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EFEITO DOS AMINOÁCIDOS DE CADEIA RAMIFICADA E SEUS CETOÁCIDOS SOBRE A ATIVIDADE DA CREATINAQUINASE DE CÉREBROS DE RATOS JOVENS

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Superar o fácil não tem mérito,
é obrigação;
vencer o difícil,
é maravilhoso;
ultrapassar o outrora impossível,
é glorificante.

.

À **D. Lourdes**, minha mãe,
que aos 92 anos continua sendo
o exemplo de dedicação e renovação.

Ao Ricardo e Eduardo, **meus filhos,**
por tudo que representam para mim e
pelo estímulo que eu possa deixar.

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ABREVIATURAS

ADP - **adenosina difosfato**
AK – **adenilatoquinase**
ANT – **translocador do nucleotídeo da adenina**
ATP – **adenosina trifosfato**
BCKD –**complexo enzimático da desidrogenase dos α-cetoácidos de cadeia ramificada**
CK – **creatinaquinase**
CK-BB – **creatinaquinase cerebral**
CK-MB - **creatinaquinase cardíaca**
CK-MM - **creatinaquinase músculo-esquelética**
CK-Mi – **creatinaquinase mitocondral**
$CK\text{-}Mi_a$ – **creatinaquinase mitocondrial ubíqua**
$CK\text{-}Mi_b$ – **creatinaquinase mitocondrial sarcomérica**
Cr – **creatina**
E1 – **2-oxoisovalerato desidrogenase, componente catalítico do complexo enzimático da desidrogenase dos α-cetoácidos de cadeia ramificada**
E2 - **di-hidrolipoamida aciltransferase, componente catalítico do complexo enzimático da desidrogenase dos α-cetoácidos de cadeia ramificada**
E3 - **di-hidrolipoamida desidrogenase, componente catalítico do complexo enzimático da desidrogenase dos α-cetoácidos de cadeia ramificada**
GABAérgicos – **substâncias com ação semelhante ao neurotransmissor ácido γ-amino-butírico**
Ile - **isoleucina**
KI**C** – **ácido α-cetoisocaproico**
KMV – **ácido α-ceto-β-metilvalérico**
KIV - **ácido α-cetoisovalérico**
Leu - **leucina**
MSUD –**doença do xarope do bordo**
NAD+ - **nicotinamida adenina dinucleotideo oxidado**
NADH - **nicotinamida adenina dinucleotídeo reduzido**
NMDA –**N-metil-D-aspartato**
~P – **grupamento fosfato capaz de transferir energia**
PCr - **fosfocreatina**
^{31}P MRS – **resonância magnética do fósforo por espectroscopia**
VDAC – **poro de membrana mitocomdrial**
Val - **valina**

RESUMO

A Doença do Xarope do Bordo é uma alteração metabólica hereditária caracterizada bioquimicamente pelo acumulo dos aminoácidos de cadeia ramificada e seus respectivos aminoácidos no sangue e nos tecidos destes pacientes. Os mecanismos patogênicos das alterações neurológicas presentes nos pacientes ainda são pouco conhecidos. Há evidências de que o metabolismo energético esteja alterado no cérebro dos pacientes. A creatinaquinase, enzima chave no metabolismo energético, está envolvida transporte e manutenção da energia cerebral.

Neste trabalho investigamos a inibição da creatinaquinase pelos aminoácidos de cadeia ramificada *in vitro* em cérebro de ratos em desenvolvimento, nas concentrações semelhantes as encontradas no plasma destes pacientes. O mesmo efeito não foi verificado com os cetoácidos de cadeia ramificada.

Verificamos ainda que este efeito também ocorreu em ratos tratados com leucina de forma crônica do 6^0 ao 21^0 dia, e de forma aguda quando tratados por 12 horas com intervalos de três horas.

Os parâmetros cinéticos estudados mostraram um Km baixo (0,8 – 1,4 mM) para a fosfocreatina como substrato, cuja variação no cérebro oscila entre 4 a 8 mM. Assim nas condições fisiopatológicas a enzima estaria saturada em ralação à fosfocreatina, sendo difícil a competição com os aminoácidos de cadeia ramificada, principalmente devido ao alto Ki encontrado (6 a 26 mM), exceto em situações de baixas concentrações deste substrato. Entretanto, o Km encontrado para o ADP como substrato (0,3 ± 0,1 mM) é semelhante à concentração do ADP do cérebro (0,2 – 0.4 mM). Como o Ki encontrado também é alto (14 – 30 mM), é possível que a competição entre o ADP como substrato e os aminoácidos de cadeia ramificada diminua a atividade da enzima alterando a homeostasia energética do cérebro, contribuindo para o dano neurológico encontrado nos pacientes com a Doença do Xarope de Bordo.

Os dados deste trabalho propõem um novo mecanismo fisiopatológico para as alterações neurológicas encontradas na Doença do Xarope do Bordo. Os aminoácidos de cadeia ramificada, acumulados no cérebro destes pacientes, ocasionando a inibição da creatinaquinase estariam competindo com o ADP, produzindo deste modo um desequilíbrio energético e conseqüentemente o dano neurológico.

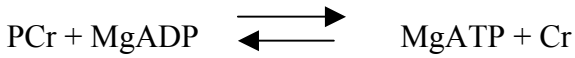
CAPÍTULO I

INTRODUÇÃO

Introdução

1 Creatinaquinase

A creatinaquinase (EC 2.7.3.2) é uma importante enzima que catalisa a transferência reversível do fosfato rico em energia (~P) da fosfocreatina (PCr) para o adenosina difosfato (ADP) originando adenosina trifosfato (ATP) e creatina (Cr). A enzima é dependente de magnésio.



Esta transferência é reversível e garante a manutenção contínua do ATP nos tecidos ou células que requerem muita energia para seu funcionamento (Wyss et al., 1992).

A creatinaquinase foi descoberta em extratos de músculo por Karl Loman, em 1934. Possui cerca de 400 aminoácidos com massa molecular de 75 a 91 kDa e 306 a 380 kDa, conforme a isoforma.

A Creatinaquinase (CK) possui cinco isoenzimas conhecidas em tecidos de aves e mamíferos, sendo três encontradas no citoplasma e duas na mitocôndria. As isoenzimas citosólicas são dímeras e compostas por dois tipos de subunidades, a M de "muscle" e B de "brain". As isoenzimas citosólicas são constituídas por dímeros e conhecidas como CK-MM, CK-BB e CK-MB, conforme o tipo de subunidade que a compõem. A isoenzima

citoplasmática CK-MM é predominantemente encontrado no músculo esquelético, a CK-BB no cérebro e tecidos neurais e a CK-MB no coração e músculo estriado durante o desenvolvimento (Eppemberg et al., 1967; e Wallimann et al., 1992). Estas isoformas são cineticamente similares, mas diferem na capacidade para associarem-se com organelas subcelulares ou estruturas protéicas. Sob a microscopia eletrônica apresenta uma forma alongada semelhante à da banana (banana-like) (figura 1) (Eder et al., 1999; e Schlegel et al., 1988).

As isoenzimas citoplasmáticas estão localizadas em compartimentos subcelulares, em subestruturas como miofibrilas, retículo sarcoplasmático, etc. Acreditava-se que as isoenzimas citosólicas da creatinaquinase fossem solúveis, mas estudos bioquímicos de fracionamento e imunoquímicos mostraram a compartimentalização subcelular das isoenzimas, acoplada ao seu funcionamento (Thompson et al., 1980; e Wallimann et al., 1992).

As isoenzimas mitocondriais, devido a sua localização, são chamadas de CK-Mi ubíqua e CK-Mi sarcomérica, ou ainda de CK-Mi_a e CK-Mi_b respectivamente. A isoforma sacomérica, é expressada nos ventrículos e no músculo esquelético enquanto a isoforma ubíqua é expressada no músculo liso e em outros tecidos. As isoenzimas mitocondriais estão localizadas no espaço intermenbrana sob forma de moléculas octaméricas (figura 2) ou diméricas (figura 1) (Schlegel et al., 1998; Eder, 1999; Wyss, 1992; e Wallimann & Hemmer, 1994).

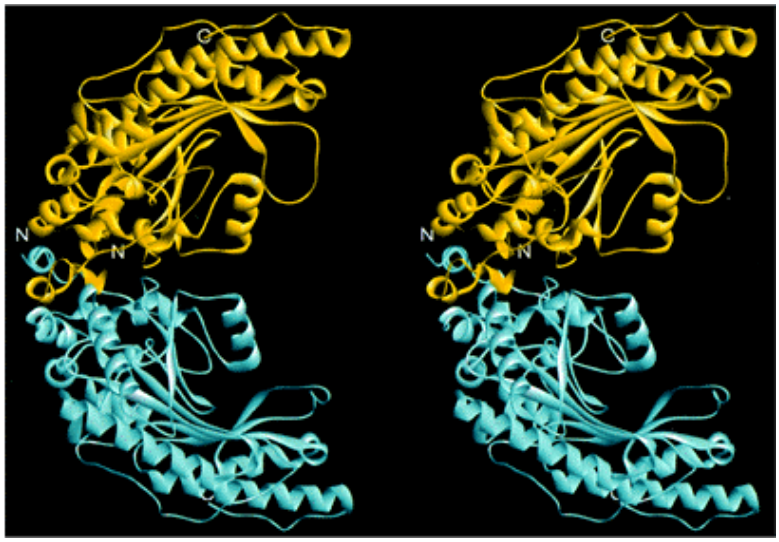


Fig 1. Estéreo representação da forma dímera da CK-BB. Os monômeros A e B são mostrados em azul e amarelo respectivamente. Estrutura tridimensional da forma dimérica da creatinaquinase, mostrando as duas subunidades. (Eder, 1999 e Schlegel, 1998).

A creatinaquinase está localizada em tecidos que exigem muita energia para o desempenho da função fisiológica como no músculo esquelético, no músculo cardíaco e no cérebro etc. (Wallimann & Hemmer, 1994, revisão).

A CK-BB foi localizada nos astrócitos e na substância branca através da microscopia eletrônica, com auxilio de coloração com imunoperoxidases e anticorpos específicos. Nenhuma coloração foi observada nos neurônios ou outros elementos gliais (Thompson et al.,1980).

A CK-BB pode estar super-expressada numa variedade de tumores sólidos e tumores de linhagens celulares, como por exemplo nas células pequenas de carcinoma de pulmão, adenocarcinoma de cólon e de reto, carcinoma de mama e de próstata e ainda em neuroblastoma. Parece que a alta atividade da CK-BB seria utilizada par sustentar a grande

quantidade de energia que as células malignas necessitam para a multiplicação intensa (Berges et al, 1996).

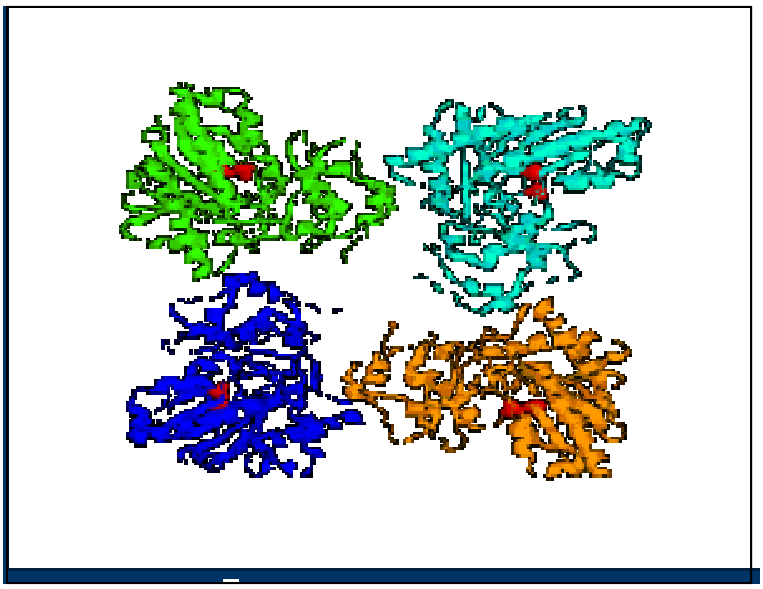


Fig 2. Estrutura tridimensional da forma octamérica da CK. Microfotografia eletrônica da CK-Mi de galinha. O ângulo observado permite visualizar a disposição concêntrica das formas diméricas, compondo a forma octamérica. Na parte central, observa-se o canal onde há a transferência do ~P

1.1 O sistema creatinaquinase/fosfocreatina

A localização privilegiada da isoenzima citosólica no interior de subestruturas citoplasmáticas, e das isoenzimas mitocondriais entre as membranas internas e externas, atribui à CK um papel importante no metabolismo energético. A creatina atua como um regulador da fosforilação oxidativa e carreador de ~P entre os locais de geração (glicólise e mitocôndria) para os locais de consumo de energia (ATPases). Forma-se uma intricada

rede de distribuição de energia, altamente regulada denominada de circuito da fosfocreatina.

Baseado no sistema CK/PCr muitos modelos foram formulados e funções atribuídas. Besmann e Carpenter (1985), Wallimann et al., (1994, revisão) formularam o modelo proposto inicialmente para o músculo, estendido após para outros tecidos, e atribuíram ao sistema as seguintes funções: tamponamento enérgico, controle metabólico e transporte de energia.

1.1.1 Tamponamento energético

A célula nervosa não possui reserva de ATP e o nível deste ATP deve ser mantido durante o trabalho celular. Em média metade dos ~P são renovados a cada três segundos. Deve haver, portanto, um mecanismo que promova a rápida renovação de ATP no cérebro. Assim, o sistema CK/PCr evita a diminuição dos níveis de ATP e o acúmulo de ADP, contribuindo ainda para impedir a acidificação intracelular devido à hidrólise do ATP durante o trabalho efetuado.

1.1.2 Controle metabólico

A creatina quinase encontra-se no meio de uma rota entre a produção e utilização de energia (Meyer et al., 1984). A atividade da CK é comparável a um circuito elétrico onde as ATPases seriam as resistências e a mitocôndria a bateria. De acordo com esta

visão, a mitocôndria sintetiza o ATP e a CK transfere uma grande quantidade de energia, atuando como um carregador de fluxo de ~P através do citoplasma, por um mecanismo de difusão facilitada. A concentração de fosfocreatina no cérebro é muito mais alta do que a concentração de ATP e de ADP. Deste modo é mantida constante a relação ATP/ADP, realizando o tamponamento da energia espacial. A manutenção do equilíbrio da creatinaquinase neste meio é feita em compartimentos celulares que conseqüentemente também armazenam a fosfocreatina (estocando energia). A combinação entre a capacidade de difundir ligações ricas em energia e a capacidade de armazenamento de energia, confere à CK a propriedade de controle metabólico.

1.1.3 Transporte de energia

A idéia do transporte de energia (energia espacial) no interior da célula surgiu há quase 40 anos com o trabalho pioneiro de Besmann (1966). A maior parte da energia cerebral (95%) é originada no interior da mitocôndria e a transferência para o citosol é feita pela fosfocreatina que atua como um carreador energético que liga o local de produção de energia e o local de sua utilização através de compartimentos subcelulares contendo a creatinaquinase. O aumento da demanda de energia exigida requer um controle efetivo da sua produção ou transferência. Alem da localização privilegiada da enzima nas entre as membranas internas e externas mitocondriais e nas subestruturas citoplasmáticas, o baixo Km da CK para ADP (entre 10 e 35 μM) pode ser considerado um sensor de ADP mantendo alta a relação ATP/ADP próximo aos locais de utilização de energia, evitando assim a diminuição da eficiência termodinâmica que poderia ser provocada pela queda dos

níveis de ATP. Este refinamento da organização e controle da CK limita o ADP celular livre, que poderia ocasionar uma inativação de ATPases e a inviabilização celular. (Wallimann et al., 1994; Saks et al., 1996).

1.2 Funcionamento do circuito creatinaquinase/fosfocreatina.

O esquema do funcionamento da creatinaquinase proposto por Walliman pode ser visto na figura 3.

O ATP celular cerebral origina-se principalmente de duas rotas metabólicas: uma da glicose através da glicólise ou glicogenólise e outra da fosforilação oxidativa que acontece no interior das mitocôndrias. Quatro tipos de compartimentos são identificados para a CK, três no citosol e um na mitocôndria.

No citosol a enzima é encontrada em "pools" de PCr/Cr e ATP/ADP, em compartimentos, que funcionam ligados à glicólise e associados a estruturas subcelulares em locais onde estão as ATPases e requerem altos níveis de energia. O sistema da CK está em equilíbrio com os substratos e os metabólitos da CK comportam-se como se estivessem livremente em solução onde os efeitos da compartimentalização são mínimos em relação a bioenergia celular e características termodinâmicas. Uma das funções da CK citosólica é manter o ADP livre em baixas concentrações e o ATP em altas concentrações. Esta parte do circuito representa a função clássica da enzima de manutenção de energia temporal. Um outro compartimento para CK é encontrado na mitocôndria ligado a fosforilação oxidativa.

Está localizado entre a membrana interna e externa das cristas mitocondriais, em locais onde elas se aproximam.

A CK-Mi é um octâmero com forma cubóide que tem um canal central onde a enzima interage com microcompartimentos formados por um translocador de nucleotídios de adenina (ANT) na membrana interna da mitocôndria e um poro íon seletivo na membrana externa (VDAC) para formar um complexo multi-enzimático trans-membrana canalizador de energia (Brdicka et al., 1994). O ATP originado da fosforilação oxidativa, é transportado através da membrana pelo ANT, transforma-se em ADP em contato com a creatinaquinase, pela transferência do ~P. A fosfocreatina formada sai da mitocôndria através do poro, quando nova molécula de creatina, entra em contato com o espaço entre membranas através do poro para nova transformação. O PCr é exportado da mitocôndria para os locais de consumo de energia no citosol, regenerando o "combustível" do ATP, mantendo alta a relação ATP/ADP e a creatina regenerada volta à mitocôndria para ser novamente "recarregada". Esta parte do modelo representa a função de tamponamento espacial. Um possível aspecto regulador da CK-Mi, pode ser visualizado com as formas octaméricas e diméricas intercovertidas, e mantidas em equilíbrio dinâmico (Schlegel, et al. 1988 b). Este equilíbrio é dependente do estado metabólico da mitocôndria e esquematicamente visto como um modulador potencial da regulação metabólica. As formas octamérica, ligada à membrana mitocondrial interna, e a oligomérica, dissociada, são dependentes de pH. A célula assim pode funcionar com pequeno pool de ATP e ADP porque recebe rapidamente reforços do sistema PCr. Este modelo acopla a funcionalidade da produção do ATP com a utilização do ATP via CK e PCr, bem como a difusão por rotas de CK e PCr para locais de trabalho na célula. Rotas paralelas envolvendo o transporte de

ATP podem operar ao mesmo tempo. Este modelo que foi desenvolvido para o músculo esquelético, estendido para o espermatozóide e fornecendo energia para a sua motilidade, células fotoreceptoras da retina, eletrócitos, bordas em escova de epitélios de transporte e cérebro.

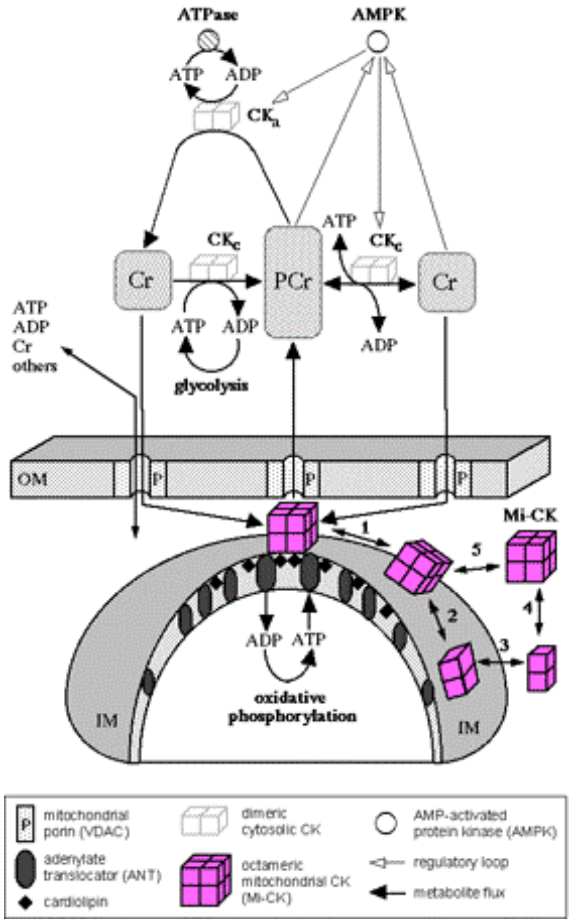


Figura 3. Modelo do circuito Cr/PCr para homeostasia da energia intracelular em células especializadas com metabolismo de alta atividade energética e armazenamento de energia flutuante. Walliman e col., 1994.

1.3 Aspectos químicos

A forma biologicamente ativa do dímero CK-BB possui uma massa molecular de duas vezes 43 000 daltons e mede aproximadamente 92 x 42 x 65 Å.

O sítio ativo da CK-Mi possui uma cisteína e dois resíduos de arginina que são essenciais para atividade catalítica e, provavelmente, uma asparagina em 335 que está envolvida na ligação ao substrato (Wyss, 1992).

A histidina faz parte de sítio ativo da enzima e atua como um extrator de próton do grupamento guanidino da creatina, facilitando assim o ataque nucleofílico do fosfato gama a ser transferido durante a reação enzimática. Estudos feitos com NMR em cérebro mostram quatro resíduos nesta área que suportam a idéia da organização de substratos.

Resíduos de glutamina 231, 232 e 233 também presentes formam uma carga compacta negativa, essencial para a catálise (Eder et al., 1999).

As isoenzimas da CK são conhecidas por conterem o grupamento sulfidrílico no sítio ativo: Cisteína 278 na CK-Mi e Cisteína 283 na CK citosólica (Furter, 1993). Modificações ocorridas nos resíduos sulfidrílicos, por espécies ativas de oxigênio e NO, diminuem a atividade mostrando que este grupamento também tem um importante envolvimento na atividade catalítica da enzima (Susuki, 1992 e Kaneko, 1993).

O pH ótimo para a catalise varia de 6,0 a 7,0 na direção do ATP (citosol) e 7.5 a 9.0 na direção da fosfocreatina (mitocôndria).

A estrutura da CK-BB possui três regiões flexíveis. Os resíduos 66-70 formam uma alça caracterizada por expor resíduos hidrofóbicos. Esta alça tem sido proposta para atuar como uma espécie de tampa próxima ao sitio ativo retirando a água da catálise (Fritz-Wolf et al., 1996). A segunda alça flexível contem os resíduos 321-331 sem interesse significativo aparentemente, e a terceira inclui uma hélice alfa, contendo os resíduos 181-190, localizada na superfície da molécula. Esta alça possui grande identidade com a adenilatoquinase, outra enzima envolvida no transporte de ~P no cérebro.

A CK é altamente sensível a espécies ativas de oxigênio (Stachowiak et al., 1998; Kaneko et al., 1993 e Gross et al, 1996). Este efeito produz deficiência energética, acúmulo de ADP e excesso de cálcio intracelular. Foi verificado que o peroxidonitrito afeta a forma octamérica da CK-Mi e evita a reoctamerização da forma dimérica (Gross et al, 1996, Wendt et al.,1998). Acredita-se que a desestabilização da energia celular por exposição crônica a espécies reativas de oxigênio pode ocorrer em muitas doenças neuromusculares, com elevação dos níveis de Ca2+ intracelular e apoptose (Mattson,1992). Foi demonstrada uma clara ligação entre o aumento das formas diméricas, o aumento de Ca2+ e doenças cardíacas (Soboll et al.,1999 e Molkentin et al., 1998). A relação fisiopatológica entre CK e patologias cérebrais foi demonstrada pelos estudos de Aksenov et al.(1997) e David et al.(1998) que descreveram a redução da atividade da CK em alterações neurodegenerativas como doença de Alzheimer e doença de Pick.

1.4 Aspectos fisiológicos

A creatinaquinase está presente em diferentes áreas do cérebro, demonstrado em vários estudos (Holtzamn et al,.1993). Alta atividade da enzima foi encontrada no cerebelo (Chandler, 1988) e no córtex cerebral (Maker et al., 1973 e Khan et al., 1976). Estudos realizados com ^{31}P-MRS, no cérebro humano *in vivo*, mostraram um alto fluxo da creatinaquinase na substância cinza, contendo concentrações mais altas de fosfocreatina do que na substância branca. Entretanto, foram encontrados altos níveis da atividade enzimática em culturas de oligodendrócitos, células gliais típicas da substância branca (Manos et al., 1991; e Walliman et al., 1994).

A entrega de ~P para ATPases nos locais de sua utilização e a restauração destes carreadores depende muito da velocidade do fluxo destes compostos na célula. Esta velocidade é determinada pelo coeficiente de difusão e conseqüentemente pela concentração dos compostos. A concentração de fosfocreatina no cérebro (~5 mM) é normalmente mais alta que a concentração do ATP (~3mM), a concentração de creatina (~6 mM) é muito mais alta que a concentração do ADP (~0.3 mM) e a concentração do AMP (~0.03 mM) (Erecisnka e Silver, 1989). Considerando estes dados, a transformação de Cr em PCr deveria ser mais efetiva que a transformação do ADP em ATP. Experimentos feitos com dinitrofluorbenzeno, um inibidor da CK, causou a perda de movimentos na cauda distal de espermatozóides (Tombes e Saphiro, 1985) e comprometeu seriamente a contração do músculo estriado (Cain e Davis, 1962).

2 A Doença do Xarope do Bordo

A Doença do Xarope do Bordo (MSUD) é uma aminoacidopatia secundária a um defeito na rota metabólica dos aminoácidos de cadeia ramificada (leucina, isoleucina e valina). O acúmulo destes três aminoácidos e seus respectivos cetoácidos, em crianças não tratadas para MSUD, leva a encefalopatia e progressiva neurodegeneração. O diagnóstico precoce e a intervenção com dieta podem prevenir complicações e permitir um desenvolvimento intelectual muitas vezes normal.

Esta doença é assim denominada porque a urina dos pacientes afetados exala um odor de açúcar queimado. Os cetoácidos da leucina (Leu), isoleucina (Ile) e valina (Val) também estão presentes na urina destes pacientes, responsáveis pela denominação de α-cetoacidúria de cadeia ramificada. O cetoácido da isoleucina, α-ceto-β-metilvalérico, é o responsável pelo odor característico da urina. Esta doença é ocasionada por uma alteração metabólica hereditária que se não tratada causa retardo mental, incapacidade física e morte. Pode atingir a todos os grupos étnicos numa incidência de 1:125 000 a 300 000 nascimentos. A freqüência nos Estados Unidos e de 1:180 000 nascimentos. Por ser uma doença autossômica recessiva, tem maior prevalência em populações com altas taxas de consangüinidade, sendo o motivo da alta prevalência (1:176 nascimentos) entre os Menonitas, numa população que vive na Pensilvânia.

A alteração metabólica é causada pela deficiência da atividade da desidrogenase dos α-cetoácidos de cadeia ramificada que catalisa a descarboxilação dos alfa-cetoácidos

da leucina, isoleucina e valina que são após metabolizados a acetil-CoA, acetoacetato e succinil-CoA (Chuang, 1998).

2.1. Aspectos históricos

Em 1954 Menkes et al. descreveram uma alteração neurológica progressiva e degenerativa em quatro filhos de uma família, que morreram com alguns meses de idade. A urina destas crianças tinha um odor de xarope de bordo ou de açúcar queimado. Ácidos orgânicos foram isolados da urina de alguns pacientes com estes sintomas (Menkes et al., 1959). Dancis et al. identificaram o aumento patogênico dos aminoácidos de cadeia ramificada e seus respectivos cetoácidos e em 1960, foi identificado o bloqueio metabólico na descarboxilação dos α-cetoácidos de cadeia ramificada: o ácido α-cetoisocaproico (KIC), o ácido α-ceto-β-metilvalérico (KMV) e o ácido α-cetoisovalérico (KIV) (Dancis et al., 1963 e 1967).

Em 1964 Snyderman iniciou com sucesso a primeira dieta com restrição de aminoácidos de cadeia ramificada. Em 1967, Dancis et al. observaram uma variante da MSUD em duas famílias de siberianos. As crianças tinham alterações neurológicas, aumento dos cetoácidos e aminoácidos de cadeia ramificada e odor característico na urina. Entretanto os sintomas eram diferentes da forma clássica de MSUD.

Em 1971, Scriver et al. descreveram o primeiro caso da doença responsiva a tiamina.

Em 1978 a enzima mitocondrial desidrogenasse dos α-cetoácidos de cadeia ramificada (BCKD) foi purificada até a homogeneidade por Pettif et al. (1978) que permitiu a clonagem do cDNA para sub-unidades do complexo BCKD humano.

2.2 Aspectos bioquímicos do metabolismo

A leucina, isoleucina e valina são aminoácidos essenciais constituídos por quatro ou cinco átomos de carbono com uma função metila que ramifica a cadeia de carbonos na posição 3 ou 4 (figura 4). Estes aminoácidos possuem uma rota catabólica em comum, participando como fonte alternativa de energia, que é constituída por quatro etapas distintas.

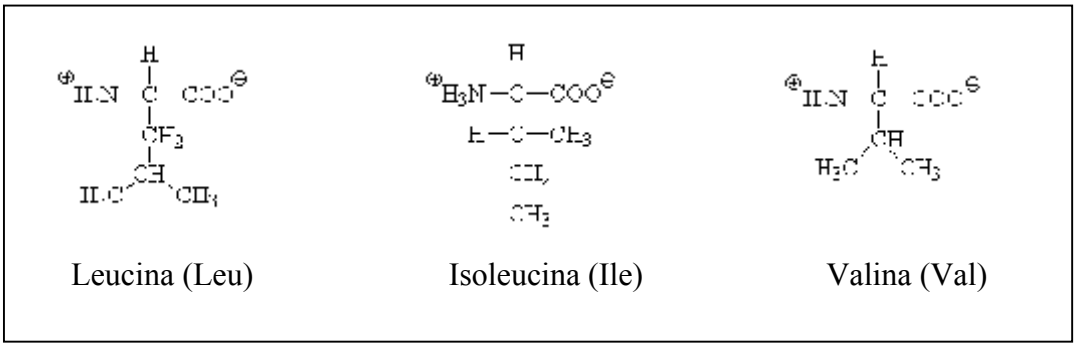


Fig 4. Estrutura dos aminoácidos de cadeia ramificada.

Após a ingestão, os aminoácidos de cadeia ramificada são metabolizados pelo músculo esquelético como fonte alternativa de energia e também oxidados nos rins, coração tecido adiposo e cérebro.

A oxidação inicia pelo transporte dos aminoácidos por um sistema L transportador situado na membrana citosólica. Inicialmente há uma transaminação onde o grupo amino é removido pela aminotransferase dos α-aminoácidos de cadeia ramificada, originando os respectivos cetoácidos: KIC, KMV e KIV.

Os cetoácidos são translocados por um transportador especifico para a mitocôndria onde sofrem uma descarboxilação oxidativa e o grupo carboxílico dos cetoácidos é removido por um complexo enzimático, a desidrogenase dos α-cetoácidos de cadeia ramificada (BCKD).

Os produtos da descarboxilização oxidativa dos cetoácidos produzidos pelo complexo mitocondrial BCKD são, respectivamente, isovaleril-CoA, α-metilbutiril-CoA e isobutiril-CoA. Estes compostos sofrem uma desidrogenação por uma acil-CoA deisdrogenase específica. Neste passo as rotas metabólicas dos aminoácidos divergem. Os derivados da leucina originam acetil-CoA e acetoacetato, os derivados da isoleucina originam acetil-CoA e succinil-CoA e os derivados da valina só succinil-CoA

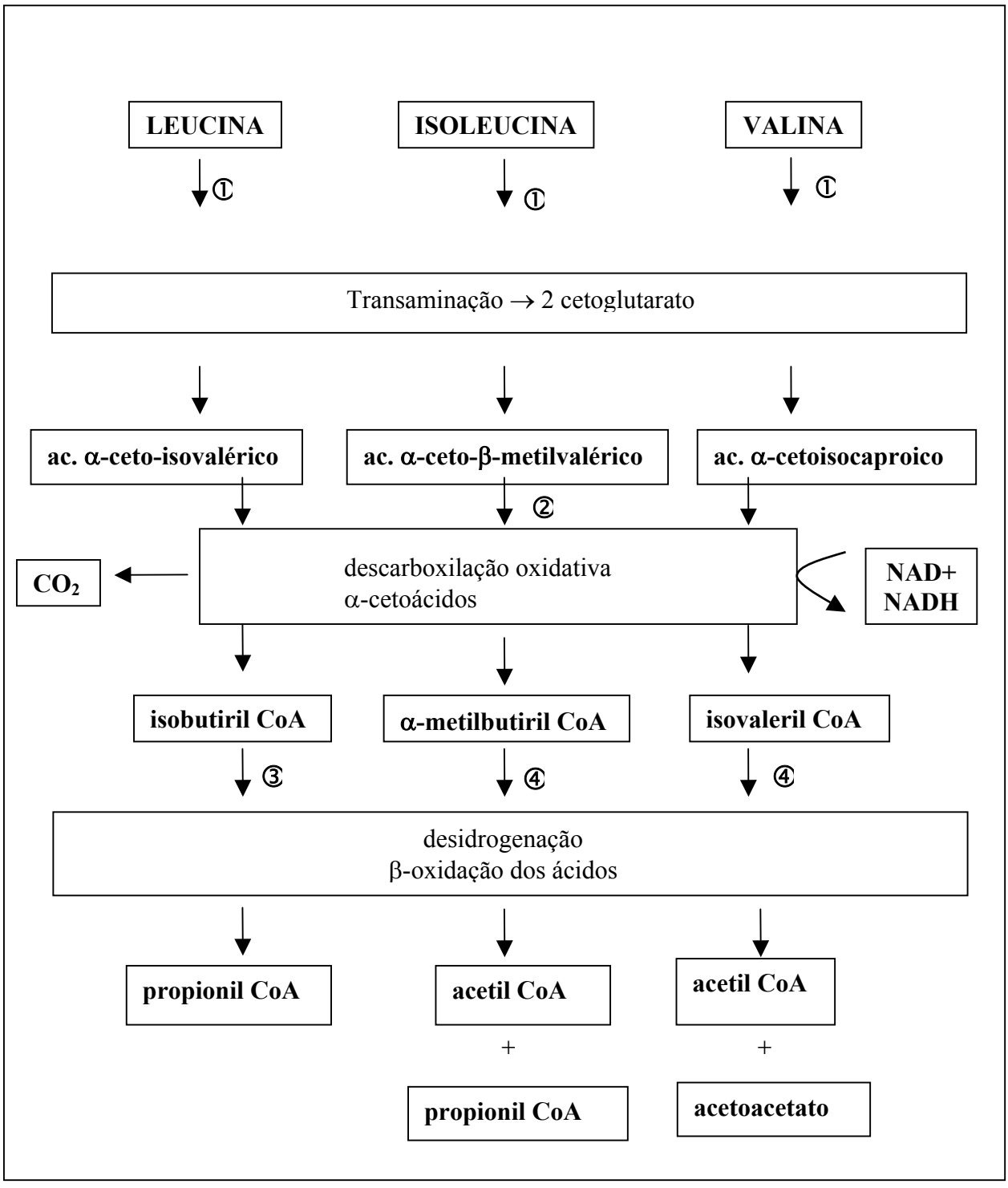


Fig. 5. Metabolismo dos aminoácidos de cadeia ramificada. ① aminotransferase dos aminoácidos de cadeia ramificada, ② desidrogenase dos α-cetoácidos de cadeia ramificada, ③ desidrogenase do isovaleril CoA, ④ desidrogenase de cadeia ramificada α-metil-acil CoA.

2.3 O complexo enzimático desidrogenase dos α-cetoácidos de cadeia ramificada (BCKD)

A Doença do Xarope do Bordo (MSUD) é causada pela deficiência na atividade do complexo enzimático da desidrogenase dos α-cetoácidos de cadeia ramificada acumulando leucina, isoleucina, valina e seus respectivos aminoácidos.

A desidrogenase dos α-cetoácidos de cadeia ramificada é um complexo enzimático caracterizado por ter múltiplas atividades. Está localizada na membrana mitocondrial interna e possui três componentes catalíticos (E1, E2, e E3). É regulada por duas enzimas (uma fosfatase e uma quinase da desidrogenase dos α-cetoácidos de cadeia ramificada). O componente catalítico E1 (2-oxoisovalerato desidrogenase) é dependente de pirofosfato de tiamina e possui duas distintas subunidades, α-E1 e β-E1, que formam o α-2 β-2 heterotetrâmero, onde ocorre a maioria das mutações. O componente catalítico E2 (di-hidrolipoamida aciltransferase) é constituído por uma transacilase. O complexo E3 (di-hidrolipoamida desidrogenase) é uma desidrogenase e está associado com dois complexos alfa-cetodesidrogenases adicionais: a piruvato desidrogenase e a alfa-cetoglutarato desidrogenase. O E3 é constituído por uma flavoproteína, regulada por uma quinase e uma fosfatase, que controlam a atividade do complexo. A mutação em E3 causa também deficiência da piruvato desidrogenase e da alfa-cetoglutarato desidrogenase.

Mutações em E1, E2 e E3 causam a Doença do Xarope do Bordo. Não há uma correlação entre fenótipos moleculares e clínicos, com exceção da mutação em E3 que causa a MSUD responsiva à tiamina. Mutações em enzimas reguladoras não são descritas.

A deficiência do complexo resulta no acúmulo dos α-cetoácidos e α-hidroxiácidos que aparecem na urina e líquor de pacientes com a Doença do Xarope do Bordo.

2.4 Classificação da Doença do Xarope do Bordo e Aspectos Clínicos

As manifestações clínicas das crianças com MSUD são bastante variáveis. Cinco fenótipos clínicos são identificados e podem ser diferenciadas conforme a idade, a severidade dos sintomas e a resposta ao tratamento com tiamina. Estes fenótipos clínicos constituem as formas: clássica, intermediaria, intermitente, responsiva à tiamina e a deficiente de E3 (Chuang e Shih, 2001).

A forma **clássica** de MSUD é a mais comum, a mais severa e desenvolve-se rapidamente. A atividade enzimática do complexo está ausente ou apresenta apenas 2 % da atividade normal. A doença manifesta-se em recém nascidos, na primeira semana de vida, e os níveis dos aminoácidos de cadeia ramificada, especialmente a leucina, estão muito aumentados no sangue, líquor e urina. Os cetoácidos derivados destes aminoácidos também estão aumentados. A presença de aloleucina confirma o diagnóstico. Os pacientes apresentam sinais neurológicos como distonia, encefalopatia e alternam episódios de hipotonia e hipertonia. A criança inicialmente tem dificuldade de alimentar-se e perde peso. Aparece o cheiro de açúcar queimado na urina, apnéia e hipoglicemia. A descoordenação motora transitória tem sido relatada em pacientes com a forma clássica de MSUD. Pancreatite foi ocasionalmente observada, cetose e o odor de açúcar queimado são

normalmente encontrados. Os recém nascidos mostram sinais neurológicos progressivos com retardo de crescimento, e na maioria das vezes morrem nos primeiros meses de vida.

A forma **intermediaria** é uma variante da forma clássica e a atividade enzimática é mais alta. Os pacientes apresentam de 3 a 30 % da atividade enzimática normal. É mais rara que a forma clássica e os pacientes toleram maior quantidade de leucina. A alteração neurológica e o retardo de crescimento são variáveis. A deficiência neurológica é mais branda. A doença manifesta-se mais tarde quando apresenta sintomas da doença clássica, entre 5 meses e 7 anos e os pacientes podem apresentar anemia por deficiência de ferro, hiperuricemia e moderada acidose. Apresenta aumento de isoleucina e dos aminoácidos e cetoacidos de cadeia ramificada.

A forma **intermitente** da Doença do Xarope do Bordo é a segunda forma mais comum. Os pacientes mostram um desenvolvimento de crescimento e inteligência quase normal. A atividade do complexo enzimático varia entre 5 e 10% do normal. Geralmente, as crianças não têm sintomas ao nascer e estes começam a aparecer no primeiro ou segundo ano de vida, embora possam aparecer mais tarde na vida adulta. Em situações de estresse catabólico, como otite média ou outras infecções, podem descompensar e apresentar episódios da doença. Durante estes episódios pode acontecer ataxia, letargias e coma, que devem ser tratados com dieta adequada. O diagnóstico correto pode prevenir os episódios de descompensação metabólica e os sintomas da doença.

A forma de MSUD **tiamina responsiva** é rara e mais branda. Somente a forma inicial foi relatada por Scriver et al. (1971). Todos os pacientes mostram uma melhora no

controle metabólico, com aumento da atividade enzimática, quando submetidos a uma dieta com adição de tiamina e restrição de aminoácidos de cadeia ramificada.

A forma **E3 deficiente** é muito rara e poucos foram os casos relatados na literatura médica. As manifestações clínicas são algumas vezes semelhantes à forma intermediaria, apresentando ainda severa acidose lática. Estes pacientes apresentam uma progressiva deterioração neurológica com perda de mielina que ocasiona hipotonia, retardo de crescimento e movimentos desordenados. Alem da deficiência de BCKD possuem ainda deficiência de piruvato desidrogenase e alfa-cetoglutarato desidrogenase.

Chuang (1995) sugeriu denominar o defeito na subunidade α-E1 de tipo IA, o defeito na subunidade β-E1 de tipo IB e o defeito na subunidade E3 de tipo III. O tipo III combina a deficiência enzimática de três enzimas mitocondriais: o complexo da desidrogenase dos alfa-cetoácidos de cadeia ramificada, a piruvato desidrogenase e a alfa-cetoglutarato desidrogenase.

Tabela 1. Fenótipos clínicos e bioquímicos na Doença do Xarope do Bordo**.**

Fenótipo	**Aspectos clínicos**	**Aspectos bioquímicos**	**Atividade da BCKDH**
Clássica	Início neonatal Dificuldade de alimentação Letargia Tônus aumentado./diminuído. Cetoacidose Convulsões	Aumento de Leu, Ile,Val Aloleucina	0 2 %
Intermediária	Deficiência de crescimento Atraso no desenvolvimento Cetoacidose pouco freqüente	Aumento de Leu, Ile,Val Aloleucina	3 – 30 %
Intermitente	No início desenvolvimento normal Episódios de ataxia Cetoacidose Precipitados por infecção ou estresse que podem ser fatais Geralmente inteligência normal	Leu, Ile, Val normais quando assintomático	5 – 20 %
Responsiva à tiamina	Similar à forma intermediária	Normalização de Leu, Ile, Val, e respectivos ceto ácidos quando respondem à terapia com tiamina	2 – 40 %
Deficiência de lipo desidrogenase (E3)	Usualmente sem sintomas neonatais Deficiência de crescimento Hipotonia Acidose láctica Atraso no desenvolvimento Movimentos desordenados Deterioração progressiva	Leu, Ile , Val e respectivos ceto-, ácidos moderadamente aumentados Aumento de piruvato e alfa-cetoglutarato	0 – 25

Extraído de Bürger (1998) e adaptado de Chuang, 1995.

2.5 Aspectos genéticos

A Doença do Xarope do Bordo é familiar e recessiva com genes localizados nos cromossomos 19q13.1 – q13.2 para o tipo 1; 1p31 para o tipo 2; 6p22 – p21 para o tipo 3. A doença é explicada pelas várias mutações que ocorrem nos loci das sub-unidades catalíticas α-E1 para o tipo1; β-E1 para o tipo 2 , E2 e E3 do complexo da desidrogenase dos α-cetoácidos de cadeia ramificada. O gene de cada subunidade está localizado em diferentes cromossomos.

A terapia gênica acena atualmente com a possibilidade de correção do tipo IA. Um paciente Menonita com MSUD teve a atividade da BCKD completamente restaurada após estabilização da subunidade α-E1 deficiente com a transdução de linfoblastos mediada por retrovirus (Chuang e Shih, 2001).

3 A energia cerebral

O ATP é a fonte de energia para muitos processos importantes na célula, e seus níveis não devem cair durante os processos de utilização, como a contração muscular e estimulação cerebral. Nestes tecidos o ATP está continuamente sendo reabastecido pela ação da creatinaquinase (Wyss et al.,1992).

O estudo do metabolismo energético cerebral é relativamente recente (Hertz e Peng, 1992; Wyss et al., 1992; Erecinska e Silver, 1994; Ames, 2000). Os processos vegetativos utilizam pouca energia, mas o metabolismo energético aumenta com a atividade mental, restaurando gradientes iônicos alterados durante a excitação nervosa (transporte de íons, por exemplo) que consomem a maior parte do ATP produzido.

O Sistema nervoso central funciona gerando, processando e transmitindo impulsos para manter o desequilíbrio iônico através da membrana neural. As interações entre os diferentes tipos de células são de suprema importância para a função do sistema nervoso central, como a liberação excitatória do glutamato pelo neurônio e sua captação pelo astrócito (Hertz e Peng, 1991). Há uma clara correlação entre o metabolismo energético, a concentração extracelular do potássio e a atividade da Na^+, K^+-ATPase (Sokoloff, 1981).

As células requerem energia para sobrevivência e realização de múltiplas tarefas biológicas, e o sistema nervoso central exige para o seu funcionamento mais energia que outros tecidos.

O metabolismo energético é altamente organizado na célula e mecanismos especiais estão envolvidos na transferência de energia do local de geração para o local de consumo. Conseqüentemente, há diferenças energéticas em diferentes locais numa mesma célula. O balanço entre a produção e o consumo é regulado de forma eficiente e econômica pelo cérebro, sendo a energia gerada rapidamente utilizada, reduzindo a reserva produzida.

O entendimento do mecanismo que envolve a energia cerebral é de extrema importância para a compreensão de vários estados patológicos.

3.1 O estudo energético

A glicólise e a fosforilação oxidativa são as principais rotas produtoras de energia no tecido nervoso dos mamíferos e estão particularmente interligadas. A Na^+,K^+-ATPase consome 50 a 60 % da energia gerada no sistema nervoso central, da qual 5 % provém da degradação da glicose e 95 % do metabolismo oxidativo (Erecinska e Silver, 1994).

O metabolismo energético é estudado através de compostos da degradação da glicose como CO_2, glicose, lactato; de nucleotídeos da adenina como ATP, ADP, AMP; de enzimas da via glicolítica como hexoquinase, fosfofrutoquinase e piruvatoquinase; de enzimas envolvidas na degradação ou síntese de ATP como a Na^+,K^+-ATPase, creatinaquinase e adenilatoquinase ou ainda com os compostos da cadeia respiratória. A idéia de que um "pool" de ATP, proveniente da glicólise ou fosforilação oxidativa, estaria a disposição no interior da célula é pouco aceita hoje em dia. A energia do ATP, gerada na mitocôndria, tem um deslocamento lento dentro da célula, muito mais lento que a difusão da água. A transferência de energia para locais de consumo seria limitada se dependesse somente do ATP e a atividade da ATPase poderia ser inibida pelo acúmulo de ADP. Um ~P deve ser levado rapidamente, através da célula, para locais onde estão as enzimas que consomem energia. Um sistema altamente organizado gera e entrega de ~P. A

compartimentalização de enzimas geradoras de ATP, a justaposição destes locais aos de consumo de ATP, aliados ao provimento de ~P para ATPases estabelecem um "feedback" resultando numa geração de energia proporcional ao consumo.

Técnicas histoquímicas demonstraram a presença de subestruturas compartilhadas, contendo locais produtores ou geradores de energia, além da habilidade do neurônio em controlar seletivamente a energia produzida (Thompson et al., 1980).

Além da cadeia respiratória há dois sistemas enzimáticos no cérebro, que auxiliam na manutenção dos níveis de ATP, um dependente de creatinaquinase e fosfocreatina, e o outro dependente de adenilatoquinase e ADP, os quais atuando em conjunto, reduzem estas limitações da reserva de ATP.

Por outro lado, já foi observado que a regulação da creatinaquinase e do ATP no cérebro altera-se na ausência do CK mitocondrial indicando que a produção de energia cerebral esta altamente relacionada com a creatinaquinase (Kekelidze et al., 2001).

4 Objetivos

4.1 Objetivo geral

O objetivo principal desta tese foi investigar se os aminoácidos e os cetoácidos de cadeia ramificada, que acumulam na Doença do Xarope do Bordo, possuem algum efeito sobre a atividade da creatinaquinase em cérebro de ratos em desenvolvimento e, em caso positivo, caracterizar este efeito.

4.2 Objetivos específicos

Estes estudos foram desenvolvidos sob a forma de quatro objetivos específicos:

1- Investigar o efeito *in vitro* dos aminoácidos de cadeia ramificada e de seus cetoácidos sobre a atividade da creatinaquinase em homogeneizado total de córtex cerebral, cerebelo e cérebro médio de ratos jovens.

2- Investigar o efeito da administração aguda e crônica de leucina sobre a atividade da creatinaquinase em homogeneizado total e nas frações citosólica e mitocondrial de córtex cerebral, cerebelo e cérebro médio de ratos jovens.

3- Caracterizar o mecanismo de inibição causado pelos aminoácidos de cadeia ramificada sobre a atividade da creatinaquinase em homogeneizado total de córtex cerebral de ratos jovens.

4- Caracterizar o mecanismo de inibição causado pelos aminoácidos de cadeia ramificada sobre a atividade da creatinaquinase em homogeneizado total de cerebelo e cérebro médio de ratos jovens.

CAPÍTULO II

RESULTADOS

Artigo 1

Objetivo 1- Investigar o efeito *in vitro* dos aminoácidos de cadeia ramificada e de seus cetoácidos sobre a atividade da creatinaquinase em homogeneizado total de córtex cerebral, cerebelo e cérebro médio de ratos jovens.

. **Creatine kinase activity from rat brain is inhibited by branched-chain amino acids *in vitro*.**

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Creatine kinase activity from rat brain is inhibited by branched-chain amino acids *in vitro*

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Running title: Branched-chain amino acids and creatine kinase

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ABSTRACT

Maple syrup urine disease (MSUD) is an inherited metabolic disorder biochemically characterized by the accumulation of branched-chain amino acids (BCAA) and their branched-chain keto acids (BCKA) in blood and other tissues. Neurological dysfunction is usually present in the affected patients, but the mechanisms of brain damage in this disease are not fully understood. Considering that brain energy metabolism seems to be altered in MSUD, the main objective of this study was to investigate the *in vitro* effect of BCAA and BCKA on creatine kinase activity, a key enzyme of energy homeostasis, in brain cortex of young rats. BCAA, but not their BCKA, significantly inhibited creatine kinase activity at concentrations similar to those found in the plasma of MSUD patients (0.5 to 5 mM). Considering the crucial role creatine kinase plays in energy homeostasis in brain, if this effect also occur in the brain of MSUD patients, it is possible that inhibition of this enzyme activity may contribute to the brain damage found in this disease.

Key words: Maple syrup urine disease, branched-chain ketoaciduria, leucine, branched-chain amino acids, branched-chain keto acids, creatine kinase

INTRODUCTION

The inability to oxidatively decarboxylate the branched-chain α-keto acids (BCKA) α-keto-isocaproic acid (KIC) , α-keto-β-methylvaleric acid (KMV) and α-keto-β-isovaleric acid (KIV) derived from the branched-chain α-amino acids (BCAA) leucine (Leu), isoleucine (Ile) and valine (Val) causes the inherited metabolic disorder maple syrup urine disease (MSUD) (1). BCAA and BCKA accumulate because there are a metabolic block of the mitochondrial branched-chain α-keto acid dehydrogenase complex (BCKD) (2). Surviving children often present a variable degree of brain damage, characterized by mental retardation, neurological difficulties in walking and speech, and seizures (3).

Leu and KIC, the main accumulating metabolites, are considered to be the most important neurotoxic substances in MSUD, but the mechanisms of brain damage in this disease are still poorly known (3). Severe brain edema, myelin deficiency, striking spongy degeneration of white matter, cerebellar necrosis and considerable nerve-cell loss in substantia nigra and pontine nuclei suggest that brain energetic homeostasis is altered in MSUD (4-6).

Creatine kinase (CK), EC 2.7.3.2, catalyzes the reversible transfer of the N-phosphoryl group from creatine phosphate to ADP regenerating ATP. This enzyme participates in a key system to maintain energy homeostasis of cells with high and fluctuating energy requirement (7). CK isoenzymes are specifically located at places of energy demand and energy production and so are linked by a creatine/creatine phosphate circuit. They are compartmentalized subcellularly and coupled to sites of energy production or energy consumption. The isoforms BB-CK (dimeric) and Mi-CK (octameric) are the cytosolic and mitochondrial forms, respectively, found in the brain (8). 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 (9). Recent findings have reinforced this hypothesis, showing that CK activity is severely reduced in several neurodegenerative diseases (10-12).

Considering that CK activity is reduced in neurodegenerative diseases, and brain energy metabolism is probably altered in MSUD, in this work we investigated the effect of BCAA and its BCKA, compounds known to accumulate in MSUD in human brain, on CK activity in homogenates of brain cortex, cerebellum and midbrain from 22-day-old rats.

EXPERIMENTAL PROCEDURES

Subjects and reagents:

Wistar rats bred in the Department of Biochemistry, UFRGS, were used in the experiments. Eight pups were randomly assigned to each dam. Rats were kept with dams while receiving the drugs until they were sacrificed at the 22nd day of life. 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 n^{o} 85-23, revised 1985) were followed in all the experiments. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Preparation of brain homogenate

Animals were sacrificed by decapitation. The brain was rapidly removed and the brain was dissected on a glass dish over ice. Time elapsed from decapitation to place the brain on the ice was less than one minute. Olfactory bulbs and pons were discarded, and cerebellum, brain cortex and midbrain (the rest of cerebrum) were separated and the tissue was disrupted in ice-cold 300 mOsm NaCl (1/10, w/v) using a ground-glass homogenizer. The homogenate was stored at -70^0 C when the assay was not carried out immediately. Before the enzyme assay, the homogenates were frozen and thawed three times and diluted a hundred times in the same buffer used in the assay.

Creatine kinase activity assay

CK activity was assayed in medium contained 5.9 mM phosphocreatine, 100 mM MgSO4-Trizma buffer pH 7.5 and approximately 1 μg of protein homogenate in a final volume of 0.1 mL. After 5 minutes of pre-incubation the reaction was initiated by the addition of 0.3 nmol of ADP-glutathione. The reaction was stopped after 10 min by the addition of 1μmol of p-hydroxymercuribenzoic acid. The creatine liberated was estimated according to Hughes (13). Color was developed by the addition of 0.1 mL 20 % α-naphtol and 0.1 mL 20 % diacetyl and read after 20 minutes at 540 nm. For the *in vitro* studies, BCAA or its BCKA were dissolved in 100 mM MgSO4-Trizma buffer pH 7.5. and added to the incubation medium at final concentrations ranging from 1 to 5 mM for Leu or KIC and from 0.5 to 2.0 mM for the other substances. These concentrations are similar to that found in plasma of MSUD patients. Results were expressed as μmol of creatine formed per min per mg of protein.

Protein determination

Protein was measured according to Lowry (14) using serum bovine albumin as standard.

Statistical analysis

Data from the experiments were analyzed by one-way analysis of variance (ANOVA) followed by the LSD test when F value was significant. For analysis of dose-dependent effect, linear regression was used. All analyses were carried out in an IBM compatible PC using the Statistical Package for the Social Sciences (SPSS) software.

RESULTS

The *in vitro* effect of BCAA, as well as the correspondent BCKA, on the enzyme activity in the brain homogenates of the brain structures from twenty two-day-old rats was investigated. Leu, Ile, and Val significantly inhibited CK activity in the homogenates of brain cortex in a dose-dependent manner (Fig 1): [$F(1,39) = 20.01$; $p< 0.001$; $\beta = -0.61$; $t = 4.47$; $p< 0.001$]; Ile: [$F(1,37) = 17.761$; $p< 0.001$; $\beta = -0.57$; $t = 4.21$; $p< 0.001$]; Val [$F(1,38) = 56.46$; $p< 0.001$; $\beta = -0.77$; $t = 4.57$; $p< 0.001$]. Similar effects were observed for midbrain (Fig 2): Leu: [$F(1,42) = 24.91$; $p< 0.001$; $\beta = -0.61$; $t = 4.99$; $p< 0.001$]; Ile [$F(1,33) = 20.86$; $p< 0.001$; $\beta = -0.62$; $t = 4.57$; $p< 0.001$]; Val [$F(1,34) = 14.69$; $p< 0.001$; $\beta = -0.55$; $t = 3.83$; $p< 0.001$]. All three BCAA also significantly inhibited CK activity in cerebellum homogenates in a dose-dependent way (Fig 3) : Leu: [$F(1,40) = 21.89$; $p< 0.001$; $\beta = -0.59$; $t = 4.68$; $p< 0.001$]; Ile: [$F(1,29) = 18.12$; $p< 0.001$; $\beta = -0.62$; $t = 4.26$; $p< 0.001$]; Val [$F(1,33) = 15.06$; $p< 0.001$; $\beta = -0.56$; $t = 3.88$; $p< 0.001$].

In contrast, BCKA did not alter CK activity in the homogenates of the brain structures studied. KIC: brain cortex [$F(5,30) = 1.32$; $p>0.3$]; cerebellum [$F(5,30) = 0.62$; $p>0.68$]; midbrain [$F(5,24) = 0.27$; $p>0.90$]; KMV: brain cortex [$F(4,25) = 0.81$; $p>0.53$]; cerebellum [$F(4,25) = 0.36$; $p>0.82$]; midbrain [$F(4,25) = 0.38$; $p>0.81$]; KIV: brain cortex [$F(4,25) = 1.57$; $p>0.23$]; cerebellum [$F(4,25) = 0.18$; $p>0.93$]; midbrain [$F(4,25) = 0.66$; $p>0.61$].

DISCUSSION

BCAA and their BCKA accumulate in plasma and tissues of MSUD affected patients. Neurological dysfunction is common in these patients but the mechanisms underlying the pathophysiology of this disorder seem to be multiple and poorly known. It has been proposed that brain cells in MSUD may be subjected to two mechanisms that limit the availability of LNAA: competition for transport of LNAA at the blood-brain barrier and trans-stimulated exchange out of neuronal cells for subsequent metabolism or sequestration in the periphery (15). In this context, we have reported a reduction of large neutral amino acid (LNAA) in plasma and brain of hyperleucinemic rats (16). On the other hand, we have reported that all amino acids, keto acis and hydroxy acids accumulating in this disease stimulate the *in vitro* lipid oxidation in rat brain (17). Other mechanisms of neurotoxicity of metabolites accumulated in MSUD proposed by us and by other investigators include: oxidation of glutamate and glutamine (18), inhibition of glutamate uptake into synaptic vesicles (19,20), apoptosis (21,22), and induction of convulsions through GABAergic and glutamatergic NMDA mechanisms. (23).

In the present study we demonstrated that the BCAA, but not their correspondent BCKA, inhibit the *in vitro* activity of CK in the brain of rats, in a dose-dependent manner,

at concentrations similar to those found in plasma of MSUD patients. This indicates that not only Leu, but also the others two BCAA accumulating in MSUD may be neurotoxic, altering brain energetic homeostasis. It is important to emphasize that the inhibition of CK activity caused by BCAA occurred in all brain structures studied, correlating with the morphologic changes found in the brain of MSUD patients (4-6).

It is possible that the inhibitory effect of BCAA on CK activity observed *in vitro*, also occurs *in vivo,* since the BCAA are rapidly transported into the brain (24) and accumulate in brain of MSUD patients (25). On the other hand, we also observed in the present study that BCKA did not affect the *in vitro* CK activity. Because KIC achieve high concentrations in the plasma of MSUD patients (0.6 to 4.6 mM), it is possible that this keto acid may reduce *in vivo* the CK activity, since it has been reported that KIC is rapidly taken up by neurons and actively transaminated to Leu, increasing the concentration of this amino acid in brain tissue (24,26). Considering that morphologic changes found in the brain of MSUD patients point to alteration in the energy metabolism in the central nervous system (4-6), it is feasible to envisage that the inhibition of CK activity caused by BCAA could contribute to the brain energy alteration in this disease.

The CK/creatine phosphate system exerts three integrated functions in Brain cells: temporary energy buffering, metabolic capacity, and energy transfer and metabolic control (27,28). This system is now recognized as an important metabolic regulator during health and disease (29). A decrease in CK activity is one of the biochemical markers of brain cell damage in age-related neurodegenerative diseases, including Alzheymer's disease (30). The decrease of CK activity in the brain correlates well with the neurodegeneration parameters in severely affected regions in Alzheimer's disease (31). Therefore, damage of CK function may be an important part of a neurodegenerative pathway that leads to

neuronal loss in the brain (9). 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 (32). On the other hand, considering that CK and the creatine-creatine phosphate energy shuttle may play a role in brain development that is associated with oligodendrocyte function and/or myelogenesis (33), the reduction of oligodendrocytes and myelin deficiency, the most prominent morphologic changes found in the brain of MSUD patients (3) may be possibly associated to reduction of brain CK activity.

In summary, the results show that BCAA, compounds known to accumulate in MSUD in human brain, inhibit CK activity in the brain of young rats. Considering that creatine kinase is a key enzyme for energy homeostasis, if this enzyme inhibition also occurs in the brain of MSUD patients, it is possible that the diminution 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. Further studies will be necessary to evaluate whether creatine supplementation may benefit MSUD patients.

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Fig 1. *In vitro* effect of leucine, isoleucine and valine on creatine kinase activity in homogenates of brain cortex from young rats.

Data are mean ± S.D. for 6-8 independent experiments performed in triplicate. Different from control, * $p < 0.05$, ** $p < 0.01$ (LSD test)

Fig.2. *In vitro* effect of leucine, isoleucine and valine on creatine kinase activity in homogenates of midbrain from young rats.

Data are mean ± S.D. for 6-8 independent experiments performed in triplicate. Different from control, * $p < 0.05$, ** $p < 0.01$ (LSD test)

Fig.3. *In vitro* effect of leucine, isoleucine and valine on creatine kinase activity in homogenates of cerebellum from young rats.

Data are mean ± S.D. for 6-8 independent experiments performed in triplicate. Different from control, * $p < 0.05$, ** $p < 0.01$ (LSD test)

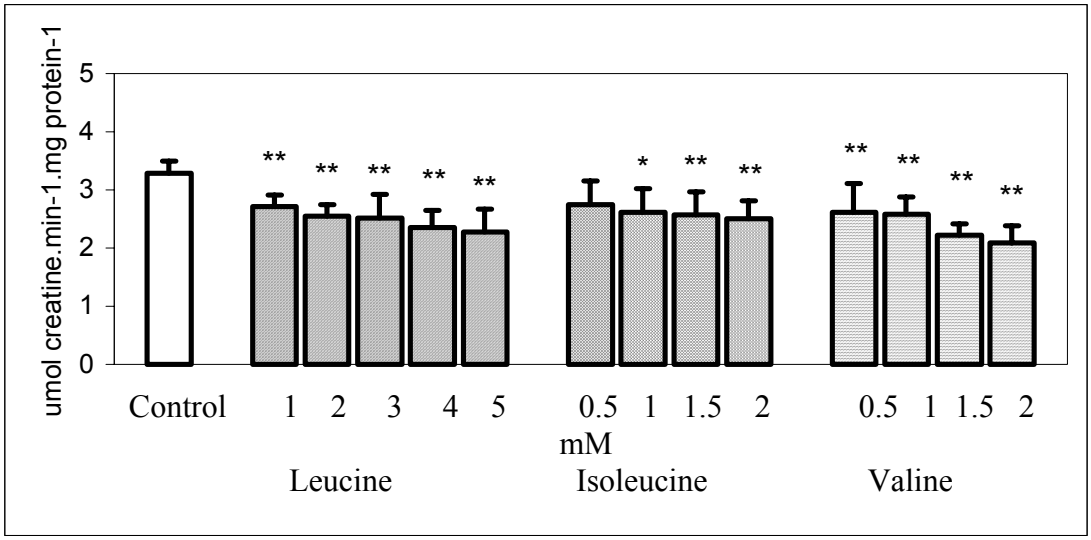
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Figure 1

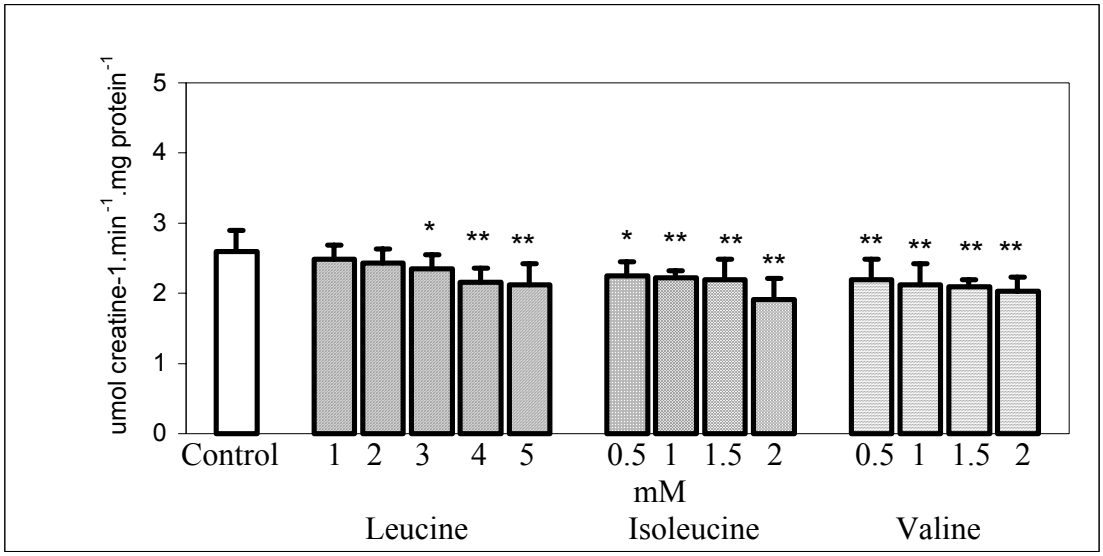
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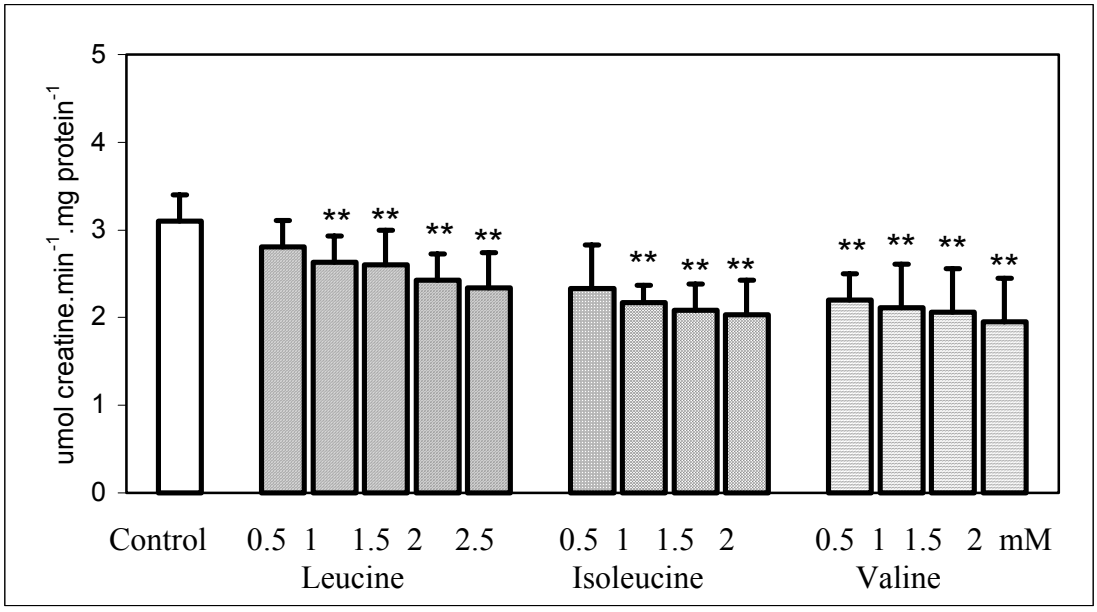
Figure 2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Control
0.5 1 1.5 2 2.5
Leucine
0.5 1 1.5 2
Isoleucine
0.5 1 1.5 2 mM
Valine

Figure 3

Artigo 2

Objetivo 2- Investigar o efeito da administração aguda e crônica de leucina sobre a atividade da creatinaquinase em homogeneizado total e nas frações citosólica e mitocondrial de córtex cerebral, cerebelo e cérebro médio de ratos jovens.

Effect of leucine administration on creatine kinase activity in rat brain.

Carmen Pilla, Rui Felipe de Oliveira Cardozo, Carlos Severo Dutra-Filho, Angela Terezinha Souza Wyse, Moacir Wajner, Clóvis Milton Duval Wannmacher.

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Effect of Leucine Administration on Creatine Kinase Activity in Rat Brain

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Maple syrup urine disease (MSUD) is a metabolic disorder biochemically characterized by the accumulation of branched-chain amino acids (BCAA) and their branched-chain keto acids (BCKA) in blood and tissues. Neurological dysfunction is usually present in the patients, but the pathophysiology of brain damage is still obscure. Considering that brain energy metabolism is possibly altered in MSUD, the main objective of this study was to determine creatine kinase activity in the brain of rats subjected to acute and chronic administration of leucine. Chronic hyperleucinemia was induced by subcutaneous administrations of 4.8 μmol leucine/g body weight, twice a day, from the 6th to the 21st postnatal day. For acute hyperleucinemia, 21-day-old rats received three administrations of the amino acid at 3 h interval. Twelve hours after the chronic treatment or 1 h after the acute one, rats were killed and creatine kinase activity measured. The results indicated that acute or chronic administration of leucine altered creatine kinase activity in the brain of leucine-treated rats. Considering the crucial role creatine kinase plays in energy homeostasis in brain, if these effects also occur in the brain of MSUD patients, it is possible that alteration of this enzyme activity may contribute to the brain damage found in this disease.

Key words: Maple syrup urine disease; branched-chain ketoaciduria; leucine; creatine kinase.

INTRODUCTION

Maple syrup urine disease (MSUD), or branched-chain ketoaciduria, is an inherited autosomal disease caused by a deficiency in the activity of branched-chain α-keto acid dehydrogenase complex (BCKD). As a result of this deficiency, the branched-chain α-amino acids (BCAA) leucine (Leu), isoleucine (Ile), and valine (Val) and their branched-chain α-keto acids (BCKA) α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV), and α-ketoisovaleric acid (KIV), respectively, accumulate in blood and tissues (Dancis *et al.*, 1960; Menkes, 1959). Surviving children often present a variable degree of brain damage, characterized by mental retardation, neurological difficulties in walking and speech, and seizures (Chuang and Shih, 2001). MSUD is predominantly a white-matter disorder, and the neuropathologic changes include severe brain edema, myelin deficiency, striking spongy degeneration of white matter, cerebellar necrosis and considerable nerve-cell loss in

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substantia nigra and pontine nuclei, suggesting that brain energetic homeostasis is altered in this disease (Kamei *et al.*, 1992; Kiil and Rokkones, 1964; Riviello *et al.*, 1991).

We have previously reported that chronic Leu administration induces behavioral deficits in rats (Mello *et al.*, 1999), in agreement with others that consider Leu and/or its keto acid KIC, as the main neurotoxic metabolites in this disease (Chuang and Shih, 2001). However, the mechanisms of brain damage in MSUD are still poorly known.

Creatine kinase (CK), EC 2.7.3.2, catalyzes the reversible transfer of the *N*-phosphoryl group from creatine phosphate to ADP, regenerating ATP. This enzyme catalyses a critical reaction to maintain energy homeostasis of cells with high and fluctuating energy requirement (Wallimann *et al.*, 1992). CK isoenzymes are specifically located at places of energy demand and energy production and so are linked by a creatine/creatine phosphate circuit. They are compartmentalized subcellularly and coupled to sites of energy production or energy consumption. The isoforms B-CK (dimeric) and Mi-CK (octameric) are the cytosolic and mitochondrial forms, respectively, founds in the brain (Wyss *et al.*, 1992). 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.*, 1993). Recent findings have reinforced this hypothesis, showing that CK activity is severely reduced in several neurodegenerative diseases (Aksenov *et al.*, 2000; David *et al.*, 1998).

Considering that CK activity is reduced in neurodegenerative diseases, brain energy metabolism is probably altered in MSUD, and Leu is considered the most probable neurotoxic BCAA, in this work we investigated the effect of acute and chronic Leu administration on CK activity in the brain of young rats.

MATERIAL AND METHODS

Subjects and Reagents

Wistar rats bred in the Department of Biochemistry, UFRGS, were used in the experiments. Eight pups were randomly assigned to each dam. 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 no. 85-23, revised 1985) were followed in all the experiments. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Leucine Treatment

Chronic Treatment

Hyperleucinemia was induced by daily subcutaneous administration of Leu, from the 6th to the 21st day of life. The animals were randomly separated into two groups and were

injected twice a day at 8 h intervals. Leucine was disolved in saline (0.85%) and buffered to pH 7.4 with NaOH and administered in doses calculated according to body weight established from pharmacokinetic parameters in our laboratory (Mello *et al.*, 1999). Half of the animals received subcutaneous administrations of 4.8 μmol leucine/g body weight, and the others received the same volume of saline solution. Twelve hours after treatment the rats were killed by decapitation without anesthesia, and the brain was rapidly removed for creatine kinase activity and protein determinations.

Acute Treatment

Twenty 2-day-old Wistar rats were used. The animals were randomly separated into two groups. Buffered Leu solution was administered to half of the animals, three times at 3 h intervals, in the same doses stated above (4.8 μmol leucine/g body weight). Control rats received the same volume of saline solution. Rats were killed by decapitation without anesthesia 1 h after the last injection and the brain was rapidly removed.

By using these doses, Leu-treated animals achieve maximal plasma Leu levels similar to that found in the plasma of MSUD patients 30 min after subcutaneous injection of the amino acid. Maximal brain Leu levels are achieved 60 min after amino acid administration. Twelve hours after injection, Leu levels return to the normal levels in plasma and brain (Mello *et al.*, 1999).

Preparation of Brain Cortex Homogenate and Fractions

The brain was dissected on a glass dish over ice. Time elapsed from decapitation to place the brain on the ice was less than 1 min. Olphatory bulbs and pons were discarded, and cerebellum, brain cortex and midbrain (the rest of cerebrum) were separated, and the tissue was disrupted in ice-cold 300 mOsm NaCl (1/10, w/v) using a ground-glass homogenizer. The homogenate was centrifuged at $800 \times g$ for 10 min, the pellet was discarded and the supernatant was centrifuged for $10{,}000 \times g$ for 15 min. The supernatant of the second centrifugation was collected for determination of cytosolic CK activity, and the pellet was washed two times with TRIS-sacarose 10 mM isotonic buffer pH 7.5 and resuspended in 100 mM MgSO4–Trizma buffer pH 7.5 for determination of mitochondrial CK activity. Homogenate, cytosolic, and mitochondrial fractions were stored at -70°C when the assay was not carried out immediately. Before the enzyme assay, the homogenate and the fractions were frozen and thawed three times.

Creatine Kinase Activity Assay

CK activity was assayed in medium containing 5.9 mM creatine phosphate, 100 mM MgSO4–Trizma buffer pH 7.5, and approximately 1 μg of protein homogenate in a final volume of 0.1 mL. After 5 min of preincubation, the reaction was initiated by the addition of 0.3 nmol of ADP-glutathione. The reaction was stopped after 10 min by the addition of 1 μmol of *p*-hydroxymercuribenzoic acid. The creatine liberated was estimated according

to Hughes (1962). Color was developed by the addition of 0.1 mL 20% α-naphtol and 0.1 mL 20% diacetyl and read after 20 min at 540 nm. Results were expressed as μmol of creatine formed per minute per mg of protein.

Protein Determination

Protein was measured according to Lowry *et al.* (1951) using bovine albumin as standard.

Statistical Analysis

Data were analyzed by the Student's t-test for independent samples. All analyses were carried out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

RESULTS

CK activity was measured in homogenates of brain cortex (Fig. 1), midbrain (Fig. 2), and cerebellum (Fig. 3) from rats subjected to acute and chronic Leu administration. CK activity was reduced in the homogenates of cerebellum ($t(14) = 5.87$; $p < 0.001$) and midbrain ($t(14) = 3.34$; $p < 0.01$), but was increased in brain cortex homogenate ($t(12) = 2.85$; $p < 0.05$) from rats subjected to acute Leu administration. The mitochondria/cytosol ratio of CK activity did not differ from those of controls in the three brain structures of acutely Leu-treated rats: cerebellum: control $= 1.01 \pm 0.11$; Leu-treated $= 1.09 \pm 0.16$; $t(14) = 1.15$; $p > 0.25$; midbrain: control $= 1.09 \pm 0.21$; Leu-treated $= 0.95 \pm 0.16$; $t(14) = 1.31$;

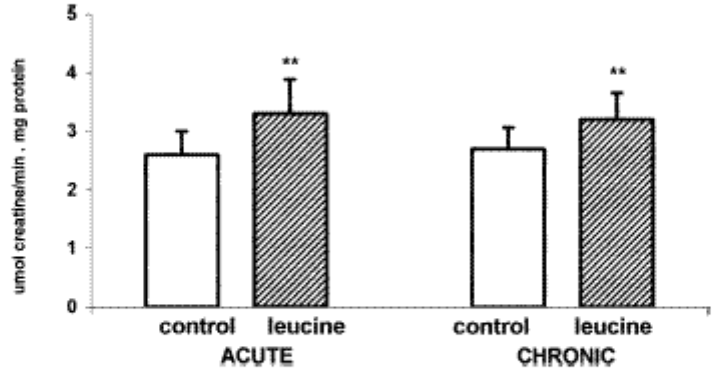

Figure 1. Effect of acute and chronic leucine administration on creatine kinase activity in homogenates from brain cortex of young rats. Data are mean ± SD for 6–8 independent experiments performed in triplicate. Different from control, $^{*}p < 0.05$, $^{**}p < 0.01$ (Student's t-test).

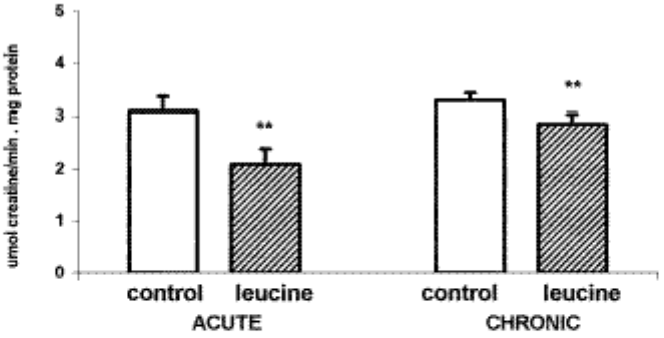

Figure 2. Effect of acute and chronic leucine administration on creatine kinase activity in homogenates from midbrain of young rats. Data are mean ± SD for 6–8 independent experiments performed in triplicate. Different from control, $^{*}p < 0.05$, $^{**}p < 0.01$ (Student's t-test).

$p > 0.20$; brain cortex: control $= 0.91 \pm 0.22$; Leu-treated $= 1.02 \pm 0.12$; $t(12) = 1.22$; $p > 0.20$), indicating that Leu effect was similar on the isoenzymes in the two subcellular fractions.

In chronically treated animals, CK activity was significantly reduced in the homogenates of cerebellum ($t(10) = 2.44$; $p < 0.05$) and midbrain ($t(10) = 3.12$; $p < 0.05$), but was increased in brain cortex homogenates ($t(10) = 3.58$; $p < 0.01$) (Fig. 2). The mitochondria/cytosol ratio of CK activity in the three brain structures of Leu-treated rats did not differ significantly from those of controls: cerebellum: control $= 0.93 \pm 0.15$; Leu-treated $= 0.84 \pm 0.13$; $t(10) = 1.37$; $p > 0.20$; midbrain: control $= 0.81 \pm 0.13$; Leu-treated $= 0.93 \pm 0.22$; $t(10) = 1.18$; $p > 0.25$; and brain cortex: control $= 1.01 \pm 0.12$; Leu-treated $= 1.02 \pm 0.11$; $t(10) = 0.69$; $p > 0.5$ indicating that Leu caused the same effect on the two isoenzymes.

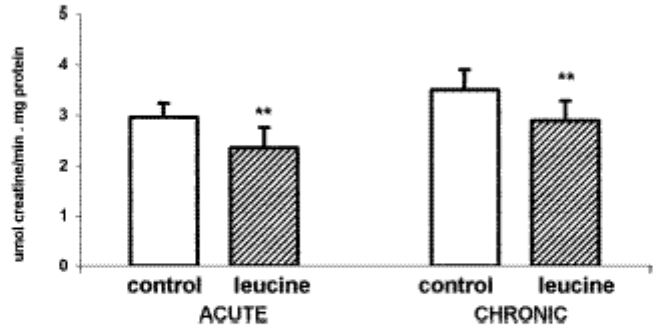

Figure 3. Effect of acute and chronic leucine administration on creatine kinase activity in homogenates from cerebellum of young rats. Data are mean ± SD for 6–8 independent experiments performed in triplicate. Different from control, $^{*}p < 0.05$, $^{**}p < 0.01$ (Student's t-test).

DISCUSSION

BCAA and their BCKA accumulate in plasma and tissues of MSUD affected patients. Neurological dysfunction is common in these patients but the mechanisms underlying the pathophysiology of this disorder seem to be multiple and poorly known. It has been proposed that brain cells in MSUD may be subjected to two mechanisms that limit the availability of large neutral amino acids (LNAA): competition for transport of LNAA at the blood–brain barrier and trans-stimulated exchange out of neuronal cells for subsequent metabolism or sequestration in the periphery (Zielke *et al.*, 2002). In this context, we have reported a reduction of large neutral amino acid (LNAA) in plasma and brain of hyperleucinemic rats (Araujo *et al.*, 2001). On the other hand, we have reported that all amino acids, keto acids, and hydroxy acids accumulating in this disease stimulate the in vitro lipid oxidation in rat brain (Fontella *et al.*, 2002). Other mechanisms of neurotoxicity of metabolites accumulated in MSUD proposed by us and by other investigators include oxidation of glutamate and glutamine (Zielke *et al.*, 1997), inhibition of glutamate uptake into synaptic vesicles (Reis *et al.*, 2000; Tavares *et al.*, 2000), apoptosis (Jouvet *et al.*, 2000), and induction of convulsions through GABAergic and glutamatergic NMDA mechanisms (Coitinho *et al.*, 2001).

We have previously demonstrated that sustained plasma Leu levels, similar to those found in the plasma of MSUD patients, induce behavior deficits in developing rats (Mello *et al.*, 1999). In the present study, we demonstrated that acute or chronic administration of Leu, the major BCAA accumulated in MSUD, increases CK activity in brain cortex and reduces this enzyme activity in midbrain and cerebellum of treated rats. The altered CK activity found in brain cortex, cerebellum, and midbrain of chronically Leu-treated rats is probably not caused by a reversible direct effect of the amino acid on the enzyme, because, at the time of animal sacrifice and brain isolation (12 h after treatment), plasma and brain Leu levels returned to normal levels (Mello *et al.*, 1999). Therefore, the alteration of CK activity may be caused by regulation of expression or posttranslational modification of existing enzyme molecules. This later mechanism was proposed by other investigators who found that the reduced level of CK, observed in several neurodegenerative disorders, is the result of posttranslational modifications of the enzyme (Aksenov *et al.*, 1997, 1999).

The hypothesis that altered CK activity observed after chronic administration of Leu was not a direct enzyme inhibition or stimulation was reinforced by the results obtained with the acute administration of Leu, which also altered the enzyme activity in the same direction. In this experiment, the concentration of Leu in the brain at the time of rat sacrifice (1 h after the last injection) is around 2.2 μmol/g tissue (approximately 3 mM), meaning that the final Leu concentration in the assay medium was as low as 0.3 μM, because the homogenates were diluted 10,000 times for the assay of CK activity.

In respect to the observed increase in CK activity in the brain cortex of Leu-treated rats, it is possible that the amino acid causes a reduction of CK activity in whole brain and, differently from midbrain and cerebellum, the mechanisms of recuperation of enzyme activity are more active in the brain cortex. In this case, CK activity would be elevated when assayed in the absence of significant amounts of Leu. This biphasic response (an initial decreased activity reflecting inactivation of the enzyme, followed by an increased activity corresponding to a response of the organism to maintain the enzyme function) was previously observed in Na^+, K^+-ATPase activity in synaptosomal membranes of brain cortex of rats subjected to phenylalanine administration (Wyse *et al.*, 1995). This hypothesis is consistent with the

neuropathology changes found in the brain cortex of MSUD patients whose neurons and axons are usually well preserved, differently from the other brain structures (Chuang and Shih, 2001). Taken together, the results of the acute and chronic Leu administration suggest that high Leu levels may decrease CK activity in the brain regions more affected by the disease.

It is possible that the inhibitory effect of Leu on CK activity observed in rats, also occurs in the patients, since BCAA are rapidly transported into the brain (Yudkoff, 1997) and accumulate in brain of MSUD patients (Prensky and Moser, 1966). On the other hand, it is possible that KIC, the α-keto acid derived from Leu, may also reduce the CK activity, because it achieves high concentrations in the plasma of MSUD patients (0.6–4.6 mM) and is rapidly taken up by neurons and actively transaminated to Leu, increasing the concentration of this amino acid in brain tissue (Yudkoff, 1997; Zielke *et al.*, 1996). Considering that morphologic changes found in the brain of MSUD patients point to alteration in the energy metabolism in the central nervous system (Kamei *et al.*, 1992; Kiil and Rokkones, 1964; Riviello *et al.*, 1991), it is feasible to envisage that the inhibition of CK activity caused by Leu could contribute to the brain energy deficit in this disease.

The CK/creatine phosphate system exerts three integrated functions in brain cells: temporary energy buffering, metabolic capacity, and energy transfer and metabolic control (Saks *et al.*, 1996; Wallimann *et al.*, 1998a). This system is now recognized as an important metabolic regulator during health and disease (Wallimann *et al.*, 1998b). 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 brain damage (Tomimoto *et al.*, 1993). These findings are reinforced by the observation that creatine and creatine phosphate have neuroprotective effects against energy deprivation and glutamate excitoxicity, attributable to an enhancement of cytosolic high-energy phosphate stores (Brustovetsky *et al.*, 2001). On the other hand, considering that CK and the creatine–creatine phosphate energy shuttle may play a role in brain development that is associated with oligodendrocyte function and/or myelogenesis (Manos *et al.*, 1991), the reduction of oligodendrocytes and myelin deficiency, which are the most prominent morphologic changes found in the brain of MSUD patients (Chuang and Shih, 2001), may be possibly associated to CK activity diminution.

In summary, our results indicate that Leu, the major BCAA accumulating in MSUD, alters the 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 MSUD patients, it is possible that the alteration of this enzyme activity may impair brain energy metabolism, contributing to the brain damage found in this disease. If this is the case, it will be important to perform more studies on CK system in MSUD to evaluate whether creatine supplementation would benefit these patients.

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Artigo 3

Objetivo 3- Caracterizar o mecanismo de inibição causado pelos aminoácidos de cadeia ramificada sobre a atividade da creatinaquinase em homogeneizado total de córtex cerebral de ratos jovens.

Kinetic studies on the inhibition of creatine kinase activity by branched-chain α-amino acids in the brain cortex of rats

Carmen Pilla, Rui Felipe de Oliveira Cardozo, Paula Karine Barcelos Dornelles, Carlos Severo Dutra-Filho, Angela Terezinha de Souza Wyse, Moacir Wajner, and Clóvis Milton Duval Wannmacher.

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Abstract

Maple syrup urine disease (MSUD) is a metabolic disorder biochemically characterized by the accumulation of branched-chain α-amino acids (BCAA) and their branched-chain α-keto acids (BCKA) in blood and tissues. Neurological dysfunction is usually present in the patients, but the mechanisms of brain damage in this disease are far from be understood. The main objective of this study was to investigate the mechanisms by which BCAA inhibit creatine kinase activity, a key enzyme of energy homeostasis, in the brain cortex of 21-day-old Wistar rats. For the kinetic studies, Lineweaver–Burk and a modification of the Chevillard et al. plots were used to characterize the mechanisms of enzyme inhibition. The results indicated that BCAA inhibit creatine kinase by competition with the substrates phosphocreatine and ADP at the active site. Considering the crucial role creatine kinase plays in energy homeostasis in brain, if these effects also occur in the brain of MSUD patients, it is possible that inhibition of this enzyme activity may contribute to the brain damage found in this disease. In this case, it is possible that creatine supplementation to the diet might benefit MSUD patients.

Keywords: Maple syrup urine disease; Branched-chain ketoaciduria; Leucine; Branched-chain α-amino acids; Creatine kinase

1. Introduction

Maple syrup urine disease (MSUD) or branched-chain ketoaciduria is a metabolic inherited disorder caused by a deficiency in the activity of branched-chain α-keto acid dehydrogenase complex (BCKD) accumulating the branched-chain α-amino acids (BCAA) leucine (Leu), isoleucine (Ile) and valine (Val) and their branched-chain α-keto acids (BCKA), α-keto-isocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV) and α-keto-isovaleric acid (KIV) in blood and tissues of the patients (Menkes, 1959; Dancis et al., 1960). Surviving children often present a variable degree of brain damage, characterized by mental retardation, neurological difficulties in walking and speech, and seizures (Chuang and Shih, 2001). MSUD is predominantly a white-matter disorder and the neuropathologic changes include severe brain edema, myelin deficiency, striking spongy degeneration of white matter, cerebellar necrosis and considerable nerve-cell loss in substantia nigra and pontine nuclei, suggesting that brain energetic homeostasis is deficient in this disease (Kiil and Rokkones, 1964; Riviello et al., 1991; Kamei et al., 1992).

Leu and KIC, the main accumulating metabolites, are considered the most important neurotoxic substances in MSUD, but the mechanisms by which this substances cause brain damage in this disease are still poorly known (Chuang and Shih, 2001).

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 participates of an important system to maintain energy homeostasis of cells with high and fluctuating energy requirement (Wallimann et al., 1992). CK isoenzymes are specifically located at places of high energy demand and production, linking energy production and energy utilization by a creatine/phosphocreatine circuit (Wyss et al., 1992). Considering that energy is critical to maintain the development and regulation of cerebral functions, it has been postulated that alteration of the creatine/phosphocreatine circuit may be an important step of a neurodegenerative pathway that leads to neuronal loss in

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the brain (Tonimoto et al., 1993). In this context, it has been reported severely reduced CK activity in several neurodegenerative diseases (David et al., 1998; Aksenov et al., 2000).

Considering that CK activity is reduced in some neurodegenerative diseases, and brain energy metabolism is probably altered in MSUD, in this work we investigated the mechanism of the inhibition caused by BCAA on CK activity in the brain of 22-day-old rats. It is important to know the type of inhibition of the enzyme CK because it is possible that this approach may be used to design drugs to avoid this inhibition.

2. Experimental procedures

2.1. Subjects and reagents

Twenty-four Wistar rats bred in the Department of Biochemistry, UFRGS, were used in the experiments. Eight pups were randomly assigned to each dam. Rats were kept with dams until they were sacrificed at the 21st day of life. 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 Ethics Committee For Animal Research of the Federal University of Rio Grande do Sul. All chemicals were purchased from Sigma.

2.2. Preparation of brain homogenate

Animals were sacrificed by decapitation. The brain was rapidly removed and dissected on a glass dish over ice. Time elapsed from decapitation to place the brain on the ice was less than 1 min. Olfactory bulbs, pons, and cerebellum were discarded, and the brain cortex tissue was disrupted in ice-cold 300 mOsm NaCl (1/10, w/v) using a ground-glass homogenizer. The homogenate was stored at −70 °C when the assay was not carried out immediately. Before the enzyme assay, the homogenate was frozen and thawed three times.

2.3. 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 0.3 μmol ADP plus 0.08 μmol 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 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 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. BCAA did not interfere with spectrometric readings or color development. Results were expressed as μmol of creatine formed per min per mg protein.

2.4. 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.

2.5. Kinetic studies

The competition studies and the K_m (Michaelis constant) values were performed according to Lineweaver and Burk (1934) and the K_i (inhibition constant) values were calculated from the Dixon and Webb (1964) plot. The interaction between Leu, Ile, and Val, 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 1. These concentrations were 3 mM for Leu and 2 mM for Ile and Val. 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.

2.6. Statistical analysis

Data from the experiments were analyzed by Student's *t*-test for independent samples or one-way analysis of variance (ANOVA) followed by the Tukey test when F-value was significant. For analysis of dose-dependent effects, linear regression was used. All analyses were carried out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

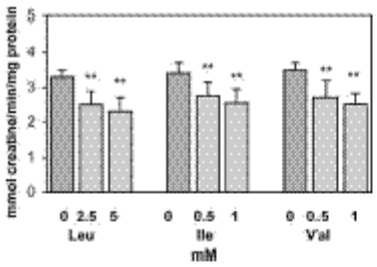

Fig. 1. In vitro effect of leucine, isoleucine and valine on creatine kinase activity in brain cortex homogenates from young rats. Data are mean ± S.D. of experiments performed in triplicate for six–eight animals in each group. Different from control, $^{**}P < 0.01$ (Tukey test).

3. Results

First, we incubated Leu at 2.5 and 5 mM, and Ile and Val at 0.5 and 1 mM final concentrations corresponding to the concentrations found in the plasma of MSUD patients. We observed a significant dose-dependent inhibition caused by Leu [$F(1, 19) = 15.49$; $\beta = -0.67$; $t = 3.94$; $P < 0.001$], Ile [$F(1, 19) = 17.42$; $\beta = -0.69$; $t = 4.17$; $P < 0.001$], and Val [$F(1, 19) = 22.31$; $\beta = -0.73$; $t = 4.72$; $P < 0.001$] (Fig. 1).

In order to better understand the mechanisms underlying the inhibition caused by BCAA on the in vitro CK activity, we first carried out competition experiments between Leu, Ile, or Val, and the enzyme substrates phosphocreatine (Fig. 2) or ADP (Fig. 3). The Lineweaver–Burk plot indicated that all three BCAA inhibited CK activity by competition with the two enzyme substrates, probably at the active site. The K_m for phosphocreatine as substrate was 0.8 ± 0.3 mM ($n = 8$), significantly higher [$t(14) = 2.78$; $P < 0.01$] than the K_m for ADP as substrate [0.3 ± 0.1 ($n = 8$)]. The K_i values for phosphocreatine as substrate, calculated by the Dixon plot, were 16 ± 3 mM ($n = 5$) for Leu, 10 ± 3 mM ($n = 5$) for Ile, and 14 ± 7 mM ($n = 5$) for Val. No significant differences were found among the K_i values [$F(2, 12) = 1.14$; $P = 0.36$].

In respect to ADP as substrate, the K_i values were 22 ± 8 mM ($n = 5$) for Leu, 21 ± 2 mM ($n = 5$) for Ile, and 23 ± 5 mM ($n = 4$) for Val. No significant differences among the K_i values [$F(2, 12) = 0.19$; $P = 0.83$] were found. Considering each of the two substrates, the similarities among the K_i values for the three amino acids, indicate that the affinities between CK and the amino acids are of the same order of magnitude.

Next, we performed interaction experiments between the BCAA plotted according to a modification of Chevillard et al. (1993) as described by us (Wyse et al., 1998). The straight line on the interaction plots, i.e. a competitive plot, showed that all three BCAA act at the same enzyme site

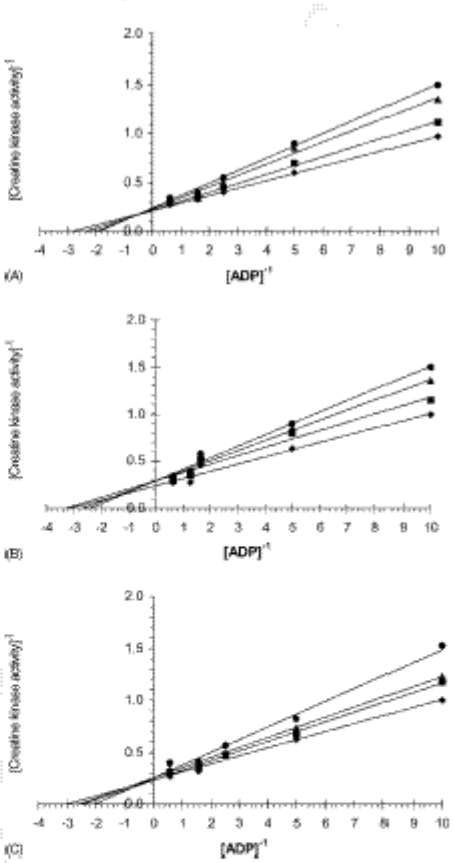

Fig. 2. Lineweaver–Burk competition plot between phosphocreatine and leucine, isoleucine, or valine on creatine kinase activity in brain cortex homogenates from young rats. Data are representative of three–five independent experiments performed in triplicate. (A) Leucine; (B) isoleucine; and (C) valine. (◆) control (without inhibitor); (■) 5 mM; (▲) 10 mM; (●) 15 mM.

(Fig. 4), reinforcing the results obtained with the Lineweaver–Burk double-reciprocal plot.

4. Discussion

BCAA and their BCKA accumulate in plasma and tissues of MSUD affected patients. Neurological dysfunction is common in these patients but the mechanisms underlying the pathophysiology of this disorder seem to be multiple and

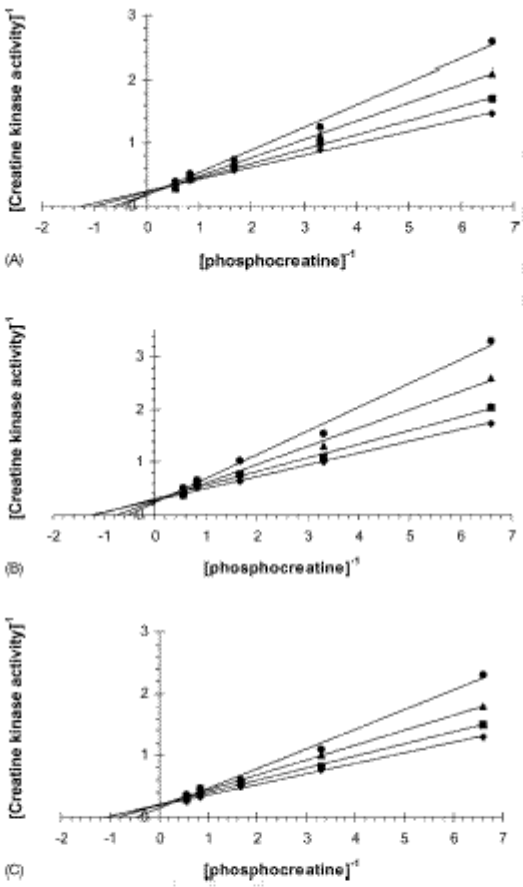

Fig. 3. Lineweaver–Burk competition plot between ADP and leucine, isoleucine, or valine on creatine kinase activity in brain cortex homogenates from young rats. Data are representative of three–five independent experiments performed in triplicate. (A) Leucine; (B) isoleucine; and (C) valine. (◆) control (without inhibitor); (■) 5 mM; (▲) 10 mM; (●) 15 mM.

poorly known. We have reported a reduction of large neutral amino acid (LNAA) in plasma and brain of hyperleucinemic rats (Araujo et al., 2001), in agreement with Zielke et al. (2002) who proposed that brain cells in MSUD may be subjected to reduced large neutral amino acids (LNAA) as a consequence of competition for transport of LNAA at the blood–brain barrier and trans-stimulated exchange out of neuronal cells for subsequent metabolism or sequestration in the periphery. On the other hand, we have reported that all amino acids, keto acids and hydroxy acids known to accumulate in this disease stimulate the in vitro lipid oxidation in rat brain (Fontella et al., 2002). Other mechanisms of neurotoxicity of metabolites accumulated in MSUD proposed by us and by other investigators include: oxidation of glutamate and glutamine (Zielke et al., 1997), inhibition of glutamate uptake into synaptic vesicles (Tavares et al., 2000; Reis et al., 2000), apoptosis (Jouvet et al., 2000a,b), and induction of convulsions through GABAergic and glutamatergic NMDA mechanisms (Coitinho et al., 2001).

In the present study we demonstrated a dose-dependent inhibition of CK activity in brain cortex homogenates from 22-day-old Wistar rats caused by BCAA at concentrations

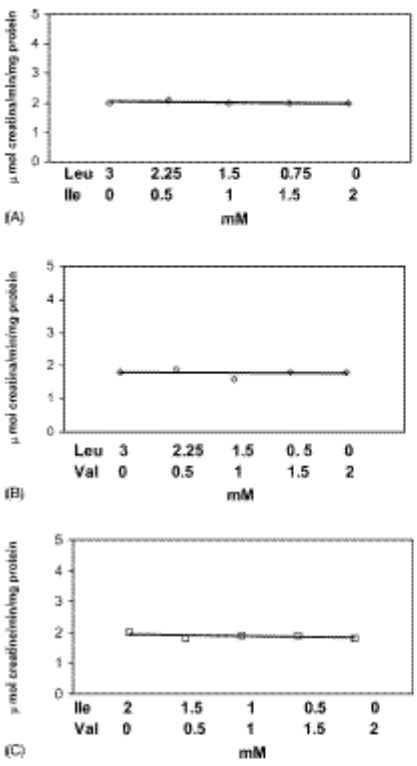

Fig. 4. (A–C) Cheviliard et al. interaction plot between leucine, isoleucine and valine on creatine kinase activity in brain cortex homogenates from young rats. Data are representative of three–five independent experiments performed in triplicate.

similar to those found in the plasma of MSUD patients. This might suggest that not only Leu, but also the others two BCAA accumulating in MSUD may be neurotoxic, impairing brain energetic homeostasis. It is possible that the inhibitory effect of BCAA on CK activity observed in vitro, also occurs in vivo, since BCAA are rapidly transported into the brain (Yudkoff, 1997) and accumulate in the brain of MSUD patients (Prensky and Moser, 1966). Considering that morphologic changes found in the brain of MSUD patients point to alteration in the energy metabolism in the central nervous system (Kiil and Rekkones, 1964; Riviello et al., 1991; Kamei et al., 1992), it is feasible to envisage that the inhibition of CK activity caused by BCAA could contribute to the brain energy alteration in this disease.

We also determined the K_m of the reaction catalyzed by CK for phosphocreatine and ADP as substrates. The 0.8 ± 0.3 mM value of the K_m for phosphocreatine as substrate, is 5–10 times lower than the concentration of phosphocreatine in rat brain, which is in the range of 4–8 mM (Nakai et al., 2000). Considering that K_m is the substrate concentration at which an enzymatic reaction proceeds at one-half its maximal velocity, brain CK is nearly saturated in respect to phosphocreatine in normal conditions. Therefore, phosphocreatine is limiting for CK activity only in situations in which severe reduction of energy production occurs with consequent dramatic drop of brain phosphocreatine levels. On the other hand, the K_i values for BCAA inhibition was in the range of 5–20 mM for phosphocreatine as substrate. Considering that K_i is the equilibrium constant for inhibitor binding, and the enzyme is saturated for the substrate, it is possible that the competition between BCAA and phosphocreatine may be of little pathophysiological significance in MSUD, unless in conditions of reduced energy production.

Regarding to ADP as substrate, the K_m for CK (0.3 ± 0.1 mM) and the ADP concentration in the brain (0.2–0.4 mM) (Plaschke et al., 1999), are in the same range, indicating that CK is far from being saturated in respect to ADP. Therefore, ADP, and not phosphocreatine content, regulates brain CK activity in normal conditions. Therefore, although the K_i values for BCAA (6–30 mM) are high, the low ADP concentrations usually found in the brain indicate that the competition between ADP and BCAA, diminishing CK activity, may alter the function of the CK/phosphocreatine system, contributing to the brain damage characteristic of the patients with MSUD.

The CK/phosphocreatine system exerts several integrated functions in brain cells, namely temporary energy buffering, metabolic capacity, energy transfer and metabolic control (Saks et al., 1996; Wallimann et al., 1998a,b). This system is now recognized as an important metabolic regulator during health and disease (Wallimann et al., 1998a,b). 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, alteration of CK function may play an important role in a 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 excitotoxicity, attributable by enhancing high-energy phosphate stores (Brustovetsky et al., 2001). Therefore, considering 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), the loss of oligodendrocytes and myelin deficiency, the most prominent morphologic changes found in the brain of MSUD patients (Chuang and Shih, 2001), may be associated with CK activity diminution.

In summary, our results indicate that BCAA inhibit competitively CK activity in cerebral cortex homogenates from 22-day-old Wistar rats. Considering that creatine kinase is a key enzyme for energy homeostasis in the brain, in case this effect also occurs in the brain of MSUD patients, it is possible to envisage that an alteration of this enzyme activity may potentially impair brain energy metabolism, contributing to the brain damage found in patients affected by this disease. Finally, considering that the current diet therapy is insufficient to maintain normal brain development in MSUD patients, that creatine easily crosses the blood–brain barrier (Hemmer and Wallimann, 1993), and that creatine administration results in significant improvement in patients with mitochondrial encephalopathy and other neurological diseases (Tarnopolsky and Beal, 2001), it may be interesting to test whether creatine supplementation to the diet would benefit MSUD patients.

Acknowledgements

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Artigo 4

Objetivo 4- Caracterizar o mecanismo de inibição causado pelos aminoácidos de cadeia ramificada sobre a atividade da creatinaquinase em homogeneizado total de cerebelo e cérebro médio de ratos jovens.

Kinetic studies on the inhibition of creatine kinase activity by branched-chain α-amino acids in cerebellum and midbrain of developing rats

Carmen Pilla, Rui Felipe de Oliveira Cardozo, Paula Karine Barcelos Dornelles, Carlos Severo Dutra-Filho, Angela Terezinha de Souza Wyse, Moacir Wajner, and Clóvis Milton Duval Wannmacher**.**.

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Running title: Branched-chain amino acids and creatine kinase

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ABSTRACT

Maple syrup urine disease (MSUD) is a metabolic disorder biochemically characterized by the accumulation of branched-chain amino acids (BCAA) and their branched-chain keto acids (BCKA) in blood and tissues. Neurological dysfunction is usually present in the patients, but the mechanisms of brain damage in this disease are far from be understood. We have previously reported that BCAA, but not BCKA inhibit CK activity in rat brain. Therefore, the main objective of the present study was to investigate the mechanisms by which BCAA inhibit creatine kinase activity in the cerebellum and midbrain of 21-day-old Wistar rats. For the kinetic studies, Lineweaver-Burk plot was used to characterize the mechanisms of enzyme inhibition. The results indicated that BCAA inhibit creatine kinase by competition with the substrates phosphocreatine and ADP at the active site. Considering the crucial role creatine kinase plays in energy homeostasis in brain, if these effects also occur in the brain of MSUD patients, it is possible that inhibition of this enzyme activity may contribute to the brain damage found in this disease.

Key words: Maple syrup urine disease, branched-chain ketoaciduria, leucine, branched-chain amino acids, creatine kinase

INTRODUCTION

Maple syrup urine disease (MSUD) or branched-chain ketoaciduria is a metabolic inherited disorder caused by a deficiency in the activity of branched-chain α-keto acid dehydrogenase complex (BCKD) accumulating the branched-chain α-amino acids (BCAA) leucine (Leu), isoleucine (Ile) and valine (Val) and their branched-chain α-keto acids (BCKA) α-keto-isocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV) and α-keto-isovaleric acid (KIV) in blood and tissues of the patients (1,2). Surviving children often present a variable degree of brain damage, characterized by mental retardation, neurological difficulties in walking and speech, and seizures (3). Neuropathologic changes in MSUD include severe brain edema, myelin deficiency, striking spongy degeneration of white matter, cerebellar necrosis and considerable nerve-cell loss in substantia nigra and pontine nuclei, suggesting that brain energetic homeostasis is deficient in this disease (4, 5). Leu and KIC, the main accumulating metabolites, are considered the most important neurotoxic substances in MSUD, but the mechanisms by which this substances cause brain damage in this disease are still poorly known (3).

Creatine kinase (CK), EC 2.7.3.2, catalyzes the reversible transfer of the N-phosphoryl group from phosphocreatine to ADP regenerating ATP. CK is the key enzyme of an important system to maintain energy homeostasis of cells with high and fluctuating energy requirement (6). CK isoenzymes are specifically located at places of high energy demand (cytosol) and production (mitochondria), linking energy production and energy utilization by a creatine/phosphocreatine circuit (7). Considering that energy is critical to maintain the development and regulation of cerebral functions, it has been postulated that alteration of the creatine/phosphocreatine circuit may be an important step of a neurodegenerative pathway that

leads to neuronal loss in the brain (8). In this context, it has been reported severely reduced CK activity in several neurodegenerative diseases (9,10).

We have previously reported that BCAA, but not their BCKA, inhibit the in vitro CK activity in rat brain in a dose-dependent way (11). We have also reported that BCAA inhibit CK activity in rat brain cortex by competition with the substrates ADP and phosphocreatine (12). Therefore, in the present work we investigated the mechanism of the inhibition caused by BCAA on CK activity in the cerebellum and midbrain of developing rats. It is important to know the type of inhibition of the enzyme CK because it is possible that this approach may be used to design drugs to avoid this inhibition.

EXPERIMENTAL PROCEDURES

Subjects and Reagents. Twenty four Wistar rats bred in the Department of Biochemistry, UFRGS, were used in the experiments. Eight pups were randomly assigned to each dam. Rats were kept with dams until they were sacrificed at the 21st day of life. 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 Ethics Committee For Animal Research of the Federal University of Rio Grande do Sul. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

Preparation of Brain Homogenate. Animals were sacrificed by decapitation. The brain was rapidly removed and dissected on a glass dish over ice. Time elapsed from decapitation to place the brain on the ice was less than one minute. Olfactory bulbs, pons, and the brain cortex were discarded and the cerebellum and the rest of the brain (midbrain) were separated and disrupted in ice-cold 300 mOsm NaCl (1/10, w/v) using a ground-glass homogenizer. The homogenate was stored at -70^0 C when the assay was not carried out immediately. Before the enzyme assay, the homogenate was frozen and thawed three times.

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 minutes of pre-incubation at 37°C, the reaction was started by the addition of 0.3 μmol ADP plus 0.08 μmol 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 (13). 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. BCAA did not interfere with spectrometric readings or color development. Results were expressed as μmol of creatine formed per min per mg protein.

Protein Determination. The protein content of the cerebral cortex homogenates was determined by the method of Lowry et al (14), using serum bovine albumin as the standard.

Kinetic Studies. The competition studies and the Km (Michaelis constant) values determination were performed according to Lineweaver and Burk (15) and the Ki (inhibition constant) values were calculated from the Dixon and Webb plot (16).

Statistical Analysis. Data from the experiments were analyzed by Student's t test for independent samples or one-way analysis of variance (ANOVA) followed by the Tukey test when F value was significant. All analyses were carried out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

RESULTS

Competition experiments between Leu, Ile, or Val, and the enzyme substrates phosphocreatine or ADP were carried out in 22-day-old rats because we have previously demonstrated that BCAA inhibited brain CK activity at this age. The Lineweaver-Burk plot performed in midbrain (Fig 1 and 2)) and in cerebellum (Fig 3 and 4) indicated that all three BCAA inhibited CK activity by competition with the two enzyme substrates, probably at the active site. The Km for phosphocreatine as substrate was significantly lower [$t(14) = 2.75$; $p < 0.01$] in midbrain [0.7 ± 0.1 mM (n= 8)] than the Km in cerebellum [1.4 ± 0.2 mM (n=8)]. In contrast, the Km for ADP as substrate did not differ significantly [$t(14) = 0.71$; $p > 0.7$] between midbrain [0.35 ± 0.1 (n=8)] and cerebellum [0.3 ± 0.1 (n=8)]. The Ki values for phosphocreatine as substrate, calculated by the Dixon plot, were 14 ± 3 mM (n=5) for Leu, 12 ± 3 mM (n=5) for Ile, and 15 ± 5 mM (n=5) for Val in midbrain and 12 ± 3 mM (n=5) for Leu, 15 ± 3 mM (n=5) for Ile, and 15 ± 4 mM (n=5) for Val in cerebellum. No significant differences were found among the Ki values in midbrain [$F(2,12) = 0.84$; $p = 0.6$] or in cerebellum [$F(2,12) = 0.64$; $p = 0.8$].

In respect to ADP as substrate,. the Ki values were 22 ± 6 mM (n=5) for Leu, 20 ± 2 mM (n=5) for Ile, and 23 ± 5 mM (n=4) for Val in midbrain, and 21 ± 6 mM (n=5) for Leu, 22 ± 4 mM (n=5) for Ile, and 22 ± 6 mM (n=4) for Val in cerebellum. No significant differences among the Ki values in midbrain [$F(2,12) = 0.28$; $p > 0.7$] or in cerebellum [$F(2,12) = 0.19$; $p > 0.8$] were found. Considering each of the two substrates, the similarities among the Ki values for the three amino acids, indicate that the affinities between CK and the amino acids are of the same order of magnitude in the two brain structures investigated.

DISCUSSION

BCAA and their BCKA accumulate in plasma and tissues of MSUD affected patients. Neurological dysfunction is common in these patients but the mechanisms underlying the pathophysiology of this disorder seem to be multiple and poorly known. We have reported a reduction of large neutral amino acid (LNAA) in plasma and brain of hyperleucinemic rats (17), in agreement with Zielke et al (18) who proposed that brain cells in MSUD may be subjected to reduced large neutral amino acids (LNAA) as a consequence of competition for transport of LNAA at the blood-brain barrier and trans-stimulated exchange out of neuronal cells for subsequent metabolism or sequestration in the periphery. On the other hand, we have reported that all amino acids, keto acids and hydroxy acids known to accumulate in this disease stimulate the *in vitro* lipid oxidation in rat brain (19). Other mechanisms of neurotoxicity of metabolites accumulated in MSUD proposed by us and by other investigators include: oxidation of glutamate and glutamine (20), inhibition of glutamate uptake into synaptic vesicles (21,22), apoptosis (23,24), and induction of convulsions through GABAergic and glutamatergic NMDA mechanisms (25).

We have previously demonstrated that BCAA inhibit CK activity in brain cortex homogenates from developing Wistar rats at concentrations similar to those found in the plasma of MSUD patients, suggesting that not only Leu, but also the others two BCAA known to accumulate in MSUD may be neurotoxic, impairing brain energetic homeostasis (11). BCAA plasma concentrations were used to investigate the in vitro effects of BCAA on CK activity because these amino acids easily penetrate in brain (26), the ratio between the CSF concentrations of BCAA in MSUD patients and normal controls are similar to those of plasma (27), and 8h after death, plasma and brain amino acid levels were similar in one MSUD patient died at the age of 25 days (28), indicating that plasma and brain concentrations are possibly similar in MSUD patients. Although proline also inhibit CK activity, it is interesting to note that the inhibition caused by BCAA on CK is not a general effect of amino acids, since glutamate do not alter the enzyme activity (29).

It is possible that the inhibitory effect of BCAA on CK activity observed *in vitro*, also occurs *in vivo,* since BCAA are rapidly transported into the brain (30) and accumulate in the brain of MSUD patients (28). Considering that morphologic changes found in the brain of MSUD patients point to alteration in the energy metabolism in the central nervous system (4,5, it is feasible to envisage that the inhibition of CK activity caused by BCAA could contribute to the brain energy alteration in this disease.

We also determined the Km of the reaction catalyzed by CK for phosphocreatine and ADP as substrates. The Km values for phosphocreatine as substrate in midbrain (0.7 ± 0.1 mM), and in cerebellum (1.4 ± 0.2 mM) are 4-10 times lower than the concentration of phosphocreatine in rat brain, which is in the range of 4-8 mM (31). Considering that Km is the substrate concentration at which an enzymatic reaction proceeds at one-half its maximal velocity, midbrain and cerebellum CK is nearly saturated in respect to phosphocreatine in

normal conditions. Therefore, phosphocreatine may be limiting for CK activity only in situations in which severe reduction of energy production occurs with consequent great drop of brain phosphocreatine levels. On the other hand, the Ki values for BCAA inhibition was in the range of 6-25 mM in midbrain, and 10-25 mM in cerebellum for phosphocreatine as substrate. Considering that Ki is the equilibrium constant for inhibitor binding, and the enzyme is saturated for the substrate, it is possible that the competition between BCAA and phosphocreatine may be of little pathophysiological significance in MSUD, unless in conditions of marked reduction of energy production.

Regarding to ADP as substrate, the Km for CK in midbrain (0.35 ± 0.1 mM) and in cerebellum (0.3 ± 0.1), and the ADP concentration in the brain (0.2-0.4 mM) (32), are in the same order of magnitude, indicating that CK is far from being saturated in respect to ADP. Therefore, ADP, and not phosphocreatine content, regulates brain CK activity in normal conditions. Therefore**,** although the Ki values of BCAA for ADP as substrate are high in midbrain and cerebellum (6-30 mM), the low ADP concentrations usually found in the brain indicate that the competition between ADP and BCAA, diminishing CK activity, may alter the function of the CK/phosphocreatine system, contributing to the brain damage characteristic of the patients with MSUD.

The CK/phosphocreatine system exerts several integrated functions in brain cells, namely temporary energy buffering, metabolic capacity, energy transfer and metabolic control (33,34). This system is considered an important metabolic regulator during health and disease (35). A decrease in CK activity is one of the biochemical markers of brain cell damage in age-related neurodegenerative diseases, including Alzheimer's disease (36). The decrease of CK activity in the brain correlates well with the neurodegeneration parameters in severely affected regions in Alzheimer's disease (37). Therefore, alteration of CK function may play an

important role in the pathway that leads to neuronal loss in the brain (8). These findings are reinforced by the observation that creatine and phosphocreatine have neuroprotective effects against energy deprivation and glutamate excitoxicity, probably because thy enhance high-energy phosphate stores (38). Therefore, considering that CK and the creatine-phosphocreatine energy shuttle may play a role in brain development that is associated with oligodendrocyte function and/or myelogenesis (39), the loss of oligodendrocytes and myelin deficiency, two morphologic changes found in the brain of MSUD patients (3), may be associated with CK activity diminution.

In summary, the results previously reported demonstrating that BCAA inhibit competitively CK activity in brain cortex of rats, and the present results indicating the same effects in midbrain and in cerebellum, in case this effect also occurs in the brain of MSUD patients, it is possible to envisage that an alteration of this enzyme activity may potentially impair brain energy metabolism, contributing to the brain damage found in the patients affected by this disease. Finally, it is well known that the current diet therapy is insufficient to maintain normal brain development in MSUD patients (3). Therefore, considering that creatine easily crosses the blood-brain barrier (40), and that creatine administration results in significant improvement in patients with mitochondrial encephalopathy and other neurological diseases (41), it may be interesting to test whether creatine supplementation to the diet would benefit MSUD patients.

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Fig. 1 Lineweaver-Burk competition plot between phosphocreatine and leucine (A), isoleucine (B), or valine (C) on creatine kinase activity in midbrain homogenates from young rats.

Data are representative of 3-5 independent experiments performed in triplicate.

♦ control (without inhibitor); ■ 5 mM; ▲ 10 mM; ● 15 mM.

Fig.2. Linewever-Burk competition plot between ADP and leucine (A), isoleucine (B), or valine (C) on creatine kinase activity in midbrain homogenates from young rats.

Data are representative of 3-5 independent experiments performed in triplicate.

♦ control (without inhibitor); ■ 5 mM; ▲ 10 mM; ● 15 mM.

Fig. 3 Lineweaver-Burk competition plot between phosphocreatine and leucine (A), isoleucine (B), or valine (C) on creatine kinase activity in cerebellum homogenates from young rats.

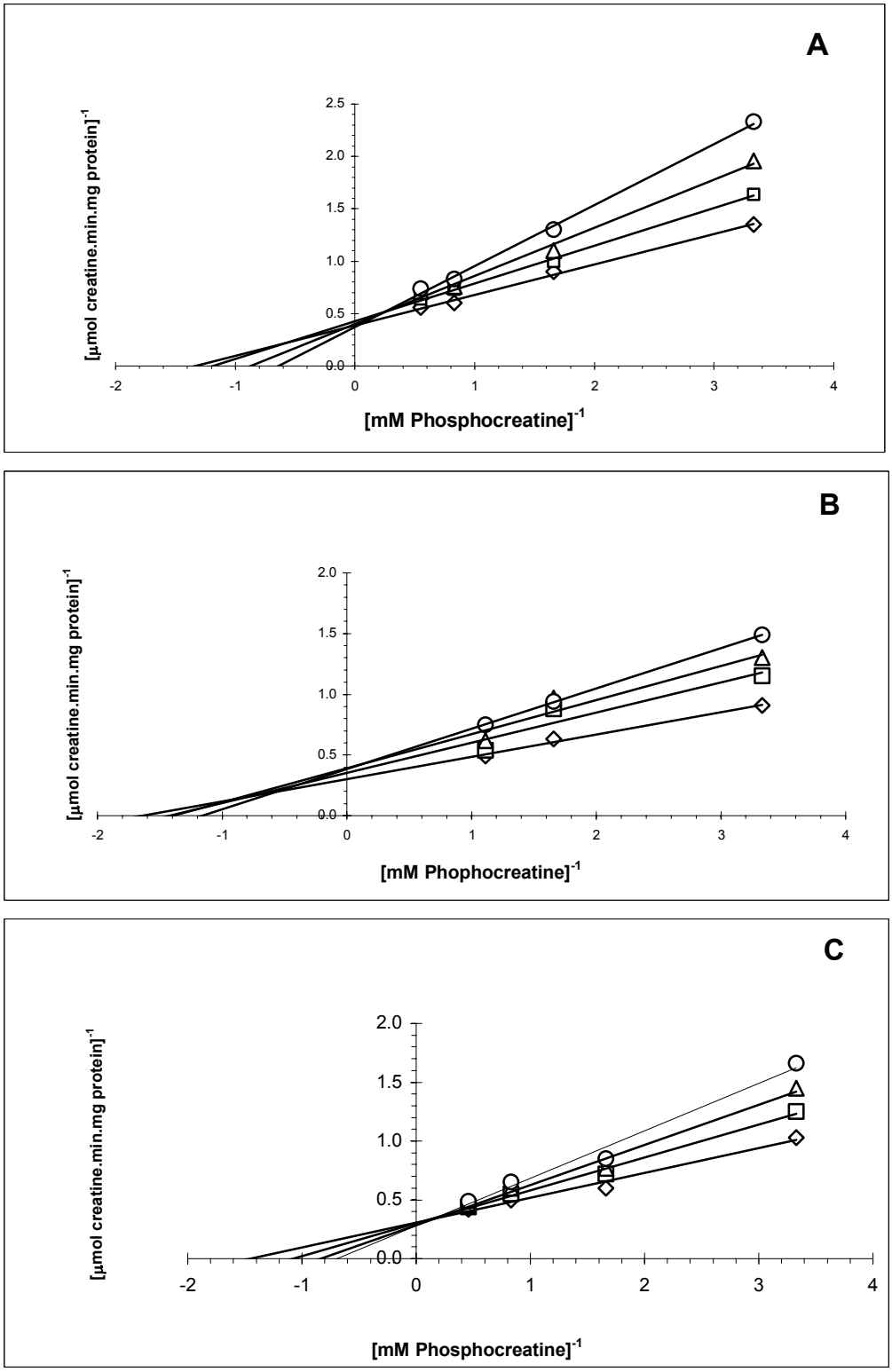
Data are representative of 3-5 independent experiments performed in triplicate.

♦ control (without inhibitor); ■ 5 mM; ▲ 10 mM; ● 15 mM.

Fig. 4 Lineweaver-Burk competition plot between ADP and leucine (A), isoleucine (B), or valine (C) on creatine kinase activity in cerebellum homogenates from young rats.

Data are representative of 3-5 independent experiments performed in triplicate.

♦ control (without inhibitor); ■ 5 mM; ▲ 10 mM; ● 15 mM.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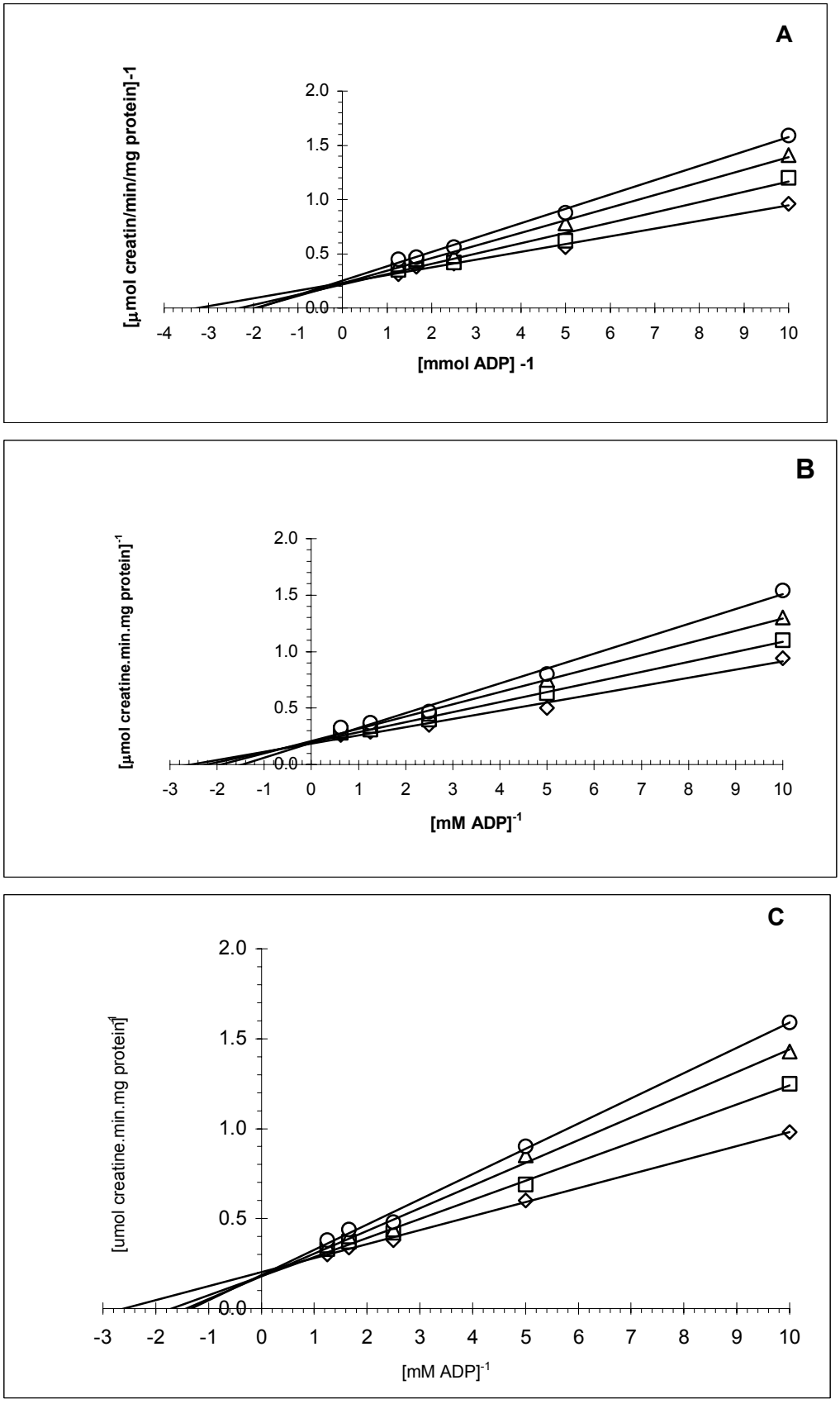
2.5
2.0
1.5
1.0
0.5
0.0
-2
-1
0
1
2
3
4
[mM Phosphocreatine]-1
B
[µmol creatine.min.mg protein]-1
2.0
1.5
1.0
0.5
0.0
-2
-1
0
1
2
3
4
[mM Phophocreatine]-1
C
[µmol creatine.min.mg protein]-1
2.0
1.5
1.0
0.5
0.0
-2
-1
0
1
2
3
4
[mM Phosphocreatine]-1

Figure 1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1.5
1.0
0.5
0.0
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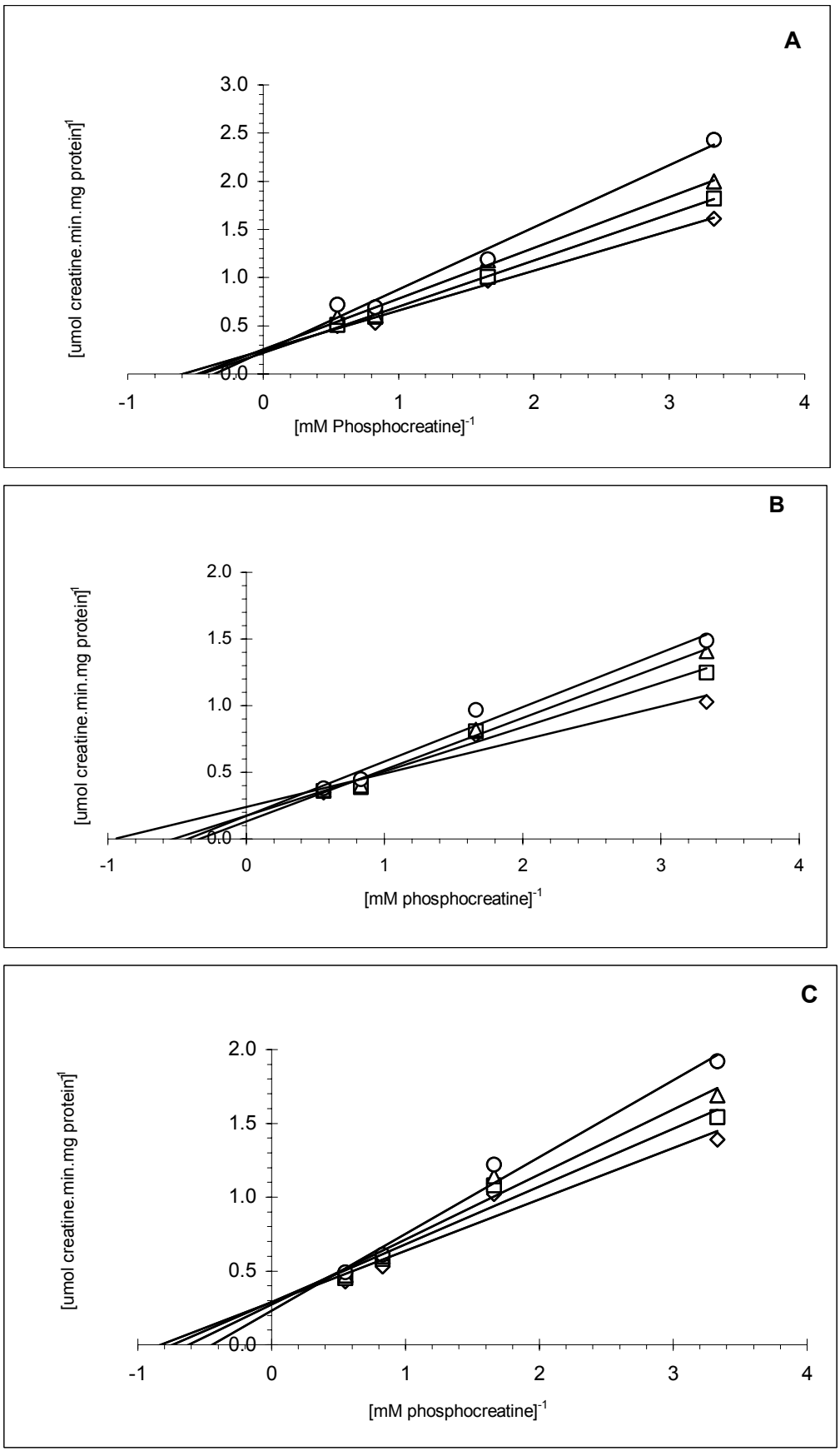
[mmol ADP] -1
B
[µmol creatine.min.mg protein]-1
2.0
1.5
1.0
0.5
0.0
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
[mM ADP]-1
C
[umol creatine.min.mg protein]-1
2.0
1.5
1.0
0.5
0.0
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
[mM ADP]-1

Figure 2


A
3.0
2.5
2.0
1.5
1.0
0.5
0.0
[umol creatine.min.mg protein]-1
-1
0
1
2
3
4
[mM Phosphocreatine]-1
B
2.0
1.5
1.0
0.5
0.0
[umol creatine.min.mg protein]-1
-1
0
1
2
3
4
[mM phosphocreatine]-1
C
2.0
1.5
1.0
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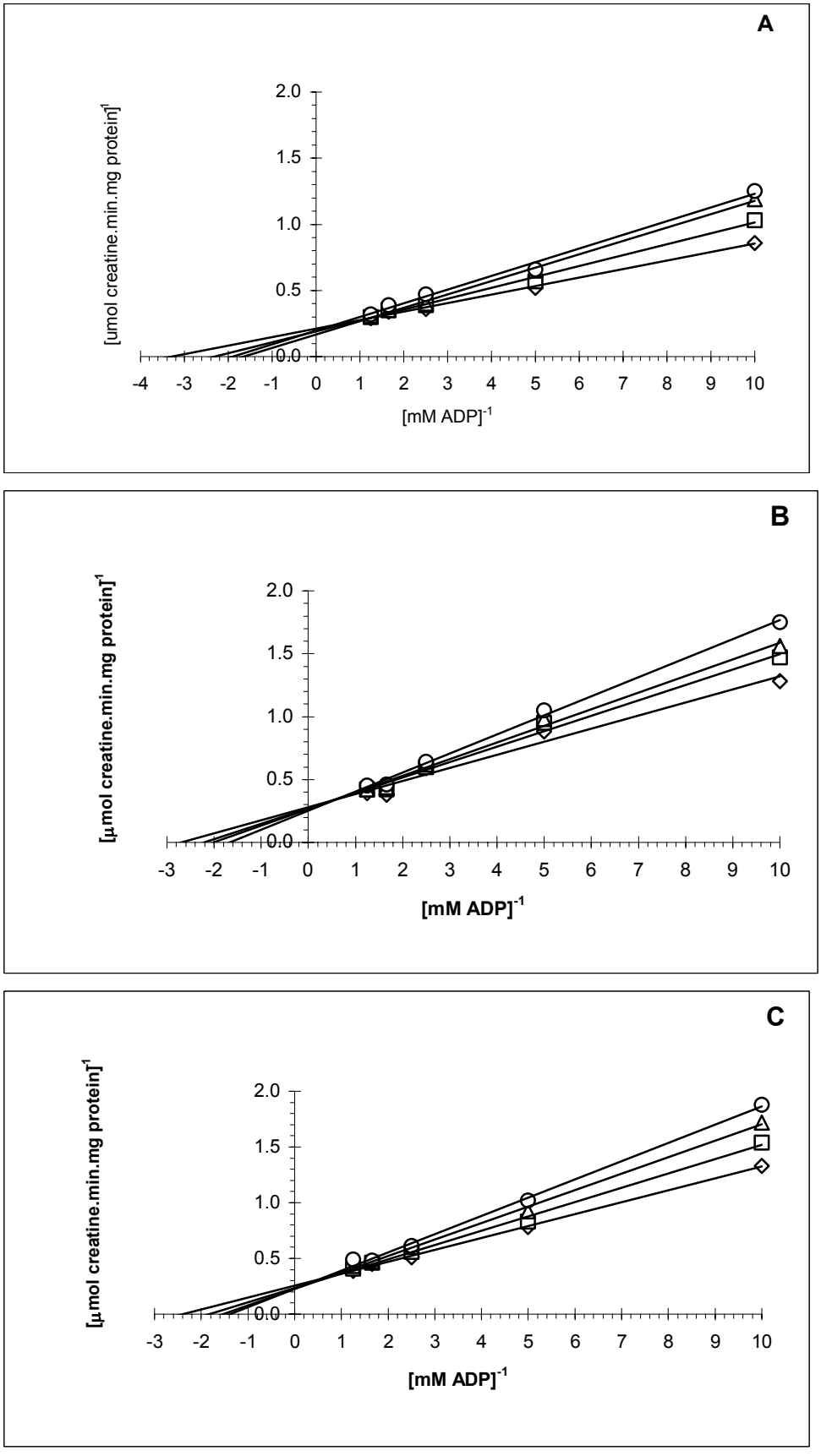
1
2
3
4
[mM phosphocreatine]-1

Figure 3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[umol creatine.min.mg protein]-1
2.0
1.5
1.0
0.5
0.0
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[mM ADP]-1
B
[µmol creatine.min.mg protein]-1
2.0
1.5
1.0
0.5
0.0
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
[mM ADP]-1
C
[µmol creatine.min.mg protein]-1
2.0
1.5
1.0
0.5
0.0
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
[mM ADP]-1

Figure 4

Capítulo III

DISCUSSÃO, CONCLUSÕES E PERSPECTIVAS FUTURAS

3.1 Discussão

Os aminoácidos e os cetoácidos de cadeia ramificada acumulam no plasma e no líquor dos pacientes com a Doença do Xarope do Bordo e pouco se sabe sobre os mecanismos que provocam a disfunção neurológica encontrada nestes pacientes. Vários mecanismos têm sido propostos através de estudos realizados no Laboratório de Erros Inatos do Metabolismo e em outros laboratórios, como por exemplo, a diminuição de aminoácidos de cadeia longa (Araújo et al., 2001), por competição com o transporte na barreira hemato-encefálica, ou seqüestração periférica (Zielke et al., 2002). Nosso laboratório demonstrou que em ratos hiperleucemicos, estes aminoácidos e cetoácidos estimulam *in vitro* a oxidação de lipídeos em cérebro de ratos (Fontella et al., 2002). Outros mecanismos também procuraram explicar o dano neurológico como a oxidação do glutamato e glutamina (Zielke, 1997), a inibição da captação do glutamato nas vesículas sinápticas (Tavares et al., 2000 e Reis et al., 2000) e a indução de convulsões por mecanismos NMDA GABAérgicos e glutamatérgicos (Coitinho et al., 2001).

Neste trabalho propomos um novo mecanismo. Demonstramos que os aminoácidos de cadeia ramificada: leucina, isoleucina e valina, que acumulam no sangue e no líquor dos pacientes com a Doença do Xarope do Bordo inibem a atividade da creatinaquinase, enzima chave para a manutenção da homeostasia energética no tecido nervoso, podendo ser em parte responsável pelo o dano neurológico observado.

Inicialmente foi demonstrado que a leucina, isoleucina e valina inibiram *in vitro* a creatinaquinase cerebral de ratos jovens, de forma dose dependente e em concentrações

semelhantes às encontradas no plasma dos pacientes com a Doença do Xarope do Bordo. Entretanto, o mesmo tipo de inibição, não foi observado *in vitro*, na presença dos respectivos cetoácidos nas concentrações já descritas nestes pacientes. É possível que estes cetoácidos também possam inibir indiretamente a enzima *in vivo*. Já foi demonstrado que o KIC, cetoácido derivado da leucina e encontrado em altas concentrações no plasma dos pacientes com a Doença do Xarope do Bordo é captado rapidamente pelos neurônios e transformado em leucina (Yudkoff, 1997), aumentando a concentração deste aminoácido no cérebro (Zielke et al., 1997). Considerando que as alterações histopatológicas encontradas no cérebro destes pacientes sugerem alterações no metabolismo energético, é possível que a inibição da creatinaquinase esteja envolvida no processo fisiopatológico.

Nosso laboratório já demonstrara que ratos tratados de forma crônica com leucina, em níveis similares aos encontrados no plasma de pacientes com a Doença do Xarope do Bordo, apresentaram déficit de comportamento (Mello et al., 1999). Portanto era importante pesquisar se o efeito dos aminoácidos de cadeia ramificada sobre a creatinaquinase, observado *in vitro,* também estaria repetindo *in vivo.* Nós demonstramos que a forma aguda e crônica de tratamento aumenta a atividade da CK no córtex, mas diminui no cérebro médio e cerebelo. É possível que as alterações encontradas nos animais tratados cronicamente não sejam devidas à ação direta do aminoácido sobre a enzima, uma vez que os animais foram sacrificados 12 horas após o último tratamento. Já foi descrito que após este tempo os níveis de leucina já retornaram ao normal (Mello, 1999). Postulou-se que estas alterações poderiam ser devidas à regulação da expressão da enzima ou mesmo uma modificação pós-traducional de moléculas existentes. A modificação pós-traducional já foi proposta por Aksenov e col. (1997, 1999 e 2000) ao observar a redução

da CK em alterações neurodenegerativas severas. Nossa hipótese de que a alteração da enzima, após tratamento crônico, não era devida a uma ação direta, foi reforçada com os resultados obtidos no tratamento agudo. Os resultados foram semelhantes aos obtidos no tratamento crônico. Os animais foram sacrificados uma hora após o último tratamento e a concentração de leucina deveria estar em torno de 3 mM (Mello, 1999), mas no meio ensaiado, devido à diluição de 10000 vezes, a concentração do aminoácido era muito baixa (0,3 μM).

Em relação ao aumento de atividade observado no córtex e diminuição no cérebro médio e cerebelo é possível que os mecanismos de recuperação da enzima no cérebro sejam mais ativos no córtex. Uma resposta bifásica, mostrando uma diminuição da atividade inicial seguida de aumento foi observada em nosso laboratório quando ratos foram tratados com fenilalanina e a atividade da Na+-K+-ATPase testada (Wyse, 1995). Esta hipótese é reforçada pelos dados obtidos por Chuang e Shih (2001) em que os neurônios de córtex de pacientes com a Doença do Xarope do Bordo são melhores preservados do que em outras estruturas do cérebro.

Para entender melhor o efeito destes aminoácidos sobre a creatinaquinase, era necessário verificar alguns parâmetros cinéticos da enzima. Portanto foram determinados o Km e o Ki para ambos os substratos da enzima.

Observamos que o Km para fosfocreatina, obtido através do gráfico de Lineweaver-Burk, foi de 0,8 ± 0,3 mM para córtex, 0,7 ± 0,1 mM para o cérebro médio e 1,4 ± 0,2 mM para cerebelo. Como o Km representa a concentração do substrato onde a enzima alcança a

metade de sua velocidade máxima, a creatinaquinase cerebral está normalmente saturada por fosfocreatina, já que os valores obtidos estão muito mais baixos do que a concentração da fosfocreatina no cérebro (4 8 mM) (Nakai et al., 2000). Deste modo, a fosfocreatina só seria limitante para a atividade da enzima em situações de grande redução de energia. Por outro lado, nós encontramos o Ki para a inibição dos aminoácidos de cadeia ramificada variando de 7-21 mM. Considerando que o Ki representa a constante de equilíbrio para a ligação do inibidor e que a enzima está saturada pela fosfocreatina, é possível que os aminoácidos não consigam competir com o substrato e que tenha pouco significado fisiopatológico na Doença do Xarope do Bordo, a não ser que exista severa diminuição de energia e portanto, de fosfocreatina.

O Km para ADP, o outro substrato da CK, foi de 0,3 ± 0,1 mM para o córtex, 0,35 + 0,1 mM para o cérebro médio e 0,3 ± 0,1 mM para o cerebelo, e a concentração de ADP no cérebro está no mesmo nível de 0,2 –0,4 mM (Plaschke et al., 2000). Os dados mostraram que a CK está longe de ser saturada pelo ADP. Os valores de Ki encontrados para o ADP variando de 6-30 mM indicam que os aminoácidos de cadeia ramificada competem com o ADP, diminuindo a atividade da enzima e alterando o sistema CK/PCr, podendo contribuir, deste modo, para as alterações neurológicas encontradas nos pacientes com a Doença do Xarope do Bordo.

A creatinaquinase exerce várias funções no cérebro como o tamponamento de energia metabólica, de capacidade metabólica e transferência de energia e é reconhecida como um importante regulador metabólico e um bio-marcador de dano celular em doenças neurodegenerativas como a doença de Alzheimer. Tem também um papel importante na

rota metabólica que leva à perda neuronal. Isto reforça a idéia de que a creatina e a fosfocreatina têm um efeito neuroprotetivo (Matthews et al., 1998) contra a diminuição de energia e contra a neurotoxicidade do glutamato (Brustovesk et al., 2001). Além disso, a CK está associada com o desenvolvimento cerebral dos oligodendrocitos e/ou a mielogenese (Manos et al., 1991). A perda de oligodendrócitos e a deficiência de mielina, são duas importantes alterações morfológicas encontradas por Chuang e Shih (2001) no cérebro de pacientes com a Doença do Xarope do Bordo.

3.2 Conclusões

Os dados experimentais realizados durante o desenvolvimento deste estudo nos permitiram verificar que:

1- Os aminoácidos de cadeia ramificada, que acumulam na Doença do Xarope do Bordo, inibem a atividade da creatina quinase *in vitro*, em cérebros de ratos jovens nas estruturas estudadas: córtex, cérebro médio e cerebelo; mas os cetoácidos respectivos não mostraram interferência na atividade da enzima.

2- O mesmo tipo de inibição foi verificado *in vivo*, no cérebro médio e cerebelo quando injetamos leucina em ratos em desenvolvimento, tratados de forma aguda e crônica. O córtex, ao contrário mostrou aumento de atividade, sugerindo que os mecanismos de recuperação da atividade enzimática sejam mais ativos no córtex cerebral do que no cerebelo e no cérebro médio.

3- A relação entre a atividade mitocondrial e citosólica manteve-se constante no tratamento agudo e crônico, indicando que o efeito exercido pelos aminoácidos de cadeia ramificada sobre a creatinaquinase é similar nas isoenzimas cerebrais localizadas nestes dois compartimentos.

4- O Km determinado no córtex cerebral (0,8 ± 0,3 mM), no cérebro médio (0,7 ± 0,1 mM) e no cerebelo (1,4 ± 0.2 mM) para a fosfocreatina como substrato, muito abaixo da concentração de fosfocreatina encontrada normalmente no cérebro (4,8 mM), e o Ki muito alto variando de 7 a 21 mM indicam que a competição entre os aminoácidos de cadeia ramificada e a fosfocreatina teria pouco valor fisiopatológico na Doença do Xarope do Bordo, exceto em condições de grande redução na produção de energia pelo cérebro.

5- O Km determinado, no córtex (0,8 ± 0,3 mM), no cérebro médio (0,7 ± 0,1 mM) e no cerebelo (1,4 ± 0,2 mM) para o ADP como substrato, é alto em relação à concentração de ADP normalmente encontrada no cérebro (0,3 ± 0,1 mM), indicando que a atividade enzimática é dependente da concentração de ADP. Neste caso, mesmo que o Ki seja muito alto (6 a 30 mM), a competição entre os aminoácidos de cadeia ramificada e o ADP pode alterar o sistema CK/PCr, ocasionando alteração da homeostasia energética cerebral, podendo contribuir para o dano neurológico nos pacientes com a Doença do Xarope do Bordo.

3.3 PERSPECTIVAS FUTURAS

O trabalho aqui apresentado demonstrou que a creatinaquinase é inibida de forma competitiva pelos aminoácidos de cadeia ramificada no cérebro de ratos e que esta inibição é provavelmente realizada no centro ativo da enzima. Foi proposto um novo mecanismo, por alteração da homeostasia energética cerebral, para contribuir com o desenvolvimento do dano neurológico presente nos pacientes com a Doença do Xarope do Bordo.

Considerando que a creatinaquinase é a enzima chave para manter homeostasia da energia cerebral, que os pacientes com a Doença do Xarope do Bordo apresentam acúmulo dos aminoácidos de cadeia ramificada no sangue e no líquor, que experimentos em nossos laboratórios já mostraram que os aminoácidos de cadeia ramificada são produtores de radicais livres (Fontella et al., 2002) e que a CK é uma enzima tiólica, seria importante investigar se os aminoácidos de cadeia ramificada podem inibir a creatinaquinase por um segundo mecanismo, não competitivo, através de radicais livres.

Considerando que os pacientes com a Doença do xarope do Bordo são tratados com dieta terapêutica hipoprotéica e que esta é insuficiente para manter o desenvolvimento normal dos pacientes, que a creatina atravessa facilmente a barreia sangue-cérebro e que em pacientes com doenças neurológicas e encefalopatias mitocondriais tratados com creatina mostraram significante melhora (Hemmer e Wallimann, 1993 e Kekelideze et al., 2000), seria importante também investigar se a suplementação de creatina na dieta dos ratos poderia prevenir os efeitos comportamentais provocados pele administração de leucina.

Considerando que tem sido sugerido para a adenilatoquinase um papel também de transferência de energia intracelular, semelhante ao atribuído a creatinaquinase

participando no tamponamento da relação ATP/ADP; que sua atividade pode substituir a creatinaquianse quando esta está diminuída (Dzeja et al., 1996), seria interessante também investigar o efeito dos aminoácidos de cadeia ramificada sobre a atividade da AK.

O processo científico é uma longa cadeia de interrogações.
Cada questão que encontra a sua resposta abre um novo campo de investigação a explorar.

George E. Palade

Premio Nobel de Medicina 1974

CAPÍTULO IV

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