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EFEITO DA FENILALANINA SOBRE A ATIVIDADE DA CREATINA QUINASE EM CÓRTEX CEREBRAL, CEREBELO E CÉREBRO MÉDIO DE RATOS

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Dedico esta Dissertação às pessoas
mais importantes da minha vida:
Renor, Juçara e Rovani.

Não imaginas
De quanto somos
Capazes
No ímpeto de nosso
Sangue
E no amor do coração!
Podemos,
Numa hora de coragem...
Audazes... Podemos,
Em lampejos de fé...
Fugazes...
Mudar o rumo do
Destino com as
Próprias mãos!

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À vida, por ter me proporcionado conhecer uma pessoa maravilhosa ... Clovis Milton Duval Wannmacher.

RESUMO

A fenilcetonúria (PKU) é um erro inato do metabolismo de aminoácidos causada pela deficiência da enzima fenilalanina hidroxilase hepática (PAH) que converte fenilalanina (Phe) em tirosina. Caracteriza-se clinicamente por retardo mental severo e, em alguns pacientes, por convulsões e eczema cutâneo. Bioquimicamente os pacientes afetados por esta doença apresentam acúmulo de Phe e seus metabólitos no sangue e nos tecidos. Phe é considerada o principal agente neurotóxico nesta doença, cujos mecanismos de neurotoxicidade são pouco conhecidos. O metabolismo energético cerebral é caracterizado por níveis altos e variáveis de síntese e de utilização de ATP. O cérebro contém altos níveis de creatinaquinase (CK), uma enzima que transferere reversivelmente um grupo fosforil entre ATP e creatina e entre ADP e fosfocreatina (PCr). Considerando que a CK parece estar envolvida em certas condições patológicas relacionadas com deficiência de energia cerebral e sabendo que a PKU está associada à redução de produção e de utilização de energia pelo cérebro, no presente trabalho verificamos a atividade da CK em homogeneizado total de córtex cerebral, cerebelo e cérebro médio de ratos Wistar submetidos aos modelos experimentais agudo e crônico de hiperfenilalaninemia (HPA) quimicamente induzida. Também investigamos o efeito *in vitro* da Phe e da α-metil-DL-fenilalanina (MePhe), um inibidor da PAH, nas mesmas estruturas de ratos Wistar de 22 dias de idade não tratados. Nossos resultados mostraram uma redução significativa na atividade da CK nas estruturas cerebrais estudadas de ratos sujeitos a HPA. Também verificamos que Phe e MePhe inibiram *in vitro* a atividade da CK nas mesmas estruturas. O estudo da interação cinética entre Phe e MePhe, sugere a existência de um único sítio de ligação na CK para os dois compostos. Considerando a importância da CK para a manutenção do metabolismo energético cerebral, nossos resultados sugerem que a

alteração da homeostasia energética pode contribuir para a neurotoxicidade da Phe na PKU.

ABSTRACT

Phenylketonuria (PKU) is a disorder of amino acid metabolism caused by a deficiency of phenylalanine hydroxylase (PAH) which converts phenylalanine (Phe) in tyrosine. PKU is clinically characterized by mental retardation and, in some patients, by convulsions and skin eczema. Biochemically, the affected patients present accumulation of Phe and its metabolites in blood and tissues. Phe is considered the main neurotoxic metabolite in this disorder, but the mechanisms of neurotoxicity are poorly known. The brain energetic metabolism is characterized by high and fluctuating levels of synthesis and utilization of ATP. The brain contain high levels of creatine kinase (CK), the enzyme that transfers a phosphoryl group between ATP and creatine and between ADP and phosphocreatine (PCr). Considering that CK seems to be related with some pathologic conditions associated with brain energy deficit and that PKU is associated with reduction of energy production and utilization by brain, in the present work was investigated CK activity in mibrain, cerebellum and brain cortex of Wistar rats subjected to acute and chronic experimental models of chemically induced hyperphenylalaninemia (HPA). We also investigated the *in vitro* effect of Phe and α-methyl-DL-phenylalanine (MePhe), a PAH inhibitor, in the same structures of non-treated 22 day old Wistar rats. Results showed reduced enzyme activity in the brain structures of HPA-treated rats. We also showed that Phe and MePhe inhibited the *in vitro* CK activity in the same structures. Kinetic studies performed on the interaction between Phe and MePhe on CK activity showed that a competition between Phe and MePhe for the same binding site of the enzyme probably occurs. Considering the importance of CK for the maintenance of energy homeostasis brain, our results suggest that alteration of this homeostasis may contribute to the Phe neurotoxicity in PKU.

SUMÁRIO

Lista de Abreviaturas

ADP	adenosina difosfato
AMPc	adenosina 3’ 5’ monofosfato cíclico
ANT	transportador adenina nucleotídeo
ATP	adenosina trifosfato
BH_4	tetraidrobiopterina
BB-CK	creatinaquinase citosólica
CK	creatinaquinase
CO_2	dióxido de carbono
Cr	creatina
EIM	erros inatos do metabolismo
HPA	hiperfenilalaninemia
H_2O	água
MePhe	α – metil-DL- fenilalanina
Mi_a-CK	creatinaquinase mitocondrial ubíqua
Mi_b-CK	creatinaquinase mitocondrial sarcomérica
MM-CK	creatinaquinase citosólica muscular
NADH	nicotinamida adenina dinucleotídeo (forma reduzida)
O_2	oxigênio molecular
PAH	fenilalanina hidroxilase hepática
PCr	fosfocreatina
Phe	fenilalanina
Pi	fosfato inorgânico
PKU	fenilcetonúria

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1 – INTRODUÇÃO

1.1 – Erros Inatos do Metabolismo

Os Erros Inatos do Metabolismo (EIM) são distúrbios hereditários devidos a mutações genéticas que resultam em anormalidades na síntese de uma proteína, geralmente uma enzima, alterando suas funções. A diminuição da atividade enzimática pode comprometer toda uma rota metabólica, acarretando um bloqueio da mesma e provocando acúmulo de substâncias tóxicas e/ou falta de substâncias essenciais (Del Rio,1962). Além de enzimas, outras proteínas, como transportadores, receptores, hormônios peptídicos, imunoglobulinas, colágeno, fatores de coagulação e fatores de transcrição também podem estar alteradas nos EIM.

Os EIM podem ser classificados de acordo com a área do metabolismo afetada. Assim, um erro inato onde a enzima deficiente seja necessária para a síntese de aminoácidos é classificado como EIM dos aminoácidos (tabela 1) (Scriver et al., 2001).

Foram descritos até o momento mais de 500 EIM (Scriver et al., 2001) a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Benson & Fenson, 1985), causando um grande número de defeitos, com quadros clínicos diversos, que podem ser desde assintomáticos até tão graves que causem morte neonatal. Os EIM podem ser situações graves, que geralmente se manifestam na infância, apresentando-se com sinais e sintomas semelhantes aos encontrados em muitas doenças infantis (Holtzman, 1978).

Estudos revelam que aproximadamente um terço dos EIM corresponde a aminoacidopatias, outro terço a acidemias orgânicas e o outro terço corresponde a todo o restante (Hoffmann,1994).

Embora individualmente sejam considerados raros, os EIM são relativamente freqüentes em seu conjunto, podendo ocorrer 1 em cada 1000 recém-nascidos vivos (Giugliani, 1988).

Tabela 1 – Classificação dos EIM de acordo com a área do metabolismo envolvida

▪ EIM dos aminoácidos
▪ EIM dos ácidos orgânicos
▪ EIM dos glicídios
▪ EIM dos glicosaminoglicanos
▪ EIM das glicoproteínas
▪ EIM das purinas e pirimidinas
▪ EIM das enzimas eritrocitárias
▪ EIM dos metais
▪ EIM das lipoproteínas
▪ EIM dos hormônios
▪ EIM das proteínas plasmáticas

Fonte: Scriver et al., 2001

1.2 – Fenilalanina

Fenilalanina (Phe) é um aminoácido essencial em seres humanos, sendo necessária uma fonte da dieta para manter sua homesostasia e para a síntese de proteínas endógenas. O passo mais importante da homeostasia da fenilalanina é a reação de hidroxilação

catalisada pela enzima fenilalanina hidroxilase hepática (EC 1.14.16.1) (PAH) (Scriver e Kaufman, 2001). Também chamada de fenilalanina-4-monooxigenase, a PAH pertence a uma classe de enzimas conhecidas por oxidases de função mista. PAH catalisa a hidroxilação simultânea do substrato por um átomo de oxigênio para O_2 e reduz o outro átomo de oxigênio para H_2O. PAH requer tetraidrobiopterina (BH_4) como cofator, o qual transporta elétrons do NADH para O_2 e torna-se oxidada para diidrobiopterina no processo. Este é subseqüentemente reduzido pela enzima diidrobiopterina redutase na reação que requer NADH (Figura 1.1) (Lehninger et al.,2000).

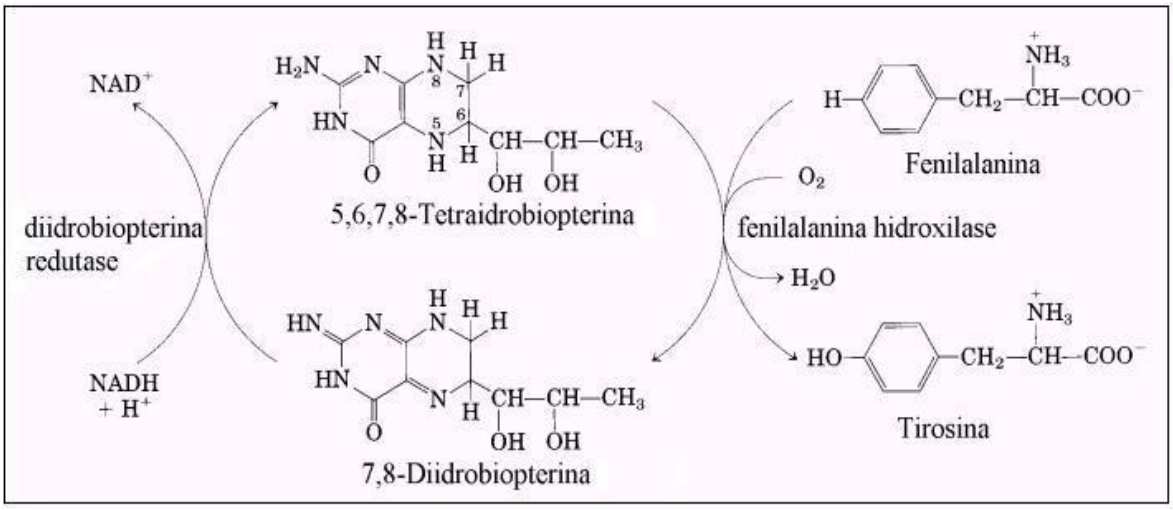

Figura 1.1 – Reação de oxidação da fenilalanina hidroxilase

1.3 – Hiperfenilalaninemia e Fenilcetonúria

Mutações na PAH que diminuem a atividade da enzima causam hiperfenilalaninemia (HPA). Quando HPA é severa (fenilalanina sanguínea > 1.2 mM, 20 vezes acima do nível normal (0.055 – 0.060 mM), ocorre a manifestação da doença conhecida por fenilcetonúria (PKU) (Scriver e Kaufman, 2001). PKU é um erro inato do metabolismo de aminoácidos transmitida por genes autossômicos recessivos com uma

incidência aproximada de 1 para 10.000 nascidos vivos. PKU certamente manifesta HPA, mas nem toda HPA é necessariamente PKU. A enzima PAH requer BH4 com cofator para conversão de fenilalanina a tirosina. Mutações no gene que controla uma das várias fases de síntese ou reciclo do BH_4 podem causar HPA.

Quando a PAH é geneticamente defeituosa, uma via secundária do metabolismo da Phe, normalmente pouco empregada, passa a ter grande atuação. Nesta, a reação inicial mais significante é a transaminação da Phe para formar fenilpiruvato, tornando-se uma via funcionalmente significante já que esta reação é induzida pelo substrato, porém, não é completamente operativa em recém-nascidos prematuros ou na primeira fase de HPA. Ambos, fenilalanina e fenilpiruvato, são acumulados no sangue e tecidos e são excretados na urina, razão pela qual esta condição patológica é chamada de fenilcetonúria (Lehninger et al., 2000; Scriver e Kaufman, 2001). A maior parte do fenilpiruvato é descarboxilada para produzir fenilacetato ou reduzida para formar fenilactato. O fenilactato tem odor tão forte e característico que foi usado para detectar PKU em crianças (Figura 1.2).

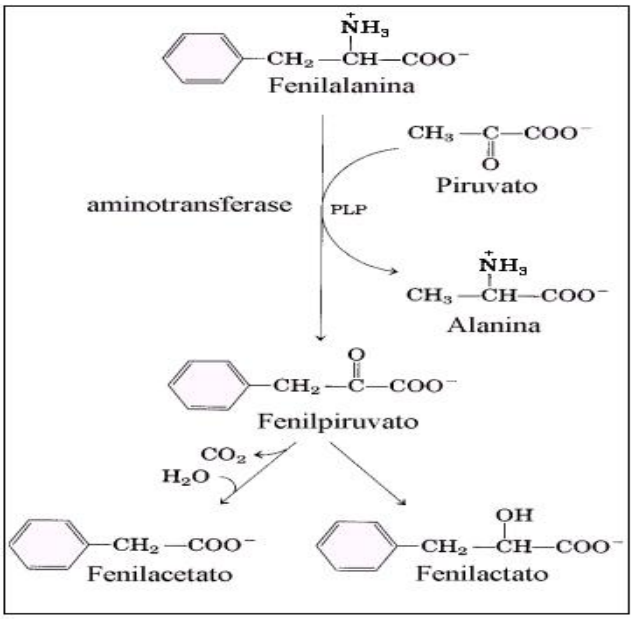

Figura 1.2 - Rota secundária da fenilalanina

A enzima PAH está ativa em hepatócitos humanos (e nos rins), mas a Phe é incorporada nas proteínas de todos os tecidos, e a conversão da Phe em vários metabólitos também ocorre em outros tecidos além do fígado. Deste modo, os fluxos entre os orgãos são parte integral da rota para este aminoácido, e o transporte da Phe através da membrana plasmática deve ser anterior a sua entrada nas vias intracelulares. A captação da Phe em células de mamíferos é mediada por transportadores que estão acoplados a um gradiente de Na^+ orientado para o interior dos ápices das membranas dos rins e epitélio intestinal, e por transportadores independentes de Na^+ em outras membranas plasmáticas. A Phe proveniente do plasma e fluido extracelular entra nas células parenquimatosas através de um transportador independente de Na^+ , o qual transporta os aminoácidos aromáticos e de cadeia ramificada e sai das células através de um sistema compartilhado pelos aminoácidos de carga neutra. Interações entre os aminoácidos nos transportadores podem perturbar estes fluxos e podem ter um papel na patogênese do fenótipo cerebral em PKU, pois o excesso de Phe pode competir com outros aminoácidos pelo transporte através da barreira hematoencefálica, resultando na depleção de alguns metabólitos necessários.

A reação de hidroxilação da Phe é uma etapa obrigatória e limitante na via catabólica que leva à completa oxidação da Phe em CO_2 e H_2O. Os produtos cetogênicos (por exemplo: acetoacetato) e gliconeogênico (por exemplo: fumarato) do catabolismo da Phe contribuem para o conjunto (“pool”) de metabólitos de 2 carbonos e glicose no organismo. Nesta visão da dependência parcial do cérebro ao suprimento periférico de glicose, a habilidade da Phe em prover substratos gliconeogênicos, neste contexto, tem outro papel no metabolismo dos mamíferos, provendo o organismo com um suprimento endógeno do aminoácido não essencial tirosina. Quando a hidroxilação é deficiente, a tirosina torna-se um aminoácido essencial. O cérebro não contém PAH, mas contém outra enzima, a tirosina hidroxilase, que catalisa a conversão da Phe em tirosina. Talvez a

tirosina hidroxilase agindo sobre a Phe, forneça uma fração significativa da tirosina necessária para a síntese protéica; se ela pode fazer o mesmo em PKU, ainda é motivo de dúvida.

A própria Phe provavelmente seja o agente neurotóxico na PKU. Os metabólitos da Phe não são encontrados na doença humana (ou de ratos) em concentrações suficientemente altas para serem relacionados com o distúrbio metabólico e químico no cérebro, pois suas concentrações, por exemplo no fluido cerebroespinhal, não têm relação com as usadas para demonstrar os efeitos tóxicos *in vitro* em experimentos animais. Estudos recentes em ratos mutantes para PKU negam a relevância de metabólitos da própria Phe (Scriver e Kaufman, 2001). Quaisquer que sejam seus mecanismos, as conseqüências neurotóxicas da Phe na PKU podem ser agudas e reversíveis ou crônicas e irreversíveis.

Em pacientes PKU clássicos não-tratados, são encontrados níveis sanguíneos de Phe que atingem até 2,4 mM. O valor de Phe livre no plasma de pessoas normais, sob condições fisiológicas, é de 58 ± 15 μM. Os valores correspondentes em crianças (com média de idade de 8 anos) e adolescentes (com média de idade de 16 anos) são 62 ± 18 μM e 60 ± 13 μM, respectivamente. Valores em recém-nascidos e crianças mais velhas são semelhantes aos dos adultos. O desenvolvimento cognitivo e as funções neurofisiológicas deficientes em PKU são complexos, mas há um consenso emergente de que a própria Phe, em concentrações elevadas, é uma molécula nociva (Scriver e Kaufman, 2001).

As principais características clínicas desta doença são retardo mental, irritabilidade, movimentos despropositados, reflexos diminuídos dos tendões, convulsões, pigmentação diminuída da pele, eczema, escleroderma, formação deficiente de mielina, microcefalia e vômitos. A deficiência de aprendizagem em pacientes PKU poderia ser devido, ao menos em parte, à produção reduzida de neurotransmissores por um transporte deficiente de

tirosina através da barreira hematoencefálica, uma vez que os níveis de tirosina plasmática são baixos em PKU (Hanley et al., 2000). No entanto, os níveis liquóricos de tirosina são altos, pois a Phe é transformada em tirosina no cérebro pela tirosina hidroxilase (Scriver e Kaufman, 2001). Com um diagnóstico precoce da PKU, o retardo mental pode ser prevenido em grande extensão através de um controle dietético rígido. A dieta precisa proporcionar as quantidades de Phe suficientes e necessárias para suprir as necessidades de tirosina e de síntese protéica do organismo. O consumo de alimentos altamente proteicos precisa ser controlado.

Trabalhos anteriormente realizados em ratos no Laboratório de Erros Inatos do Metabolismo evidenciaram que a Phe inibe a captação de glicose (Rodrigues et al., 1990), a produção de energia (Lütz et al, 2003) a atividade da Na^+, K^+-ATPase (Wyse et al., 1995) e da piruvatoquinase (Feksa et al, 2002), e a fosforilação de proteínas do citoesqueleto (De Freitas et al., 1995). Além disso, também foi reportada uma redução significativa do metabolismo da glicose no cérebro de pacientes PKU, principalmente na substância branca (Hasselbach et al., 1996). Juntos, estes dados sugerem que este aminoácido compromete a produção energética cerebral e sua utilização. Deficiências na energia cerebral poderiam explicar as anormalidades na mielinização, na densidade e organização celular, arborização dendrítica e número de espinhas sinápticas encontradas no cérebro de pacientes PKU não tratados (Bauman e Kemper, 1982).

1.4- Creatinaquinase

A enzima creatinaquinase (CK; ATP: creatine N-phosphoryl-transferase, EC 2.7.3.2) catalisa a transferência reversível do grupo N-fosforil da fosfocreatina (PCr) para ADP regenerando ATP (figura 1.3). Possui papel essencial na homeostasia energética de

células com necessidades energéticas variáveis e intermitentes, como por exemplo, músculo esquelético e cardíaco, tecidos neurais como cérebro e retina, fotoreceptores e eletrócitos (Wallimann et al., 1998 a,b).

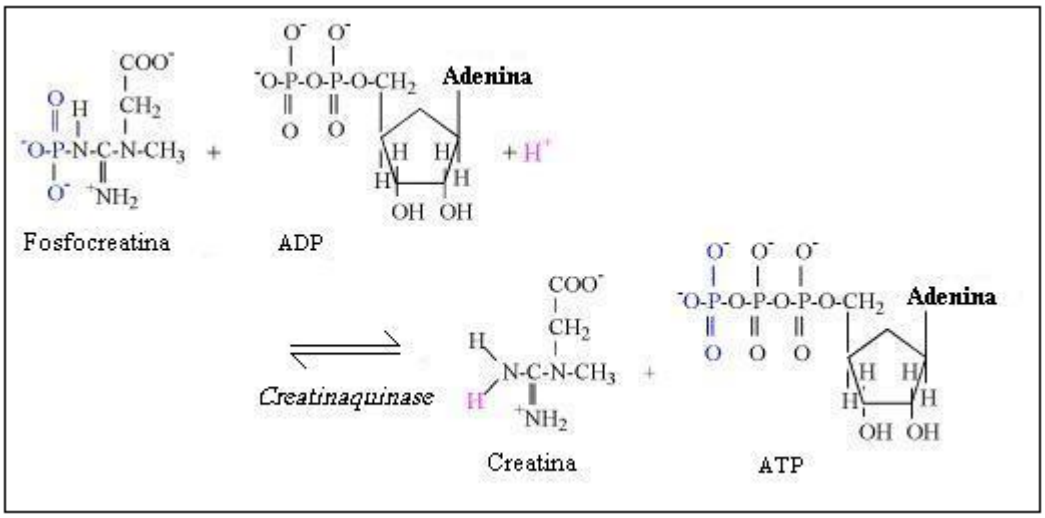

Figura 1.3 – Reação de transfosforilação catalizada pela enzima creatinaquinase (Wallimann et al, 1992).

As células contém um número de isoformas diferentes de creatinaquinase : 2 isoformas, M-CK (músculo) e B-CK (cérebro), são citosólicas formando moléculas dímeras e duas outras isoformas são mitocondrias : Mi_a-CK (ubíqua) e Mi_b-CK (sarcomérica), as quais são expressas predominantemente no cérebro e no músculo estriado respectivamente, formando moléculas dímeras e octaméricas.

As isoenzimas da CK citosólica (MM-, MB- e BB-CK) são sempre co-expressadas em tecido específico junto com a isoforma mitocondrial. Usando fracionamento bioquímico e localização in situ, foi demonstrado que as isoenzimas da CK são estritamente solúveis e de fato compartimentalizadas subcelularmente e unidas funcionalmente 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), formando um sistema de distribuição de energia altamente regulado e intricado - o circuito da PCr (Wallimann et al., 1992).

Mi-CK, localizada na parte externa da membrana mitocondrial interna, assim como em sítios de contato da membrana interna e externa, serve para facilitar, por união funcional com carreador ATP/ADP (translocador adenina nucleotídeo – ANT): a exportação eletrogênica da matrix mitocondrial geradora de ATP em troca por ADP através da membrana mitocondrial interna e a transfosforilação do ATP em PCr e assim conservar a energia livre da hidrólise do ATP apenas exportado por ANT. Em adição, por ser unida funcionalmente ao poro da membrana mitocondrial externa, Mi-CK está provavelmente envolvida na regulação da importação da creatina (Cr) para mitocôndria e exportação da PCr para o citosol. Portanto, através da união cinética das três enzimas ANT, Mi-CK e porina (proteína poro-mitocondrial), é formado um importante microcompartimento para o transporte de energia mitocondrial e metabolismo energético celular (figura 1.4) (Wallimann et al., 1992).

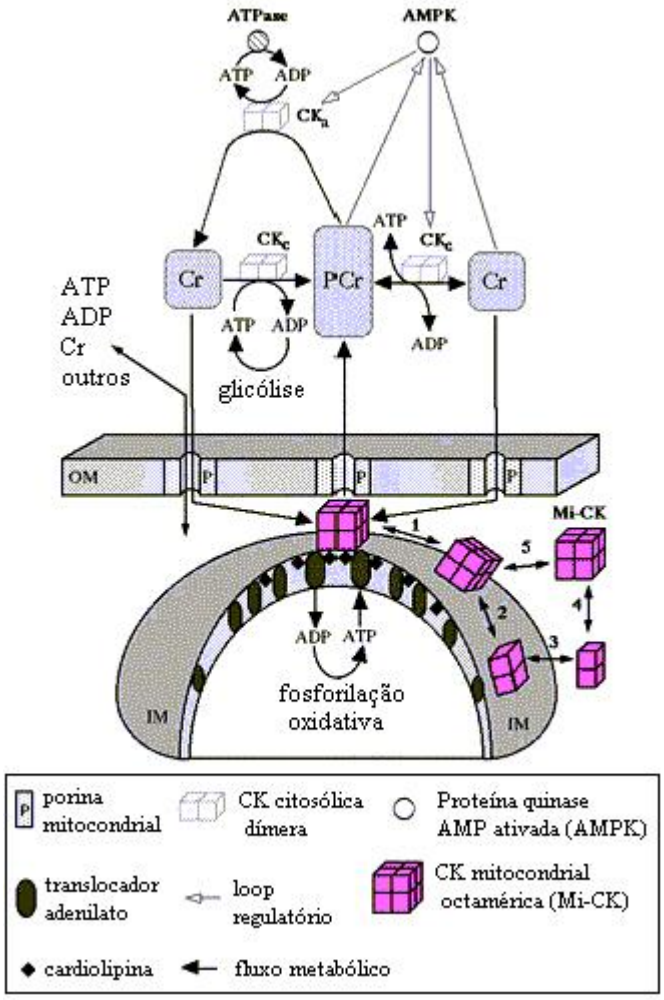

Figura 1.4 – Circuito da fosfocreatina (Wallimann et al., 1994)

Um número de diferentes funções tem sido sugerido para a comunicação entre as isoformas de creatinaquinase citosólica e mitocondrial por fosfocreatina e creatina (Wallimann, 1994), levando à proposta do modelo de circuito da fosfocreatina. (Rojo et al., 1991; Wallimann et al., 1992; Wyss et al., 1992).

Primeiro, o sistema CK/PCr serve como tampão de energia flutuante, mantendo [ATP] e [ADP] estáveis e tamponando [H^+]. Essa função tamponante ao longo do tempo impede a rápida queda de [ATP] durante o trabalho celular e ao mesmo tempo evita uma acidificação intracelular esperada na hidrólise do ATP durante o trabalho. Esse tamponamento de próton do sistema CK/PCr parece especialmente importante na fase

inicial do exercício intenso, antes da glicogenólise ser ativada. Em adição, a liberação de fosfato inorgânico (Pi) é outra função metabólica do sistema CK/PCr (Meyer et al., 1984).

Segundo, o circuito PCr serve para melhorar a eficiência termodinâmica da hidrólise do ATP para manter [ADP] baixa e para manter a razão ATP/ADP alta nos sítios subcelulares onde CK é funcionalmente associada aos processos que requeiram ATP, como por exemplo, bombas de íons (Wallimann et al., 1994).

Terceiro, o circuito PCr também serve como tampão de energia transportada entre compartimentos. Nesse papel, PCr tem a função de transportador de energia conectando sítios de produção de energia, como fosforilação oxidativa mitocondrial, com sítios de utilização de energia, no qual 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 músculo, eletrócitos, células fotoreceptoras e espermatozóides ; por compartimentalização subcelular da PCr/Cr, ATP/ADP e Pi e pela localização, estrutura e propriedades funcionais da Mi-CK octamérica (Saks et al., 1984; Gellerich et al., 1987; Savabi et al., 1988; Saks et al.,1991; Zeleznikar et al., 1991; Wallimann et al., 1992).

A respeito dos resultados da localização subcelular e proporções relativas das isoenzimas da CK, assim como o nível da PCr em diferentes tecidos de demanda energética alta e variada, levam a sugerir 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 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. Dependendo das necessidades

metabólicas da célula ou tecido, uma dessas diferentes funções do circuito CK/PCr pode estar dominante. Por exemplo, em fibras musculares glicolíticas de contração rápida, a função de tamponamento pode ser mais proeminente do que o função de transporte, já na contração lenta, ou no músculo cardíaco, assim como em espermatozóides ou células fotoreceptoras, a função de transporte pode ter maior relevância do que a função de tamponamento. O circuito CK/PCr representa uma rede de distribuição de energia conectando sítios intracelulares de produção de ATP (mitocôndria e glicólise) com sítios de consumo de ATP (ATPases) (Wallimann et al., 1992).

No tecido nervoso, a CK encontra-se em duas isoformas, uma mitocondrial (Mi_a-CK) e outra citosólica (B-CK), acoplada funcionalmente à Na^+, K^+ – ATPase (Blum et al., 1991). A atividade da B-CK é maior na substância branca do que na substância cinzenta do cérebro de porcos (Holtzman et al., 1996) e maior na substância branca cerebelar do que no córtex cerebelar de ratos (Kato et al.,1986). As atividades da Mi_a-CK e da B-CK são equivalentes na substância cinza, enquanto a atividade da B-CK predomina na substância branca (Hemmer e Wallimann, 1993). O cérebro tem capacidade de sintetizar parte da creatina de que necessita (Defalco e Davies, 1961), sendo o restante captado através de um sistema de co-transporte Na^+/ creatina (Guimbal e Kilimann, 1993).

A maturação funcional da CK durante o desenvolvimento do cérebro do rato com expressão seqüencial das isoenzimas (Holtzman et al., 1993), a regulação da expressão da B-CK via AMPc nas células gliais (Kuzhikandathil e Molly, 1994), o papel da B-CK na regeneração do ATP necessário para o transporte de íons e neurotransmissores, e a atividade significativa da B-CK nos neurônios, astrócitos e oligodentrócitos (Manos et al., 1991), são compatíveis com um papel importante exercido pelo sistema CK, principalmente pela B-CK, no processo de mielinização e nas diversas atividades do sistema nervoso.

A CK parece estar envolvida em certas condições patológicas relacionadas com deficiência de energia cerebral. Em condições anóxicas, a adição de creatina ao meio de incubação contendo fatias de cérebro protege a transmissão sináptica e mantém o potencial de ação via Na^+, K^+- ATPase (Whittingham e Lipton, 1981); a adição de creatina aumenta os níveis de PCr reduzindo a queda de ATP, a liberação de Ca^{+2} e a morte celular (Carter et al., 1995). A deficiência congênita de creatina cerebral está associada a disfunção extrapiramidal, convulsões e fraqueza muscular (Stöckler et al., 1994).

Devido a ser a energia necessária para manutenção do desenvolvimento e regulação das funções cerebrais, tem sido postulado que o prejuízo na função da CK pode ser um importante passo no processo neurodegenerativo que leva à perda neuronal no cérebro (Tomomoto et al., 1993). De fato, a atividade da CK está severamente reduzida em várias doenças neurodegenerativas (David et al., 1998; Aksenov et al., 2000).

1.5 - Objetivos:

Considerando as alterações neurológicas encontradas na fenilcetonúria, as elevadas concentrações de fenilalanina encontradas no sangue e tecidos de fenilcetonúricos e a importância da atividade da creatinaquinase para o funcionamento normal do sistema nervoso central, este trabalho teve como ojetivos:

1.5.1 – Verificar os efeitos *in vitro* e *in vivo* da Phe e da MePhe sobre a atividade da CK em córtex cerebral de ratos jovens.

1.5.2 – Verificar os efeitos *in vitro* e *in vivo* da Phe e da MePhe sobre a atividade da CK em cerebelo e cérebro médio (núcleos da base, diencéfalo e mesencefalo) de ratos jovens.

2 - RESULTADOS

OBJETIVO 1

Verificar os efeitos *in vitro* e *in vivo* da Phe e da MePhe sobre a atividade da CK em córtex cerebral de ratos jovens.

RESULTADO 1- Artigo 1

Elisa Costabeber, Adriana Kessler, Carlos Severo Dutra-Filho, Angela Terezinha de Souza Wyse, Moacir Wajner, Clóvis Milton Duval Wannmacher (2003). Hyperphenylalaninemia reduces creatine kinase activity in the cerebral cortex of rats. *International Journal of Developmental Neuroscience* 21 : 111-116.

OBJETIVO 2

Verificar os efeitos *in vitro* e *in vivo* da Phe e da MePhe sobre a atividade da CK em cerebelo e cérebro médio de ratos jovens.

RESULTADO 2- Artigo 2

Elisa Costabeber, Adriana Kessler, Carlos Severo Dutra-Filho, Angela Terezinha de Souza Wyse, Moacir Wajner, Clóvis Milton Duval Wannmacher. Reduced creatine kinase activity in midbrain and cerebellum of hyperphenylalaninemic rats. Recebido em 31/01/03 pela *Neurochemical Research*.

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Hyperphenylalaninemia reduces creatine kinase activity in the cerebral cortex of rats

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Abstract

Phenylketonuria (PKU) is a metabolic disorder accumulating phenylalanine (Phe) and its metabolites in plasma and tissues of the patients. Considering that phenylalanine is the main neurotoxic metabolite, and brain energy homeostasis seems to be affected in phenylketonuria, our main objective was to investigate the effect of acute and chronic hyperphenylalaninemia (HPA) on creatine kinase (CK) activity in brain cortex of Wistar rats. Hyperphenylalaninemia was induced by subcutaneous administration of 5.2 μmol phenylalanine + 2.4 μmol α-methylphenylalanine (phenylalanine hydroxylase (PAH) inhibitor)/g of body weight. We also investigated the in vitro effect of phenylalanine and/or α-methylphenylalanine on creatine kinase activity in the brain cortex of non-treated rats. The results showed that phenylalanine significantly inhibited creatine kinase activity in vitro and reduced the enzyme activity in vivo. Considering the importance of creatine kinase for the maintenance of energy homeostasis in brain, if this enzyme inhibition also occurs in phenylketonuric patients, it is possible that creatine kinase inhibition may be one of the mechanisms by which phenylalanine is neurotoxic in phenylketonuria.
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Keywords: Phenylalanine; Phenylketonuria; Hyperphenylalaninemia; Creatine kinase

1. Introduction

Phenylketonuria (PKU), one of the most frequent inborn errors of metabolism, is an autosomal recessive disease caused by a deficiency of phenylalanine hydroxylase (PAH), the enzyme catalyzing the first step of phenylalanine (Phe) catabolism in the liver. As a result, phenylalanine (Phe) and its metabolites accumulate in blood and other tissues. Untreated PKU patients may present blood levels as high as 2.5 mM. Severe mental and psychomotor delay are the clinical hallmarks in this disease. Phe itself is probably the main neurotoxic agent in PKU, although the mechanisms of neurotoxicity are far from be understood. (Scriver and Kaufman, 2001).

We previously reported that Phe inhibits the in vitro glucose uptake (Rodrigues et al., 1990), as well as Na^+, K^+-ATPase activity in vitro and in vivo (Wyse et al., 1995) and also decreases phosphorylation of cytoskeletal proteins (De Freitas et al., 1995) in brain cortex of rats. In this context, a reduction of glucose metabolism in brain of PKU patients has also been reported, mainly in white matter (Hasselbach et al., 1996). Taken together, these data suggest that Phe may compromise brain energy production and/or utilization. This alteration of brain energy metabolism could explain, at least in part, the abnormalities in myelination, cell density and organization, dendritic arborization, and number of synaptic spines found in the brain of untreated PKU patients (Bauman and Kemper, 1982).

Creatine kinase (CK) plays a key role in the energy metabolism of tissues that have intermittently high and fluctuating energy requirements, such as skeletal and cardiac muscle, and nervous tissue (Wallimann and Hemmer, 1994). CK catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP, to regenerate ATP. Cells contain a number of different creatine kinase isoforms. Two isoforms, sarcomeric M-CK and ubiquitous B-CK, are cytosolic, and two other isoforms, sarcomeric Mib-CK and ubiquitous Mia-CK are mitochondrial (Wallimann et al., 1992). CK isoenzymes are in part compartmentalized specifically at those places where energy is produced or utilized (Wallimann et al., 1998a,b). Because energy is necessary to maintain the development and regulation of cerebral functions, it has been postulated that damage of CK function may be an important step of a neurodegenerative pathway that leads to neuronal loss in the brain (Tomimoto et al.,

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1993). In fact, CK activity is severely reduced in several neurodegenerative diseases (David et al., 1998; Aksenov et al., 2000).

The main objective of the present study was to investigate the acute and chronic effect of chemically induced hyperphenylalaninemia (HPA) on CK activity in cerebral cortex of rats. We also investigated the in vitro effects of Phe and α-methyl-DL-phenylalanine (MePhe), a PAH inhibitor used together with Phe to induce HPA, on CK activity of cerebral cortex of non-treated rats.

2. Experimental procedures

2.1. Subjects and reagents

Wistar rats bred in the Department of Biochemistry, UFRGS, were used in the experiments. Rats were kept with dams while receiving the drugs until they were sacrificed. The dams had free access to water and to a standard commercial chow (Germani, Porto Alegre, RS, Brazil) containing 20.5% protein (predominantly soybean), 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*" (NIH publication 85-23, revised 1985) 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.

2.2. Hyperphenylalaninemia induction

HPA was induced by chronic or acute treatments, as follows.

2.2.1. Chronic treatment

Hyperphenylalaninemia was induced by daily subcutaneous administration of Phe + MePhe, from the 6th to the 21st day of life. Fourteen animals were randomly assigned to two groups as previously reported (Wyse et al., 1994): (1) HPA group: rats received subcutaneous administration of 5.2 μmol Phe/g body weight twice a day at 8 h intervals, and 2.4 μmol MePhe/g body weight once a day. These doses were calculated from pharmacokinetic parameters to achieve plasma levels between 1 and 4 mM; (2) control group: rats received the same volume of 0.85% saline twice a day. All drugs were dissolved in 0.85% NaCl (saline) and the pH was adjusted to 7.4 by NaOH addition. Twelve hours after the last injection the rats were killed by decapitation without anesthesia and the cerebral cortex was separated for creatine kinase activity assay and protein determination.

2.2.2. Acute treatment

Twenty 2-day-old rats were randomly separated into four groups of six animals each and treated with one single subcutaneous injection as follows: (1) HPA group: rats received subcutaneous administration of Phe + MePhe; (2) Phe group: rats received Phe administration; (3) MePhe group: rats received MePhe administration; (4) saline group: rats received saline. Doses were calculated according to body weight and prepared as stated above. One hour after the subcutaneous administration the animals were killed by decapitation without anesthesia and the brain cortex was separated for creatine kinase activity assay and protein determination.

Treated animals achieved maximal plasma Phe levels 30 min after subcutaneous injection of Phe (3.6 ± 0.2 mmol/l). Maximal brain levels were achieved 60 min after Phe administration (1.1 ± 0.1 mmol/kg wet weight tissue). Twelve hours after treatment, plasma and brain Phe concentrations returned to normal levels (0.1 ± 0.01 mmol/l and 0.15 ± 0.01 mmol/kg wet weight tissue, respectively).

2.2.3. In vitro experiments

For the in vitro experiments, 36 non-treated rats aging 22 days were used. Phe and MePhe were dissolved in SET buffer pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base) and added to the assay at 0.5–5.0 mM final concentrations. The interaction between Phe and MePhe was characterized by an adaptation of the competitive plot of Chevillard et al. (1993) according to Wyse et al. (1998). The competitive plot is a method for determining whether or not two enzyme-catalyzed reactions with two different substrates occur at the same active site. This method was adapted for determining whether or not two different inhibitors act at the same site on the enzyme. It is a plot of total rate against p, where p varies from 0 to 1, and specifies the concentrations $(1-p)a_0$ and pb_0 of the two amino acids in terms of reference concentrations a_0 and b_0 chosen so as to give the same rates at $p = 0$ and $p = 1$. These concentrations were 1 mM for Phe and 1 mM for MePhe. If the two inhibitors act at the same site, the competitive plot gives a horizontal straight line, i.e. the total rate is independent of p. Independent reactions at two separate sites give a curve with a minimum; separate reactions with cross-inhibition generate curves with either maxima or minima according to whether the inhibition constants of the two inhibitors at one site are smaller or higher than the inhibition constants at the other site.

2.2.4. Preparation of brain tissue homogenate

The cerebral cortex was isolated and homogenized with a Teflon-glass homogenizer (1:20, w/v) in SET buffer pH 7.4. The homogenate was stored at −70 °C when the assay was not carried out immediately.

2.2.5. Creatine kinase activity assay

The reaction mixture contained the following final concentrations: 60 mM Tris–HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, and approximately 1 μg protein in a final volume of 0.1 ml. After 15 min of pre-incubation at 37 °C, the reaction was started by the addition of

2 nmol ADP + 8 nmol reduced glutathione. The reaction was stopped after 10 min by the addition of 1 μmol *p*-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were choose 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 2% α-napthol and 0.1 ml 0.05% diacetyl in a final volume of 1 ml and read after 20 min at 540 nm. Phe or MePhe did not interfere with the color development or spectrometric readings. Results were expressed as micromoles of creatine formed per minute per milligram of protein.

2.2.6. Protein determination

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

2.3. Statistical analysis

Data from the chronic treatment were analyzed by the Student's *t*-test. Data from the acute treatment and from the in vitro experiments were analyzed by one-way ANOVA followed by the Tukey test when the F values were significant. Data were analyzed through the Statistical Package for Social Sciences software inserted in an IBM-compatible PC computer.

3. Results

First, we subjected the animals to chronic and acute treatments. Chronic treatment had no effect on body weight (control = 32.6 ± 6.6 g; HPA = 32.3 ± 5.7 g), brain weight (control = 1.2 ± 0.1 g; HPA = 1.1 ± 0.1 g) and brain cortex weight (control = 0.58 ± 0.1 g; HPA = 0.57 ± 0.1 g), indicating that treatment did not cause undernutrition in the animals. The acute treatment did not affect the same parameters.

CK activity was reduced by approximately 11% in cerebral cortex homogenates from rats subjected to chronic treatment [$t(12) = 2.95$; $P < 0.05$] (Fig. 1). Acute administration of Phe, MePhe, or Phe + MePhe also reduced CK activity by around 15, 16, and 19%, respectively [$F(3, 20) = 15.2$; $P < 0.001$]. No differences were found among the three treatments (Fig. 2).

Next, we investigated the in vitro effects of various concentrations of Phe and MePhe on CK activity of cerebral cortex homogenates from non-treated rats. CK activity was significantly inhibited by approximately 25% [$F(4, 30) = 16.61$; $P < 0.001$] and 20% [$F(4, 30) = 11.12$; $P < 0.001$] by Phe and MePhe, respectively (Fig. 3).

In an attempt to better investigate whether MePhe could affect the inhibition caused by Phe on CK activity in the animals subjected to chronic HPA, we compared the inhibition caused by the association of Phe + MePhe with the isolated effects of each one of the two substances at the same concentration (2.5 mM). The significant inhibition of CK activity caused by Phe, MePhe, or Phe + MePhe [$F(3, 24) = 16.22$; $P < 0.001$] was of the same magnitude

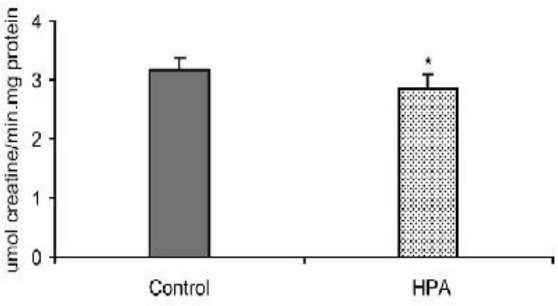

Fig. 1. Effect of chronically induced hyperphenylalaninemia on creatine kinase activity in the cerebral cortex homogenates from young rats. HPA: hyperphenylalaninemia. Data are mean±S.D. for seven animals per group. Different from control, $^{*}P < 0.05$ (Student's *t*-test).

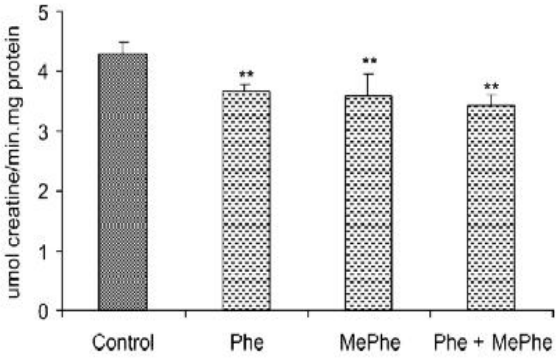

Fig. 2. Effect of acute administration of phenylalanine and α-methylphenylalanine on creatine kinase activity in the cerebral cortex homogenates from young rats. Phe: phenylalanine; MePhe: α-methylphenylalanine. Data are mean±S.D. for six animals per group. $^{**}P < 0.01$ compared to control (Tukey test).

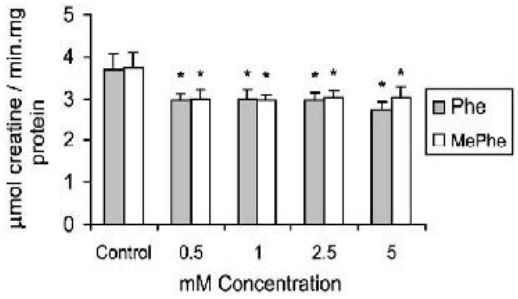

Fig. 3. In vitro effect of phenylalanine and α-methylphenylalanine on creatine kinase activity in the cerebral cortex homogenates from young rats. Phe: phenylalanine; MePhe: α-methylphenylalanine. Data are expressed as mean ± S.D. for six animals per group. $^{*}P < 0.01$ compared to control (Tukey test).

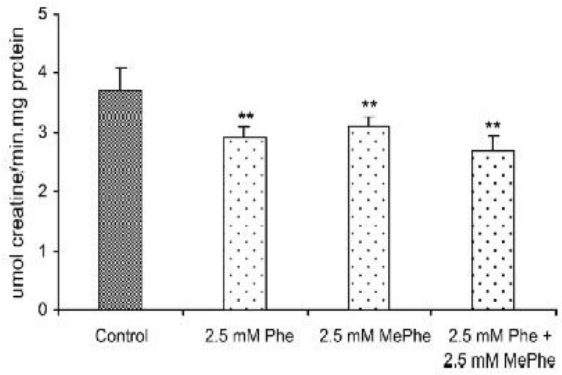

Fig. 4. In vitro effect of phenylalanine and α-methylphenylalanine on creatine kinase in the cerebral cortex homogenates from young rats. Phe: phenylalanine; MePhe: α-methylphenylalanine. Data are expressed as mean ± S.D. for six animals per group. $^{**}P < 0.01$ compared to control (Tukey test).

(Fig. 4). However, considering that at 2.5 mM concentrations of Phe and MePhe the binding of the two substances seems to be saturated, we measured CK activity in the presence of various proportions between Phe and MePhe at concentrations ranging from 0.2 to 1 mM for each substance, similar to Phe concentrations found in the brain of non-treated PKU patients. At this range, the inhibition caused by Phe [$F(1, 19) = 14.61$; $\beta = -0.66$; $P < 0.001$] and by MePhe [$F(1, 19) = 23.07$; $\beta = -0.74$; $P < 0.001$] are dose-dependent. The straight line in the interaction plot indicated a competition between Phe and MePhe for the same site of the enzyme (Fig. 5). Therefore, considering that both substances present approximately the same effect, it is feasible to presume that Phe itself is able to affect CK activity in vivo.

4. Discussion

In the present investigation we demonstrated that Phe inhibits the in vitro CK activity in cerebral cortex homogenates from non-treated rats and also reduces this enzyme activity in the brain cortex homogenates from rats subjected to acute or chronic HPA. Since Phe has a direct inhibitory action on CK activity, this effect observed in vitro, probably also occurs in vivo. However, when CK activity was measured in the cerebral cortex homogenates from Phe-treated rats, this enzyme activity was reduced. In this case, we cannot explain the reduced CK activity by an inhibition caused by significant amounts of Phe in the enzyme assay, because the brain tissue was diluted 10,000 times in the incubation medium. Therefore, the diminution of CK activity might be caused by down-regulation of expression or post-translational modification of existing enzyme molecules. This latter mechanism was proposed by other investigators who found that the reduced level of CK, observed in several neurodegenerative disorders, was the result of post-translational modifications of the enzyme (Aksenov et al., 1997; Aksenova et al., 1999).

However, considering that MePhe crosses the blood–brain barrier (Dougan et al., 1983), we cannot rule out that reduced CK activity could be caused by MePhe, the PAH inhibitor co-administered with Phe. It is difficult to test this hypothesis in chronically induced HPA because long-term administration of MePhe causes accumulation of Phe in blood and tissues. Therefore, we investigated the effects of a single administration of Phe and/or MePhe on CK activity. Our results indicated that Phe and MePhe reduced CK activity and the magnitude of the reduction was similar to that caused by the administration of Phe + MePhe. In addition, we also observed that Phe, MePhe, and Phe + MePhe inhibited the in vitro CK activity, and the magnitude of the inhibition was similar for the three groups, suggesting that the two

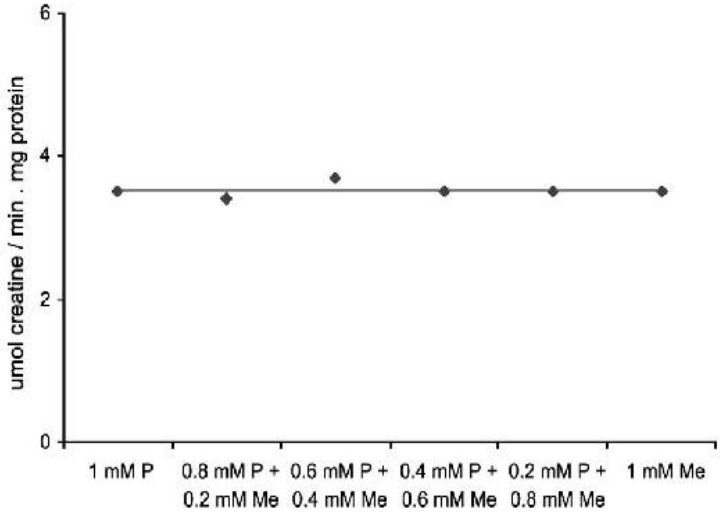

Fig. 5. Interaction plot between phenylalanine and α-methylphenylalanine on creatine kinase activity in the cerebral cortex homogenates from young rats. Phe: phenylalanine; MePhe: α-methylphenylalanine. Data are representative of three independent experiments performed in triplicate.

substances act on the enzyme through the same mechanism. Reinforcing these results, the competition plot indicated a competition between Phe and MePhe for the same site at the enzyme. Taken together, the results of the in vivo and the in vitro experiments suggest that highly sustained levels of Phe may diminish CK activity through enzyme inhibition and reduction of the number of active enzyme molecules.

PKU patients approximately show a 10-fold increase of brain Phe levels as compared with healthy controls (Möller et al., 1997). Phe is probably the main responsible by the brain damage in this disease, but the pathophysiological mechanisms of brain injury are multiple and not fully understood. The reduction of oxygen consumption in the brain may be an important factor contributing to the neurological defect in PKU, since it has been reported that oxygen consumption is lower in the PKU brain than in the normal one (Himwich, 1951). Therefore, it is feasible to presume that early increased brain Phe levels in PKU could reduce brain respiration at a critical time during brain development. In this context, our results showing an in vivo reduction of CK activity, an important enzyme for energy homeostasis, in the brain of rats subjected to chemically induced HPA suggest that this inhibition may be one of the mechanisms responsible for the alteration of energy metabolism in the brain of PKU patients, impairing cell function and inducing brain damage through distinct mechanisms, including secondary excitotoxicity (Schinder et al., 1996; Beal, 2000).

The CK/phosphocreatine system exerts several integrated functions in brain cells, such as temporary energy buffering, metabolic capacity, energy transfer, and metabolic control (Saks et al., 1996). This system is now recognized as an important metabolic regulator during health and disease (Wallimann et al., 1998a,b). It has been postulated that CK and the creatine–phosphocreatine energy shuttle may play a role in brain development that is associated with oligodendrocyte function and/or myelogenesis (Manos et al., 1991).

A decrease of 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 step of a neurodegenerative pathway that leads to brain damage (Wallimann et al., 1998a,b). These findings are reinforced by the observation that creatine and phosphocreatine have neuroprotective effects against energy deprivation and glutamate excitotoxicity, attributable to an enhancement of cytosolic high-energy phosphate stores (Brustovetsky et al., 2001). Taken together these observations and the reduction of brain CK activity found in the present study, it is possible that Phe at high concentrations may induce excitotoxicity.

In summary, our results indicate that Phe alters in vitro and in vivo CK activity in the brain cortex of rats. Considering that creatine kinase is a key enzyme for energy homeostasis in brain, if this effect also occurs in the brain of PKU patients, it is possible that the alteration of this enzyme activity may impair brain energy metabolism, contributing to the brain damage characteristic of this disease. Considering that creatine easily crosses the blood-brain barrier (Hemmer and Wallimann, 1993) and that creatine administration improves patients with some neurological diseases (Tarnopolsky and Beal, 2001), it might be important to perform more studies on the CK system in PKU to evaluate whether creatine supplementation to the Phe-restricted diet would benefit these patients.

Acknowledgements

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REDUCED CREATINE KINASE ACTIVITY IN MIDBRAIN AND CEREBELLUM OF HYPERPHENYLALANINEMIC RATS

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ABSTRACT

Phenylketonuria (PKU) is a metabolic disorder accumulating phenylalanine (Phe) and its metabolites in plasma and tissues of the patients. Unless treated, PKU patients present a variable degree of brain damage. Phe has been considered the main neurotoxic metabolite, but the mechanisms of neurotoxicity are multiple and not fully understood. Considering that creatine kinase (CK) is a key enzyme for energy homeostasis of brain cells, our main objective was to investigate the effect of acute and chronic chemically induced hyperphenylalaninemia on CK activity in cerebellum and midbrain of developing Wistar rats. We also investigated the *in vitro* effect of Phe and α-methyl-DL-phenylalanine, a phenylalanine hydroxylase inhibitor, on CK activity in the same brain structures of 22-day-old non-treated rats. The results showed that Phe significantly inhibited CK activity *in vitro* and reduced the enzyme activity *in vivo* in the two brain structures. Considering the importance of CK for the maintenance of energy homeostasis in brain, if this enzyme inhibition also occurs in PKU patients, its a possible that the reduction of CK activity may be one of the mechanisms by which Phe is neurotoxic in PKU.

Key words: phenylalanine, phenylketonuria, hyperphenylalaninemia, creatine kinase

INTRODUCTION

Phenylketonuria (PKU), the most frequent inborn error of amino acid metabolism, is an autosomal recessive disorder caused by a deficiency of hepatic phenylalanine hydroxylase (PAH), the key enzyme controlling phenylalanine (Phe) catabolism. PKU is biochemically characterized by the accumulation of phenylalanine and their metabolites in blood and other tissues. In untreated classical PKU, Phe blood levels may achieve 2.5

mmol/L, and the patients develop severe mental and psychomotor retardation. Phenylalanine itself is considered the main neurotoxic agent in PKU, although the mechanisms of neurotoxicity are still unclear (1).

We have previously reported that Phe inhibits by approximately 50 % the *in vitro* glucose uptake by brain cortex of rats (2), as well as Na^+,K^+-ATPase activity *in vitro* and *in vivo* (3) and phosphorylation of cytoskeletal proteins (4). In this context, it has also been reported a reduction of glucose metabolism in the brain of PKU patients, mainly in white matter (5). Taken together, these data suggest that Phe may alter brain energy metabolism. Brain energy impairment could explain the abnormalities in myelination, cell density and organization, dendritic arborization, and number of synaptic spines found in the brain of untreated PKU patients (6).

The enzyme creatine kinase (CK) plays a key role in the energy metabolism of cells that have intermittently high and fluctuating energy requirements, such as skeletal and cardiac muscle, and neuronal tissues like brain and retina. This enzyme catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP, to regenerate ATP. CK is compartmentalized specifically at those places where energy is produced (mitochondria) or utilized (cytosol) (7,8). Because energy is necessary to maintain the development and regulation of cerebral functions, it has been postulated that damage of CK function may result in neuronal loss in the brain (9). Reinforcing this hypothesis, it has been reported severely reduced CK activity in several neurodegenerative diseases (10,11).

We have recently reported that chemically induced hyperphenylalaninemia reduces CK activity in the brain cortex of rats (12). In the present work, our main objective was to investigate the chronic and acute effects of chemically induced hyperphenylalaninemia (HPA) on CK activity in cerebellum and midbrain of developing rats. We also investigated

the *in vitro* effects of Phe and α-methyl-DL-phenylalanine (MePhe), a PAH inhibitor, on CK activity in the same brain structures of non-treated rats.

EXPERIMENTAL PROCEDURE

Subjects and Reagents. Seventy-six Wistar rats bred in the Department of Biochemistry, UFRGS, were used in the experiments. Rats were kept with dams while receiving the drugs until they were sacrificed. The dams 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" (NIH publication 85-23, revised 1985) were followed in all the experiments, and the experimental protocol was approved by the Ethical Committee for Animal Research of the Federal University of Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Chronic Treatment. Hyperphenylalaninemia was induced by daily subcutaneous administration of Phe plus α-methyl-DL-phenylalanine (MePhe), a PAH inhibitor, to developing rats, from the 6th to the 21st day of life. Sixteen animals were randomly assigned to two groups of eight animals each as previously reported (13): 1- HPA group: rats received subcutaneous administration of 5.2 μmol Phe/g body weight twice a day at 8 h intervals and 2.4 μmol MePhe/g body weight once a day. These doses were calculated from pharmacokinetic parameters to achieve plasma levels between 1 and 4 mM; 2 – Control group: rats received the same volume of 0.85% saline twice a day. All drugs were dissolved in 0.85% saline and pH was adjusted to 7.4 by NaOH addition. Twelve hours after treatment the rats were killed by decapitation without anesthesia and the cerebellum

and midbrain (cerebrum minus brain cortex and pons) were separated for creatine kinase activity assay and protein determination.

Acute Treatment. Twenty two-day-old rats were randomly separated into four groups of six animals each and treated with one single subcutaneous injection as follows: 1- HPA group: rats received subcutaneous administration of Phe + MePhe; 2- Phe group: rats received Phe administration; 3- MePhe group: rats received MePhe administration; 4- Saline group: rats received 0.85% saline. Doses were calculated according to body weight and prepared as stated above. One hour after the subcutaneous administration the animals were killed by decapitation without anesthesia and the cerebellum and midbrain were separated for creatine kinase activity assay and protein determination.

Treated animals achieved maximal plasma Phe levels 30 min after subcutaneous injection of Phe (3.6 ± 0.2 mmol/L). Maximal brain levels were achieved 60 min after Phe administration (1.1 ± 0.1 mmol/kg wet weight tissue). Twelve hours after treatment, plasma and brain Phe concentrations returned to normal levels (0.1 ± 0.01 mmol/L and 0.15 ± 0.01 mmol/kg wet weight tissue, respectively).

In Vitro Experiments. For the *in vitro* experiments, 36 non-treated rats aging 22 days were used. Phe and MePhe were dissolved in SET buffer pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base) and added to the assay at 0.5 – 5.0 mM final concentrations. The interaction between Phe and MePhe was characterized by an adaptation of the competitive plot of Chevillard et al., (14) according to Wyse et al., (15). The competitive plot is a method for determining whether or not two enzyme-catalyzed reactions with two different substrates occur at the same active site. This method was adapted for determining whether or not two different inhibitors act at the same site on the enzyme. It is a plot of total rate against p, where p varies from 0 to 1, and specifies the concentrations $(1-p)a_0$ and pb_0 of the two amino acids in terms of reference concentrations a_0 and b_0 chosen so as to

give the same rates at p=0 and p=1. These concentrations were 1 mM for Phe and 1 mM for MePhe. If the two inhibitors act at the same site, the competitive plot gives a horizontal straight line, i.e., the total rate is independent of p. Independent reactions at two separate sites give a curve with a minimum; separate reactions with cross-inhibition generate curves with either maxima or minima according to whether the inhibition constants of the two inhibitors at one site are smaller or higher than the inhibition constants at the other site.

Preparation of Brain Tissue Homogenate. The cerebellum and midbrain were isolated and homogenized with a Teflon-glass homogenizer (1:20, w/v) in SET buffer pH 7.4. The homogenate was stored at – 70°C when the assay was not carried out immediately.

Creatine Kinase Activity Assay. The reaction mixture contained the following final concentrations: 60 mM Tris-HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, 0.02% triton X-100, and approximately 1 μg protein in a final volume of 0.1 mL. After 15 minutes of pre-incubation at 37°C, the reaction was started by the addition of 2 nmol ADP plus 8 nmol reduced glutathione. The reaction was stopped after 10 minutes 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 (16). The color was developed by the addition of 0.1 mL 2 % α-naphtol and 0.1 mL 0.05 % diacetyl in a final volume of 1 mL and read after 20 minutes at 540 nm. Phe or MePhe did not interfere with the color development or spectrometric readings. Results were expressed as μmol of creatine formed per min per mg protein.

Protein Determination. The protein content of the homogenates was determined by the method of Lowry et al., (17), using serum bovine albumin as the standard.

Statistical Analysis. Data from the chronic treatment were analyzed by the Student's t test. Data from the acute treatment and from the *in vitro* experiments were analyzed by one-way ANOVA followed by the Tukey test when the F values were significant. Dose-dependent effect was analyzed by linear regression. Data were analyzed through the Statistical Package for Social Sciences software inserted in an IBM-compatible PC computer.

RESULTS

Chronic treatment had no effect on body weight (control = 32.6 ± 6.6 g; HPA = 32.3 ± 5.7 g), brain weight (control = 1.21 ± 0.1 g; HPA = 1.12 ± 0.1 g), midbrain weight (control = 0.29 ± 0.01 g; HPA = 0.27 ± 0.03 g), and cerebellum weight (control = 0.16 ± 0.02 g; HPA = 0.14 ± 0.03 g), indicating that treatment did not cause undernutrition in the animals. The acute treatment did not affect the same parameters.

Creatine kinase activity was in the range of 3 – 4 μmol creatine per min per mg protein and 4 - 6 μmol creatine per min per mg protein in midbrain and cerebellum of 22-day-old control rats, respectively. Chronically induced HPA significantly reduced CK activity by approximately 15% in midbrain [$t(12) = 3.19$; $p<0.01$] and 30 % in cerebellum [$t(12) = 8.81$; $p<0.001$] (Fig 1). Acute administration of Phe or Phe + MePhe significantly reduced CK activity by approximately 20 **%** in midbrain, [$F(3,20) = 11.71$; $p<0.01$], and 25% in cerebellum [$F(3,20) = 89,021$; $p<0.001$] (Fig 2).

Next, we investigated the i*n vitro* effects of Phe and MePhe on CK activity in midbrain and cerebellum of 22-day-old rats (fig 3). Phe significantly inhibited CK activity

in all concentrations tested by approximately 21 % in midbrain [F(4,30) = 28,91; p<0.001), and 23 % [F(4,30) = 43,98; p<0.001 in cerebellum. MePhe also significantly inhibited CK activity in all concentrations tested by approximately 20 % in midbrain [F (4,30) = 15,74; p < 0.001], and 20% in cerebellum [F (4,30) = 29,73; p < 0.001].

In order to evaluate whether the inhibition was specific or not, we tested the *in vitro* effect of glutamate on CK activity. Our results indicated that glutamate did not alter CK activity in midbrain (control = 3.4 ± 0.2; glutamate 1mM = 3.3 ± 0.1; glutamate 5 mM = 3.5 ± 0.1 μmol / min.mg protein for 7 animals; [F(2,18) = 1.21; p> 0.3] or in cerebellum (control = 4.3 ± 0.3; glutamate 1 mM = 4.3 ± 0.2; glutamate 5 mM = 4.2 ± 0.3 μmol / min.mg protein for 7 animals; [F(2,18) = 0.28; p > 0.7].

In an attempt to better investigate whether MePhe could affect the inhibition caused by Phe on CK activity in the animals subjected to chronic HPA, we compared the inhibition caused by the association of Phe plus MePhe with the isolated effects of each one of the two substances at the same concentration (2.5.mM). The significant inhibitions of CK activity caused by Phe, MePhe, or Phe plus MePhe in midbrain [F(3,24) = 19.49; p < 0.001] and in cerebellum [F(3,24) = 70.54; p < 0.001] were of the same magnitude (Fig 4). However, considering that at 2.5 mM concentrations of Phe and MePhe the binding of the two substances seems to be saturated, we measured CK activity in the presence of various proportions between Phe and MePhe at concentrations ranging from 0.2 to 1 mM for each substance, similar to Phe concentrations found in the brain of non-treated PKU patients. At this range, the inhibition caused by Phe in midbrain [F(1,19) = 28.82; β = -0.87; p < 0.001] and in cerebellum [F(1,19) = 57.08; β = -0.87; p < 0.001], and by MePhe in midbrain [F(1,19) = 36.98; β = -0.81; p < 0.001] and in cerebellum [F(1,19) = 29.86; β = -0.78; p < 0.001] are dose-dependent. The straight lines in the interaction plots indicated a

competition between Phe and MePhe for the same CK site (Fig 5). Therefore, considering that Phe and MePhe present approximately the same effect, it is feasible to presume that Phe itself is able to affect CK activity in vivo.

DISCUSSION

We have previously demonstrated that Phe inhibits the in vitro CK activity in cerebral cortex and also reduces this enzyme activity in vivo in the same brain structure (12). In the present investigation we demonstrated that Phe also inhibits the *in vitro* CK activity in midbrain and cerebellum from non-treated rats and also reduces this enzyme activity in the same brain structures from rats subjected to acute or chronic HPA, indicating that these effects occur in whole brain. Considering that Phe has a direct inhibitory action on CK activity, this effect observed in vitro, probably also occurs in vivo. However, when CK activity was measured in the cerebral structures from Phe-treated rats, this enzyme activity was reduced. In this case, we cannot explain the reduced CK activity by an inhibition caused by significant amounts of Phe in the enzyme assay, because the brain tissue was diluted 10,000 times in the incubation medium. Therefore, it is possible that the diminution of CK activity might be caused by down-regulation of expression or posttranslational modification of existing enzyme molecules. This latter mechanism was proposed by other investigators who found that the reduced level of CK, observed in several neurodegenerative disorders, was the result of posttranslational modifications of the enzyme (18).

Considering that MePhe crosses the blood brain barrier (19), we cannot rule out that reduced CK activity found in animals subjected to chronic HPA could be caused by MePhe, the PAH inhibitor co-administered with Phe. It is difficult to test this hypothesis in

chronically induced HPA because long-term administration of MePhe causes inhibition of PAH, accumulating Phe in blood and tissues. Therefore, we investigated the effects of a single administration of Phe and/or MePhe on CK activity. Our results indicated that Phe and MePhe reduced CK activity and the magnitude of the reduction was similar to that caused by the administration of Phe plus MePhe. In addition, we also observed that Phe, MePhe, and Phe plus MePhe inhibited the *in vitro* CK activity, and the magnitude of the inhibition was similar for the three groups, suggesting that the two substances act on the enzyme through the same mechanism. Reinforcing these results, the competition plot indicated a competition between Phe and MePhe for the same site at the enzyme. Taken together, the results of the *in vivo* and the *in vitro* experiments suggest that highly sustained levels of Phe may diminish CK activity through enzyme inhibition and reduction of the number of active enzyme molecules.

We have reported that leucine inhibits in vivo and in vitro CK activity in rat brain (20). However, this is not an unspecific effect for amino acids, since glutamate did not alter the in vitro CK activity in rat brain.

PKU patients usually show a ten-fold increase of brain Phe levels as compared with healthy controls (21). Phe is probably the main responsible by the brain damage in this disease, but the pathophysiological mechanisms of brain injury are multiple and not fully understood. We have reported that Phe inhibits mitochondrial respiratory chain (22), and reduces glycolysis and increases ADP levels (23), in rat brain. Therefore, it is feasible to presume that early increased brain Phe levels in PKU could reduce brain respiration and energy production at a critical time during brain development. In this context, our results showing an *in vivo* reduction of CK activity, an important enzyme for energy homeostasis, in the brain of rats subjected to chemically induced HPA, suggest that this inhibition may be one of the mechanisms responsible for the alteration of energy metabolism in the brain

of PKU patients, impairing cell function and inducing brain damage through distinct mechanisms, including secondary excitotoxicity (24,25).

The CK / phosphocreatine system exerts several integrated functions in brain cells, such as temporary energy buffering, metabolic capacity, energy transfer, and metabolic control (26). This system is now recognized as an important metabolic regulator during health and disease (27,28). It has been postulated that CK and the creatine-phosphocreatine energy shuttle may play a role in brain development that is associated with oligodendrocyte function and/or myelogenesis (29).

A decrease of CK activity is one of the biochemical markers of brain cell damage in age-related neurodegenerative diseases, such as Alzheimer's and Pick's diseases (18). The decrease of CK activity in the brain correlates well with the neurodegeneration parameters in severely affected regions in Alzheimer's disease (30). Therefore, damage of CK function may be an important step of a neurodegenerative pathway that leads to brain damage (27,28). 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 (31). However, It is not possible to assure that the observed decrease in creatine kinase activity provoked by Phe (15 - 30%) is relevant to the pathology of PKU. Nevertheless, considering that the Km (Michaelis constant) of CK for ADP as substrate (0.3 ± 0.1 mM) indicates that the enzyme is far from be saturated at normal brain ADP levels (02-04 mM) (32), we may presume that a 30% reduction on CK activity may lead to increase in ADP and decrease in ATP concentrations. Taken together these observations, and the reduction of brain CK activity found in the present study, it is possible that Phe at high concentrations may induce excitotoxicity.

In summary, our results indicate that Phe alters *in vitro* and *in vivo* CK activity in rat brain. Considering that creatine kinase is a key enzyme for energy homeostasis in brain, if this effect also occurs in the brain of PKU patients, it is possible that the alteration of this enzyme activity may impair brain energy metabolism, contributing to the brain damage characteristic of this disease. Considering that creatine easily crosses the blood-brain barrier (33) and that creatine administration improves patients with some neurological diseases (34), it might be important to perform more studies on the CK system in PKU to evaluate whether creatine supplementation to the Phe-restricted diet would benefit these patients.

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Figure 1- Effect of chronic hyperphenylalaninemia on creatine kinase activity in midbrain and cerebellum of developing rats.

Data are mean ± SD for 7 animals in each group

**p<0.01 compared to the control.

Figure 2- Effect of acute administration of phenylalanine, α-methylphenylalanine and phenylalanine plus α-methylphenylalanine on creatine kinase activity in midbrain (A) and cerebellum (B) of developing rats.

Data are mean ± SD for 6 animals in each group

**p<0.01 compared to the control.

Figure 3- In vitro effect of phenylalanine on creatine kinase activity in midbrain (A) and cerebellum (B) of developing rats.

Data are mean ± SD for 7 animals in each group

**p<0.01 compared to the control.

Figure 4- In vitro effect of α-methylphenylalanine on creatine kinase activity in midbrain (A) and cerebellum (B) of developing rats.

Data are mean ± SD for 7 animals in each group

**p<0.01 compared to the control.

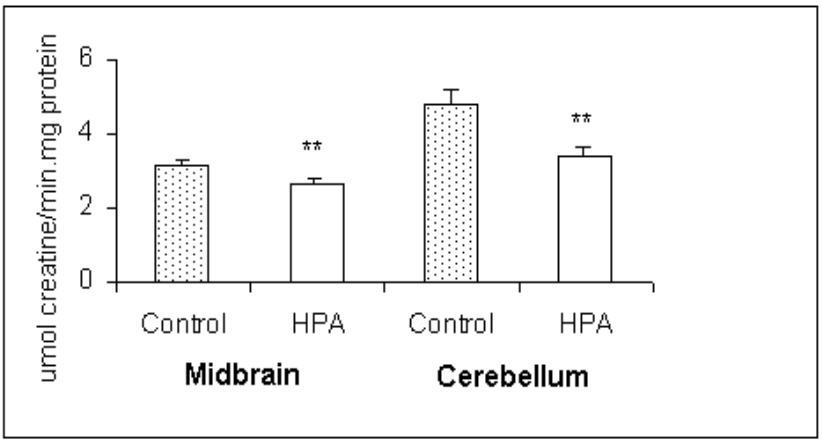
Figure 5- In vitro effect of phenylalanine, α-methylphenylalanine and phenylalanine plus α-methylphenylalanine, on creatine kinase activity in midbrain (A) and cerebellum (B) of developing rats.

Data are mean ± SD for 7 animals in each group

**p<0.01 compared to the control.

Fig. 6. Chevillard et al interaction plot between phenylalanine and α-methylphenylalanine on creatine kinase activity in midbrain (A) and cerebellum (B) of developing rats.

Data are representative of 3-5 independent experiments performed in triplicate.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6
4
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0
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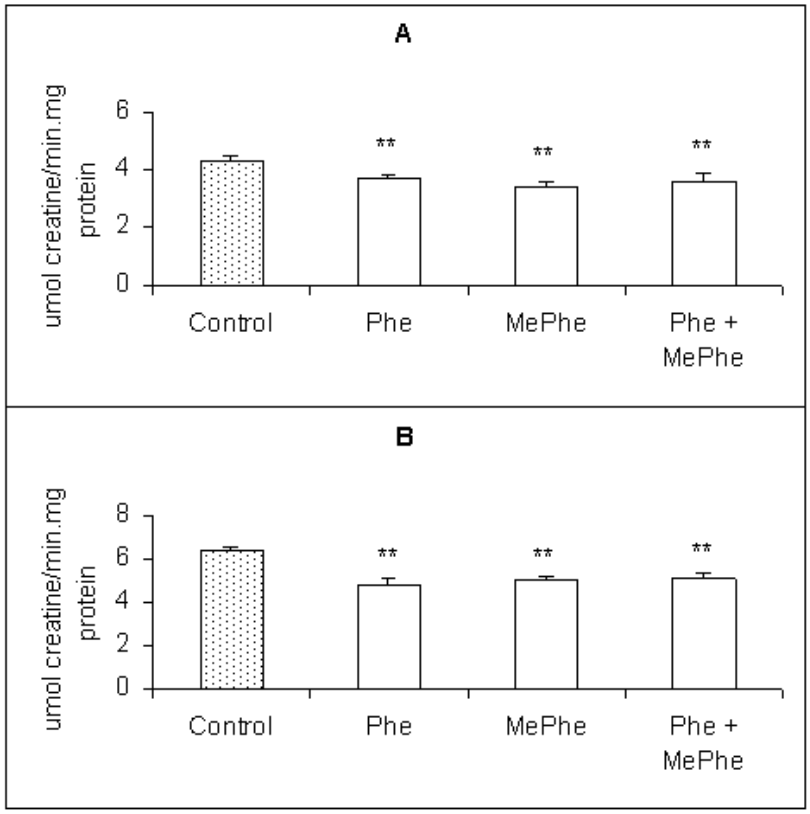
Control
HPA
Control
HPA
Midbrain
Cerebellum


A
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Control
Phe
MePhe
Phe + MePhe
B
umol creatine/min.mg protein
8
6
4
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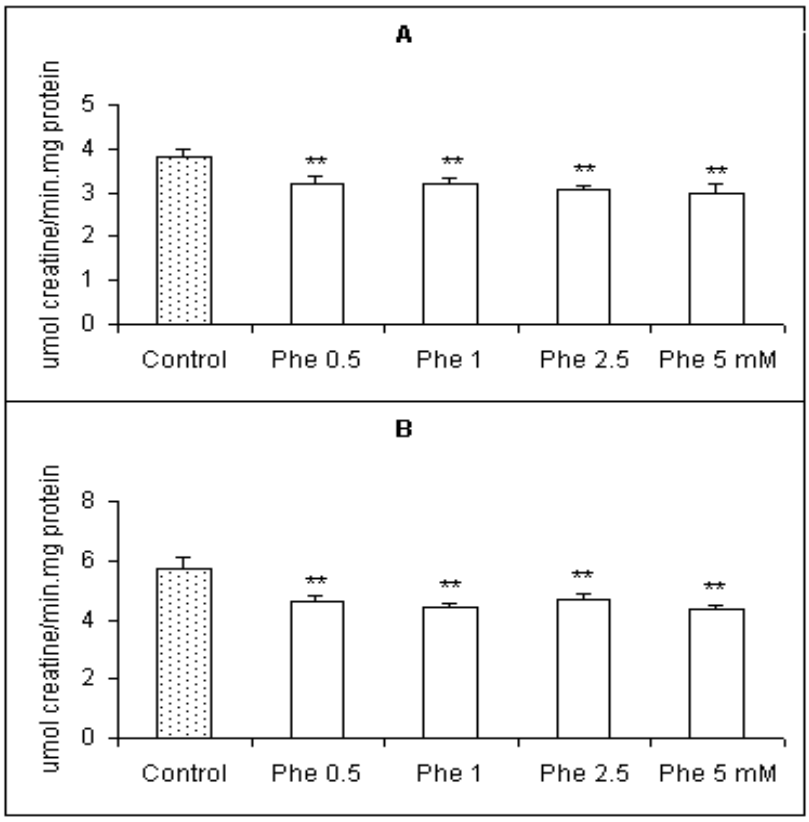
**
**
Control
Phe
MePhe
Phe + MePhe


A
umol creatine/min.mg protein
5
4
3
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**
**
**
Control
Phe 0.5
Phe 1
Phe 2.5
Phe 5 mM
B
umol creatine/min.mg protein
8
6
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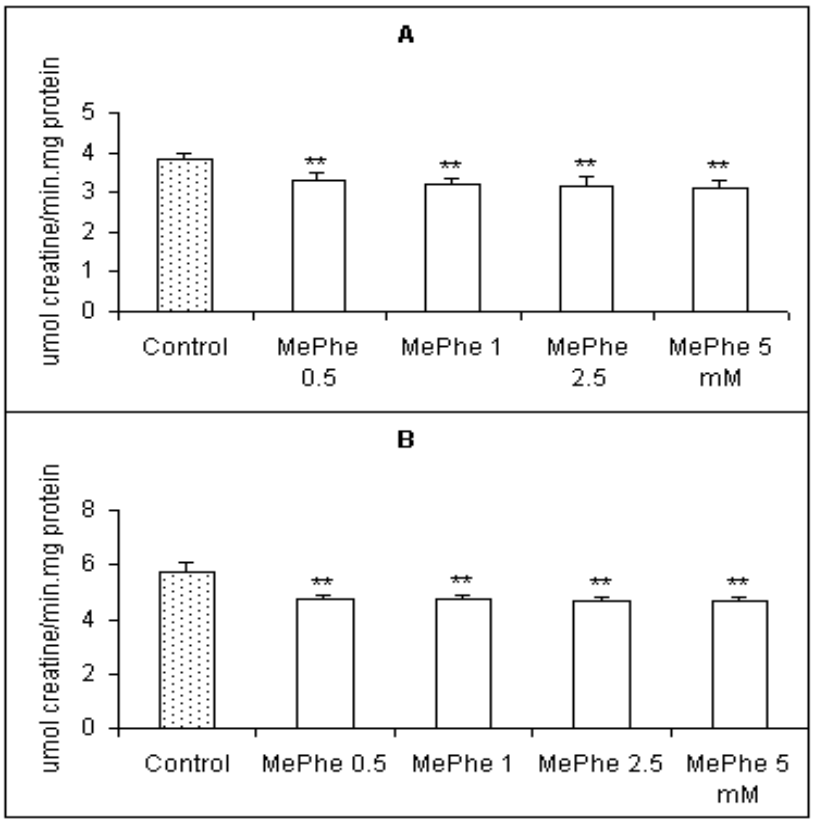
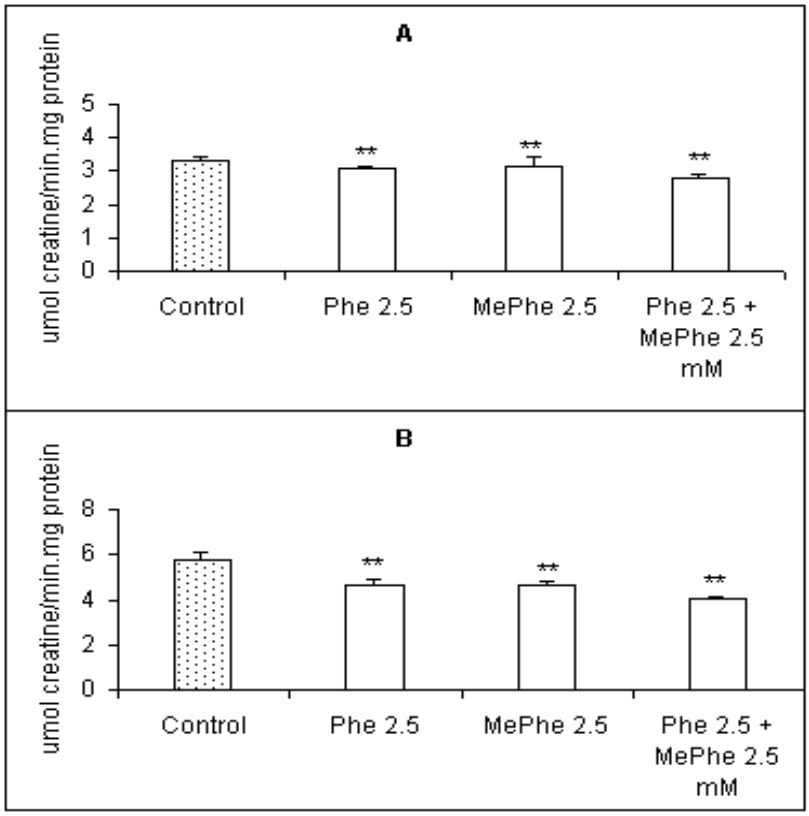
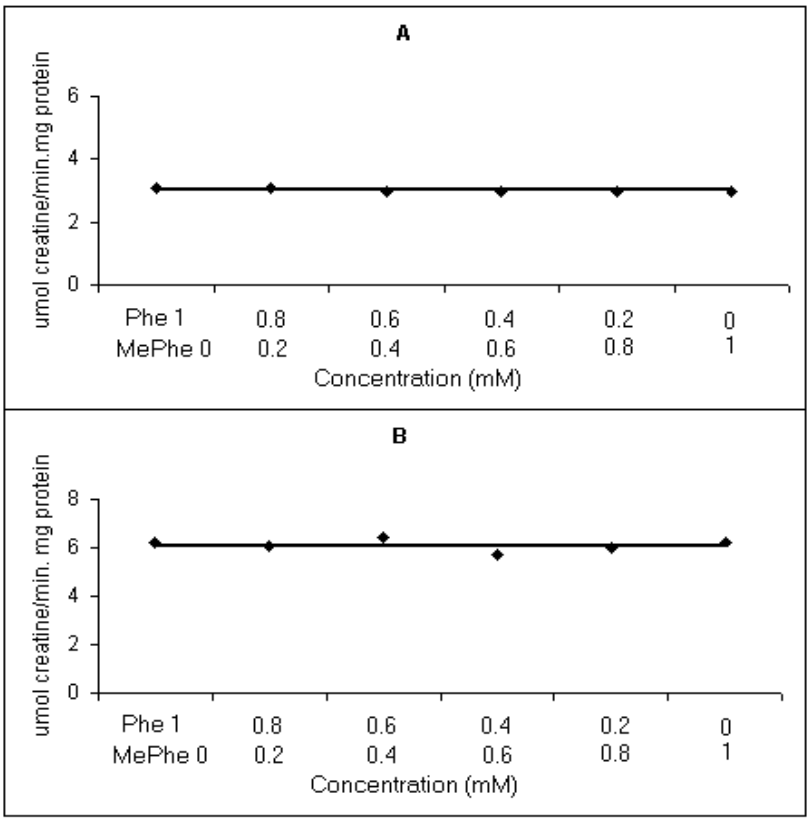
**
**
**
Control
Phe 0.5
Phe 1
Phe 2.5
Phe 5 mM


A
umol creatine/min.mg protein
5
4
3
2
1
0
**
**
**
**
Control
MePhe 0.5
MePhe 1
MePhe 2.5
MePhe 5 mM
B
umol creatine/min.mg protein
8
6
4
2
0
**
**
**
**
Control
MePhe 0.5
MePhe 1
MePhe 2.5
MePhe 5 mM


A
umol creatine/min.mg protein
5
4
3
2
1
0
**
**
**
Control
Phe 2.5
MePhe 2.5
Phe 2.5 + MePhe 2.5 mM
B
umol creatine/min.mg protein
8
6
4
2
0
**
**
**
Control
Phe 2.5
MePhe 2.5
Phe 2.5 + MePhe 2.5 mM


A
umol creatine/min.mg protein
6
4
2
0
Phe 1 0.8 0.6 0.4 0.2 0
MePhe 0 0.2 0.4 0.6 0.8 1
Concentration (mM)
B
umol creatine/min. mg protein
8
6
4
2
0
Phe 1 0.8 0.6 0.4 0.2 0
MePhe 0 0.2 0.4 0.6 0.8 1
Concentration (mM)

3 - DISCUSSÃO

A fenilcetonúria (PKU) é um erro inato do metabolismo de aminoácidos onde ocorre acúmulo de fenilalanina (Phe) e seus metabólitos no plasma e tecidos de pacientes afetados devido à deficiência da enzima fenilalanina hidroxilase hepática (HPA), que converte fenilalanina em tirosina. Pacientes não tratados com uma dieta restrita em Phe apresentam um quadro de retardo mental, convulsões e eczema cutâneo. Phe é considerada o principal metabólito neurotóxico desta doença, mas os mecanismos da neurotoxicidade são múltiplos e não completamente compreendidos.

Creatinaquinase (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). Células excitáveis e tecidos, como por exemplo o cérebro, dependem da vasta quantidade de energia disponível que pode ser usada em pulso ou de maneira variável. ATP é continuamente e eficientemente reposto por grandes pools de PCr através da reação catalisada por CK (Wallimann et al., 1992).

A glicólise aeróbica é a fonte primária da síntese de ATP no cérebro (Sokoloff, 1989). Com pequenos estoques de glicose, glicogênio e oxigênio, a taxa de glicólise no cérebro é dependente do fluxo sanguíneo cerebral e da captação de oxigênio. Essas taxas estão interligadas à utilização de ATP por fatores como [ADP], pH local, ou $[K^+]$ extracelular (Holtzman & Olson, 1983). Uma fonte disponível rapidamente para síntese de ATP no cérebro é o sistema CK/PCr. A alta atividade da CK no cérebro (Norwood et al., 1983) justifica a suposição de que a CK seja uma enzima chave no metabolismo energético deste tecido.

Todos os processos envolvidos no crescimento e metabolismo das células requerem entrada de energia. Na vida celular, produção, transporte, conversão e utilização de energia são processos fundamentais que são facilitados via rotas metabólicas envolvidas em um grande número de reações catalisadas por enzimas firmemente reguladas. A regulação de um sistema enzimático pode ser alcançada através de diferentes processos, como por exemplo, por modulação de concentrações de substrato, por moléculas regulatórias específicas, por modificações pós-traducionais ou por compartimentalizaçao subcelular. Um exemplo do último modelo é a CK que representa um sistema enzimático com isoenzimas, as quais são em parte compartimentalizadas especificamente em lugares onde a energia é produzida ou utilizada. Recentes dados experimentais sugerem que CK está localizada perto dos sítios onde a ação real ocorre, por exemplo, onde forneça energia para proteínas motoras, transporte de íons através das membranas por bomba de íons e processos ATP dependentes. O sistema CK/PCr parece ter um papel complexo e multifacetado na homeostase energética celular. (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 (Sacks et al., 1996). O sistema é reconhecido como um importante regulador metabólico durante a saúde e a doença (Wallimann et al., 1998 a,b). Tem sido postulado que CK e o circuito creatina-fosfocreatina podem ter um papel no desenvolvimento cerebral que é associado com a função de oligodendrócitos e/ou mielogênese (Manos et al., 1991).

CK está ligada a vesículas sinápticas e membrana plasmática e provavelmente na liberação de acetilcolina (Dunant et al., 1988) e em conjunção com Na^+ K^+ - ATPase, na manutenção de potenciais de membrana (Blum et al., 1991).

Razões de ATP/ADP intracelular regulam uma variedade de processos celulares e, considerando que o sistema CK está envolvido na regulação de razões de ATP/ADP local (Watts, 1971; Newsholme et al.,1978; Rossi et al., 1990), é interessante considerar que CK pode ser por si só sujeita a regulação, como por exemplo, por modificação pós-traducional (Wallimann et al., 1992).

No presente trabalho, utilizamos o modelo experimental de hiperfenilalaninemia (Wyse et al., 1995) onde a administração de Phe e MePhe inicia no 6° dia de vida, período em que o cérebro apresenta maturação equivalente à de um ser humano recém-nascido e finaliza no 21° dia de vida, período em que o cérebro apresenta maturação equivalente à de uma criança de 2 a 8 anos de idade (Loo, 1980).

Nossos resultados mostraram que os animais tratados cronicamente e agudamente com Phe e MePhe não apresentaram alterações nos pesos corporal, do cérebro, do córtex cerebral, do cerebelo e do cérebro médio. Também não observamos diferenças significativas na concentração de proteínas do homogeneizado total do córtex cerebral, cerebelo e cérebro médio de ratos, em relação aos grupos controles.

No tratamento crônico, onde os animais receberam subcutaneamente uma dose de MePhe e duas de Phe por dia, do 6° ao 21° dia e foram sacrificados 12 horas após a última administração da droga, houve uma redução significativa na atividade da CK em homogeneizado total em torno de 11% em córtex cerebral, de 15% em cérebro médio e de 30% em cerebelo.

Na administração aguda, os animais foram divididos em quatro grupos: 1- Phe + MePhe (HPA), 2- Phe, 3- MePhe e 4- Salina. Os animais com 22 dias de vida receberam uma única dose da droga e foram sacrificados 1 hora após a administração. Houve redução significativa na atividade da CK em torno de 15, 16 e 19% respectivamente para Phe, MePhe e Phe + MePhe (HPA) em córtex cerebral, de 14, 21 e 16% para Phe, MePhe e Phe

+ MePhe (HPA) respectivamente em cérebro médio e de 25, 21 e 20% respectivamente para Phe, MePhe e Phe + MePhe (HPA) em cerebelo.

De acordo com o estudo farmacocinético realizado em nosso laboratório, as doses foram calculadas para obter níveis plasmáticos entre 1 e 4 mM (5,2 μmol Phe e 2,4 μmol MePhe por grama de peso corporal), o que corresponde, aproximadamente, aos níveis de Phe encontradas no plasma de pacientes fenilcetonúricos.

Nos experimentos *in vitro*, houve inibição significativa na atividade da CK em todas as concentrações utilizadas tanto para Phe como para MePhe, nas estruturas cerebrais investigadas. Na tentativa de melhor investigar se MePhe pode afetar a inibição causada por Phe na atividade da CK em animais sujeitos a HPA crônica, foi comparada a inibição causada pela associação de Phe + MePhe com efeitos isolados de cada um dos dois compostos na mesma concentração (2.5 mM). A inibição da atividade da CK causada por Phe, MePhe ou Phe + MePhe teve a mesma magnitude nas três estruturas cerebrais investigadas.

A inibição da atividade da CK por Phe e MePhe, nos levou a um estudo cinético de interação entre estes dois compostos. Utilizando a adaptação de Wyse et al. (1995) do modelo de interação proposto por Chevillard et al. (1993), Phe e MePhe foram testadas simultaneamente, utilizando diferentes concentrações de cada um, de 0,2 a 1mM, proporcionalmente crescente para um e decrescente para outro, similar às concentrações de Phe encontradas no cérebro de pacientes fenilcetonúricos não tratados. A análise dos gráficos indicaram que Phe e MePhe provavelmente competem por um mesmo sítio de ligação da enzima nas três estruturas cerebrais investigadas. Portanto, considerando que ambas substâncias têm aproximadamente o mesmo efeito, é viável presumir que a Phe por si só seja capaz de afetar a atividade da CK *in vivo*. Os resultados dos experimentos *in vivo*

e *in vitro* sugerem que altos níveis de Phe podem diminuir a atividade da CK através da inibição enzimática e redução do número de moléculas ativas da enzima.

A diminuição da atividade da CK é um dos marcadores bioquímicos do dano cerebral em doenças neurodegenerativas, incluindo doença de Alzheimer (Askenov et al., 1997). A diminuição da atividade da CK no cérebro correlacionado com parâmetros neurodegenerativos está severamente afetada em regiões da doença de Alzheimer (Hensley et al., 1995). Portanto, o dano na função da CK pode ser uma importante etapa no processo neurodegenerativo que leva ao prejuízo cerebral (Wallimann et al., 1998 a,b).

Muitas desordens neurodegenerativas no cérebro são acompanhadas por deterioração da energia celular (Wallace et al., 1995). A deficiência de energia pode levar à despolarização de membrana, aumento da concentração intracelular de cálcio livre, e finalmente à morte celular por apoptose (Whittingham, 1984). Portanto, na tentativa de manter o alto potencial intracelular de fosforilação, a suplementação de creatina, por aumentar os níveis de creatina e PCr no cérebro (Carter et al., 1995), pode ter um efeito benéfico como adjuvante na terapia de doenças neurodegenerativas.

Em resumo, nossos resultados indicam que Phe altera *in vitro* e *in vivo* a atividade da CK nas três estruturas cerebrais investigadas. Considerando que a CK é uma enzima chave para homeostasia energética do cérebro, se esse efeito também ocorrer no cérebro de pacientes fenilcetonúricos, é possível que esta alteração na atividade enzimática possa prejudicar o metabolismo energético cerebral contribuindo para o prejuízo cerebral característico dessa doença. Considerando que a creatina facilmente atravessa a barreira hematoencefálica (Hemmer and Wallimann, 1993) e a administração de creatina melhora pacientes com algumas doenças neurológicas (Tarnopolsky and Beal, 2001), pode ser importante realizar mais estudos no sistema CK na fenilcetonúria para avaliar se a suplementação com creatina na dieta restrita de Phe pode beneficiar estes pacientes.

4 - CONCLUSÕES

1. Os animais tratados cronicamente e agudamente não apresentaram alterações no peso corporal, peso total do cérebro, do córtex cerebral, do cerebelo e do cérebro médio, quando comparados com os animais controles. Também não foram encontradas alterações nas concentrações de proteínas do homogeneizado total.

2. A atividade da CK em homogeneizado total de córtex cerebral reduziu significativamente em 11% nos animais submetidos ao modelo experimental crônico de hiperfenilalaninemia.

3. A atividade da CK em homogeneizado total de cérebro médio reduziu significativamente em 15% nos animais submetidos ao modelo experimental crônico de hiperfenilalaninemia.

4. A atividade da CK em homogeneizado total de cerebelo reduziu significativamente em 30% nos animais submetidos ao modelo experimental crônico de hiperfenilalaninemia.

5. Os animais submetidos à administração aguda apresentaram uma redução significativa na atividade da CK em córtex cerebral em torno de 15, 16 e 19% respectivamente para o grupo da Phe, da MePhe e da Phe + MePhe (HPA).

6. Os animais submetidos à administração aguda apresentaram uma redução significativa na atividade da CK em cérebro médio em torno de 14, 21 e 16% respectivamente para o grupo da Phe, MePhe e da Phe + MePhe (HPA).

7. Os animais submetidos à administração aguda apresentaram uma redução significativa na atividade da CK em cerebelo em torno de 25, 21 e 20% respectivamente para o grupo da Phe, da MePhe e da Phe + MePhe (HPA).

8. A Phe nas concentrações 0,5 – 1,0 – 2,5 e 5,0 mM, inibiu significativamente a atividade da CK em homogeneizado total de córtex cerebral, cérebro médio e cerebelo.

9. A MePhe nas concentrações 0,5 – 1,0 – 2,5 e 5,0 mM, inibiu significativamente a atividade da CK em homogeneizado total de córtex cerebral, cérebro médio e cerebelo.

10. A Phe + MePhe na concentração 2,5 mM inibiu significativamente a atividade da CK em homogeneizado total de córtex cerebral, cérebro médio e cerebelo na mesma magnitude de Phe 2.5 mM e MePhe 2.5 mM.

11. A análise dos gráficos com os resultados do estudo cinético da interação entre Phe e MePhe sobre a atividade da CK em homogeneizado total de córtex cerebral, cérebro médio e cerebelo mostrou que a inibição da atividade enzimática encontrada é quantitativamente semelhante para os dois compostos, sem efeito aditivo, indicando que Phe e MePhe competem por um mesmo sítio de ligação na enzima.

É possível que a inibição na atividade da CK possa estar envolvida nos mecanismos pelos quais a fenilalanina é neurotóxica. Acreditamos que nossos resultados possam contribuir na compreensão da disfunção neurológica encontrada em pacientes fenilcetonúricos.

5 - PERSPECTIVAS

Nossos resultados abrem a perspectiva de continuarmos a investigação com os seguintes objetivos:

1. Verificar os efeitos da hiperfenilalaninemia quimicamente induzida, bem como os efeitos *in vitro* da Phe e da MePhe sobre a atividade da CK nas frações citosólica e mitocondrial purificadas em córtex cerebral, cérebro médio e cerebelo de ratos jovens.

2. Verificar os mecanismos de ação da Phe sobre a atividade da CK citosólica e mitocondrial em córtex, cérebro médio e cerebelo de ratos jovens *in vivo* e *in vitro*, através de dois tipos de estudo: 1- estudos cinéticos de competição entre Phe e ADP ou PCr de acordo com Lineweaver-Burk; 2- estudos de prevenção e reversão da inibição na presença de antioxidantes.

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