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Efeito, *in vitro* e *in vivo*, da tirosina sobre algumas enzimas do metabolismo energético em córtex cerebral de ratos

Rodrigo Binkowski de Andrade

Orientador: Prof. Dr. Clovis Milton Duval Wannmacher

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Dedico este trabalho a meus pais, minha namorada Tanise e amigos.

"Toda a nossa ciência, comparada com a realidade, é primitiva e infantil - e, no entanto, é a coisa mais preciosa que temos."

(Albert Einstein)

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PARTE I

INTRODUÇÃO E OBJETIVOS

RESUMO

A tirosinemia tipo II é um erro inato do metabolismo que se caracteriza por altos níveis plasmáticos e teciduais de tirosina, cujo efeito sobre o Sistema Nervoso Central pode variar de retardo mental leve a grave, podendo estar associado com outras anomalias neurológicas. No presente estudo nosso objetivo foi investigar *in vitro* e *in vivo* os efeitos de elevadas concentrações de tirosina sobre importantes parâmetros do metabolismo energético em córtex cerebral de ratos de 14 dias de idade. Os resultados mostraram que 1,0-2,0 mM de tirosina inibe *in vitro* a atividade da creatinaquinase (CK) citosólica e mitocondrial em um padrão dependente da concentração e que esta inibição é prevenida por glutationa reduzida (GSH), sugerindo a alteração de grupos tiólicos essenciais para o funcionamento da enzima. Os resultados também indicam que somente a atividade da CK mitocondrial é inibida pela tirosina em uma maneira dependente do tempo. Além disso, uma única injeção de L-tirosina metil éster (TME) diminuiu a atividade da CK citosólica e mitocondrial em córtex cerebral de ratos. Observou-se que 2,0 mM de tirosina inibe *in vitro* a atividade da piruvatoquinase (PK) e que esta inibição é prevenida por GSH nos tempos de 30, 60 e 90 min de pré-incubação. Além disso, a administração TME reduziu a atividade da PK e essa redução é prevenida pelo pré-tratamento com creatina. Por outro lado, a tirosina não alterou a atividade da adenilatoquinase (AK) *in vitro*, mas a administração de TME aumentou a atividade da AK. Finalmente, administração de TME diminuiu a atividade da CK da fração citosólica e mitocondrial e esta diminuição é prevenida pelo pré-tratamento com creatina. Considerando os resultados em conjunto, podemos presumir que a tirosina altera grupos sulfidrilas essenciais para a atividade da CK e PK, possivelmente por estresse oxidativo. Se estes efeitos também ocorrerem nos pacientes com tirosinemia tipo II, é possível que alterações no metabolismo energético contribuem, pelo menos em parte, para a disfunção neurológica característica dessa doença metabólica.

ABSTRACT

Tyrosinemia type II is an inborn error of metabolism, where tyrosine levels are highly elevated in tissues and physiological fluids of affected patients. The central nervous system involvement can range from mild to severe mental retardation and may be associated with other neurologic abnormalities. The present study investigated the *in vitro* and *in vivo* effects of high concentrations of tyrosine on important parameters of energy metabolism in cerebral cortex homogenates from 14-day-old Wistar rats. It was observed that 1.0-2.0 mM tyrosine inhibit *in vitro* the enzymatic activity of mitochondrial and cytosolic creatine kinase (CK) in a concentration-dependent pattern and that this inhibition is prevented by reduced glutathione (GSH), suggesting that creatine kinase inhibition was caused by altering essential sulfhydryl groups necessary for the enzyme function. Results also indicate that mitochondrial, but not cytosolic CK activity is inhibited by tyrosine in a time-dependent way. In addition, a single injection of L-tyrosine methyl ester (TME) decreased cytosolic and mitochondrial CK activities of brain cortex from rats. It was observed that 2.0 mM tyrosine inhibit the pyruvate kinase (PK) activity *in vitro* and that this inhibition is prevented by GSH at 30 min, 60 and 90 min of pre-incubation. Moreover, TME administration reduced the PK activity and this reduction is prevented by pre-treatment with creatine. On the other hand, tyrosine did not alter the adenylate kinase (AK) activity *in vitro*, but the administration of TME increased the activity of AK. Finally, TME administration decreased the CK activity of cytosolic and mitochondrial fractions and this decrease is prevented by creatine pre-treatment. Taken together all the results, it can be presumed that tyrosine alters sulfhydryl groups essential for CK and PK functions possibly through oxidative stress. If these effects also occur in patients with tyrosinemia type II, it is possible that energy metabolism alterations contribute, at least in part, to the neurological dysfunction characteristic of this metabolic disease.

LISTA DE ABREVIATURAS

ADP – difosfato de adenosina

AMP - adenosina monofosfato

AK - adenilatoquinase

ATP - adenosina trifosfato

ATPase - enzimas que catalisam a hidrólise do ATP

ANOVA – análise de variância

ANT – translocador adenina nucleotídeo

CAT – catalase

CK – creatinaquinase

CK-Cy– creatinaquinase citosólica

CK-BB – isoforma citosólica da CK cerebral

CK-MB – isoforma citosólica da CK do coração

CK-MM – isoforma citosólica da CK muscular

CK-Mi - CK mitocondrial

CK-Mis - creatinaquinase mitocondrial sarcomérica

CK-Miu - creatinaquinase mitocondrial ubíqua

Cr - creatina

ERO – espécies reativas de oxigênio

ERN – espécies reativas de nitrogênio

ERON - espécies reativas de oxigênio e de nitrogênio

FBP - frutose-1,6-bifosfato

GPx – glutationa peroxidase

GSSH – glutationa oxidada

GSH – glutationa reduzida

GTP – guanosina trisfosfato

H_2O_2 - peróxido de hidrogênio

IM - membrana mitocondrial interna

i.p - via intraperitoneal

LDH - lactato-desidrogenase

MO - membrana mitocondrial externa

PCr– fosfocreatina

$O_2^{\cdot -}$ - radical ânion superóxido

OONO- - peroxinitrito

PK – piruvatoquinase

RNA - Ácido ribonucléico

SLC6A8 - transportador de creatina

SNC – Sistema Nervoso Central

SOD – superóxido dismutase

TAT – tirosina aminotransferase

TBARS – espécies reativas ao ácido tiobarbitúrico

TME – tirosina metil éster

TYR - tirosina

VDAC - canal aniônico dependente de voltagem

I.1 – INTRODUÇÃO

I. 1.1 – Erros Inatos do Metabolismo

A primeira menção aos erros inatos do metabolismo (EIM) ocorreu em 1908, quando Sir Archibald Garrod usou este termo para designar doenças como a alcaptonúria, patologia na qual os pacientes afetados excretam grandes quantidades do ácido homogentísico na urina (Scriver et al., 2001). Garrod observou uma maior frequência desta alteração em indivíduos de uma mesma família e maior incidência de consanguinidade entre os pais dos pacientes afetados. Sabendo disso e baseado nas leis de Mendel, Garrod propôs um modelo de herança autossômica recessiva para este distúrbio. Através da observação de que o ácido homogentísico, excretado em altas concentrações na urina dos pacientes com alcaptonúria e sendo um metabólito normal da degradação protéica, ele relacionou este distúrbio com um bloqueio na rota do catabolismo da tirosina (Scriver et al., 2001).

Com o surgimento de novos distúrbios relacionados a alterações genéticas e que envolviam o acúmulo de outras substâncias nos líquidos biológicos dos pacientes, postulou-se que estas doenças resultavam da síntese qualitativa ou quantitativamente anormal de uma proteína, enzimática ou não, pertencente ao metabolismo (Scriver et al., 2001). Portanto, em consequência deste bloqueio metabólico, pode ocorrer o acúmulo de precursores da reação catalisada pela enzima envolvida, com a formação de rotas metabólicas alternativas e a deficiência de produtos essenciais ao organismo (Bickel 1987).

Desde o estudo inicial de Garrod, o conhecimento destas doenças metabólicas tem crescido geometricamente. Foram descritos até o momento mais de 500 EIM (Scriver et al., 2001), a maioria envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo, causando um grande número de defeitos, com quadros clínicos diversos, que podem ser desde assintomáticos até tão graves que causem morte neonatal (Benson e Fenson, 1985).

Os EIM manifestam-se geralmente na infância, apresentando sinais e sintomas semelhantes aos encontrados em muitas doenças infantis (Holtzman, 1978). Apesar de raros quando considerados individualmente, os EIM são relativamente frequentes em seu conjunto, podendo ocorrer aproximadamente um em cada mil recém-nascidos vivos. As técnicas de investigação bioquímica têm colaborado para a descoberta de novos erros inatos do metabolismo, esclarecendo cada vez mais os já conhecidos.

I.1.1.2 – Hipertirosinemias

A tirosina é um aminoácido não-essencial classificado no grupo dos aromáticos sendo sintetizado a partir da hidroxilação da fenilalanina. Este aminoácido participa da síntese de hormônios da tireóide, catecolaminas e melanina, e apresenta dois principais destinos metabólicos: síntese protéica ou degradação em fumarato e acetoacetato através de cinco reações enzimáticas. Em seres humanos, são encontradas deficiências em quatro das cinco enzimas responsáveis pela degradação da tirosina: deficiência da fumarilacetoacetase (tirosinemia tipo I), deficiência da tirosina aminotransferase (TAT) (tirosinemia tipo II),

deficiência da 4-hidroxifenilpiruvato dioxigenase (tirosinemia tipo III) e deficiência da homogentizato 1,2-dioxigenase (alcaptonúria) (Mitchell et al., 2001; Held, 2006) (Figura 1). Portanto, o acúmulo de tirosina nos tecidos e fluidos fisiológicos dos pacientes pode ocorrer nas tirosinemias tipo I, II e III, na tirosinemia transitória do recém-nascido e nos distúrbios hepáticos (Mitchell et al., 2001).

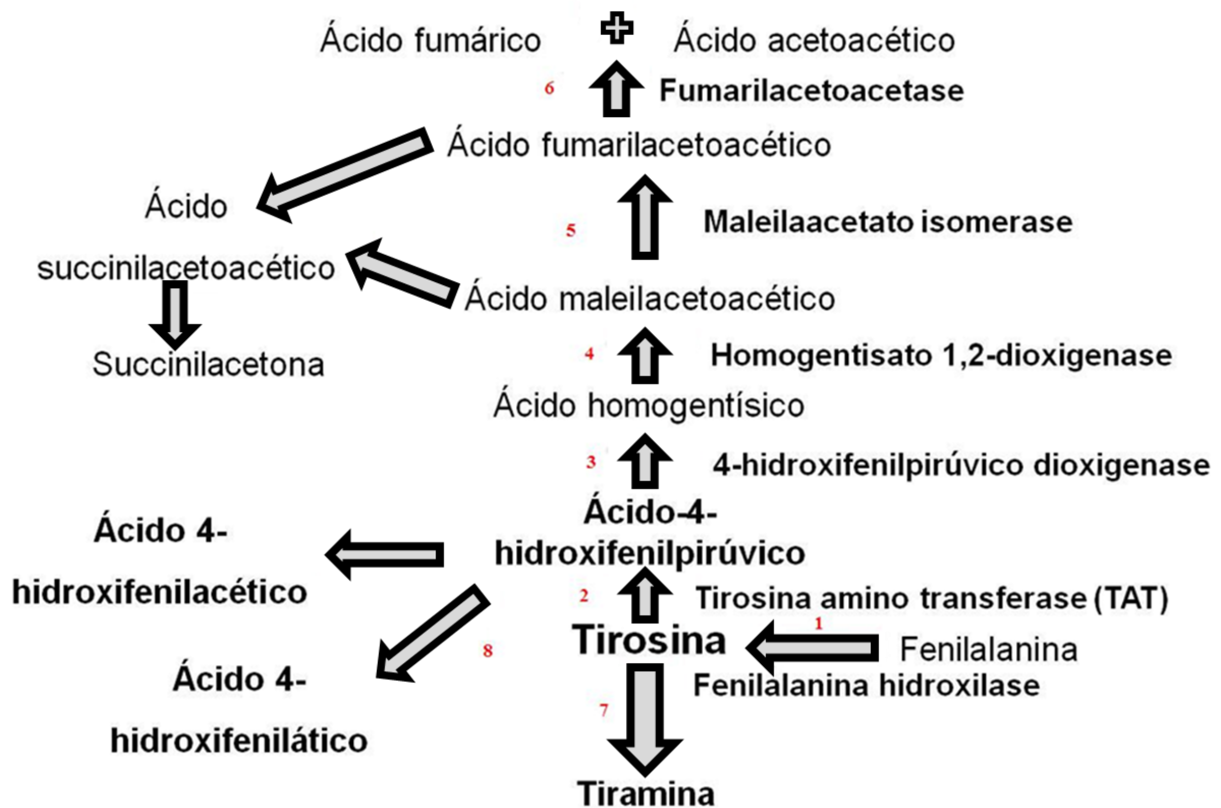

Figura 1 - Catabolismo da tirosina. As enzimas 7 e 8 ainda não foram identificadas. Adaptado de Mitchell et al., 2001.

I.1.1.2 –Tirosinemia tipo II

A tirosinemia tipo II, também denominada tirosinemia oculocutânea ou síndrome de Richner-Hanhart, é causada pela deficiência autossômica recessiva da enzima hepática TAT. Esta deficiência enzimática da TAT causa um acúmulo de tirosina no plasma e nos tecidos dos indivíduos e está associada à consanguinidade e apresenta uma frequência de 1 caso em aproximadamente 250.000 nascidos vivos (Macsai et al., 2001). As concentrações plasmáticas de tirosina variam entre 0,3 e 3,0 mM em pacientes não tratados enquanto que as concentrações normais são inferiores a 0,1 mM (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).

As manifestações clínicas associadas à tirosinemia tipo II envolvem basicamente lesões oculares, lesões cutâneas e alterações neurológicas (Mitchell et al., 2001; Held, 2006). As lesões oculares podem gerar conjuntivites e ulcerações na córnea, que podem causar uma diminuição na visão e glaucoma (Mitchell et al., 2001). Além disso, essas lesões oculares caracterizam-se por fotofobia, lacrimejamento, vermelhidão e dores intensas que também são freqüentemente observadas nesses pacientes (Rabinowitz et al., 1995). As lesões cutâneas são do tipo hiperceratóticas e atingem as plantas dos pés e as palmas das mãos nesses indivíduos (Valikhani et al., 2005). 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 (Lemonnier et al., 1979; Mitchell et al., 200).

O mecanismo das lesões oculares e cutâneas na tirosinemia tipo II está relacionada ao envolvimento de cristais intracelulares de tirosina e o desenvolvimento de uma resposta inflamatória acentuada (Held, 2006). Resultados prévios do nosso grupo de Erros Inatos do Metabolismo demonstraram a administração de tirosina a ratos induz estresse oxidativo, observado pelo aumento da lipoperoxidação, carbonilação de proteínas, diminuição de GSH e da relação tiol/dissulfeto (razão SH / SS) em córtex cerebral de ratos Wistar de 14 dias de idade (Sgaravatti et al., 2008; Sgaravatti et al., 2009). Acredita-se que os altos níveis plasmáticos de tirosina sejam os responsáveis pelo comprometimento do Sistema Nervoso Central (SNC) na tirosinemia tipo II (Mitchell et al., 2001; Held, 2006).

O diagnóstico da tirosinemia tipo II consiste tanto na dosagem de altas concentrações plasmáticas e urinárias de tirosina, bem como na detecção de altos níveis de seus metabólitos na urina (Mitchell et al., 2001). Para a confirmação do diagnóstico é feita pela determinação da atividade enzimática da TAT em biópsia hepática ou por análise de mutações (Mitchell et al., 2001). O tratamento é baseado na restrição nutricional de fenilalanina e tirosina para amenizar as lesões oculares e cutâneas dos pacientes com tirosinemia tipo II (Held, 2006). Entretanto, não é conhecido o momento em que o controle dietético deve ser iniciado e terminado, e quais 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 tirosinemia tipo II não é específico, o entendimento dos mecanismos fisiopatológicos é importante para desenvolver abordagens

terapêuticas mais eficientes. Neste sentido, nosso grupo de pesquisa tem demonstrado alterações em parâmetros do metabolismo energético na neuropatologia de erros inatos do metabolismo (Wajner et al., 2004).

I.1.2 - Rede de fosforiltransferência

Os mecanismos responsáveis envolvidos no equilíbrio entre o consumo e a produção de ATP são finamente regulados por processos intracelulares (Dzeja et al., 2003). A operação otimizada do sistema celular bioenergético exige que compostos ricos em energia, como ATP, sejam produzidos e entregues aos consumidores de ATP à taxa correspondente à velocidade de consumo (Dzeja et al, 2000).

Para estabelecer esta função de transferência de grupos fosforil, uma rede intracelular enzimática, catalisada principalmente pela creatinaquinase (CK), adenilatoquinase (AK), e enzimas glicolíticas (especialmente a piruvatoquinase - PK), atuam em processos de produção e consumos de ATP (Figura 2) (Wallimann et al, 1992;. Saks et al, 1994;. Dzeja et al, 1998). A sinalização metabólica é dinâmica e através das enzimas da rede de transferência de grupos fosforil contribui para a eficiente comunicação energética intracelular na manutenção dos altos níveis de ATP celular (Dzeja et al, 2000; Neumann et al., 2003).

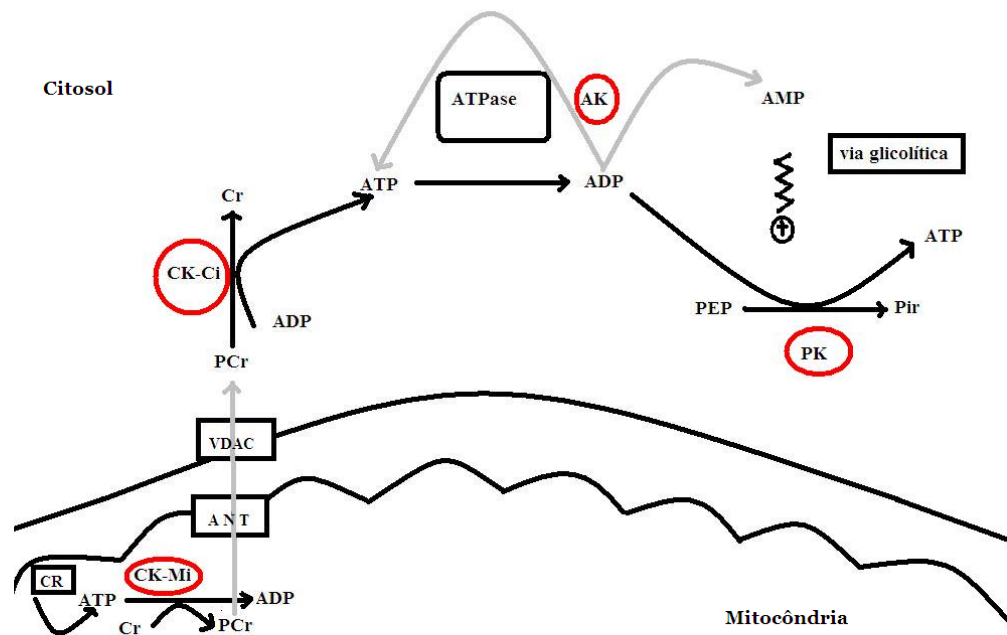

Figura 2 – Esquema das principais enzimas da rede de transferência de grupos fosforil em tecidos que consomem grande quantidade energética como, por exemplo, músculo esquelético, coração é cérebro. A entrega de fosfocreatina (PCr) é facilitada através de creatinaquinase mitocondrial (CK-Mi) a partir de ATP gerado pela fosforilação oxidativa através da cadeia respiratória. A PCr é exportada para o citosol e a creatinaquinase citosólica (CK-Ci) é capaz de transferir o grupo fosfato da PCr para o ADP, formando ATP. A adenilatoquinase (AK) é capaz de regenerar o ATP a partir de duas moléculas de ADP. Finalmente, a via glicolítica é estimulada por AMP proveniente da reação da AK e a piruvatoquinase (PK) é capaz de sintetizar ATP a partir de fosfoenol piruvato (PEP) e ADP. Como esses sistemas de transporte operam em conjunto, uma atividade diminuída de uma única enzima pode ser compensada. Entretanto, uma alteração da atividade de duas ou mais enzimas pode levar a um comprometimento cumulativo na comunicação entre sítios de geração e consumo de ATP.

A AK gera 10% do total da renovação de ATP no tecido muscular cardíaco, enquanto no músculo esquelético em repouso gera cerca de 4%. A CK perfaz a maior parte da renovação do ATP (Dzeja et al., 1999). Apesar da porcentagem diminuída em relação à CK, a AK pode transferir e utilizar duas moléculas de ADP, podendo assim, em momentos de excessiva necessidade energética fornecer ATP, além de AMP que estimula a via glicolítica por alosteria na enzima fosfofrutoquinase (Pucar et al., 2000). Além disso, as

enzimas glicolíticas como a hexoquinase, gliceraldeído3-fosfato desidrogenase e PK podem contribuir para a transferência intracelular de grupos fosforil e sua distribuição espacial está sendo cada vez mais reconhecida (Dzeja et al, 2003). Em média, as concentrações de ATP, ADP e AMP são, respectivamente, 5, 1 e 0,1 mM (Noma, 2005). Modificações nas concentrações destes compostos podem alterar enzimas importantes e regulatórias em diversas rotas biossintéticas e oxidativas, que percebem mudanças no estado metabólico e energético celular (Dzeja et al, 2000). A simples difusão do ATP não parece ser responsável pelo fluxo de energia na célula. O mecanismo que supre a demanda energética é a rede de fosforiltransferência, da qual fazem parte as enzimas AK, CK e PK (Dzeja et al., 1999).

I.1.2.1 – Creatinaquinase

A creatinaquinase (CK) - EC 2.7.3.2 - catalisa a transferência reversível de um grupo N-fosforil da fosfocreatina (PCr) para o ADP regenerando ATP e creatina (Cr) (Figura 3). A enzima é formada por 400 aminoácidos com massa molecular de 306 a 380 kDa, conforme a isoforma. A CK participa na homeostasia de células com necessidades energéticas variáveis e intermitentes como, por exemplo, músculo esquelético e cardíaco, tecidos neurais como cérebro e retina, fotorreceptores e eritrócitos (Wallimann, 1994). O pH ótimo para a catálise enzimática varia de 6,0 a 7,0 na direção de formação de ATP e 7,5 a 9,0 na direção de formação de PCr.

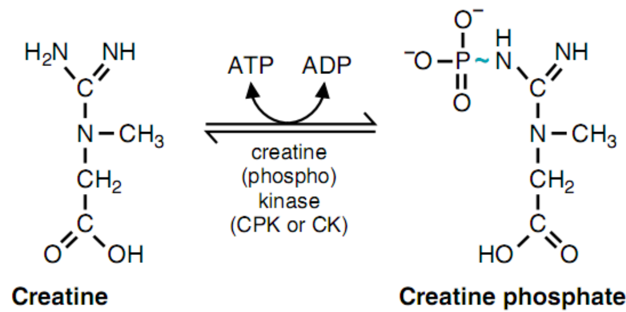

Figura 3 - Reação reversível catalisada pela creatinaquinase (CK). Fonte: MARKS et al., 2007.

As células contêm várias isoformas da CK que são identificadas por mobilidade eletroforética, distribuição tecidual e subcelular e sua sequência primária (Villarreal-Levy et al., 1987; Perryman et al., 1986). Há quatro principais isoenzimas da CK e os nomes são dados em função dos tecidos em que foram historicamente isoladas. Há duas isoformas citosólicas, a muscular (CK-MM) e a cerebral (CK-BB), ambas existindo como homodímeros sob condições fisiológicas, podendo se apresentar como um heterodímero CK-MB no coração. As isoformas mitocondriais também são duas, a forma ubíqua (CK-Miu) e a sarcomérica (CK-Mis), as quais são expressas predominantemente no cérebro e no músculo estriado, respectivamente (Wallimann et al., 1992).

As isoformas da creatinaquinase mitocondrial (CK-Mi), geralmente existem como octâmeros, mas podem ser transformadas rapidamente em dímeros (McLeish et al., 2005). As isoenzimas da CK citosólica (CK MM-, MB- e BB-) são sempre co-expressadas em tecido específico junto com a isoforma mitocondrial. A interação entre as isoformas citosólica e

mitocondrial é de fundamental importância na homeostasia energética celular (Silva et al., 2003).

As isoenzimas da CK são conhecidas por conterem o grupamento sulfidrílico no sítio ativo: cisteína 278 na CK mitocondrial e cisteína 283 na CK citosólica (Furter, 1993). Modificações ocorridas nos resíduos sulfidrílicos por espécies reativas de oxigênio e nitrogênio (ERON)/ diminuem a atividade da enzima, mostrando que este grupamento também tem um importante envolvimento na sua atividade catalítica (Kaneko, 1993; Sukuki et al., 1992). Esta diminuição de atividade produz deficiência energética, acúmulo de ADP e excesso de cálcio intracelular (Wallimann et al., 1992). Foi verificado que o peroxinitrito afeta a forma octamérica da CK-Mi e evita a re-octamerização da forma dimérica (Gross et al., 1996). As ERON elevam os níveis de cálcio intracelular ($Ca^{2+)}$ e induzem apoptose (Mattson, 1992).

Usando fracionamento bioquímico e localização *in situ*, foi demonstrado que as isoenzimas da CK são estritamente solúveis. De fato são compartimentadas subcelularmente e unidas funcional e/ou estruturalmente com sítios de produção de energia (glicólise e mitocôndria) ou consumo de energia (ATPases celulares, como ATPase actinomiosina e ATPase retículo sarcoplasmático). Formam um sistema de distribuição de energia altamente regulado e intrincado - o circuito da fosfocreatina (PCr) (Figura 4) - (Wallimann et al., 1992). Dependendo das necessidades metabólicas da célula ou tecido, uma dessas diferentes funções do circuito CK/PCr pode estar dominante.

As isoenzimas CK-Mi estão localizadas na parte externa da membrana mitocondrial interna, assim como em sítios de contato com a membrana externa e interna, no espaço

intermembranas, por união funcional com o carreador ATP/ADP (translocador adenina nucleotídeo - ANT) (Wallimann et al., 1992). Esta ligação funcional ocorre por meio da exportação eletrogênica da matriz mitocondrial geradora de ATP em troca de ADP através da membrana mitocondrial interna e a transfosforilação do ATP a PCr, conservando a energia livre da hidrólise do ATP exportado pelo ANT (Wyss et al., 1992). A CK-Mi, por ser funcionalmente unida ao poro da membrana mitocondrial externa, provavelmente esteja envolvida na regulação da importação da creatina pela mitocôndria e exportação da PCr para o citosol (Wallimann et al., 1992; Wyss et al., 1992). Portanto, através da união cinética das três proteínas ANT, CK-Mi, e o canal aniônico dependente de voltagem (VDAC), também conhecido como porina, forma-se um importante microcompartimento para o transporte de energia mitocondrial e metabolismo energético celular (Wallimann et al., 1992).

Diferentes funções têm sido sugeridas para a comunicação entre as isoformas de creatinaquinase citosólica e mitocondrial por PCr e Cr (Wallimann, 1994), levando à proposta do modelo de circuito da PCr (Rojo et al., 1991; Wallimann et al., 1992; Wyss et al., 1992). O circuito CK/PCr parece cumprir todas as necessidades de um sistema altamente organizado de transporte e tamponamente de energia, assim como um sistema regulatório para controle das razões subcelulares de ATP/ADP, levando juntos a uma utilização de energia mais eficiente em termos termodinâmicos.

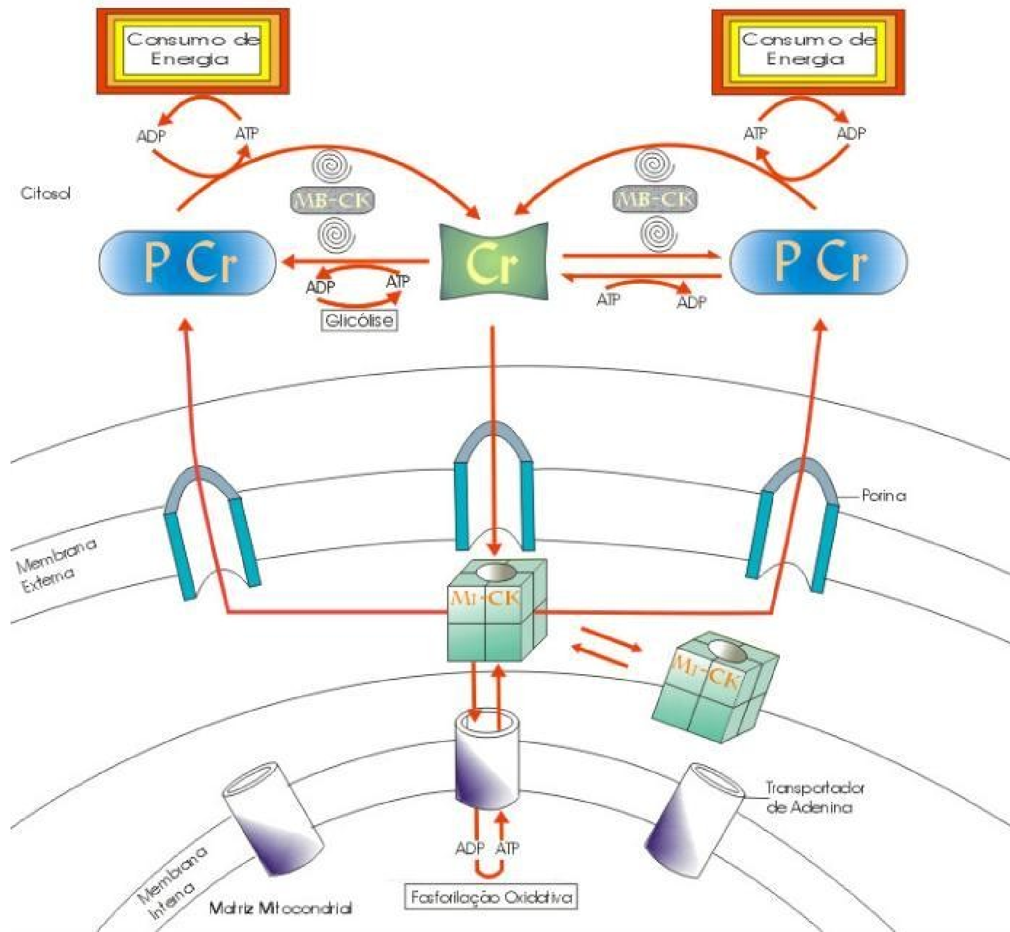

Figura 4 – Modelo do circuito creatina (Cr) / fosfocreatina (PCr) na homeostase energética intracelular. São mostradas duas isoformas da creatinaquinase, um mitocondrial (CK-Mi) e outra citosólica (CK-MB). A CK-Mi está ligada funcionalmente à fosforilação oxidativa, enquanto a CK-MB pode estar associada à glicólise, ao armazenamento de energia mantendo o equilíbrio entre as relações PCr/Cr e ATP (trifosfato de adenosina) / ADP (difosfato de adenosina) ou então sítios subcelulares de consumos altos e flutuantes de ATP.

O circuito PCr serve para melhorar a eficiência termodinâmica da hidrólise do ATP para manter baixa a concentração de ADP e manter alta a razão ATP/ADP nos sítios subcelulares onde a CK está funcionalmente associada aos processos que requeiram ATP, como por exemplo, bombas de íons (Wallimann et al., 1994). Esse circuito serve também como tampão de energia transportada entre compartimentos. Nesse papel, a PCr tem a função

de transportadora de energia, conectando sítios de produção de energia, como fosforilação oxidativa mitocondrial, com sítios de utilização de energia, onde a CK mitocondrial tem um papel eminente. Essa função da CK é sustentada por compartimentalização subcelular específica de diferentes isoenzimas da CK em uma variedade de tecidos, como músculos, eritrócitos, células fotorreceptoras e espermatozóides, por compartimentalização subcelular da PCr/Cr, ATP/ADP e Pi e pela localização, estrutura e propriedades funcionais da CK-Mi octamérica (Wallimann et al., 1992).

A CK parece estar envolvida em certas condições patológicas relacionadas com déficit de energia cerebral. Em condições anóxicas, a adição de creatina ao meio de incubação contendo fatias de cérebro de ratos protege a transmissão sináptica e mantém o potencial de ação via Na+, K+- ATPase (Whittingham, 1981); a adição de creatina aumenta os níveis de PCr reduzindo a queda de ATP, a liberação de Ca^{2+} e a morte celular no SNC(Carter et al., 1995). Quantidades significativas da atividade da CK-Mi e de seu RNA mensageiro (RNAm) também foram observadas nos rins, onde a CK-Mi junto com a CK-BB, foi encontrada no córtex e na medula renal, provavelmente participando do transporte de sódio. O RNAm para a CK-Mi ubíqua foi a principal forma encontrada nos rins, placenta, intestino e cérebro (Wallimann et al., 1992).

Por fim, foi observado que em ratos o processo de mielinização, bem como as demais atividades do sistema nervoso, depende da maturação funcional da CK durante o desenvolvimento do encéfalo do rato com expressão sequencial das isoenzimas (Holtzman et al., 1993).

I.1.2.2 – Adenilatoquinase

A enzima adenilatoquinase (EC 2.7.4.3 – AK) também conhecida como mioquinase é uma fosfotransferase com uma única cadeia polipeptídica de aproximadamente 200 aminoácidos que catalisa a interconversão de nucleotídeos adenina, e desempenha um papel importante na homeostase da energia celular (Dzeja et al., 1999). A reação reversível catalisada pela AK:

A AK é responsável pela interconversão de ATP, ADP e AMP (Pucar et al., 2000). Essa enzima potencializa o papel energético do ATP, por ter habilidade de regenerar o ATP, a partir de dois ADP, e pela regulação dos processos envolvendo os nucleotídeos de adenina. A AK gera sinais metabólicos para a célula, como o AMP, ativando rotas dependentes de AMP (Dzeja et al., 2002). A estrutura da AK sofre grandes mudanças conformacionais durante seu ciclo catalítico. Possui três domínios bem descritos: o CORE, o domínio de ligação do AMP e o domínio de ligação do ATP. Os dois domínios de ligação do AMP e ATP são descritos como passo limitante da catálise da AK e o CORE é responsável pelas propriedades alostéricas da enzima (Noma et al., 2005; Dzeja et al., 2002).

Existem descritas até o momento seis isoenzimas da AK (AK1 a AK6) (Noma et al., 2005). A AK1 existe no citoplasma e AK2 existe principalmente no espaço intermembranas mitocondrial, ambas utilizam os nucleotídeos de adenina (Dzeja et al., 1999). A AK3 utiliza GTP para formação de ADP ou GDP, devido à localização subcelular, pois é encontrada na

matriz mitocondrial, onde há a formação de GTP no ciclo do ácido cítrico. A AK4 está localizada na matriz mitocondrial, e AK5 no citosol. A AK6 é a única a ser encontrada no núcleo celular (Noma et al., 2005).

1.1.2.3 – Piruvatoquinase

A última etapa da via glicolítica é a transferência do grupo fosforil do fosfoenolpiruvato (PEP) ao ADP, catalisada pela piruvatoquinase (EC 2.7.1.40 - PK), que requer K^+ e Mg^{2+} ou Mn^{2+} (Oberfelder et al., 1984). Considerando que a via glicolítica é um sistema fundamental para o metabolismo energético nos organismos, as enzimas glicolíticas estão presentes em todas as células ou tecidos dos mamíferos (Heyduk et al., 1992). Entretanto, cada tipo de tecido utiliza essas enzimas de uma maneira específica para seu metabolismo (Friesen et al., 1998). A manutenção do fluxo de energia e de nutrientes para dentro da célula é essencial para a homeostasia e o funcionamento celular. Nesse contexto a PK de mamíferos desempenha um papel fundamental nas células eucarióticas. A PK é uma enzima homo-tetrâmera e cada subunidade (monômero) consiste de 4 domínios (A, B, C e N), cada um possuindo um sítio ativo (Figura 5) (Consler et al., 1992). Nos mamíferos há 4 isoenzimas da PK que são referidas como tipo M1, L, R e M2 (Consler et al.,1989). Estas formas de isoenzimas diferem em suas propriedades químicas, físicas, cinéticas, eletroforéticas e imunológicas, bem como na distribuição nos tecidos: a isoenzima tipo M1 é o principal tipo nos tecidos especialmente diferenciados, tais como músculo esquelético, coração e cérebro adulto (Consler et al., 1988). O tipo L é predominante em tecidos

gliconeogênicos, especialmente no fígado, onde se constitui no maior componente, e no rim, onde é o menor componente (Staal et al., 1985). O tipo R está presente nos eritrócitos e tecidos hematopoiéticos. A M2–PK é predominante no feto, em neoplasias e nos tecidos indiferenciados e em proliferação, embora seja encontrada também em alguns tecidos adultos, em menor quantidade no fígado e em maior quantidade no rim (Dabrowska et al., 1998). As expressões dessas isoenzimas são reguladas durante o desenvolvimento. A M2–PK é a única detectável precocemente em tecido fetal, sendo gradualmente substituída pelos tipos L-, R-, M1-, durante o desenvolvimento. Em contraste, em células transformadas e na regeneração do fígado, as isoenzimas tecido-específicas estão presentes em níveis diminuídos ou completamente ausentes e são substituídas pela isoenzima M2–PK (Heyduk et al., 1992; COnsler et al., 1992.

A reação catalisada pela PK requer, para sua atividade, cátions monovalente e divalente (Friesen et al., 1998). A PK é tipicamente uma enzima alostérica e participa com uma maior função no controle do fluxo metabólico da frutose-1,6-bifosfato (FBP) a piruvato, o qual está envolvido em uma variedade de rotas metabólicas, indicando que a PK pode ser considerada uma enzima chave não somente para a rota glicolítica, mas também para o metabolismo celular (Mattevi et al., 1996).

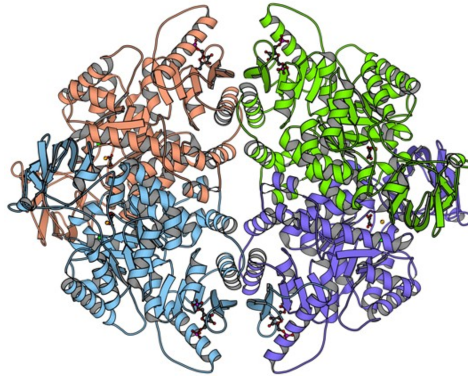

Figura 5 - PK de mamíferos é homo-tetrâmera, e cada subunidade (monômero) consiste de 4 domínios (A, B, C e N), cada um possuindo um sítio ativo.

I.1.3 - Glutationa reduzida (GSH)

Homem de Bittencourt e colaboradores (1998) sugerem que os mecanismos homeostáticos de manutenção da concentração de GSH sejam fundamentais para defesa celular contra agentes oxidantes e eletrofílicos. Devido ao fato da tirosina induzir o estresse oxidativo (Sgaravatti et al., 2008), a GSH serviu em nossos estudos como um protetor do estresse oxidativo causado pela tirosina (Sgaravatti et al., 2008; Sgaravatti et al., 2009) nos ensaios enzimáticos, pois ela mantém os grupos sulfidrilas reduzidos das enzimas. Estudo envolvendo estresse oxidativo e GSH demonstrou que ocorre depleção intracelular da GSH em presença de injúria oxidativa (Aucoin et al., 1995), sendo que a relação [GSSG]/[GSH] é considerada um índice do estado redox intracelular.

A glutationa é um tripeptídeo (γ-glutamilcisteinilglicina – Figura 6) presente em praticamente todas as células vivas e mantida em altas concentrações. No sistema nervoso central, por exemplo, em sua forma reduzida (GSH), pode ser importante para a manutenção dos grupos sulfidrila reduzidos em proteínas, e para remoção de peróxido de hidrogênio (H_2O_2) e reparo dos lipideos peroxidados por superóxido e radicais hidroxila (Newsholme & Leech, 1995).

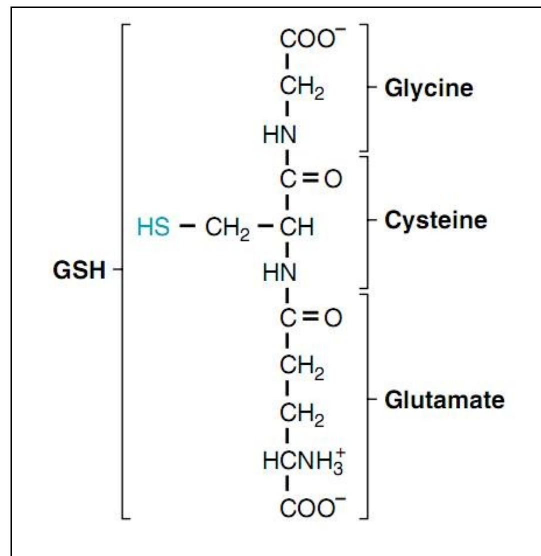

Figura 6 – Estrutura do tripeptídeo glutationa. Fonte: Marks et al, 2007.

I.1.4 – Creatina

A creatina é um composto que ocorre naturalmente no organismo humano encontrada primariamente no músculo esquelético e sintetizada endogenamente pelo fígado, rins e pâncreas a partir dos aminoácidos glicina e arginina (Wyss & Schulze, 2002). Nas células humanas, 60 a 70%, aproximadamente, correspondem à forma livre e 30 a 40%, à forma

fosforilada (fosfocreatina) (Wallimann et al., 1998). A creatina tem um papel crítico no metabolismo energético, pois sua principal função ocorre quando se encontra na forma fosforilada, agindo como uma doadora de grupo fosforil para moléculas de ADP, ressintetizando o ATP, principalmente no músculo esquelético, onde é degradado em condições de alta demanda energética dentro da célula (Wallimann et al., 1998). Além disso, essa amina é armazenada em outros tecidos como coração, músculos lisos, testículos e cérebro (Wyss & Schulze, 2002). O transportador de creatina (SLC6A8) é expresso na barreira hematoencefálica e regula a concentração de creatina no cérebro, representando uma importante via para o fornecimento de creatina a partir da circulação sanguínea para o cérebro (Tarnopolsky, 2007).

Estudos recentes envolvendo a suplementação com creatina demonstram propriedades antioxidantes desse composto (Sestili et al., 2006; Hersch et al., 2006. Lawler e colaboradores (2002) concluíram que a creatina desempenha um papel antioxidante primário significativo, havendo uma relação direta da concentração de creatina com a neutralização do radical ânion superóxido $O_2^{\cdot -}$ e com o peroxinitrito (OONO-). Além disso, a creatina não mostrou uma significante atividade antioxidante contra hidroperóxidos como o H_2O_2, sugerindo que a creatina apresenta uma capacidade antioxidante seletiva (Wyss & Schulze, 2002). A creatina exerce uma atividade citoprotetora antioxidante direta em diferentes linhagens de células contra diversos agentes oxidativos (Sestili et al., 2006). Outros efeitos benéficos da creatina foram extensivamente estudados em doenças neurodegenerativas, neuromusculares e neurometabólicas (Bender et al., 2006; Hersch et al., 2006; Tarnopolsky, 2007; Bolaños et al., 2009).

Uma vez que a tirosina é capaz de alterar parâmetros do estresse oxidativo como causar dano oxidativo ao DNA, diminuir as defesas antioxidantes não-enzimáticas e enzimáticas e alterar o estado redox (razão SH/SS), em córtex cerebral de ratos (Sgaravatti et al., 2008; Sgaravatti et al., 2009) e a creatina é um agente neuroprotetor, um dos objetivos deste trabalho foi determinar se a administração de creatina poderia prevenir as alterações causadas pela tirosina.

2. OBJETIVOS

I.2.1. – Objetivos geral

Considerando que pacientes com tirosinemia tipo II manifestam sintomas neurológicos na infância, o objetivo geral do presente trabalho foi investigar o efeito, *in vitro* e *in vivo,* de elevadas concentrações de tirosina sobre parâmetros bioquímicos do metabolismo energético em córtex cerebral de ratos de 14 dias de idade. Também investigamos o papel protetor da creatina sobre alterações bioquímicas observadas no modelo.

I.2.2. – Objetivos específicos

1 - Determinar o efeito *in vitro* de 0,1-2,0 mM de tirosina e/ou de 1,0 mM de GSH sobre a atividade da CK, em frações citosólica e mitocondrial e de córtex cerebral de ratos Wistar de 14 dias de idade.

2 - Determinar o efeito *in vitro* de 0,1-2,0 mM tirosina e/ou de 1,0 mM de GSH sobre a atividade da AK e PK em córtex cerebral de ratos Wistar de 14 dias de idade.

3 - Determinar o efeito da administração de 500mg/Kg de L-tirosina metil éster e/ou 400mg/Kg de creatina sobre a atividade da CK, AK e PK em córtex cerebral de ratos Wistar de 14 dias de idade.

PARTE II

ARTIGOS CIENTÍFICOS

II. CAPÍTULO I – Artigo 1

Tyrosine inhibits creatine kinase activity in cerebral cortex of young rats

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ORIGINAL PAPER

Tyrosine inhibits creatine kinase activity in cerebral cortex of young rats

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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. Tyrosinemia type II is a disorder of autosomal recessive inheritance characterized by neurological symptoms similar to those observed in patients with creatine deficiency syndromes. Considering that the mechanisms of brain damage in these disorders are poorly known, in the present study our main objective was to investigate the in vivo and in vitro effects of different concentrations and preincubation times of tyrosine on cytosolic and mitochondrial creatine kinase activities of the cerebral cortex from 14-day-old Wistar rats. The cytosolic CK was reduced by 15% at 1 mM and 32% at 2 mM tyrosine. Similarly, the mitochondrial CK was inhibited by 15% at 1 mM and 22% at 2 mM tyrosine. We observed that the inhibition caused by tyrosine was concentration-dependent and was prevented by reduced glutathione. Results also indicated that mitochondrial, but not cytosolic creatine kinase activity was inhibited by tyrosine in a time-dependent way. Finally, a single injection of L-Tyrosine methyl ester administered i.p. decreased cytosolic (31%) and mitochondrial (18%) creatine kinase activities of brain cortex from rats. Considering that creatine kinase is an enzyme dependent of thiol residues for its function and tyrosine induces oxidative stress, the results suggest that the inhibition caused by tyrosine might occur by oxidation of essential sulfhydryl groups of the enzyme. In case this also occurs in patients with tyrosinemia, it is possible that creatine kinase inhibition may contribute to the neurological dysfunction characteristic of tyrosinemia.

Keywords Tyrosine · Tyrosinemia · Cerebral cortex · Creatine kinase · Hypertyrosinemia

R. B. de Andrade · T. Gemelli · D. B. Rojas · C. S. Dutra-Filho · C. M. D. Wannmacher (✉)
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul,
Rua Ramiro Barcelos 2600-Anexo,
Porto Alegre, RS, Brasil 90035-003
e-mail: clovisdw@ufrgs.br

C. Funchal
Centro Universitário Metodista IPA,
Rua Cel. Joaquim Pedro Salgado, 80,
CEP 90420-060 Porto Alegre, RS, Brazil

Introduction

Tyrosinaemia type II, also known as Richner–Hanhart syndrome or oculocutaneous tyrosinaemia, is an autosomal recessive inherited disorder caused by deficiency of hepatic cytosolic tyrosine aminotransferase (TAT) (Ruetschi et al. 2000). This disease is often associated with consanguinity and the incidence is less than 1 in 250,000 (Macsai et al. 2001). It is characterized by bilateral pseudodendritic keratitis, painful palmoplantar hyperkeratotic lesions and mental retardation (Mitchell et al. 2001). The involvement of the central nervous system (CNS) is variable and ranges from severe mental retardation to slight decrease in intelligence and may be associated with microcephaly, tremor, ataxia, self-mutilating behavior, fine motor coordination disturbances, language deficits and convulsions (Rabinowitz et al. 1995; Mitchell et al. 2001).

Plasma concentrations of tyrosine are raised and the disorder is successfully treated with a low-phenylalanine and low-tyrosine diet (Mitchell et al. 2001). Moreover, plasma tyrosine levels are higher in TAT-deficiency

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comparing to the other causes of hypertyrosinemia and range from 370 to 3,420 μM (normal <90 μM) along with the accumulation of some tyrosine derivatives, namely 4-hydroxyphenylpyruvic acid, 4-hydroxy-phenyllactic acid and 4-hydroxyphenylacetic acid (Rabinowitz et al. 1995; Mitchell et al. 2001; Held 2006). 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 in the lesion sites. 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 (Uylings 2000).

Creatine kinase (CK), EC 2.7.3.2, catalyzes the reversible transfer of the N-phosphoryl group from phosphocreatine to ADP regenerating ATP. This enzyme is part of an important system maintaining energy homeostasis of cells with high and fluctuating energy requirement (Wallimann et al., 1992). CK-deficient mice showed mutation-dependent abnormalities in brain morphology and behavior including impaired spatial learning, reduced nestbuilding activity, diminished acoustic startle reflex responses (Jost et al., 2002; Streijger et al., 2005) and reduced brain energy (Kekelidze et al, 2001; In 't Zandt et al, 2004; Streijger et al, 2010). On the other hand, reduced brain creatine levels and CK activity are implicated in some neurodegenerative diseases partially treated with creatine supplementation (Béard and Braissant, 2010).

CK isoenzymes have been repeatedly considered sensitive targets of reactive species such as H_2O_2, and peroxynitrite (Yuan et al., 1992; Konorev et al., 1998). Recently, our research group reported that high concentrations of tyrosine provoke in vitro oxidative stress (decrease of enzymatic and non-enzymatic antioxidant defenses, change of the redox state and increase of DNA damage) in the cerebral cortex of young rats (Sgaravatti et al. 2008). Thus, considering that CK is a thiolic enzyme crucial for brain energy metabolism and function, CK deficiency is associated to neurodegenerative diseases and that the mechanisms underlying the neurological dysfunction in hypertyrosinemic patients are poorly known, in this work we investigated the in vitro and in vivo (ex-vivo) effects of tyrosine on CK activity in homogenates of cerebral cortex from 14-day-old rats in order to clarify its participation in the brain damage mechanisms responsible for the neurological impairment observed in hypertyrosinemic patients.

Materials and methods

Reagents and equipments

All chemicals were purchased from Sigma (St. Louis, MO, USA). 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 and 2.0 mM for the in vitro experiments. L-Tyrosine methyl ester was dissolved in 0.1% Tween 20 in saline solution for the in vivo experiments. A double-beam spectrophotometer with temperature control (Hitachi U-2001) was used for the measurements. Eppendorf 5417R was used for centrifugation procedures.

Animals

Fourteen-day-old Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, Porto Alegre, RS, Brazil were used in the experiments. Rats were kept with dams until they were sacrificed without anesthesia. The rats had free access to water and to a standard commercial chow (Germani, Porto Alegre, RS, Brazil) containing 20.5% protein (predominantly soybean supplemented with methionine), 54% carbohydrate, 4.5% fiber, 4% lipids, 7% ash and 10% moisture. Temperature was maintained at 24±1°C, with a 12–12 h light–dark cycle. The "Principles of Laboratory Animal Care" (Guide for the Care and Use of Laboratory Animals, NIH publication no. 80–23, revised 1996; http://www.nap.edu/readingroom/books/labrats/) were followed in all the experiments, and the experimental protocol was approved by the Ethics Committee For Animal Research of the Federal University of Rio Grande do Sul.

In vitro experiment

First, assays were performed to determine the in vitro influence of tyrosine (0.1, 1.0 and 2.0 mM) in cerebral cortex homogenates. Next, the effect of 1.0 mM GSH alone or associated to tyrosine 2.0 mM was tested. The brain cortical fractions were incubated for 30–90 min at 37°C depending on the experimental design with the same reaction mixture described below with tyrosine and/or GSH, with the exception of control.

Acute administration of L-tyrosine methyl ester

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 L-Tyrosine was administered

intraperitoneally. This dose was chosen in order to obtain tyrosine concentrations about 10 times normal 1 h after administration (Morre et al. 1980; Bongiovanni et al. 2003), which are similar variations of plasma tyrosine concentration observed in patients affected by tyrosinemia type II (Mitchell et al. 2001). Controls received 0.1% Tween 20 in saline solution. One hour after injection rats were killed and the brain was rapidly removed.

Preparation of brain cortical fractions

Animals were sacrificed by decapitation without anesthesia. The brain was rapidly removed and dissected on a glass dish over ice for in vitro and in vivo experiments. Time elapsed from decapitation to place the brain on the ice was less than 1 min. After dissection, the cerebral cortex was washed in SET buffer (0.32 M sucrose/1 mM EGTA/ 10 mM Tris–HCl, pH 7.4), minced finely and homogenized in the same SET buffer (1:10, w/v with a Potter-Elvehjem glass homogenizer). At least two homogenizations of 30 s duration were performed at approximately 1000 rpm with an electrically driven Teflon pestle. The homogenate was collected for determination of cytosolic and mitochondrial fractions. A portion of the homogenate was centrifuged at 800 X g for 10 min at 4°C, the pellet was discarded and the supernatant was centrifuged at 10,000 X g for 15 min at 4°C. The supernatant of the second centrifugation, containing cytosol and other cellular components as endoplasmic reticulum, was collected for determination of cytosolic CK activity (Cy-CK). The pellet, containing mitochondria, myelin, synaptosomes, and membrane fragments, was washed twice with the same Tris–sucrose isotonic buffer, resuspended in 100 mM MgSO4–Trizma buffer, pH 7.5, for determination of mitochondrial CK activity (Mi-CK). Homogenate, cytosolic and mitochondrial fractions were stored for no longer than one week at −70°C when the assay was not carried out immediately.

Creatine kinase activity assay

The reaction mixture contained the following final concentrations: 65 mM Tris–HCl buffer, pH 7.5, 7.0 mM phosphocreatine, 9.0 mM MgSO4, and approximately 1 µg protein in a final volume of 0.1 ml. After 30–90 min of pre-incubation at 37°C, the reaction was started by the addition of 0.3 µmol of ADP. The reaction was stopped after 10 min by the addition of 1 µmol p-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 ml of 2% α-naphtol and 0.1 ml of 0.05% diacetyl in a final volume of 1 mL and read after 20 min at 540 nm. None of the substances added to the assay medium interfered with the color development or spectrophotometric readings. Results were expressed as µmol of creatine formed per min per mg protein.

Protein determination

The protein content of cerebral cortex fractions was determined by the method of Lowry et al. (1951), using serum bovine albumin as the standard.

Statistical analysis

Data were analyzed by Student's t-test or by ANOVA, depending on the experimental design. Data from concentration effect was performed by one-way ANOVA followed by the Tukey test when the F value was significant. Data from time effect were analyzed by three-way ANOVA with repeated measures (saline/ tyrosine, saline/GSH and time – 30, 60, 90 min - as factors). Comparison between means, when appropriate, was performed by one-way ANOVA followed by the Tukey test when the F value was significant. Linear regression was used to evaluate dose or time dependency. Data from the in vivo experiments were analyzed by the independent Student t test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. Values of $p<0.05$ were considered to be significant.

Results

First, Cy-CK and Mi-CK from cerebral cortex cytosolic fraction were assayed in vitro in the presence of 0.1, 1.0 and 2.0 mM tyrosine concentration (Fig. 1). Cy-CK activity was markedly reduced by 2.0 mM tyrosine (32%) and linear regression showed that the amino acid inhibited this activity in a concentration-dependent way ($F(1,26)=22.39$; $\beta=-0.68$; $p<0.001$). Similarly, Mi-CK activity was inhibited at 1.0 (15%) and 2.0 (22%) mM in a concentration-dependent way ($F(1,26)=26.54$; $\beta=-0.71$; $p<0.001$).

Next, CK activity was measured in the presence of 2.0 mM tyrosine and/or 1.0 mM GSH. Tyrosine was added to the incubation medium containing the cytosolic or the mitochondrial cerebral cortex fractions obtained from non-treated rats and preincubated for 30, 60 or 90 min with or without GSH. Mi-CK showed a significant saline/Tyr by

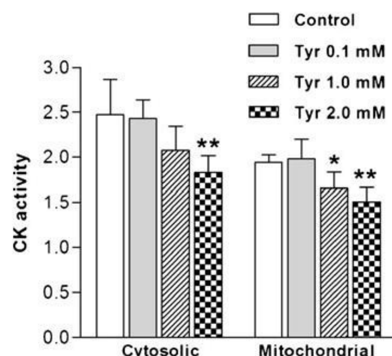

Fig. 1 In vitro effects of different concentration of tyrosine on CK activity of cytosolic (Cy-CK) and mitochondrial (Mi-CK) fractions of the cerebral cortex from young Wistar rats. Results are expressed as µmol of creatine per min per mg of protein. Data are mean ± SD for 7 independent experiments performed in triplicate. * $p<0.05$; ** $p<0.01$ compared to the controls (Tukey test)

saline/GSH by time interaction ($F(2,48)=4.13$; $p<0.05$), indicating that Tyr inhibits CK activity along time and GSH prevents this inhibition. Furthermore, regression analysis showed that the inhibition of Mi-CK activity by tyrosine was time-dependent ($F(1,19)=33.66$; $\beta=-0.79$; $p<0.0001$) (Fig. 2).

On the other hand, Cy-CK showed significant time effect ($F(2,48)=19.9$; $p<0.05$) but not saline/Tyr by saline/GSH by time interaction. Analyzing after colapsing time, saline/Tyr by saline/GSH interaction was significant ($F(1,24)=21.55$; $p<0.05$), indicating that Tyr and GSH had different effects on the enzyme activity, GSH preventing part of the inhibition caused by Tyr. Linear regression analysis showed that the inhibition caused by tyrosine was not time-dependent ($F(1,19)=0.16$; $\beta=-0.09$; $p>0.05$), reinforcing the observation that the effects of Tyr on Cy-CK and Mi-CK are different (Fig. 3).

Finally, the effect of a single administration of L-tyrosine methyl ester on Cy-CK and Mi-CK (Fig. 4) of cerebral cortex were studied. Tyrosine administration significantly reduced Cy-CK ($t(12)=3.38$; $p<0.05$) and Mi-CK ($t(10)=2.38$; $p<0.05$) activities.

Discussion

Inherited tyrosinemia is classified into type I (deficiency of fumarylacetoacetate hydrolase) with hepatorenal diseases, type II (deficiency of hepatic TAT) with oculocutaneous and CNS disturbances, and type III (deficiency of 4-hydroxyphenylpyruvate) with normal liver function and intermittent neurologic anomalies (Mitchell et al. 2001). Furthermore, all types demonstrate elevated tyrosine levels in tissues and physiological fluids of patients with tyrosinemia, but plasma tyrosine levels are higher in TAT-deficiency comparing to the other causes of hypertyrosinemias. Mental retardation and other neurological findings have been reported in patients affected by tyrosinemia type II (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 CK is a target for other amino acids accumulating in some metabolic diseases of amino acid metabolism, CK deficiency has been observed in some neurodegenerative diseases and patients with creatine deficiency syndromes present symptoms similar to those observed in patients affected by tyrosinemia (Béard and Braissant, 2010), in the present work, we investigated the in vitro and in vivo effect of tyrosine on CK activity of cerebral cortex from 14-day-old Wistar rats.

Cerebral cortex was used to investigate the effect of tyrosine on CK activity because tyrosine concentration into this brain region showed the highest relative increase compared to other regions when tyrosine is administered to rats (Morre et al. 1980; Bongiovanni et al. 2003). 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 2–3 years (Uylings 2000). In this regard, tyrosinemic patients,

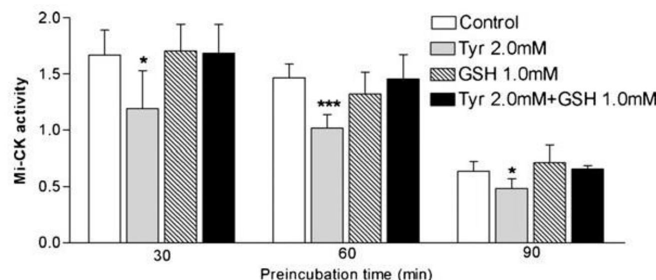

Fig. 2 In vitro effect of GSH on the inhibition of CK activity caused by tyrosine in mitochondrial fraction of the cerebral cortex from young Wistar rats. Results are expressed as µmol of creatine per min per mg of protein. Data are mean ± SD for 7 independent experiments performed in triplicate. * $p<0.05$; *** $p<0.001$ compared to the other groups (Tukey test)

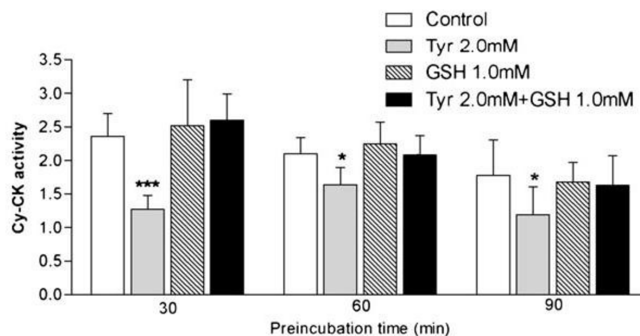

Fig. 3 In vitro effect of GSH on the inhibition of CK activity caused by tyrosine in cytosolic fraction of the cerebral cortex from young Wistar rats. Results are expressed as μmol of creatine per min per mg of protein. Data are mean ± SD for 7 independent experiments performed in triplicate. $^{*}p<0.05$; $^{***}p<0.001$ compared to the other groups (Tukey test)

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 (Mitchell et al. 2001).

In the present study, we demonstrated that high concentrations of tyrosine, similar to those found in the brain cortex of tyrosinemia type II patients, inhibited the in vitro CK activity of cytosolic and mitochondrial fractions of cerebral cortex homogenates from rats in concentration-dependent way. We observed that tyrosine inhibited in vitro the Cy-CK and that this inhibition was prevented by reduced glutathione (GSH) at all tested preincubation times, but the inhibition was not time-dependent. On the other hand, Mi-CK also was inhibited by tyrosine and the inhibition was prevented by GSH at all tested preincubation times in a time-dependent pattern. These results suggest that oxidative stress is a possible mechanism of the enzyme activity inhibition, altering crucial thiol residues for the enzyme activity. The time-dependent inhibition of Mi-CK and not in Cy-CK is in agreement with this hypothesis, since mitochondria is the main source of free radicals generation (Halliwell 2001; Halliwell 2006; Sas et al.

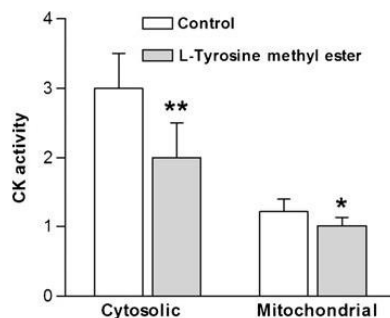

Fig. 4 Effect of acute administration of L-tyrosine methyl ester on CK activity of cytosolic and mitochondrial fractions in cerebral cortex from young Wistar rats. Results are expressed as μmol of creatine per min per mg of protein. Data are mean ± SD for 7 independent experiments performed in triplicate. $^{*}p<0.05$; $^{**}p<0.01$ compared to control (Student's t-test for unpaired samples)

2007). So, the time-dependent inhibition of Mi-CK could at least in part be caused by generation of oxidative stress induced by tyrosine, because it is well known that CK activity decreases after exposure to agents promoting generation of free radicals, probably by oxidation of the sulfhydryl residues of the enzyme (Burmistrov et al. 1992; Yuan et al. 1992; Mekhfi et al. 1996; Wolosker et al. 1996; Arstall et al. 1998; Konorev et al. 1998; Stachowiak et al. 1998; Wallimann et al. 1998; Koufen and Stark 2000) and reagents reacting with thiols (Gross et al. 1996; Wolosker et al. 1996). This may explain our present results showing that GSH prevented the inhibitory effect of tyrosine on CK activity.

Previous findings from our laboratory demonstrated that rats subjected to L-tyrosine acute administration increases oxidative stress, observed by increase of lipoperoxidation, carbonylation of proteins, decrease of GSH and thiol-disulfide redox state (SH/SS ratio) (Sgaravatti et al. 2009). It is known that numerous processes are dependent upon cellular redox state, which may be related to the reduction of CK activity in neurodegenerative diseases such as Alzheimer and Pick (Wolosker et al. 1996; Aksenov et al. 1997).

The influence of acute administration of L-tyrosine methyl ester on CK activity in cerebral cortex of rats was also investigated. We demonstrated that tyrosine reduced Cy-CK and Mi-CK activities, probably by a direct action of tyrosine on CK, or by reactive species (Sgaravatti et al. 2008). However, we cannot rule out that tyrosine derivatives might contribute 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 (Rabinowitz et al. 1995; Mitchell et al. 2001; Held 2006).

The CK/creatine phosphate system exerts three integrated functions in brain cells: temporary energy buffering, metabolic capacity, energy transfer and metabolic control (Saks et al. 1996; Wallimann et al. 1992). This system is now recognized as an important metabolic regulator during health and disease (Wyss et al. 1992; Walliman et al. 1998). A decrease in CK

activity is one of the biochemical markers of brain cell damage in age-related neurodegenerative diseases, including Alzheimer's disease (Aksenov et al. 1997). The decrease of CK activity in the brain correlates well with the neurodegeneration parameters in severely affected regions in Alzheimer's disease (Hensley et al. 1995). Therefore, damage of CK function may be an important part of a neurodegenerative pathway that leads to neuronal loss in the brain (Tomimoto et al. 1993). These findings are reinforced by the observation that creatine and phosphocreatine have neuroprotective effects against energy deprivation and glutamate excitoxicity, attributable to an enhancement of cytosolic high-energy phosphate stores (Brustovetsky et al. 2001). Besides, CK is also important to inhibit the Ca^{+2}-induced opening of the mitochondrial permeability transition pore, which leads to apoptosis (O'Gorman et al. 1997).

Some of the in vitro effects of tyrosine on CK activity were elicited only at the highest concentration studied (2.0 mM). To our knowledge, the actual concentration that is reached into the brain of type II tyrosinemic patients is not known. It was reported that plasma tyrosine levels may exceed 1 mM in untreated type II patients (Scott 2006) and achieve up to 3.4 mM (Goldsmith et al. 1973; Lemonnier et al. 1979; Rabinowitz et al. 1995; Mitchell et al. 2001; Macsai et al. 2001; Viglizzo et al. 2006). So, it is quite possible that the effects presented here may have pathophysiological relevance to tyrosinemia type II

Taken together, the results show that tyrosine inhibits CK activity in the brain of young rats. Considering that CK is a key enzyme for energy homeostasis, if this enzyme inhibition also occurs in the brain of tyrosinemia type II patients, it is possible that the decrease of this enzyme activity may alter energy metabolism and function in the brain of the patients and contribute to the brain damage characteristic of this disease.

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II. CAPÍTULO II – Artigo 2

Tyrosine impairs enzymes of energy metabolism in cerebral cortex of rats

Rodrigo Binkowski de Andrade, Tanise Gemelli, Denise Bertin Rojas, Cláudia Funchal, Carlos Severo Dutra-Filho, Clovis Milton Duval Wannmacher

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Editor-in-Chief

Molecular and Cellular Biochemistry

Tyrosine impairs enzymes of energy metabolism in cerebral cortex of rats

Rodrigo Binkowski de Andrade[1], Tanise Gemelli[1], Denise Bertin Rojas[1], Cláudia Funchal[2], Carlos Severo Dutra-Filho[1], Clovis Milton Duval Wannmacher[1]

1 - Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, UFRGS, Rua Ramiro Barcelos 2600, CEP 90.035-003 Porto Alegre, RS, Brazil.

2 - Centro Universitário Metodista IPA, Rua Cel. Joaquim Pedro Salgado, 80, CEP 90420-060 Porto Alegre, RS, Brazil.

CORRESPONDING AUTHOR: Clovis Milton Duval Wannmacher, Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Departamento de Bioquímica. Rua Ramiro Barcelos 2600-Anexo - Porto Alegre – RS, Brasil, 90035-003. Tel: 5551 33085575

E-mail: clovisdw@ufrgs.br

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 and provokes 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 the in vivo and in vitro effects of tyrosine on some parameters of energy metabolism in cerebral cortex of 14-day-old Wistar rats. We observed that 2 mM tyrosine inhibited in vitro the pyruvate kinase (PK) activity and that this inhibition was prevented by 1 mM reduced glutathione (GSH) with 30, 60 and 90 min of

preincubation. Moreover, administration of tyrosine methyl ester (TME) (0.5 mg/g of body weight) decreased the activity of PK and this reduction was prevented by pre-treatment with creatine. On the other hand, tyrosine did not alter adenylate kinase (AK) activity in vitro, but administration of TME enhanced AK activity not prevented by creatine pre-treatment. Finally, TME administration decreased the activity of CK from cytosolic and mitochondrial fractions and this diminution was prevented by creatine pre-treatment. The results suggest that tyrosine alters essential sulfhydryl groups necessary for CK and PK functions, possibly through oxidative stress. In case this also occurs in the patients, it is possible that energy metabolism alterations may contribute, along with other mechanisms, to the neurological dysfunction of hypertyrosinemias.

Keywords: tyrosine; tyrosinemia; energy metabolism; adenylate kinase, pyruvate kinase; creatine kinase

INTRODUCTION

Tyrosinemia type II (Richner–Hanhart syndrome) is a rare autosomal recessive disease with deficiency of hepatic tyrosine aminotransferase (TAT:EC 2.6.1.5) and subsequently increased level of serum tyrosine [1]. 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[1]. The incidence of this disease is less than 1 in 250,000 [2]. It is characterized by bilateral pseudodendritic keratitis, painful palmoplantar hyperkeratotic lesions, mental retardation, and variable manifestations of central nervous system involvement. Delay in diagnosis may result in permanent visual impairment and mental retardation [3]. Children who had presented with transient neonatal tyrosinaemia were compared with a group of unaffected controls at 7-9 years of age. A comprehensive psychometric assessment revealed significant differences between the groups in adaptive behaviour, psycholinguistic abilities, and speed of learning. In nearly all components of the tests used, higher levels of TNT were associated with lower levels of performance. Therefore, transientneonatal tyrosinemia, a condition commonly regarded as benign in the short term, has long-term effects which may be detrimental to the child in school. [4]

In rats, the inhibition of TAT associated with tyrosine treatment 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 [5]. These findings suggest that high levels of tyrosine may not be innocuous to the central nervous system. It has been reported that neuropathologic findings of tyrosinemic patients include metabolic astrocytosis and delay in myelination [6]. Moreover, it has been recently reported that high concentrations of tyrosine provoke oxidative stress in vitro and in vivo in cerebral cortex of rats [7,8] and we have reported that 2.0mM tyrosine reduces in vitro the activities of cytosolic and mitochondrial CK and that this inhibition was prevented by reduced glutathione (GSH) [9].

Pyruvate kinase (PK), a crucial enzyme of glycolysis pathway, is a thiolic enzyme present in all tissues. Thiol groups are necessary for its functioning, and the enzyme is characterized by lack of activation by fructose 1,6-biphosphate, and a hyperbolic kinetics for phosphoenolpyruvate (PEP) as the substrate and Mg^{2+} as the cofactor. Adenylate kinase (AK) (EC 2.7.4.3) catalyzes the reversible phosphoryltransfer between ATP and AMP [10]. This enzyme, along with creatine kinase (CK), is responsible for the enzymatic phosphoryltransfer network, in other words, responsible for the transfer of the phosphoryl of ATP where it is produced (mitochondria) to the place where it is consumed (cytosol) [11].

GSH is potential physiologic target for both superoxide and hydrogen peroxide. Scavenging of superoxide is followed by thiol oxidation and can deplete defenses against other oxidants, or shift the redox balance toward a more oxidized status [12]. The major cellular thiol/disulfide systems, including GSH/GSSG, are not in redox equilibrium and respond differently to chemical toxicants and physiologic stimuli [13]. Moreover, creatine (Cr) is other antioxidant compound that is based mostly on the function of the enzyme creatine kinase (CK) and its high-energy product phosphocreatine. Moreover, there are evidences that creatine may help in treating depression and traumatic brain injury, slow the ageing process, and possess antioxidant properties which could protect against illness [14]. Normal brain levels of creatine are 6 to10 mM and may increase 5-15% after creatine supplementation [15,16].

Considering that the mechanisms underlying the neurological dysfunction in hypertyrosinemic patients are poorly known, tyrosine induces oxidative stress and inhibits CK activity in vitro, our hypothesis was that tyrosine could also inhibit PK and AK activities, disrupting the phosphoryltransfer network, impairing brain energy homeostasis, situation that has been implicated in a variety of diseases of the CNS, including inherited metabolic disorders [17,18]. Therefore, considering that transiemt neonatal tyrosinemia may induce brain damage, the main objective of the present study was to investigate the acute in vivo effects of tyrosine on PK, AK and CK activities in brain cortex of young Wistar rats, in order to identify a possible mechanism contributing to initiate biochemical alterations that lead to brain damage observed in hypertyrosinemic patients.

MATERIALS AND METHODS

Subjects and reagents

Fourteen-day-old Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, Porto Alegre, RS, Brazil were used in the experiments. Rats were kept with dams until they were weaned. The dams and the pups after wining had free access to water and to a standard commercial chow (Germani, Porto Alegre, RS, Brazil) containing 20.5% protein (predominantly soybean supplemented with methionine), 54% carbohydrate, 4.5% fiber, 4% lipids, 7% ash and 10% moisture. Temperature was maintained at 24 ± 1 ºC, with a 12–12 h light–dark cycle. The ''Principles of Laboratory Animal Care'' (Guide for the Care and Use of Laboratory Animals, NIH publication no. 80-23, revised 1996; http://www.nap.edu/readingroom/books/labrats/) were followed in all the experiments, and the experimental protocol was approved by the Ethics Committee For Animal Research of the Federal University of Rio Grande do Sul. All chemicals were purchased from Sigma.

In vitro experiment

For the in vitro assays, 28 non-treated 14-day-old rats were used. First, L-tyrosine was prepared on the day of the in vitro experiment in distilled water and added to cerebral cortex homogenates at final concentrations of 0.1, 1 and 2 mM. Next, the effect of 1 mM GSH alone or associated to tyrosine was tested. The homogenates were incubated for 0-90 min at 37°C with the same reaction mixture described below, in the presence of tyrosine and/or GSH, with the exception of control.

In vivo experiment

First, 28 nine-day-old rats were divided into two groups. Half of the animals were pre-treated with 0.4 mg/g of body weight of creatine administered intraperitoneally for five days in volumes of 10 µL/g of body weight and the others received the same volume of saline solution (0.9%). In the sixth day each group was divided into two groups of seven rats. Therefore, at the sixth day four groups were formed: group 1 (control), rats pre-treated with saline solution that received 20 µL/g of body weight of saline solution containing 0.1% Tween 20; group 2 (tyrosine-treated), rats pre-treated with saline that received 0.5 mg/g of body weight of L-tyrosine methyl ester dissolved in saline solution containing 0.1% Tween 20 in volume of 20 µL/g of body weight; group 3 (creatine-treated) rats pre-treated with creatine that received 20 µL/g of body weight of saline solution containing 0.1% Tween 20; and group 4 (creatine and tyrosine-treated) rats pre-treated with creatine that received 0.5 mg/g of body

weight of L-tyrosine methyl ester dissolved in saline solution containing 0.1% Tween 20 in volume of 20 μL/g of body weight. All solutions were buffered to pH 7.4. This dose of tyrosine methyl ester was chosen in order to obtain plasma and brain cortex tyrosine concentrations about 10 times the normal 1 h after administration [19,20], which are similar to plasma tyrosine concentration observed in patients affected by tyrosinemia type II [1, 21]. One hour after injection rats were killed and the brain was rapidly removed.

Tissue preparation and incubation

Treated (in vivo) and non-treated (in vitro) animals were sacrificed by decapitation without anesthesia and the brain was rapidly removed and dissected 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 washed in SET buffer (0.32 M sucrose/1mM EGTA/10 mM Tris–HCl, pH 7.4), minced finely and homogenized in the same SET buffer (1:10, w/v with a Potter-Elvehjem glass homogenizer). At least two homogenizations of 30 s duration were performed at approximately 1000 rpm with an electrically driven Teflon pestle. A portion of the homogenate was centrifuged at 800 X g for 10 min at 4 ºC for determination of AK activity, the pellet was discarded and the supernatant was centrifuged at 10,000 X g for 15 min at 4 ◦C. The supernatant of the second centrifugation, containing cytosol and other cellular components as endoplasmic reticulum, was collected for determination of PK and cytosolic CK activity (BB-CK). The pellet, containing mitochondria, myelin, synaptosomes, and membrane fragments, was washed twice with the same Tris–sucrose isotonic buffer, resuspended in 100 mM $MgSO_4$–Trizma buffer, pH 7.5, for determination of mitochondrial CK activity (Mi-CK). The supernatants were stored for no more than 1 week at −70ºC when the assay was not carried out immediately.

Enzyme assays

Pyruvate kinase activity was assayed essentially as described by Leong et al. [22]. The incubation medium consisted of 0.1 M Tris /HCl buffer, pH 7.5, 10 mM $MgCl_2$, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7 units of L-lactate dehydrogenase, 0.1% (v/v) Triton X-100, and 10 mL of the mitochondria-free supernatant in a final volume of 0.5 mL. After 0-90 min of pre-incubation at 37◦ C, the reaction was started by the addition of 1.0 mM phosphoenolpyruvate (PEP). All assays were performed in duplicate at 25˚C. Results were expressed as μmol of pyruvate per min per mg of protein.

Adenylate kinase activity was measured with a coupled enzyme assay with hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD), according to Dzeja et al.

[23]. The reaction mixture contained 100 mM KCl, 20 mM HEPES, 20 mM glucose, 4 mM $MgCl_2$, 2 mM NADP+, 1 mM EDTA, 4.5 U/mL of HK, 2 U/mL of G6PD and 1 µg of protein homogenate. After 0-90 min of pre-incubation at 37◦ C, the reaction was initiated by the addition of 2 mM ADP and the reduction of $NADP^+$ was followed at 340 nm for 3 min in a spectrophotometer (Spectronic Genesys 8, Spectronic Instruments, Rochester, New York, USA). ADP, NADP+, tyrosine, CSH, G6PD and HK were dissolved in water. Reagents concentration and assay time (3 min) were chosen to assure the linearity of the reaction. GSH did not interfere with HK or G6PD activities or with spectrophotometric readings. The results were expressed in µmol of ATP formed per min per mg of protein.

Creatine kinase activity was assayed in the reaction mixture contained the following final concentrations: 65 mM Tris–HCl buffer, pH 7.5, 7.0 mM phosphocreatine, 9.0 mM MgSO4, and approximately 1 µg of protein in a final volume of 0.1 mL. After 0-90 min of pre-incubation at 37◦ C, the reaction was started by the addition of 0.3 µmol of ADP. The reaction was stopped after 10 min by the addition of 1 µmol of p-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes [24]. The color was developed by the addition of 0.1 mL 2% α-naphthol and 0.1 mL 0.05% diacetyl in a final volume of 1 mL and read after 20 min at 540 nm. None of the substances added to the assay medium interfered with the color development or spectrophotometer readings. Results were expressed as µmol of creatine formed per min per mg of protein.

Protein determination

Protein content of cerebral cortex homogenates was determined by the method of Lowry et al. [25], using serum bovine albumin as the standard.

Statistical analysis

Data were analyzed by one or two-way ANOVA, depending on the experimental design. The effect of different concentrations of tyrosine on the enzymes activities was analyzed by one-way ANOVA, because different animals were used for different concentrations. In vitro effect of tyrosine and GSH and the in vivo (ex-vivo) effect of tyrosine and creatine were analyzed by two-way ANOVA. Post hoc analysis of significant values of F was performed by the Tukey test when required. Concentration-dependence or time-dependence was analyzed by linear regression. All data were analyzed by the Statistical

Package for the Social Sciences software (SPSS 15.0 for Windows). Values of $p<0.05$ were considered significant.

RESULTS

First, the homogenate was pre-incubated with tyrosine at 0.1, 1 and 2 mM final concentrations. The results showed that tyrosine inhibited PK activity [$F(3,25) = 4.93$; $p< 0.05$], in a dose-dependent way [$F(1,24) = 10.25$; $p< 0.05$; $\beta = -0.54$] (Fig. 1). Tyrosine did not inhibited AK activity [$F(3,23) = 0.69$; $p> 0.05$] (Fig. 2).

Next, the homogenate was pre-incubated for 0, 30, 60, or 90 min, in the presence of 2 mM tyrosine and/or 1 mM GSH on PK and AK activity. In respect to PK activity, interaction between GSH and tyrosine was significant for 30 [$F(1,27) = 5.81$; $p< 0.05$], 60 [$F(1,27) = 5.98$; $p< 0.05$], or 90 [$F(1,27) = 5.26$; $p< 0.05$], min of preincubation were significant, but not without pre-incubation [$F(1,27) = 0.21$; $p> 0.05$]. Two-way ANOVA followed by the Tukey test showed that tyrosine inhibited PK activity and pre-treatment with creatine prevented the enzyme inhibition (Fig 3). PK activity was inhibited in a time-dependent way [$F(1,23) = 6.08$; $p< 0.05$; $\beta = - 0.45$]. Considering that GSH is a strong physiological protector of thiol groups, these results suggest that tyrosine inhibited the PK activity probably related to the alteration of the sulfhydryl groups of the enzyme. In respect to AK activity, interaction between GSH and tyrosine was not significant for 30 [$F(1,27) = 0.01$; $p> 0.05$], 60 [$F(1,27) = 0.01$; $p> 0.05$], or 90 [$F(1,27) = 0.01$; $p> 0.05$], min of preincubation or without pre-incubation [$F(1,27) = 0.03$; $p> 0.05$],). AK activity was not altered along the preincubation time [$F(1,25)= 0.03$; $\beta=-0.03$; $p>0.05$] (Fig. 4). Therefore, in these experiments tyrosine and GSH did not alter the AK activity in all times tested.

Finally, considering that tyrosine inhibited the in vitro mitochondrial (Mi-CK) and cytosolic (BB-CK) activities [9], we investigated the effect of administration of L-tyrosine methyl ester and/or creatine on PK, AK and CK activities in the cerebral cortex of rats. Two-way ANOVA followed by the Tukey test showed significant interaction between tyrosine and creatine for PK activity [$F(1,27)=17.17$;$p<0.05$], indicating that tyrosine methyl ester administration significantly reduced PK activity and creatine pre-treatment prevented the PK inhibition (Fig 5). In respect to AK activity, two-way ANOVA followed by the Tukey test did not show significant interaction between tyrosine and creatine [$F(1,25)=3.43$;$p>0.05$], indicating that tyrosine methyl ester administration significantly increased AK activity but creatine pre-treatment did not prevent the AK increase (Fig 6). Moreover, interaction between tyrosine and creatine was significant for BB-CK [$F(1,27)=6.61$; $p<0.05$] and Mi-CK

[F(1,27)=4.74; p<0.05] (Fig 7). Tukey test showed that tyrosine inhibited the two CK activities and creatine pre-treatment prevented the enzymes inhibition.

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 [21]. Mental retardation and other neurological findings have been reported in patients affected by tyrosinemia type II [21]. 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 [8, 20].

Animal models do not completely imitate human diseases in all its complexity. However, chemical animal models have been largely used because they have the advantage of isolate every substance known to accumulate in human disease and study against adequate control. Therefore, animal models are important in the investigation of pathophysiologic mechanisms of the diseases, especially in brain metabolism, helping to suggest preventive measures and new drugs for treatment. However, tyrosine methy eater load do not reproduces entirely tyrosinemia, because the enzyme TAT is active in the rats, increasing the pathway of tyrosine catabolism, differently from the human disease. Therefore, the chemical model used in the present work has some limitations. After administration of 0.5 mg/g of body weight of tyrosine, this amino acid achieve 1.2 mM level in plasma after 15 min and the same concentration in brain cortex after 60 min, ten times higher than the basal concentrations [19, 20]. On the other hand, tyrosine may achieve 3.8 mM plasma levels in tyrosinemic patients [18], suggesting that a similar concentration may occur in the brain, indicating that 1-2 mM tyrosine concentration used in the present work may be of pathological significance for this disease.

Considering that tyrosine induces oxidative stress and inhibits CK activity in vitro, a thiol-containing enzyme, in the present work we investigated the in vitro effects of tyrosine on the activities of PK and AK, two thiol-containing enzymes that are crucial for energy homeostasis in cerebral cortex from 14-day-old Wistar rats. Besides, we investigated the effects of tyrosine methyl ester administration, a precursor of tyrosine by metabolic demethylation, and the possible preventive effect of pre-treatment with creatine, on AK, PK and CK activities, three important enzymes of the phosphoryltransfer network.

Coupling of spatially separated intracellular ATP-producing and ATP-consuming processes is fundamental to the function of living organisms. However, the spatial arrangements are insufficient for all cellular energetic needs [26]. Therefore, it is necessary an enzymatic network, catalyzed by CK, AK and glycolytic enzymes, in especial PK, to support high-energy phosphoryl transfer between ATP-generating and ATP-consuming processes [27-29]. This dynamic metabolic signaling maintain the balance between cellular ATP consumption and production, the energetic homeostasis for preserving cell survival [11,27]. Enzymatic capacities, isoform distribution and the dynamics of net phosphoryl flux through the integrated phosphoryltransfer systems maintain the cellular energetic homeostasis [11]. Energy-rich phosphoryls from ATP in glycolytic pathway can be used to phosphorylate ADP through the pyruvate kinase-catalyzed reaction at remote ATP utilization sites.

We have previously reported that 2 mM tyrosine reduces in vitro BB-CK and Mi-CK activities and that these inhibitions were prevented by reduced glutathione (GSH), suggesting that the enzymes inhibition occurred by alteration of sulfhydryl groups of the enzymes [9]. It has been demonstrated that tyrosine is a compound that induces oxidative stress by increasing free radical formation and decreasing brain antioxidant defenses [6,7]. The increase in reactive species generation promoted by tyrosine can alter groups that are essential for enzymatic activity of PK, AK, BB-CK and Mi-CK, such as sulfhydryl groups, or components of the plasma membrane, where enzymes are anchored [28]. So, in the present study, we extended our investigation to verify the influence of tyrosine on PK and AK, other important parameters of brain energy metabolism.

Pyruvate kinase is a rate-controlling glycolytic enzyme that catalyses the irreversible conversion of phosphoenolpyruvate (PEP) to pyruvate, coupled to the synthesis of one molecule of ATP [29]. We observed that high concentrations of tyrosine, similar to those found in the cerebral cortex of tyrosinemia type II patients, inhibited the in vitro PK activity of cerebral cortex homogenates from rats in concentration-dependent way. Furthermore, tyrosine inhibited the enzyme activity along the preincubation times and the inhibition was prevented by GSH, a strong protector of thiol groups. The time-dependent inhibition of PK could, at least in part, be caused by generation of oxidative stress induced by tyrosine [8], because it is well known that PK activity decreases after exposure to agents promoting generation of free radicals, like some amino acids, and substances reacting with thiols, probably by alteration of the sulfhydryl residues of the enzyme [6, 14, 15]. As a consequence of PK inhibition, synthesis of pyruvate is diminished, and it has been reported that pyruvate deficit may induce cell death [32]. In the present investigation, PK activity was inhibited by tyrosine methyl ester administration and pre-treatment with creatine prevented PK inhibition. The protective effect of creatine has been attributed to its antioxidant properties, which may result from different mechanisms of action: direct scavenging of radical species, iron chelation [33], and amelioration of cellular energetics [34-37]. Therefore, the preventive effect of

creatine reinforces the hypothesis that the inhibition of PK caused by tyrosine methyl ester administration was possibly due to alteration of essential thiol groups of the enzyme by oxidative stress.

Adenylate kinase (AK) (EC 2.7.4.3) catalyzes the reversible transfer of phosphoryl between ATP and AMP [10]. AK, along with CK, PK and other enzymes is responsible for the enzymatic phosphoryltransfer network [38]. Both enzymes, CK and AK, are intimately associated in such a way that when one enzyme activity is reduced, the activity of the other enzyme is enhanced, but the mechanism of this interrelation is unknown [23, 39]. In the present study, we showed that in vivo BB-CK and Mi-CK activities were inhibited by tyrosine and AK was increased, in agreement with this interrelation [39].

Creatine kinase-catalyzed phosphoryltransfer is the major component of the high-energy phosphoryltransfer and distribution network, coupling ATP production in mitochondria with ATP utilization sites in excitable tissues [27, 28, 40]. CK isoenzymes are specifically located at places of high energy demand and production, linking energy production and energy utilization by a Cr/PCr circuit [24]. Moreover, this circuit represents an efficient regulator of energy flux and uses metabolite channelling as a fine-tuning device of local ATP levels. BB-CK isoenzymes occur as dimers, in contrast to Mi-CK, which may appear as dimers or octamers [27]. The octameric Mi-CK isoform, localized in the intermembrane space of mitochondria, functionally interacts with the transmembrane proteins adenylate translocator (ANT) of the inner mitochondrial membrane and porin of the outer mitochondrial membrane [27, 28, 41], transporting phosphphocreatine formed in mitochondria to cytosol. The CK–PCr system exerts several integrated functions in brain cells, such as temporary energy buffering, metabolic capacity, energy transfer and metabolic control [42]. This system is recognized as an important metabolic regulator during health and disease [27, 43]. It has been postulated that CK and the Cr–PCr energy shuttle may play a role in brain development that is associated with oligodendrocyte function and/or myelogenesis [27, 42,43].

Considering that tyrosine induces oxidative stress [7,8], and inhibits the two CK activities in vitro [9 8] and in vivo (ex-vivo), it is possible that CK inhibition occurred by alteration of important cysteinyl residues of the enzyme [9, 44]. Prevention of the in vitro inhibition by GSH [9] and by creatine pre-treatment in the present work reinforces this possibility. Creatine is a critical component in maintaining cellular energy homeostasis. While the role of creatine in the muscular system is well recognized, there is growing evidence that it also plays an important role in the central nervous system [45]. Although many of the molecular mechanisms are not well understood, creatine supplementation has been effective in a variety of animal/cellular models of neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease [46]. Therefore, it is possible that the ability of creatine

to prevent the alterations observed in PK, BB-CK and Mi-CK activities may be mediated by its antioxidant capacity. On the other hand, the lack of in vitro effect of tyrosine, on AK activity, and the lack of prevention by creatine pre-treatment of the increased AK activity caused by tyrosine methyl ester administration suggest that the increased AK activity was not caused by direct effect of tyrosine, reinforcing the hypothesis that the increase of AK activity was caused by mechanisms involving reduction of CK activity. Studies have shown that creatine administration may reduce the frequency of epileptic seizures. Pre-treatment of rats with creatine (10-days old), 3 days before exposure to hypoxia, has been shown to lead to a recovery in the levels of ATP and phosphocreatine and decrease convulsions induced by hypoxia [13]. Magni et al. [47] showed that creatine administration (300 mg/kg, i.p.) decreased seizures induced by glutaric acid in rats, increasing the latency for the first convulsive episode and decreasing the duration of convulsive episodes. In addition to its functions in the buffering and transport of high energy phosphates, new roles for creatine have recently been suggested in CNS, such as direct anti-apoptotic effects [41, 48], alterations in vasculature, leading to improved circulation in the brain [49 46] and beneficial effects on cognitive processes [50]. In summary, in vivo experiments showed that creatine was able to prevent bioenergetic disturbances like CK and PK inhibitions, suggesting that this compound may have a protective role in the brain alterations caused by tyrosine. Oxidative stress caused by tyrosine is a possible mechanism for the decreased enzymes activity. This may impair the phosphoryltransfer network and reduce creatine and pyruvate content, two important antioxidants and neuroprotectors. As a consequence, a vicious circle is established, because diminution of antioxidant defenses increases PK and AK inhibition which decreases pyruvate and creatine content, and so on.

In this work, we have injected tyrosine methyl ester, inducing a situation similar to the observed in transient neonatal tyrosinemia, a condition commonly regarded as benign in the short term, that have long-term effects which may be detrimental to the child in school [4]. Taken together, our results show that tyrosine alters some crucial enzymes of the phosphoryltransfer network, altering brain energy homeostasis and natural antioxidants. If the alteration of these enzymes activities also occurs in the brain of the patients, it is possible that energy metabolism and function may be altered, contributing to the brain damage observed in patients with tyrosinemia type II. If this is the case, it is possible that creatine supplementation may benefit these patients. However, more studies are needed to elucidate other processes and mechanisms involved in the neurological dysfunction presented by these patients.

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Figures

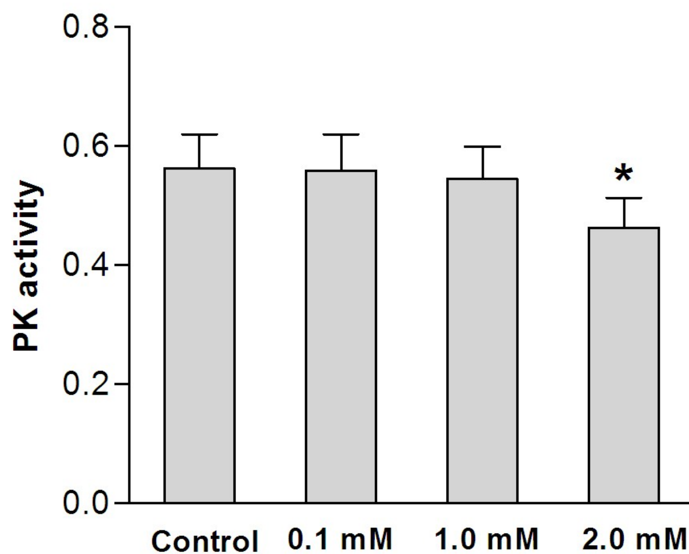

Fig. 1 In vitro effect of tyrosine on PK activity in cerebral cortex of young rats.

Results are expressed as µmol of pyruvate per min per mg of protein. Data are mean ± SD for 7 independent experiments performed in triplicate. *$p<0.05$ compared to the other groups (Tukey test)

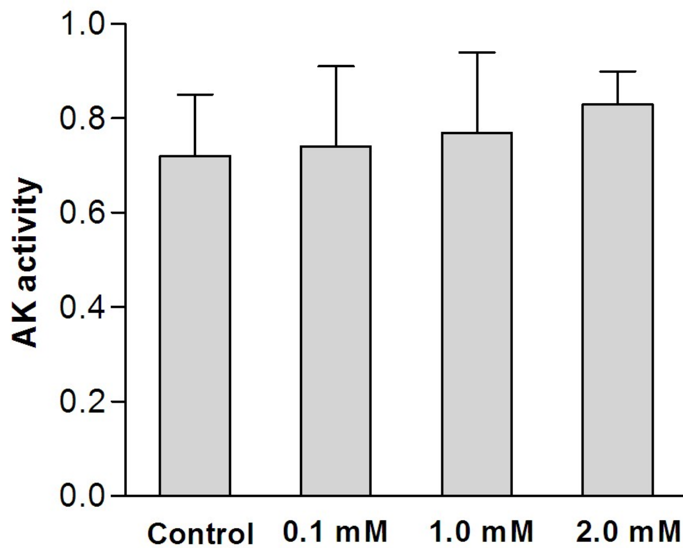

Fig. 2 In vitro effect of tyrosine on AK activity in cerebral cortex of young rats.

Results are expressed as µmol of ATP per min per mg of protein. Data are mean ± SD for 7 independent experiments performed in triplicate

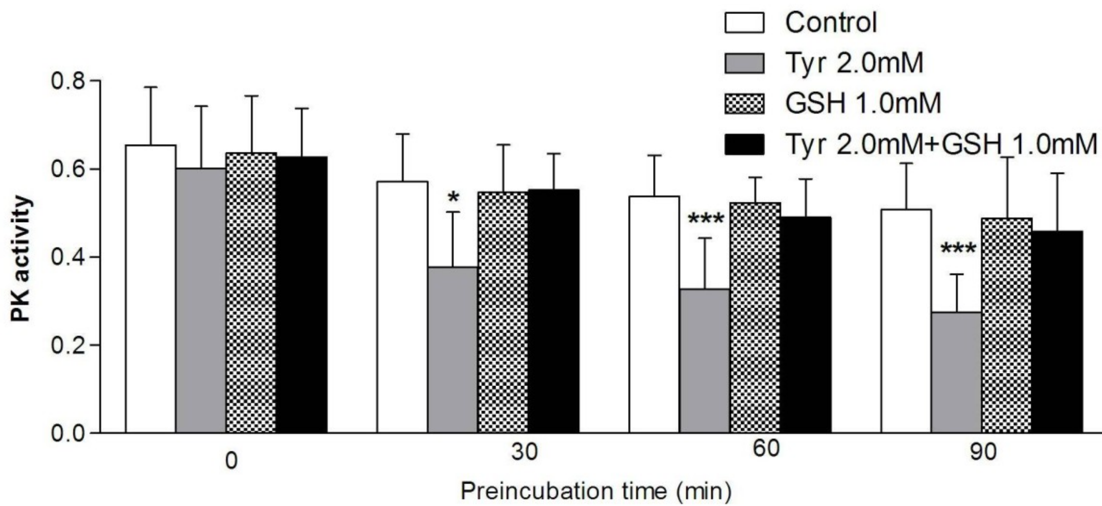

Fig. 3 In vitro effect of GSH on the inhibition of PK activity caused by tyrosine in cerebral cortex of young rats. Results are expressed as µmol of pyruvate per min per mg of protein. Data are expressed as mean ± SD for 7 independent experiments performed in triplicate. *$p<0.05$ compared to the other groups (Tukey test)

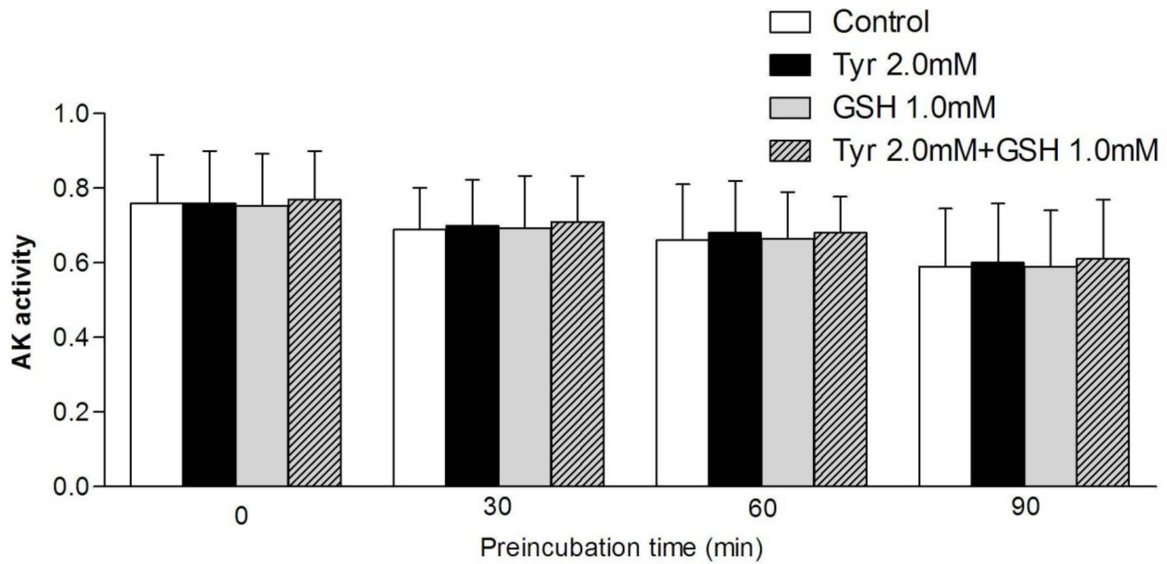

Fig. 4 In vitro effect of GSH and tyrosine on AK activity in cerebral cortex of young rats. Results are expressed as μmol of ATP per min per mg of protein. Data are expressed as mean ± SD for 7 independent experiments performed in triplicate

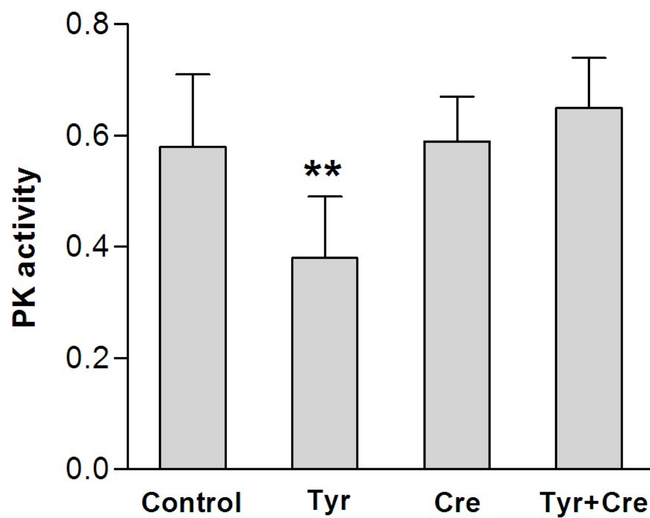

Fig. 5 Effect of L-tyrosine and / or creatine administration on PK activity in cerebral cortex of young rats. Results are expressed as μmol of pyruvate per min per mg of protein. Data are expressed as mean ± SD for 7 independent experiments performed in triplicate. *p<0.05 compared to the other groups (Tukey test)

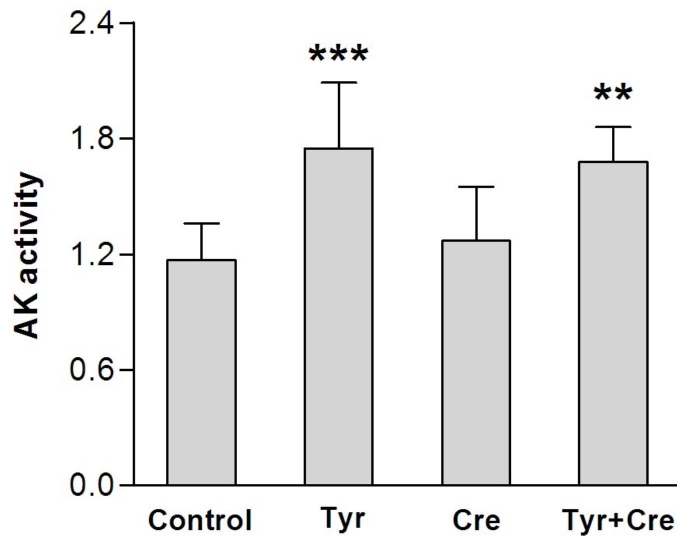

Fig. 6 Effect of L-tyrosine and / or creatine administration on AK activity in cerebral cortex of young rats. Results are expressed as μmol of ATP per min per mg of protein. Data are expressed as mean ± SD for 7 independent experiments performed in triplicate. *$p<0.05$ compared to the other groups (Tukey test)

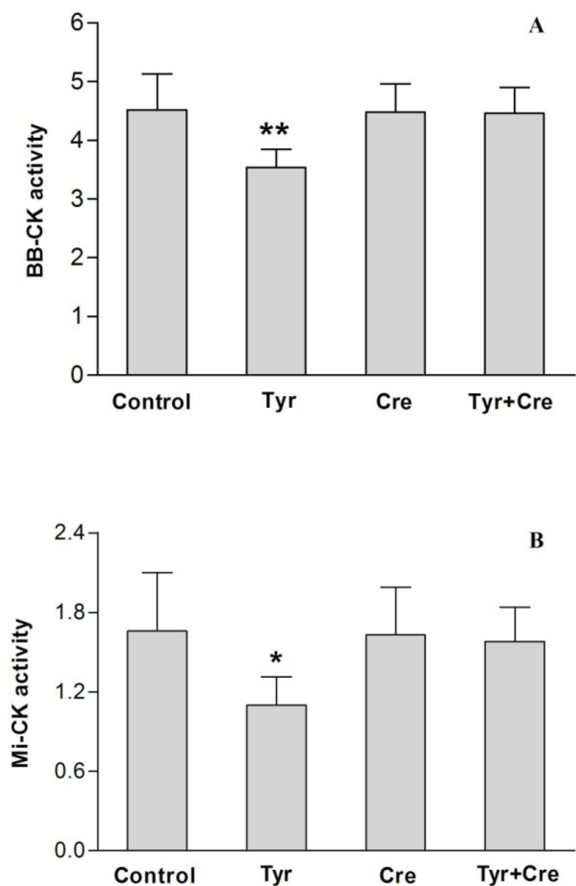

Fig. 7 Effect of acute administration of L-tyrosine or creatine on BB-CK (A) and Mi-CK (B) activities in cerebral cortex of young rats. Results are expressed as μmol of creatine per min per mg of protein. Data are expressed as mean ± SD for 7 independent experiments performed in triplicate. *$p<0.05$ compared to the other groups (Tukey test)

PARTE III
DISCUSSÃO E CONCLUSÕES

III. 1. DISCUSSÃO

Primeiramente, discutiremos os resultados da Parte I deste trabalho como segue.

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). Na tirosinemia tipo II, os elevados níveis plasmáticos de titosina são devidos à deficiência enzimática da TAT (Valikhani et al., 2005; Mitchell et al., 2001). O acúmulo de tirosina em tecidos dos pacientes com tirosinemia tipo II pode causar danos severos nos olhos, pele e sistema nervoso, cujos mecanismos responsáveis pela lesão não são completamente entendidos. As manifestações clínicas associadas à tirosinemia tipo II envolvem basicamente lesões oculares, lesões cutâneas e alterações neurológicas (Mitchell et al., 2001; Held, 2006). Considerando-se que a CK é alvo de outros aminoácidos acumulados em algumas doenças metabólicas e a deficiência da CK tem sido observada em algumas doenças neurodegenerativas, o objetivo principal desta etapa do trabalho foi investigar *in vivo* e *in vitro* o efeito de diferentes concentrações e tempos de pré-incubação de tirosina atividade da creatinaquinase nas frações citosólica e mitocondrial do córtex cerebral de ratos de 14 dias de idade Wistar.

A CK é uma enzima chave na homeostase do metabolismo energético cerebral, catalisando a transferência reversível do grupo fosforil da fosfocreatina (PCr) para ADP regenerando ATP. O ATP é a fonte imediata de energia para muitos processos que requerem energia nos sistemas biológicos (Lehninger, 2000). Além disso, a localização subcelular e proporções relativas das isoenzimas da CK, assim como os níveis da PCr em diferentes tecidos de demanda energética alta e intermitente, sugerem que o sistema CK/PCr trabalha

não somente em paralelo com a difusão de ATP e ADP, mas dependendo do tecido, também funciona como um elo obrigatório entre sítios de produção e consumo de ATP (Wallimann et al., 1992). O sistema CK/PCr exerce várias funções integradas no cérebro, como tamponamento de energia temporal, capacidade metabólica, transferência de energia e controle metabólico (Saks et al., 1996). O sistema é reconhecido como um importante regulador metabólico durante a saúde e a doença (Wallimann et al., 1998). Tem sido postulado que CK e o circuito ou sistema creatina-fosfocreatina podem ter um papel no desenvolvimento cerebral associado com a função de oligodendrócitos e/ou mielogênese (Manos et al., 1991).

Os resultados *in vitro* mostraram que a tirosina reduz a atividade da CK de forma dependente da concentração nas frações citosólica e mitocondrial. Além disso, a CK mitocondrial é inibida dependente do tempo de pré-incubação no ensaio enzimático, além de poder ser prevenida pela adição de GSH ao ensaio. Por outro lado, a fração citosólica da CK não é inibida ao longo do tempo, mas a adição de GSH também preveniu a inibição da enzima. Portanto, a adição de GSH ao ensaio enzimático preveniu a redução da atividade, possivelmente preservando os grupos tiólicos essenciais da enzima. Estes resultados sugerem que o estresse oxidativo é um possível mecanismo de inibição da atividade enzimática, alterando resíduos cruciais contendo tiol para a atividade enzimática (Sgaravatti et al., 2009). A inibição dependente do tempo da CK mitocondrial e não em na fração citosólica está de acordo com esta hipótese, pois as mitocôndrias são as principais fontes de geração de espécies reativas (Saks et al., 1996;. Halliwell, 2001; Halliwell, 2006). Assim, a inibição dependente do tempo na fração mitocondrial da CK poderia, pelo menos em parte, ser causada pela indução de estresse oxidativo pela tirosina, porque é conhecido que a

atividade da CK diminui após exposição a agentes que promovam a geração de espécies reativas, provavelmente por oxidação dos resíduos sulfidrila da enzima. Finalmente, a administração única de L-tirosina metil éster reduziu significativamente a atividade da fração citosólica e mitocondrial da CK, provavelmente pela ação direta da tirosina sobre a CK ou por espécies reativas (Sgaravatti et al., 2008). No entanto, não podemos descartar que os metabólitos derivados da tirosina possam contribuir para os efeitos in vivo desde a elevada excreção urinária do ácido 4-hidroxifenilpirúvico, ácido 4-hidroxifenilacético, ácido 4-hidroxifenilático, N-acetiltirosina e 4-tiramina (Rabinowitz et al 1995;. Mitchell et al 2001;. Held2006).

Consideramos que um dos possíveis efeitos da toxicidade da tirosina, seja via aumento de espécies reativas como descrito por Sgaravatti et al (2008), que resulta do desequilíbrio entre uma produção excessiva de compostos oxidantes e mecanismos de defesa antioxidante ineficiente. Como a CK é uma enzima tiólica crucial para a homeostasia energética, sua inibição, por acúmulo de tirosina, pode causar um desequilíbrio energético intracelular em pacientes com tirosinemia tipo II.

A seguir, discutiremos os resultados da Parte II deste trabalho.

Considerando que a tirosina induz o estresse oxidativo e inibe *in vitro* a atividade de CK, uma enzima tiólica, nessa etapa do trabalho foram investigados os efeitos *in vitro* da tirosina sobre as atividades da PK e AK, duas enzimas contendo grupos tióis que são cruciais para a homeostase energética no córtex cerebral de ratos Wistar de 14 dias de idade. Além disso, nós investigamos os efeitos da administração de TME, um precursor

da tirosina por desmetilação metabólica e os possíveis efeitos de prevenção do pré-tratamento com creatina sobre as atividades da AK, PK e CK, três importantes enzimas da rede de transferência de grupos fosforil.

Observou-se que altas concentrações de tirosina, semelhantes às encontradas no córtex cerebral de pacientes com tirosinemia tipo II, inibiu *in vitro* a atividade da PK em homogeneizados de córtex cerebral de ratos de forma dependente da concentração. Além disso, a tirosina inibiu a atividade da enzima ao longo do tempo de pré-incubação e a inibição foi prevenida por GSH, um protetor de grupos tiol. A inibição dependente do tempo da PK poderia, pelo menos em parte, ser causada por geração de estresse oxidativo induzido pela tirosina , porque é conhecido que a atividade da PK diminui após a exposição de agentes que promovam a geração de espécies reativas, como alguns aminoácidos e substâncias que reagem com tióis, provavelmente por alteração dos resíduos sulfidrilas da enzima (Feksa et al., 2003). Além disso, como conseqüência da inibição da PK, a síntese de piruvato é diminuída e tem sido relatado que o déficit de piruvato pode induzir a morte celular, pois além de ser um substrato energético o piruvato tem propriedades antioxidantes (Dzeka et al., 1998).

Na administração aguda, 28 ratos Wistar de ambos os sexos com nove dias de idade foram divididos em dois grupos. Metade dos animais foram pré-tratados com 0.4 mg/g de peso corporal de creatina administrada por via intraperitoneal por cinco dias em volumes de 10 μL/g de peso corporal e a outra metade dos animais recebeu o mesmo volume de solução salina (0.9%). Deste modo os animais foram divididos em quatro grupos: 1- Sal, 2- Tyr, 3- Cr e 4- Tyr+Cr. Uma hora após a injeção os ratos foram mortos e o córtex cerebral foi removido rapidamente e colocado em uma placa de Petri invertida sobre o gelo.

No tratamento agudo na presença ou ausência de tirosina e/ou creatina, a atividade da PK foi inibida pela administração de L-tirosina metil éster e o pré-tratamento com creatina impediu a inibição da PK. O efeito protetor da creatina tem sido atribuído às suas propriedades antioxidantes que podem resultar de diferentes mecanismos de ação: "scanvenger" de espécies reativas, quelante de ferro e melhora da energética celular. Portanto, o efeito preventivo da creatina reforça a hipótese de que a inibição da PK causado pela administração de L-tirosina metil éster foi possivelmente devido à alteração de grupos tióis essenciais da enzima pelo estresse oxidativo. A CK e AK estão intimamente associadas de tal forma que quando uma atividade enzimática é reduzida, a atividade da outra enzima é aumentada, mas o mecanismo desta inter-relação é desconhecido. No presente estudo, mostramos que as atividades *in vivo* da fração citosólica e mitocondrial da CK foram inibidas por tirosina e a atividade da AK aumentou, de acordo com esta inter-relação. Por outro lado, a falta de efeito *in vitro* da L-tirosina na atividade AK e a falta de prevenção do pré-tratamento com creatina na administração aguda sobre a atividade da AK sugerem que o aumento da atividade da AK não foi causada por efeito direto da tirosina, reforçando a hipótese de que o aumento da atividade da AK foi causada por mecanismos que envolvem a redução da atividade da CK. A creatina é um componente crítico para a manutenção da homeostase energética da célula. Embora o papel da creatina no sistema muscular é mais bem descrito e bem reconhecido, existem evidências crescentes de que ela também desempenha um papel importante no SNC (Andres et al., 2008). O efeito neuroprotetor da creatina tem sido atribuído a suas propriedades antioxidantes, que podem resultar a partir de diferentes mecanismos de ação. Pesquisadores têm testado o potencial da creatina em agir de forma direta na remoção de espécies reativas de oxigênio, sugerindo que ela pode atenuar danos

oxidativos induzidos pelo exercício em células musculares pela manutenção da homeostase energética mitocondrial (Matthews et al., 1998; Lawler et al., 2002); além disso pode agir como quelante de ferro (Azzi et al., 2004), e melhorar a energética celular (Persky & Brazeau, 2001; Wyss & Schulze, 2002). Além disso, o aumento das concentrações de fosfocreatina pode facilitar a geração de ATP, e promover a manutenção da homeostase de Ca^{2+} intracelular, impedindo danos às células e insuficiência de energia em algumas doenças neurodegenerativas (Wyss & Schulze, 2002). Considerando os resultados em conjunto, podemos presumir que a tirosina altera grupos sulfidrilas essenciais para a atividade da CK e PK, possivelmente por estresse oxidativo. Se estes efeitos também ocorrerem nos pacientes com tirosinemia tipo II, é possível que alterações no metabolismo energético possam contribuir, pelo menos em parte, para a disfunção neurológica característica dessa doença metabólica.

III.2. CONCLUSÕES

Objetivo 1 - Determinar o efeito in vitro de elevadas concentrações de tirosina e/ou da GSH sobre a atividade da CK, em frações citosólica e mitocondrial de córtex cerebral de ratos Wistar de 14 dias de idade.

Conclusões

1) A inibição da atividade da CK é dose-dependente;

2) GSH previne os efeitos inibitórios causados pela tirosina sobre a atividade da CK quando pré-incubados, sugerindo que a tirosina inibe a atividade das enzimas por oxidação de grupos sulfidrilas das enzimas;

3) A administração de L-tirosina metil éster reduziu a atividade da CK na fração citosólica e da fração mitocondrial no córtex cerebral dos ratos;

Objetivo 2 - Determinar o efeito in vitro de elevadas concentrações de tirosina e/ou da glutationa reduzida (GSH) sobre a atividade da AK e PK em córtex cerebral de ratos Wistar de 14 dias de idade.

1) A inibição da atividade da PK é dose-dependente, mas não houve efeito sobre a atividade da AK;

2) GSH previne os efeitos inibitórios causados pela tirosina sobre a atividade da PK quando pré-incubados, sugerindo que a tirosina inibe a atividade da enzima por oxidação de

grupos sulfidrilas das enzimas, mas a GSH e/ou tirosina não apresentam nenhum efeito sobre a atividade da AK;

3 - Determinar o efeito da administração de L-tirosina metil éster e/ou creatina sobre a atividade da CK, AK e PK em córtex cerebral de ratos Wistar de 14 dias de idade.

3) A administração L-tirosina metil éster reduziu a atividade da PK, CK-BB e CK-Mi e a creatina preveniu esta inibição no córtex cerebral dos ratos. Entretanto, a administração de tirosina aumentou a atividade da AK e a creatina não alterou a atividade da AK em relação ao controle, provavelmente devido às atividades normais da AK e da CK.

Concluindo, nossos resultados demonstraram que a creatina foi capaz de evitar perturbações bioenergéticas como as inibições da CK e PK causadas pela tirosina, sugerindo que a creatina possa ter um papel protetor no cérebro. Estes dados são muito encorajadores, uma vez que a suplementação de creatina poderá vir a se constituir numa boa alternativa terapêutica para pacientes com tirosinemia tipo II. Entretanto, mais estudos são necessários para elucidar outros processos e mecanismos envolvidos nas disfunções neurológicas apresentadas por esses pacientes.

III. 3. PERSPECTIVAS

Esse trabalho abre oportunidades para novos estudos, tais como:

1 - Investigar o efeito da administração crônica de L-tirosina metil éster sobre parâmetros enzimáticos do metabolismo energético e de parâmetros de estresse oxidativo no córtex cerebral de ratos Wistar de 14 dias de idade;

2 – Investigar os efeitos da infusão de tirosina na região CA1 do hipocampo dorsal sobre o comportamento dos ratos;

3 - Analisar, *in vitro e in vivo*, os efeitos dos metabólitos que acumulam (4-hidroxifenilpirúvico e 4-hidroxilático) na tirosinemia tipo II sobre parâmetros enzimáticos do metabolismo energético em córtex cerebral de ratos Wistar de 14 dias de idade.

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