o protocolo metodológico usado para medir $^\bullet$OH é um método indireto, os resultados nos levam a sugerir que a HPA não aumentou $^\bullet$OH em ratos córtex cerebral e que o dano lipídico observado no primeiro capítulo pode ser devido a uma possível ação de radicais ROO$^\bullet$ formada por outras ER de cadeia de iniciação. Embora não observemos uma diferença significante entre os grupos de animais quanto $^\bullet$OH, não podemos descartar a possibilidade de um aumento de geração de $^\bullet$OH no início ou durante os procedimentos com injeções. Por outro lado, de acordo com os nossos resultados, $O_2^{\bullet-}$ e NO$^\bullet$ estavam aumentados no grupo HPA. Nenhuma dessas ER é suficientemente ativa para iniciar a LPO diretamente, mas pode produzir rapidamente o ONOO$^-$ o qual pode dar início a reações em cadeia de LPO (Pacher et al., 2007). Assim, tanto $O_2^{\bullet-}$ e NO$^\bullet$ aumentados devido ao acúmulo de Phe nos animais do grupo HPA pode ser um indicativo de que estas ER podem estar envolvidas no aumento da LPO relatado na doença.
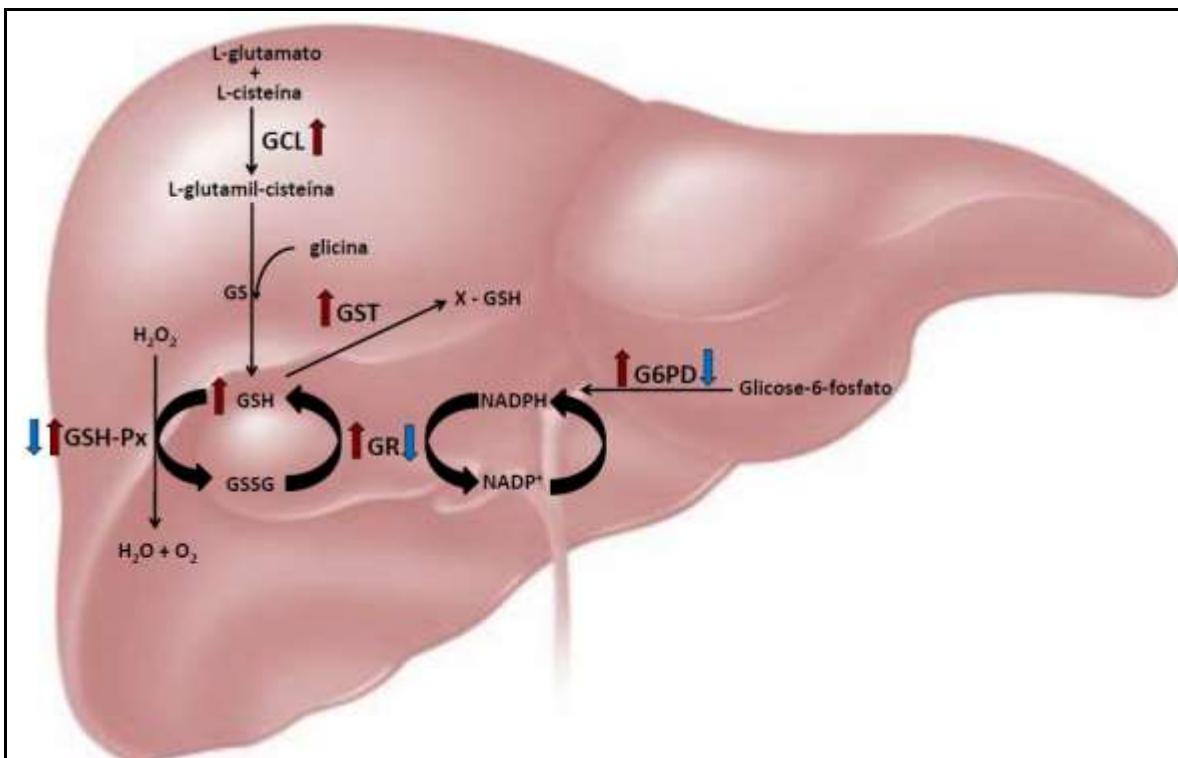
Figura 4. Resumo dos resultados observados no fígado dos animais em relação aos tratamentos de hiperfenilalaninemia com ou sem ácido lipoico. Setas indicam aumento ou diminuição. = indica que não houve diferença dos grupos experimentais em relação ao controle. Em vermelho (efeito do HPA) e em azul (efeito do HPA+AL quando houve prevenção antioxidante).

No terceiro capítulo da tese, para avaliação do perfil do metabolismo da GSH, foram analisadas as atividades de algumas importantes enzimas envolvidas na sua utilização, regeneração ou síntese, tanto em amostras de cérebro quanto em fígado dos animais. A GSH é um tiol não proteico importante na proteção contra o EO e sua regulação e síntese pode ser restringida pelo transporte de aminoácidos tioicos na BHE (Valdovinos-Flores e Gosenbatt, 2012). O fígado possui um papel fundamental para a homeostase de GSH entre diferentes órgãos (Ookhtens and Kaplowitz, 1998) e por isso foi um dos alvos de estudo no último capítulo, mesmo não sendo um órgão afetado na doença.

Inicialmente, observou-se que as atividades de GSH-Px, GR e G6PD foram inibidas no cérebro de animais do grupo HPA. GSH-Px catalisa a reação de GSH com hidroperóxidos e estudos recentes em eritrócitos de pacientes com PKU e modelo animal HPA demonstraram uma inibição da atividade da GSH-Px (Kienzle-Hagen et al., 2002; Sirtori et al., 2005; Sitta et al., 2006), e ainda, que esta inibição está correlacionada com as baixas concentrações plasmáticas de selênio em pacientes com PKU (Sitta et al., 2009b). Além disso, há evidencias quanto a alterações na atividade da GSH-Px e conteúdo de GSH em pacientes com PKU e o EO pode estar associado à neuropatologia da doença (Sanayama et al., 2011; Sitta et al., 2009c). G6PD fornece NADPH necessário para a atividade de GR para reduzir GSSG em GSH (Brigelius-Flohe, 1999). Assim, a inibição da atividade de G6PD e GR pode prejudicar a reciclagem de GSH a partir de GSSG, tornando o tecido cerebral em um meio mais oxidante. Por outro lado, Ercal e colaboradores (2002) mostraram um aumento de atividade de G6PD em células sanguíneas de BTBR-Pah[(enu2)] e uma diminuição concomitante dos níveis de GSH no sangue e cérebro destes camundongos.

Com já mencionado, a conversão de GSSG a GSH é realizada pela enzima GR utilizando NADPH como doador de elétrons, que é gerado principalmente pela atividade de G6PD. Assim, os níveis de GSH são mais elevados do que a GSSG em condições fisiológicas. Neste estudo, o grupo de AL apresentou um aumento significativo da GSH, e sabe-se que a administração de AL é capaz de aumentar os níveis de GSH intracelular até cerca de 70% (Packer et al., 1997). Por conseguinte, os elevados níveis de GSH encontrados nos animais do grupo AL podem promover uma inibição da atividade de GR e, eventualmente, acumulando NADPH que, por sua vez, também pode regular a atividade da G6PD (Chung et al., 1991; Singh et al., 2012).

A atividade da GCL também foi avaliada no cérebro dos animais e um aumento significativo foi observado no grupo de HPA. Este resultado sugere que, possivelmente, possa haver um mecanismo de *feedback* na tentativa de manter os níveis de GSH intracelular, esta indução de GCL pode representar o principal meio de manter os níveis cerebrais de GSH, já que GR foi inibida no cérebro desses animais (Aoyama et al., 2008; Lu, 2009). Já a enzima GST catalisa a conjugação de compostos contendo carbono e nitrogênio eletrofílico com GSH, contribuindo para a eliminação celular de substâncias tóxicas (Wojtkowiak-Giera et al., 2011). No entanto, não houve nenhuma alteração na atividade de GST em qualquer grupo de animais quando em comparação com o controle e, possivelmente, o mecanismo pelo qual o EO se manifesta no cérebro de animais com HPA não é através de reações do tipo fase II de desintoxicação (Townsend et al., 2008). Reforçando este achado, Artuch e outros pesquisadores (2004) também descreveram não haver nenhuma diferença na atividade de GST em eritrócitos de pacientes com PKU.

O fígado é um órgão importante de desintoxicação com altos níveis de antioxidantes, especialmente GSH, servindo também de reservatório de cisteína para o organismo (Reed et al., 2008). Apesar de a PAH ser uma enzima hepática, o fígado não é um órgão afetado na PKU, a maioria dos sintomas dos pacientes acomete o SNC. Porém, o objetivo foi avaliar se a condição de HPA, que sabidamente afeta o tecido cerebral, teria algum efeito no fígado sobre parâmetros do metabolismo da GSH. Sendo assim, analisamos a quantidade deste tripeptídeo no fígado, bem como a atividade das enzimas envolvidas em sua síntese, regeneração e utilização como resposta antioxidante. Os resultados observados nas amostras de fígado dos animais HPA mostraram um aumento da atividade de todas as enzimas analisadas que estão envolvidas no metabolismo de GSH, além de uma elevação no conteúdo total de GSH. Esses dados indicam uma possível participação efetiva deste

órgão na desintoxicação de ER e na manutenção da homeostase redox com a utilização da GSH como um antioxidante contra o EO em HPA. Com efeito, o fígado desempenha um papel importante na homeostase corporal do GSH, pois é o principal órgão que libera GSH no sangue (Kretzschmar, 1996; Ookhtens e Kaplowitz, 1998; Lu, 1999). Além disso, as alterações na homeostase GSH estão envolvidas na fisiopatologia de doenças neurodegenerativas e sabe-se que a GSH chega ao cérebro através de transportadores específicos da BHE (Favilli et al., 1997; Johnson et al., 2012; Valdovinos-Flores e Gosenbatt, 2012), embora esses mecanismos específicos de transporte ainda não são completamente compreendidos. Além disso, o reservatório de GSH do fígado é uma fonte de cisteína, que é um aminoácido limitante para a síntese de novas moléculas de GSH e pode ultrapassar essa barreira através dos mesmos transportadores de aminoácidos (Tateishi et al., 1977). Assim, é possível que o fígado possa contribuir para a manutenção dos níveis de GSH no cérebro de ratos sujeitos a tratamento crônico de HPA. Contudo, mais estudos são necessários para entender melhor essa hipótese.

A respeito da ação do tratamento com o antioxidante obtivemos alguns resultados interessantes. Os animais hiperfenilalaninêmicos que receberam o tratamento com AL apresentaram capacidade de prevenir as alterações na atividade das enzimas CAT e SOD. Isto mostra que a ação antioxidante do AL é eficaz na manutenção da atividade dessas enzimas evitando o acúmulo de H_2O_2 provocado pela HPA nesta situação observada em cérebro de ratos jovens. Além disso, o aumento da atividade de SOD, verificado no grupo de animais submetidos somente ao AL, está de acordo com investigações de outros pesquisadores que utilizam um tratamento semelhante de AL em ratos (Kim et al., 2011; Perera et al., 2011). É interessante destacar que o AL pode neutralizar o EO por indução da expressão do gene da SOD a partir da regulação do fator de crescimento do nervo (NGF –

do inglês, *nerve growth factor*) (Garrett et al., 1997; Nistico et al., 1992). Esse efeito indutor de expressão de uma enzima antioxidante pode estar ocorrendo nesses animais que receberam injeções diárias de AL.

Ainda referente ao primeiro trabalho descrito, o tratamento com AL foi capaz de prevenir o dano encontrado no cérebro dos animais com HPA. Estes resultados corroboram estudos anteriores que mostraram, em modelo agudo e experimentos *in vitro* com homogeneizados de cérebro de animais incubados com Phe (Moraes et al., 2010), que o AL foi eficaz para impedir a peroxidação lipídica provocada pela HPA. Além disso, o AL preveniu dano proteico induzido pela toxicidade do ácido N-acetilaspártico, acumulado na doença de Canavan (Pederzolli et al., 2010). A HPA alterou todos os marcadores de danos às biomoléculas estudados, lipídeos, proteínas e DNA, no cérebro dos animais e todos esses efeitos foram inibidos pelo AL, possivelmente a partir de capacidade *scavenger* deste antioxidante, induzindo formação e ação de outras substâncias antioxidantes, bem como algumas enzimas. Entretanto, referente à capacidade de ação nas ER estudadas no segundo capítulo (H_2O_2; $NO^{\bullet}$; $O_2^{\bullet-}$ e $^{\bullet}OH$), foi possível identificar uma melhora do tratamento com AL somente em relação à total eliminação de H_2O_2 acumulado no modelo HPA e parcialmente do $NO^{\bullet}$. Sendo assim, é possível que a ação do AL seja indireta através de indução de outros sistemas de defesa antioxidante, como sugerido anteriormente.

Com relação à interação do AL no metabolismo da GSH, foi verificado que o tratamento com este antioxidante preveniu algumas alterações no cérebro e fígado dos animais, provocadas pela HPA. No cérebro, o AL preveniu a inibição da GSH-Px e a diminuição do conteúdo de GSH total. Já no fígado a prevenção foi mais evidente, mantendo a atividade das enzimas GSH-Px, GR e G6PD em níveis semelhantes do controle. É importante notar que o AL *per se* também altera a atividade dessas enzimas,

porém quando em uma condição de HPA, que também altera a homeostase redox do meio, ele age de forma a atenuar os efeitos gerados pelo acúmulo de Phe. Tem sido proposto que o AL induz expressão de Nrf2 via ARE (do inglês – *antioxidant response element*) e, consequentemente, aumenta a atividade da síntese de GSH e da GCL, além de outras enzimas antioxidantes (Suh et al., 2004; Wagner et al., 2012). O Nrf2 regula a expressão de enzimas antioxidantes como resposta ao EO e o aumento da concentração de GSH está acompanhado pelo aumento Nrf2, que regula os níveis de GCL e expressão do gene que codifica para a GST (Satoh et al., 2013). Além disso, é importante salientar que o potencial de redução (E^0) do ADHL é de -0,32V, sendo um potente agente redutor capaz de reduzir GSSG a GSH. Entre 25% a 45% do AL é convertido a ADHL pela lipoamida desidrogenase, com gasto de NADPH ou NADH, ou também pelas enzimas glutationa e tioredoxina redutases (Packer et al., 1997; Halliwell e Gutteridge, 2007). Sendo assim, é possível que parte do AL injetado seja convertida em ADHL e que as alterações nos níveis de GSH podem estar relacionadas a esta característica do ADHL.

Tendo em vista que muitos estudos e pesquisas vêm sendo realizado na tentativa de propor alternativas ao tratamento da PKU incluindo uso de antioxidantes, este trabalho cumpre seu papel como pesquisa básica a fim de procurar responder algumas perguntas importantes acerca do uso do AL como antioxidante em HPA, que são: Como o AL age? Seria eliminando ER diretamente ou induzindo sistemas de defesa? Quais sistemas de defesa são mais prontamente induzidos pelo AL? Quais biomoléculas o AL protege contra dano? Essas biomoléculas são alvos importantes na HPA? Claro que além dessas perguntas outras surgem a partir de agora como, por exemplo, qual a ação do AL quanto ao metabolismo energético celular, já que alguns antioxidantes já utilizados na clínica também possuem um papel fundamental quanto a este aspecto. Outra questão que se deve levar em

conta é relativa à ação pró-oxidante dessas substâncias, no caso de antioxidantes tioicos há possibilidade de formação de radicais derivados do grupamento sulfidril.

Porém, mesmo procurando ter a cautela necessária para abordar a proposta de uso de antioxidantes no tratamento da HPA, este trabalho mostra que o AL pode ser eficaz para normalizar o estado redox alterado pelo acúmulo de Phe e/ou seus metabólitos. Se estes resultados forem confirmados em pacientes, antioxidantes como o AL pode ser uma terapia adjuvante no tratamento da PKU.

CONCLUSÕES

- O modelo crônico de HPA provocou dano a lipídeos, proteína e DNA no cérebro dos animais além de alterar a razão SOD/CAT promovendo aumento de ER.

- O AL foi capaz de prevenir todos esses efeitos provocados pela HPA.

- As enzimas CAT e SOD parecem ter um papel importante no estresse oxidativo envolvido na HPA, já que suas atividades são alteradas pela Phe e seus metabólitos.

- O acúmulo de Phe no modelo aumentou H_2O_2, $O_2^{\cdot-}$ e $NO^{\cdot}$, enquanto que o tratamento com AL foi capaz de impedir o aumento de H_2O_2 e $NO^{\cdot}$ no cérebro dos animais.

- As atividades das enzimas envolvidas no metabolismo da GSH foram alteradas tanto em cérebro quanto no fígado dos animais pelo modelo crônico de HPA, parecendo haver um mecanismo compensatório para manter os níveis de GSH no cérebro com aumento da atividade de GCL e das enzimas hepáticas.

- O AL normalizou os níveis de GSH no cérebro dos animais que foram diminuídos pelo modelo HPA.

REFERÊNCIAS

Aseervatham G.S., Sivasudha T., Jeyadevi R., Arul Ananth D. (2013). Environmental factors and unhealthy lifestyle influence oxidative stress in humans – an overview. Environ Sci Pollut Res Int 20(7): 4356-4369.

Aoyama K., Watabe M., Nakaki T. (2008). Regulation of Neuronal Glutathione Synthesis. J Pharmacol Sci 108, 227-238.

Artuch R., Colomé C., Sierra C., Brandi N., Lambruschini J.C., Urgate D., Vilaseca M.A. (2004). A longitudinal study of antioxidant status in phenylketonuric patients. Clin Bioch 37, 198-203.

Baric I., Fumic K., Hoffmann G.F. (2001). Inborn errors of metabolism at the turn of the millennium. Croat Med J 42: 379-383.

Bast A. (1993). Oxidative stress and calcium homeostasis. In: Halliwell B, Aruoma OI, eds. DNA and free radicals. Chichester: Ellis Horwood 95-108.

Beadle G.W., Tatum E.L. (1941). Genetic Control of Biochemical Reactions in Neurospora. PNAS 27 (11): 499–506. DOI:10.1073/pnas.27.11.499

Brigelius-Flohe´ R. (1999). Tissue-specific functions of individual glutathione peroxidases. Free Radic Biol Med 27:951-965.

Bertini E., Federici G., Pastore A., Piemonte F. (2003). Analysis of glutathione: implication in redox and detoxification. Elsevier, Clinica Chimica Acta. (333):19-36.

Blass J.P. (1983). Inborn errors of pyruvate metabolism. In: Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein J.L., Brown M.S. (Ed.) The metabolic basis of inherited disease. New York: Mc Graw Hill 193-203.

Bonnefoy M., Drai J., Kostka T. (2002). Antioxidants to slow aging, facts and perspectives. Press Med 31(25): 1174-84.

Boveris A., Chance B. (1973). The mitocondrial generation of hydrogen peroxide. Biochem J 134: 707-716.

Callegari-Jacques S.I. Bioestatística – Princípios e Aplicações (2003). 1ª Ed. Porto Alegre. Ed Artmed 146-152.

Channon S., German E., Cassina C., Lee P. (2004). Executive functioning, memory, and learning in phenylketonuria. Neuropsy 18: 613-620.

Chung P.M., Cappel R.E., Gilbert H.F. (1991). Inhibition of glutathione disulfide reductase by glutathione. Arch Biochem Biophys 288(1): 48-53.

Clague A., Thomas A. (2002). Neonatal biochemical screening for disease. Clin Chim Acta 315: 99-110.

Clark J.B., Bates T.E., Cullingford T., Land J. M. (1993). Development of enzymes of energy metabolism in neonatal mammalian brain. Dev Neurosci 15: 174-180.

Delanty N., Dichter M.A. (1998). Oxidative injury in the nervous system. Acta Neurol Scand 98: 145-153.

Dringen, R. (1999). Metabolism and functions of glutathione in brain. Progress in Neurobiol 62: 649-669.

Ercal N., Aykin-Burns N., Gurer-Orhan H., Mcdonald J.D. (2002). Oxidative stress in a phenylketonuria animal model. Free Rad Biol Med 32: 906-911.

Favilli F., Marraccini P., Iantomasi T., Vincenzini M.T. (1997). Effect of orally administered glutathione on glutathione levels in some organs of rats: role of specific transporters. Br J Nutr 78: 293-300.

Fernandes C.G., Leipnitz G., Seminotti B., Amaral A.U., Zanatta A., Vargas C.R., Dutra Filho C.S., Wajner M. (2010). Experimental evidence that phenylalanine provokes oxidative stress in hippocampus and cerebral cortex of developing rats. Cell Mol Neurobiol 30:317–326.

Forman H.J., Rinna A., Zhang H. (2009). Glutathione: Overview of its protective roles, measurement, and biosynthesis. Mol Aspects Med. 30:1-12.

Franklin C.C. (2010). Enhanced glutathione: biosynthetic capacity promotes resistence to AS3+- induced apoptosis. Toxicol Let. 193(1): 33-40.

Fuchs J., Schofer H., Milbradt R., Freisleben H.J., Buhl R., Siems W., Grune T. (1993). Studies on lipoate effects on blood redox state in human immunodeficiency virus infected patients. Arzneimit Forsch 43: 1359–1362.

Garrett N.E., Malcangio M., Dewhurst M., Tomlinson D.R. (1997). α-Lipoic acid corrects neuropeptide deficits in diabetic rats via induction of trophic support. Neurosci Lett 222:191-194.

Gassió R., Artuch R., Vilaseca M.A., Fusté E., Colome R., Campistol J. (2008). Cognitive Functions and the Antioxidant System in Phenylketonuric Patients. Neuropsyc 22 (4): 426–431.

Ghibu S., Lauzier B., Delemasure S., Amoureux S., Sicard P., Vergely C., Muresan A., Mogosan C., Rochette L. (2009). Antioxidant pro Antioxidant properties of alpha-lipoic acid: effects on red blood membrane permeability and adaptation of isolated rat heart to reversible ischemia. Mol Cell Biochem 320(1-2):141-8.

Gorąca A., Huk-Kolega H., Piechota A., Kleniewska P., Ciejka E., Skibska B. (2011). Lipoic acid – biological activity and therapeutic potential. Pharmacol Rep 63:849-858.

Greengard O., Yoss M.S., Delvalle J.A. (1976). α-Methylphenylalanine, a new inducer of chronic hyperphenylalaninemia in suckling rats. Sci 192: 1007-1008.

Hagen T.M., Ingersoll R.T., Lykkesfeldt J., Liu J., Wehr C.M., VinarskyL V., Bartholomew J.C., Ames B.N. (1999). (R)-α-lipoic acid-supplemented old rats have improved mitochondrial function, decreased oxidative damage, and increased metabolic rate. Faseb J 13: 411-418.

Huijbregts S.C., de Sonneville L.M., Licht R., van Spronsen F.J., Verkerk P.H., Sergeant J.A. (2002). Sustained attention and inhibition of cognitive interference in treated phenylketonuria: associations with concurrent and lifetime phenylalanine concentrations. Neuropsy 40: 7-15.

Halliwell B. (1994). Free radicals, antioxidants and human disease: curiosity, cause or consequence? The Lancet 344: 721-724.

Halliwell B. (2001). Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 18: 685-716.

Halliwell B. (2006). Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life. Plant Physiol 141:312-322.

Halliwell B., Gutteridge J.M.C. (2007). Free Radicals in Biology and Medicine. 4º edição. New York: Oxford University Press.

Hanley W.B. (2004) Adult Phenylketonuria. Am J Med 117: 590-595.

Hoeksma M., Reijngoud D.J., Pruim J., de Valk H.W., Paans A.M., van Spronsen F.J. (2009). Phenylketonuria: High plasma phenylalanine decreases cerebral protein synthesis. Mol Genet Metab 96(4): 177-182.

Hoffmann G.F. (1984). Selective screening for inborn errors of metabolism: past, present and future. Eur J Pediatr 153: 2-8.

Hommes F.A., Polman H.A., Patrick A.D. (1968). Leigh's encephalomyelopathy: An inborn error of gluconeogenesis. Arch Dis Childhood 43: 423-426.

Hommes F.A., Eller R.G. and Taylor E.H. (1982). The effects of phenylalanine on myelin metabolism in adolescent rats. In: Cockburn F., Gitzelmann R. (editores). Inborn Errors of Metabolism in Human: MTP Press, Lancaster, 193-199.

Johnson W.M., Wilson-Delfosse A.L., Mieyal J.J. (2012). Dysregulation of Glutathione Homeostasis in Neurodegenerative Diseases. Nutrients 4: 1399-1440.

Kaufman S. (1989). An evaluation of the possible neurotoxicity of metabolities of phenylalanine. J Pediatr 114(5):895-900.

Kienzle-Hagen M.E., Pederzolli C.D., Sgaravatti A.M., Bridi R., Wajner M., Wannmacher C.M., Wyse A.T., Dutra-Filho C.S. (2002). Experimental hyperphenylalaninemia provokes oxidative stress in rat brain. Biochem Biophys Acta 1586: 344-352.

Killian D.M., Chikhale P.J. (2001). Predominant functional activity of the large, neutral amino acid transporter (LAT 1) isoform at the cerebrovascularture. Neurosci Lett 306(1-2): 1-4

Kim M.Y., Kim E.J., Kim Y., Choi C., Lee B. (2011). Effects of α-lipoic acid and L-carnosine supplementation on antioxidant activities and lipid profiles in rats. Nutrition Research and Practice Nutr Res Pract 5(5):421-428.

Knox W.E. (1972). Phenylketonuria. In Stanbury J.B., Wyngaarden J.B., Fredrickson D.S. The Metabolic Basis of Inherited Disease. MacGraw-Hill, New York.

Kohli S., Saxena R., Thomas E., Rao P. and Verma I.C. (2005). Prenatal diagnosis of phenylketonuria. Indian. J Med Res 122: 400-403.

Kretzschmar M. (1996). Regulation of hepatic glutathione metabolism and its role in hepatotoxicity. Exp Toxic Pathol 48: 439-446.

Lehninger A. L., NELSON D. L., COX, M. M. (1993) Principles of biochemistry. New York, Worth Publishers, 1993. pp 530 - 531.

Le Masurier M., Oldenzeil W., Lehman C., Cowen P., Sharp T. (2006). Effect of acute tyrosine depletion in using a branched chain aminoacids mixture on dopamine neurotransmission in the rat brain. Neuropsychopharmacol 31(2):310-317

Lindegren M.L., Krishnaswami S., Fonnesbeck C., Reimschisel T., Fisher J., Jackson K., Shields T., Sathe N.A., McPheeters ML (2012).

Lu S.C. (1999). Regulation of hepatic glutathione synthesis: current concepts and controversies. The FASEB Journal 13(10): 1169-1183.

Lu S.C. (2009). Regulation of glutathione synthesis. Mol Aspects Med30: 42-59.

Lu L., Gu X., Li D., Liang L., Zhao Z., Gao J. (2011). Mechanisms regulating superoxide generation in experimental models of phenylketonuria: An essential role of NADPH oxidase. Mol Gent and Metabol 104: 241-248.

Manning P., Cookson M.R., McNeil C.J., Figlewicz D., Shaw P.J. (2001). Superoxide-induced nitric oxide release from cultured glial cells. Brain Res 911:203-210.

Martinez-Cruz F., Pozo D., Osuna C., Espinar A., Marchante C., Guerrero J.M. (2002). Oxidative stress induced by phenylketonuria in the rat: prevention by melatonin, vitamin E and Vitamin C. J Neurosci Res 69: 550-558.

Matsugo S., Bito T., Konishi T. (2011). Photochemical stability of lipoic acid and its impact on skin ageing. Free Radic Res. 45(8):918-24.

Maxwell S.R. (1995). Prospects for the use of antioxidant therapies. Drugs 49: 345-361.

Mazzola P.N., Terra M., Rosa A.P., Mescka C.P., Moraes T.B., Piccoli B., Jacques C.E., Dalazen G.R., Cortes M.X., Coelho J.C., Dutra-Filho C.S. (2011). Regular exercise prevents oxidative stress in the brain of hyperphenylalaninemic rats Metab Brain Dis 26:291–297.

Mazzola P.N., Karikas G.A., Schulpis K.H., Dutra-Filho C.S. (2013). Antioxidant treatment strategies for hyperphenylalaninemia. Metab Brain Dis DOI 10.1007/s11011-013-9414-2

McLain A.L., Szweda P.A., Szweda L.I. (2011). α-Ketoglutarate dehydrogenase: a mitochondrial redox sensor. Free Radic Res 45(1): 29-36.

Monteiro L.T.B. e Candido L.M.B. (2006). Fenilcetonuria no Brasil: evolução e casos. Rev Nutr Campinas 19(3):381-387.

Moraes T.B., Zanin F., da Rosa A., de Oliveira A., Coelho J., Petrillo F., Wajner M., Dutra-Filho C.S. (2010). Lipoic acid prevents oxidative stress in vitro and in vivo by an acute hyperphenylalaninemia chemically-induced in rat brain. J Neurol Sci 292:89-95.

Niki E., Yoshida Y., Saito Y., Noguchi N. (2005). Lipid peroxidation: mechanisms, inhibition, and biological effects. Biochem Biophys Res Commun 338: 668-676.

Nistico G., Ciriolo M.R., Fiskin K., Iannone M., De Martino A., Rotilio G. (1992). NGF restores decrease in catalase activity and increases superoxide dismutase and glutathione peroxidase activity in the brain of aged rats. Free Radical Biol Med 12: 177–181.

Nyhan W.l. (1984) Abnormalities in amino acid metabolism in clinical medicine. Appleton-century_crofts, Norwalk. 129-148.

Ookhtens O., Kaplowitz N. (1998). Role of the liver in intergorgan homeostasis of glutathione and cyst(e)ine. Semin Liver Dis 18: 313-329.

Packer L., Wiltf E.H., Tritschler H.J. (1995). Alpha-lipoic acid as a biological antioxidant. Free Rad Biol and Med 19 (2): 227-250.

Packer L., Tritschler H.J., Wessel K. (1997). Neuroprotection by the metabolic antioxidant α-lipoic acid. Free Rad Biol and Med 22, 359-378.

Pederzolli C.D., Rosa A.P., de Oliveira A.S., Coelho J.G., Becker D.L., Dalazen G.R., Moraes T.B. Dutra-Filho C.S. (2010). Neuroprotective role of lipoic acid against acute toxicity of N-acetylaspartic acid. Mol Cell Biochem 344:231-9.

Perera J., Tan J.H., Jeevathayaparan S., Chakravarthi S., Haleagrahara N. (2011). Neuroprotective Effects of Alpha Lipoic Acid on Haloperidol-Induced Oxidative Stress in the Rat Brain. Cell & Biosc 1:12.

Prast H., Philippu A. (2001). Nitric oxide as a modulator of neuronal function. Prog Neurobiol 64: 51-68.

Rech V.C., Feksa L.R., Dutra-Filho C.S., Wyse A.T., Wajner M., Wannmacher C.M. (2002). Inhibition of the mitochondrial respiratory chain by phenylalanine in rat cerebral cortex. Neurochem Res 27:353–357.

Reed M.C., Thomas R.L., Pavisic J., James S.J., Ulrich C.M., Nijhon H.F. (2008). A mathematical model of glutathione metabolism. Theor Biol and Med Model 5:8 doi:10.1186/1742-4682-5-8.

Ribas G.S., Sitta A., Wajner M., Vargas C.R. (2011). Oxidative stress in phenylketonuria: What is the evidence? Cell Mol Neurobiol 31(5): 653-62.

Ribas G.S., Biancini G.B., Mescka C.P., Wayhs C.Y., Sitta A., Wajner M., Vargas C.R. (2012). Oxidative Stress Parameters in Urine from Patients with Disorders of Propionate Metabolism: a Beneficial Effect of L-Carnitine Supplementation Cell Mol Neurobiol 32:77–82

Ristow M., Zarse K. (2010). How increased oxidative stress promotes longevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis). Exper Gerontol 45: 410–418.

Rocha J.C., Martins M.J. (2011). Oxidative stress in Phenylketonuria: future directions J Inherit Metab Dis DOI 10.1007/s10545-011-9417-2

Sanayama Y., Nagasaka H., Takayanagi M., Ohura T., Sakamoto O., Ito T., Ishige-Wada M., Usui H., Yoshino M., Ohtake A., Yorifuji T., Tsukahara H., Hirayama S., Miida T., Fukui M., Okano Y. (2011). Experimental evidence that phenylalanine is strongly associated to oxidative stress in adolescents and adults with phenylketonuria. Molecular Genetic and Metabol 103(3): 220-225.

Samuel S., Kathirvel R., Jayavelu T., Chinnakkannu P. (2005). Protein oxidative damage in arsenic induced rat brain: influence of DL-α-lipoic acid. Toxicol Lett 155, 27-34.

Satoh T., McKercher S.R., Lipton S.A. (2013). NrF2/ARE-mediated antioxidant actions of pro-electrophilic drugs. Free Radic Biol Med doi: 10.1016/j.freeradbiomed.2013.07.022

Saudubray J.M., Charpentier C. (2001). Clinical phenotypes: diagnosis/algorithms. In: Scirver C.R., Beaudet A.L., Sly W.S., Walle D. (editores). The Metabolic & Molecular Inherited Disease. 8ª ed. Volume II. New York: McGraw-Hill.

Schulpis K.H., Papassotiriou I., Vounatsou M., Karikas G.A., Tsakiris S., Chrousos G.P. (2004). Morning preprandial plasma ghrelin and catecholamine concentrations in patients with phenylketonuria and normal controls: evidence for catecholamine-mediated ghrelin regulation. J Clin Endocrinol Metab 89(8): 3983-3987.

Schulpis K.H., Tsakiris S., Traeger-Synodinos J. and Papassotiriou I. (2005). Low total antioxidant status in implicated with high 8-hydroxy-2-deoxyguanosine serum concentrations in phenylketonuria. Clin. Biochem. 38: 239-242.

Schulpis K.H., Kalogerakou M., Gioni V., Papastamataki M., Papassotiriou I. (2011). Glutamine, ornithine, citrulline and arginine levels in children with phenylketonuria: The diet effect. Clin Biochem 44(10-11):821-825.

Scriver C.R., Beaudet A.L., Sly W.S., Valle D. eds. (2001). The metabolic and molecular basis of inherited disease. 8 edição. New York. McGraw-Hill

Scriver C.R., Kaufman S. (2001). Hyperphenylalaninemia: Phenylalnine Hydroxylase Deficiency. In Scriver C.R., Beaudet A.L., Sly W.S., Walle D. (editores). The Metabolic & Molecular Inherited Disease. 8ª ed. Volume II. New York: McGraw-Hill.

Sierra C., Vilaseca M.A., Moyano D., Brandi N., Campistol J., Lambruschini N., Cambra F.J., Deulofeu R., Mira A. (1998). Antioxidant status in hyperphenylalaninemia. Clin Chim Acta 276: 1–9.

Sies H. (1991). Oxidative Stress II. Oxidants and antioxidants. Acadamic Press, London.

Singh S., Anand A., Srivastava P.K. (2012). Regulation and properties of glucose-6-phosphate dehydrogenase: A review. International Journal of Plant Physiology and Biochemistry Vol. 4(1): 1-19.

Simon E., Fingerhut R., Baumkötter J., Konstantopoulou V., Ratschmann R., Wendel U. (2006). Maple Syrup Disease: favourable effect of early diagnosis by newborn screening on the neonatal course of disease. J. Inherit Metab Dis 29:532-537.

Sirtori L.R., Dutra-Filho C.S., Fitarelli D., Sitta A., Haeser A., Barschak A.G., Wajner M., Coelho D.M., Llesuy S., Bello´-Klein A., Giugliani R., Deon M., Vargas C.R. (2005). Oxidative stress in patients with phenylketonuria. Biochim Biophys Acta 1740:68–73.

Sitta A., Barschak A.G., Deon M., Terroso T., Pires R., Giugliani R., Dutra-Filho C.S., Wajner M., Vargas C.R. (2006). Investigation of oxidative stress parameters in treated phenylketonuric patients. Metab Brain Dis 4: 287-296.

Sitta A., Manfredini V., Biasi L., Treme´a R., Schwartz I.V., Wajner M., Vargas C.R. (2009a). Evidence that DNA damage is associated to phenylalanine blood levels in leukocytes from phenylketonuric patients. Mutat Res 679:13–16.

Sitta A., Barschak A.G., Deon M., de Mari J.F., Barden A.T., Vanzin C.S., Biancini G.B., Schwartz I.V., Wajner M., Vargas C.R. (2009b). L-carnitine blood levels and oxidative stress in treated phenylketonuric patients. Cell Mol Neurobiol 29:211–218.

Sitta A., Barschak A.G., Deon M., Barden A.T., Biancini G.B., Vargas P.R., de Souza C.F., Netto C., Wajner M., Vargas C.R. (2009c). Effect of short- and long-term exposition to high phenylalanine blood levels on oxidative damage in phenylketonuric patients Inter J of Develop Neurosc 27:243–247.

Suh J.H., Shenvi S.V., Dixon B.M., Liu H., Jaiswal A.K., Hagen T.M. (2004). Decline in transcriptional activity of Nrf2 causes age related loss of glutathione synthesis, which is reversible with lipoic acid. Proc Natl Acad Sci 101:3381-3386.

Tam S.Y., Roth R.H. (1997). Mesofrontal dopaminergic neurons: can tyrosine avaibility influence their functions? Biochem Pharmacol 53:441-453.

Townsend D.M., Manevich Y., He L., Hutchens S., Pazoles C.J., Tew K.D. (2008). Novel Role for Glutathione S-Transferase: Regulator of protein S-glutathionylation following oxidative and nitrosative stress. J of Biol Chem 284(1): 436-445.

Valdovinos-Flores C., Gosenbatt M.E. (2012). The role of amino acids transporters in GSH synthesis in the blood brain barrier and central nervous system. Neurochem Intern 61: 405-414.

van Backel M.M.E., Printzen G., Wermuth B., Wiesmann U.N. (2000). Antioxidant and thyroid hormone status in selenium-deficient phenylketonuric and hyperphenylalaninemic patients. Am J Clin Nutr 72:976–981

van Spronsen F.J., van Dijk T., Smit G.P., van Rijn M., Reijngoud D.J., Berger R., Heymans H.S. (1996). Large daily fluctuations in plasma tyrosine in treated patients with phenylketonuria. Am J Clin Nutr 64(6): 916-921.

Vanzin C.S., Biancini G.B., Sitta A., Wayhs C.A.Y., Pereira Y.N., Rockenbach F., Garcia S.C., Wyse A.T.S., Schwartz I.V.D., Wajner M., Vargas C.R. (2011). Experimental evidence of oxidative stress in plasma of homocystinuric patients: A possible role for homocysteine. Mol Gene and Metabol 104: 112–117.

Vargas C.R., Wajner M., Sitta A. (2011). Oxidative stress in phenylketonuric patients. Mol Gene and Metabol 104: S97–S99.

Veal D. e Day A. (2011). Hydrogene peroxide as a signaling molecule. Antioxid Redox Signal 15(1): 147-151.

Vilaseca M.A., Lambruschini N., Gomez-Lopez L., Gutierrez A., Fuste E., Gassio R., Artuch R., Campistol J. (2010). Quality of dietary control in phenylketonuric patients and its relationship with general intelligence. Nutr Hosp 25(1):60-66.

Wagner A.E., Ernst I.M.A., Birringer M., Sancak Ö., Barella L., Rimbach G. (2012). A Combination of Lipoic Acid Plus Coenzyme Q10 Induces PGC1α, a Master Switch of Energy Metabolism, Improves Stress Response, and Increases Cellular Glutathione Levels in Cultured C2C12 Skeletal Muscle Cells. Oxi Med and Cell Longevity doi:10.1155/2012/835970

Wajner M., Latini A., Wyse A.T. and Dutra-Filho C.S. (2004). The role of oxidative damage in the neuropathology of organic acidurias: insights from animal studies. J Inherit Metabol Dis 27: 427-448.

Wannmacher C.M.D. (1995). Estudos experimentais sobre a patogênese de erros inatos do metabolismo. Tese apresentada para a obtenção de grau de Doutor ao Curso de Pós-Graduação em Ciências Biológicas-Bioquímica. UFRGS, Porto Alegre, RS.

Wink D.A., Miranda K.M., Espey M.G., Pluta R.M., Hewett S.J., Colton C., Vitek M., Feelisch M., Grisham M.B. (2001). Mechanisms of the antioxidant effects of nitric oxide. Antiox Redox Signal 3: 203-213.

Williams R.A., Mamotte C.D.S., Burnett, J.R. (2008). Phenylketonuria: An inborn error of phenylalanine metabolism. Clin Biochem Rev 29: 31-41.

Wojtkowiak-Giera A., Wandurska-Nowak E., Michalak M. (2011). Changes in the activity and kinetics of mouse intestinal glutathione transferase during experimental trichinellosis. Parasitol Res 108: 1309-1313.

Woo S.L., Lidsky A.S., Guttler F., Chandra T., Robson K.J. (1983). Cloned human phenylalanine hydroxylase gene allows prenatal diagnosis and carrier detection of classical phenylketonuria. Nature 306:151-155.

Wyse A.T.S., Bolognese G., Brusque A.M., Wajner M., Wannmacher C.M.D. (1995). Na^+, K^+-APTase activity in the synaptic plasma membrane from the cerebral cortex of rats subjected to chemically induced phenylketonuria. Med Sci Res 23, 261-263.

Zeman J., Pijackova A., Behulova J., Urge O., Saligova D., Hyanek J. (1996). Intellectual and school performance in adolescents with phenylketonuria according to their dietary compliance. Eur J Pediatr 155: 56-58.

Zielonka J., Sikora A., Joseph J., Kalyanaraman B. (2010). Peroxynitrite is the major species formed from different flux ratios of co-generated nitric oxide and superoxide: direct reaction with boronate-based fluorescent probe. J Biol Chem 285: 14210-14216.

ANEXO I – *Lista de figuras e tabelas*

ANEXO II – *Metodologia*

ANIMAIS

Para estes modelos ratos Wistar são apropriados, pois o desenvolvimento diário do sistema nervoso do rato é equivalente ao que ocorre a cada mês no ser humano (Clark et al., 1993), atingindo o desenvolvimento cerebral equivalente ao recém nascido humano, entre o 6º e o 8º dia de vida pós-natal (Hommes, 1982). Ainda que os modelos químicos experimentais com animais não correspondam exatamente à doença humana, permitem reproduzir alterações metabólicas específicas semelhantes às encontradas nos EIM, sendo, portanto, úteis para o estudo de suas repercussões, como o entendimento da fisiopatologia da doença e a avaliação de medidas terapêuticas a serem propostas para os pacientes.

Foram necessários para o desenvolvimento do projeto aproximadamente 130 animais, considerando um n=8 para as medidas de parâmetros de EO. O número de animais foi calculado de acordo com Callegari-Jacques (2003) e estimado pela experiência anterior do grupo na determinação dos mesmos parâmetros (Kienzle-Hagen et al., 2002). Os animais foram obtidos do biotério do Departamento de Bioquímica, ICBS, UFRGS, e mantidos dentro de gaiolas apropriadas em salas climatizadas à temperatura constante (22±1ºC), e ciclos de claro/escuro de 12 horas, com acesso à ração comercial padrão e água *ad libitum*. Os cuidados com os animais foram seguidos de acordo com as diretrizes governamentais oficiais conforme a Federação das Sociedades Brasileiras para Biologia Experimental e o projeto foi previamente submetido à Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal do Rio Grande do Sul.

MODELO EXPERIMENTAL E PREPARAÇÃO DOS TECIDOS

O modelo animal de HPA já foi estabelecido por nosso grupo de pesquisa (Wannmacher, 1995; Wyse et al., 1995; Kienzle-Hagen et al., 2002) e está baseado no modelo desenvolvido por Greengard e colaboradores (1976) em ratos de 6 dias de vida. Através da administração de Phe (2,1 µmol/g) duas vezes ao dia e de α-metilfenilalanina (α-MePhe) (1,6 µmol/g), um inibidor da PAH, uma vez ao dia, durante oito dias, com intervalo de 9h entre cada injeção. Este modelo experimental foi capaz de reproduzir quimicamente, em ratos, níveis de Phe cerebrais semelhantes aos encontrados em pacientes com PKU.

Para avaliar o efeito do pré-tratamento do AL no modelo crônico de HPA, ratos Wistar foram divididos em quatro grupos: Grupo controle (solução salina); grupo AL (40 mg/kg de AL a partir do 1º dia); Grupo HPA (salina no 1º dia e uma solução de 1,6 mmol/g de α-MePhe e 2,1 mmol/g de Phe a partir do 2º dia de injeções) e grupo HPA + AL (40 mg/kg de AL a partir do 1º dia e 1,6 mmol/g de α-MePhe e 2,1µmol/g de Phe a partir do 2º dia de injeções). As soluções foram preparadas no dia e administradas por injeção subcutânea durante oito dias. Após 12 horas da última injeção os animais foram eutanasiados por decapitação, sem anestesia, sendo os cérebros (*forebrain*) imediatamente removidos e limpos, descartando-se o cerebelo, bulbos olfatórios, ponte e medula. Para avaliação do perfil do metabolismo da GSH foi utilizado o fígado dos animais a fim de comparar com as amostras de cérebro total.

Os tecidos foram então pesados e mantidos no gelo até serem homogeneizados em tampão de homogeneização (tampão fosfato de sódio 20 mM com KCl 140 mM, pH 7,4). Em seguida, o homogeneizado foi centrifugado a 800g por 10 minutos a 4ºC, sendo o sobrenadante usado para a medida dos parâmetros de EO e o pellet contendo núcleos celulares foi separado para análise de dano ao DNA.

ANEXO III – *Parecer da Comissão de Ética na Utilização de Animais*

U F R G S   **PRÓ-REITORIA DE PESQUISA**   PROPEQ

UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL   Comissão De Ética Na Utilização De Animais

CARTA DE APROVAÇÃO

Comissão De Ética Na Utilização De Animais analisou o projeto:

Número: 18851

Título:
Efeito neuroprotetor do ácido lipóico sobre parâmetros de estresse oxidativo em modelos animais de fenilcetonúria e de doença do xarope de bordo

Pesquisadores:

Equipe UFRGS:

CARLOS SEVERO DUTRA FILHO - coordenador desde 01/08/2010
TARSILA BARROS MORAES - Aluno de Doutorado desde 01/08/2010

Comissão De Ética Na Utilização De Animais aprovou o mesmo, em seus aspectos éticos e metolodológicos de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Sexta-Feira, 12 de Novembro de 2010
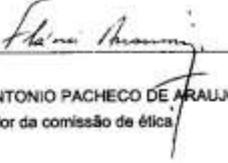
FLAVIO ANTONIO PACHECO DE ARAUJO
Coordenador da comissão de ética

1

ANEXO IV – *Considerações Éticas e tratamento dos resíduos biológicos e químicos*

Os resíduos biológicos gerados pelos experimentos, incluindo luvas, ependorfes, ponteiras, papel higiênico com sangue dos animais, foram descartados em sacos de lixo branco e posteriormente depositados em recipientes próprios localizados no Departamento de Bioquímica (UFRGS). As agulhas e laminas de bisturi foram descartadas em caixas amarelas (Descarpack®) e as carcaças de animais são colocadas em sacos brancos e armazenadas em freezer próprio no departamento até serem recolhidos. Esses resíduos serão periodicamente recolhidos por uma empresa especializada e terceirizada (contratada pela universidade através de licitações), que realiza o descarte do material de acordo com as normas vigentes de biossegurança.

Já os resíduos químicos foram armazenados em bombonas plásticas, devidamente identificadas e separados de acordo com a seguinte classificação estabelecida pelo departamento: resíduos orgânicos halogenados, orgânicos não-halogenados, solventes aquosos, solventes orgânicos passíveis de purificação e resíduos sólidos. As embalagens contendo estes resíduos serão periodicamente coletadas e enviadas para o Centro de Gestão e Tratamento de Resíduos Químicos do Instituto de Química da UFRGS.