UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

EFEITO DO ÁCIDO γ-HIDROXIBUTÍRICO E DA TIROSINA SOBRE PARÂMETROS DE ESTRESSE OXIDATIVO EM CÓRTEX CEREBRAL DE RATOS JOVENS

ÂNGELA MALYSZ SGARAVATTI

Porto Alegre, 2008

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ÂNGELA MALYSZ SGARAVATTI

Orientador: Dr. Carlos Severo Dutra Filho

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Doutor em Ciências Biológicas: Bioquímica.

Porto Alegre, 2008

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RESUMO

A deficiência da semi-aldeído succínico desidrogenase (SSADH) e a tirosinemia tipo II caracterizam-se pela presença de elevadas concentrações teciduais e plasmáticas de ácido γ-hidroxibutírico (GHB) e tirosina, respectivamente. Tanto os pacientes afetados pela deficiência da SSADH quanto aqueles com tirosinemia tipo II apresentam sinais e sintomas neurológicos. Embora os mecanismos responsáveis pela disfunção neurológica sejam pouco conhecidos, sabe-se que podem estar relacionados ao acúmulo de GHB e tirosina e seus possíveis efeitos tóxicos sobre o sistema nervoso central (SNC). Considerando ainda que o estresse oxidativo parece estar envolvido em diversas doenças ou alterações patológicas que afetam o SNC, os efeitos do GHB e da L-tirosina sobre vários parâmetros de estresse oxidativo foram investigados em homogeneizados de córtex cerebral de ratos jovens. Os efeitos *in vivo* do GHB, ou de seu precursor 1,4-butanodiol, foram semelhantes. Ambos mostraram comprometimento das defesas antioxidantes não-enzimáticas e indução da lipoperoxidação. Os efeitos *in vitro* e *in vivo* da L-tirosina, por sua vez, foram diferentes: *in vitro*, a L-tirosina provocou dano oxidativo ao DNA, diminuiu as defesas antioxidantes não-enzimáticas e enzimáticas e alterou o estado redox (razão SH/SS), enquanto que a administração aguda de L-tirosina causou dano oxidativo a lipídios e proteínas, modificou a razão SH/SS, diminuiu as defesas antioxidantes não-enzimáticas e estimulou a atividade da glicose-6-fosfato desidrogenase. Esses resultados sugerem que o GHB e a L-tirosina são capazes de promover o estresse oxidativo em córtex cerebral de ratos jovens. Desta forma, caso esses mesmos efeitos ocorrerem em seres humanos, é provável que o estresse oxidativo seja um dos mecanismos responsáveis pela neuropatologia característica da deficiência da SSADH e tirosinemia tipo II.

PALAVRAS-CHAVES: ácido γ-hidroxibutírico; deficiência da semi-aldeído succínico desidrogenase; tirosina; tirosinemia tipo II; estresse oxidativo.

ABSTRACT

Succinic semialdehyde dehydrogenase (SSADH) deficiency and tyrosinemia type II are characterized by predominant tissue and blood accumulation of γ-hydroxybutyric acid (GHB) and tyrosine, respectively. Patients with SSADH deficiency and tyrosinemia type II present neurological signs and symptoms. Although mechanisms of brain damage remain unclear, they are probably related to the accumulation of GHB or tyrosine leading to possible noxious effects on central nervous system (CNS) development in those patients. Considering that the damaging consequences of oxidative stress have been implicated in a variety of disorders of CNS, the effect of GHB and L-tyrosine were investigated on some oxidative stress parameters in cerebral cortex homogenates of young rats. The *in vitro* and *in vivo* effects of GHB, or its precursor 1,4-butanediol (1,4-BD), were similar. It was observed that GHB or 1,4-BD impairs non-enzymatic antioxidant defenses and induces lipid peroxidation. On the other hand, the *in vitro* and *in vivo* effects of L-tyrosine were different. Oxidative damage to DNA was promoted while non-enzymatic and enzymatic antioxidant defenses, and thiol-disulfide redox state (SH/SS ratio) were markedly diminished by L-tyrosine *in vitro*. In contrast, the acute administration of L-tyrosine causes lipid peroxidation and protein oxidation, decreases non-enzymatic antioxidant defenses, alters SH/SS ratio and stimulates glucose-6-phosphate dehydrogenase activity. Taken together, it may be presumed that GHB and L-tyrosine elicit oxidative stress in cerebral cortex of young rats. If these effects also occur in the brain of patients affected by SSADH deficiency or tyrosinemia type II, it is possible that oxidative stress may contribute, at least in part, to the neurological dysfunction characteristic of these diseases.

KEYWORDS: γ-hydroxybutyric acid; succinic semialdehyde dehydrogenase deficiency; tyrosine; tyrosinemia type II; oxidative stress.

LISTA DE FIGURAS

Figura 1. Metabolismo do GABA e a sua relação com a deficiência da semi-aldeído succínico desidrogenase. 3

Figura 2. Metabolismo da tirosina. 10

LISTA DE TABELAS

Tabela 1. Principais características clínicas dos pacientes com deficiência da semi-aldeído succínico desidrogenase. 4

Tabela 2. Efeitos do GHB e do 1,4-BD em homogeneizados de córtex cerebral de ratos jovens. 146

Tabela 3. Efeitos da L-tirosina em homogeneizados de córtex cerebral de ratos jovens. 148

Tabela 4. Efeitos *in vitro* dos metabólitos da L-tirosina em homogeneizados de córtex cerebral de ratos jovens. 149

LISTA DE ABREVIATURAS

1,4-BD 1,4-Butanodiol

CAT Catalase

EIM Erros inatos do metabolismo

ERN Espécies reativas de nitrogênio

ERO Espécies reativas de oxigênio

GABA Ácido γ-aminobutírico

GBL γ-Butirolactona

GHB Ácido γ-hidroxibutírico

G6PDH Glicose-6-fosfato desidrogenase

GPx Glutationa peroxidase

GR Glutationa redutase

GSH Glutationa reduzida

GSSG Dissulfeto de glutationa

4-HNE 4-Hidróxi-2-nonenal

H_2O_2 Peróxido de hidrogênio

$LO^•$ Radical alcoxil

$LOO^•$ Radical peroxil

LOOH Hidroperóxido orgânico

$NADP^+$ Nicotinamida adenina dinucleotídeo fosfato oxidada

NADPH Nicotinamida adenina dinucleotídeo fosfato reduzida

$NO^{\bullet}$ Óxido nítrico

$O_2^{\bullet -}$ Radical superóxido

$OH^{\bullet}$ Radical hidroxila

$ONOO^-$ Peroxinitrito

ONOOH Ácido peroxinitroso

SNC Sistema nervoso central

SOD Superóxido dismutase

SSA Semi-aldeído succínico

SSADH Semi-aldeído succínico desidrogenase

TAR Reatividade antioxidante total

TAT Tirosina aminotransferase

TBA-RS Substâncias reativas ao ácido tiobarbitúrico

TRAP Potencial antioxidante total

SUMÁRIO

1. INTRODUÇÃO 1

1.1. ERROS INATOS DO METABOLISMO 1

1.2. DEFICIÊNCIA DA SEMI-ALDEÍDO SUCCÍNICO DESIDROGENASE 2

1.2.1. Características Clínicas 3

1.2.2. Diagnóstico 4

1.2.3. Tratamento 5

1.2.4. Modelo Animal 5

1.3. EFEITOS DO ÁCIDO γ-HIDROXIBUTÍRICO 6

1.4. HIPERTIROSINEMIAS 8

1.4.1. Tirosinemia Tipo II 8

1.4.1.1. Características Clínicas 9

1.4.1.2. Diagnóstico 11

1.4.1.3. Tratamento 11

1.5. ESPÉCIES REATIVAS 11

1.5.1. Defesas Antioxidantes 15

1.5.1.1. Defesas Antioxidantes Enzimáticas 15

1.5.1.1.1. Superóxido Dismutase 15

1.5.1.1.2. Catalase 16

1.5.1.1.3. Glutationa Peroxidase 17

1.5.1.2. Defesas Antioxidantes Não-Enzimáticas 17

1.5.1.2.1. *Glutationa Reduzida* 17

1.5.1.2.2. α-Tocoferol 18

1.5.1.2.3. *Ácido Ascórbico* 18

1.5.2. Estresse Oxidativo 19

1.5.3. Estresse Oxidativo e o Sistema Nervoso Central 19

2. OBJETIVO 21

2.1. GERAL 21

2.2. ESPECÍFICO 21

3. ARTIGOS CIENTÍFICOS 23

3.1. CAPÍTULO I 24

3.2. CAPÍTULO II 32

3.3. CAPÍTULO III 63

3.4. CAPÍTULO IV 100

4. DISCUSSÃO 129

4.1. EFEITOS DO ÁCIDO γ-HIDROXIBUTÍRICO E DO 1,4-BUTANODIOL 129

4.2. EFEITOS *IN VITRO* E *IN VIVO* DA L-TIROSINA 135

5. CONCLUSÃO 145

5.1. EFEITOS DO ÁCIDO γ-HIDROXIBUTÍRICO E DO 1,4-BUTANODIOL EM HOMOGENEIZADOS DE CÓRTEX CEREBRAL DE RATOS JOVENS 145

5.2. EFEITOS DA L-TIROSINA EM HOMOGENEIZADOS DE CÓRTEX CEREBRAL DE RATOS JOVENS 147

5.2.1. *In vitro* 147

5.2.1. *In vivo* 147

5.3. EFEITOS *IN VITRO* DOS METABÓLITOS DA L-TIROSINA EM HOMOGENEIZADOS DE CÓRTEX CEREBRAL DE RATOS JOVENS 149

6. PERSPECTIVAS 150

REFERÊNCIAS BIBLIOGRÁFICAS 151

1. INTRODUÇÃO

1.1. ERROS INATOS DO METABOLISMO

Os erros inatos do metabolismo (EIM) são distúrbios hereditários causados pela deficiência parcial ou total de uma proteína, geralmente uma enzima. A ausência ou deficiência severa na atividade enzimática pode levar ao bloqueio de uma rota metabólica, ocasionando tanto o acúmulo de substratos tóxicos quanto a falta de produtos essenciais. O bloqueio metabólico pode, ainda, originar outros derivados por rotas metabólicas alternativas (Scriver *et al.*, 2001).

O grande número de alterações genéticas resulta em quadros clínicos extremamente diversos. Enquanto alguns EIM são assintomáticos, outros são tão graves que implicam em morte neonatal. De maneira geral, os EIM são situações graves que se manifestam na infância (Giugliani, 1988).

Saudubray *et al.* (2002) classificaram os EIM em três grandes grupos conforme o fenótipo clínico dos pacientes:

Grupo I: desordens de síntese ou catabolismo de moléculas complexas, como as doenças lisossômicas de depósito.

Grupo II: desordens do metabolismo intermediário, como as aminoacidopatias e as acidemias orgânicas.

Grupo III: desordens na produção ou utilização de energia, como as doenças mitocondriais da cadeia respiratória.

Este trabalho enfoca dois EIM do metabolismo intermediário: a deficiência da semi-aldeído succínico desidrogenase e a tirosinemia tipo II.

1.2. DEFICIÊNCIA DA SEMI-ALDEÍDO SUCCÍNICO DESIDROGENASE

A deficiência da semi-aldeído succínico desidrogenase (SSADH), ou acidúria γ-hidroxibutírica, é um EIM autossômico recessivo do ácido γ-aminobutírico (GABA). Jakobs e colaboradores (1981) identificaram o primeiro caso dessa doença em um menino turco que apresentava retardo no desenvolvimento motor e mental, ataxia não-progressiva e hipotonia muscular. Atualmente, existem aproximadamente 400 casos descritos da deficiência da SSADH (GUPTA *et al.*, 2003).

Em condições fisiológicas, as enzimas GABA transaminase e SSADH convertem o GABA em ácido succínico, que pode ser utilizado como fonte de energia através de sua entrada no ciclo de Krebs. Entretanto, quando a atividade da SSADH está deficiente, o semi-aldeído succínico (SSA) se acumula e é reduzido a ácido γ-hidroxibutírico (GHB) (Figura 1). Conseqüentemente, ocorre um aumento nas concentrações do GHB nos tecidos e fluidos fisiológicos dos pacientes afetados por essa doença (GIBSON E JAKOBS, 2001). Os níveis de GHB podem atingir 0,6 mM no líquido cefalorraquidiano e 1,0 mM no sangue (JAKOBS *et al.*, 1981; GIBSON *et al.*, 1984; GIBSON E JAKOBS, 2001).

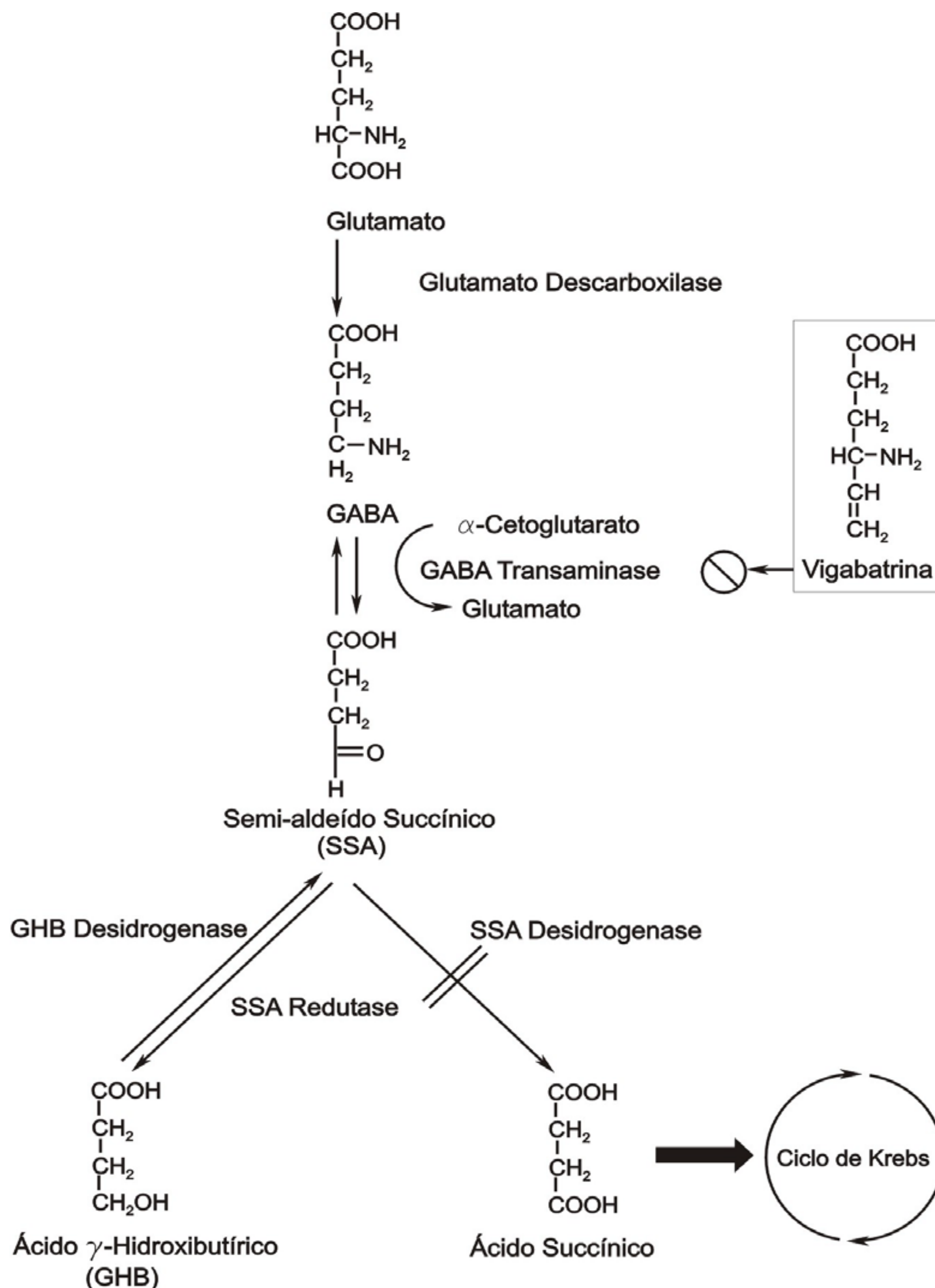


Figura 1. Metabolismo do GABA e a sua relação com a deficiência da semialdeído succínico desidrogenase. Adaptada de GROPMAN, 2003.

1.2.1. Características Clínicas

As características clínicas descritas em pacientes com a deficiência da SSADH são muito variadas e inespecíficas (Tabela 1), porém observa-se que os principais sintomas e sinais clínicos são neurológicos.

Tabela 1. Principais características clínicas dos pacientes com deficiência da SSADH.

Característica clínica	Freqüência (n=60)
Retardo	
▪ mental	50 (83%)
▪ da fala	49 (82%)
▪ motor	45 (75%)
Hipotonia	48 (80%)
Convulsões	38 (63%)
Problemas de comportamento	30 (50%)
Ataxia	29 (48%)
Hiporreflexia	27 (45%)
Problemas oculares	15 (25%)
Sintomas gastrointestinais	13 (22%)
Problemas neonatais	08 (13%)

Adaptada de PEARL *et al.*, 2003.

1.2.2. Diagnóstico

Devido à heterogeneidade dos sintomas, o diagnóstico da deficiência da SSADH não pode ser feito baseado nas manifestações clínicas. Entretanto, a identificação e a quantificação do GHB, através de métodos cromatográficos nos fluidos fisiológicos, são de crucial importância para a confirmação diagnóstica, além da análise da deficiência da SSADH em leucócitos e/ou fibroblastos dos pacientes (GIBSON E JAKOBS, 2001).

O diagnóstico pré-natal é realizado pela determinação da atividade da SSADH em punção de cordão umbilical (coleta de sangue fetal) ou amniocentese, juntamente com a análise do GHB no líquido amniótico (THORBURN *et al.*, 1993) e de mutações em cultura de leucócitos ou linfoblastos (HOGEMA *et al.*, 2001a).

1.2.3. Tratamento

Embora não exista um tratamento eficaz para a deficiência da SSADH, o anticonvulsivante vigabatrina, inibidor irreversível da GABA transaminase, é amplamente utilizado (GIBSON E JAKOBS, 2001). Teoricamente, a vigabatrina deveria impedir a formação do SSA e do GHB com concomitante aumento nas concentrações cerebrais do GABA. Porém, a redução dos níveis do GHB no líquido cefalorraquidiano foi insignificante nos pacientes tratados com vigabatrina, favorecendo a idéia de que ocorre um suprimento periférico do GHB (HOWELLS *et al.*, 1992). Além disso, os resultados clínicos variaram de melhoras no comportamento e ataxia em alguns casos, até exacerbação, ou mesmo indução, de crises convulsivas em outros (GIBSON E JAKOBS, 2001; GROPMAN, 2003).

Outros anticonvulsivantes usados no tratamento da deficiência da SSADH são a carbamazepina e a lamotrigina. No entanto, os agentes terapêuticos promissores são os antagonistas dos receptores do GHB (NCS-382) ou GABAérgicos (CPG 35348) (GIBSON E JAKOBS, 2001; GROPMAN, 2003; PEARL *et al.*, 2003).

1.2.4. Modelo Animal

Hogema e colaboradores (2001b) desenvolveram um modelo da deficiência da SSADH em camundongos através da deleção do gene que codifica o sítio ativo dessa enzima. Conseqüentemente, não só a ausência completa da atividade da SSADH foi observada nos tecidos analisados (cérebro, fígado, coração e rins), mas também o aumento nas concentrações teciduais de GHB e GABA. Os animais mutantes apresentam problemas neurológicos como, por exemplo, ataxia, hipotonia e convulsões, da mesma forma que ocorre em pacientes com a deficiência da

SSADH. Portanto, esse modelo animal fornece uma excelente oportunidade para que os mecanismos fisiopatológicos da deficiência da SSADH sejam elucidados.

1.3. EFEITOS DO ÁCIDO γ-HIDROXIBUTÍRICO

O GHB é um constituinte endógeno do sistema nervoso central sintetizado a partir do GABA através das enzimas GABA transaminase e SSA redutase em neurônios GABAérgicos ou naqueles neurônios capazes de formar GABA (MAITRE, 1997).

A sua localização sinaptossomal, a presença de mecanismos próprios de liberação e recaptação, e a distribuição cerebral heterogênea dos receptores do GHB de baixa e alta afinidade sugerem que o GHB atue como um neurotransmissor ou neuromodulador (MAITRE, 1997).

A administração exógena do GHB causa alterações eletrofisiológicas, bioquímicas e comportamentais de forma dose-dependente. Estudos eletrofisiológicos sobre o efeito farmacológico de baixas e altas doses do GHB indicaram a existência de uma resposta mediada por receptores do GHB e GABAérgicos, respectivamente. Essa ação GABAérgica poderia ser induzida pelo controle do GHB sobre a liberação do GABA ou através da síntese do GABA a partir do GHB (MAITRE, 1997).

Achados bioquímicos mostram que o GHB influencia a atividade de diferentes neurotransmissores. Os efeitos desse ácido sobre a atividade do sistema dopaminérgico estão entre os mais estudados. A administração de baixas doses de GHB hiperpolariza as estruturas dopaminérgicas, causando uma redução na liberação de dopamina, enquanto que doses maiores estimulam a síntese e o acúmulo tecidual de dopamina com subseqüente aumento de sua liberação. Mesmo

assim, é importante salientar que o GHB não inibe as enzimas responsáveis pela degradação da dopamina - monoamino oxidase e catecol-o-metil-transferase (MAMELAK, 1989; CASH, 1994; MAITRE, 1997).

Aliado a isso, experimentos *in vitro* demonstraram que 1,0 mM do GHB não só aumentou a liberação de dopamina após uma inibição inicial (HECHLER *et al.*, 1991), mas também comprometeu o metabolismo energético mitocondrial através da diminuição da produção de CO_2 e, como conseqüência, inibiu a síntese lipídica (SILVA *et al.*, 1999).

Além disso, a administração do GHB em animais provocou modificações comportamentais e eletroencefalográficas semelhantes àquelas comumente observadas nas convulsões de ausência. De fato, o GHB apresenta propriedades sedativas e, por isso, foi utilizado como adjuvante em anestesias gerais.

Atualmente, o GHB tem sido utilizado no tratamento de pacientes narcolépticos, catalépticos e viciados em drogas, apesar de também ser usado como droga de abuso (MAMELAK, 1989; CASH, 1994; MAITRE, 1997; WONG *et al.*, 2004). Por essa razão, o acesso ao GHB foi restringido pelo *Food and Drug Administration* e, conseqüentemente, houve um aumento na utilização ilícita de seus precursores: γ-butirolactona (GBL) e 1,4-butanodiol (1,4-BD). A GBL é metabolizada em GHB através de lactonases periféricas ou hidrólise não-enzimática (ROTH E GIARMAN, 1966), enquanto que a conversão do 1,4-BD em GHB é feita pelas enzimas hepáticas álcool desidrogenase e aldeído desidrogenase (ROTH E GIARMAN, 1968; SNEAD *et al.*, 1989). Tanto a GBL quanto o 1,4-BD têm causado diversos efeitos agudos e crônicos (SHANNON E QUANG, 2000; TETER E GUTHRIE, 2001). Entretanto, várias evidências sugerem que a GBL e o 1,4-BD atuam como pró-fármacos do GHB, ou seja, o GHB é a substância farmacologicamente ativa (POLDRUGO E SNEAD,

1984; SNEAD *et al.*, 1989, CARAI *et al.*, 2002; QUANG *et al.*, 2002; CARTER *et al.*, 2006).

1.4. HIPERTIROSINEMIAS

A tirosina é um aminoácido aromático não-essencial sintetizado a partir da hidroxilação da fenilalanina. Além de estar envolvida diretamente na biossíntese de catecolaminas, hormônios da tireóide e melanina, a tirosina apresenta dois principais destinos metabólicos: síntese proteica ou degradação em fumarato e acetoacetato através de cinco reações enzimáticas. Em humanos, deficiências em quatro das cinco enzimas responsáveis pela degradação da tirosina foram identificadas: deficiência da tirosina aminotransferase (TAT) (tirosinemia tipo II), deficiência da 4-hidroxifenilpiruvato dioxigenase (tirosinemia tipo III), deficiência da homogentizato 1,2-dioxigenase (alcaptonúria) e deficiência da fumarilacetoacetase (tirosinemia tipo I) (MITCHELL *et al.*, 2001; HELD, 2006) (Figura 2). Tanto as tirosinemias tipo I, II e III quanto à tirosinemia transitória do recém-nascido e os distúrbios hepáticos provocam hipertirosinemia, ou seja, o acúmulo de tirosina nos tecidos e fluidos fisiológicos dos indivíduos afetados (MITCHELL *et al.*, 2001).

1.4.1. Tirosinemia Tipo II

A tirosinemia tipo II, também denominada tirosinemia oculocutânea ou síndrome de Richner-Hanhart, parece estar associada à consangüinidade e apresenta uma freqüência de 1 caso em aproximadamente 250.000 nascidos vivos (MACSAI *et al.*, 2001). A tirosinemia tipo II é causada pela deficiência autossômica recessiva da enzima hepática TAT. Conseqüentemente, altos níveis de tirosina são encontrados nos tecidos e fluidos fisiológicos desses pacientes. As concentrações

plasmáticas de tirosina variam entre 370 e 3420 μM em pacientes não tratados enquanto que as concentrações normais são inferiores a 103 μM (GOLDSMITH *et al.*, 1973; RABINOWITZ *et al.*, 1995; MACSAI *et al.*, 2001; MITCHELL *et al.*, 2001; VALIKHANI *et al.*, 2005; HELD, 2006). A elevada excreção urinária de tirosina e seus metabólitos (ácido 4-hidroxifenilpirúvico, ácido 4-hidroxifenilacético, ácido 4-hidroxifenilático, N-acetiltirosina e 4-tiramina) também caracterizam a tirosinemia tipo II (MITCHELL *et al.*, 2001).

1.4.1.1. Características Clínicas

As manifestações clínicas associadas à tirosinemia tipo II envolvem lesões oculares, lesões cutâneas e alterações neurológicas (MITCHELL *et al.*, 2001; HELD, 2006).

As lesões oculares caracterizam-se por fotofobia, lacrimejamento, vermelhidão e dores intensas. Conjuntivites e ulcerações na córnea, que podem causar uma diminuição na visão e glaucoma, também são freqüentemente observadas nesses pacientes. As lesões cutâneas são hiperceratóticas e atingem principalmente as plantas dos pés e as palmas das mãos. As alterações neurológicas variam desde leve decréscimo na inteligência até retardo mental severo associado à microcefalia entre outras anormalidades como automutilação, hiperatividade, distúrbios motores e no desenvolvimento da fala (GOLDSMITH *et al.*, 1973; LEMONNIER *et al.*, 1979; RABINOWITZ *et al.*, 1995; MACSAI *et al.*, 2001; MITCHELL *et al.*, 2001; VALIKHANI *et al.*, 2005).

A patogênese das lesões oculares e cutâneas na tirosinemia tipo II está relacionada com a formação de cristais intracelulares de tirosina e o desenvolvimento de uma resposta inflamatória. Provavelmente, os altos níveis

plasmáticos de tirosina são os responsáveis pelo comprometimento do sistema nervoso central na tirosinemia tipo II (MITCHELL *et al.*, 2001; HELD, 2006).

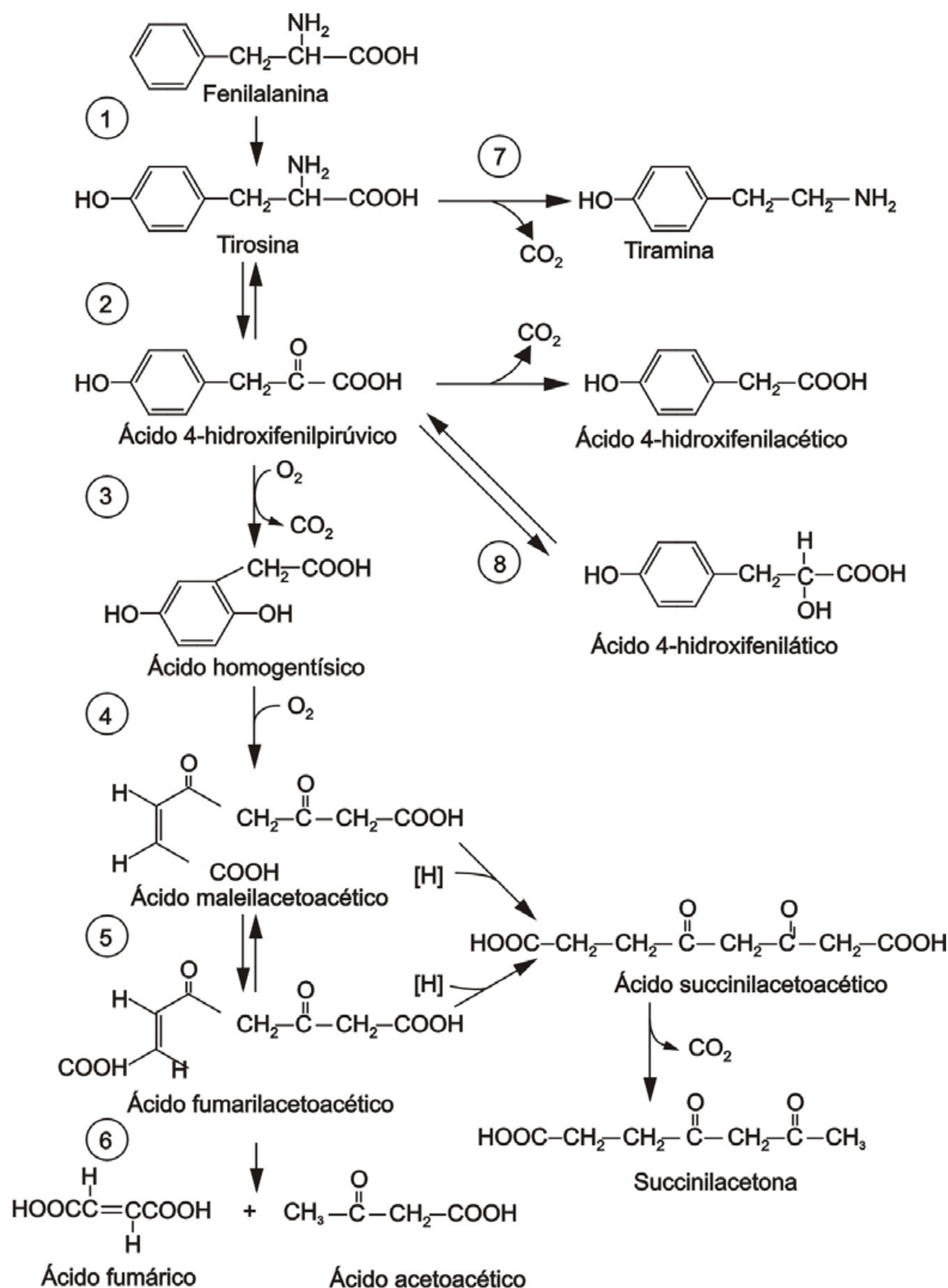


Figura 2. Metabolismo da tirosina. Enzimas envolvidas: ① = fenilalanina hidroxilase; ② = tirosina aminotransferase; ③ = 4-hidroxifenilpiruvato dioxigenase; ④ = homogentizato 1,2-dioxigenase; ⑤ = maleilacetato isomerase; ⑥ = fumarilacetoacetase. As enzimas ⑦ e ⑧ ainda não foram identificadas. Adaptada de MITCHELL *et al.*, 2001.

1.4.1.2. Diagnóstico

O diagnóstico da tirosinemia tipo II consiste tanto na determinação de elevadas concentrações plasmáticas e urinárias de tirosina como na detecção de altos níveis de seus metabólitos na urina. A confirmação do diagnóstico é feita pela avaliação da atividade enzimática em biópsia hepática ou da análise de mutações no gen da TAT (MITCHELL *et al.*, 2001; HELD, 2006).

1.4.1.3. Tratamento

A restrição nutricional de fenilalanina e tirosina tem sido indicada para amenizar as lesões oculares e cutâneas dos pacientes com tirosinemia tipo II. Entretanto, ainda não foi estabelecido em que momento o controle dietético deve ser iniciado e quais seriam as concentrações plasmáticas de tirosina apropriadas para evitar os problemas neurológicos (MITCHELL *et al.*, 2001; HELD, 2006).

Tendo em vista que o tratamento da deficiência da SSADH e da tirosinemia tipo II não é específico, o entendimento dos mecanismos fisiopatológicos é importante para a implementação de abordagens terapêuticas mais eficientes. Neste sentido, nosso grupo de pesquisa tem demonstrado o papel de espécies reativas na neuropatologia de erros inatos do metabolismo (WAJNER *et al.*, 2004).

1.5. ESPÉCIES REATIVAS

O termo espécies reativas de oxigênio (ERO) refere-se aos radicais livres formados pela redução do oxigênio e aos não-radicais dele derivados, como o peróxido de hidrogênio, o oxigênio *singlet*, o ácido hipocloroso e o ozônio (HALLIWELL, 2006; HALLIWELL E GUTTERIDGE, 2007). Um radical livre é definido como

qualquer espécie química capaz de ter existência independente e que contém um ou mais elétrons desemparelhados (SOUTHORN E POWIS, 1988; HALLIWELL E GUTTERIDGE, 2007). Os radicais livres são formados em um cenário de oxirredução ou se, no rompimento de uma ligação covalente, cada um dos átomos envolvidos ficar com um elétron (fissão homolítica) (HALLIWELL E GUTTERIDGE, 2007).

Em condições fisiológicas do metabolismo celular aeróbio, pelo menos 95% do oxigênio molecular sofre redução tetravalente, resultando na geração de água. Durante esse processo, as espécies reativas superóxido, hidroperoxila, hidroxila e peróxido de hidrogênio são produzidas em pequenas quantidades na mitocôndria (cadeia respiratória) (COHEN, 1989). Outras fontes celulares de ERO são o retículo endoplasmático, a membrana nuclear, a membrana plasmática (lipoxigenase, prostaglandina sintetase) e algumas substâncias encontradas no citosol (HALLIWELL E GUTTERIDGE, 2007).

Além de ser formado durante a respiração celular, o radical superóxido ($O_2^{\bullet -}$) pode ser produzido em reações de auto-oxidação e por certas enzimas, como a xantina oxidase (FRIDOVICH, 1995; HALLIWELL E GUTTERIDGE, 2007). Apesar de não causar, diretamente, danos ao DNA, proteínas e lipídios, a citotoxidade do $O_2^{\bullet -}$ está relacionada a sua capacidade de gerar outras ERO. Contudo, em concentrações elevadas, o $O_2^{\bullet -}$ tem condições de mobilizar pequenas quantidades de ferro da proteína ferritina (BOLANN E ULVIK, 1990), além de atacar o sítio ativo de enzimas que contenham centros ferro-enxofre, causando a liberação do ferro e a inativação das mesmas (LIOCHEV, 1996).

O peróxido de hidrogênio (H_2O_2) pode ser formado pela dismutação do $O_2^{\bullet -}$ (FRIDOVICH, 1995) ou por outras reações (HALLIWELL, 2001; HALLIWELL, 2006). O H_2O_2 é pouco reativo e parece ser incapaz de oxidar o DNA, lipídios e proteínas,

exceto aquelas proteínas que possuem grupos tiólicos hiper-reativos e resíduos de metionina (LEVINE *et al.*, 1999; BORUTAITE E BROWN, 2001; HALLIWELL E GUTTERIDGE, 2007). Essa baixa reatividade do H_2O_2 pode ser vantajosa, se for considerado que essa ERO é gerada em processos inflamatórios e está presente no ar exalado, na urina e na maioria das bebidas quentes (café solúvel, por exemplo) (HALLIWELL *et al.*, 2000). Porém, em níveis milimolares, o H_2O_2 pode promover a liberação de íons ferro das hemeproteínas, como a mioglobina e os citocromos (PUPPO E HALLIWELL, 1988; HAREL *et al.*, 1988).

O ferro, por sua vez, pode converter o H_2O_2 em radical hidroxila ($OH^{\bullet}$) através das reações de Fenton e Haber-Weiss devido a sua habilidade de transferir um único elétron (HALLIWELL E GUTTERIDGE, 1984; HALLIWELL E GUTTERIDGE, 2007). Por essa razão, qualquer agente redutor capaz de transformar o íon férrico (Fe^{+3}) em ferroso (Fe^{+2}), como o radical superóxido, pode ser responsável pela continuidade dessas reações.

O radical $OH^{\bullet}$ reage com DNA, carboidratos, proteínas e lipídios, danificando-os. Assim, se o $OH^{\bullet}$ for produzido próximo ao DNA, poderão ocorrer modificações em bases nitrogenadas e resíduos de açúcar, levando à inativação ou mutação do DNA (HALLIWELL, 2001; HALLIWELL E GUTTERIDGE, 2007). Além disso, o $OH^{\bullet}$ tanto compromete a atividade biológica das proteínas ao oxidá-las (DEAN *et al.*, 1997), quanto induz a oxidação dos ácidos graxos poliinsaturados das membranas celulares (lipoperoxidação) (HALLIWELL, 2001; HALLIWELL E GUTTERIDGE, 2007). Os efeitos da lipoperoxidação sobre as membranas podem ser de quatro tipos:

(1) mudanças no microambiente lipídico;

(2) formação de novos canais de membrana, alterando a sua permeabilidade;

(3) formação de ligações cruzadas entre proteínas e fosfolipídios, inativando-os irreversivelmente;

(4) oxidação de grupos sulfidrilas nos sítios ativos de enzimas ligadas à membrana, acarretando a perda de suas propriedades funcionais.

Dependendo da intensidade desses efeitos, a lipoperoxidação pode causar desde pequenas alterações na permeabilidade das membranas afetadas até morte celular (MEERSON *et al.*, 1982).

Além das ERO, as espécies reativas de nitrogênio (ERN) desempenham um papel importante na fisiologia e patologia humana (BREDT, 1999; HOBBS *et al.*, 1999). O óxido nítrico ($NO^{\bullet}$), por exemplo, é um radical livre pouco reativo que, em concentrações iguais ou superiores a 1 μM (MASTRONICOLA *et al.*, 2003), inibe a enzima mitocondrial citocromo oxidase e, conseqüentemente, aumenta a formação do $O_2^{\bullet -}$ (FORFIA *et al.*, 1999). O $NO^{\bullet}$ pode reagir com o $O_2^{\bullet -}$, gerando o peroxinitrito ($ONOO^-$) (BECKMAN E KOPPENOL, 1996).

O $ONOO^-$ é protonado em pH fisiológico e o ácido peroxinitroso (ONOOH) formado favorece a produção de agentes citotóxicos com capacidade de nitração, hidroxilação e oxidação (BECKMAN E KOPPENOL, 1996; HALLIWELL *et al.*, 1999). Por essa razão, a geração *in vivo* do $ONOO^-$ contribui para a oxidação e nitração de lipídios, DNA e proteínas (BECKMAN E KOPPENOL, 1996; ISCHIROPOULOS, 1998; HALLIWELL *et al.*, 1999).

Diversos estudos sugerem que a decomposição do $ONOO^-$ consiste em um mecanismo independente de metais de transição para formar o radical $OH^{\bullet}$ (HALLIWELL *et al.*, 1999). Porém, a participação do radical $OH^{\bullet}$ na toxicidade do $ONOO^-$ é pequena, visto que a sua forma protonada é, por si só, muito mais reativa (HALLIWELL, 2001).

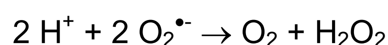
1.5.1. Defesas Antioxidantes

Os seres vivos dispõem de mecanismos protetores para retardar e/ou prevenir o acúmulo de espécies reativas e seus efeitos deletérios. Os sistemas de defesa podem ser enzimáticos ou não. As principais enzimas que exercem esse papel são a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx). Essas enzimas evitam o acúmulo de radical hidroxila, contra o qual não existe nenhum sistema enzimático de defesa. As defesas não-enzimáticas incluem antioxidantes lipofílicos (tocoferóis, carotenóides e bioflavonóides) e hidrofílicos (glutationa, ácido ascórbico, indóis e catecóis) (HALLIWELL E GUTTERIDGE, 2007; FANG *et al.*, 2002).

1.5.1.1. Defesas Antioxidantes Enzimáticas

1.5.1.1.1. Superóxido Dismutase

A superóxido dismutase (SOD) catalisa a reação de dismutação do radical superóxido até peróxido de hidrogênio e, conseqüentemente, diminui a formação das ERO e ERN dele derivadas, assim como qualquer dano direto por ele causado (FRIDOVICH, 1995; HALLIWELL E GUTTERIDGE, 2007).



$$2\ H^+ + 2\ O_2^{\bullet -} \rightarrow O_2 + H_2O_2$$

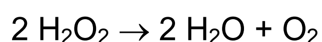
Existem duas formas de superóxido dismutase nas células eucarióticas: a manganês-SOD (Mn-SOD) e a cobre-zinco-SOD (CuZn-SOD). A importância da Mn-SOD, presente na matriz mitocondrial (FRIDOVICH, 1995; WARD E PETERS, 1995), foi demonstrada em estudos onde camundongos geneticamente modificados não a sintetizavam e morriam precocemente com danos mitocondriais severos (LI *et al.*,

1995; LEBOVITZ *et al.*, 1996; MELOV *et al.*, 1999). A CuZn-SOD está localizada no citosol, mas também pode ser encontrada nos lisossomas, núcleo celular, espaço intermembrana da mitocôndria, peroxissomas e fluido extracelular (EC-SOD) (ABRAHAMSSON *et al.*, 1992; FRIDOVICH, 1995; WARD E PETERS, 1995).

Embora a remoção do $O_2^{\bullet-}$ previna a formação de ERO e ERN, o H_2O_2 produzido pela SOD deve ser eliminado pelas enzimas catalase, glutationa peroxidase e também por outras peroxidases para impedi-lo de reagir com metais de transição e gerar o $OH^{\bullet}$ (CADENAS E DAVIES, 2000; HALLIWELL E GUTTERIDGE, 2007).

1.5.1.1.2. Catalase

A catalase (CAT) é uma hemeproteína que acelera a decomposição do peróxido de hidrogênio em água e oxigênio (CHANCE *et al.*, 1979; WARD E PETERS, 1995).



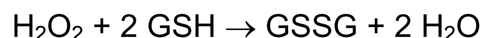
$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

A catalase é encontrada na maioria das células humanas, principalmente nos peroxissomas (CHANCE *et al.*, 1979). Alguns órgãos como o coração, o músculo esquelético e o cérebro, contêm menor quantidade e atividade de catalase que o fígado, o que os tornam mais vulneráveis à ação das ERO e ERN (HALLIWELL E GUTTERIDGE, 2007).

1.5.1.1.3. Glutationa Peroxidase

A glutationa peroxidase (GPx) catalisa a redução do peróxido de hidrogênio e hidroperóxidos orgânicos (LOOH) para seus álcoois correspondentes às custas da

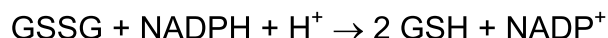
conversão da glutationa reduzida (GSH) a dissulfeto de glutationa (GSSG) (CHANCE *et al.*, 1979; SHAN *et al.*, 1990).



$$H_2O_2 + 2\ GSH \rightarrow GSSG + 2\ H_2O$$

$$LOOH + 2\ GSH \rightarrow GSSG + H_2O + LOH$$

A glutationa redutase (GR), que regenera a GSH a partir do GSSG, é uma flavoproteína dependente de NADPH e, portanto, também dependente da integridade da via das pentoses (CHANCE *et al.*, 1979; GILBERT, 1990).



$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$$

A GPx e a GR estão presentes em diversos compartimentos celulares, inclusive na mitocôndria (CHANCE *et al.*, 1979; LASH *et al.*, 1998; ESPOSITO *et al.*, 2000).

1.5.1.2. <u>Defesas Antioxidantes Não-Enzimáticas</u>

1.5.1.2.1. Glutationa Reduzida

A glutationa reduzida (GSH) é um tripeptídio formado pelos resíduos de glicina, glutamato e cisteína. A capacidade redutora da GSH é determinada pela presença do grupamento tiólico (-SH) da cisteína. Na maioria das células, a GSH é encontrada em elevadas concentrações (mM) no meio intracelular e atua como transportadora e reservatório de cisteína, além de participar da detoxificação de ERO, ERN e produtos da lipoperoxidação. A GSH, ainda, é requerida para a síntese de DNA, proteínas e algumas prostaglandinas (DENEKE E FANBURG, 1989; ANDERSON, 1998; HALLIWELL E GUTTERIDGE, 2007).

1.5.1.2.2. α-Tocoferol

Os tocoferóis (α, β, γ, δ) e os tocotrienos (α, β, γ, δ) provenientes da dieta são os responsáveis pela atividade da vitamina E. A forma biologicamente ativa, o α-tocoferol, é selecionada pelo fígado, incorporada nas lipoproteínas plasmáticas e distribuída pelo organismo, enquanto que os outros tocoferóis são eliminados na bile (HALLIWELL, 2001; HALLIWELL E GUTTERIDGE, 2007). O α-tocoferol confere proteção à membrana celular por reagir com as ERO produzidas durante a lipoperoxidação e o radical α-tocoferil formado pode ser regenerado pelo ácido ascórbico, ubiquinol ou por mecanismos enzimáticos (HALLIWELL E GUTTERIDGE, 2007).

1.5.1.2.3. Ácido Ascórbico

Os seres humanos são incapazes de sintetizar o ácido ascórbico (vitamina C) e devem obtê-lo a partir da dieta (HALLIWELL E GUTTERIDGE, 2007). O ácido ascórbico reage diretamente com várias espécies reativas (oxigênio "singlet", $O_2^{\bullet -}$, $OH^\bullet$, $LO^\bullet$, $LOO^\bullet$, $NOO^\bullet$) e regenera o α-tocoferol a partir do radical α-tocoferil. Contudo, o ácido ascórbico pode agir como pró-oxidante na presença de metais de transição e estimular danos oxidativos às biomoléculas através da formação do $OH^\bullet$ (HALLIWELL, 1999). Além disso, o ácido ascórbico está envolvido em algumas reações de hidroxilação, como aquelas necessárias para a síntese do colágeno, onde atua como cofator. Por esse motivo, a deficiência prolongada de ácido ascórbico pode levar a uma condição patológica denominada escorbuto, em que a incapacidade de sintetizar o colágeno de forma adequada reflete-se no aparecimento de edema e sangramento (HALLIWELL E GUTTERIDGE, 2007).

1.5.2. Estresse Oxidativo

Os sistemas de defesa antioxidante controlam os níveis de espécies reativas, mas não as eliminam e, conseqüentemente, o dano oxidativo às biomoléculas não é completamente evitado. Deste modo, os sistemas de reparo são fundamentais para prevenir o acúmulo de biomoléculas danificadas oxidativamente (HALLIWELL E GUTTERIDGE, 2007).

O termo estresse oxidativo refere-se a qualquer situação em que a geração de espécies reativas excede as defesas antioxidantes e a capacidade de reparo do organismo. Portanto, o estresse oxidativo pode resultar da produção excessiva de espécies reativas, diminuição das defesas antioxidantes, liberação de metais de transição ou da combinação desses fatores (HALLIWELL, 2001).

As células expostas ao estresse oxidativo podem se adaptar através de um aumento das defesas antioxidantes e/ou do sistema de reparo, tornando-se mais resistentes às injúrias subseqüentes (HALLIWELL E GUTTERIDGE, 2007). Além da adaptação celular, o estresse oxidativo pode ocasionar a morte da célula. O comprometimento da função mitocondrial, por exemplo, é capaz de liberar citocromo c, ativar caspases e desencadear a apoptose. Entretanto, o estresse oxidativo severo pode inativar as caspases por oxidação e inibir a apoptose. Nessa situação, provavelmente ocorreria à necrose celular, o que iria resultar na liberação de agentes tóxicos (HALLIWELL, 2001; HALLIWELL, 2006; HALLIWELL E GUTTERIDGE, 2007).

1.5.3. Estresse Oxidativo e o Sistema Nervoso Central

A suscetibilidade do sistema nervoso central ao estresse oxidativo se deve a diversos fatores. Dentre esses, cinco merecem destaque. Primeiro, o alto consumo de oxigênio por unidade de massa tecidual. Segundo, os íons ferro encontram-se em

altas concentrações em certas áreas cerebrais e, quando liberados, são capazes de catalisar reações de produção de ERO. Terceiro, vários neurotransmissores são moléculas auto-oxidáveis e podem reagir com o oxigênio molecular, gerando ERO. Quarto, o cérebro apresenta um conteúdo lipídico alto em relação aos outros tecidos, principalmente quanto ao nível de ácidos graxos poliinsaturados, que são sensíveis ao ataque de ERO. E quinto, os baixos níveis de enzimas antioxidantes e sua localização nas células gliais fazem com que os neurônios estejam menos protegidos contra os oxidantes presentes no cérebro (HALLIWELL, 2006; HALLIWELL E GUTTERIDGE, 2007).

O estresse oxidativo parece estar envolvido em várias doenças ou alterações patológicas que afetam o sistema nervoso central. Entre elas, destacam-se doença de Parkinson, doença de Alzheimer, esclerose múltipla, convulsões e demência (REZNICK E PACKER, 1993; HALLIWELL, 2006; HALLIWELL E GUTTERIDGE, 2007). Porém, existem ainda incertezas e controvérsias no que diz respeito ao estresse oxidativo ser a causa ou a conseqüência das doenças nas quais está envolvido (HALLIWELL, 1994).

Diversos estudos têm demonstrado que o estresse oxidativo pode estar implicado na patogênese dos danos neurológicos de alguns EIM como hiperprolinemia tipo II (DELWING *et al.*, 2003a; DELWING *et al.*, 2003b), fenilcetonúria (KIENZLE-HAGEN *et al.*, 2002) e doença do xarope do bordo (FONTELLA *et al.*, 2002; BRIDI *et al.*, 2003), entre outros. Portanto, torna-se importante investigar o possível papel do estresse oxidativo na neurotoxicidade do GHB e da tirosina a fim de avaliar sua participação nos mecanismos responsáveis pela disfunção neurológica observada nos pacientes com a deficiência da SSADH e a tirosinemia tipo II, respectivamente.

2. OBJETIVO

2.1. GERAL

Investigar o efeito do GHB e da L-tirosina sobre vários parâmetros de estresse oxidativo em homogeneizados de córtex cerebral de ratos jovens a fim de melhor compreender a etiopatogenia dos sintomas neurológicos da deficiência da SSADH e da tirosinemia tipo II.

2.2. ESPECÍFICO

Capítulo I: Estudar o efeito da administração aguda de GHB sobre os níveis de substâncias reativas ao ácido tiobarbitúrico (TBA-RS), o potencial antioxidante total (TRAP) e a reatividade antioxidante total (TAR) em homogeneizados de córtex cerebral de ratos jovens.

Capítulo II: Estudar o efeito da administração aguda de duas doses de 1,4-BD (20 ou 120 mg/Kg) sobre os níveis de TBA-RS, a quimiluminescência espontânea, o TAR, o conteúdo de carbonilas protéicas, o conteúdo de sulfidrilas e as atividades das enzimas antioxidantes SOD, CAT e GPx em homogeneizados de córtex cerebral de ratos jovens.

Capítulo III: Estudar o efeito *in vitro* da L-tirosina sobre diversos parâmetros de estresse oxidativo, tais como TRAP, TAR, conteúdo de ácido ascórbico, conteúdo

de GSH, níveis de TBA-RS, quimiluminescência espontânea, conteúdo de tióis totais e dissulfetos (razão SH/SS), conteúdo de carbonilas protéicas, ligações cruzadas entre DNA e proteínas e as atividades das enzimas SOD, CAT, GPx e glucose-6-fosfato desidrogenase (G6PDH), em homogeneizados de córtex cerebral de ratos jovens.

Capítulo IV: Estudar o efeito da administração aguda de L-tirosina sobre os níveis de TBA-RS, o conteúdo de carbonilas protéicas, o conteúdo de tióis totais e dissulfetos (razão SH/SS), o conteúdo de ácido ascórbico, o conteúdo de GSH e as atividades das enzimas SOD, CAT, GPx e G6PDH em homogeneizados de córtex cerebral de ratos jovens.

3. ARTIGOS CIENTÍFICOS

3.1. CAPÍTULO I

Gamma-hydroxybutyric acid induces oxidative stress in cerebral cortex of young rats.

3.2. CAPÍTULO II

Effects of 1,4-butanediol administration on oxidative stress in rat brain: study of the neurotoxicity of γ-hydroxybutyric acid *in vivo.*

3.3. CAPÍTULO III

Tyrosine promotes oxidative stress in cerebral cortex of young rats.

3.4. CAPÍTULO IV

Tyrosine administration decreases glutathione and stimulates lipid and protein oxidation in rat cerebral cortex.

3.1. CAPÍTULO I

Gamma-hydroxybutyric acid induces oxidative stress in cerebral cortex of young rats

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Os resultados referentes a esta tese são os apresentados na Figura 4.

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γ-Hydroxybutyric acid induces oxidative stress in cerebral cortex of young rats

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Abstract

GHB is a naturally occurring compound in the central nervous system (CNS) whose tissue concentration are highly increased during drug abuse and in the inherited deficiency of succinic semialdehyde dehydrogenase (SSADH) activity. SSADH deficiency is a neurometabolic-inherited disorder of the degradation pathway of γ-aminobutyric acid (GABA). It is biochemically characterized by increased concentrations of γ-hydroxybutyric acid (GHB) in tissues, cerebrospinal fluid (CSF), blood and urine of affected patients. Clinical manifestations are variable, ranging from mild retardation of mental, motor, and language development to more severe neurological symptoms, such as hypotonia, ataxia and seizures, whose underlying mechanisms are practically unknown. In the present study, the *in vitro* and *in vivo* effects of GHB was investigated on some parameters of oxidative stress, such as chemiluminescence, thiobarbituric acid-reactive substances (TBA-RS), total radical-trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), as well as the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) in homogenates from cerebral cortex of 15-day-old Wistar rats. *In vitro*, GHB significantly increased chemiluminescence and TBA-RS levels, while TRAP and TAR measurements were markedly diminished. In contrast, the activities of the antioxidant enzymes SOD, CAT and GPX were not altered by GHB *in vitro*. Acute administration of GHB provoked a significant enhance of TBA-RS levels and a decrease of TRAP and TAR measurements. These results indicate that GHB induces oxidative stress by stimulating lipid peroxidation and decreasing the non-enzymatic antioxidant defenses in cerebral cortex of young rats. If these effects also occur in humans, it is possible that they might contribute to the brain damage found in SSADH-deficient patients and possibly in individuals who consume GHB or its prodrug γ-butyrolactone.

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Keywords: Succinic semialdehyde dehydrogenase deficiency; γ-Hydroxybutyric acid; Oxidative stress

1. Introduction

γ-Hydroxybutyric acid (GHB) is an endogenous constituent of the central nervous system, where it is believed to act as a neurotransmitter or neuromodulator (Cash, 1994; Maitre, 1997). When administered exogenously, GHB is able to cross the blood–brain barrier and produce a number of neuropharmacological effects, including changes in dopamine synthesis and release, anxiolysis, sedation, anesthesia, and electroencephalographic alterations, which resemble those of non-convulsive epilepsy (Cash, 1994; Tunnicliff, 1992; Maitre, 1997). Multiple receptor systems, including the $GABA_B$ receptor, the $GABA_A$-benzodiazepine receptor complex, the GHB-specific binding site, and the opioid receptor, appear to be involved in the expression of the pharmacologic effects of GHB. In this regard, some effects of GHB can be blocked by $GABA_B$ receptor antagonists and mimicked by the $GABA_B$

Abbreviations: ABAP, 2,2′-azo-bis(2-amidinopropane); CAT, catalase; CNS, central nervous system; CSF, cerebrospinal fluid; GABA, γ-aminobutyric acid; GHB, γ-hydroxybutyric acid or 4-hydroxybutyric acid; GPX, glutathione peroxidase; GSH, glutathione; SOD, superoxide dismutase; SSA, succinic semialdehyde; SSADH, succinic semialdehyde dehydrogenase; TAR, total antioxidant reactivity; TBA-RS, thiobarbituric acid-reactive substances; TRAP, total radical-trapping antioxidant potential

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receptor agonist baclofen (Gupta et al., 2002; Quang et al., 2002; Carai et al., 2005). Furthermore, GHB has been clinically used as a general anesthetic and hypnotic agent, as well as in the treatment of narcolepsy and alcoholism, although it has emerged recently as a major recreational drug of abuse (Cash, 1994; Maitre, 1997; Wong et al., 2004).

High levels of GHB are detected in tissues and biological fluids of patients with succinic semialdehyde dehydrogenase (SSADH) deficiency, or γ-hydroxybutyric aciduria (MIM 279180; McKusick, 1998), an autosomal-recessively inherited metabolic disorder in the degradative pathway of the inhibitory neurotransmitter GABA. This enzymatic defect blocks the oxidation of succinic semialdehyde (SSA) to succinic acid, resulting in conversion of SSA to γ-hydroxybutyric acid (GHB), which accumulates and is the biochemical hallmark of this disorder (Gibson and Jakobs, 2001). Clinical manifestations of SSADH deficiency are variable including mild to severe retardation of intellectual, motor, and language development. Many patients also present with hypotonia and non-progressive ataxia, whereas others succumb to a fatal outcome, despite the fact that there is no metabolic acidosis, hyperammonemia, hypoglycemia, or other signs of clinical decompensation with vomiting and/or lethargy (Jakobs et al., 1993; Gibson et al., 1997; Gibson and Jakobs, 2001). Despite an expanding patient population, there is no effective treatment for SSADH deficiency. Vigabatrin, an irreversible GABA transaminase inhibitor, remains the most widely used drug in SSADH deficiency with a variable success (Uziel et al., 1993; Gibson et al., 1995; Gibson and Jakobs, 2001; Ergezinger et al., 2003; Gropman, 2003). The lack of a therapeutic efficacy of this antiepileptic drug reflects heterogeneity at the genetic or enzymatic level (Gibson et al., 1997). On the other hand, Howells et al. (1992) have suggested that vigabatrin has limited use in SSADH deficiency because of differential effects on organ specific GABA transaminases. These investigators postulated that, although vigabatrin effectively inhibits brain GABA transaminase, limited inhibition of peripheral organ transaminases could lead to resupply of GHB across the blood–brain barrier, specially from the liver (Gupta et al., 2004), decreasing the clinical efficacy of therapeutic intervention in the CNS. Although neurological symptoms predominate in SSADH-deficient patients, the mechanisms underlying the neurological dysfunction are poorly known.

Interestingly, elevated levels of GHB were detected *post-mortem* in brain obtained from patients with Huntington's disease (Ando et al., 1979) where the damaging consequences of oxidative stress have been well demonstrated (Halliwell and Gutteridge, 1985; Reznick and Packer, 1993; Halliwell, 2001). In addition, *in vitro* GHB may promote brain energy impairment (Silva et al., 1999), and inhibit dopamine release that is associated with an intracellular accumulation of dopamine (Hechler et al., 1991). Furthermore, enhanced catecholamines turnover was observed in a murine model of SSADH deficiency (Gupta et al., 2003), and in various SSADH-deficient patients (Gibson and Jakobs, 2001; Gibson et al., 2003; Pearl et al., 2003), which could suggest that increased levels of free radicals are related to high concentrations of GHB in brain tissue.

Therefore, in the present work we studied whether GHB can induce oxidative stress in the hope to contribute to better understand the mechanisms underlying the actions of GHB in the central nervous system, including the neurological damage present in SSADH-deficient patients. Thus, we investigated the *in vitro* and *in vivo* effects of GHB on some parameters of oxidative stress in cerebral cortex of young Wistar rats. Oxidative stress was studied by evaluating lipid peroxidation, assessed by chemiluminescence and thiobarbituric acid-reactive substances (TBA-RS), and the antioxidant defenses, represented by the total radical-trapping antioxidant potential (TRAP), the total antioxidant reactivity (TAR), and the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX).

2. Experimental procedures

2.1. Reagents

All chemicals were purchased from Sigma (St. Louis, MO, USA) except thiobarbituric acid and 2,2′-azo-bis(2-amidinopropane) (ABAP), which were purchased from Merck (Darmstadt, Germany) and Wako Chemicals (Richmond, VA, USA), respectively. GHB was prepared on the day of the experiment in the incubation medium for each technique with the pH adjusted when necessary and added to homogenates at final concentrations of 0.1, 0.5 and 1.0 mM. These concentrations were within the average levels found in CSF of SSADH deficient patients (Jakobs et al., 1981, 1990).

2.2. Animals

Fifteen-day-old Wistar rats bred in the Central Animal House of the Department of Biochemistry, ICBS, UFRGS were used. Rats were kept with dams until they were sacrificed. The dams had free access to water and a 20% (w/w) protein commercial chow (Supra, Porto Alegre, RS, Brazil). They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air-conditioned constant temperature (22 ± 1 °C) colony room. The "Principles of Laboratory Animal Care" (NIH publication number 80-23, revised 1996) were followed throughout the experiments.

2.3. *In vitro experiments*

2.3.1. *Tissue preparation and incubation*

Rats were sacrificed by decapitation without anesthesia, and the brain was rapidly removed and kept on an ice-plate. The olfactory bulb, pons and medulla were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization. Cerebral cortex was homogenized (1:10, w/v) in 20 mM sodium phosphate buffer containing 140 mM KCl (pH 7.4) for chemiluminescence, TBA-RS, TRAP and TAR. For the antioxidant enzyme activity determinations, cerebral cortex was homogenized in 10 volumes (1:10, w/v) of the specific medium for each technique. Homogenates were centrifuged at 750 × *g* for 10 min at 4 °C to remove nuclei and cell debris (Llesuy et al., 1985). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, was separated and immediately used for analyses.

Cerebral cortex supernatants were incubated at 37 °C for 1 h in the absence (controls) or presence of GHB, at final concentrations ranging from 0.1 to 1.0 mM. After incubation, aliquots were taken to measure chemiluminescence, TBA-RS, TRAP and TAR. To test the effect of GHB on the activity of the antioxidant enzymes SOD, CAT and GPX, the organic acid was added to the cerebral cortex homogenates at the time of assay of each enzyme activity without previous incubation.

2.4. *In vivo experiments*

2.4.1. *GHB administration*

GHB was dissolved in physiological saline solution with pH adjusted to 7.2–7.4. Animals received one intraperitoneal injection of GHB solution (10 mg/kg body weight) or an equal volume of saline (controls). Sixty minutes after the administration rats were killed by decapitation without anesthesia as described above. Cerebral cortex homogenates were prepared as mentioned for the *in vitro* studies and used to measure TBA-RS, TRAP and TAR.

2.4.2. *Chemiluminescence*

Samples were assayed for chemiluminescence in a dark room using a beta liquid scintillation spectrometer Tri-Carb 2100TR (Lissi et al., 1986). Incubation flasks contained 3.5 mL of the medium, and the background chemiluminescence was measured for 5 min. An aliquot of 0.5 mL of supernatant was added and chemiluminescence was measured for 10 min at room temperature. The background chemiluminescence was subtracted from the total value. Chemiluminescence was calculated as cps/mg protein.

2.4.3. *Thiobarbituric acid-reactive substances (TBA-RS)*

For the *in vitro* experiments, TBA-RS were measured as described as Esterbauer and Cheeseman (1990) using a Beckman DU® 640 Spectrophotometer. Briefly, 300 μL of cold 10% (w/v) trichloroacetic acid were added to 150 μL of supernatant and centrifuged at 300 × *g* for 10 min. Three hundred microliters of the supernatant were transferred to a Pyrex tube and incubated with 300 μL of 0.67% (w/v) thiobarbituric acid in 7.1% (w/v) sodium sulfate in a boiling water bath for 25 min. The mixture was allowed to cool on water for 5 min. The resulting pink stained TBA-RS was determined in a spectrophotometer at 532 nm. Calibration curve was performed using 1,1,3,3-tetramethoxypropane as standard. TBA-RS was calculated as nmol TBA-RS/mg protein.

For the *in vivo* experiments, TBA-RS were determined according to the method described by Ohkawa et al. (1979). Tissue homogenates (1:10) were prepared in 1.15% KCl. Briefly, 50 μL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid solution pH 3.5, 1.5 mL of 0.8% aqueous solution of TBA, and 700 μL of distilled water were added to 500 μL of homogenate in a Pyrex tube and then heated in a boiling water bath for 60 min. After cooling, the mixture was centrifugated at 1000 × *g* for 10 min. The resulting pink stained TBA-RS was determined in a spectrophotometer at 532 nm. The results were represented as nmol of TBA-RS/mg protein.

2.4.4. *Total radical-trapping antioxidant potential (TRAP)*

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by ABAP thermolysis in a Wallac 1409 Scintillation Counter (Lissi et al., 1992; Evelson et al., 2001). The reaction mixture contained 3 mL of 10 mM ABAP and 10 μL of 4 mM luminol dissolved in 50 mM sodium phosphate buffer (pH 7.4) and 0.1 M NaOH, respectively. Incubation of this mixture generates an almost constant light intensity at room temperature. Ten microliters of 300 μM Trolox (water-soluble α-tocopherol analogue) or tissue supernatant was added to asses their antioxidant content. At this point, the luminescence intensity is practically abolished. The time required to consume the active antioxidants present in homogenates was evaluated by observing the return of the luminescence and this was compared to that obtained employing Trolox under identical experimental conditions. TRAP values were calculated as nmol Trolox/mg protein.

2.4.5. *Total antioxidant reactivity (TAR)*

TAR was determined by measuring the luminol chemiluminescence intensity induced by ABAP using a Wallac 1409 Scintillation Counter (Lissi et al., 1995). The background chemiluminescence was measured by adding 4 mL of 2 mM ABAP plus 15 μL of luminol (4 mM) into a glass scintillation vial. This was considered to be the basal value. Ten microliters of 10–100 μM Trolox (curve calibration) or tissue supernatant was then added and the chemiluminescence was measured during 60 s. The rapid reduction in luminol intensity is considered as a measure of its TAR capacity. The results were calculated as nmol Trolox/mg protein.

2.4.6. *Superoxide dismutase assay*

The assay of SOD activity was carried out as described by Marklund (1985). Cerebral tissue was homogenized 1:10 (w/v) in 50 mM Tris–HCl buffer containing 1 mM EDTA (pH 8.2). 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 spectrophotometrically at 420 nm, using a double-beam spectrophotometer with temperature control (Hitachi U-2001). 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.

2.4.7. *Catalase assay*

CAT activity was assayed by the method of Aebi (1984) using a double-beam spectrophotometer with temperature control (Hitachi U-2001). Cerebral cortex was homogenized 1:10 (w/v) in 10 mM potassium phosphate buffer (pH 7.0). This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer (pH 7.0), and 0.1–0.3 mg protein/mL. One CAT unit is defined as 1 μmol of H_2O_2 consumed per minute and the specific activity is represented as units per mg protein.

2.4.8. *Glutathione peroxidase assay*

GPX activity was measured according to the method of Wendel (1981) using *tert*-butyl hydroperoxide as substrate. Tissue was homogenized in 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.7). NADPH disappearance was monitored at 340 nm using a double-beam spectrophotometer with temperature control (Hitachi U-2001). The reaction medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl hydroperoxide and 0.1 mM NADPH. One GPX unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as units per mg protein.

2.4.9. *Protein determination*

Protein concentration was determined in cerebral cortex supernatants using bovine serum albumin as standard (Lowry et al., 1951).

2.4.10. *Statistical analysis*

Data were analyzed by the Student's *t*-test or one-way analysis of variance (ANOVA) followed by the post hoc Tukey multiple range test when the F value was significant. Linear regression analysis was also used to verify dose-dependent effects and the Pearson correlation to test for associations between parameters of oxidative stress. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p < 0.05$ was considered to be significant.

3. Results

For the *in vitro* studies, cerebral cortex homogenates were incubated with different concentrations of GHB and after 1 h aliquots were taken out to measure the parameters of lipid peroxidation and non-enzymatic antioxidant defenses.

Firstly, the *in vitro* effect of GHB on lipid peroxidation was investigated by measuring chemiluminescence and TBA-RS levels in rat cerebral cortex (Fig. 1). Fig. 1A shows that GHB concentrations strongly increased chemiluminescence [$F(3, 16) = 33.38$; $p < 0.001$] in a dose-dependent way ($\beta = 0.92$; $p < 0.001$). In addition, TBA-RS levels were significantly increased by the presence of GHB [$F(3, 12) = 13.51$; $p < 0.001$], also in a dose-dependent manner ($\beta = 0.88$; $p < 0.001$) (Fig. 1B). These results show that GHB stimulates lipid peroxidation in cerebral cortex homogenates.

Next, the antioxidant capacity of cerebral cortex homogenates was studied by determining TRAP and TAR, which

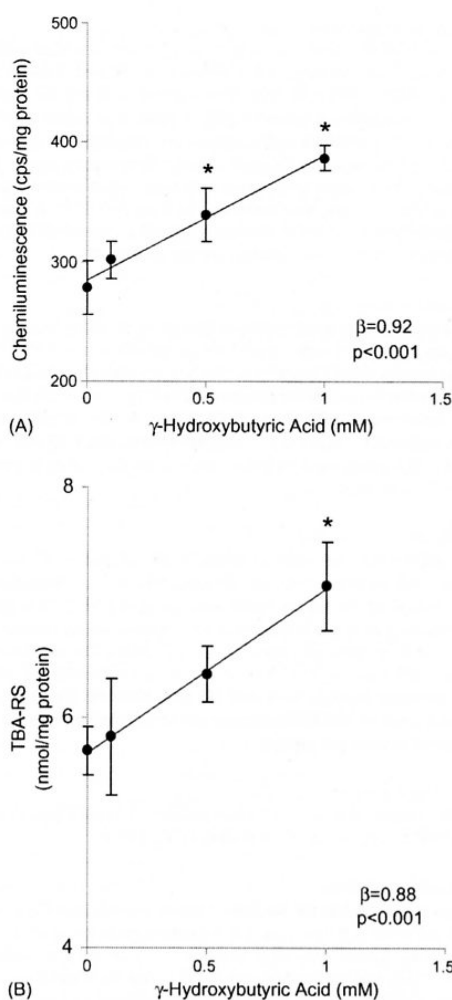

Fig. 1. *In vitro* effect of γ-hydroxybutyric acid on chemiluminescence (A) and thiobarbituric acid-reactive substances (TBA-RS) (B) in homogenates from cerebral cortex of rats. Data are expressed as mean ± S.D. for four to five independent experiments performed in duplicate. $^*p < 0.01$ compared to control (Tukey multiple range test).

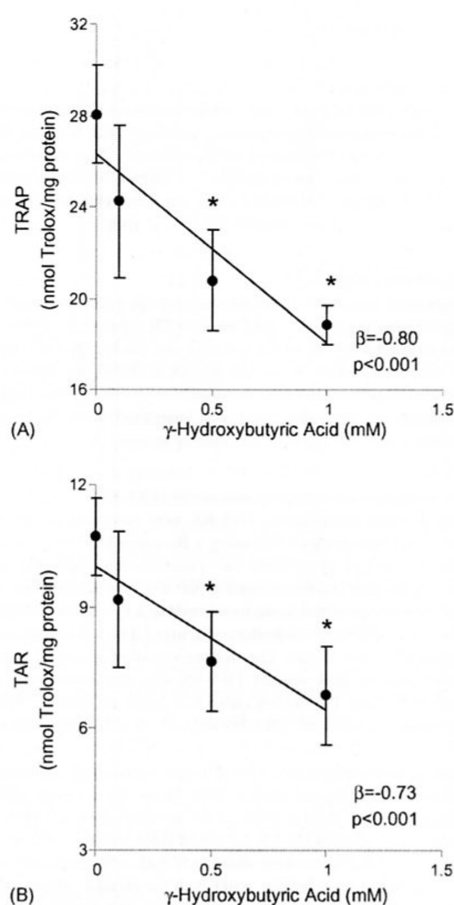

Fig. 2. *In vitro* effect of γ-hydroxybutyric acid on total radical-trapping antioxidant potential (TRAP) (A) and total antioxidant reactivity (TAR) (B) in homogenates from cerebral cortex of rats. Data are expressed as mean ± S.D. for four to five independent experiments performed in duplicate. $^*p < 0.01$ compared to control (Tukey multiple range test).

represent non-enzymatic antioxidant quantity and reactivity, respectively. It can be observed in Fig. 2A that 0.5 and 1.0 mM GHB markedly decreased TRAP [$F(3, 12) = 12.374$; $p < 0.001$] in a concentration-dependent way ($\beta = -0.80$; $p < 0.001$). Similarly, TAR measurement was significantly reduced in a dose-related fashion when cerebral cortex was exposed to GHB [$F(3, 16) = 8.86$; $p < 0.001$] ($\beta = -0.73$; $p < 0.001$) (Fig. 2B). These findings suggest that the non-enzymatic antioxidant defenses of cerebral cortex homogenates are significantly diminished by GHB.

These *in vitro* effects of GHB are strengthened by a significantly inverse correlation between chemiluminescence and TRAP (Pearson correlation = −0.834; $p < 0.001$) as illustrated in Fig. 3.

The effect of GHB was also evaluated on the activities of the antioxidant enzymes SOD, CAT and GPX (Table 1). This set of experiments was carried out without pre-incubation of GHB with the homogenates previously to the enzymatic activity assays. This organic acid did not alter the activities of SOD [$F(3, 8) = 1.4$; $p > 0.05$], CAT [$F(3, 24) = 0.64$; $p > 0.05$] and GPX [$F(3, 8) = 1.59$; $p > 0.05$]. These results indicate that GHB does not directly affect the activities of enzymatic antioxidant defenses.

Finally, the effect of the acute administration of GHB on lipid peroxidation and non-enzymatic antioxidant defenses was investigated in cerebral cortex homogenates. This was achieved by measuring TBA-RS, TRAP and TAR. Fig. 4A shows that rats subjected to acute administration of GHB significantly increased TBA-RS levels [$t(20) = -2.47$; $p < 0.05$], whereas TRAP [$t(22) = 2.53$; $p < 0.05$] (Fig. 4B) and TAR [$t(22) = 2.69$; $p < 0.05$] (Fig. 4C) measurements were markedly reduced.

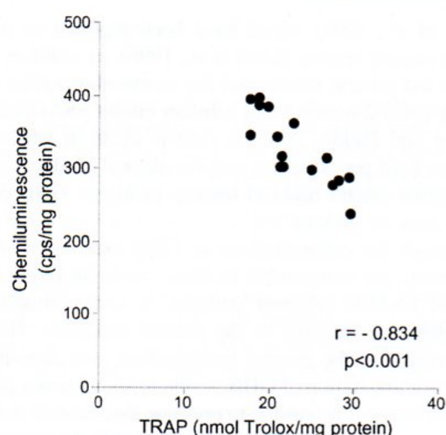

Fig. 3. Correlation between chemiluminescence and total radical-trapping antioxidant potential (TRAP) in homogenates from cerebral cortex of rats. Data were taken from experiments on the *in vitro* effect of GHB (Figs. 1A and 2A).

Taken together, these findings clearly show that GHB induces oxidative stress in rat cerebral cortex.

4. Discussion

GHB is a naturally occurring compound in the CNS whose tissue concentration are highly increased during drug abuse and in the inherited deficiency of SSADH activity. The understanding of the mechanisms by which GHB elicits its actions might contribute to a better therapeutic management in such situations, especially of SSADH-deficient patients in which the current therapy with vigabatrin is not uniformly successful (Uziel et al., 1993; Gibson et al., 1995; Gibson and Jakobs, 2001; Ergezinger et al., 2003; Gropman, 2003).

Recently, Gupta et al. (2003) observed that SSADH-knockout mice present elevated levels of homovanilic acid, a product of dopamine metabolism, suggesting that oxidative stress may be involved of in SSADH deficiency. Therefore, to test this hypothesis and to better understand the mechanisms of GHB neurotoxicity, in the present study we evaluated the *in vitro* and *in vivo* effects of GHB on some parameters of oxidative stress in homogenates from cerebral cortex of young rats.

Initially, the *in vitro* influence of GHB on lipid peroxidation was investigated by measuring chemiluminescence and

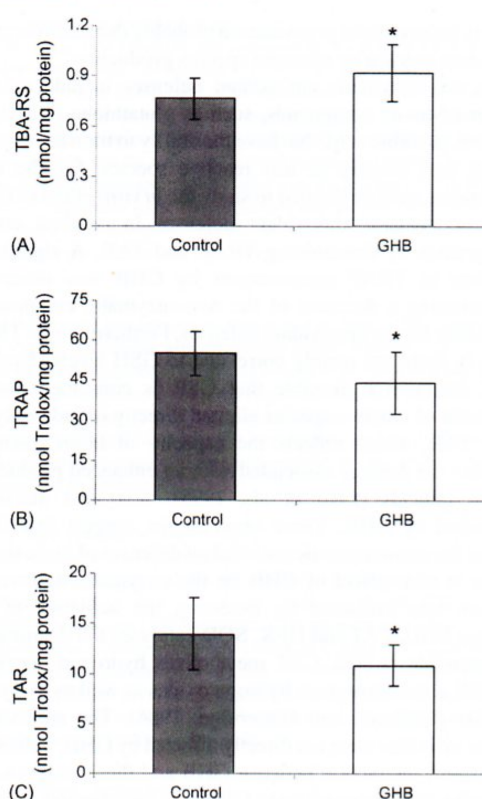

Fig. 4. Effect of acute administration of γ-hydroxybutyric acid (GHB) on thiobarbituric acid-reactive substances (TBA-RS) (A), total radical-trapping antioxidant potential (TRAP) (B) and total antioxidant reactivity (TAR) (C) in homogenates from cerebral cortex of rats. Data are expressed as mean ± S.D. (n = 10–13). $^{*}p < 0.05$ compared to control (Student's t-test).

TBA-RS levels. GHB, at concentrations similar to those found in CSF (0.6 mM) and blood (1.0 mM) of patients affected by SSADH deficiency (Jakobs et al., 1981, 1990), significantly increased chemiluminescence and TBA-RS levels in cerebral cortex homogenates of young rats. Considering that the light emitted in chemiluminescence assay arises from peroxidizing lipids (Halliwell and Gutteridge, 1999) and that TBA-RS reflects the content of malondialdehyde, the most abundant individual aldehyde resulting from lipid peroxidation (Esterbauer and Cheeseman, 1990), these findings indicate that GHB

Table 1
In vitro effect of γ-hydroxybutyric acid on catalase (CAT), superoxide dismutase (SOD) and gluthatione peroxidase (GPX) activities in homogenates from cerebral cortex of rats

Antioxidant enzyme activity (U/mg protein)	γ-Hydroxybutyric acid (mM)			
	0	0.1	0.5	1.0
CAT (n = 7)	12.30 ± 1.90	11.52 ± 1.92	11.10 ± 1.45	11.34 ± 1.54
GPX (n = 3)	18.91 ± 2.34	19.91 ± 0.51	17.65 ± 0.95	19.50 ± 0.82
SOD (n = 3)	4.87 ± 0.37	5.84 ± 0.46	5.16 ± 0.87	5.29 ± 0.63

Data are expressed as mean ± S.D. for three to seven independent experiments performed in duplicate. One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute. One GPX unit is defined as 1 μmol of NADPH consumed per minute. One SOD unit is defined as 50% inhibition of pyrogallol autoxidation. No significant differences from controls were detected by ANOVA.

strongly induces lipid peroxidation probably due to an increase in reactive oxygen or nitrogen species production.

The non-enzymatic antioxidant defenses include a large number of tissue compounds, such as glutathione (GSH), α-tocoferol, ascorbic acid, that have the ability to transform active radicals into inactive or less reactive species. So, the next experiments were performed to study the *in vitro* effect of GHB on non-enzymatic antioxidant defenses in cerebral cortex homogenates by determining TRAP and TAR. A significant reduction in TRAP measurement by GHB was observed, demonstrating a decrease of the non-enzymatic components responsible for the antioxidant defenses. Furthermore, as TRAP values in brain are mainly correlated to GSH levels (Evelson et al., 2001), it is feasible that GSH is consumed due to generation of reactive species elicited directly or indirectly by GHB. TAR, which reflects the capacity of brain tissue to modulate the damage associated with an enhanced production of free radicals (Lissi et al., 1995), was also markedly diminished by GHB. These observations suggest that GHB reduces the non-enzymatic antioxidant defenses of brain tissue.

The *in vitro* effect of GHB on the enzymatic antioxidant defenses was evaluated by assessing the activities of the enzymes SOD, CAT and GPX. SOD catalyses the dismutation of superoxide radical, CAT metabolizes hydrogen peroxide, and GPX removes organic hydroperoxides as well as hydrogen peroxide (Halliwell and Gutteridge, 1985). The antioxidant enzyme activities were not directly affected by GHB, indicating no apparent interaction between GHB and these enzymes.

Finally, the *in vivo* effect of GHB was investigated on some parameters of oxidative stress. The acute administration of GHB increased TBA-RS levels while TRAP and TAR measurements were reduced by the treatment. These results are in agreement with our previous *in vitro* experiments and strongly indicate that GHB stimulates lipid peroxidation and decreases the non-enzymatic defenses in the SNC.

It is considered that oxidative stress can result from distinct situations, such as generation of reactive species at abnormally high rate, insufficient antioxidant defenses, releasing of transition metal ions, or due to a combination of these conditions (Halliwell, 2001). Therefore, the results presented here clearly demonstrate that GHB induces oxidative stress by stimulating lipid peroxidation and by decreasing the non-enzymatic antioxidant defenses in homogenates from cerebral cortex of young rats. The inverse correlation between lipid peroxidation and the antioxidant capacity of cerebral cortex homogenates *in vitro* (Fig. 3) suggests that GHB either by stimulating lipid peroxidation consumes and decreases the amount tissue antioxidants or by decreasing the antioxidant capacity due to activation of free radical synthesis enhances lipid peroxidation. On the other hand, we cannot rule out that a derivative of GHB may be the species leading to oxidative stress in our experiments, since the samples used (cerebral cortex homogenates) have the enzymatic machinery needed to convert GHB to SSA, GABA or even D-α-hydroxyglutarate (Kaufman and Nelson, 1991; Cash, 1994; Kaufman et al., 1998). Moreover, increased guanidine compounds were recently reported in murine and human SSADH deficiency (Jansen et al., 2006) which have been reported to produce reactive oxygen species (Mori et al., 1996). In addition, taking together the present results and the recent observation that 4-hydroxy-*trans*-2-nonenal and acrolein inhibit SSADH activity (Nguyen and Picklo, 2003) a vicious cycle in which GHB enhances lipid peroxidation and the alkenal products of lipid peroxidation inhibit SSADH leading to higher GHB concentrations may be present.

Although the concentrations of GHB used in the *in vitro* experiments are comparable to those found in physiological fluids of SSADH-deficient patients, it seems simplistic to extrapolate our findings to the human condition. However, considering that the present investigation also demonstrated that the administration of GHB is able to significantly promote oxidative stress, it is feasible to presume that oxidative damage may be one of the mechanisms contributing to the neurological dysfunction characteristic of SSADH deficiency. On the other hand, other mechanisms should be taken into account when we consider our *in vivo* results, specially those mediated by receptors. Since GHB produces oxidative stress *in vivo*, the administration of antioxidants should be considered as an adjuvant therapy for patients affected by SSADH deficiency. Furthermore, considering the use of GHB therapeutically or as a recreative drug, even though we do not know the concentrations of GHB reached in the brain in these situations, the participation of free radicals in the mechanisms of action and/or toxicity of GHB in the central nervous system should be considered. Therefore, the findings of the present work may have implications for SSADH-deficient patients and possibly to individuals who consume GHB, or its prodrug γ-butyrolactone.

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3.2. CAPÍTULO II

Effects of 1,4-butanediol administration on oxidative stress in rat brain: study of the neurotoxicity of γ-hydroxybutyric acid *in vivo*

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Effects of 1,4-butanediol administration on oxidative stress in rat brain: study of the neurotoxicity of γ-hydroxybutyric acid *in vivo*

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Running title: γ-Hydroxybutyric acid and oxidative stress in rat brain

Abstract

γ-Hydroxybutyric acid (GHB) is a naturally occurring compound in the central nervous system (CNS) whose tissue concentration are highly increased in the neurometabolic-inherited deficiency of succinic semialdehyde dehydrogenase (SSADH) activity or during drug abuse. SSADH deficiency is biochemically characterized by increased concentrations of GHB in tissues, cerebrospinal fluid, blood and urine of affected patients. Clinical manifestations are variable and include retardation of mental, motor, and language development along with other neurological symptoms, such as hypotonia, ataxia and seizures, whose underlying mechanisms are practically unknown. The precursor of GHB, 1,4-butanediol (1,4-BD) has been used to study the mechanisms of *in vivo* GHB neurotoxicity. Therefore, in the present work, the effect of acute administration of 20 or 120 mg/Kg 1,4-BD was investigated on various parameters of oxidative stress, such as spontaneous chemiluminescence, thiobarbituric acid-reactive substances (TBA-RS), total antioxidant reactivity (TAR), sulfhydryl and protein carbonyl contents, as well as the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in homogenates from cerebral cortex of 14-day-old Wistar rats. Acute administration of 120 mg/Kg 1,4-BD significantly increased spontaneous chemiluminescence and TBA-RS levels, while TAR measurement was markedly diminished, whereas injection of a lower dose (20 mg/Kg) did not change the parameters examined. Other parameters of oxidative stress evaluated were not affected by administration of 1,4-BD. These results indicate that 1,4-BD induces *in vivo* oxidative stress by stimulating lipid peroxidation and decreasing the non-enzymatic antioxidant defenses in cerebral cortex of young rats. If these effects also

occur in humans, it is possible that they might contribute to the brain damage found in SSADH-deficient patients and possibly in individuals intoxicated by GHB or its prodrugs (γ-butyrolactone or 1,4-BD).

Introduction

The aliphatic diol 1,4-butanediol (1,4-BD) is a naturally occurring alcohol and a largely used industrial solvent. In recent years, 1,4-BD has been marketed in the USA as an alternative to the popular drug of abuse γ-hydroxybutyric acid (GHB). 1,4-BD is metabolized by alcohol dehydrogenase to γ-hydroxybutyraldehyde, which is then converted by aldehyde dehydrogenase to GHB (Roth and Giarman, 1968; Snead *et al.*, 1989). 1,4-BD has been found to have a number of acute and chronic effects. Acute effects include euphoria, ataxia, confusions, hallucinations and loss of consciousness (Shannon and Quang, 2000; Teter and Guthrie, 2001). In animals, 1,4-BD causes alterations in locomotor activity, sedation/hypnosis and hypothermia (Kaufman *et al.*, 1990; Carai *et al.*, 2002; de Fiebre *et al.*, 2004). Much evidence suggests that 1,4-BD acts primarily as a prodrug of GHB. Since 1,4-BD itself does not bind to GHB, γ-aminobutyric acid $(GABA)_A$, or $GABA_B$ receptors (Roth and Giarman, 1968; Beanvides *et al.*, 1982; Snead and Liu, 1984) it is assumed that GHB is the pharmacologically active species when 1,4-BD is used (Poldrugo and Snead, 1984; Snead *et al.*, 1989, Carai *et al.*, 2002; Quang *et al.*, 2002; Carter *et al.*, 2006).

GHB is a neurotransmitter or a neuromodulator that occurs in mammalian central nervous system (CNS) and possesses different physiological and pharmacological properties (Cash, 1994; Maitre, 1997). GHB has been clinically used as an intravenous anesthetic, as well as in the treatment of narcolepsy-associated catalepsy. More recently, GHB has been proposed as an effective agent in pharmacotherapy of alcohol and opiate dependence (Cash, 1994; Maitre, 1997; Wong *et al.*, 2004; Snead and Gibson., 2005). When administered exogenously, GHB crosses the blood brain barrier and produces behavioral, eletrophysiological

and biochemical effects, including changes in dopaminergic, GABAergic and opioid systems, anxiolysis, sedation and eventually anesthesia accompanied in some animal species by electroencephalographic alterations, which resemble those of nonconvulsive epilepsy (Tunnicliff, 1992; Cash, 1994; Maitre, 1997; Maitre *et al.*, 2000). Available biochemical data suggest that the intrinsic neurobiological activity of GHB is mediated through the GHB receptor while many of the pharmacologic, clinical, behavioral, and toxicological effects of exogenously administered GHB appear to be mediated through the $GABA_B$ receptor, where GHB may act both directly and indirectly (Maitre, 1997; Snead and Gibson, 2005).

GHB accumulates at supraphysiological concentrations in succinic semialdehyde dehydrogenase (SSADH) deficiency, which is an inborn error of GABA metabolism caused by a defect in the enzymatic oxidation of succinic semialdehyde (SSA) to succinic acid, resulting in conversion of SSA to GHB (Gibson and Jakobs, 2001). SSADH-deficient patients present a heterogeneous and nonspecific neurological phenotype, including delayed intellectual, motor, and speech and language development in conjunction with hypotonia, hyporeflexia, seizures, non-progressive ataxia, and neuroradiological abnormalities (Jakobs *et al.*, 1993; Gibson *et al.*, 1997; Gibson and Jakobs, 2001). Although neurological symptoms predominate in SSADH-deficient patients, the mechanisms of brain damage remain not fully established. Treatment options in SSADH deficiency are few, and primarily symptomatic.

Previous data suggested that oxidative stress might contribute to the neurodegeneration in SSADH deficiency. Enhanced catecholamine turnover that is associated with intracellular free radical accumulation and mitochondrial dysfunction (Berman and Hastings, 1999; Graham *et al.,* 1978; Sulzer and Zecca, 2000) was

observed in a murine model of SSADH deficiency (Gupta *et al.*, 2003), and in various SSADH-deficient patients (Gibson and Jakobs, 2001; Gibson *et al.*, 2003; Pearl *et al.*, 2003). Furthermore, Sauer *et al.* (2007) also observed a decrease of glutathione content and decreased activities of the respiratory chain complexes I-IV in CNS of SSADH-deficient mice, suggesting increased oxidative stress and mitochondrial damage. In this context, we have recently shown that oxidative stress indeed occurs in the murine knockout model of SSADH deficiency (Latini *et al.*, 2007) but the neurotoxin responsible for this deleterious mechanism is yet unknown since increased levels of guanidinoacetate and guanidinobutyrate have been also found in the SSADH-null mice (Jansen *et al.*, 2006). Interestingly, these compounds are able to induce oxidative stress *in vitro* and *in vivo* (Zugno *et al.,* 2004, Zugno *et al.,* 2006). GHB also inhibited lipid synthesis and CO_2 production *in vitro*, indicating impairment of mitochondrial metabolism which may secondarily inhibit lipid synthesis (Silva *et al.,* 1999).

In the present work we studied a possible role of GHB inducing oxidative stress in the brain in order to better understand the mechanisms underlying the actions of GHB in the central nervous system. Considering that the availability of GHB is restricted, we determine the effects of acute treatment with 1,4-BD, a GHB precursor, on the following oxidative stress parameters in cerebral cortex of 14-day-old rats: spontaneous chemiluminescence and thiobarbituric acid-reactive substances (TBA-RS), to access lipid peroxidation; total antioxidant reactivity (TAR), to evaluate non-enzymatic antioxidant defenses; sulfhydryl and protein carbonyl contents, to measure protein oxidation; and activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), to assess enzymatic antioxidant defenses.

Experimental Procedures

Reagents and Equipments

All chemicals were purchased from Sigma (St. Louis, MO, USA) except for thiobarbituric acid and 2,2'-azo-bis(2-amidinopropane) (ABAP), which were purchased from Merck (Darmstadt, Germany) and Wako Chemicals (Richmond, VA, USA), respectively. A double-beam spectrophotometer with temperature control (Hitachi U-2001), Beckman DU® 640 spectrophotometer and Wallac 1409 Scintillation Counter were used for the measurements. Eppendorf 5417R (refrigerated version) and Eppendorf 5403 were used for the centrifugation procedures.

Animals

Fourteen-day-old Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, were used. Rats were kept with dams until they were killed. The dams had free access to water and 20% (w/w) protein commercial chow (Supra, Porto Alegre, RS, Brazil). They were maintained in a room with a 12:12 h light/dark cycle (lights on 7:00-19:00 h) and with air-conditioned controlled temperature (22 ± 1°C). The Principles of Laboratory Animal Care (NIH publication # 80-23, revised 1996) were followed throughout the experiments.

Acute administration of 1,4-BD and tissue preparation

1,4-BD was dissolved in saline solution and the pH was adjusted to 7.4. 1,4-BD was administered intraperitoneally in the following doses: 20 or 120 mg/Kg body weight. Controls received saline solution. One hour after injection, rats were killed by decapitation without anesthesia, and the brains were rapidly removed and

kept on an ice-plate. The olfactory bulb, pons and medulla were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization. Cerebral cortex was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4ºC to discard nuclei and cell debris (Llesuy *et al.*, 1985; Lissi *et al.*, 1986). The pellet was discarded and the supernatant was immediately separated and used for the measurements.

Spontaneous chemiluminescence

Samples were assayed for spontaneous chemiluminescence in a dark room using a scintillation counter (Lissi *et al.*, 1986). The background chemiluminescence was measured for 5 min in vials containing 3.5 mL of the same buffer used for homogenization. An aliquot of 0.5 mL of sample was then added and chemiluminescence was measured for 10 min at room temperature. The background chemiluminescence was subtracted from the final value. Spontaneous chemiluminescence was represented as cps/mg protein.

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS were measured according to Ohkawa *et al.* (1979). Briefly, to glass tubes were added, in order of appearance: 500 μL of sample; 50 μL of sodium dodecyl sulfate 8.1%; 1500 μL of 20% acetic acid in aqueous solution (v/v) pH 3.5; 1500 μL of 0.8 % thiobarbituric acid; and 700 μL of distilled water. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 hour. The mixture was allowed to cool on water for 5 min and was centrifuged 750 *g* for 10 min. The resulting pink stained TBA-RS were determined in a spectrophotometer at 532

nm. TBA-RS were calculated as nmol/mg protein. A calibration curve was performed using 1,1,3,3-tetramethoxypropane as a standard. TBA-RS were represented as nmol TBA-RS/mg protein.

Total antioxidant reactivity (TAR)

TAR was determined by measuring the luminol chemiluminescence intensity induced by ABAP (free radical source) using a scintillation counter (Lissi *et al*., 1995). The chemiluminescence was measured by adding 4 mL of 2 mM ABAP plus 15 μL of luminol (4 mM) into a glass scintillation vial. This was considered to be the basal value. Ten μL of 10 to 100 μM Trolox (standard curve) or samples were then added and the chemiluminescence was measured during 60 s. The rapid initial reduction of luminescence is considered as a measure of TAR capacity of the samples. The results were calculated as Trolox equivalents and were represented as nmol Trolox/mg protein.

Sulfhydryl Content

This assay is based on the reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, which in turn become oxidized (disulfide), generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). Briefly, 50 μL of homogenate were added to 1 mL of PBS buffer pH 7.4 containing 1 mM EDTA. Then 30 μL of 10 mM DTNB, prepared in a 0.2 M potassium phosphate solution pH 8.0, were added. Subsequently, 30 min incubation at room temperature in a dark room was performed. Absorption was measured at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were represented as nmol TNB/mg protein.

Protein carbonyl content

Protein carbonyl content was assayed by a method based on the reaction of carbonyls with dinitrophenylhydrazine (DNPH) forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer, 1994). Briefly, 200 μL of sample were added to 400 μL 10 mM DNPH in 2 M HCl, or to 2 M HCl (blank). This mixture was kept in dark room for 1 hour and vortexed each 15 min. After that, 500 μL of 20% trichloroacetic acid were added to each tube. The mixture was vortexed and centrifuged at 20,000 *g* for 3 min. The supernatant obtained was discarded. The pellet was washed with 1 mL ethanol:ethyl acetate (1:1, v/v), vortexed and centrifuged at 20,000 *g* for 3 min to remove the free DNPH. The supernatant was discarded and the pellet was resuspended in 600 μL of 6 M guanidine (prepared in a 20 mM potassium phosphate pH 2.3), vortexed and incubated at 60 ^{o}C for 15 min. After that, it was centrifuged at 20,000 *g* for 3 min and the supernatant was used to measure absorbance in a spectrophotometer at 370 nm. The molar extinction coefficient of 22,000 for DNPH was used to calculate the concentration of protein carbonyls. Results were represented as carbonyl content (nmol/mg protein).

Superoxide dismutase (SOD) assay

The assay of SOD activity was carried out as described by Marklund (1985). 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 spectrophotometrically at 420 nm, using a double-beam spectrophotometer with temperature control (Hitachi U-2001). A calibration curve was performed with purified

SOD as a 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.

Catalase (CAT) assay

CAT activity was assayed by the method of Aebi (1984) using a spectrophotometer. This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0. One CAT unit is defined as one μmol of H_2O_2 consumed per minute and the specific activity is represented as units per mg protein.

Glutathione peroxidase (GPx) assay

GPx activity was measured according to the method of Wendel (1981) using *tert*-butyl hydroperoxide as substrate. NADPH disappearance was monitored at 340 nm using a spectrophotometer. The reaction medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as one μmol of NADPH consumed per minute and the specific activity is represented as units per mg protein.

Protein Determination

Protein concentration was determined in cerebral cortex homogenates using bovine serum albumin as a standard (Lowry *et al.*, 1951).

Statistical analysis

Data were analyzed by the Student's t-test for unpaired samples. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

Results

Lipid peroxidation

Lipid peroxidation was investigated by measuring spontaneous chemiluminescence and TBA-RS levels in rat cerebral cortex homogenates after acute administration of 1,4-BD. Figures 1 and 2 show that the acute administration of 1,4-BD at the dose of 120 mg/Kg was able to increase spontaneous chemiluminescence [$t(10)=2.84$; $p<0.05$] and TBA-RS levels [$t(23)=2.11$; $p<0.05$], respectively, in rat cerebral cortex. These results demonstrate that *in vivo* 1,4-BD administration promotes lipid peroxidation in cerebral cortex homogenates from 14-day-old rats.

Non-enzymatic antioxidant defenses

The effect of acute administration of 1,4-BD on the antioxidant capacity of cerebral cortex homogenates was studied by determining TAR, which represents non-enzymatic antioxidant reactivity. It can be observed in Figure 3 that rats subjected to acute administration of 1,4-BD 120 mg/Kg significantly decreased TAR [$t(12)=4.13$; $p<0.05$]. These data indicate that the non-enzymatic antioxidant defenses may be compromised by acute administration of 1,4-BD 120 mg/Kg.

Protein oxidation

The effect of acute administration of 1,4-BD on tissue proteins was also evaluated. To accomplish this, two different parameters of oxidative protein damage were measured: sulfhydryl and carbonyl content. Table 1 shows that 1,4-BD had no effect on sulfhydryl and protein carbonyl content in cerebral cortex. These findings suggest that oxidative protein damage in rat cerebral cortex is not affected by acute administration of 1,4-BD.

Enzymatic antioxidant defenses

Finally, the activities of the enzymes SOD, CAT, and GPx were assayed 1 h after the administration of 1,4-BD at doses of 20 or 120 mg/Kg. The activity of these enzymes were not affected by 1,4-BD (Table 2). These results indicate that the enzymatic antioxidant defenses are not altered in cerebral cortex from rats subjected to acute administration 1,4-BD.

Discussion

GHB is a natural constituent of CNS whose tissue concentration is increased due to drug abuse and in the inherited deficiency of SSADH activity. The understanding of the mechanisms by which GHB elicits its action might contribute to a better therapeutic management in such situations, especially in SSADH-deficient patients in which the treatment possibilities are limited.

Previous findings from our laboratory revealed that GHB alters various parameters of oxidative stress in rat brain *in vitro* (Sgaravatti *et al.*, 2007) indicating the necessity to study its *in vivo* effects. So, in the present work we studied the effect

of the acute administration of 1,4-BD, which is converted to GHB *in vivo* (Snead *et al.*, 1989), on various parameters of oxidative stress in rat brain.

The effect of the acute administration of 1,4-BD (20 or 120 mg/Kg) on lipid peroxidation was investigated by measuring spontaneous chemiluminescence and TBA-RS levels. Spontaneous chemiluminescence and TBA-RS levels were enhanced by the administration of 120 mg/Kg 1,4-BD but not of 20 mg/Kg. Considering that the light emitted in spontaneous chemiluminescence assay arises from peroxidizing lipids (Halliwell and Gutteridge, 2007) and that TBA-RS reflects the amount of malondialdehyde formation, the most abundant individual aldehyde resulting from lipid peroxidation (Esterbauer and Cheeseman, 1990), these findings indicate that lipid oxidative damage is present in cerebral cortex from Wistar rats receiving 1,4-BD. Although the experimental conditions are not the same, these results are in agreement with our previous data that GHB induces lipid peroxidation *in vitro* and *in vivo* (Sgaravatti *et al.*, 2007).

The next experiments were performed to study the effect of 1,4-BD treatment on non-enzymatic antioxidant defenses in cerebral cortex homogenates by determining TAR. According to Lissi *et al.* (1995), in a complex biological sample, TAR represents the capacity of a given tissue to avoid the damage associated with an increased production of reactive species. So, TAR may express the “quality” of the non-enzymatic antioxidants of a sample. Therefore, the significant reduction in TAR measurement in cerebral cortex from rats subjected to acute administration of 120 mg/Kg 1,4-BD suggests that brain tissue is more vulnerable to reactive species elicited by this compound or its byproduct GHB.

In contrast, the acute administration of 1,4-BD at all doses studied did not modify sulfhydryl and protein carbonyl content suggesting that the overall pool of cortical proteins is preserved from oxidative modification.

Similarly, no significant changes were observed on the antioxidant enzymatic defenses SOD, CAT, and GPx activities following 1,4-BD administration, reinforcing our previous *in vitro* experiments showing that GHB, the pharmacologically active species of 1,4-BD, did not alter the activities of these antioxidant enzymes (Sgaravatti *et al.*, 2007).

In a previous work, we have shown that administration of 10 mg/Kg body weight GHB stimulated lipid peroxidation and decreased the non-enzymatic antioxidant defenses in cerebral cortex of 14-day-old Wistar rats (Sgaravatti *et al.*, 2007). The present investigation revealed that a higher dose of 1,4-BD (120 mg/Kg body weight) was necessary to elicit similar effects obtained with GHB *in vivo* (Sgaravatti *et al.*, 2007). Although we cannot rule out that 1,4-BD induce oxidative stress itself, it is unlikely that this occurred with doses similar or even higher (20 mg/Kg body weight) than that observed for GHB (10 mg/Kg body weight) (Sgaravatti *et al.*, 2007). Therefore, it is possible that the pharmacokinetic of 1,4-BD, including its initial distribution, rate of conversion to GHB and disappearance, could influence its actions on the brain and produce the different dose effects observed. Furthermore, it is also conceivable that a derivative of GHB may be the species leading to oxidative stress in our experiments, since the samples used (cerebral cortex homogenates) have the enzymatic machinery necessary to convert GHB to SSA, GABA, or even D-2-hydroxyglutarate (Kaufman and Nelson, 1991; Cash, 1994; Kaufman *et al.*, 1998; Struys *et al.,* 2006a).

It is considered that oxidative stress can result from distinct situations, such as generation of reactive species at abnormally high rate, insufficient antioxidant defenses, release of transition metal ions, or due to a combination of these conditions (Halliwell, 2001). Therefore, the present results demonstrate that acute administration of 120 mg/Kg 1,4-BD promotes oxidative stress by stimulating oxidative damage to lipids and decreasing the reactivity of non-enzymatic antioxidant defenses. Our data are in line with those of Latini *et al.* (2007) who studied the association between oxidative stress and SSADH-deficient mice and found a reduction in non-enzymatic antioxidant defenses (decreased GSH content), an increase in lipid peroxidation (enhanced TBA-RS levels) and no alteration in antioxidant enzymatic defenses in cerebral cortex from SSADH-null animals.

Additional factors that may contribute to oxidative stress in SSADH deficiency is the accumulation of guanidine compounds (Jansen *et al.*, 2006) and D-2-hydroxyglutaric acid (Struys *et al.*, 2006a; Struys *et al.*, 2006b) observed in this disorder since these compounds have been reported to produce reactive oxygen species (Latini *et al.*, 2003; Zugno *et al.*, 2004; Zugno *et al.*, 2006). In addition, it is known that, in the brain, SSADH is the main oxidizing enzyme of 4-hydroxy-*trans*-2-nonenal (HNE), a strong reactive lipid peroxidation aldehyde, converting it to a nontoxic product (Murphy *et al.*, 2003). Moreover, HNE inhibits SSADH activity leading to even higher GHB concentrations (Nguyen and Picklo, 2003).

Considering that the present investigation demonstrated that 1,4-BD is able to significantly stimulate oxidative stress and that 1,4-BD is converted to GHB to a great extent *in vivo*, it is feasible to presume that oxidative damage may be, at least in part, one of the mechanisms contributing to the neurological dysfunction characteristic of SSADH deficiency. So, the administration of antioxidants should be considered in

situations that GHB is increased such as in patients affected by SSADH deficiency and in the use of GHB therapeutically or as a recreative drug even though it is not known the concentrations of GHB reached in the brain in these situations.

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Table 1. Effect of acute administration of 20 or 120 mg/Kg 1,4-butanediol (1,4-BD) on protein oxidation in cerebral cortex homogenates from 14-day-old rats.

Protein oxidation parameters	20 mg/Kg		120 mg/Kg	
	Control	1,4-BD	Control	1,4-BD
Sulfhydryl content (nmol TNB/mg protein)	47.4 ± 7.8	43.2 ± 7.1	46.2 ± 10.2	44.1 ± 10.4
Protein carbonyl content (nmol/mg protein)	4.2 ± 1.4	3.8 ± 1.0	4.2 ± 1.0	4.0 ± 0.5

Results are mean ± SD (n=6-11) for experiments performed in triplicate. No significant differences were detected by Student's t-test for unpaired samples.

Table 2. Effect of acute administration of 20 or 120 mg/Kg 1,4-butanediol (1,4-BD) on superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities in cerebral cortex homogenates from 14-day-old rats.

Antioxidant enzyme activity (U/mg protein)	20 mg/Kg		120 mg/Kg	
	Control	1,4-BD	Control	1,4-BD
SOD (n=7)	5.2 ± 0.6	5.6 ± 0.8	4.7 ± 1.3	4.2 ± 0.5
CAT (n=6-7)	5.8 ± 0.9	5.6 ± 0.9	4.2 ± 0.4	4.1 ± 0.5
GPx (n=5-7)	15.7 ± 2.8	13.5 ± 3.9	15.7 ± 1.3	14.2 ± 1.5

Data are expressed as mean ± SD for independent experiments performed in duplicate. One SOD unit is defined as 50% inhibition of pyrogallol autoxidation. One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute. One GPx unit is defined as 1 μmol of NADPH consumed per minute. No significant differences were detected by Student's t-test for unpaired samples.

FIGURE LEGENDS

Figure 1. Effect of acute administration of 20 or 120 mg/Kg 1,4-butanediol (1,4-BD) on spontaneous chemiluminescence in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean + SD (n=5-7) for independent experiments performed in duplicate. *$p < 0.05$ compared to control (Student's t-test for unpaired samples).

Figure 2. Effect of acute administration of 20 or 120 mg/Kg 1,4-butanediol (1,4-BD) on thiobarbituric acid-reactive substances (TBA-RS) levels in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean + SD (n=7-14) for independent experiments performed in duplicate. *$p < 0.05$ compared to control (Student's t-test for unpaired samples).

Figure 3. Effect of acute administration of 20 or 120 mg/Kg 1,4-butanediol (1,4-BD) on total antioxidant reactivity (TAR) in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean + SD (n=7-11) for independent experiments performed in duplicate. *$p < 0.05$ compared to control (Student's t-test for unpaired samples).

Figure 1

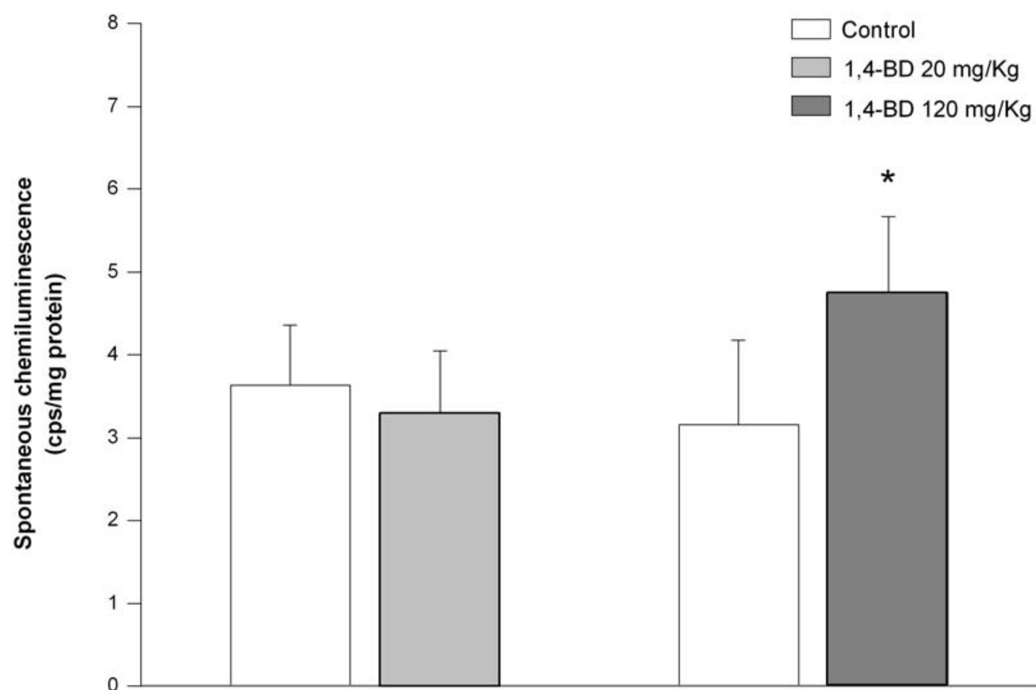


Figure 2

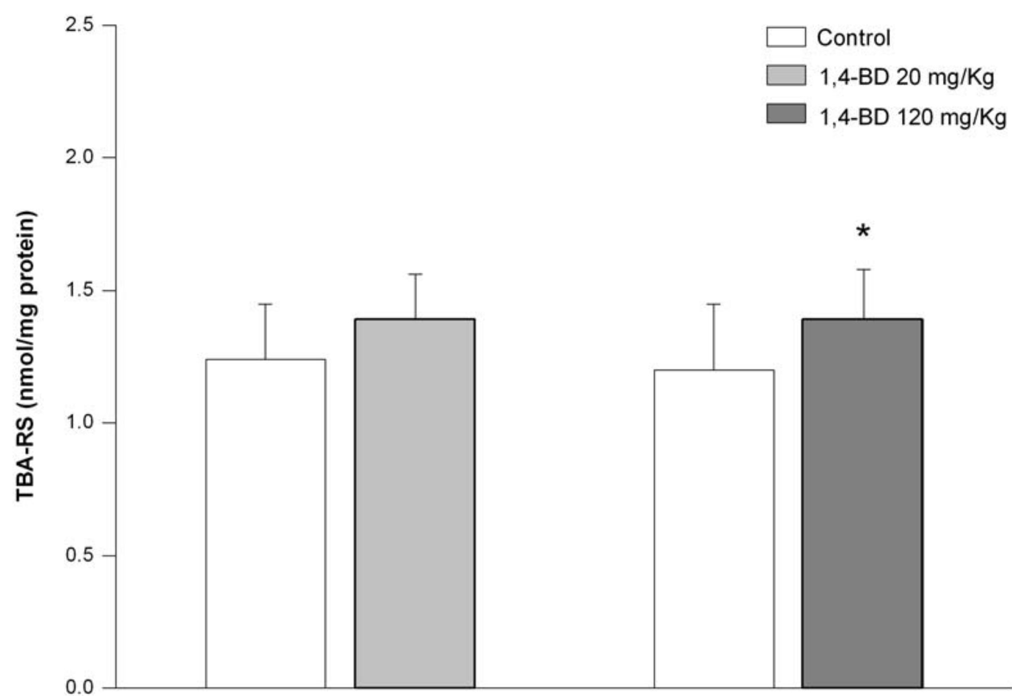
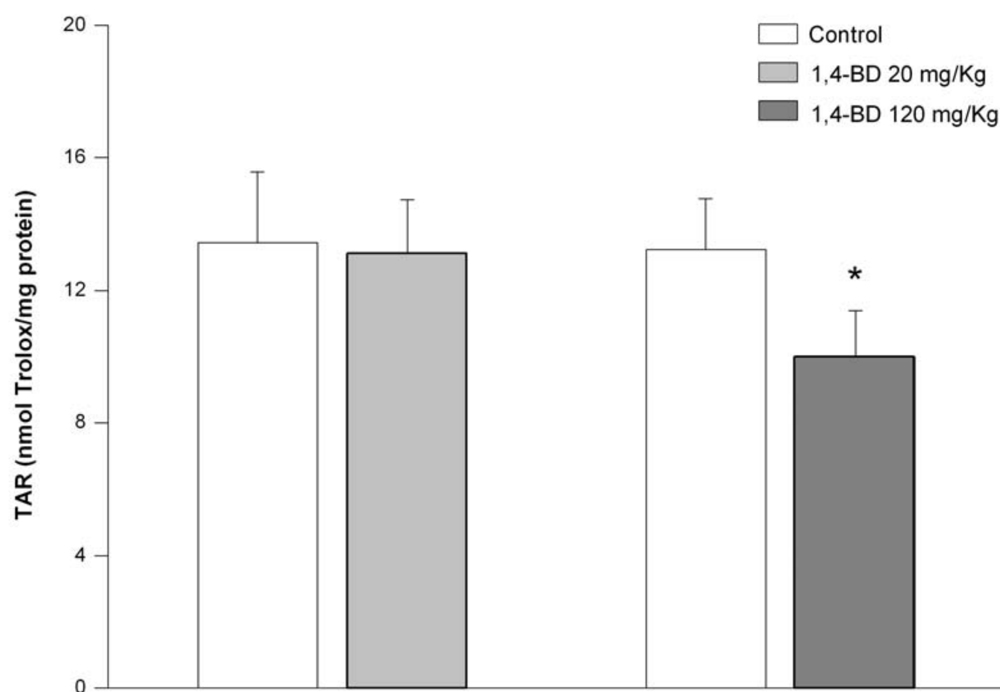


Figure 3



3.3. CAPÍTULO III

Tyrosine promotes oxidative stress in cerebral cortex of young rats

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Tyrosine promotes oxidative stress in cerebral cortex of young rats

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Running title: Tyrosine and oxidative stress in brain

List of abbreviations: ABAP, 2,2'-azo-bis(2-amidinopropane); CAT, catalase; CNS, central nervous system; CSF, cerebrospinal fluid; DCIP, 2,6-dichlorophnolindophenol; DNPH, dinitrophenylhydrazine; DNA-PC, DNA-protein cross-links; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; G6PDH, glucose-6-phosphate dehydrogenase; GSH, reduced glutathione; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; 4-HPLA, 4-hydroxyphenyllactic acid; 4-HPPA, 4-hydroxyphenylpyruvic acid, 4-HPPD, 4-hydroxyphenylpyruvate dioxygenase; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TAR, total antioxidant reactivity; TAT, tyrosine aminotransferase; TBA-RS, thiobarbituric acid-reactive substances; TCA, trichloroacetic acid; TRAP, total radical-trapping antioxidant potential.

Abstract

Tyrosine accumulates in inborn errors of tyrosine catabolism, especially in tyrosinemia type II, where tyrosine levels are highly elevated in tissues and physiological fluids of affected patients. In tyrosinemia type II, high levels of tyrosine are correlated with eyes, skin and central nervous system disturbances. Considering that the mechanisms of brain damage in these disorders are poorly known, in the present study, we investigated whether oxidative stress is elicited by L-tyrosine in cerebral cortex homogenates of 14-day-old Wistar rats. The *in vitro* effect of 0.1-4.0 mM L-tyrosine was studied on the following oxidative stress parameters: total radical-trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), ascorbic acid content, reduced glutathione (GSH) content, spontaneous chemiluminescence, thiobarbituric acid-reactive substances (TBA-RS), thiol-disulfide redox state (SH/SS ratio), protein carbonyl content, formation of DNA-protein cross-links, and the activities of the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glucose-6-phosphate dehydrogenase (G6PDH). TRAP, TAR, ascorbic acid content, SH/SS ratio and CAT activity were significantly diminished, while formation of DNA-protein cross-link was significantly enhanced by L-tyrosine *in vitro*. In contrast, L-tyrosine did not affect the other parameters of oxidative stress evaluated. These results indicate that L-tyrosine decreases enzymatic and non-enzymatic antioxidant defenses, changes the redox state and stimulates DNA damage in cerebral cortex of young rats *in vitro*. This suggests that oxidative stress may represent a pathophysiological mechanism in tyrosinemic patients, in which this amino acid accumulates.

Keywords: Tyrosine; hypertyrosinemias; tyrosinemia type II; rat brain; oxidative stress.

Tyrosine is a semi-essential aromatic amino acid that can be derived either from hydrolysis of dietary or tissue protein, or from hydroxylation of phenylalanine. Tyrosine has two major metabolic fates. It can be incorporated into protein or degraded into two products, fumarate (a Krebs cycle intermediate) and acetoacetate (a ketone body). Tyrosine is metabolized by a series of enzymatic reactions that occurs within the cytoplasm of hepatocytes and proximal tubule epithelium of the kidney (Held, 2006). A deficiency of any one of the catabolic enzymes involved will lead to an accumulation of tyrosine in tissues, cerebrospinal fluid (CSF), blood and urine (Scott, 2006). In humans, three distinct autosomal recessive diseases of tyrosine catabolism have been identified: tyrosinemia type I (deficiency of fumarylacetoacetate hydrolase), tyrosinemia type II (deficiency of tyrosine aminotransferase (TAT)), and tyrosinemia type III (deficiency of 4-hydroxyphenylpyruvate dioxygenase (4-HPPD)) (Mitchell *et al.*, 2001; Held, 2006). Besides, fasting hypertyrosinemia may occur in other situations including liver immaturity of premature newborns and liver disease (Mitchell *et al.*, 2001).

Interestingly, hypertyrosinemia is associated with neurologic and development difficulties in numerous patients with TAT deficiency and some others with 4-HPPD deficiency and transient hypertyrosinemia (Light *et al.*, 1973; Mamunes *et al.*, 1976; Rice *et al.*, 1989). Although a causal link with hypertyrosinemia is not formally established, current data do not eliminate the possibility that elevated levels of tyrosine and/or its derivatives may have noxious effects on CNS development in these patients.

Among the tyrosinemias, tyrosinemia type II is characterized by the highest level of tyrosine. Plasma tyrosine levels in TAT-deficient patients range from 370 to 3,420 μM (normal < 90 μM) along with the accumulation of some tyrosine derivatives,

namely 4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllactic acid and 4-hydroxyphenylacetic acid (Goldsmith *et al.*, 1973; Rabinowitz *et al.*, 1995; Macsai *et al.*, 2001; Mitchell *et al.*, 2001; Valikhani *et al.*, 2005; Held, 2006). The eyes, skin and nervous system are the only organs affected by TAT deficiency. The involvement of the central nervous system (CNS) is variable and ranges from severe mental retardation to slight decreases in intelligence and may be associated with microcephaly, nystagmus, tremor, ataxia, language deficits, convulsions and other organ abnormalities in some cases (Goldsmith *et al.*, 1973; Lemonnier *et al.*, 1979; Rabinowitz *et al.*, 1995; Macsai *et al.*, 2001; Mitchell *et al.*, 2001; Valikhani *et al.*, 2005).

Neuropathologic findings of tyrosinemic patients include metabolic astrocytosis and delay in myelination. Sener (2005) also reported intramyelinic edema attributable to status spongious in a 5-month-old boy with tyrosinemia type I and Proton MR spectroscopy confirmed the presence of tyrosine into the lesion sites.

Considering that the mechanisms underlying the neurological dysfunction in hypertyrosinemic patients are poorly known and that the damaging consequences of oxidative stress have been implicated in a variety of diseases of the CNS including inherited metabolic disorders (Reznick and Parker, 1993; Halliwell, 2001; Wajner *et al.*, 2004; Halliwell, 2006), in the present study the possible role of oxidative stress in tyrosine neurotoxicity was investigated in order to clarify its participation in the brain damage mechanisms responsible for the neurological impairment observed in hypertyrosinemic patients. Thus, the *in vitro* effect of L-tyrosine was investigated on the following oxidative stress parameters in cerebral cortex homogenates of young Wistar rats: total radical-trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), ascorbic acid and reduced glutathione (GSH) levels, to evaluate

non-enzymatic antioxidant defenses; spontaneous chemiluminescence and thiobarbituric acid-reactive substances (TBA-RS), to assess lipid peroxidation; thiol and disulfide contents, to determine SH/SS redox state; protein carbonyl content, to evaluate protein oxidation; and DNA-protein cross-links, to measure DNA damage; the activities of the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), to evaluate enzymatic antioxidant defenses; and glucose-6-phosphate dehydrogenase (G6PDH), to measure the NADPH-producing system.

Experimental procedures

Reagents and equipments

All chemicals were purchased from Sigma (St. Louis, MO, USA) except thiobarbituric acid and 2,2'-azo-bis(2-amidinopropane) (ABAP), which were purchased from Merck (Darmstadt, Germany) and Wako Chemicals (Richmond, VA, USA), respectively. L-Tyrosine was prepared on the day of the experiment in distilled water and added to homogenates at final concentrations of 0.1, 1.0, 2.0 and 4.0 mM. A double-beam spectrophotometer with temperature control (Hitachi U-2001), Wallac 1409 Scintillation Counter and Perkin Elmer Victor 2 Fluorometer were used for the measurements. Eppendorf 5417R (refrigerated version) and Eppendorf 5403 were used for the centrifugation procedures.

Animals

Fourteen-day-old Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, were used. Rats were kept with dams until they were sacrificed. The dams

had free access to water and 20% (w/w) protein commercial chow (Supra, Porto Alegre, RS, Brazil). They were maintained in a room with a 12-h light:12-h darkcycle (lights on 7:00-19:00 h) and with air conditioned controlled temperature (22 ± 1 ºC). The “Principles of Laboratory Animal Care” (NIH publication # 80-23, revised 1996) were followed throughout the experiments.

Tissue preparation and incubation

Rats were killed by decapitation without anesthesia, and the brain was rapidly removed and kept on an ice-plate. The olfactory bulb, pons and medulla were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization.

Cerebral cortex was homogenized 1:6 w/v (for protein carbonyl determination) or 1:10 w/v (for all other measurements) in 50 mM sodium phosphate buffer pH 7.4 Homogenates were centrifuged at 750 *g* for 10 min at 4 ºC. The pellet was used to measure DNA-protein cross-links and the supernatant (a suspension of mixed and preserved organelles) was immediately separated and utilized for the other analyses.

Samples were pre-incubated for 1 hour at 37°C in the presence of L-tyrosine at final concentrations ranging from 0.1 to 4.0 mM. Controls were incubated with distilled water. After incubation, aliquots were taken to measure TRAP, TAR, ascorbic acid levels, spontaneous chemiluminescence, TBA-RS, thiol-disulfide redox state, protein carbonyl content, and DNA-protein cross-links. To test the effect of L-tyrosine on the activity of the enzymes SOD, CAT, GPx and G6PDH, the amino acid was added to the cerebral cortex homogenates at the time of assay of each enzyme activity without previous incubation.

Total radical-trapping antioxidant potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by ABAP thermolysis (free radical source) in a scintillation counter (Lissi *et al.*, 1992; Evelson *et al.*, 2001). After adding 3 mL of 10 mM ABAP and 10 μL of 5.6 mM luminol to scintillation vials, the initial light intensity was obtained. Ten μL of 160 μM Trolox (water-soluble α-tocopherol analogue) or 30 μL of samples were added to assess their antioxidant content and at this point, the luminescence intensity is practically abolished. The consumption of active antioxidants present in samples produces the return of the luminescence and the time required to this was compared to those obtained employing Trolox under identical experimental conditions. TRAP values were calculated as Trolox equivalents and were represented as nmol Trolox/mg protein.

Total antioxidant reactivity (TAR)

TAR was determined by measuring the luminol chemiluminescence intensity induced by ABAP (free radical source) using a scintillation counter (Lissi *et al.*, 1995). The chemiluminescence was measured by adding 4 mL of 2 mM ABAP plus 15 μL of luminol (4 mM) into a glass scintillation vial. This was considered to be the basal value. Ten μL of 10 to 100 μM Trolox (curve calibration) or samples were then added and the chemiluminescence was measured during 60 s. The rapid initial reduction of luminescence is considered as a measure of TAR capacity of the samples. The results were calculated as Trolox equivalents and were represented as nmol Trolox/mg protein.

Ascorbic acid content

Ascorbic acid was measured according the method of Omaye *et al.* (1979). Samples were deproteinized by addition of metaphosphoric acid 10% (1:1) and, after thorough mixing, were centrifuged for 20 min at 3,500 *g*. To an aliquot of 300 μL of the previous supernatant, 300 μL of citrate/acetate buffer pH 4.15 and 300 μL of 2,6-dichlorophenolindophenol (DCIP) solution were added and this mixture was read in a spectrophotometer. Ascorbic acid reduces the DCIP causing a decrease in its absorption at 520 nm. A standard curve, including a reagent blank, was constructed with standards ranging between 0 and 15 μg of ascorbic acid per milliliter of 5% metaphosphoric acid. Ascorbic acid content was represented as μg ascorbic acid/mg protein.

Reduced glutathione (GSH) content

To determine GSH, samples were deproteinized with 2 M perchloric acid and centrifuged at 1,000 *g* for 10 min. The pH of supernatant was adjusted to 7.0 with 2 M potassium hydroxide and the samples were centrifuged at 1,000 g for 10 min. The supernatant obtained reacted with 6 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and the mixture was read at 420 nm in a spectrophotometer (Akerboom and Sies, 1981). The results were represented in nmol GSH/mg protein.

Spontaneous chemiluminescence

Samples were assayed for spontaneous chemiluminescence in a dark room using a scintillation counter (Lissi *et al.*, 1986). The background chemiluminescence was measured for 5 min in vials containing 3.5 mL of the 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. An aliquot of 0.5 mL of sample was added and

chemiluminescence was measured for 10 min at room temperature. The background chemiluminescence was subtracted from the final value. Spontaneous chemiluminescence was represented as cps/mg protein.

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS were measured as described by Esterbauer and Chesseman (1990). Briefly, 300 μL of cold 10% trichloroacetic acid (TCA) were added to 150 μL of sample and centrifuged at 300 *g* for 10 min. Three hundred μL of the supernatant were transferred to a Pyrex tube and incubated with 300 μL of 0.67% thiobarbituric acid in 7.1% sodium sulfate in a boiling water bath for 25 min. The mixture was allowed to cool on water for 5 min. The resulting pink stained TBA-RS was determined in a spectrophotometer at 532 nm. Calibration curve was performed using 1,1,3,3-tetramethoxypropane as standard. TBA-RS were represented as nmol TBA-RS/mg protein.

Thiol and disulfide contents (SH/SS redox state)

Thiol and disulfide contents were determined essentially according to Zahler and Cleland (1968). For disulfide measurement, 100 μL of sample were mixed with 25 μL of 0.05 M Tris pH 9.0 and 25 μL of 3 M dithiothreitol (DTT). After 20 min at room temperature, 50 μL of 1.0 M Tris pH 8.1, 375 μL of 5 mM sodium arsenite, and enough water to give a final volume of 925 μL were added, and the solutions were mixed and allowed to stand for 3 min. For thiol determination, DTT and sodium arsenite were omitted of the procedure. To start the reaction, 25 μL of 3 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.05 M acetate pH 5.0 were added. After 15 min, the absorbance of TNB formed (equivalent to the amount of SH groups) was

measured in a spectrophotometer at 412 nm. Thiol and disulfide groups were represented as nmol TBN/mg protein.

Protein carbonyl content

Protein carbonyl content was assayed by a method based on the reaction of carbonyls with dinitrophenylhydrazine (DNPH) forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer, 1994). Briefly, 200 μL of sample were added to 400 μL 10 mM DNPH in 2 M HCl, or to 2 M HCl (blank). This mixture was kept in dark room for 1 hour and vortexed each 15 min. After that, 500 μL of 20% TCA were added to each tube. The mixture was vortexed and centrifuged at 14,000 *g* for 3 min. The supernatant obtained was discarded. The pellet was washed with 1 mL ethanol: ethyl acetate (1:1, v/v), vortexed and centrifuged at 14,000 rpm for 3 min to remove the free DNPH. The supernatant was discarded and the pellet was resuspended in 600 μL of 6 M guanidine (prepared in a 20 mM potassium phosphate pH 2.3), vortexed and incubated at 60 ^{o}C for 15 min. After that, it was centrifuged at 14,000 rpm for 3 min and the supernatant was used to measure absorbance in a spectrophotometer at 370 nm. The molar extinction coefficient of 22,000 for DNPH was used to calculate the concentration of carbonyls content and the results were reported as nmol/mg protein.

DNA-protein cross-links (DNA-PC) assay

DNA-protein cross-links were measured 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

200 μL of sample and this mixture was vortexed and frozen at -20 °C overnight. After thawing, 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 *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 *g* for 3 min. The washing step was repeated three times more. 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 *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 Syber Gold. Fluorescent measurements were performed using a fluorometer with 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 Log %DNA-PC.

Superoxide dismutase assay

The assay of SOD activity was carried out with the RANSOD kit (Randox, USA). This method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical (produced in the incubation medium from xanthine oxidase reaction). Red formazan was measured spectrophotometrically at 505 nm. The inhibition of the produced chromogen is proportional to the activity of the SOD present in the sample. A 50%

inhibition is defined as one unit of SOD and specific activity is represented as units per mg protein.

Catalase assay

CAT activity was assayed by the method of Aebi (1984) using a spectrophotometer. This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0. One CAT unit is defined as 1 μmol of H_2O_2 consumed per minute and the specific activity is represented as units per mg protein.

Glutathione peroxidase assay

GPx activity was measured according to the method of Wendel (1981) using *tert*-butyl hydroperoxide as substrate. NADPH disappearance was monitored at 340 nm using a spectrophotometer. The reaction medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as units per mg protein.

Glucose-6-phosphate dehydrogenase assay

Glucose-6-phosphate dehydrogenase was measured by the method of Leong and Clark (1984), in which the reaction mixture (1 mL) contained: 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$, and sample. The reaction was started by the addition of 1 mM glucose-6-phosphate and was followed in a spectrophotometer at 340 nm. One G6PDH unit corresponds to 1 μmol of substrate transformed per minute and the specific activity is represented as units per mg protein.

Protein determination

Protein concentration was determined in cerebral cortex samples using bovine serum albumin as a standard (Lowry *et al.*, 1951).

Statistical analysis

Data were analyzed by the one-way analysis of variance (ANOVA) followed by the post hoc Tukey test when the F value was significant. Linear regression analysis was also used to verify dose-dependent effects. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p<0.05$ was considered to be significant.

Results

Firstly, the *in vitro* effect of L-tyrosine on the antioxidant capacity of cerebral cortex homogenates was studied by determining TRAP and TAR, which represent non-enzymatic antioxidant quantity and reactivity, respectively. It can be observed in Figure 1A that 2.0 and 4.0 mM L-tyrosine markedly decreased TRAP as compared to control [$F(4,15)=8.38$; $p<0.01$] in a concentration-dependent way ($\beta=-0.81$; $p<0.01$). Similarly, TAR measurement was significantly reduced in a dose-related fashion when cerebral cortex was exposed to L-tyrosine [$F(4,15)=11.35$; $p<0.01$] ($\beta=-0.83$; $p<0.01$) (Figure 1B).

Next, ascorbic acid and GSH levels were measured to evaluate whether TRAP and TAR reduction occurred by means of these antioxidants levels diminution. Figure 2A shows that ascorbic acid content was significantly reduced in the presence of L-

tyrosine $[F(4,25)=3.13; p<0.05]$ in a dose-dependent manner $[\beta=-0.57; p<0.01)$, while GSH levels were not significantly altered $[F(4,25)=1.56; p>0.05]$ (Figure 2B).

Lipid peroxidation was investigated by measuring spontaneous chemiluminescence and TBA-RS levels in rat cerebral cortex homogenates (Table 1). L-Tyrosine caused no effect on spontaneous chemiluminescence $[F(4,25)=0.58; p>0.05]$ or TBA-RS levels $[F(4,25)=0.55; p>0.05]$ in cerebral cortex.

The *in vitro* effect of L-tyrosine on SH/SS redox status was studied by measuring thiol and disulfide contents. Table 2 shows that L-tyrosine significantly decreased the total thiol content $[F(4,20)=24.90; p<0.01]$ in a dose-dependent manner $(\beta=-0.906; p<0.01)$, while disulfide levels were strongly enhanced $[F(4,20)=9.23; p<0.01]$ in a concentration-dependent way $(\beta=0.80; p<0.01)$. In addition, the SH/SS ratio was calculated and was significantly diminished $[F(4,20)=12.24; p<0.01]$ $(\beta=-0.82; p<0.01)$.

The effect of L-tyrosine on tissue proteins was also evaluated. To accomplish this, carbonyl content, a parameter of oxidative protein damage, was measured. Figure 3 shows that L-tyrosine had no effect on protein carbonyl content in cerebral cortex $[F(4,25)=0.56; p>0.05]$.

DNA damage was also determined by measuring DNA-protein cross-links formation. Significant amounts of DNA-protein cross-links were detected as a result of exposure cerebral cortex to increasing concentration of L-tyrosine $[F(4,23)=6.25; p<0.01]$ (Figure 4).

Finally, the activities of the enzymes SOD, CAT, GPx and G6PDH were assayed in the presence of L-tyrosine (Figure 5). The measurements of SOD, CAT, GPx and G6PDH activities were carried out without previous incubation of L-tyrosine with the homogenates. L-Tyrosine markedly reduced CAT activity in cerebral cortex

homogenates $[F(4,25)=12.12; p<0.01]$ in a dose-dependent way ($\beta=-0.79; p<0.01$), but did not alter the activities of SOD $[F(4,30)=0.32; p>0.05]$, GPx $[F(4,20)=0.99; p>0.05]$ and G6PDH $[F(4,15)=2.35; p>0.05]$.

Additionally, the effect of two tyrosine derivatives, namely 4-hydroxyphenylpyruvic acid (4-HPPA) and 4-hydroxyphenyllactic acid (4-HPLA), at concentrations of 0.02 and 0.2 mM were tested on some parameters of oxidative stress. 4-HPPA caused no effect on TRAP $[F(2,9)=0.15; p>0.05]$, spontaneous chemiluminescence $[F(2,15)=0.05; p>0.05]$, TBA-RS $[F(2,15)=0.26; p>0.05]$, and protein carbonyl content $[F(2,21)=0.69; p>0.05]$ as compared to control. 4-HPLA also did not alter TRAP $[F(2,9)=0.03; p>0.05]$, spontaneous chemiluminescence $[F(2,18)=0.09; p>0.05]$, TBA-RS $[F(2,15)=0.025; p>0.05]$, and protein carbonyl content $[F(2,12)=1.08; p>0.05]$ (Table 3).

Discussion

Tyrosine accumulates in inborn errors of tyrosine catabolism, especially in tyrosinemia type II, where tyrosine levels are highly elevated in tissues and physiological fluids of TAT-deficient patients (Mitchell *et al.*, 2001). Although the molecular deficiency and the symptoms of TAT deficiency are well described, the mechanisms responsible for the neuropathophysiology of this metabolic disorder are largely unknown. Considering that very limited information is available on the possible role of tyrosine on oxidative stress, in the present work, the *in vitro* effect of this amino acid on some oxidative stress parameters was investigated in cerebral cortex homogenates of 14-day-old Wistar rats.

Cerebral cortex was used to investigate the effect of tyrosine on oxidative stress status because tyrosine concentration into this brain region showed the highest relative increase (related to controls) compared to other regions when tyrosine is administered to rats (Morre *et al.*, 1980). Furthermore, as the development of CNS occurs in phases, which follow a precise sequence (Morgane *et al.*, 2002), we have chosen to study 14-day-old rats because during the second postnatal week, Slotkin *et al.* (2005) identified a crucial phase of vulnerability of neuronal cells, corresponding to peak periods of differentiation when metabolic demands are especially high. In addition, the period of fastest dendritic outgrowth in rat cerebral cortex is between 8 and 14 postnatal days, whereas in the human is during the first two to three years (Uylings, 2000). In this regard, tyrosinemic patients, like many other inherited metabolic diseases, are subjected to high levels of accumulated metabolites (in this case, tyrosine) in postnatal period presenting symptoms during critical stages of CNS development.

A significant reduction in TRAP and TAR measurements by L-tyrosine in cerebral cortex of 14-day-old rats was observed (Figure 1). TRAP measures the content of non-enzymatic antioxidant defenses, while TAR reflects how promptly a given tissue can handle an increased production of reactive species. So, TRAP and TAR may respectively express the “quantity” and the “quality” of the non-enzymatic antioxidants of a sample (Lissi *et al.*, 1995). Our results indicate that L-tyrosine reduces the non-enzymatic antioxidant capacity in cerebral cortex homogenates, by diminishing non-enzymatic antioxidant content (TRAP) and the antioxidant reactivity (TAR), which became the CNS prone to reactive species. As ascorbic acid and GSH are the most abundant antioxidants of low molecular weight in the CNS (Rice and Russo-Menna, 1998), their contents were measured to evaluate whether the

reduction of TRAP and TAR promoted by L-tyrosine was in fact caused by a decrease in the level of these antioxidants in cerebral cortex homogenates. L-Tyrosine did not change GSH levels but significantly reduced ascorbic acid content (Figure 2), which may be related to the reduction in TRAP and TAR measurements observed in the presence of L-tyrosine. In addition, reduced levels of ascorbic acid in the brain may hinder α-tocopherol action against oxidative stress, although it is not clear whether the synergic action of these antioxidant vitamins occurs *in vivo* (Halliwell and Gutteridge, 2007a).

In contrast, spontaneous chemiluminescence and TBA-RS levels (parameters of lipid oxidation) were not altered in our experimental conditions (Table 1), suggesting that oxidative damage to lipids is not induced by L-tyrosine in cerebral cortex homogenates. As lipid peroxidation is a process avoided by α-tocopherol, it seems that the action of this vitamin is preserved despite the reduction of ascorbic acid levels observed in the experiments (Halliwell and Gutteridge, 2007a).

Enhanced generation of reactive species and/or impaired antioxidant detoxification functions contribute to an imbalance between oxidative and reductive reactions, which alters thiol/disulfide redox (Moriarty-Craige and Jones, 2004). Considering that the total thiol content was significantly diminished by L-tyrosine while disulfide levels were significantly enhanced, a significant reduction in SH/SS ratio was observed (Table 2), suggesting that L-tyrosine was able to disturb the thiol redox status in homogenates from cerebral cortex of young rats. Given the numerous processes that are dependent upon cellular redox state, alteration of SH/SS ratio is considered an important general parameter indicative of oxidative stress.

The presence of L-tyrosine in the incubation medium did not change the carbonyl content (Figure 3), a parameter of oxidative damage to proteins, indicating

that, in general, proteins may be preserved from oxidative modification and/or fast degradation of oxidized proteins. However, we cannot rule out that specific proteins were affected.

Free radicals generate a large number of modifications in DNA by a variety of mechanisms. These include sugar and base modifications, strand breaks and DNA-protein cross-links (Dizdaroglu *et al.*, 2002). The latter are common DNA lesions created when a protein is covalently bound to DNA (Reardon *et al.*, 2006). L-tyrosine was able to cause oxidative damage to DNA, as verified by the significant alteration in DNA-protein cross-links formation (Figure 4). Since protein carbonyl content was not affected by tyrosine at any concentration studied, a speculative mechanism that may be related to our results is that tyrosyl radical is being produced during the incubations. Tyrosyl radical may elicit the abstraction of hydrogen atoms from DNA bases and sugars which, in turn, may produce more radicals and trigger DNA-protein cross-links. This was in line with the fact that tyrosyl radical-mediated damage includes the formation of protein cross-links (Ban *et al.*, 2002).

Finally, the effect of L-tyrosine on SOD, CAT, GPx and G6PDH was evaluated. SOD catalyses the dismutation of superoxide radical, CAT metabolizes hydrogen peroxide, GPx removes organic hydroperoxides as well as hydrogen peroxide (Halliwell and Gutteridge, 1985), and G6PDH is the main enzyme which have the capacity to generate reducing power in the form of NADPH (Halliwell and Gutteridge, 2007a). Although G6PDH is not a classical antioxidant enzyme (in other words, its substrate is not itself a reactive species), the activity of this enzyme seems to be crucial in hydrogen peroxide (H_2O_2) elimination and antioxidant function as stated by other authors (Hashida *et al.*, 2002; Makino *et al.*, 2004). In fact, H_2O_2 reduction by glutathione peroxidase (GPx) requires the recycling of oxidized glutathione by

glutathione reductase, which is dependent on the supply of NADPH. L-Tyrosine significantly diminished CAT activity, but did not change SOD, GPx and G6PDH activities (Figure 5). These results indicate that L-tyrosine directly affects the CAT activity since the amino acid was not pre-incubated with the sample and rather was present during measurement of the enzyme activity. As a consequence of L-tyrosine effect, CAT inhibition could conceivably lead to excessive formation of H_2O_2, which in turn may decrease CAT activity since H_2O_2 is capable of inactivating enzymes by oxidation of hyper-reactive sulfhydryl groups, essential for catalysis (Halliwell and Gutteridge, 2007b), resulting in a vicious cycle.

It is considered that oxidative stress can result from distinct situations, such as generation of reactive species at abnormally high rate, insufficient antioxidant defenses, releasing of transition metal ions, or due to a combination of these conditions (Halliwell, 2001). Taken together, the results presented here demonstrate that L-tyrosine may promote oxidative stress by decreasing non-enzymatic and enzymatic antioxidant defenses, changing the redox state and stimulating oxidative damage to DNA in cerebral cortex of young rats. However, we cannot rule out that tyrosyl radical is being produced during the incubations. Tyrosyl radical can be generated by any free radical reactive enough to abstract a hydrogen atom from hydroxyl group of tyrosine. Considering that tyrosyl radical can react with ascorbate, cysteine and GSH (Halliwell and Gutteridge, 2007b), it is possible that this reactive species participates on the mechanisms by which the effects are being produced in our experiments.

Some of the effects of tyrosine on the oxidative stress parameters were elicited only at the highest concentration studied (4 mM) which is possible that may not occur in the brain of tyrosinemic patients. To our knowledge, the actual

concentration that is reached into the brain of type II patients is not known. It was reported that plasma tyrosine levels may exceed 1,000 μM in untreated type II patients (Scott, 2006), but other studies found that plasma tyrosine values range from 370 to 3,420 μM (Goldsmith *et al.*, 1973; Lemonnier *et al.*, 1979; Rabinowitz *et al.*, 1995; Mitchell *et al.*, 2001; Macsai *et al.*, 2001; Viglizzo *et al.*, 2006). Although some effects presented here may have pathophysiological relevance to tyrosinemia type II, we cannot rule out that these effects were suprapathological.

It is difficult to extrapolate these *in vitro* findings to the human condition but if these effects also occur in the brain of patients affected by these disorders, it is possible that oxidative stress may contribute, along with other mechanisms, to the neurological dysfunction characteristic of tyrosinemias. Further experiments performed *in vivo* should be carried out to confirm these results. If similar findings are achieved, it is proposed that administration of antioxidants, particularly vitamin C, should be considered as a potential adjuvant therapy for patients affected by tyrosinemias, especially type II.

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Table 1. *In vitro* effect of L-tyrosine on lipid peroxidation in cerebral cortex homogenates from 14-day-old rats.

Lipid peroxidation parameter	L-Tyrosine (mM)				
	0.0	0.1	1.0	2.0	4.0
Spontaneous chemiluminescence (cps/mg protein)	46.96 ± 12.8	48.41 ± 14.30	50.19 ± 12.88	53.06 ± 13.30	57.06 ± 11.42
TBA-RS (nmol/mg protein)	5.16 ± 0.77	5.12 ± 0.54	4.95 ± 0.58	4.99 ± 0.61	4.70 ± 0.44

Results are mean ± SD (n=6) for independent experiments performed in duplicate. No significant differences were detected by ANOVA.

Table 2. *In vitro* effect of L-tyrosine on thiol (SH) and disulfide (SS) contents, and SH/SS ratio in cerebral cortex homogenates from 14-day-old rats.

	L-Tyrosine (mM)				
	0.0	0.1	1.0	2.0	4.0
Thiol content (SH) (nmol of TNB /mg protein)	58.38 ± 1.11	57.79 ± 2.35	55.15 ± 1.27*	53.10 ± 1.10*	49.32 ± 1.98*
Disulfide content (SS) (nmol of TNB /mg protein)	16.72 ± 2.62	18.14 ± 1.33	20.46 ± 2.86	22.60 ± 4.50	27.9 ± 3.86*
SH/SS ratio	3.56 ± 0.57	3.19 ± 0.19	2.73 ± 0.35	2.45 ± 0.61	1.80 ± 0.29*

Results are mean ± SD (n=5) for independent experiments performed in duplicate. *p<0.01 compared to control (Tukey test).

Table 3. *In vitro* effect of 4-hydroxyphenylpyruvic acid (4-HPPA) and 4-hydroxyphenyllactic acid (4-HPLA) on oxidative stress parameters in cerebral cortex homogenates from 14-day-old rats.

Oxidative stress parameter	4-HPPA (mM)			4-HPLA (mM)		
	0.0	0.02	0.2	0.0	0.02	0.2
TRAP (nmol Trolox/mg protein)	40.7 ± 12.8	40.5 ± 10.1	37.1 ± 8.4	41.8 ± 8.4	40.6 ± 4.8	41.72 ± 6.8
Spontaneous chemiluminescence (cps/mg protein)	45.7 ± 6.31	45.1 ± 5.1	46.1 ± 4.6	46.7 ± 6.14	45.7 ± 5.66	45.5 ± 5.04
TBA-RS (nmol/mg protein)	4.96 ± 0.55	4.99 ± 0.48	5.20 ± 0.77	4.69 ± 0.37	4.74 ± 0.42	4.69 ± 0.41
Protein carbonyl content (nmol/mg protein)	5.37± 0.99	5.84 ± 1.58	5.02 ± 1.55	5.19 ± 0.58	6.68 ± 1.31	5.93 ± 2.39

Results are mean ± SD (n=4-8) for independent experiments performed in duplicate.

No significant differences were detected by ANOVA.

FIGURE LEGENDS

Figure 1. *In vitro* effect of L-tyrosine on total radical-trapping antioxidant potential (TRAP) (A) and total antioxidant reactivity (TAR) (B) in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean ± SD (n=4) for independent experiments performed in duplicate. $^*p<0.05$ and $^{**}p<0.01$ compared to control (Tukey test).

Figure 2. *In vitro* effect of L-tyrosine on ascorbic acid content (A) and reduced glutathione content (B) in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean ± SD (n=6) for independent experiments performed in duplicate. $^*p<0.05$ compared to control (Tukey test).

Figure 3. *In vitro* effect of L-tyrosine on protein carbonyl content in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean ± SD (n=6) for independent experiments performed in triplicate. No significant differences were detected by ANOVA.

Figure 4. *In vitro* effect of L-tyrosine on DNA-protein cross-links (DNA-PC) formation in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean ± SD (n=5-6) for independent experiments performed in duplicate. $^*p<0.01$ compared to control (Tukey test).

Figure 5. *In vitro* effect of L-tyrosine on superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione peroxidase (GPx) (C) and glucose-6-phosphate

dehydrogenase (G6PDH) activities in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean ± SD (n=4-7) for independent experiments performed in duplicate. One SOD unit is defined as 50% inhibition of red formazan formation. One CAT unit is defined as 1 μmol of H_2O_2 consumed per minute. One GPx unit is defined as 1 μmol of NADPH consumed per minute. One G6PDH unit is defined as 1 μmol of substrate transformed per minute. *$p<0.01$ compared to control (Tukey test).

Figure 1

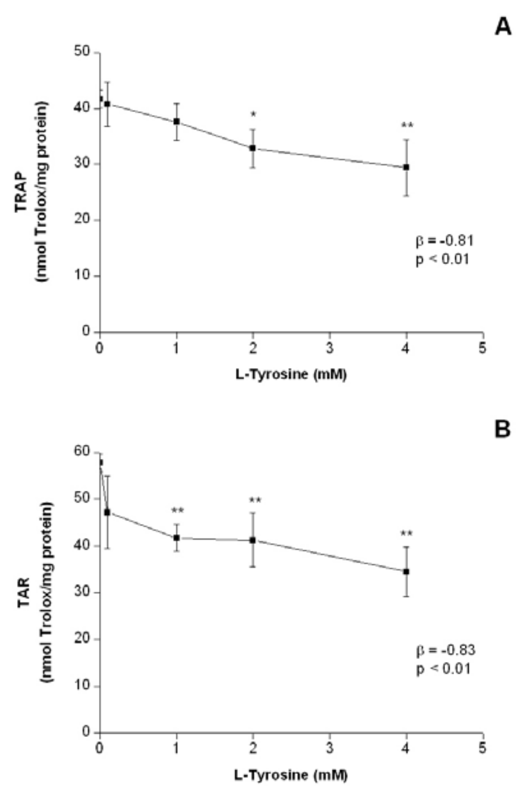


Figure 2

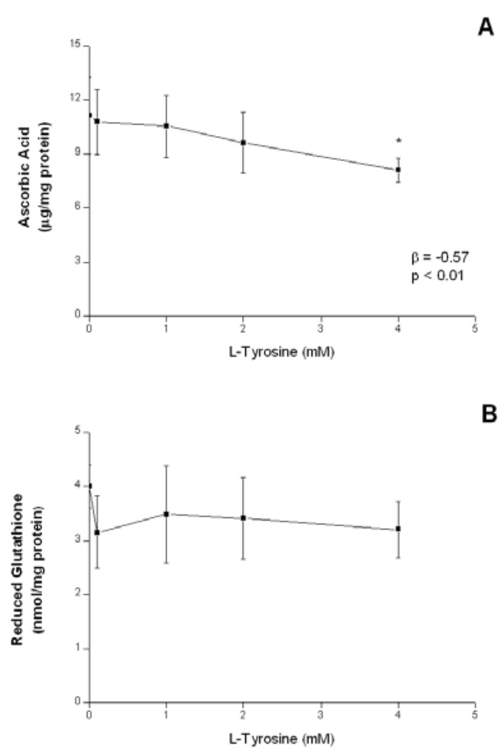


Figure 3

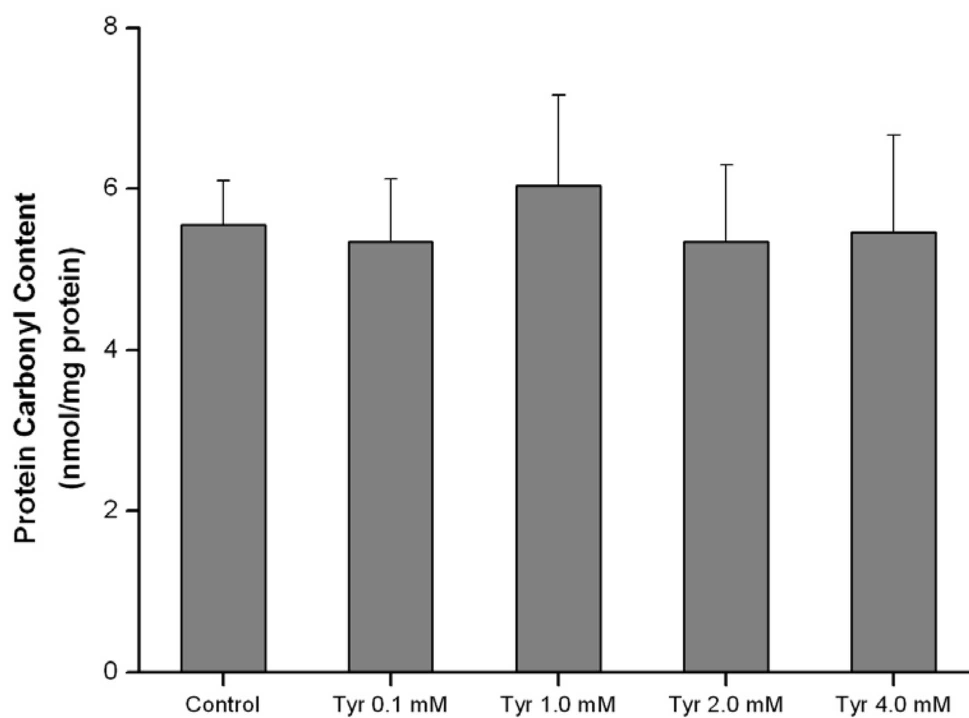


Figure 4

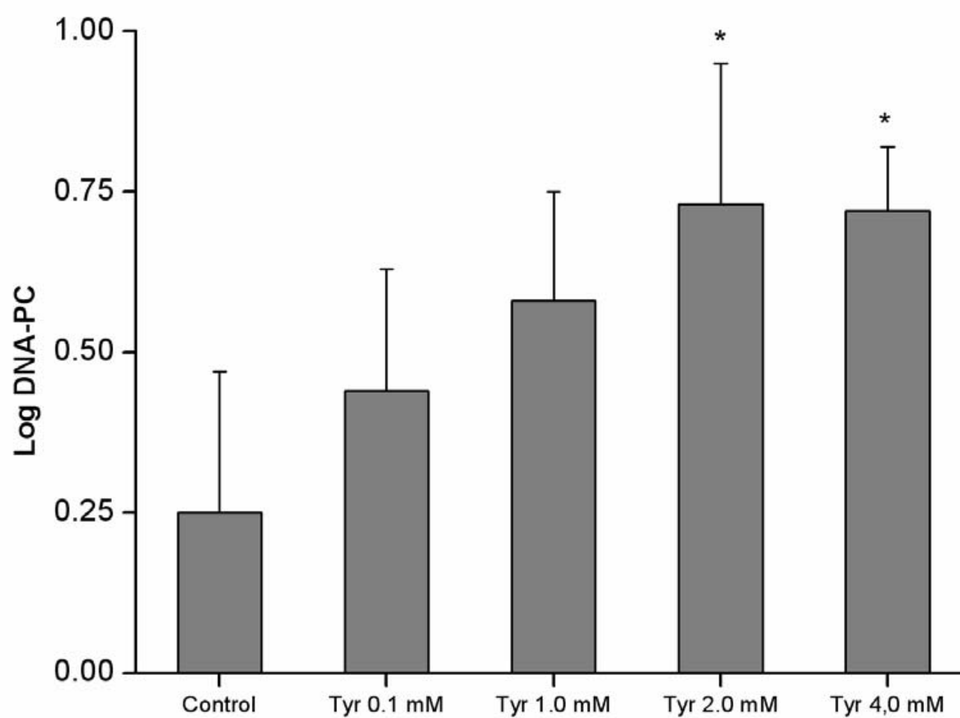
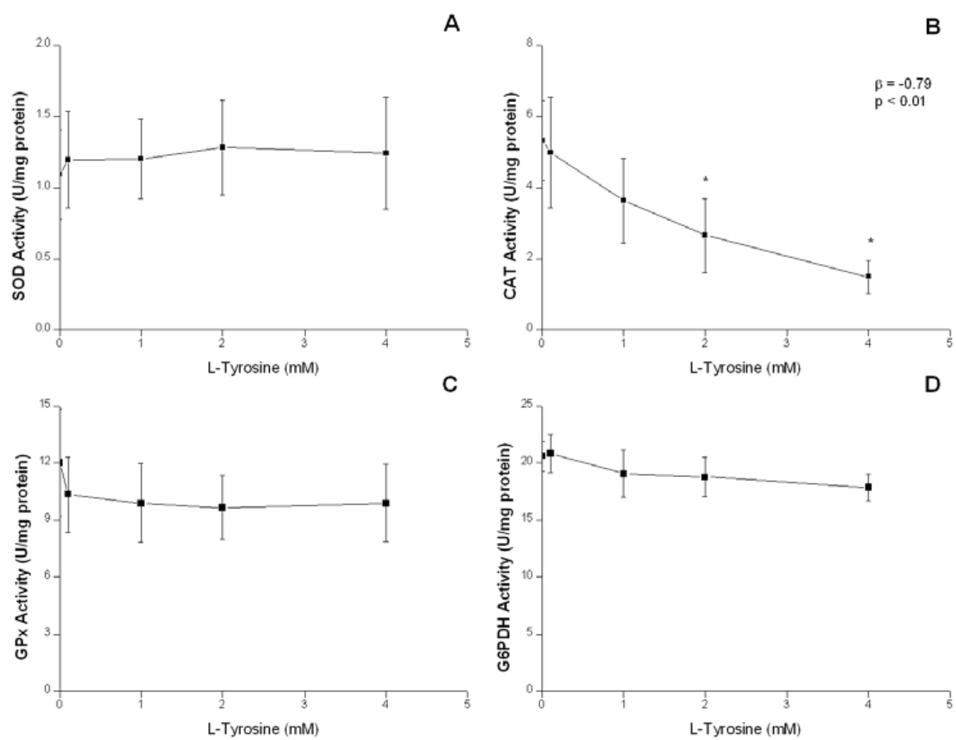


Figure 5



3.4. CAPÍTULO IV

Tyrosine administration decreases glutathione and stimulates lipid and protein oxidation in rat cerebral cortex

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Tyrosine administration decreases glutathione and stimulates lipid and protein oxidation in rat cerebral cortex

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Running title: Tyrosine and oxidative stress in rat brain.

Abbreviations: CAT, catalase; CNS, central nervous system; DCIP, 2,6-dichlorophnolindophenol; DNPH, dinitrophenylhydrazine; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; FAH, fumarylacetoacetate hydrolase; G6PDH, glucose-6-phosphate dehydrogenase; GSH, reduced glutathione; GPx, glutathione peroxidase; 4-HPPD, 4-hydroxyphenylpyruvate dioxygenase; OPT, o-phtalaldeyde; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TAT, tyrosine aminotransferase; TBA-RS, thiobarbituric acid-reactive substances; TCA, trichloroacetic acid.

Abstract

Tyrosine levels are abnormally elevated in tissues and physiological fluids of patients with inborn errors of tyrosine catabolism especially in tyrosinemia type II which is caused by deficiency of tyrosine aminotransferase (TAT) and provokes eyes, skin and central nervous system (CNS) disturbances. We have recently reported that tyrosine promoted oxidative stress *in vitro* but the exact mechanisms of brain damage in these disorders are poorly known. In the present study, we investigated the *in vivo* effect of L-tyrosine (500 mg/Kg) on oxidative stress indices in cerebral cortex homogenates of 14-day-old Wistar rats. A single injection of L-tyrosine decreased glutathione and thiol-disulfide redox state (SH/SS ratio) while thiobarbituric acid-reactive substances, protein carbonyl content and glucose-6-phosphate dehydrogenase activity were enhanced. In contrast, the treatment did not affect ascorbic acid content, and the activities of superoxide dismutase, catalase and glutathione peroxidase. These results indicate that acute administration of L-tyrosine may impair antioxidant defenses and stimulate oxidative damage to lipids and proteins in cerebral cortex of young rats *in vivo.* This suggests that oxidative stress may represent a pathophysiological mechanism in hypetyrosinemic patients.

Keywords: Tyrosine; tyrosine administration; hypertyrosinemias; tyrosinemia type II; rat brain; oxidative stress.

1. Introduction

Inherited deficiencies of enzymes involved in tyrosine catabolism leads to hypertyrosinemia. Three distinct autosomal recessive diseases of tyrosine metabolism have been identified in humans: tyrosinemia type I (deficiency of fumarylacetoacetate hydrolase (FAH)), tyrosinemia type II (deficiency of tyrosine aminotransferase (TAT)), and tyrosinemia type III (deficiency of 4-hydroxyphenylpyruvate dioxygenase (4-HPPD)) (Mitchell *et al.*, 2001; Held, 2006). Hypertyrosinemia is associated with neurologic and development difficulties in several patients with TAT deficiency and, less frequently, in patients with 4-HPPD deficiency and transient hypertyrosinemia (Light *et al.*, 1973; Mamunes *et al.*, 1976; Rice *et al.*, 1989). Interestingly, plasma tyrosine levels are higher in TAT-deficiency comparing to the other causes of hypertyrosinemias and range from 370 to 3,420 μM (normal < 90 μM) in untreated patients. Tyrosinemia type II is characterized by a triad of painful palmo-plantar keratoderma, ocular involvement with photophobia and bilateral keratitis, and mental retardation. The involvement of the CNS is variable and ranges from severe mental retardation to slight decreases in intelligence and may be associated with microcephaly, nystagmus, tremor, ataxia, self-mutilating behavior, fine motor coordination disturbances, language deficits, and convulsions (Goldsmith *et al.*, 1973; Lemonnier *et al.*, 1979; Rabinowitz *et al.*, 1995; Macsai *et al.*, 2001; Mitchell *et al.*, 2001; Valikhani *et al.*, 2005).

In rats, the administration of 4-HPPD inhibitors have been associated with the development of exudative keratites and painful edema of the paws (Lock *et al.*, 1996), and the authors suggested that the signs of oculocutaneous tyrosinemia may be related to increased tissue levels of tyrosine. On the other hand, cognitive impairment is reported in patients with 4-HPPD deficiency (tyrosinemia type III) and

FAH deficiency (tyrosinemia type I) (Ellaway *et al.*, 2001; Masurel-Paulet *et al.*, 2008). The inhibition of TAT associated with pretreatment of tyrosine produced typical neurotoxic symptoms in rats, such as, head retraction, hyperextension of the tail, turning behavior, uncoordinated gait, walking backwards, and bouts of convulsive seizures (Shasi Vardhan *et al.*, 1997). These findings suggest that high levels of tyrosine may not be innocuous to the central nervous system.

We have recently demonstrated that high concentrations of tyrosine provoke oxidative stress *in vitro* in cerebral cortex of rats (Sgaravatti *et al.*, 2008). So, in the present study we investigated the *in vivo* effect of acute administration of L-tyrosine on the following oxidative stress parameters in cerebral cortex homogenates of young rats: thiobarbituric acid-reactive substances (TBA-RS) levels, to assess lipid peroxidation; protein carbonyl content, to evaluate protein oxidation; thiol and disulfide contents, to determine SH/SS redox state; reduced glutathione (GSH) and ascorbic acid levels, to estimate the main non-enzymatic antioxidant defenses in the brain; the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), to evaluate enzymatic antioxidant defenses; and glucose-6-phosphate dehydrogenase (G6PDH) activity, to measure the main NADPH-producing system.

2. Materials and Methods

2.1. Reagents and equipments

All chemicals were purchased from Sigma (St. Louis, MO, USA) except thiobarbituric acid, which was purchased from Merck (Darmstadt, Germany). A double-beam spectrophotometer with temperature control (Hitachi U-2001), a spectrofluorometer SpectraMax Gemini XPS (Molecular Devices) and a Wallac 1409

Scintillation Counter were used for the measurements. Eppendorf 5417R and Eppendorf 5403 were used for centrifugation procedures.

2.2. Animals

Fourteen-day-old Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, were used. Rats were kept with dams until they were sacrificed. The dams had free access to water and 20% (w/w) protein commercial chow (Supra, Porto Alegre, RS, Brazil). They were maintained in a room with a 12:12 h light/dark cycle (lights on 7:00-19:00 h) and with air conditioned controlled temperature (22 ± 1°C). The Principles of Laboratory Animal Care (NIH publication # 80-23, revised 1996) were followed throughout the experiments.

2.3. Acute administration of L-tyrosine methyl ester and tissue preparation

L-Tyrosine methyl ester was dissolved in 0.1% Tween 20 in saline solution (pH was adjusted to 7.4) and the equivalent to 500 mg/Kg body weight of free L-tyrosine was administered intraperitoneally. Controls received 0.1% Tween 20 in saline solution. One hour after injections, rats were killed by decapitation without anesthesia, and the brain was rapidly removed and kept on an ice-plate. The olfactory bulb, pons and medulla were discarded and the cerebral cortex was dissected, weighed and kept chilled until homogenization. Cerebral cortex was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4ºC to discard nuclei and cell debris (Llesuy *et al.*, 1985). The pellet was discarded and the supernatant was immediately separated and used for the measurements.

2.4. Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS were measured according to Ohkawa *et al.* (1979). Briefly, to glass tubes were added, in order of appearance: 200 μL of sample; 50 μL of sodium dodecyl sulfate (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 hour. The mixture was allowed to cool on water for 5 min, and was centrifuged 750 *g* for 10 min. The resulting pink stained TBA-RS obtained was determined in a spectrophotometer at 532 nm. Calibration curve was performed using 1,1,3,3-tetramethoxypropane as standard, being subjected to the same treatment as that of the samples. TBA-RS were represented as nmol TBA-RS/mg protein.

2.5. Protein carbonyl content

Protein carbonyl content was assayed by a method based on the reaction of carbonyls with dinitrophenylhydrazine (DNPH) forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer, 1994). Briefly, 200 μL of sample were added to 400 μL 10 mM DNPH in 2 M HCl, or to 2 M HCl (blank). This mixture was kept in dark room for 1 hour and vortexed each 15 min. After that, 500 μL of 20% trichloroacetic acid (TCA) were added to each tube. The mixture was vortexed and centrifuged at 20,000 *g* for 3 min. The supernatant obtained was discarded. The pellet was washed with 1 mL ethanol:ethyl acetate (1:1, v/v), vortexed and centrifuged at 20,000 *g* for 3 min to remove the free DNPH. The supernatant was discarded and the pellet was resuspended in 600 μL of 6 M guanidine (prepared in a 20 mM potassium phosphate pH 2.3), vortexed and incubated at 60 ^{o}C for 15 min. After that, it was centrifuged at 20,000 *g* for 3 min and

the supernatant was used to measure absorbance in a spectrophotometer at 370 nm. The molar extinction coefficient of 22,000 for DNPH was used to calculate the concentration of carbonyls content and the results were reported as nmol/mg protein.

2.6. Thiol and disulfide contents (SH/SS redox state)

Thiol and disulfide contents were determined essentially according to Zahler and Cleland (1968). For disulfide measurement, 50 μL of sample were mixed with 25 μL of 0.05 M Tris pH 9.0 and 25 μL of 3 M dithiothreitol (DTT). After 15 min at room temperature, 50 μL of 1.0 M Tris pH 8.1, 375 μL of 5 mM sodium arsenite, and enough water to give a final volume of 850 μL were added, and the solutions were mixed and allowed to stand for 3 min. For thiol determination, DTT and sodium arsenite were omitted of the procedure. To start the reaction, 25 μL of 3 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.05 M acetate pH 5.0 were added. After 15 min, the absorbance of TNB formed (equivalent to the amount of SH groups) was measured in a spectrophotometer at 412 nm. Thiol and disulfide groups were represented as nmol TBN/mg protein.

2.7. Reduced glutathione (GSH) content

This method is based on the reaction of GSH with the fluorophore o-phtalaldeyde (OPT) after deproteinizing the samples, and was measured according to Browne and Armstrong (1998). Initially, 25% metaphosphoric acid was used to deproteinize the samples, which were centrifuged at 1,000 *g* for 10 min. Briefly, to 15 μL of each supernatant were added 270 μL of sodium phosphate buffer pH 8.0 and 15 μL OPT 1 mg/mL (prepared in metanol). The mixture was vortexed and allowed to stand in the dark at room temperature for 15 min. After that, fluorescence was

measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curves were established with standard GSH (0.01–1 mmol/L). The results were represented in nmol GSH/mg protein.

2.8. Ascorbic acid content

Ascorbic acid was measured according the method of Omaye *et al.* (1979). Samples were deproteinized by addition of metaphosphoric acid 10% (1:1) and, after thorough mixing, were centrifuged for 20 min at 3,500 *g*. To an aliquot of 300 μL of the previous supernatant, 300 μL of citrate/acetate buffer pH 4.15 and 300 μL of 2,6-dichlorophenolindophenol (DCIP) solution were added and this mixture was read in a spectrophotometer. Ascorbic acid reduces the DCIP causing a decrease in its absorption at 520 nm. A standard curve, including a reagent blank, was constructed with standards ranging between 0 and 15 μg of ascorbic acid per milliliter of 5% metaphosphoric acid. Ascorbic acid content was represented as μg ascorbic acid/mg protein.

2.9. Superoxide dismutase (SOD) assay

The assay of SOD activity was carried out as described by Marklund (1985). 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 spectrophotometrically 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.

2.10. Catalase (CAT) assay

CAT activity was assayed by the method of Aebi (1984) using a spectrophotometer. This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0. One CAT unit is defined as one μmol of H_2O_2 consumed per minute and the specific activity is represented as units per mg protein.

2.11. Glutathione peroxidase (GPx) assay

GPx activity was measured according to the method of Wendel (1981) using *tert*-butyl hydroperoxide as substrate. NADPH disappearance was monitored at 340 nm using a spectrophotometer. The reaction medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as one μmol of NADPH consumed per minute and the specific activity is represented as units per mg protein.

2.12. Glucose-6-phosphate dehydrogenase (G6PDH) assay

G6PDH activity was measured by the method of Leong and Clark (1984), in which the reaction mixture (1 mL) contained: 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$, and sample. The reaction was started by the addition of 1 mM glucose-6-phosphate and was followed in a spectrophotometer at 340 nm. One G6PDH unit corresponds to 1 μmol of substrate transformed per min and the specific activity is represented as units per mg protein.

2.13. Protein determination

Protein concentration was determined in cerebral cortex samples using bovine serum albumin as a standard (Lowry *et al.*, 1951).

2.14. Statistical analysis

Data were analyzed by the Student's t-test for unpaired samples. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

3. Results

Lipid peroxidation was investigated by measuring TBA-RS levels in rat cerebral cortex homogenates after acute administration of L-tyrosine. Figure 1 shows that the acute administration of L-tyrosine 500 mg/Kg was able to increase TBA-RS levels in rats cerebral cortex [$t(11)=2.82$; $p<0.05$]. The results demonstrate that acute administration of L-tyrosine may promote lipid peroxidation in cerebral cortex homogenates from 14-day-old rats.

The effect of acute administration of L-tyrosine on tissue proteins was also evaluated. To accomplish this, carbonyl content was measured as a parameter of oxidative protein damage. Figure 2 shows that the carbonyl content was significantly enhanced by the acute administration of L-tyrosine [$t(12)=2.82$; $p<0.05$] in cerebral cortex homogenates, indicating an increased protein carbonylation.

The effect of acute administration of L-tyrosine on thiol/disulfide redox status was studied by measuring SH/SS ratio. Figure 3 shows that acute administration of L-tyrosine significantly decreased SH/SS ratio [$t(8)=3.63$; $p<0.05$], suggesting alteration of the redox state.

The effect of acute administration of L-tyrosine on non-enzymatic antioxidant defenses of cerebral cortex homogenates was studied by determining GSH (Figure 4) and ascorbic acid levels (Figure 5). It can be observed that rats subjected to acute administration of L-tyrosine significantly decreased GSH levels in cerebral cortex homogenates [$t(23)=3.46$; $p<0.05$], while ascorbic acid content was not altered [$t(10)=0.59$; $p>0.05$]. These data indicate that the main non-enzymatic antioxidant defense in the brain, represented by GSH, is compromised by acute administration of L-tyrosine.

The activities of the enzymes SOD, CAT, GPx and G6PDH were assayed in cerebral cortex after L-tyrosine treatment (Table 1). The administration of L-tyrosine markedly increased G6PDH activity in cerebral cortex homogenates [$t(11)=2.45$; $p<0.05$], while did not alter the activities of SOD [$t(12)=0.73$; $p>0.05$], CAT [$t(8)=0.68$; $p>0.05$] and GPx [$t(12)=1.36$; $p>0.05$]. These results indicate that the main NADPH-producing system is stimulated in cerebral cortex from rats subjected to acute administration of L-tyrosine.

4. Discussion

Inborn errors of tyrosine catabolism lead to hypertyrosinemia, especially tyrosinemia type II or TAT deficiency, in which tyrosine levels are highly elevated in tissues and physiological fluids of these patients (Mitchell *et al.*, 2001). Tyrosinemia type II is characterized by skin and eye lesions and, occasionally, neurological complications. Mental retardation and other neurological findings have been reported in patients affected by tyrosinemia type II. Although the underlying mechanisms of brain damage in this disorder remain unclear, it has been speculated that the degree of CNS involvement may be correlated to abnormal high levels of plasma tyrosine

(Mitchell *et al.*, 2001). In addition, we have recently demonstrated that *in vitro* tyrosine altered oxidative stress indices in cerebral cortex of rats (Sgaravatti *et al.*, 2008).

Plasma tyrosine levels in TAT-deficient patients are approximately 3 to 30 times higher than normal (Mitchell *et al.*, 2001) and the administration of L-tyrosine 100 mg/Kg enhanced twofold tyrosine concentration in cerebral cortex 1 hour after injection (Morre *et al.*, 1980). So, we have chosen to study the effect of acute administration of 500 mg L-tyrosine per Kg body weight in order to obtain cerebral cortex tyrosine concentrations about 10 times normal, which are similar variations of tyrosine concentration observed in patients affected by tyrosinemia type II. In addition, the highest relative increase (related to controls) of tyrosine concentration was reported in cerebral cortex compared to other brain regions when L-tyrosine is administered to rats (Morre *et al.*, 1980).

The influence of acute administration of L-tyrosine on lipid and protein oxidation in cerebral cortex of rats was investigated. TBA-RS reflects the content of malondialdehyde, the most abundant individual aldehyde resulting from lipid peroxidation (Esterbauer and Cheeseman, 1990). On the other hand, protein carbonylation is a useful index of protein oxidation (Reznick and Packer, 1994; Stadtman and Levine, 2003). We have demonstrated that L-tyrosine is able to increase TBA-RS levels and protein carbonyl content in cerebral cortex suggesting that oxidative damage to lipids and proteins has occurred *in vivo*.

Enhanced generation of reactive species and/or impaired antioxidant detoxification functions contribute to an imbalance between oxidative and reductive reactions, which alters thiol/disulfide redox state (Moriarty-Craige and Jones, 2004). A significant reduction in SH/SS ratio was observed, suggesting that L-tyrosine

treatment was able to disturb the thiol redox status *in vivo*. In addition, tyrosine also decrease SH/SS ratio *in vitro* (Sgaravatti *et al.*, 2008). Given the numerous processes that are dependent upon cellular redox state, alteration of SH/SS ratio is considered an important general parameter indicative of oxidative stress (Moriarty-Craige and Jones, 2004).

GSH and ascorbic acid are considered the most abundant antioxidants of low molecular weight in the CNS (Rice and Russo-Menna, 1998). It was demonstrated that the acute administration of L-tyrosine significantly decreased GSH levels, indicating that the non-enzymatic antioxidant defenses may be compromised in cerebral cortex. Considering that the GSH protects against oxidative damage caused by various reactive oxygen species and that this antioxidant is involved in the maintenance of the thiol moieties of proteins and low molecular weight compounds (Anderson, 1998), it is possible that the low GSH levels found became the CNS prone to reactive species and oxidative stress.

In contrast, our previous findings showed that TBA-RS levels, protein carbonyls were not altered by tyrosine *in vitro* and, interestingly, GSH content was also unaffected (Sgaravatti *et al.*, 2008). This may indicate that separated mechanisms are responsible by the differences between *in vitro* and *in vivo* effects of tyrosine. Moreover, we cannot rule out that other tyrosine derivatives are responsible for the present *in vivo* effects since increased urinary excretion of 4-hydroxyphenylpyruvic acid, 4-hydroxyphenyllatic acid, 4-hydroxyphenylacetic acid, N-acetyltyrosine, and 4-tyramine were observed in hypertyrosinemic patients (Goldsmith *et al.*, 1973; Rabinowitz *et al.*, 1995; Macsai *et al.*, 2001; Mitchell *et al.*, 2001; Valikhani *et al.*, 2005; Held, 2006).

The *in vivo* effect of L-tyrosine on SOD, CAT, GPx and G6PDH was also evaluated. SOD catalyses the dismutation of superoxide radical, CAT metabolizes hydrogen peroxide, GPx removes organic hydroperoxides as well as hydrogen peroxide (Halliwell and Gutteridge, 1985), and G6PDH is the main enzyme that generates reducing power in the form of NADPH (Halliwell and Gutteridge, 2007). The acute administration of L-tyrosine did not change enzymatic antioxidant defenses, as determined by the activities of SOD, CAT and GPx. On the other hand, G6PDH activity was increased in cerebral cortex from rats subjected to acute administration of L-tyrosine. This result suggests that an increased necessity of NADPH occurred possibly trying to recycle GSH/GSSG system by glutathione reductase. This finding is in line with the present result of low GSH levels produced by the same treatment. Furthermore, G6PDH activity was not altered by tyrosine *in vitro* and no alteration of GSH levels was observed (Sgaravatti *et al.*, 2008).

It is considered that oxidative stress can result from distinct situations, such as generation of reactive species at abnormally high rate, insufficient antioxidant defenses, releasing of transition metal ions, or due to a combination of these conditions (Halliwell, 2001). In addition, the damaging consequences of oxidative stress have been implicated in a variety of diseases of the CNS including inherited metabolic disorders (Reznick and Parker, 1993; Halliwell, 2001; Wajner *et al.*, 2004; Halliwell, 2006). Taken together, the present results indicate that acute administration of L-tyrosine may promote oxidative stress in cerebral cortex of 14-day-old rats by changing the redox state, decreasing GSH levels, and enhancing G6PDH activity, and eventually stimulating oxidative damage to lipids and proteins.

Although the exact tyrosine brain levels of patients with TAT deficiency are not yet established, it is possible that oxidative stress may contribute, along with other

mechanisms, to the neurological dysfunction characteristic of hypertyrosinemias, especially tyrosinemia type II. If similar findings were observed in humans, administration of antioxidants should be considered as a potential adjuvant therapy for patients presenting hypertyrosinemia.

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Table 1. Effect of acute administration of L-tyrosine on superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glucose-6-phosphate dehydrogenase (G6PDH) activities in cerebral cortex homogenates from 14-day-old rats.

Antioxidant enzyme activity (U/mg protein)	Control	L-Tyrosine
SOD (n=6-8)	3.82 ± 0.38	4.03 ± 0.59
CAT (n=5)	2.49 ± 0.19	2.34 ± 0.46
GPx (n=6-8)	20.21 ± 2.66	17.83 ± 3.57
G6PDH (n=6-7)	18.87 ± 1.62	20.52 ± 0.72*

Data are expressed as mean ± SD for independent experiments performed in duplicate. One SOD unit is defined as 50% inhibition of pyrogallol autoxidation. One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute. One GPx unit is defined as 1 μmol of NADPH consumed per minute. One G6PDH unit is defined as 1 μmol of substrate transformed per minute. *$p<0.05$ compared to control (Student's t-test for unpaired samples).

FIGURE LEGENDS

Figure 1. Effect of acute administration of L-tyrosine on thiobarbituric acid-reactive substances (TBA-RS) levels in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean ± SD (n=5-8) for independent experiments performed in duplicate. * $p<0.05$ compared to control (Student's t-test for unpaired samples).

Figure 2. Effect of acute administration of L-tyrosine on protein carbonyl content in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean ± SD (n=6-8) for independent experiments performed in triplicate. * $p<0.05$ compared to control (Student's t-test for unpaired samples).

Figure 3. Effect of acute administration of L-tyrosine on SH/SS ratio in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean ± SD (n=4-6) for independent experiments performed in duplicate. * $p<0.05$ compared to control (Student's t-test for unpaired samples).

Figure 4. Effect of acute administration of L-tyrosine on reduced glutathione (GSH) content in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean ± SD (n=12-13) for independent experiments performed in duplicate. * $p<0.05$ compared to control (Student's t-test for unpaired samples).

Figure 5. Effect of acute administration of L-tyrosine on ascorbic acid content in cerebral cortex homogenates from 14-day-old rats. Data are expressed as mean ±

SD (n=4-6) for independent experiments performed in duplicate. * $p<0.05$ compared to control (Student's t-test for unpaired samples).

Figure 1

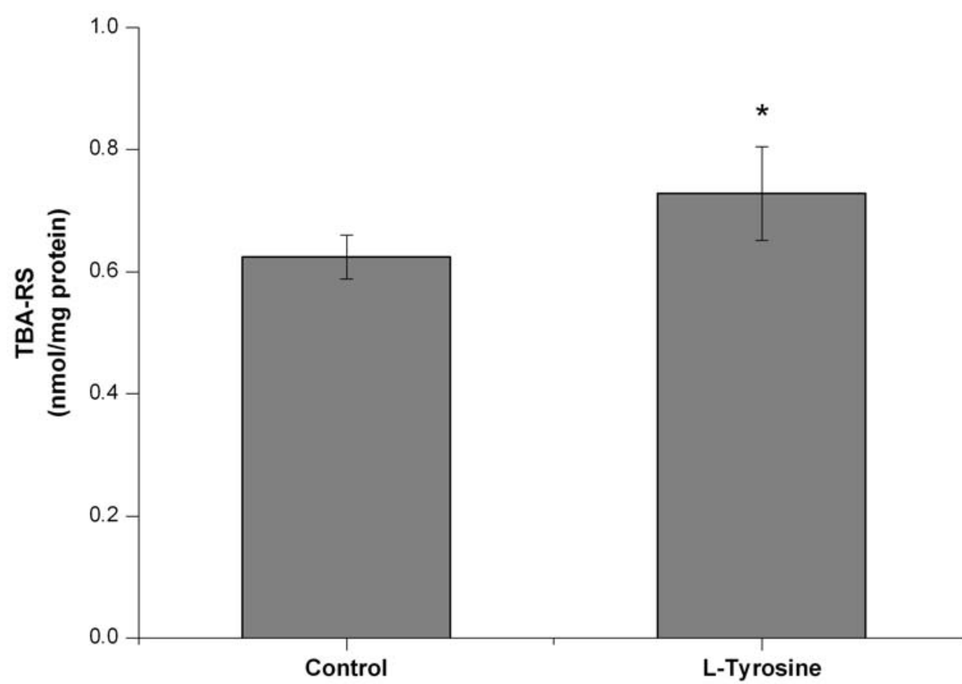


Figure 2

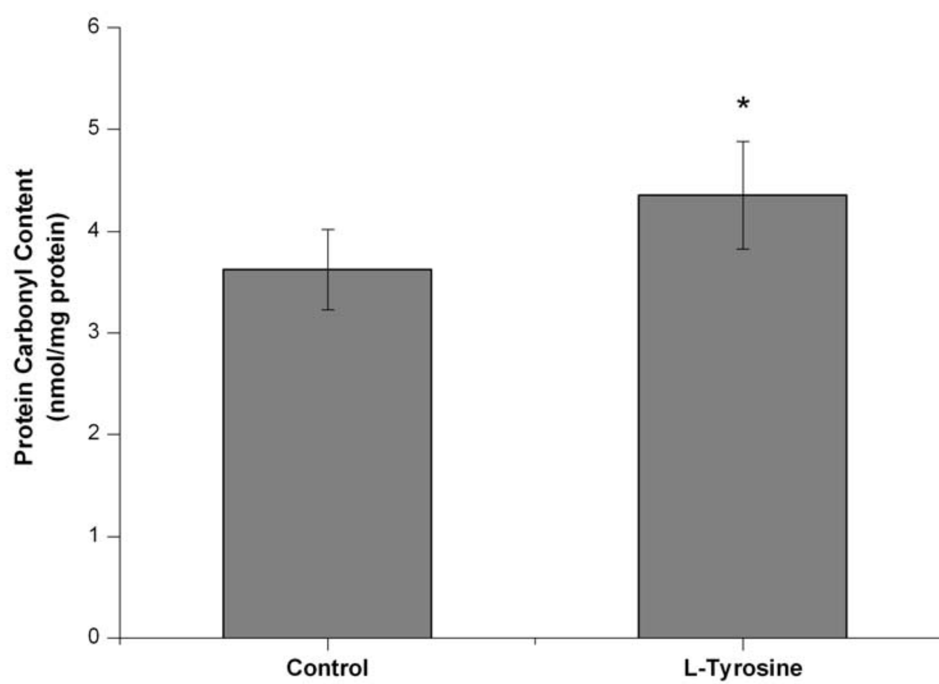


Figure 3

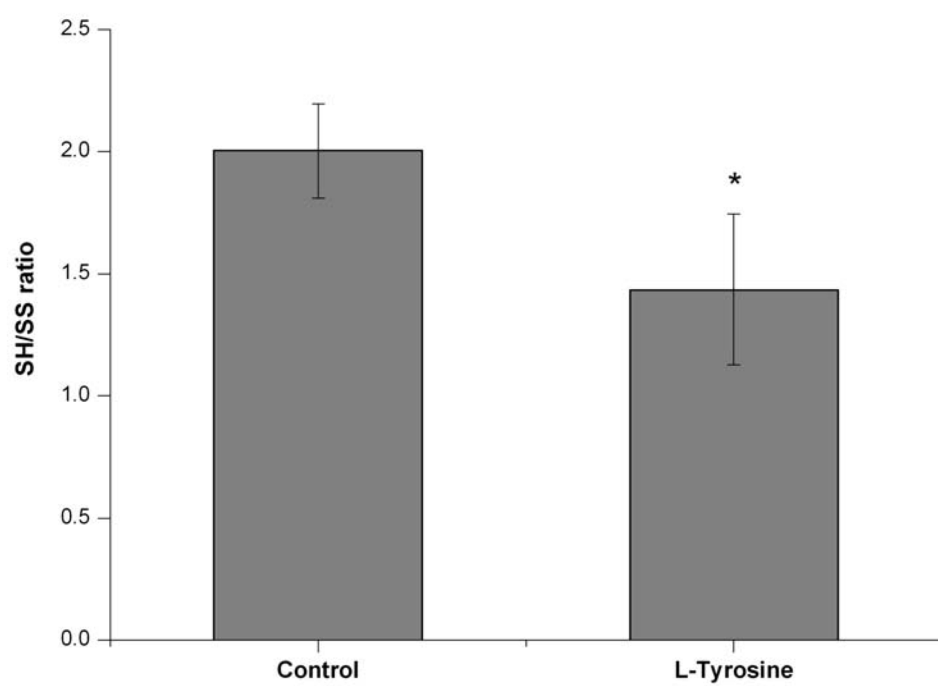


Figure 4

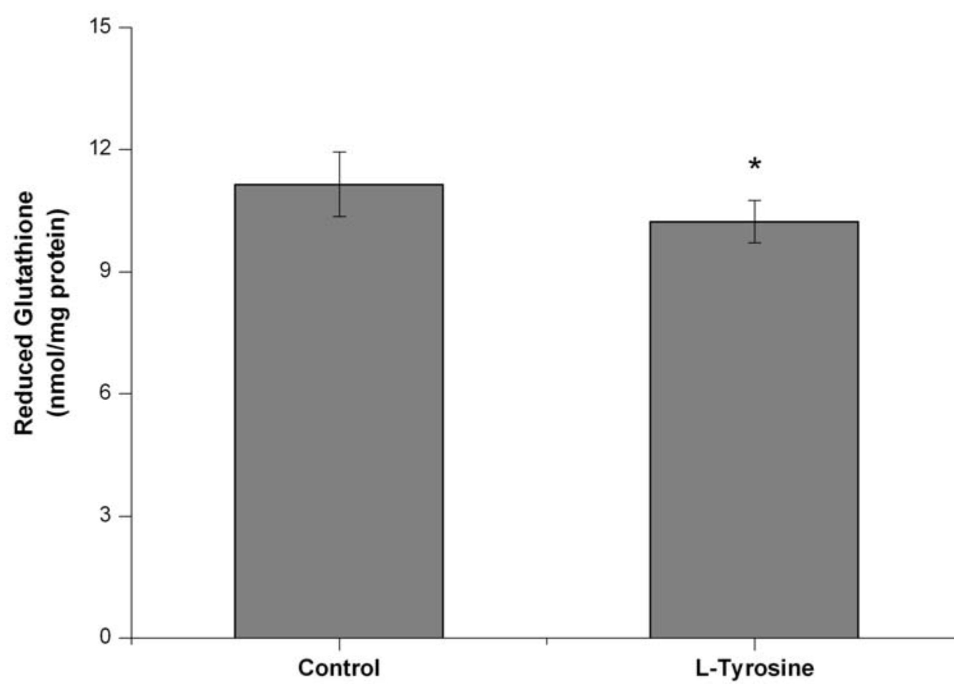
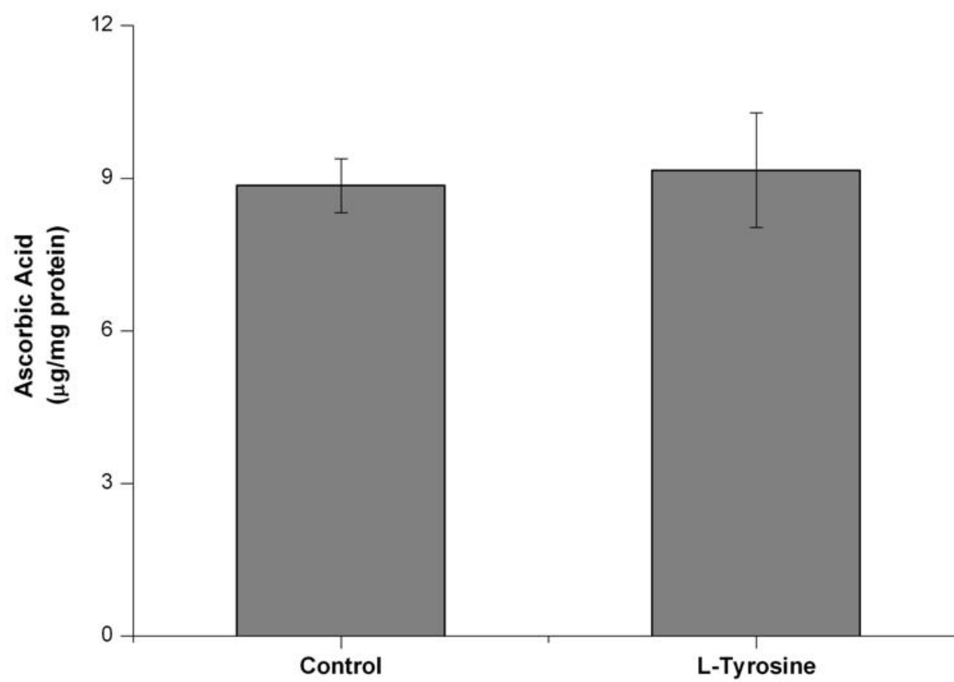


Figure 5



4. DISCUSSÃO

4.1. EFEITOS DO ÁCIDO γ-HIDROXIBUTÍRICO E DO 1,4-BUTANODIOL

O GHB é um constituinte endógeno do sistema nervoso central que atua como neurotransmissor ou neuromodulador (CASH, 1994; MAITRE, 1997). Altos níveis de GHB são encontrados nos tecidos e fluidos fisiológicos de indivíduos que o utilizam, para fins terapêuticos ou recreativos, ou naqueles com a deficiência da SSADH (GIBSON E JAKOBS, 2001; WONG *et al.*, 2004; SNEAD E GIBSON, 2005). Além do acúmulo de GHB, os pacientes afetados pela deficiência da SSADH apresentam sinais e sintomas neurológicos (GIBSON *et al.,* 1997*;* GIBSON E JAKOBS, 2001; GIBSON *et al.*, 2003). No entanto, os mecanismos responsáveis pelo dano cerebral permanecem ainda não esclarecidos. A comparação de achados bioquímicos e clínicos desses pacientes com os efeitos neurofarmacológicos do GHB observados em animais e seres humanos indica que o agente neurotóxico primário da deficiência da SSADH é o GHB.

Vários efeitos do GHB sugerem uma possível relação entre o estresse oxidativo e a neurodegeneração observada nos pacientes com a deficiência da SSADH. Um desses efeitos é que o GHB *in vitro* e *in vivo* altera a síntese e liberação de dopamina (HEDNER E LUNDBORG, 1982; HECHLER *et al.*, 1991; CASH, 1994; MAITRE, 1997). Considerando que a dopamina é uma molécula auto-oxidável capaz de formar ERO (HALLIWELL E GUTTERIDGE, 2007), é provável que o aumento na síntese e liberação desse neurotransmissor causado pelo GHB favoreça uma maior produção de radicais livres. Sabe-se também que tanto os pacientes com a deficiência da

SSADH quanto os animais geneticamente modificados apresentam um aumento no metabolismo da dopamina (GIBSON E JAKOBS, 2001; GIBSON *et al.*, 2003; GUPTA *et al.*, 2003; PEARL *et al.*, 2003), o que reforça tais efeitos do GHB. O comprometimento do metabolismo energético cerebral através da diminuição da produção de CO_2 e inibição da síntese lipídica (SILVA *et al.*, 1999) é outro efeito do GHB que pode estar relacionado ao estresse oxidativo. Aliado a esses efeitos do GHB, está o fato de que o estresse oxidativo é um dos mecanismos responsáveis pela neurodegeneração observada na doença de Huntington (REZNICK E PACKER, 1993; HALLIWELL, 2006; HALLIWELL E GUTTERIDGE, 2007), em que altas concentrações de GHB foram detectadas *post-mortem* no cérebro de alguns pacientes (ANDO *et al.*, 1979).

Diante dessas evidências, o efeito do GHB foi avaliado sobre alguns parâmetros de estresse oxidativo em homogeneizados de córtex cerebral de ratos jovens. Dados preliminares mostraram a influência *in vitro* do GHB sobre a lipoperoxidação e as defesas antioxidantes. O GHB aumentou significativamente a medida de quimiluminescência espontânea e os níveis de TBA-RS, enquanto que o TRAP e o TAR foram marcadamente diminuídos. No entanto, as atividades das enzimas antioxidantes SOD, CAT e GPx não foram alteradas pelo GHB. Esses resultados indicam que *in vitro* o GHB pode estimular a lipoperoxidação e reduzir as defesas antioxidantes não-enzimáticas em homogeneizados de córtex cerebral de ratos jovens.

Dando continuidade a esses estudos, o efeito da administração aguda de 10 mg/Kg de GHB foi investigado sobre indicativos de lipoperoxidação e defesas antioxidantes não-enzimáticas. A lipoperoxidação foi avaliada através da medida dos níveis de TBA-RS. Observou-se que a administração aguda de GHB aumentou significativamente os níveis de TBA-RS. Considerando que os níveis de TBA-RS

refletem a quantidade de malondialdeído, um dos principais aldeídos formados no processo de lipoperoxidação (ESTERBAUER E CHEESEMAN, 1990), esse achado sugere que a administração aguda de GHB induz a lipoperoxidação em homogeneizados de córtex cerebral de ratos jovens. As defesas antioxidantes não-enzimáticas foram analisadas através da determinação do TRAP e TAR. Verificou-se uma redução significativa do TRAP e do TAR nos animais submetidos ao tratamento agudo com o GHB. Enquanto que o TRAP avalia o conteúdo das defesas antioxidantes não-enzimáticas, o TAR reflete a capacidade de certo tecido modular o dano associado a uma produção aumentada de radicais livres. Ou seja, o TRAP é um parâmetro quantitativo e o TAR é uma medida qualitativa, que representa a reatividade antioxidante do tecido (LISSI *et al.*, 1995). Portanto, os resultados anteriormente descritos indicam que houve um decréscimo tanto dos componentes não-enzimáticos responsáveis pela defesa antioxidante cerebral (TRAP) quanto na velocidade com que esses antioxidantes são capazes de reagir com os radicais livres (TAR). Assim, a administração aguda de GHB compromete as defesas antioxidantes não-enzimáticas em homogeneizados de córtex cerebral de ratos jovens. Tais resultados estão de acordo com os efeitos *in vitro* do GHB sobre esses mesmos parâmetros de estresse oxidativo e confirmam que o GHB pode induzir a lipoperoxidação e diminuir as defesas antioxidantes não-enzimáticas.

A utilização indiscriminada do GHB como droga de abuso provocou uma restrição na sua produção e comercialização (WONG *et al.*, 2004; SNEAD E GIBSON, 2005). Conseqüentemente, a alternativa encontrada para melhor esclarecer o papel do estresse oxidativo na neurotoxicidade do GHB foi estudar o efeito da administração aguda do 1,4-BD sobre alguns parâmetros de estresse oxidativo. O 1,4-BD é biotransformado em GHB através das enzimas hepáticas álcool

desidrogenase e aldeído desidrogenase (ROTH E GIARMAN, 1968; SNEAD *et al.*, 1989) e seus efeitos farmacológicos são a ele atribuídos (POLDRUGO E SNEAD, 1984; SNEAD *et al.*, 1989, CARAI *et al.*, 2002; QUANG *et al.*, 2002; CARTER *et al.*, 2006).

Inicialmente, o efeito da administração aguda de 20 ou 120 mg/Kg de GHB foi determinado sobre dois índices de lipoperoxidação, a quimiluminecência espontânea e os níveis de TBA-RS. A administração aguda de 120 mg/Kg de 1,4-BD aumentou significativamente tanto a quimiluminescência espontânea quanto os níveis de TBA-RS. A quimiluminescência espontânea mede a luz emitida pelos lipídios peroxidados resultantes de um aumento na produção de ERO e ERN (HALLIWELL E GUTTERIDGE, 2007), enquanto que os níveis de TBA-RS, como citado anteriormente, refletem o conteúdo de malondialdeído formado durante a lipoperoxidação (ESTERBAUER E CHEESEMAN, 1990). Esses resultados mostram que a administração aguda de 120 mg/Kg de 1,4-BD pode causar dano oxidativo a lipídios em homogeneizados de córtex cerebral de ratos jovens.

Para avaliar as defesas antioxidantes não-enzimáticas, estudou-se o efeito da administração aguda de duas diferentes doses de 1,4-BD sobre o TAR. Observou-se uma redução significativa na medida do TAR em homogeneizados de córtex cerebral de ratos submetidos ao tratamento agudo com 120 mg/Kg de 1,4-BD. O decréscimo na medida do TAR demonstra uma menor capacidade do tecido cerebral modular os danos associados a uma produção aumentada de espécies reativas, demonstrando que a administração de 120 mg/Kg de 1,4-BD pode prejudicar as defesas antioxidantes não-enzimáticas.

O efeito da administração aguda de 1,4-BD também foi investigado sobre parâmetros de dano oxidativo a proteínas: conteúdo de tióis totais e conteúdo de carbonilas protéicas. Nenhum desses parâmetros foi alterado pela administração

aguda de 1,4-BD, indicando que as proteínas foram preservadas da oxidação ou as proteínas oxidadas foram rapidamente degradadas.

Finalmente, o efeito do 1,4-BD sobre as atividades das enzimas antioxidantes SOD, CAT e GPx foi determinado em homogeneizados de córtex cerebral de ratos jovens. A SOD catalisa a dismutação do radical superóxido; a CAT acelera a decomposição do peróxido de hidrogênio; a GPx catalisa a redução do peróxido de hidrogênio e de outros hidroperóxidos orgânicos às custas de GSH (HALLIWELL E GUTTERIDGE, 1985). Nenhuma das enzimas antioxidantes estudadas teve a sua atividade alterada pela administração aguda de 20 ou 120 mg/Kg de 1,4-BD. Esses resultados confirmam tanto os efeitos *in vitro* do GHB como aqueles observados por Latini e colaboradores (2007) em córtex cerebral de camundongos geneticamente modificados.

Apesar do 1,4-BD ser metabolizado em GHB, os resultados anteriormente apresentados sugerem que o 1,4-BD não produz efeitos idênticos ao GHB: a dose de 10 mg/Kg de GHB estimulou a lipoperoxidação e diminuiu as defesas antioxidantes não-enzimáticas, enquanto que nenhum dos parâmetros de estresse oxidativos foi alterado pela administração de 20 mg/Kg de 1,4-BD. É possível que tanto diferenças experimentais quanto as características farmacocinéticas do 1,4-BD possam interferir nos efeitos farmacológicos e, dessa forma, contribuir para que essas variações fossem observadas.

De forma semelhante aos efeitos *in vitro* e *in vivo* do GHB, a administração aguda de 120 mg/Kg de 1,4-BD também foi capaz de estimular a lipoperoxidação e diminuir as defesas antioxidantes não-enzimáticas. Como o termo estresse oxidativo é usado para designar situações em que a geração de espécies reativas ultrapassa a capacidade de reparo das defesas antioxidantes (HALLIWELL, 2001), esses

resultados sugerem a participação do estresse oxidativo na neurotoxicidade do GHB e, provavelmente, nos mecanismos etiopatogênicos da deficiência da SSADH. Corroborando com tais achados, Latini e colaboradores (2007) mostraram o comprometimento das defesas antioxidantes não-enzimáticas (diminuição do conteúdo de GSH) e a indução da lipoperoxidação (aumento dos níveis de TBA-RS) em homogeneizados de córtex cerebral de camundongos com a deficiência da SSADH.

Outras evidências também indicam o envolvimento do estresse oxidativo na deficiência da SSADH. Tem sido relatado que tanto compostos guanidínicos quanto o ácido D-2-hidroxiglutárico induzem dano oxidativo (LATINI *et al.*, 2003; ZUGNO *et al.*, 2004; ZUGNO *et al.*, 2006) e que níveis elevados dessas substâncias são encontrados no modelo animal e em pacientes com a deficiência da SSADH (JANSEN *et al.*, 2006; STRUYS *et al.*, 2006a; STRUYS *et al.*, 2006b). Sabe-se, ainda, que o 4-hidróxi-2-nonenal (4-HNE), um dos produtos finais da lipoperoxidação, não só é transformado em um composto não-tóxico pela SSADH (MURPHY *et al.*, 2003), mas também é capaz de inibi-la (NGUYEN E PICKLO, 2003). Entretanto, quando há a deficiência da SSADH, o acúmulo de GHB pode estimular a lipoperoxidação e, conseqüentemente, a formação de 4-HNE. Nessa situação, o 4-HNE não será detoxificado através da SSADH e, provavelmente, contribuirá tanto para uma maior inativação dessa enzima quanto para uma produção excessiva de GHB, o que resultará em um ciclo vicioso. Por fim, dados na literatura também mostram que ocorre acúmulo de peróxido de hidrogênio no modelo da deficiência da SSADH em plantas (FAIT *et al.*, 2005).

Tanto os efeitos *in vitro* e *in vivo* do GHB quanto aqueles da administração aguda de 1,4-BD sugerem a participação do estresse oxidativo na neurotoxicidade do GHB. No entanto, é muito difícil extrapolar tais resultados para a condição

humana. Porém, se esses efeitos também ocorrerem no cérebro dos pacientes afetados, é possível que possam contribuir, ao menos em parte, para a disfunção neurológica observada na deficiência da SSADH. E, sendo assim, a administração de antioxidantes deveria ser testada como terapia auxiliar no tratamento desses pacientes. Além disso, a participação dos radicais livres nos mecanismos de ação e toxicidade do GHB também deve ser considerada em situações em que o GHB é utilizado para fins terapêuticos ou ilícitos. Portanto, esses resultados são relevantes não só para os pacientes com a deficiência da SSADH, mas também para aqueles indivíduos que utilizam o GHB ou seus precursores, GBL e 1,4-BD.

4.2. EFEITOS *IN VITRO* E *IN VIVO* DA L-TIROSINA

As tirosinemias tipo I, II e III são caracterizadas pela presença de elevadas concentrações de tirosina nos tecidos e fluidos fisiológicos dos pacientes afetados (MITCHELL *et al.*, 2001; HELD, 2006). Entretanto, os maiores níveis de tirosina são encontrados em indivíduos com a deficiência da TAT, ou seja, a tirosinemia tipo II (MITCHELL *et al.*, 2001). Esses indivíduos desenvolvem lesões oculares, cutâneas e, ocasionalmente, complicações neurológicas que podem variar desde leve decréscimo na inteligência até retardo mental severo associado à microcefalia (GOLDSMITH *et al.*, 1973; LEMONNIER *et al.*, 1979; RABINOWITZ *et al.*, 1995; MACSAI *et al.*, 2001; MITCHELL *et al.*, 2001; VALIKHANI *et al.*, 2005). No entanto, os mecanismos responsáveis pelo dano cerebral ainda não estão esclarecidos.

Sabe-se que a hipertirosinemia está associada aos transtornos neurológicos de diversos pacientes com tirosinemia tipo II, além de alguns outros com tirosinemia tipo III e tirosinemia transitória do recém-nascido (LIGHT *et al.*, 1973; MAMUNES *et al.*, 1976; RICE *et al.*, 1989). Também tem sido descrito que, tanto em seres humanos

quanto em animais experimentais, a administração do inibidor da 4-hidroxifenilpiruvato dioxigenase tem causado o aparecimento de sinais e sintomas característicos da tirosinemia tipo II provavelmente por aumentar a concentração de tirosina (LOCK *et al.*, 1996; MASUREL-PAULET *et al.*, 2008). Além disso, Shasi Vardhan e colaboradores (1997) demonstraram que a inibição da TAT pela neurotoxina da *Lathyrus sativus* causou um aumento de até 55% nos níveis hepáticos de tirosina e que ratos Wistar, pré-tratados com tirosina ou fenilalanina, apresentaram distúrbios neurológicos como retração da cabeça, hiperextensão da cauda, descoordenação motora e convulsões, entre outros. Apesar dos mecanismos pelos quais a hipertirosinemia compromete o SNC não estejam estabelecidos, é provável que altas concentrações de tirosina e/ou seus metabólitos tenham efeitos deletérios sobre o desenvolvimento cerebral.

Considerando que o estresse oxidativo parece estar envolvido em diversas doenças que afetam o sistema nervoso central, inclusive EIM (REZNICK E PARKER, 1993; HALLIWELL, 2001; WAJNER *et al.*, 2004; HALLIWELL, 2006), e que há pouca informação disponível sobre o possível papel do estresse oxidativo nos mecanismos de neurotoxicidade da tirosina e, indiretamente, do dano neurológico observado em pacientes com tirosinemia tipo II, os efeitos *in vitro* e *in vivo* da L-tirosina sobre vários parâmetros de estresse oxidativo foram avaliados em homogeneizados de córtex cerebral de ratos Wistar.

In vitro, os parâmetros de estresse oxidativo analisados na presença de 0,1; 1,0; 2,0 ou 4,0 mM de L-tirosina foram o TRAP, o TAR, o conteúdo de ácido ascórbico, o conteúdo de GSH, a quimiluminescência espontânea, os níveis de TBA-RS, o conteúdo de tióis totais e dissulfetos, o conteúdo de carbonilas protéicas,

a formação de ligações cruzadas entre DNA e proteínas e as atividades das enzimas SOD, CAT, GPx e G6PDH.

O efeito *in vitro* da L-tirosina sobre as defesas antioxidantes não-enzimáticas cerebrais foi estudado através da determinação do TRAP e do TAR. O TRAP mede o conteúdo das defesas antioxidantes não-enzimáticas, enquanto que o TAR avalia a velocidade com que os antioxidantes são capazes de neutralizar os radicais livres (LISSI *et al.*, 1995). Verificou-se que a L-tirosina causou uma redução significativa e dose-dependente desses dois parâmetros de estresse oxidativo em homogeneizados de córtex cerebral de ratos. Esses resultados indicam que a L- tirosina pode diminuir tanto a quantidade (TRAP) quanto a reatividade (TAR) dos antioxidantes não-enzimáticos e, conseqüentemente, tornar o tecido cerebral mais vulnerável aos radicais livres. Com o objetivo de esclarecer o prejuízo nas defesas antioxidantes não-enzimáticas (TRAP e TAR) causado pela L-tirosina, os níveis dos principais antioxidantes de baixo peso molecular presentes no SNC, GSH e ácido ascórbico (RICE E RUSSO-MENNA, 1998), foram avaliados. A L-tirosina não alterou o conteúdo de GSH, mas diminuiu significativamente os níveis de ácido ascórbico em homogeneizados de córtex cerebral de ratos. Portanto, a redução do TRAP e TAR observada pode estar relacionada à depleção do conteúdo de ácido ascórbico.

A influência da L-tirosina sobre a lipoperoxidação foi investigada através da medida da quimiluminescência espontânea e dos níveis de TBA-RS. A quimiluminescência espontânea mede a emissão de luz visível quando espécies excitadas (oxigênio *singlet* e carbonilas) geradas durante a lipoperoxidação retornam ao seu estado fundamental (HALLIWELL E GUTTERIDGE, 2007), enquanto os níveis de TBA-RS refletem o conteúdo de malondialdeído formado através da decomposição de hidroperóxidos lipídicos (ESTERBAUER E CHEESEMAN, 1990). Observou-se que a

medida de quimiluminescência espontânea e os níveis de TBA-RS não foram alterados pela presença de L-tirosina no meio de incubação. Esses resultados mostram que a L-tirosina *in vitro* não provoca dano oxidativo a lipídios em homogeneizados de córtex cerebral de ratos jovens.

A produção excessiva de espécies reativas e/ou a diminuição de defesas antioxidantes contribuem para o desequilíbrio entre as reações de oxirredução através de modificações na razão SH/SS (MORIARTY-CRAIGE E JONES, 2004). Como a razão SH/SS é um importante parâmetro indicativo de estresse oxidativo, o efeito *in vitro* da L-tirosina sobre o estado redox (razão SH/SS) foi determinado através da quantificação dos níveis de tióis totais (SH) e dissulfetos (SS). Considerando que a L-tirosina foi capaz de diminuir o conteúdo de tióis totais e, ao mesmo tempo, aumentar os níveis de dissulfetos, um decréscimo significativo na razão SH/SS foi constatado. Tais resultados sugerem que a L-tirosina pode promover o estresse oxidativo por alterar a razão SH/SS em homogeneizados de córtex cerebral de ratos.

O conteúdo de carbonilas protéicas foi o índice utilizado para estudar o efeito *in vitro* da L-tirosina sobre o dano oxidativo a proteínas. A formação das carbonilas protéicas é uma conseqüência da reação de oxidação entre a cadeia lateral de diversos aminoácidos e espécies reativas (REZNICK E PACKER, 1994; STADTMAN E LEVINE, 2003). Entretanto, a L-tirosina não modificou o conteúdo de carbonilas protéicas em homogeneizados de córtex cerebral de ratos, indicando que as proteínas foram preservadas da oxidação ou as proteínas oxidadas foram rapidamente degradadas.

Os radicais livres também são capazes de reagir com o DNA e danificá-lo por modificar bases nitrogenadas e resíduos de açúcar, romper a dupla fita e formar ligações cruzadas entre DNA e proteínas (DIZDAROGLU *et al.*, 2002; REARDON *et al.*,

2006). O dano oxidativo ao DNA foi analisado através da medida de ligações cruzadas entre o DNA e proteínas. A L-tirosina *in vitro* aumentou significativamente a formação de ligações cruzadas entre o DNA e proteínas em homogeneizados de córtex cerebral de ratos, mostrando que a L-tirosina pode causar dano oxidativo ao DNA.

O efeito *in vitro* da L-tirosina sobre as atividades das enzimas SOD, CAT, GPx e G6PDH também foi determinado. Os produtos primários da redução parcial do oxigênio, como $O_2^{\bullet -}$ e H_2O_2, são mantidos em baixas concentrações nas células e tecidos através das enzimas antioxidantes SOD, CAT e GPx, enquanto que a G6PDH é a principal enzima capaz de produzir poder redutor forma de NADPH (HALLIWELL E GUTTERIDGE, 2007). Diferentemente dos experimentos anteriores, nos quais o homogeneizado foi incubado na presença de L-tirosina por 1 hora a 37 °C antes das determinações, nessa etapa, a adição de L-tirosina ao homogeneizado ocorreu apenas no momento da medida das atividades enzimáticas, ou seja, sem incubação prévia. A L-tirosina inibiu significativamente a atividade da CAT, porém não alterou a atividade das outras enzimas estudadas (SOD, GPx e G6PDH), o que indica uma interação direta entre a L-tirosina e a CAT em homogeneizados de córtex cerebral de ratos. É possível que ocorra a formação de um ciclo vicioso em conseqüência do efeito da L-tirosina sobre a CAT: a inibição da CAT provocaria o acúmulo de peróxido de hidrogênio e esse último poderia causar um decréscimo ainda maior na atividade da CAT por oxidar grupos sulfidrílicos essenciais para a catálise (HALLIWELL E GUTTERIDGE, 2007).

Os resultados obtidos, até o momento, indicam que *in vitro* a L-tirosina pode induzir o estresse oxidativo por diminuir as defesas antioxidantes não-enzimáticas e enzimáticas, alterar o estado redox (razão SH/SS) e estimular o dano oxidativo ao

DNA em homogeneizados de córtex cerebral de ratos jovens. Entretanto, não pode ser excluída a hipótese de que, durante a incubação do homogeneizado com as diferentes concentrações de L-tirosina, ocorra à produção do radical tirosil, uma vez que qualquer radical livre suficientemente reativo para abstrair o átomo de hidrogênio da hidroxila presente na molécula da tirosina é capaz de formá-lo. Sendo assim, é possível que o radical tirosil contribua para os efeitos aqui observados por reagir com o ascorbato, a cisteína e a GSH (HALLIWELL E GUTTERIDGE, 2007).

Os efeitos *in vitro* dos metabólitos da tirosina, ácido 4-hidroxifenilpirúvico e ácido 4-hidroxifenilático, também foram estudados sobre alguns parâmetros de estresse oxidativo (TRAP, TBA-RS, quimiluminescência espontânea e conteúdo de carbonilas protéicas). Verificou-se que tanto o ácido 4-hidroxifenilpirúvico quanto o ácido 4-hidroxifenilático não alteraram tais parâmetros, o que reforça o papel da tirosina na patogênese dos distúrbios neurológicos característicos dos pacientes com tirosinemia tipo II.

Com o propósito de melhor compreender a neurotoxicidade da tirosina sobre o córtex cerebral e também confirmar os efeitos *in vitro* descritos anteriormente, o efeito da administração aguda de L-tirosina foi avaliado sobre os níveis de TBA-RS, o conteúdo de carbonilas protéicas, o estado redox (razão SH/SS), o conteúdo de ácido ascórbico, o conteúdo de GSH e as atividades das enzimas SOD, CAT, GPx e G6PDH.

Tanto a estrutura cerebral utilizada quanto a dose administrada foram estabelecidas a partir do trabalho realizado por Morre e colaboradores (1980). Esses pesquisadores verificaram que os maiores níveis relativos de tirosina são encontrados no córtex cerebral de ratos após a administração intraperitoneal de 100 mg/Kg de L-tirosina. Sendo assim, o córtex cerebral foi a estrutura escolhida

para testar os efeitos da L-tirosina. Considerando que os níveis plasmáticos de tirosina nos pacientes afetados pela tirosinemia tipo II são aproximadamente três a trinta vezes superiores aos normais (MITCHELL *et al.*, 2001) e que a quantidade de tirosina no córtex cerebral dos animais dobrou transcorrida 1 hora da injeção de 100 mg/Kg de L-tirosina, a dose de 500 mg/Kg de L-tirosina foi selecionada para que a concentração cerebral de tirosina permanecesse dentro daquele intervalo de variação observado nos pacientes com tirosinemia tipo II.

Apesar da L-tirosina não ter causado alterações nos índices de dano oxidativo a lipídios e proteínas *in vitro*, a administração aguda de 500 mg/Kg de L-tirosina aumentou significativamente tanto os níveis de TBA-RS quanto o conteúdo de carbonilas protéicas em homogeneizados de córtex cerebral de ratos jovens. Tais discrepâncias entre os níveis de TBA-RS *in vitro* e *in vivo* poderiam ser explicadas pelo fato de que *in vivo* a tirosina é capaz de agir sobre as membranas plasmáticas íntegras, o que favoreceria a oxidação dos lipídios poliinsaturados e a formação do malondialdeído, um dos produtos finais da cascata da lipoperoxidação determinado através da medida dos níveis de TBA-RS. Já a diferença entre o conteúdo de carbonilas protéicas *in vitro* e *in vivo* pode ter sido ocasionada por uma rápida degradação das proteinas oxidadas *in vitro*.

Tanto *in vitro* quanto *in vivo*, a L-tirosina causou uma alteração significativa no estado redox por diminuir a razão entre a concentração de tióis totais e a concentração de dissulfetos em homogeneizados de córtex cerebral de ratos jovens. Como citado anteriormente, essa razão é um forte indicativo de estresse oxidativo por refletir o desequilíbrio entre o aumento na produção de espécies reativas e/ou a redução das defesas antioxidantes (MORIARTY-CRAIGE E JONES, 2004).

A GSH e o ácido ascórbico são capazes de inativar várias espécies reativas. A GSH é particularmente efetiva sobre $OH^{\bullet}$ (BAINS E SHAW, 1997), enquanto que o ácido ascórbico pode atuar como *scavenger* de $O_2^{\bullet -}$, $OH^{\bullet}$, $LO^{\bullet}$, $LOO^{\bullet}$, $NOO^{\bullet}$, entre outros (HALLIWELL, 1999; HALLIWELL, 2001; HALLIWELL E GUTTERIDGE, 2007). Como *in vitro* uma redução significativa nas defesas antioxidantes não-enzimáticas (TRAP, TAR e conteúdo de ácido ascórbico) foi causada pela L-tirosina, tanto a concentração de ácido ascórbico quanto a de GSH também foram medidas após a administração intraperitoneal de 500 mg/Kg de L-tirosina. Verificou-se que esse tratamento agudo diminuiu o conteúdo de GSH, mas não modificou os níveis de ácido áscórbico em homogeneizados de córtex cerebral de ratos jovens. Além de proteger contra o dano oxidativo causado por espécies reativas, a GSH também ajuda a manter os grupos sulfidrílicos de proteínas e compostos de baixo peso molecular na forma reduzida (ANDERSON, 1998). Dessa forma, é possível que os baixos níveis de GSH encontrados após a administração de L-tirosina aumentem a suscetibilidade do SNC as espécies reativas e ao estresse oxidativo. Entretanto, as divergências entre o conteúdo de GSH *in vitro* e *in vivo* podem ter sido causadas não só pela utilização de dois métodos analíticos (um espectrofotométrico e o outro fluorimétrico), mas também por diferenças na especificidade dessas metodologias. Tanto a maior seletividade do o-ftaldialdeído em pH 8,0 para a GSH quanto a menor manipulação das amostras (BROWNE E ARMSTRONG, 1998) devem ter contribuído para que as alterações nos níveis de GSH entre os grupos controle e experimental pudessem ser observadas no método fluorimétrico. A administração aguda de L-tirosina não alterou os níveis de ácido ascórbico, embora os tenha reduzido *in vitro*. O homogeneizado de córtex cerebral foi incubado na ausência ou presença de diferentes concentrações de L-tirosina durante 1 hora nos experimentos *in vitro*,

enquanto que *in vivo* 1 hora foi o intervalo de tempo entre a administração de L-tirosina e a decapitação dos animais. Ou seja, o período que a L-tirosina agiu sobre o córtex cerebral *in vivo* foi além de inferior ao dos experimentos *in vitro*, provavelmente insuficiente para que a mesma alteração no conteúdo de ácido ascórbico fosse verificada.

E, por fim, as atividades das enzimas SOD, CAT, GPx e G6PDH foram avaliadas. A administração aguda de L-tirosina não modificou as atividades das enzimas antioxidantes SOD, CAT e GPx, embora tenha aumentado significativamente a atividade da G6PDH em homogeneizados de córtex cerebral de ratos. A G6PDH é a principal enzima responsável pela produção de NADPH, que é o cofator da reação catalisada pela GR para regenerar a GSH a partir do GSSG (HASHIDA *et al.*, 2002; MAKINO *et al.*, 2004; HALLIWELL E GUTTERIDGE, 2007). Então, o aumento na atividade da G6PDH provavelmente está relacionado à maior utilização de NADPH na tentativa de restabelecer os baixos níveis de GSH também observados após a administração aguda de L-tirosina. Dados na literatura têm demonstrado que as enzimas antioxidantes, inclusive a G6PDH, podem responder ao estresse oxidativo através do aumento de suas atividades a fim de reduzir o dano (TRAVACIO E LLESUY, 1996; TSUBAI E MATSUO, 2002; ORDONEZ *et al.*, 2006; VALDERRAMA *et al.*, 2006).

Assim como nos experimentos *in vitro*, a administração aguda de L-tirosina foi capaz de alterar vários parâmetros de estresse oxidativo. Tais alterações demonstram que o tratamento agudo com a L-tirosina pode favorecer o dano oxidativo a lipídios e a proteínas, modificar o estado redox (razão SH/SS), diminuir o conteúdo de GSH e induzir a atividade da G6PDH.

Considerando que o estresse oxidativo pode resultar da produção excessiva de espécies reativas, da diminuição das defesas antioxidantes, da liberação de metais de transição ou da combinação desses fatores (HALLIWELL, 2001), os efeitos *in vitro* e *in vivo* da L-tirosina sugerem o envolvimento do estresse oxidativo na toxicidade de altas concentrações de tirosina e, provavelmente, na neuropatologia da tirosinemia tipo II, na qual o acúmulo de tirosina é a principal característica bioquímica.

5. CONCLUSÃO

5.1. EFEITOS DO ÁCIDO γ-HIDROXIBUTÍRICO E DO 1,4-BUTANODIOL EM HOMOGENEIZADOS DE CÓRTEX CEREBRAL DE RATOS JOVENS

- A administração aguda de GHB 10 mg/Kg aumentou significativamente os níveis de TBA-RS;
- A administração aguda de GHB 10 mg/Kg reduziu significativamente o TRAP e o TAR;
- A administração aguda de 1,4-BD 20 mg/Kg não alterou os parâmetros de estresse oxidativo: quimiluminescência espontânea, níveis de TBA-RS, TAR, conteúdo de tióis totais, conteúdo de carbonilas protéicas e as atividades das enzimas antioxidantes SOD, CAT e GPx;
- A administração aguda de 1,4-BD 120 mg/Kg aumentou significativamente a quimiluminescência espontânea e os níveis de TBA-RS;
- A administração aguda de 1,4-BD 120 mg/Kg diminuiu significativamente a medida do TAR;
- A administração aguda de 1,4-BD 120 mg/Kg não alterou os parâmetros de dano oxidativo a proteínas (conteúdo de tióis totais e carbonilas protéicas);
- A administração aguda de 1,4-BD 120 mg/Kg não alterou as atividades das enzimas antioxidantes SOD, CAT e GPx;

A Tabela 2 apresenta os resultados acima descritos da administração aguda de GHB 10 mg/Kg e 1,4-BD nas doses de 20 ou 120 mg/Kg.

Tabela 2. Efeitos do GHB e do 1,4-BD em homogeneizados de córtex cerebral de ratos jovens.

Parâmetro de estresse oxidativo	Administração aguda		
	GHB 10 mg/Kg	1,4-BD 20 mg/Kg	1,4-BD 120 mg/Kg
Quimiluminescência espontânea	NA	=	↑
TBA-RS	↑	=	↑
TRAP	↓	NA	NA
TAR	↓	=	↓
Conteúdo de carbonilas protéicas	NA	=	=
Conteúdo de tióis totais	NA	=	=
Superóxido dismutase	NA	=	=
Catalase	NA	=	=
Glutationa peroxidase	NA	=	=

NA: não avaliado.

Tanto os efeitos *in vivo* do GHB quanto aqueles da administração aguda de 120 mg/Kg de 1,4-BD mostraram indução da lipoperoxidação e comprometimento das defesas antioxidantes não-enzimáticas em homogeneizados de córtex cerebral de ratos jovens. Tais resultados sugerem a participação do estresse oxidativo na neurotoxicidade do GHB e, possivelmente, nos mecanismos responsáveis pelas disfunções neurológicas características da deficiência da SSADH.

5.2. EFEITOS DA L-TIROSINA EM HOMOGENEIZADOS DE CÓRTEX CEREBRAL DE RATOS JOVENS

5.2.1. *In vitro*

- A L-tirosina reduziu significativamente o TRAP e o TAR;
- A L-tirosina diminuiu significativamente o conteúdo de ácido ascórbico, mas não alterou os níveis de GSH;
- A L-tirosina não alterou os parâmetros de dano oxidativo a lipídios (quimiluminescência espontânea e níveis de TBA-RS) e proteínas (conteúdo de proteínas protéicas);
- A L-tirosina aumentou significativamente o conteúdo de dissulfetos (SS), enquanto que os níveis de tióis totais (SH) e a razão SH/SS foram marcadamente reduzidos;
- A L-tirosina aumentou significativamente a formação de ligações cruzadas entre DNA e proteínas;
- A L-tirosina inibiu significativamente a atividade da enzima antioxidante CAT, mas não alterou as atividades das enzimas SOD, GPx e G6PDH;

5.2.2. *In vivo*

- A administração aguda de L-tirosina aumentou significativamente os níveis de TBA-RS;
- A administração aguda de L-tirosina aumentou significativamente conteúdo de carbonilas protéicas;
- A administração aguda de L-tirosina reduziu significativamente a razão SH/SS;

- A administração aguda de L-tirosina diminuiu significativamente os níveis de GSH, mas não alterou o conteúdo de ácido ascórbico;
- A administração aguda de L-tirosina aumentou significativamente a atividade da G6PDH, porém não alterou as atividades das enzimas antioxidantes SOD, CAT e GPx;

A Tabela 3 esquematiza os efeitos *in vitro* e *in vivo* da L-tirosina.

Tabela 3. Efeitos da L-tirosina em homogeneizados de córtex cerebral de ratos jovens.

Parâmetro de estresse oxidativo	L-Tirosina	
	In vitro	*In vivo*
TRAP	↓	NA
TAR	↓	NA
Conteúdo de GSH	=	↓
Conteúdo de ácido ascórbico	↓	=
Quimiluminescência espontânea	=	NA
TBA-RS	=	↑
Conteúdo de tióis totais	↓	NA
Conteúdo de dissulfetos	↑	NA
Razão SH/SS	↓	↓
Conteúdo de carbonilas protéicas	=	↑
Ligações cruzadas entre DNA e proteínas	↑	NA
Superóxido dismutase	=	=
Catalase	↓	=
Glutationa peroxidase	=	=
Glicose-6-fosfato desidrogenase	=	↑

NA: não avaliado.

Os efeitos *in vitro* e *in vivo* da L-tirosina sugerem o envolvimento do estresse oxidativo na toxicidade de altas concentrações de tirosina e, provavelmente, na neuropatologia da tirosinemia tipo II.

5.3. EFEITOS *IN VITRO* DOS METABÓLITOS DA L-TIROSINA EM HOMOGENEIZADOS DE CÓRTEX CEREBRAL DE RATOS JOVENS

- O ácido 4-hidroxifenilpirúvico e ácido 4-hidroxifenilático não alteraram os parâmetros de estresse oxidativo: TRAP, quimiluminescência espontânea, níveis de TBA-RS e conteúdo de carbonilas protéicas;

A Tabela 4 mostra os efeitos *in vitro* dos ácidos 4-hidroxifenilpirúvico e ácido 4-hidroxifenilacético.

Tabela 4. Efeitos *in vitro* dos metabólitos da L-tirosina em homogeneizados de córtex cerebral de ratos jovens.

Parâmetro de estresse oxidativo	Metabólitos da tirosina	
	Ácido 4-hidroxifenilpirúvico	Ácido 4-hidroxifenilático
TRAP	=	=
Quimiluminescência espontânea	=	=
TBA-RS	=	=
Conteúdo de carbonilas protéicas	=	=

Tais resultados reforçam o papel da tirosina na patogênese dos distúrbios neurológicos característicos dos pacientes com tirosinemia tipo II.

6. PERSPECTIVAS

Estudar o efeito da administração aguda de duas doses (20 e 120 mg/Kg) de 1,4-BD sobre o TRAP em homogeneizados de córtex cerebral de ratos jovens.

Estudar o efeito da administração aguda de 500 mg/Kg de L-tirosina sobre alguns parâmetros de estresse oxidativo, tais como quimiluminescência espontânea, TRAP, TAR e ligações cruzadas entre DNA e proteínas em homogeneizados de córtex cerebral de ratos jovens.

Estudar o efeito do pré-tratamento com antioxidantes sobre parâmetros de estresse oxidativo em ratos submetidos à administração intraperitoneal de 500 mg/Kg de L-tirosina.

Desenvolver um modelo experimental de tirosinemia tipo II através da administração intraperitoneal de L-tirosina.

Estudar o efeito da administração crônica de L-tirosina sobre parâmetros de estresse oxidativo em cérebro de ratos jovens.

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