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PARÂMETROS BIOQUÍMICOS DE ALTERAÇÕES DO CITOESQUELETO NO MODELO EXPERIMENTAL DA DOENÇA DO XAROPE DO BORDO

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"Uma longa viagem começa com um único passo".

Lao-Tsé

"Eterno, é tudo aquilo que dura uma fração de segundo, mas com tamanha intensidade, que se petrifica, e nenhuma força jamais o resgata".

Carlos Drummond de Andrade

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LISTA DE ABREVIATURAS

AACR	Aminoácidos de cadeia ramificada
AMPA	Ácido α-amino-3-hidroxi-5metil-4-isoxazolepropionato
AMPc	3’,5’- Adenosina monofosfato cíclico
ATP	Adenosina trifosfato
BAPTA-AM	Ácido tetra acético 1,2-bis(2-aminofenóxi)etano-éster acetoximetil
Ca^{2+}	Íon cálcio
CACR	α-cetoácidos de cadeia ramificada
CIC	Ácido α-cetoisocapróico
CIV	Ácido α-cetoisovalérico
Cl^{-}	Íon cloro
CQ	Creatina quinase
CMV	Ácido α-ceto-β-metilvalérico
DXB	Doença do Xarope do Bordo
EIM	Erros inatos do metabolismo
FI	Filamentos intermediários
GABA	Ácido γ- aminobutírico
GABA-T	GABA-transaminase
GAD	Ácido glutâmico descarboxilase
DAG	Diacilglicerol
GAP	Proteínas ativadoras da GTPase
GDI	Inibidores da dissociação de GDP
GDP	Guanosina monofosfato
GEF	Fator de troca de GDP/GTP
GFAP	Proteína glial fibrilar ácida
GluRs	Receptores glutamatérgicos
GTP	Guanosina trifosfato
GMPc	3’, 5’-Guanosina monofosfato cíclico
IDPN	β, β- iminodipropilnitrila

iGluRs	Receptores glutamatérgicos ionotrópicos
IP3	Inositol trifosfato
K^+	Íon potássio
kDa	Quilodálton
LPA	Ácido lisofosfatídico
mGluRs	Receptores glutamatérgicos metabotrópicos
LTD	Depressão de longa duração
LTP	Potenciação de longa duração
MTOC	Centro organizador de microtúbulos
Na^+	Íon sódio
NF-H	Subunidade protéica de 200 kDa dos neurofilamentos
NF-L	Subunidade protéica de 68 kDa dos neurofilamentos
NF-M	Subunidade protéica de 150 kDa
NMDA	N-metil-D-aspartato
PKA	Proteína quinase dependente de AMPc
PKCaM	Proteína quinase dependente de cálcio e calmodulina
PKC	Proteína quinase dependente de cálcio e fosfolipídios
PKG	Proteína quinase dependente de GMPc
PP1	Proteína fosfatase do tipo 1
PP2A	Proteína fosfatase do tipo 2A
PP2B	Proteína fosfatase do tipo 2B
PP2C	Proteína fosfatase do tipo 2
SNC	Sistema nervoso central

RESUMO

A Doença do Xarope do Bordo (DXB) é um erro inato do metabolismo causado pela deficiência na atividade do complexo desidrogenase dos cetoácidos de cadeia ramificada, levando ao acúmulo de concentrações milimolares dos seguintes α-cetoácidos de cadeia ramificada (CACR): ácidos α-cetoisocapróico (CIC), α-ceto-β-metilvalérico (CMV), α-cetoisovalérico (CIV) e dos seus aminoácidos precursores, leucina, isoleucina e valina em tecidos de pacientes afetados. Essa doença é caracterizada por severos sintomas neurológicos que incluem edema e atrofia cerebral, entretanto, os mecanismos envolvidos na neuropatologia da DXB ainda não são bem estabelecidos. Neste trabalho utilizamos um modelo experimental de DXB com o objetivo de verificar os efeitos dos CACR que se acumulam nessa desordem neurodegenerativa sobre o citoesqueleto de células neurais de ratos. Nesse modelo fatias de córtex cerebral de ratos de diferentes idades, ou culturas de células neurais foram incubados com concentrações variando de 0,1 a 10 mM de cada metabólito.

Inicialmente demonstramos que o CIC, CMV e CIV inibiram a captação de glutamato em fatias de córtex cerebral de ratos durante o desenvolvimento. O CIC inibiu a captação de glutamato em ratos de 9, 21 e 60 dias de idade, enquanto o CMV e o CIV inibiram a captação de glutamato em animais de 21 e 60 dias.

Observamos que o CIC alterou a fosforilação de proteínas do citoesqueleto de um modo dependente do desenvolvimento através de receptores glutamatérgicos ionotrópicos em fatias de córtex cerebral de ratos. O metabólito causou diminuição da fosforilação dos filamentos intermediários (FI) em ratos de 9 dias de idade e aumento dessa fosforilação em animais de 21 dias de vida. Também demonstramos que em animais de 9 dias de idade o efeito do CIC foi mediado pelas proteínas fosfatases PP2A e principalmente pela PP2B, uma proteína fosfatase dependente de cálcio, enquanto que em animais de 21 dias de idade o efeito deste metabólito foi mediado pela proteína quinase dependente de AMP cíclico (PKA) e pela proteína quinase dependente de cálcio e calmodulina (PKCaMII). Além disso, verificamos que o CIC promoveu um aumento nos níveis

intracelulares dos segundos mensageiros AMPc e Ca^{2+}. O aumento do Ca^{2+} intracelular provocado pelo CIC foi demonstrado pelo uso de bloqueadores específicos de canais de cálcio dependentes de voltagem tipo L, por exemplo, nifedipina, canais de cálcio dependentes de ligantes, por exemplo, NMDA e de quelantes de cálcio intracelular, por exemplo, BAPTA-AM. Por outro lado, o CMV aumentou a fosforilação de FI somente em ratos de 12 dias de idade, sendo esse efeito mediado por receptores GABAérgicos do tipo A e B, desencadeando a ativação das proteínas quinases PKA e PKCaMII. É importante salientar que o CIV não alterou a atividade do sistema fosforilante em nenhuma das idades estudadas.

Além dos efeitos causados pelos CACR que se acumulam na DXB sobre a atividade do sistema fosforilante associado aos FI em fatias de córtex cerebral de ratos, demonstramos que esses metabólitos foram capazes de alterar a fosforilação da proteína glial fibrilar ácida (GFAP) na linhagem de glioma C6. Essa alteração de fosforilação causou uma reorganização do citoesqueleto de GFAP e um aumento no imunoconteúdo da GFAP na fração citoesquelética.

Também verificamos que o CIC, o CMV e o CIV, em concentrações encontradas em pacientes portadores de DXB, levaram a uma reorganização dos filamentos de GFAP e do citoesqueleto de actina de astrócitos em cultura, causando uma importante alteração na morfologia destas células. As alterações do citoesqueleto levaram a morte celular progressiva quando os astrócitos foram expostos por várias horas aos metabólitos. Demonstramos também que os efeitos dos CACR sobre a morfologia dos astrócitos foram desencadeados por mecanismos de membrana que diminuíram a atividade da Rho GTPase. Esse mecanismo foi evidenciado utilizando-se ácido lisofosfatídico (LPA), um ativador específico da RhoA, o qual preveniu os efeitos causados pelos CACR em culturas de astrócitos.

É importante salientar que as alterações morfológicas e a morte celular induzida pelos CACR em culturas de astrócitos foram totalmente evitadas com a suplementação de creatina às culturas. Também verificamos que a atividade da creatina quinase foi inibida pelos metabólitos, indicando que a homeostase energética provavelmente estaria envolvida nos efeitos causados pelos CACR.

Sabe-se que as alterações do citoesqueleto estão relacionadas com inúmeras doenças neurodegenerativas. Portanto, é provável que as alterações causadas pelos CACR nos mecanismos de membrana que regulam níveis de segundos mensageiros intracelulares e cascatas de sinalização celular, na perda do equilíbrio fisiológico do sistema fosforilante associado ao citoesqueleto e conseqüentemente na sua reorganização, possam ter importantes conseqüências para a função neural. Com base nos presentes resultados demonstramos que os CACR que se acumulam na DXB levam à desorganização do citoesqueleto em um modelo experimental podendo ser uma contribuição importante para o estudo da patogênese do sistema nervoso central característica dos pacientes portadores de DXB.

I- INTRODUÇÃO

1. ERROS INATOS DO METABOLISMO

Os erros inatos do metabolismo (EIM) são alterações genéticas que se traduzem na ausência ou na síntese anormal, qualitativa ou quantitativa, de uma proteína, geralmente uma enzima. A ausência ou deficiência severa na atividade enzimática leva a um bloqueio metabólico com repercussão variável no organismo, dependendo principalmente da rota metabólica afetada (Chalmers, 1989; Ozand e Gascon, 1991a; Ozand e Gascon, 1991b; Gascon et al., 1994; Vilaseca-Brusca et al., 2002). A deficiência na atividade de um enzima localizada em uma rota metabólica de degradação de aminoácidos, carboidratos ou lipídios é a principal causa de EIM já descritos.

Em 1982 Siclair classificou os EIM em quatro grandes grupos, conforme o tipo de função exercida pela proteína deficiente e o tecido envolvido, considerando ainda aspectos clínicos, bioquímicos, patológicos e terapêuticos.

1- Doenças de transporte: afetam o transporte renal e/ou intestinal de moléculas inorgânicas e orgânicas. Determinam graus variáveis de deficiência tecidual de substâncias essenciais. A deficiência de dissacaridases e defeito no transporte de magnésio são exemplos desse grupo.

2- Doenças de armazenamento, degradação e secreção: determinam o acúmulo de macromoléculas e, conseqüentemente, alterações patológicas nos tecidos afetados, envolvendo principalmente o complexo de Golgi ou os lisossomas. As doenças lisossômicas de depósito são exemplos desse grupo.

3- Doenças de síntese: envolvem a síntese de moléculas biologicamente importantes como hormônios, moléculas de função estrutural ou imunológica, proteínas plasmáticas e enzimas secretadas para o plasma. A hiperplasia adrenal congênita por deficiência na atividade da enzima 21- hidroxilase e a hipogamaglobulinemia são exemplos desse grupo.

4- Doenças do metabolismo intermediário: envolvem deficiências enzimáticas nas rotas de degradação de moléculas pequenas (aminoácidos,

glicídios, neurotransmissores), podendo comprometer rotas metabólicas importantes. Têm como característica o acúmulo de metabólitos tóxicos nas células, provocando alterações bioquímicas e dano tecidual. Constituem o maior grupo de EIM e têm como exemplos as acidúrias orgânicas, as desordens do metabolismo de aminoácidos e das purinas e as porfirias.

A disponibilidade das técnicas de quantificação desses compostos nos líquidos biológicos (cromatografia gasosa/espectrometria de massa) (Tanaka et al., 1966) permitiu a descoberta de muitos desses distúrbios que hoje somam mais de 60 (Scriver et al, 2001). Exemplos de algumas dessas desordens, com sua respectiva prevalência (incidência na população geral) são a acidúria metilmalônica (1:29.000), a acidemia glutárica (1:40.000), a acidemia propiônica (1:48.000), as deficiências de biotinidase (1:60.000), da desidrogenase de acilas de ácidos graxos de cadeia média (MCAD, 1:10.000), a doença do xarope do bordo (1:70.000) e a deficiência de piruvato desidrogenase (1:250.000). No início da década de 80 foi demonstrado que essas entidades eram os EIM mais freqüentes em crianças hospitalizadas (Chalmers et al, 1980), o que se refletiu em intensos estudos clínico/laboratoriais e epidemiológicos nos anos que se seguiram.

Clinicamente os pacientes afetados por esses distúrbios apresentam como sintomatologia mais comum a disfunção neurológica em suas mais diversas formas de expressão tais como regressão neurológica, convulsões, coma, ataxia, hipotonia, hipertonia, irritabilidade, tremores, tetraparesia espástica, atraso no desenvolvimento neuropsicomotor, etc. Sem tratamento, um grande número de casos tem desenlace fatal ainda no primeiro ano de vida, enquanto que os pacientes que sobrevivem aos estágios iniciais da doença apresentam um grau variável de retardo mental e outras sequelas neurológicas (Scriver, 2001).

1.1. DOENÇA DO XAROPE DO BORDO

Em 1954, Menkes, Hurst e Craig descreveram quatro casos de uma doença degenerativa cerebral com início nas primeiras semanas de vida e morte dentro de três meses. O fenótipo mais proeminente foi um odor forte de açúcar queimado na urina que deu origem ao nome “Doença do Xarope do Bordo”. Entre 1957 e 1959, Dancis e colaboradores identificaram os compostos acumulados na urina e plasma dos pacientes como os aminoácidos de cadeia ramificada leucina, isoleucina e valina e seus α-cetoácidos (Dancis, 1959; Dancis et al, 1959).

A doença do Xarope do Bordo (DXB) é um distúrbio de herança autossômica recessiva (Danner e Elsas, 1989; Nobukuni et al, 1991) causado pela deficiência na atividade do complexo α-cetoácido desidrogenase de cadeia ramificada. Este bloqueio resulta no acúmulo dos aminoácidos de cadeia ramificada (AACR) leucina, isoleucina e valina e de seus respectivos α-cetoácidos de cadeia ramificada (CACR), ácido α-cetoisocapróico (CIC), ácido α-ceto-β-metilvalérico (CMV) e ácido α-cetoisovalérico (CIV) (Figura 1) (Chuang e Shih, 2001).

Os AACR leucina, valina e isoleucina compreendem em torno de 40% dos aminoácidos essenciais da dieta nos indivíduos normais. O principal destino metabólico destes AACR é a incorporação em proteínas corporais (Schadewaldt e Wendel, 1997).

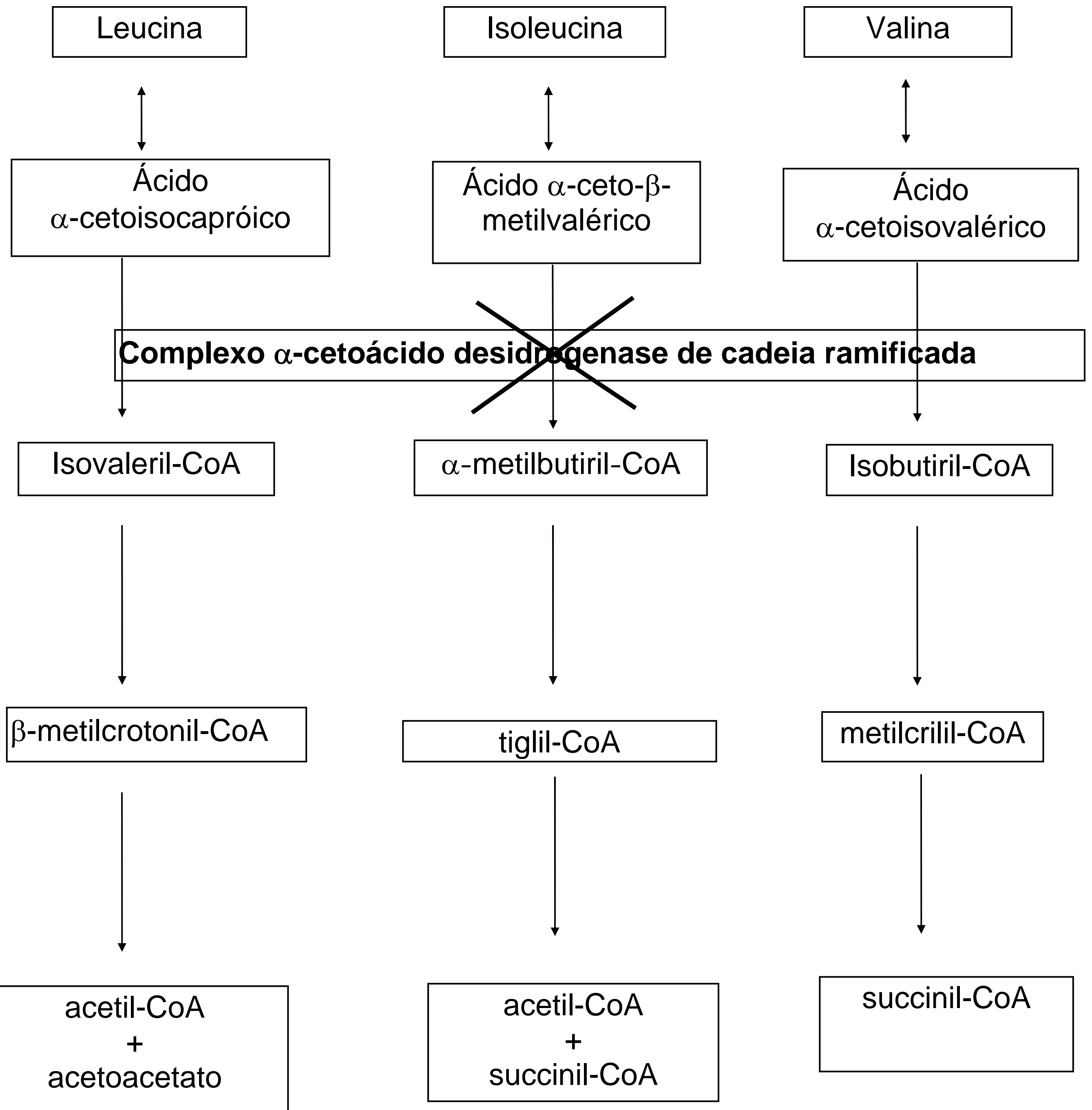

Figura 1 - Rota metabólica dos aminoácidos de cadeia ramificada leucina, isoleucina e valina, mostrando a etapa em que a enzima é deficiente nos pacientes com DXB. Adaptado de Scriver et al, 2001.

A via catabólica dos AACR inicia com o transporte destes aminoácidos para dentro da célula através do sistema de transporte L localizado no lado citosólico da membrana. Dentro da célula, os AACR sofrem transaminação reversível pela aminotranferase de cadeia ramificada produzindo os CACR, α-cetoisocapróico da leucina, α-ceto-β-metilvalérico da isoleucina e α-cetoisovalérico da valina. Os CACR são translocados para dentro da mitocôndria por um transportador específico onde sofrem descarboxilação oxidativa catalisada pelo complexo multienzimático α-cetoácido desidrogenase de cadeia ramificada. Estas reações produzem os respectivos acil CoA de cadeia ramificada que são metabolizados por vias distintas. Os produtos finais do catabolismo da leucina são a acetil CoA e o acetoacetato, da valina, succinil CoA e da isoleucina, acetil CoA e succinil CoA. Os AACR são tanto cetogênicos quanto glicogênicos, servindo como precursores para a síntese de ácidos graxos e do colesterol e também servindo como substrato para a produção de energia via succinil CoA e acetoacetato (Chuang e Shih, 2001).

O diagnóstico da DXB é fundamentalmente laboratorial. A identificação de concentrações plasmáticas e urinárias elevadas de leucina, isoleucina e valina e de seus respectivos α-cetoácidos, através de cromatografia de aminoácidos e ácidos orgânicos, caracterizam a doença. No entanto, a leucina é o principal metabólito acumulado na doença, atingindo níveis plasmáticos de até 5 mM enquanto a isoleucina e a valina atingem 1 mM em pacientes não tratados (Zielke et al, 1996). O perfil dos aminoácidos é determinado no plasma e a urina é analisada para os cetoácidos. Muitos estados nos Estados Unidos e outros países utilizam um programa de triagem em massa para identificar neonatos com níveis plasmáticos de leucina acima de 2 mg/100 ml (153 μmol/l) indicativos da doença (Danner e Elsas, 1989).

A presença de quantidades mensuráveis de L-aloisoleucina no plasma, um produto da racemização da isoleucina, é considerada patognomônica para a doença. No entanto, demonstrou-se que a formação da L-aloisoleucina não está associada somente a DXB, pois ocorre tanto em cultura de células normais *in vitro* como também em pessoas normais *in vivo*. Na análise de rotina dos aminoácidos,

porém, a L-aloisoleucina é indetectável no plasma de pacientes saudáveis devido à sua baixa concentração e à presença de compostos interferentes (Shadewaldt et al, 1990).

A confirmação do diagnóstico é feita através da medida da atividade do complexo α-cetoácido desidrogenase de cadeia ramificada em leucócitos periféricos dos pacientes. Esta determinação é feita em cultura de células (Peinemann e Danner, 1994).

O diagnóstico pré-natal pode ser realizado em células do fluido amniótico retiradas por amniocentese entre a 14° a 18° semana de gestação ou por análise direta do tecido de vilosidades coriônicas e cultura de células vilosidades coriônicas (Kleijer et al, 1985; Chuang e Shih, 2001).

Os pacientes com DXB podem ser classificados em cinco fenótipos, dependendo da apresentação clínica, da tolerância à leucina e da atividade residual da enzima medida *in vitro* em fibroblastos da pele ou de leucócitos do sangue (Schadewaldt et al, 1998).

1) Forma Clássica

Representa a forma mais severa e comum da doença. Os níveis dos AACR, particularmente leucina, estão muito aumentados no sangue, líquido cefaloraquidiano e urina. Na DXB clássica 50% ou mais dos AACR são derivados da leucina. Os recém-nascidos afetados parecem normais no nascimento e os sintomas começam a se desenvolver entre os 4-7 dias após o nascimento. Letargia e perda de apetite são os primeiros sintomas, seguidos por perda de peso e alteração progressiva dos sinais neurológicos. Cetoacidose e odor de açúcar queimado são observados. A maioria dos pacientes morre nos primeiros meses de vida devido as crises metabólicas e deterioração neurológica (Pienemann e Danner, 1994; Chuang e Shih, 2001).

2) Forma Intermediária

Os pacientes com a forma intermediária da doença apresentam elevação persistente dos AACR, porém em níveis mais baixos que os da forma clássica. Geralmente não possuem sintomatologia aguda no período neonatal. A atividade

residual da enzima é geralmente maior que a da forma clássica, em torno de 3 a 30% do normal (Chuang e Shih, 2001).

3) <u>Forma Intermitente</u>

Os pacientes com a forma intermitente da doença apresentam desenvolvimento normal com crescimento e desenvolvimento cognitivos normais. Apresentam risco de descompensação metabólica aguda durante situações de estresse. Quando assintomáticos, os dados laboratoriais desse pacientes são normais, incluindo os níveis plasmáticos dos AACR. A atividade do complexo enzimático encontra-se em torno de 5 a 20% do normal. Os sintomas geralmente aparecem entre 5 meses e 2 anos de idade associados, geralmente, a otite média ou outras infecções (Chuang e Shih, 2001).

4) <u>Forma Tiamina-Responsível</u>

Geralmente, estes pacientes não apresentam doença neonatal aguda. O distúrbio é detectado devido a um atraso no desenvolvimento psicomotor. A atividade da enzima está em torno de 30 a 40% do normal. A concentração plasmática dos AACR é cerca de 5 vezes maior que o normal, essa concentração pode retornar aos níveis normais com doses de 10 a 1000 mg/dia de tiamina e com dieta restrita em proteínas (Chuang e Shih, 2001).

5) <u>Forma Di-hidrolipoil Desidrogenase (E_3)-Deficiente</u>

A deficiência de E_3 é um distúrbio raro. O fenótipo clínico é similar ao da forma intermediária, mas é acompanhada de acidose lática severa. Os níveis de lactato, piruvato, α-cetoglutarato, α-hidroxiisovalerato e α-hidroxiglutarato estão aumentados. Os níveis dos AACR estão levemente ou moderadamente aumentados no plasma se comparados com os pacientes com a forma clássica. Esses pacientes têm uma deficiência combinada das desidrogenases do piruvato, α-cetoglutarato e AACR. Na presença de acidose láctica persistente ocorre deterioração neurológica progressiva, incluindo hipotonia (Chuang e Shih, 2001).

Um dos achados mais comum dos pacientes com DXB é a encefalopatia. Observa-se também edema cerebral envolvendo o tronco cerebral dorsal, os pedúnculos cerebrais e a cápsula interna, que se acentua durante as crises de descompensação metabólica. Além disso, estudos relataram hipodensidade difusa

no globo pálido e tálamo afetando a substância branca destas regiões, o que é um indicativo de hipomielinização (Treacy et al, 1992). O trato piramidal da medula espinal, a mielina que circunda o núcleo dentado, o corpo caloso e os hemisférios cerebrais são os mais afetados. Paralelamente à deficiência de mielina, observa-se redução dos oligodendrócitos. Outra estrutura que apresenta alterações expressivas é o cerebelo, pois relatos mostram panecrose da camada de células granulares com preservação das células de Purkinje. Nos núcleos da base e substância negra observou-se perda neuronal considerável (Chuang e Shih, 2001).

O tratamento dos pacientes com DXB consiste basicamente em restrição dos AACR da dieta para minimizar os efeitos do acúmulo destes metabólitos, principalmente no sistema nervoso central (SNC). O objetivo do tratamento é normalizar as concentrações dos AACR sem prejudicar o crescimento e desenvolvimento dos jovens pacientes e para tanto, deve ser iniciado o mais cedo possível e ainda no período neonatal. Para tanto, administra-se um leite especial com concentrações reduzidas de leucina, valina e isoleucina. Outras preparações dietéticas específicas para pacientes com DXB também foram desenvolvidas (Snyderman et al, 1964). A terapia com tiamina (50 a 300 mg/dia) por três semanas é empregada no início do tratamento para a detecção de pacientes com a doença tiamina-responsíveis (Chuang e Shih, 2001).

Em casos mais graves tem sido utilizado o transplante de fígado, substituindo o órgão com gene alterado por um órgão normal. Desta forma, os aminoácidos de cadeia ramificada podem ser metabolizados normalmente.

Na fase aguda, emprega-se um tratamento mais agressivo, pois o aumento dos AACR e CACR, freqüentemente precipitados por infecções ou outros estresses, leva à deterioração das funções cerebrais. Existem três medidas a serem tomadas para o controle das crises metabólicas: remoção dos metabólitos tóxicos; promover suporte nutricional adequado e minimizar o catabolismo e/ou promover o anabolismo. Supõe-se que promover o anabolismo pode ser um dos mais importantes fatores no controle da descompensação metabólica (Chuang e Shih, 2001).

A diálise peritonial e a transfusão exsanguínea são utilizadas para a remoção dos metabólitos com melhora significativa e rápida do quadro neurológico. No entanto, a hemodiálise é mais eficaz na depuração dos AACR e dos CACR do plasma do que a diálise peritonial. A diálise peritonial é um procedimento relativamente simples, enquanto que a hemodiálise requer um equipamento sofisticado e treinamento especializado. Desenvolveu-se, para os pacientes com DXB, uma terapia com nutrição parenteral que inclui uma mistura de AACR em combinação com glicose, lipídios, eletrólitos e vitaminas a fim de proporcionar uma nutrição balanceada. Esta preparação é utilizada nos casos de descompensação metabólica moderada ou em combinação com outras terapias na descompensação metabólica severa. Uma terapia alternativa consiste em tratar o estado catabólico com insulina e glicose (Rogers et al, 1962).

Os mecanismos tóxicos dos AACR e CACR sobre o cérebro são pouco conhecidos. Alguns fatores, tais como a complexidade do desenvolvimento do cérebro, as concentrações alcançadas pelas toxinas e o estágio do desenvolvimento cerebral em que elas atuam prejudicam o esclarecimento destes efeitos.

Tashian, em 1961, demonstrou que os ácidos CIC e CIV inibem competitivamente a glutamato descarboxilase em homogeneizados de cérebro de rato. Os hidroxiácidos α-hidroxi-isocapróico e α-hidroxi-isovalérico também apresentaram o mesmo efeito, mas em menor grau. Desta forma, estes metabólitos poderiam reduzir a quantidade de produção de ácido γ-aminobutírico (GABA), o principal neurotransmissor inibitório do sistema nervoso central (SNC). Foi também verificado em um modelo animal de DXB em bovinos, que eles apresentaram uma perda marcante no número de receptores pós-sinápticos $GABA_A$ demonstrada por ligação de $[H^3]$diazepam (Dodd et al, 1992). Por outro lado, foi demonstrado que os cetoácidos CIC, CIV e CMV inibem a oxidação da glicose e a síntese de acetilcolina, lipídios, proteínas e ácidos nucléicos em fatias de cérebro de ratos (Gibson e Blass, 1976). Além disso, outros investigadores demonstraram que estes cetoácidos também inibem a enzima ácido graxo sintetase (Gibson e Blass, 1976). Outros estudos observaram que os cetoácidos

inibem a oxidação do piruvato em cérebro de ratos, o transporte mitocondrial de piruvato, a atividade do complexo piruvato desidrogenase e a atividade da enzima α-cetoglutarato desidrogenase (Gibson e Blass, 1976). Os aminoácidos valina e isoleucina inibiram a piruvato quinase alostericamente em cérebro de ratos (Gibson e Blass, 1976). Outros achados mostraram que o excesso de leucina na dieta provoca uma diminuição da serotonina cerebral (Yuwiler e Geller, 1965). Finalmente, como uma propriedade da leucina é estimular a secreção de insulina, Panten (1972) propôs que essa ação poderia explicar os episódios de hipoglicemia apresentados pelos pacientes com a DXB.

Dentre os cetoácidos acumulados na doença, o CIC é usualmente considerado o mais tóxico. O CIC inibe o consumo de oxigênio em cérebro de ratos, a oxidação do 3-hidroxibutirato (Gibson e Blass, 1976) e provoca deficiência na formação da mielina no cerebelo de ratos. Alguns estudos demonstraram que a elevação sérica de leucina e de CIC no espaço extracelular altera a concentração no SNC dos aminoácidos transportados pelo transportador dos aminoácidos neutros de cadeia longa (sistema L) que incluem a metionina, valina, isoleucina, triptofânio, tirosina, fenilalanina e glutamina. Desta forma, a biossíntese celular de alguns neurotransmissores cerebrais como as catecolaminas e a serotonina poderia estar afetada pela diminuição na concentração cerebral de alguns destes aminoácidos (Zielke et al, 1996; Huang et al, 1996). Estes mesmos autores observaram que a adição de CIC a cultura de astrócitos de rato diminui o conteúdo de glutamato aumentando a taxa de oxidação do glutamato. O mecanismo sugerido foi a transferência do grupo amino do glutamato para o CIC levando a formação de leucina e α-cetoglutarato. Também se demonstrou que, além de aumentar a taxa de oxidação de glutamato, níveis elevados de CIC também aumentam a oxidação da glutamina (Zielke et al, 1997). Na presença de um excesso de CIC, o conteúdo de glutamato intracelular em cultura de astrócitos diminui em 50% (YudKoff et al, 1993; Yudkoff et al, 1994). Outro efeito da leucina é o de ativar alostericamente a enzima glutamato desidrogenase (Zielke et al, 1997). Outros estudos demonstram ativação semelhante em mitocôndrias de ratos (Coueé e Tipton, 1989) e em sinaptossomas (Erecinska et al, 1990). Por outro

lado, o CIC inibe a síntese de glutamina em cultura de astrócitos (Yudkoff et al, 1994) e os cetoácidos CIC e CMV e o hidroxi-ácido α-hidroxi-isovalérico inibem o crecimento celular *in vitro* (Wajner et al, 1995).

2. CITOEQUELETO

A capacidade que as células eucarióticas têm de adotar uma variedade de formas e de executar movimentos coordenados e direcionados depende de uma rede complexa de filamentos de proteínas fibrosas que se estendem por todo o citoplasma. Esta rede é chamada de citoesqueleto, o qual é uma estrutura altamente dinâmica que se reorganiza continuamente sempre que a célula altera a forma, se divide ou responde ao seu ambiente. O citoesqueleto poderia ser denominado "citomusculatura", pois ele é o responsável direto por movimentos tais como: deslocamento das células sobre um substrato, contração muscular e muitas alterações na forma em embriões de vertebrados em desenvolvimento. O citoesqueleto também fornece a maquinaria necessária para movimentos intracelulares tais como: transporte de organelas de uma região para outra no citoplasma e a segregação de cromossomos na mitose (Alberts et al, 2002).

As mudanças citoesqueléticas são a chave para a transmissão de sinais que levam a uma resposta celular apropriada. As interações entre as proteínas de membrana e as proteínas do citoesqueleto têm papéis centrais nas respostas celulares. O citoesqueleto também está envolvido na organização e reorganização dos receptores da membrana plasmática e é essencial para os mecanismos de reconhecimento celulares (Carraway, 2000).

As diferentes atividades do citoesqueleto dependem de três diferentes tipos de filamentos protéicos: filamentos de actina (microfilamentos), microtúbulos e filamentos intermediários. Cada tipo é formado pela associação ou polimerização de monômeros específicos: nos microfilamentos, por polímeros de actina globular, nos filamentos intermediários por associações de famílias de proteínas fibrosas célula-específicas e nos microtúbulos por associações de dímeros de tubulina α e β (Figura 2) (Carraway, 2000; Alberts et al, 2002; Helfand et al, 2004).

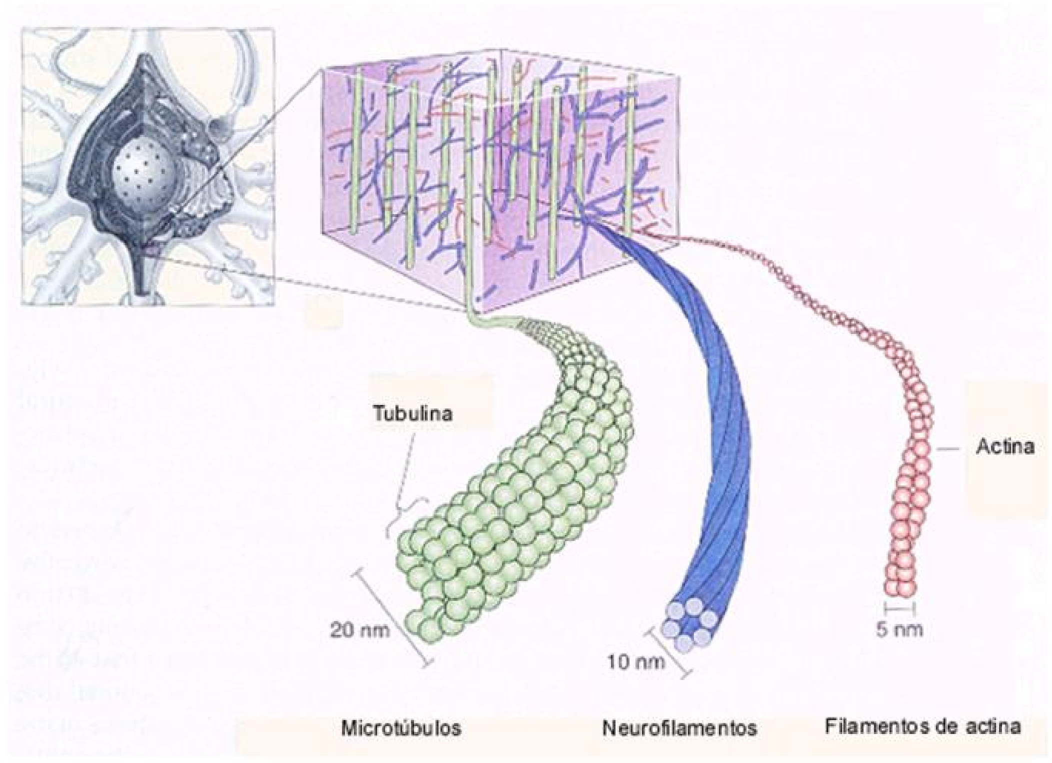

Figura 2. Representação esquemática dos constituintes do citoesqueleto. Adaptado de Bear et al, 1996.

2.1. FILAMENTOS DE ACTINA

Os filamentos de actina, também conhecidos como microfilamentos, são estruturas flexíveis com 5 a 9 nm, consistindo de uma hélice compacta de moléculas de actina (actina globular ou actina G) uniformemente orientadas (Figura 3). É uma estrutura polar com duas extremidades diferentes - a extremidade "menos" relativamente inerte e de crescimento lento, e a extremidade "mais" de crescimento rápido. Estas estruturas flexíveis são organizadas na forma de feixes lineares, redes bidimensionais ou géis tridimensionais. Embora os filamentos de actina estejam distribuídos por toda a célula, eles estão mais concentrados na região cortical, logo abaixo da membrana plasmática (Alberts et al, 2002).

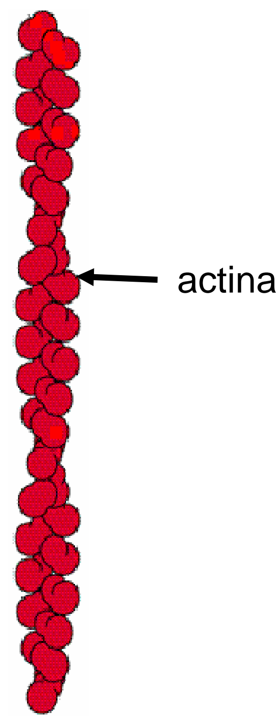

Figura 3. Representação esquemática de um microfilamento. Adaptada de Alberts et al, 2002.

As principais funções dos filamentos de actina nas células eucarióticas são a manutenção da forma bem como a participação nos movimentos de divisão celular (Albets et al, 2002). Além disso, nas células nervosas têm importante papel no crescimento de neuritos (Lin e Forscher,1995) e na liberação de neurotransmissores (Marinowich et al, 1997; Small et al, 1999).

Várias proteínas que interagem com microfilamentos têm sido descritas, tais como a proteína motora miosina. Exemplos de proteínas associadas aos microfilamentos são: a filamina, que une os filamentos por meio de ligações transversais formando um gel frouxo; a fimbrina e a α-actinina que formam feixes de filamentos paralelos; a gelsolina que induz a fragmentação da actina na presença de cálcio (Ca^{2+}). As espectrinas formam o complexo de sustentação da membrana plasmática, sendo importantes para as interações célula-célula, além

disso, parecem estar relacionadas com a modulação da transmissão sináptica por sua associação com as vesículas sinápticas (Kirkpatrick e Brady, 1999; Alberts et al, 2002). O efeito coordenado das várias proteínas associadas à actina, gerando uma resposta do citoesqueleto de actina a um sinal extracelular, só é possivel graças a complexos mecanismos cooperativos de interação destas proteínas, tanto com a actina monomérica, regulando sua capacidade de polimerização, quanto com os microfilamentos, regulando a sua organização citoplasmática e consequentemente os movimentos de superfície celular (Alberts et al, 2002).

2.2. MICROTÚBULOS

Os microtúbulos são polímeros longos e rígidos, com diâmetro em torno de 25 nm, que se estendem ao longo de todo o citoplasma e coordenam a localização intracelular de outros componentes celulares. São filamentos ocos formados pela polimerização dos dímeros α e β tubulina, formando filamentos mais rígidos que os filamentos de actina. São longos e retos tendo uma extremidade ligada ao único centro organizador de microtúbulos (MTOC) da célula, chamado centrossomo. A partir desta estrutura os microtúbulos emanam por toda a célula. As tubulinas α e β são proteínas globulares que formam uma estrutura linear chamada protofilamento. Treze protofilamentos se dispõem lado a lado formando um microtúbulo (Figura 4). O microtúbulo é uma estrutura polar, uma vez que os protofilamentos estão paralelamente alinhados com a mesma polaridade (Díaz et al, 1998; Dowing, 2000).

As subunidades de tubulina podem sofrer várias modificações pós-traducionais como, por exemplo, acetilação, fosforilação e detirosinação. Essas modificações podem influenciar a estabilidade dos microtúbulos e interação com outras proteínas (Ludena, 1998; Rosenbaum, 2000).

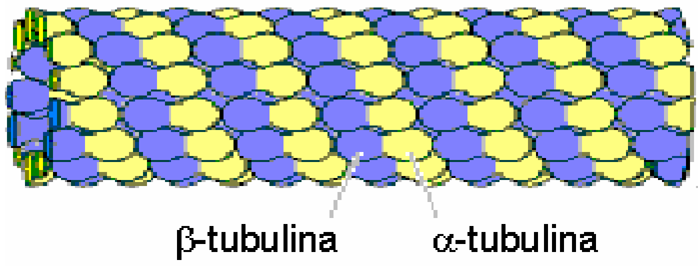

Figura 4. Representação esquemática de um microtúbulo. Adaptado de Cooper et al, 2001.

Os microtúbulos possuem uma variedade de funções em diferentes células. Eles têm papel fundamental nos movimentos celulares, transporte intracelular de organelas e formação do fuso mitótico durante a divisão celular (Schulze et al, 1987; Nogales, 2000, Sánchez et al, 2000). No cérebro, estão envolvidos em várias funções celulares, incluindo transporte axonal, sinaptogênese (Vale et al, 1985), crescimento e elongação de neuritos (Rieder et al, 1997).

2.3. FILAMENTOS INTERMEDIÁRIOS

Os filamentos intermediários (FI) são polímeros de proteínas fibrosas, com diâmetro aproximado de 10 nm, tamanho intermediário entre os filamentos de actina (5 nm) e os microtúbulos (25 nm). São filamentos longos que possuem subtipos específicos em diferentes tecidos e grande diversidade em sua seqüência. Desempenham papel estrutural na célula, mantendo sua integridade (Fuchs e Cleveland, 1998; Alberts et al, 2002).

Na formação de um FI um monômero interage com outro monômero idêntico para formar um dímero, no qual os domínios centrais se alinham paralelamente enrolando-se em uma estrutura torcida. Dois dímeros alinham-se lado a lado para formar um tetrâmero antiparalelo com quatro cadeias

polipeptídicas. Oito tetrâmeros enrolam-se para formar a estrutura do FI com 10 nm de diâmetro (Figura 5) (Alberts et al, 2002).

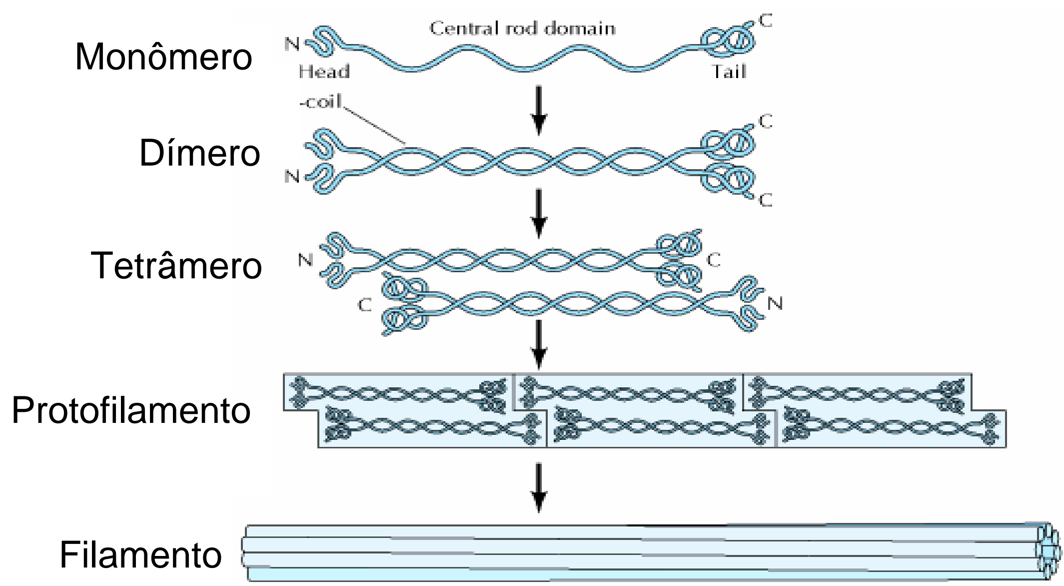

Figura 5. Representação esquemática da formação de um filamento intermediário. Adaptado de Cooper et al, 2001.

A maioria das proteínas que formam os FI possuem um domínio central, em forma de α-hélice altamente conservado enquanto as regiões amino-terminal e carbóxi-terminal apresentam diferenças que permitem uma subclassificação em seis tipos diferentes: subtipo I- queratinas ácidas; subtipo II- queratinas básicas e neutras; subtipo III- desmina, vimentina, periferina e proteína glial fibrilar ácida (GFAP); subtipo IV- neurofilamentos; subtipo V- laminas nucleares e subtipo VI- nestina (Fuchs e Weber, 1994; Elder et al, 1999; Inada et al, 2000; Herrmann e Aebi, 2000; Lariviere e Julien, 2004).

A classificação dos FI bem como sua ocorrência em células de mamíferos estão apresentadas na tabela 1.

Classe	Exemplos	Ocorrência em mamíferos
I e II	Citoqueratinas ácidas e básicas	Células epiteliais
III	Vimentina Desmina Periferina GFAP	Células mesenquimais Células musculares Células neurais diversas Astrócitos
IV	Neurofilamentos α-internexina	Neurônios Neurônios
V	Laminas A, B e C	Lâmina nuclear de células eucarióticas
VI	Nestina	Células musculares e neuroepiteliais

Tabela 1. Classificação e ocorrência dos filamentos intermediários. Adaptado de Herrmann e Aebi, 2000.

2.3.1. NEUROFILAMENTOS

Os neurofilamentos constituem o principal tipo de FI em neurônios adultos (Lee e Cleveland, 1996). São formados pela polimerização de três subunidades: os neurofilamentos de alto peso molecular (NF-H), de médio peso molecular (NF-M) e de baixo peso molecular (NF-L), com pesos moleculares aparentes de 200, 150 e 68 kDa, respectivamente, determinados por eletroforese em gel de poliacrilamida. As três subunidades possuem os mesmos 310 aminoácidos no domínio central e diferem entre si pelos seus domínios carbóxi e amino-terminal (Betts et al, 1997; Li et al, 1999; Ackerley, 2000; Al-Chalabi e Miller, 2003).

Embora as três subunidades polimerizem contribuindo para a formação do neurofilamento, a mais abundante delas é a NF-L que foi descrita como sendo a responsável, juntamente com a extremidade amino-terminal, pela formação do filamento propriamente dito (Geisler e Weber, 1981), enquanto as regiões carbóxi-

terminais das subunidades NF-M e NF-H são responsáveis pela formação das projeções laterais. Estas se estendem perpendicularmente ao filamento central, permitindo a interação dos neurofilamentos entre si e com os demais constituintes do citoesqueleto (Figura 6) (Hisanga e Hirokawa, 1988; Gotow et al, 1992; Kirkpatrick e Brady, 1999).

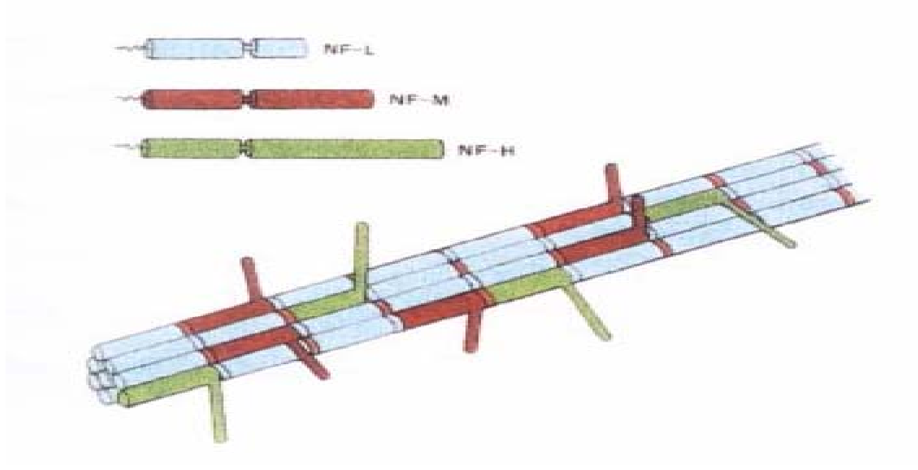

Figura 6. Representação esquemática de um neurofilamento. Adaptado de Nixon e Sihag, 1991.

Os neurofilamentos determinam o calibre axonal, auxiliam na manutenção da morfologia neuronal e participam do transporte axonal de metabólitos do corpo celular até a sinapse (Kirkpatrick e Brady, 1999; Elder et al, 1999). Estudos com camundongos transgênicos demonstram que animais que super expressam NF-L apresentam um aumento na densidade de neurofilamentos, mas nenhum aumento no calibre axonal (Monteiro et al, 1990). Enquanto que animais que não expressam NF-L e NF-M têm o número de neurofilamentos e o calibre axonal significativamente diminuídos (Zhu et al, 1997; Elder et al, 1998). Nos animais que não expressam NF-H foram observadas modestas reduções no calibre axonal, mas os efeitos da depleção de NF-H foram em parte compensados pelo aumento dos níveis de NF-M e do número de microtúbulos (Zhu et al, 1998).

Os neurofilamentos são sintetizados no corpo celular e transportados para o axônio em processo denominado transporte axonal, o qual é regulado por fosforilação (Ackerley et al, 2000; Jung et al, 2000).

O acúmulo de neurofilamentos é descrito em várias doenças neurodegenarativas, tais como: esclerose amiotrófica lateral, doença de Parkinson e doença de Alzheimer. Ainda não está claro como esse acúmulo de neurofilamentos contribui para o processo neurodegenerativo nessas doenças, mas sugere-se que o transporte de neurofilamentos esteja interrompido nos neurônios afetados (Ackerley et al, 2000; Lariviere e Julien, 2004).

2.3.2. PROTEÍNA GLIAL FIBRILAR ÁCIDA (GFAP)

A GFAP é uma proteína estrutural sintetizada nos astrócitos e algumas células de Schwanm (Kaneko et al, 1994; Kosako et al, 1997; Guo-Ross et al, 1999). Possui um peso molecular aparente de 50 kDa, sendo composta por três regiões distintas. A região amino-terminal é composta de 35 resíduos de aminoácidos e possui uma estrutura em conformação β que contém oito resíduos de arginina, caracterizando esta região como básica. A região carbóxi-terminal contém cerca de 50 resíduos de aminoácidos, possuindo uma estrutura globular que pode estar envolvida em interações da GFAP com outras proteínas (Inagaki et al, 1990; Feintein et al, 1992).

A GFAP é considerada marcador de astrócitos e é importante na modulação da motilidade e forma celular por fornecer estabilidade estrutural aos astrócitos. No SNC depois de uma injúria, seja ela resultado de um trauma, doença, insulto químico ou desordens genéticas, os astrócitos ficam reativos e respondem de uma maneira típica, processo chamado de astrogliose. A astrogliose é caracterizada pela rápida síntese de GFAP (Pekny et al, 1999; Eng et al, 2000).

A GFAP também é importante para as interações astrócito-neurônio, (McCall et al, 1996; Eliasson et al, 1999), as quais apresentam um sofisticado sistema de comunicação recíproca que pode regular a liberação de

neurotransmissores, a excitabilidade neuronal e a transmissão sináptica (Carmignoto, 2000).

2.3.3. VIMENTINA

A vimentina é uma subunidade de FI de 54 kDa, sendo a proteína estrutural mais amplamente distribuída entre as células dos FI, ocorrendo em muitas células de origem mesenquimal, incluindo fibroblastos, células endoteliais e glóbulos brancos, além disso, muitas células expressam vimentina de forma transitória durante o desenvolvimento (Alberts et al, 2002). A vimentina também é encontrada em astrócitos imaturos, em alguns astrócitos maduros, como a glia de Bergmann no cerebelo e em subpopulações de astrócitos do corpo caloso (Galou et al, 1996; Alberts et al, 2002).

A vimentina tem papel importante nas funções de deformabilidade, migração e contractilidade dos fibroblastos. Células deficientes em vimentina são menos contrácteis e com menos motilidade (Ecker et al, 1998; Wang e Stamenovic, 2000). A vimentina, assim como a GFAP, é necessária no processo de cicatrização da glia após traumas no SNC (Eliasson et al, 1999).

2.4. FATORES QUE MODIFICAM O METABOLISMO DAS PROTEÍNAS DO CITOESQUELETO

As proteínas do citoesqueleto têm se mostrado sensíveis à ação de agentes externos (drogas), a patologias e à alteração do estado nutricional do organismo. Estes fatores levam, por mecanismos distintos, a alterações do funcionamento normal destas proteínas acarretando danos reversíveis ou irreversíveis à célula. Modelos experimentais produzidos *in vivo* e *in vitro* por agentes neurotóxicos são utilizados para induzir modificações no citoesqueleto neural. Vários estudos têm demonstrado alterações das proteínas do citoesqueleto em doenças neurodegenerativas e sua sensibilidade a agentes químicos neurotóxicos.

Diferentes agentes neurotóxicos alteram as propriedades físico-químicas ou o estado conformacional destas proteínas causando alterações na organização e função do citoesqueleto: alumínio (Trancoso et al, 1990; Sivaguru et al, 2003); acrilamida (Lapadula et al, 1989; Gupta e Abou-Donia, 1997); β,β´-aminodipropionitrila (IDPN) (Gold e Austin, 1991; Zhu et al, 1997; Zhu et al, 1998); 2,5-hexanediona (Heijink et al, 1996); dissulfeto de carbono (Wilmarth et al, 1993; Gupta e Abou-Donia, 1997), peroxinitritos (Landino et al, 2002) e organofosforados (Abou-Donia et al, 1988) têm sido associados com o acúmulo de neurofilamentos e com a alteração na dinâmica dos microtúbulos. A exposição ao lítio causa a degradação de subunidades de tubulinas e a inibição da fosforilação da subunidade NF-M de neurofilamentos (Bennett et al, 1991; Perez et al, 2003). Estas alterações podem estar envolvidas com os efeitos tóxicos do lítio. Recentemente demonstramos que o metilmercúrio inibe a fosforilação de filamentos intermediários de córtex cerebral de ratos jovens (Moretto et al, 2004). Alterações citoesqueléticas também têm sido relacionadas à degeneração axonal observada em patologias como a doença de Parkinson, a doença do neurônio motor, a doença de Alzheimer e a esclerose amiotrófica lateral (Julien e Mushinski, 1998; Fitzpatrick et al, 1998; Lariviere e Julien, 2004).

Modelos experimentais de erros inatos do metabolismo, como a hiperfenilalaninemia têm mostrado alterações específicas no sistema fosforilante endógeno associado ao citoesqueleto do córtex cerebral de ratos. A hiperfenilalaninemia experimental produziu um efeito inibitório sobre o sistema responsável pela fosforilação e desfosforilação in vitro da subunidade NF-M dos neurofilamentos e das tubulinas α e β e este efeito envolve a inibição das atividades da PKCaMII e da PP1 (de Freitas et al, 1995; de Freitas et al, 1997).

Demonstramos em nosso laboratório que fatias de córtex cerebral de ratos de 17 dias tratadas com os ácidos propiônico e metilmalônico em concentrações encontradas no sangue de pacientes com acidemia propiônica e metilmalônica, respectivamente, apresentam um aumento de incorporação de ^{32}P ortofosfato em proteínas do citoesqueleto e que este efeito é mediado por receptores glutamatérgicos do tipo N-metil-D-aspartato (NMDA) (de Mattos-Dutra et al, 2000).

Além disso, verificamos que os ácidos propiônico e metilmalônico alteram a fosforilação de FI em fatias de córtex cerebral de ratos de um modo dependente do desenvolvimento. Diminuem a fosforilação protéica em animais de 12 dias de vida e aumentam essa fosforilação em ratos de 17 e 21 dias de idade (Vieira de Almeida, 2003).

3. FOSFORILAÇÃO DE PROTEÍNAS NO SISTEMA NERVOSO CENTRAL

A fosforilação de proteínas é um processo molecular reversível que desempenha um papel fundamental na regulação de diversas funções celulares, sendo o principal mecanismo utilizado no processo de transdução de sinal (Hunter, 1995). Comum a todos os organismos eucariontes, o mecanismo de fosforilação protéica envolve pelo menos três elementos: (1) proteína quinase; (2) proteína fosfatase; (3) substrato protéico, o qual tem suas propriedades conformacionais e funcionais alteradas em decorrência de seu estado fosforilado/desfosforilado (Walaas e Greengard, 1991; Rodnight et al, 1997). As proteínas quinases são fosforiltransferases que catalisam a transferência do fosfato γ do ATP ou GTP para o grupo hidroxila de resíduos serina, treonina ou tirosina do substrato protéico. As proteínas fosfatases são fosforiltransferases que catalisam a reação inversa, ou seja, a hidrólise do grupo fosfato ligado ao substrato protéico (Nestler e Greengard, 1999) (Figura 7). Então, a fosforilação de uma determinada proteína ocorre em sítios específicos, pela ação de uma ou mais quinases e a desfosforilação pela atividade específica de fosfatases. Deste modo, o processo de fosforilação depende de uma ação coordenada de atividades quinásicas e fosfatásicas, ambas sujeitas à regulação por diversos mensageiros celulares (Hubbard e Cohen, 1993; MacKintosh e MacKintosh, 1994; Hunter, 1995).

O sistema nervoso é um tecido muito rico em sistemas fosforilantes e este processo desempenha um papel fundamental nas funções neuronal e glial. Desta forma, uma diversidade de funções podem ser reguladas através do processo de fosforilação/desfosforilação de proteínas no SNC, incluindo: expressão de genes, atividade de enzimas, condutância de canais iônicos, atividade de receptores,

biossíntese de neurotransmissores, modulação da transmissão sináptica, ancoramento e transporte de vesículas sinápticas, (Nixon e Sihag, 1991; Walaas e Greengard, 1991) plasticidade das células no SNC e a dinâmica remodelagem da arquitetura citoesquelética das células nervosas (Rodnight e Wofchuk, 1992; Nairn e Shenolikar, 1992; Inagaki et al, 1994; Pasqualotto e Shaw, 1996).

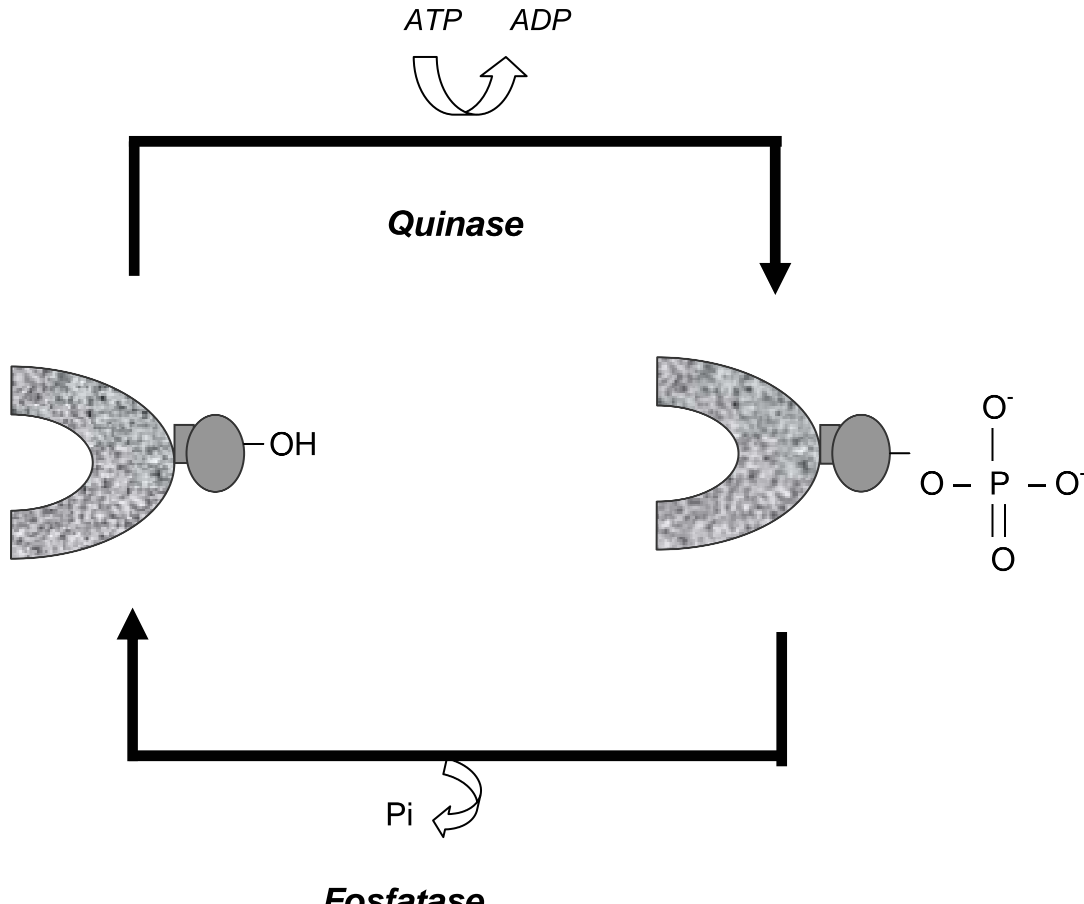

Figura 7. Representação esquemática de um sistema fosforilante. A reação de fosforilação é catalisada por uma quinase que transfere o grupo fosfato de uma molécula de ATP para o grupo hidroxila da cadeia lateral de um resíduo de serina, treonina ou tirosina do substrato protéico. A remoção do grupo fosfato é catalisada por uma fosfatase. Adaptado de Alberts et al, 2002.

A fosforilação protéica também está intimamente envolvida nas várias formas de plasticidade sináptica, exercendo um importante papel no início da LTP e da LTD (Schulman, 1995; Adams et al, 2000) e em eventos de aprendizado e memória (Izquierdo e Medina, 1997; Viola et al, 2000).

As proteínas quinases podem ser classificadas de acordo com os resíduos de aminoácidos que fosforilam em dois grupos principais: serina/treonina quinases e tirosina quinases. Aproximadamente 95% dos resíduos fosforilados são em serina e de 3 a 4% em treonina, enquanto que apenas 1% das fosfoproteínas no SNC são fosforiladas em resíduos de tirosina (Greengard, 1987; Nestler e Greengard, 1999). O mecanismo de ativação mais comum das serina/treonina quinases é mediado por segundos mensageiros, tais como: AMPc, GMPc, Ca^{2+}, fosfolipídios e ácido araquidônico. As tirosina quinases são freqüentemente associadas a receptores de superfície e ativadas diretamente por ligação ao primeiro mensageiro. Além desses mecanismos, sabe-se que a maioria das tirosina quinases apresentam sítios autofosforiláveis que atuam na regulação de sua atividade (Nestler e Greengard, 1999; Lau e Hugani, 1999).

As principais serina/treonina quinases dependentes de segundos mensageiros são: proteína quinase dependente de AMPc (PKA), proteína quinase dependente de GMPc (PKG), proteína quinase dependente de cálcio e calmodulina (PKCaM) e proteína quinase dependente de cálcio e fosfolipídios (PKC) (Walaas e Greengard, 1991) .

As proteínas fosfatases promovem a reversibilidade da ação quinásica através da hidrólise do grupo fosfato ligado ao substrato protéico. Seu papel na regulação do estado fosforilado/desfosforilado de proteínas tem sido cada vez mais evidente (Cohen, 1992; Hunter, 1995; Douglas et al, 2001).

As proteínas fosfatases podem ser de três tipos: ácidas, básicas ou neutras. Os dois primeiros tipos estão presentes em pequenas quantidades no SNC, de modo que não chegam a ter um papel muito significativo. No entanto, as fosfatases neutras apresentam-se em grandes quantidades no SNC e são divididas em duas classes, de acordo com a especificidade pelo substrato: serina/treonina fosfatases (que desfosforilam resíduos de fosfoserina e

fosfotreonina) e tirosina fosfatases (que atuam sobre resíduos de fosfotirosina) (Nestler e Greengard, 1999). Um terceiro grupo é constituído por fosfatases que possuem especificidade dupla no que diz respeito aos resíduos de aminoácidos que desfosforilam, atuando sobre fosfoserina, fosfotreonina e fosfotirosina. Estas atualmente encontram-se no grupo das tirosina fosfatases (Jia, 1997). Apesar desta classificação, algumas fosfatases são capazes de atuar sobre outros sítios, de modo que ocorre uma certa sobreposição de sítios desfosforiláveis em um mesmo substrato.

Algumas fosfatases têm sua atividade regulada no SNC, direta ou indiretamente, por segundos mensageiros, tais como: AMPc, GMPc e Ca^{2+}. Estes diferentes sistemas regulatórios possuem distintas distribuições celulares. Além disso, certos neurotransmissores podem produzir alguns de seus efeitos fisiológicos no cérebro regulando os inibidores de fosfatases específicas (Walaas e Greengard, 1991).

As principais fosfatases com atividade no SNC são: proteína fosfatase do tipo 1 (PP1), proteína fosfatase do tipo 2A (PP2A), proteína fosfatase do tipo 2B (PP2B) e proteína fosfatase do tipo 2C (PP2C) (Walaas e Greengard, 1991).

3.1. FOSFORILAÇÃO DOS FILAMENTOS INTERMEDIÁRIOS EM CÉLULAS DO SISTEMA NERVOSO CENTRAL

A fosforilação das subunidades dos FI parece ser um dos mecanismos predominantes na coordenação de sua organização celular (Inagaki et al, 1996) estando relacionada com a sua capacidade de polimerização e de interação com outras proteínas do citoesqueleto (Kirkpatrick e Brady, 1999; Inada et al, 2000).

Os neurofilamentos são altamente fosforilados *in vivo*, porém o grau de fosforilação do NF-H, NF-M e especialmente do NF-L, é diferente em cada compartimento neuronal, sendo altamente fosforilados no axônio, mas contendo poucos grupos fosfatos no corpo celular e dendritos (Gotow et al, 1994; Gotow e Tanaka, 1994).

As regiões amino e carboxi-terminais das três subunidades dos neurofilamentos são sítios em potencial para fosforilação. O domínio amino-terminal é fosforilado por proteínas quinases dependentes de segundos mensageiros - incluindo PKC, PKA, PKCaM -, sendo que a fosforilação desse domínio está relacionada com a capacidade de polimerização dos neurofilamentos (Nixon e Sihag, 1990; Nixon et al, 1994; Aranda-Espinoza et al, 2002). No entanto, o domínio carboxi-terminal é substrato para quinases independentes de segundos mensageiros, sendo que nas subunidades NF-M e NF-H estes domínios são considerados os sítios de interação entre neurofilamentos, microtúbulos e outras organelas (Nixon e Sihag, 1991; Jung et al, 2000). Por outro lado, pouco se sabe sobre as fosfatases que agem nos neurofilamentos, mas provavelmente a PP2A esteja envolvida, uma vez que desfosforila sítios nos domínios carbóxi e amino-terminal de neurofilamentos *in vivo*. Além da PP2A, a PP1 também participa da desfosforilação dos neurofilamentos (Veeranna et al, 1995; Saito et al, 1995; Strack et al, 1997).

É descrito que os neurofilamentos são os maiores constituintes do citoesqueleto dos axônios mielinizados, sendo sua fosforilação o principal determinante da velocidade de condução dos impulsos nervosos ao longo do axônio (Hirokawa e Takeda, 1998).

No entanto, as subunidades NF-M e NF-H localizam-se perifericamente nos neurofilamentos, sendo que as extremidades carboxi-terminal destas subunidades, quando extensivamente fosforiladas, formam projeções laterais que se estendem perpendicularmente ao filamento central, sendo responsáveis pela manutenção da distância entre os filamentos e conseqüentemente, da manutenção do calibre axonal (Hirokawa e Takeda, 1998; Martin et al, 1998). Essas projeções não são encontradas em outras proteínas de filamento intermediário como a desmina, a GFAP ou a vimentina (Elder et al, 1998).

Estudos *in vitro* com GFAP de porco mostraram a presença de seis sítios fosforiláveis, cinco deles na porção N-terminal (Thr 7, Ser 8, Ser 13, Ser 17 e Ser 34) e um na porção C-terminal (Ser 389). Estes sítios mostraram-se fosforiláveis pelas quinases PKA, PKCaM II e PKC (Inagaki et al, 1994). Os sítios fosforiláveis

de GFAP de ratos ainda não foram identificados, mas a grande homologia entre os domínios N-terminal de ambas as espécies é suficiente para assumir que possivelmente estes sítios estejam presentes na GFAP de ratos e que neles atuem as mesmas quinases indicadas para GFAP de porco (Rodnight et al, 1997). Por outro lado, as fosfatases PP1 e PP2B possivelmente estejam envolvidas na desfosforilação da GFAP (Vinadé et al, 1997).

Estudos *in vitro* demonstram que a vimentina pode ser fosforilada pela PKA, PKC e PKCaM e desfosforilada de uma maneira dependente de cálcio (Inada et al, 2000).

Reações de fosforilação/desfosforilação (Inagaki et al, 1994), regulam o equilíbrio dinâmico entre o estado polimerizado e o despolimerizado dos filamentos de GFAP e de vimentina contribuindo para a remodelação da arquitetura glial (Rodnight et al, 1997).

4. TRANSDUÇÃO DE SINAL

Todas as células recebem os sinais a sua volta e respondem aos mesmos. Entretanto, é nos organismos multicelulares que a comunicação célula-célula atinge o seu mais alto nível de sofisticação. A maior parte dos sinalizadores celulares age através de receptores específicos. A ligação das moléculas sinalizadoras aos seus receptores causa uma alteração na concentração de segundos mensageiros intracelulares que iniciam uma série de reações, modificando a atividade de enzimas intermediárias de cascatas de sinalização, propagando e amplificando o sinal iniciado pelo ligante extracelular. Este processo é chamado de transdução de sinal. Esses sinais regulam todos os aspectos do comportamento celular, incluindo metabolismo, movimento, proliferação e diferenciação celular (Cooper et al, 2001).

4.1. NEUROTRANSMISSÃO GLUTAMATÉRGICA

O glutamato é considerado o principal neurotransmissor excitatório do SNC de mamíferos e exerce um importante papel na plasticidade neural e neurotoxicidade (Nakanishi, 1992). O glutamato medeia vários processos vitais, tais como: desenvolvimento das células nervosas, incluindo proliferação e migração (McDonald e Johnson, 1990), modulação de mecanismos de aprendizado e memória (Izquierdo e Medina, 1997) e envelhecimento (Segovia et al, 2001). Os receptores glutamatérgicos (GluRs) têm papel fundamental na plasticidade e no desenvolvimento neural, bem como nos processos de neurodegeneração e transmissão sináptica. A ativação excessiva dos GluRs durante episódios de estresse cerebral tais como: isquemia, traumatismo craniano e surtos epilépticos levam à morte de neurônios (neurotoxicidade). Os GluRs estão envolvidos intimamente na fisiologia e na patologia das funções cerebrais (Nakanishi et al, 1998; Ozawa et al, 1998).

Os GluRs são divididos em duas classes distintas: receptores ionotrópicos e metabotrópicos (Conn e Pin, 1997; Ozawa et al, 1998). Os receptores inonotrópicos (iGluR) são assim denominados pois são canais que permitem a passagem de um cátion específico quando ativados por um agonista e foram subdivididos em NMDA (N-metil-D-aspartato) e não NMDA, que compreendem os receptores AMPA (ácido α-amino-3-hidróxi-5-metil-isoxazolenopropionato) e cainato (Ozawa et al, 1998). Os receptores metabotrópicos (mGluR) pertencem a uma família de receptores que estão acoplados a proteínas ligantes de nucleotídeos da guanina (proteínas G), promovendo então a modulação de efetores intracelulares que por sua vez ativam e/ou inibem diversos eventos de transdução de sinal celular (Conn e Pin, 1997; Ozawa et al, 1998).

Os receptores NMDA são altamente permeáveis a cálcio, sendo a alta condutância deste íon responsável pela possível neurotoxicidade causada pela superativação dos receptores glutamatérgicos (Sans et al, 2000; Constantible, 2000). Além disso, estes receptores medeiam respostas sinápticas voltagem dependentes, propriedade relacionada com o bloqueio transitório e efetivo do

canal pelo íon magnésio (Ozawa et al, 1998). Os receptores AMPA e cainato medeiam a transmissão excitatória rápida, respostas sinápticas voltagem independentes e são permeáveis aos íons sódio e potássio (Ozawa et al, 1998).

4.1.1. CAPTAÇÃO DE GLUTAMATO

O glutamato, após ser sintetizado, é estocado pelo sistema de transporte presente nas vesículas que se encontram no terminal pré-sináptico. Quando ocorre a despolarização dos terminais sinápticos glutamatégicos, o glutamato que se encontra nas vesículas é liberado para o meio extracelular (fenda sináptica) processo dependente de cálcio citosólico (Nicholls e Atweel, 1990), para interagir com seus receptores ionotrópicos e/ou metabotrópicos que estão localizados nas membranas pré e pós-sinápticas e também nas membranas gliais (Gallo e Ghiani, 2000; Scannevin e Huganir, 2000). Após a promoção de influxo iônico nestas células e a produção de segundos mensageiros, o glutamato é removido da fenda sináptica principalmente por sistemas de transporte que são dependentes de sódio, localizados nos neurônios e principalmente nas células gliais (Robinson e Down, 1997; Anderson e Swanson, 2000; Danboldt, 2001; Amara e Fontana, 2002). Esses sistemas de captação de glutamato são responsáveis pela inativação da transmissão glutamatérgica, pois não existem enzimas no meio extracelular que metabolizem o glutamato (Figura 8) (Danboldt, 2001).

Atualmente cinco tipos de transportadores de glutamato dependentes de sódio estão bem identificados e caracterizados: GLAST –1 (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4, EAAT5 (Amara e Fontana, 2002). GLT-1 e GLAST-1 estão distribuídos nas membranas plasmáticas astrocitárias (Chaudhry et al, 1995) enquanto os EAATs estão predominantemente localizados nos neurônios (Amara e Fontana, 2002). Além disso, está bem estabelecido que os transportadores de glutamato localizados nas membranas das células gliais são de fato os responsáveis pela manutenção dos baixos níveis extracelulares de glutamato e, desta maneira, garantem a homeostase celular (Anderson e Swanson, 2000; Tanaka, 2000; Gegelashvili et al, 2001; Amara e Fontana, 2002).

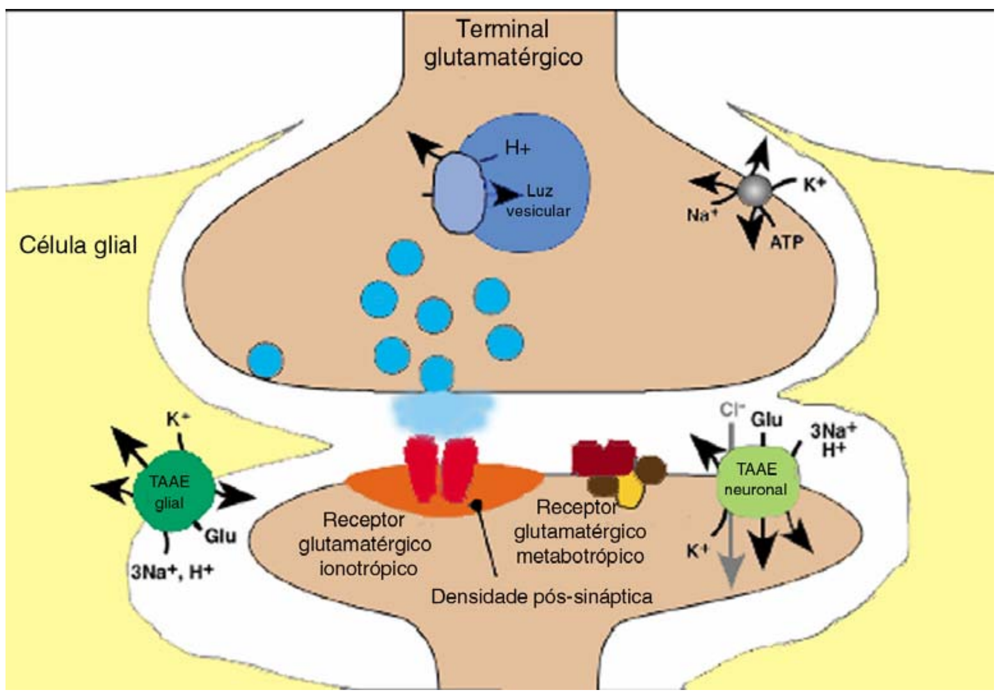

Figura 8. Representação esquemática de uma sinapse glutamatérgica. Adaptado de Amara e Fontana, 2002.

4.2. NEUROTRANSMISSÃO GABAÉRGICA

O ácido γ-aminobutírico (GABA) é considerado o principal neurotransmissor inibitório do sistema nervoso central (Roberts, 1976). O GABA é particularmente abundante no cérebro, onde está presente uniformemente (Rang et al, 2003).

O GABA é formado a partir de glutamato pela ação da enzima ácido glutâmico descarboxilase (GAD) e é metabolizado por uma reação de transaminação catalisada pela GABA-transaminase (GABA-T). A ação desse neurotransmissor na fenda sináptica é cessada pela recaptação nos terminais pré-sinápticos dos neurônios e nas células gliais. Também pode ser inativado pela ação da GABA-T (Olsen e DeLorey, 1999).

O GABA exerce sua ação através de receptores GABAérgicos. Os receptores GABAérgicos diferem em suas propriedades farmacológicas, eletrofisiológicas e bioquímicas (Olsen e Tobin, 1990; Tillakaratne et al, 1995). Os

receptores $GABA_A$ são membros de uma classe de canais iônicos cujo mecanismo é controlado pelo ligante (Unwin, 1993). A ligação de GABA nos receptores $GABA_A$ é responsável pela abertura de canais de cloreto (Cl^-) o que pode ativar a entrada de Ca^{2+} via canais dependentes de voltagem. Os receptores $GABA_B$ são indiretamente acoplados a canais de potássio (K^+) e quando ativados podem diminuir a condutância de Ca^{2+} e inibir a produção de AMPc via mecanismos intracelulares mediados por proteínas G (Olsen e DeLorey, 1999).

A ativação de receptores $GABA_A$ resulta em um aumento da inibição em neurônios pós-sinápticos enquanto a ativação de receptores $GABA_B$ medeia a inibição tanto pós-sináptica quanto pré-sináptica (Velisek e Mares, 1995).

4.3. CÁLCIO COMO SEGUNDO MENSAGEIRO

O Ca^{2+} é um segundo mensageiro que tem papel fundamental na regulação de vários processos biológicos. Um aumento na concentração de Ca^{2+} citoplasmático representa um passo crucial para a liberação de neurotransmissores e para a modulação da excitabilidade de membranas celulares. Esses processos dependem basicamente da passagem de Ca^{2+} através de canais presentes na membrana plasmática, do seu transporte por bombas iônicas ou da sua liberação a partir das reservas intracelulares (Prado, 2001).

Embora o Ca^{2+} seja fundamental à manutenção da vida, um aumento prolongado na concentração deste íon pode levar a célula à morte. Ao contrário das outras moléculas de segundos mensageiros, o Ca^{2+} não pode ser metabolizado. Desta forma, os níveis de cálcio dentro da célula são regulados por proteínas ligadoras de Ca^{2+} e por bombas que transportam ativamente este íon, tanto para as reservas intracelulares (as SERCAs – “sarcoplasmic-endoplasmic reticulum Ca^{2+}–ATPases”), quanto para o espaço extracelular (as bombas PMCA) (Putney, 1999; Cooper et al, 2001).

Existem pelo menos três mecanismos fundamentais que regulam a entrada de cálcio através da membrana plasmática: canais dependentes de voltagem,

canais dependentes de ligantes e processos capacitativos de entrada de cálcio (Putney, 1999).

Os canais de Ca^{2+} dependentes de voltagem são capazes de aumentar muito os níveis de Ca^{2+} citosólico e são essenciais para muitas funções celulares, tais como contração muscular, propagação de potenciais de ação, manutenção da atividade elétrica e regulação de neurotransmissores (Hui, 1991). A despolarização da membrana plasmática desencadeia uma mudança conformacional na região da molécula sensível à voltagem, promovendo um fluxo de Ca^{2+} através da membrana plasmática. Estes canais apresentam uma atividade auto-limitante que comanda o fechamento do canal. Até o momento, cinco tipos de canais de Ca^{2+} dependentes de voltagem foram descritos: tipo -L, -N, -P, -Q e -T (Récasens e Vignes, 1994). Estes tipos diferem entre si em suas respostas a neuromoduladores, na distribuição entre neurônios e células gliais e também quanto a sublocalização dentro de uma mesma célula (Clapham, 1995). Os canais dependentes de ligantes são numerosos e quando há ligação do seu agonista permitem a entrada do segundo mensageiro. Os receptores NMDA constituem uma família de receptores altamente permeáveis ao Ca^{2+} e são considerados um exemplo de canais dependentes de ligantes (Putney, 1999).

Os mecanismos de liberação de Ca^{2+} intracelular envolvem a enzima fosfolipase C e o segundo mensageiro inositol trifosfato (IP_3). O IP_3 age através da ligação a um receptor específico presente no retículo endoplasmático (Cooper et al, 2001). No evento de transdução de sinal envolvendo o Ca^{2+}, um primeiro mensageiro liga-se ao receptor na membrana plasmática e este, por sua vez, estimula a fosfolipase C a converter fosfolipídios de membrana (PIP_2) em IP_3 e diacilglicerol (DAG) (Berridge e Irvine, 1989). O IP_3 atua como segundo mensageiro ligando-se ao seu receptor no retículo endoplasmático, promovendo a liberação do íon cálcio das reservas intracelulares associadas a receptores de IP_3. O receptor de IP_3 é complexo e sua regulação é apenas parcialmente compreendida. A cinética complicada do receptor IP_3 se deve em parte às ondas de cálcio, as quais refletem períodos de flutuação na atividade elétrica da membrana (Putney, 1999).

4.4. Rho GTPases

As pequenas proteínas ligantes de GTP são proteínas G monoméricas com massas moleculares que variam entre 20-40 kDa. Atualmente mais de 100 pequenas proteínas G foram identificadas em eucarióticos desde leveduras até humanos e elas compreendem uma superfamília: a superfamília Ras. Os membros desta superfamília estão estruturalmente classificados em pelo menos 5 famílias: Ras, Rho, Rab, Sarf/Arf e Ran (Takai et al, 2001).

As pequenas proteínas G são proteínas que ligam nucleotídeos da guanina e possuem mecanismo de funcionamento cíclico. Quando estão ativas apresentam-se ligadas a GTP e quando estão inativas apresentam-se ligadas a GDP. No estado inativo as proteínas estão associadas com uma classe de reguladores negativos, os GDIs (inibidores da dissociação de GDP) que inibem a dissociação de GDP e estabilizam a forma da proteína ligada a GDP, além de a seqüestrarem da membrana para o citoplasma (Olofsson, 1999). O estado ativo das proteínas é promovido por reguladores positivos chamados GEFs (fatores de troca GDP/GTP) que funcionam como fatores de troca GDP/GTP, mantêm uma dada GTPase em uma localização subcelular distinta e trocam as moléculas de GDP por moléculas de GTP (Zheng, 2001). Como conseqüência, uma mudança conformacional é induzida, levando a uma proteína ativa. Isto permite iniciar uma série de complexos eventos de sinalização celular com as diversas proteínas alvo culminando com diferentes respostas fisiológicas (Hall, 1998). Os eventos são então finalizados através da atividade das GAPs (proteínas ativadoras de GTPase), as quais ativam a atividade GTPásica intrínseca da pequena proteína G. As GAPs podem aumentar a velocidade de reação em até 8 ordens de grandeza (Paduch et al, 2001). Desta forma, um ciclo de ativação e inativação é obtido e a pequena proteína G age como um interruptor molecular (Moon e Zheng, 2003).

O gene Rho foi identificado em 1985, mas foram as observações publicadas na década de 1990 que forneceram os primeiros indícios sobre as funções celulares das Rho GTPases (Ridley, 2001). A família das Rho GTPases de

mamíferos consiste de 7 classes de proteínas distintas: Rho (isoformas A, B e C), Rac (isoformas 1 e 2), cdc42 (isoformas cdc42Hs e G25K), RhoD, RhoG, RhoE e TC10 (Mackay e Hall, 1998). As proteínas Rho controlam o citoesqueleto de actina em todas as células eucarióticas (Mackay e Hall, 1998). A ativação da Rho em fibroblastos induz a organização dos filamentos contráteis de actina e miosina (fibras de estresse), a ativação da Rac promove a polimerização da actina na periferia das células formando os lamelipódios (Ridley e Hall, 1992; Ridley et al, 1992) e a ativação da cdc42 induz a formação de extensões de membrana em forma de dedos, ricas em actina e denominadas filipódia (Kozma et al, 1995).

O interesse atual nas Rho GTPases tem sido alimentado pelo fato de que elas regulam muitas outras vias de transdução de sinal além daquelas ligadas ao citoesqueleto de actina. Foram publicados trabalhos mostrando o envolvimento das proteínas Rho/Rac/Cdc42 em diversos eventos celulares, como crescimento celular (Khosravi-Far et al, 1995; Qiu et al, 1997), orientação (Luo et al, 1994) e extensão do axônio (Kozma et al, 1997). Além disso, elas participam na regulação da polaridade celular (Pruyne, 2000), na transcrição gênica (Westwick et al, 1997), no controle da progressão da fase G_1 do ciclo celular (Olson et al, 1995), na dinâmica de microtúbulos (Ishizaki et al, 2001) e em vias de transporte vesicular (Kroschewski et al, 1999).

O ácido lisofosfatídico (LPA) é um dos mais simples fosfolipídios de ocorrência natural. O LPA age em diferentes tipos celulares e tecidos como um mensageiro intercelular e pode atuar como fator de crescimento (Moolenaar, 2000). Este composto é produzido e liberado principalmente por plaquetas ativadas (Eichholtz et al, 1993). A estimulação dos receptores para LPA promove a regulação de diversas vias de sinalização, incluindo a inibição da adenilato ciclase, mobilização de Ca^{2+}, ativação de MAPKs, síntese de DNA e mudanças no citoesqueleto dependentes de RhoA. De acordo com a diversidade nas vias de sinalização, o LPA está envolvido na regulação de numerosas respostas celulares, incluindo agregação plaquetária, contração do músculo liso, proliferação e diferenciação celular, proteção contra a apoptose, formação de fibras de estresse e invasão de tecidos por células tumorais (Fishman et al, 2001).

5. ASTRÓCITOS

Diversos tipos celulares atuam de forma integrada no SNC para o adequado funcionamento do organismo como um todo. Essas células se dividem em dois grupos: as células neuronais e as células gliais, as quais compreendem os astrócitos, os oligodendrócitos e as células da microglia. Atualmente reconhece-se que a glia modula a função neuronal de forma dinâmica, em condições fisiológicas e patológicas, além de exercer uma função de suporte ao funcionamento dessas células. Uma das mais recentes funções atribuídas à glia é a modulação de sinais dolorosos (nocicepção) através da liberação de substâncias neuroativas, o que transforma as células gliais em novos alvos para descoberta de drogas pela indústria farmacêutica (Watkins et al, 2003).

Dentre as células gliais, as células mais abundantes no SNC dos mamíferos são os astrócitos. Até a década de 50 os astrócitos eram vistos como mero componente de suporte estrutural para as células neuronais. Essa imagem começou a ser modificada na década de 70 quando foram descobertos receptores β-adrenérgicos em astrócitos. As pesquisas atuais apontam para um papel ativo dos astrócitos frente aos neurônios, mas sim uma dinâmica interação metabólica e funcional entre esses dois tipos celulares (Nedergaard et al, 2003). A amplitude de funções dos astrócitos sugere que estas células estão envolvidas em quase todas as funções cerebrais (Nedergaard et al, 2003).

5.1. FUNÇÕES DOS ASTRÓCITOS

5.1.1. FUNÇÕES ESTABELECIDAS

Em condições normais, os astrócitos são responsáveis pela captação e/ou redistribuição do K+ durante a atividade neural (Barres, 1991; Walz, 1997), detoxificação da amônia, remoção do glutamato e do GABA das sinapses (Kimelberg e Norenberg, 1989; Anderson e Swanson, 2000), síntese de

precursores para produção de GABA e glutamato (Tansey et al, 1991; Sonnewald et al, 1996; Waagepetersen et al, 2003) e pelo auxílio na migração e no direcionamento dos neurônios até sua região definitiva durante o desenvolvimento do SNC (Hunter e Hatten, 1995; Hof et al, 1999).

Os astrócitos estão envolvidos na patogênese da Doença de Alexander, uma condição rara que inicia na infância causada por uma mutação na GFAP (Brenner et al, 2001), na formação dos gliomas (Holland, 2001) e do edema cerebral citotóxico (Mueller et al, 2000). Lesões cerebrais agudas, como isquemia ou trauma, estão associadas à acidose tecidual, o que leva ao edema citotóxico (acúmulo de água intracelular), afetando principalmente os astrócitos. Esse inchamento celular pode ser causado, entre outros, por ácido lático, glutamato e ácido araquidônico (Plesnila et al, 1999; Mueller et al, 2000; Badaut et al, 2003).

5.1.2. FUNÇÕES PROVÁVEIS

Além das funções comprovadas dos astrócitos descritas acima, inúmeras funções prováveis tem sido atribuídas a estas células. Entre elas, considera-se que em condições normais os astrócitos fornecem substratos energéticos, como o lactato, para os neurônios (Tsacopoulos e Magistretti, 1996) e funcionam como reservatório energético cerebral por estocarem glicogênio, que pode servir de fonte de combustível para as células vizinhas (Dringen et al, 1993). Além disso, os astrócitos liberam agentes neuroativos, como neurotransmissores, eicosanóides, esteróides e neuropeptídeos (Martin, 1992), e também fatores tróficos que regulam a sobrevivência, proliferação, morfologia e diferenciação de neurônios e de outras células gliais (Matsushima et al, 1998; Semkova e Krieglstein, 1999; Hof et al, 1999). Os astrócitos provavelmente influenciam na integridade da barreira hemato-encefálica, através da liberação de fatores que promovem uma forte adesão entre as células endoteliais, apesar de este ser um tema controverso (Del Zoppo e Hallenbeck, 2000; Abbott, 2002). Outra provável função importante dos astrocitos é a regulação do pH extracelular (Bradford, 1985).

Os astrócitos podem estar envolvidos também na patogênese da encefalopatia hepática (Jalan et al, 2003; Lizardi-Cervera et al, 2003) e na resolução do acidente vascular cerebral (AVC), através da detoxificação de radicais livres (Neal et al, 1996; Yamashita et al, 2000), da homeostase do glutamato e da expressão de conexina, uma proteína presente nas junções GAP que está implicada na capacidade tamponante dos astrócitos, sendo assim neuroprotetora (Siushansian et al, 2001).

5.1.3. . FUNÇÕES EMERGENTES

Sugere-se que, em condições normais, os astrócitos possam modular as sinapses excitatórias e inibitórias (Newman, 2003), influenciar na formação e maturação das sinapses (Slezak e Pfrieger, 2003), determinar a arquitetura estrutural e funcional do cérebro adulto (Nedergaard et al, 2003), regular a neurogênese a partir de células precursoras endógenas em giro denteado de ratos adultos (Horner e Palmer, 2003) e detoxificar espécies reativas de oxigênio no cérebro; os astrócitos possuem altos níveis de glutationa, um importante antioxidante no SNC (Juurlink, 1997).

Sugere-se também que os astrócitos atuem na modulação trófica do reparo neuronal e re-crescimento de axônios após lesão, na liberação de citocinas mediadoras da dor (Wieseler-Frank et al, 2004) e na modulação da neuroinflamação, presente em várias patologias do SNC, como doença da Alzheimer, isquemia cerebral, infecções virais e bacterianas, e esclerose múltipla (Bajetto et al, 2001; Kielian e Esen, 2004).

II. OBJETIVOS

OBJETIVO GERAL

Nosso grupo recentemente demonstrou que o α-cetoisocapróico, o α-cetoácido considerado o mais tóxico na Doença do Xarope do Bordo, altera a fosforilação das proteínas do citoesqueleto em fatias de córtex cerebral de ratos de um modo dependente do desenvolvimento. O α-cetoisocapróico diminui a fosforilação dos filamentos intermediários em animais de até 12 dias de vida e aumenta essa fosforilação em ratos de 17 e 21 dias de idade. Além disso, observamos que esse efeito é mediado por receptores glutamatégicos ionotrópicos (Funchal et al, 2002). Considerando que a fosforilação protéica é um importante mecanismo molecular através do qual a função protéica é regulada em resposta a estímulos extracelulares e que o estado de fosforilação das diferentes proteínas do citoesqueleto parece estar diretamente envolvido com a morfologia e plasticidade do SNC, o *objetivo geral* deste trabalho é verificar o efeito dos α-cetoácidos de cadeia ramificada que se acumulam na Doença do Xarope do Bordo sobre alguns parâmetros bioquímicos do citoesqueleto e os prováveis mecanismos envolvidos nesses efeitos.

OBJETIVOS ESPECÍFICOS

1. Verificar o envolvimento de proteínas quinases e fosfatases no efeito do ácido α-cetoisocapróico sobre a incorporação *in vitro* de ^{32}P em proteínas de filamento intermediário - NF-M, NF-L, vimentina e GFAP – em fatias de córtex cerebral de ratos em ratos de 9 e 21 dias de idade.

2. Estudar o envolvimento do cálcio como segundo mensageiro no efeito do ácido α-cetoisocapróico sobre a incorporação *in vitro* de ^{32}P em proteínas de filamento intermediário - NF-M, NF-L, vimentina e GFAP – em fatias de córtex cerebral de ratos de 9 e 21 dias de idade.

3. Realizar um estudo ontogenético dos efeitos dos ácidos α-ceto-β-metilvalérico e α-cetoisovalérico sobre a incorporação *in vitro* de ^{32}P em proteínas de filamento intermediário - NF-M, NF-L, vimentina e GFAP – em fatias de córtex cerebral de ratos de 9, 12, 17 e 21 dias de idade.

4. Identificar os mecanismos de membrana envolvidos nos efeitos mediados pelos ácidos α-ceto-β-metilvalérico e α-cetoisovalérico sobre o sistema fosforilante associado a proteínas de filamento intermediário - NF-M, NF-L, vimentina e GFAP – em fatias de córtex cerebral de ratos.

5. Realizar um estudo ontogenético dos efeitos dos ácidos α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico sobre a captação de glutamato em fatias de córtex cerebral de ratos de 9, 21 e 60 dias de idade.

6. Investigar os efeitos dos ácidos α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico sobre a morfologia e a reorganização do citoesqueleto de astrócitos em cultura.

7. Investigar mecanismos moleculares envolvidos nos efeitos dos ácidos α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico sobre reorganização do citoesqueleto de astrócitos em cultura.

8. Verificar os efeitos dos ácidos α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico sobre a morfologia, fosforilação e polimerização da proteína glial fibrilar ácida em células C6.

III - ANEXO 1

Artigo submetido ao periódico **Neurochemistry International**

Título: Evidence that Ca^{2+} currents mediate the effect of alpha-ketoisocaproic acid on the phosphorylating system of cytosketetal proteins from cerebral cortex of rats

Autores: Cláudia Funchal, Ariane Zamoner, André Quincozes dos Santos, Samanta Oliveira Loureiro, Moacir Wajner e Regina Pessoa-Pureur

NEUROCHEMISTRY International

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Montreal, 16th September, 2004

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EVIDENCE THAT Ca^{2+} CURRENTS MEDIATE THE EFFECT OF ALPHA-KETOISOCAPROIC ACID ON THE PHOSPHORYLATING SYSTEM OF CYTOSKELETAL PROTEINS FROM CEREBRAL CORTEX OF RATS

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ABSTRACT

In this study we investigated the involvement of Ca^{2+} on the effects of α-ketoisocaproic acid (KIC), the main metabolite accumulating in the inherited metabolic disorder maple syrup urine disease (MSUD), on the phosphorylating system associated with the intermediate filament (IF) proteins in slices from cerebral cortex of 9- and 21-day-old rats. We observed that KIC significantly decreased the in vitro phosphorylation of IF proteins in tissue slices of 9-day-old animals through PP2A and PP2B. In contrast, KIC increased the in vitro incorporation of ^{32}P into the IF proteins in cortical slices of 21-day-old rats through PKA and PKCaMII. Furthermore, KIC was able to increase cAMP levels, supporting the involvement of PKA on its effects. We also demonstrated the involvement of Ca^{2+} in KIC effects using the specific L-voltage-dependent Ca^{2+} channels (L-VDCC) inhibitor nifedipine, the NMDA antagonist DL-AP5 and the intracellular Ca^{2+} chelator BAPTA-AM. Results showed that blocking Ca^{2+} channels or chelating intracellular Ca^{2+} completely prevented the effects of KIC on the phosphorylating system associated with IF proteins. We also verified that blockage of Ca^{2+} currents was more evident in 9-day-old rats as compared to 21-day-old animals. Taken together, our present data indicate that the alterations of IF phosphorylation caused by KIC are dependent on intracellular Ca^{2+} that regulate calcineurin, PKCaMII and PKA activities in a developmentally regulated manner.

Keywords: α-ketoisocaproic acid, calcium, cAMP, phosphorylation, intermediate filaments

Running title: Ca^{2+} and the phosphorylation system of the cytoskeleton

INTRODUCTION

Protein phosphorylation is an important mechanism of cell regulation in the central nervous system (Dunkley, 1992; Nestler and Greengard, 1999). We have previously reported that α-ketoisocaproic acid (KIC), the principal metabolite accumulating in the neurometabolic inherited disorder maple syrup urine disease (MSUD) (Chuang and Shih, 2001), altered the in vitro incorporation of ^{32}P into IF proteins after 30 min incubation in slices of cerebral cortex of rats in a developmentally regulated manner. KIC decreased the in vitro phosphorylation of IF proteins in rats of up to 12 days of life and increased this phosphorylation in tissue slices from 17- and 21-day-old rats. Furthermore, we also observed that this effect was mediated by the glutamate ionotropic receptors NMDA, AMPA and kainate (Funchal et al, 2002).

Changes in the phosphorylation of neurofilaments and other cytoskeletal proteins have been described in various neurodegenerative diseases, including amyotrophic lateral sclerosis, Parkinson´s disease, dementia with Lewy bodies and Alzheimer´s disease, as well as in neuronal injury and regeneration (Hirano, 1991; Schmidt et al, 1996; Trojanowski et al, 1993).

Many extracellular signals induce an increase in cytosolic Ca^{2+} levels, triggering Ca^{2+} waves that are responsible for many cellular responses, ranging from secretion to changes in cellular metabolism. Generally, the initial target of calcium action is a specific calcium-binding protein, being calmodulin one of the most well characterized (Klee and Newton, 1985). The binding of calcium to calmodulin induces a conformational change which imparts signaling information to a number of different molecules, including protein

kinases and phosphatases. Among the most prominent Ca^{2+}-dependent protein kinases and phosphatases are calcium/calmodulin-dependent protein kinase (PKCaM) and protein phosphatase 2 B (PP2B) or calcineurin. Among the six types of known CaM kinases, CaM kinase II has been the most extensively characterized (Kasahara et al, 1999). It is abundantly expressed in the brain and plays important roles in regulating cytoskeletal functions. On the other hand, calcineurin is a member of the serine/threonine protein phosphatase family and the only known phosphatase activated by Ca^{2+} and calmodulin (Shibasaki et al, 2002). Calcineurin is highly concentrated in mammalian brain, where it binds to membrane or cytoskeletal elements (Shibasaki et al, 2002; Vieira de Almeida et al, 2003). In this context, we have previously described that CaMKII and calcineurin activities are associated to cytoskeletal proteins in cerebral cortex of rats and are altered in the experimental models of propionic and methylmalonic acidemias (Vieira de Almeida et al, 2003).

Therefore, in the present investigation we studied the involvement of Ca^{2+} signaling mechanisms on the activity of protein kinases and phosphatases mediating the effects of KIC on the phosphorylation of IF proteins in slices of cerebral cortex from 9- and 21-day-old rats.

EXPERIMENTAL PROCEDURES

Radiochemicals and compounds- [^{32}P] orthophosphate was purchased from CNEN, São Paulo, Brazil, [^{3}H] cyclic AMP (23 Ci/mmol) was from Amersham International (UK).

α-ketoisocaproic acid, benzamidine, leupeptin, antipain, pepstatin, chymostatin, nifedipine, 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis (BAPTA-AM), D-2-amino-5-phosphonopentanoic acid (DL-AP5), cyclic AMP, acrylamide and bis-acrylamide were obtained from Sigma (St. Louis, MO, USA). KN-93 and H-89 were purchased from Calbiochem (La Jolla, CA, USA). Okadaic acid was obtained from Tocris Neuramin (Bristol, UK).

Animals- Wistar rats (9 and 21 days of age) were obtained from our breeding stock. Rats were maintained on a 12-h light/12-h dark cycle in a constant temperature (22° C) colony room. On the day of birth the litter size was culled to eight pups. Litters smaller than eight pups were not included in the experiments. Water and a 20% (w/w) protein commercial chow were provided ad libitum. The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul and followed by the "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985).

Preparation and labelling of slices- Rats were killed by decapitation, the cerebral cortex was dissected onto Petri dishes placed on ice and cut into 400 μm thick slices with a McIlwain chopper.

Preincubation- Tissue slices were initially preincubated at 30°C for 10 min in a medium containing 124 mM NaCl, 4mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES (pH 7.4), 12 mM glucose, 1mM $CaCl_2$ (Krebs-Hepes), and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin and 0.7 μM chymostatin. In some experiments 100 μM D-amino-5-phosphonopentanoic acid (DL-

AP5), 100 μM nifedipine or 50 μM BAPTA-AM was added to the medium during preincubation.

Incubation- After preincubation, the medium was changed and incubation was carried out at 30 ^{o}C with 100 μl of the basic medium containing 80 μCi of [^{32}P] orthophosphate with or without addition of the different drugs. When indicated 1.0 mM α-ketoisocaproic acid, 100 μM nifedipine, 50 μM BAPTA-AM or 100 μM DL-AP5 was added to the incubation medium. The labelling reaction was normally allowed to proceed for 30 min at 30 ^{o}C and stopped with 1 ml of cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, Tris-HCl 50 mM, pH 6.5, and the protease inhibitors described above). When specified, incubation was carried out for 5 min. Slices were then washed twice with stop buffer to remove excess radioactivity. In some experiments, nifedipine or BAPTA-AM or DL-AP5 plus α-ketoisocaproic acid were added to the incubation medium.

Phosphatase activity assays-. The phosphatase assays were carried out in tissue slices of 9-day-old rats. The standard assay system was essentially as described above. Phosphatase inhibitors were added to the medium at the concentrations decribed below and the preincubation occurred for 20 min at 30°C. After preincubation, the medium was changed and incubation was carried out for 30 min at 30 ^{o}C with 100 μl of the basic medium containing 80 μCi of [^{32}P] orthophosphate, with or without addition of 1.0 mM KIC in the presence or absence of one of the following phosphatase inhibitors: okadaic acid (OA) added at the concentrations described to inhibit protein phosphatase 2A (PP2A), 1 (PP1) or 2B (PP2B) respectively (0.05, 0.5 or 5 μM) (Ishihara et al, 1989) or 100 μM FK 506, an immunosuppressant described to inhibit PP2B (Griffith et al, 1995).

Kinase activity assays- Tissue slices of 21-day-old rats were initially preincubated at 30 ^{o}C for 20 min with 10 μM KN-93, a Ca^{2+}/calmodulin dependent protein kinase (PKCaMII) inhibitor or 10 μM H-89, a PKA inhibitor. After preincubation, the medium was changed and incubation was carried out at 30 ^{o}C with 100 μl of the basic medium containing 80 μCi of [^{32}P] orthophosphate, with or without addition of 1.0 mM KIC, in the presence or absence of one of the kinase inhibitors described above.

Preparation of the high salt-Triton insoluble cytoskeletal fraction from slices of cerebral cortex- After treatment, preparations of total IF were obtained from cerebral cortex of 9- and 21-day-old rats as described by Funchal et al (2003). Briefly, after the labelling reaction, slices were homogenized in 400 μl of ice-cold high salt buffer containing 5 mM KH_2PO_4, (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1 % Triton X-100 and the protease inhibitors described above. The homogenate was centrifuged at 15800 x g for 10 min at 4 °C, in an Eppendorf centrifuge, the supernatant discarded and the pellet homogenized with the same volume of the high salt medium. The resuspended homogenate was centrifuged as described and the supernatant was discarded. The Triton-insoluble intermediate filament-enriched pellet, containing neurofilament subunits, vimentin and GFAP, was dissolved in 1% SDS and protein concentration was determined by the method of Lowry et al (1951).

Polyacrylamide gel electrophoresis (SDS-PAGE) - The cytoskeletal fraction was prepared as described above. Equal protein concentrations were loaded onto 10 % polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). After drying, the gels were exposed to X-ray films (X-Omat XK1) at -70 ^{o}C with intensifying screens and finally the autoradiograph was obtained. Cytoskeletal

proteins were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studied proteins.

Measurement of cyclic AMP levels - Slices were preincubated in 500 μl of Krebs-Hepes buffer, pH 7.4, at 37°C for 60 min, the Krebs-Hepes buffer was changed twice during this period. Incubation was then started by adding 1 mM KIC during 5 or 30 minutes. Incubation was stopped by placing the tubes in an ice-cold bath and samples were processed as previously described (Tasca et al, 1995). In brief, incubation medium was replaced by 0.5 M perchloric acid, slices were homogenized and an aliquot was used for protein measurement by the method of Lowry et al. (1951), using bovine serum albumin as standard. The homogenate was centrifuged (12 800 X g for 2 min) and the supernatant was neutralized with 2 M KOH and 1 M Tris/HCl. The pellet was removed by centrifugation (15 800 X g for 3 min) and an aliquot from the supernatant was evaporated under a stream of air in a 50°C bath according to a modification of the procedure of Baba et al (1982). Residues were dissolved in 50 mM Tris-HCl, pH 7.4, containing 4 mM EDTA. Cyclic AMP content was measured by the protein binding method of Tovey et al (1974), using [^{3}H] cyclic-AMP (23 Ci/mmol) and protein kinase A as the binding protein. In some experiments we used purified protein kinase A isolated from bovine heart as decribed by Gliman, (1970). Radioactivity was counted by liquid scintillation.

Statistical analysis- Data were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey the test when the F-test was significant. All analyses were performed using the SPSS software program on an IBM-PC compatible computer.

RESULTS

We have previously described that the IF-enriched cytoskeletal fraction extracted from slices of cerebral cortex of rats contained phosphoproteins corresponding to the molecular weight of the neurofilament subunits (NF-M and NF-L) along with GFAP and vimentin, as previously identified by us using monoclonal antibodies. In addition, the 150- (NF-M) and 68-kDa (NF-L) neurofilament subunits, as well as vimentin and GFAP, were good substrates for the endogenous phosphorylation system. (Branco et al., 2000).

In the present report we describe that, when slices of cerebral cortex of 9- and 21-day-old rats were treated with 1 mM KIC for 5 and 30 min, the in vitro ^{32}P incorporation was decreased in the younger animals and increased in the older ones (Figure 1). The next step was to elucidate the mechanisms responsible for the inhibitory effect of KIC on the phosphorylating system associated tothe high salt Triton insoluble cytoskeletal proteins. Thus, we co-incubated tissue slices from 9-day-old rats for 30 min with 1 mM KIC and different concentrations of okadaic acid (OA), a known inhibitor of PP2A, PP1 and PP2B (Cohen et al, 1989). Results showed that the inhibitory effect of KIC on the in vitro phosphorylation of the proteins studied was not observed when tissue slices were incubated with KIC in the presence of 0.05 μM OA (Figure 2A), a concentration which only inhibits PP2A, indicating that KIC effect is probably mediated by PP2A. In addition, when tissue slices were exposed to KIC in the presence of 0.5 μM OA (Figure 2A), a concentration that inhibits PP2A and PP1, the inhibitory effect of the acid was also prevented. We cannot establish at this time whether PP1 was involved in the effect elicited by KIC, since PP2A already prevented this effect at this concentration and we did not use a cell permeable

specific PP1 inhibitor. On the other hand, incubation of cortical slices with KIC in the presence of 5 μM OA, which inhibits PP2B, not only prevented the effect of KIC (Figure 2A) but also provoked a significant increase of the in vitro ^{32}P incorporation into the cytoskeletal proteins, as compared with controls. Furthermore, when the slices were incubated with KIC in the presence of 100 μM FK506, an immunosuppressant that inhibits PP2B (Liu et al, 1991), the effect of the acid was also totally prevented (Figure 2B). These data suggest an important role of PP2B in our cytoskeletal preparation mediating the effect of KIC on the phosphorylating system associated to the high-salt Triton insoluble IF proteins in the cerebral cortex slices of 9-day-old rats.

We also investigated whether PKA and PKCaMII were involved in the activating effect of KIC on the phosphorylating system associated with the high-salt Triton insoluble IF proteins from cerebral cortex of 21-day-old rats. For this purpose, we added the specific protein kinase inhibitors of PKA and PKCaMII, H-89 and KN-93, respectively, to the incubation system in the presence of KIC. Results showed that KN-93 fully prevented the increased phosphorylation induced by KIC, whereas H-89 not only prevented but also decreased this phosphorylation, as compared to controls, indicating an important role of PKA in the cytoskeletal preparation (Figure 3). These results suggested that KIC activating effect on the phosphorylation of the cytoskeletal proteins from cerebral cortex of 21-day-old rats was mediated by PKA and PKCaMII.

In order to verify the involvement of voltage- or ligand-dependent Ca^{2+} channels on the effect of KIC, tissue slices of 9- and 21-day-old rats were co-incubated with 1 mM KIC and the specific L-calcium channel (L-VDCC) inhibitor nifedipine or the competitive NMDA ionotropic antagonist L-AP5. We first observed that tissue slices from 9-day-old

rats were more sensitive to nifedipine than those of 21-day-old animals, since 100 μM nifedipine itself inhibited the phosphorylating system in the younger animals (Figure 4A), without affecting this system in the older ones (Figure 4B). In addition, co-incubation of tissue slices with 1 mM KIC plus 10 μM nifedipine (9-day-old rats) or 1 mM KIC plus 100 μM nifedipine (21-day-old rats) totally prevented the effect of KIC on the phosphorylating system (Figure 4A and B). Furthermore, 100 μM DL-AP5 (a concentration that does not disturb the phosphorylating system) also prevented the effect of KIC on the phosphorylating system associated to the cytoskeleton (Figure 4A and B), suggesting that Ca^{2+} entry via voltage- and ligand- gated channels are involved in the ability of KIC to alter the phosphorylating/dephosphorylating equilibrium of the cytoskeletal proteins studied. To further investigate the role of intracellular Ca^{2+} in this process we performed experiments using the membrane-permeable form of BAPTA, namely BAPTA-AM, which is freely taken up into cells were it is hydrolyzed by cytosolic esterases and trapped intracellularly as the active Ca^{2+} chelator BAPTA (Mafra et al, 2002). Tissue slices of 9- and 21-day-old rats were incubated with 1 mM KIC plus 50 μM BAPTA-AM, a concentration that does not alter the phosphorylating system per se. Results showed that BAPTA prevented both the inhibitory effect of KIC observed in tissue slices of 9-day-old rats and the stimulatory effect induced in slices of 21-day-old animals, indicating that an increase in the intracellular Ca^{2+} concentration could be one of the mechanisms regulating the effects of KIC on the phosphorylating system associated to the cytoskeleton.

Finally, considering that the expression of type I adenylyl cyclase is activated by Ca^{2+} and calmodulin in the cerebral cortex (Cooper et al, 1995), we investigated the effect of KIC on cAMP levels. In these experiments, slices of cerebral cortex of 9- and 21-day-old

rats were incubated with 1.0 mM KIC during 5 and 30 min. Results showed that KIC significantly increased cAMP levels after 5 min exposure in both ages studied (Figure 5).

DISCUSSION

We have previously reported that KIC was able to induce alterations in the phosphorylating system associated to the cytoskeleton in a developmentally regulated manner (Funchal et al, 2002).Our previous observations were obtained incubating tissue slices for 30 min with KIC. In the present report we demonstrated that KIC was able to produce a similar effect at a shorter incubation period (5 min). Furthermore, we also provided an insight on the mechanisms involved in such effects. We first identified the phosphatases and kinases involved in the effects elicited by KIC on IF proteins by using specific protein phosphatase and kinase inhibitors.

The major phosphatases identified in eukariotic cells are type 1 (PP1) and type 2, which comprise three enzymes (PP2A, PP2B and PP2C) that can be distinguished by their sensitivity to the marine toxin OA. At a low concentration of OA (<1.0 μM), PP1 and PP2A are inhibited, whereas higher concentrations (5.0 μM) are required to inhibit PP2B. In contrast, protein phosphatase 2C (PP2C) is insensitive to OA (Cohen et al, 1989). By using different doses of OA, we demonstrated that the decreased phosphorylation induced by KIC in cortical slices of 9-day-old animals was mediated mainly by PP2A and PP2B. The involvement of PP2B was confirmed using the specific PP2B inhibitor FK506 (MacKintosh et al, 1990; Cohen et al, 1990; Liu et al, 1991). We cannot however rule out that at least part of the effect verified in cortical slices of 9-day-old rats could be due to PP1

since we did not use specific PP1 inhibitors. It is interesting to note that tissue slices incubated with 5 μM OA alone or combined with KIC resulted in a 30% increased ^{32}P incorporation into IF proteins over controls, pointing to a prominent role of calcineurin, mediating the dephosphorylation of IF proteins. These data are supported by our previous findings indicating calcineurin as the main phosphatase activity mediating the effects of propionic and methylmalonic acids in slices from cerebral cortex of 12-day-old rats (Vieira de Almeida et al, 2003).

It was also observed that KIC-induced stimulatory effect on the phosphorylating system associated to IF cytoskeletal proteins in cerebral cortex of 21-day-old rats probably occurs via protein kinases PKA and PKCaMII, since the cell-permeable, selective and potent inhibitor of PKA (H-89) (Chijiwa et al, 1990) and the specific PKCaMII inhibitor (KN-93) (Tokumitsu et al, 1990) totally prevented the effect of KIC on the in vitro phosphorylation of the proteins studied. This is consistent with our previous reports demonstrating that in our experimental conditions these protein kinases are associated to the cytoskeletal fraction (de Freitas et al, 1995; Vieira de Almeida et al, 2003).

Taken into account that our findings point to a prominent action of Ca^{2+}-dependent kinase and phosphatase activities mediating the phosphorylation/dephosphorylation effects of KIC, we further investigated the roles of Ca^{2+} currents in such effects. Considering that Ca^{2+} enters cells via voltage- or ligand-dependent channels or by means of capacitative entry (Putney, 1999), we concentrated our efforts investigating the role of voltage-dependent Ca^{2+} channels (VDCC) and ligand-dependent Ca^{2+} channels mediating the effects of KIC on the cytoskeletal proteins. VDCC are designated L-, T-, P-, Q- and R-type. Each channel has different voltage ranges and rates for activation (Catterall, 1996). The L-

type channels are found in the skeletal as well as in neuronal tissue and are sensitive to dihydropyridines such as the agonist BayK-2844 and the inhibitor nifedipine (Hockerman et al, 1997). Ligand-gated Ca^{2+} channels refer to channels gated directly by binding receptor agonists. In this context, the ionotropic glutamate receptor NMDA function as a ligand-gated Ca^{2+} channel. Our results showed that the effects of KIC on the IF associated phosphorylating system in 9- and 21-day-old rats were totally prevented by pre-incubation of tissue slices with the specific L-channel blocker nifedipine, or the competitive NMDA antagonist DL-AP5, supporting important roles of Ca^{2+} currents mediating the effects of KIC on the cytoskeletal proteins. The involvement of intracellular Ca^{2+} in such effects was further evidenced using the intracellular Ca^{2+} chelator BAPTA, since KIC was unable to cause any effect on the phosphorylating system when tissue slices were preincubated with this powerful calcium chelator. In this context, it is interesting to emphasize that our results point to a greater activity of L-VDCC currents in tissue slices of 9-day-old as compared to slices of 21-day-old animals, as evidenced by the higher sensibility to nifedipine observed in the younger animals.

Additionally, the evidence that the stimulatory effect of KIC on the IF-associated phosphorylating system in cerebral cortex of 21-day-old rats was also mediated by PKA lead us to evaluate whether KIC could alter the intracellular cAMP levels. Results showed that after 5 min incubation KIC was able to transiently induce increased cAMP levels in both 9- and in 21-day-old animals, returning to control values after 30 min incubation. These findings are consistent with the expression in the cerebral cortex of type I adenylyl cyclase which is activated by Ca^{2+} and calmodulin (Cooper et al 1995). The loss of sensitivity of the adenylyl cyclase/cAMP system to KIC at 30 min incubation could be possibly due to desensitization of G-protein-linked receptors, which frequently involves

receptor phosphorylation/dephosphorylation cycles by G protein-coupled receptor kinases (GRKs) (Vásquez-Prado et al, 2003).

Taken together, these results demonstrate that intracellular Ca^{2+} currents via VDCC and NMDA receptors are involved in the effects of KIC on IF phosphorylating system in cerebral cortex of both 9- and 21-day-old rats. Interestingly, calcium activated various cytoskeletal associated proteins in a developmentally regulated manner, i.e. protein phosphatases (PP2A and calcineurin) in 9-day-old rats and protein kinases (PKCaMII and PKA) in 21-day-old animals. Although we can not at the present precisely explain the differential effects of KIC on the phosphorylating system at these two animal ages, they may be possibly due to the increased VDCC activity reflected by the increased sensibility of these channels to nifedipine in 9-day-old animals, as compared to 21-day-old rats. In this context, N- (Jones et al, 1997; Vance et al, 1998) and L- (Drean et al, 1995) type VDCC appear to be expressed early in the developing nervous system, playing critical roles in many aspects of neuronal function, such as neuronal migration (Komuro and Rakic, 1992; Komuro and Rakic, 1998), gene activation (Brosenisch and Katz, 2001; Finkbeiner and Greenberg, 1998) and neurotransmitter release (Scholz and Miller, 1995; Tojima and Ito, 2004). Otherwise, we can not exclude the possibility that the differential effect of KIC over the phosphorylation level of the IF proteins studied could be due to developmental changes in the expression of PKCaMII/PKA and calcineurin/PP2A. Further experiments will be necessary to define the expression levels of these kinases/phosphatases in the different aged-slices.

Our results showing that Ca^{2+} probably mediates the effects elicited by KIC on the phosphorylating system via VDCC is in agreement with previous reports evidencing that VDCC subunits are important molecular substrates for neurological diseases, including

cerebral ishemia (Guo et al, 2004), Alzheimer´s disease (Porter et al, 1997), Lambert-Easton myasthenic syndrome (Greenberg, 1999; Takamori et al 2000), hemiplegic migraine (Ophoff et al, 1998; Terwindt et al, 1998), cerebellar ataxia (Jen, 1999), and epilepsy (Fletcher et al, 1996; Zwingman et al, 2001).

Considering that the phosphorylating system is important for neural cell function, that a great body of evidence in the literature shows that alterations of cytoskeletal proteins may lead to disorganization of cellular structure and that alterations of protein phosphorylation can be involved in brain damage (Brandt, 2001), it is tempting to speculate that this may be at least one of the factors associated with the neurodegeneration and cerebral atrophy characteristic of MSUD patients.

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Figure 1. Effect of time exposure to KIC on the phosphorylation of intermediate filament subunits from 9- and 21-day-old rat cerebral cortex. Slices of cerebral cortex of 9- and 21-day-old rats were incubated for 5 and 30 min with 1 mM KIC in the presence of ^{32}P-orthophosphate. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into intermediate filament subunits was measured, as described in Material and Methods. NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit; Vim, vimentin; GFAP, glial fibrillary acidic protein. Data are reported as means ± S.E.M. of 6-8 animals expressed as percentage of controls. Statistically significant differences from controls, as determined by ANOVA followed by Tukey test are indicated: *P<0.001.

Figure 2. Effect of phosphatase inhibitors on the inhibitory effect elicited by KIC on the phosphorylation system associated to IFs in the cytoskeletal fraction of cerebral cortex of 9-day-old rats. Slices of cerebral cortex were preincubated and incubated for 30 min with 1 mM KIC and ^{32}P-orthophosphate, in the presence or absence of 0.05, 0.5 or 5 µM okadaic acid (A); or 100 µM FK506 (B), as described in Material and Methods. The cytoskeletal fraction was extracted and the radioactivity incorporated into middle molecular weight neurofilament subunit (NF-M), low molecular weight eurofilament subunit (NF-L), vimentin (Vim) and glial fibrillary acidic protein (GFAP) was measured. Data are reported as means ± SEM of 6-10 animals in each group expressed as percent of controls. Statistically significant differences from controls, as determined by one-way ANOVA followed by Tukey test are indicated. Control: *P<0.001. 1 mM KIC $^{\#}$ P<0.001.

Figure 3. Effect of cAMP- or Ca^{2+}/calmodulin-dependent protein kinases inhibitors on KIC-induced alterations on IF-associated phosphorylating system in cerebral cortex of 21-day-old rats. Slices of cerebral cortex were preincubated and incubated for 30 min with 1 mM KIC and ^{32}P-orthophosphate in the presence or absence of 10µM H-89 (PKA inhibitor) or 10µM KN-93 (PKCaMII inhibitor). The cytoskeletal fraction was extracted and the radioactivity incorporated into middle molecular weight neurofilament subunit (NF-M), low molecular weight neurofilament subunit (NF-L), vimentin (Vim) and glial fibrillary acidic protein (GFAP) was measured as described in Material and Methods. Data are reported as means ± SEM of 6-8 animals in each group and expressed as percent of controls. Statistically significant differences from controls, as determined by Tukey test are indicated. Control: $*P<0.001$. 1 mM KIC $^{\#}P<0.001$.

Figure 4. Effects of inhibitors of Ca^{2+} currents and of the intracellular Ca^{2+} chelator BAPTA-AM on KIC-induced alterations on IF-associated phosphorylating system in cerebral cortex of 9- (A) and 21- (B) day-old rats. Slices of cerebral cortex were preincubated and incubated for 30 min with 1 mM KIC and ^{32}P-orthophosphate in the presence or absence of 10 or 100 µM nifedipine (L-VDCC inhibitor), 100 µM DL-AP5 (NMDA antagonist) or 50 µM BAPTA-AM (intracellular Ca^{2+} chelator). The cytoskeletal fraction was extracted and the radioactivity incorporated into middle molecular weight neurofilament subunit (NF-M), low molecular weight neurofilament subunit (NF-L), vimentin (Vim) and glial fibrillary acidic protein (GFAP) was measured as described in Material and Methods. Data are reported as means ± SEM of 6-8 animals in each group and

expressed as percent of controls. Statistically significant differences from controls, as determined by Tukey test, are indicated. Control: *P<0.001. 1 mM KIC # P<0.001.

Figure 5. Effect of KIC on cAMP levels in slices from cerebral cortex of 9- and 21-day-old rats at different incubation times. Slices from cerebral cortex were incubated for 5, or 30 min with 1 mM KIC and cAMP levels were measured as described in Material and Methods. Results were calculated as pmol cAMP/mg protein and expressed as percentage of control for 6-8 animals in each group. Data are reported as means ± SEM. Statistically significant differences from controls, as determined by one-way ANOVA followed by Tukey test are indicated: *P<0.01.

Figure 1

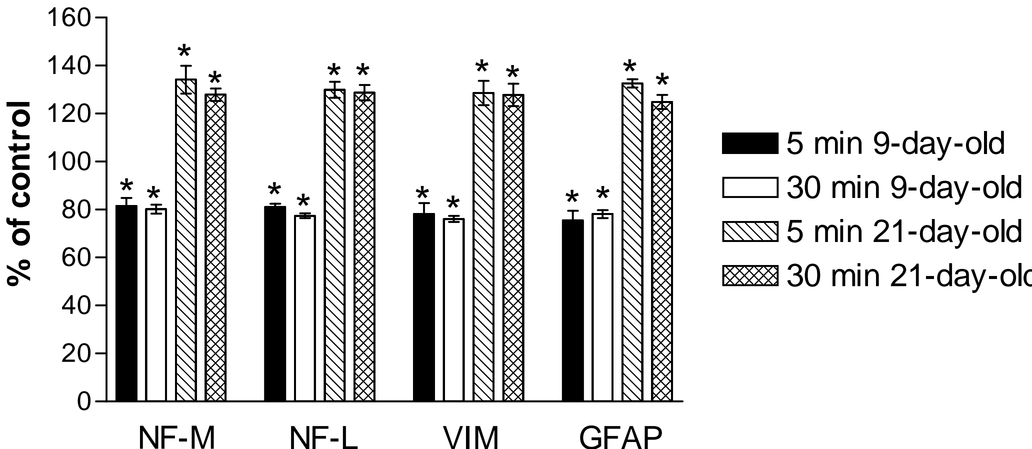
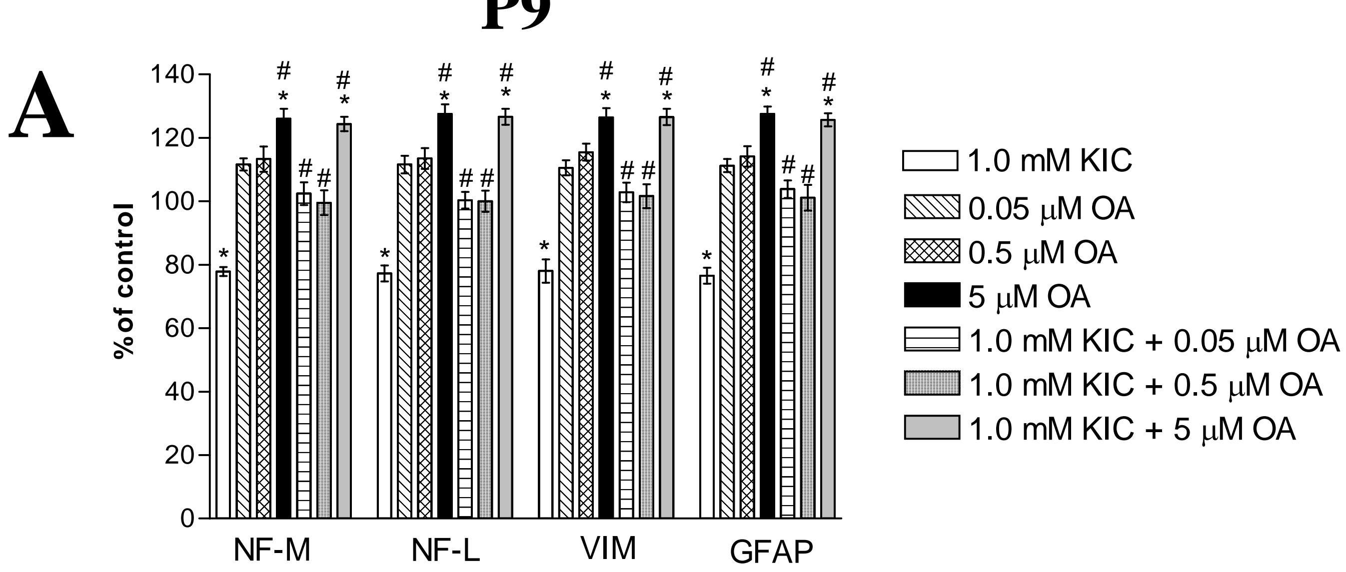
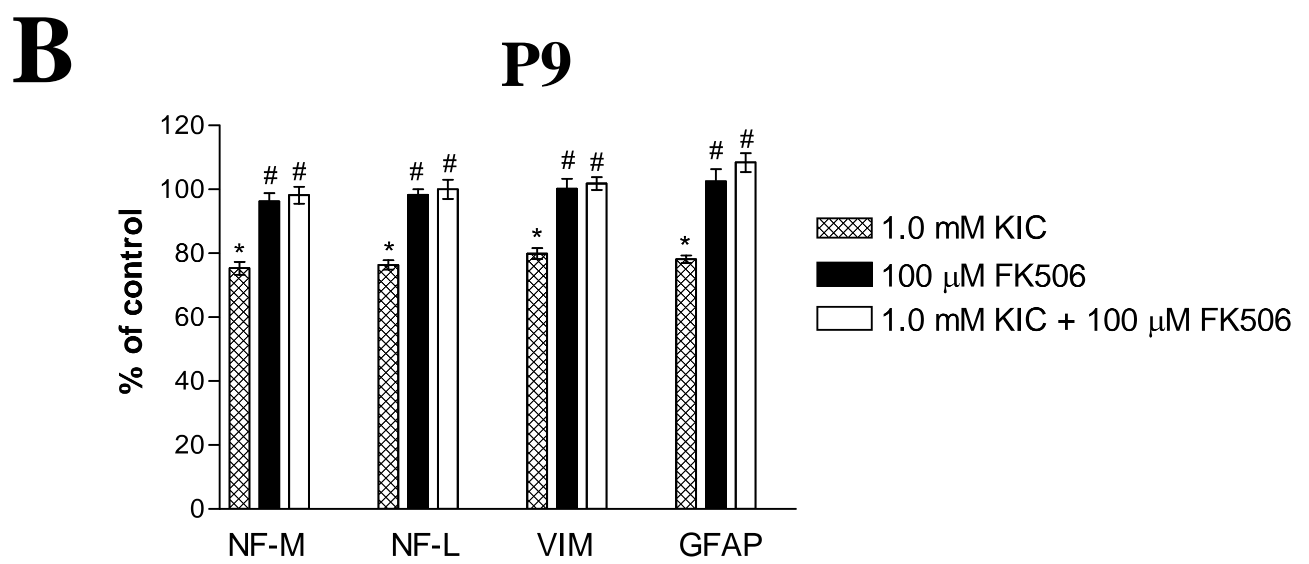


Figure 2





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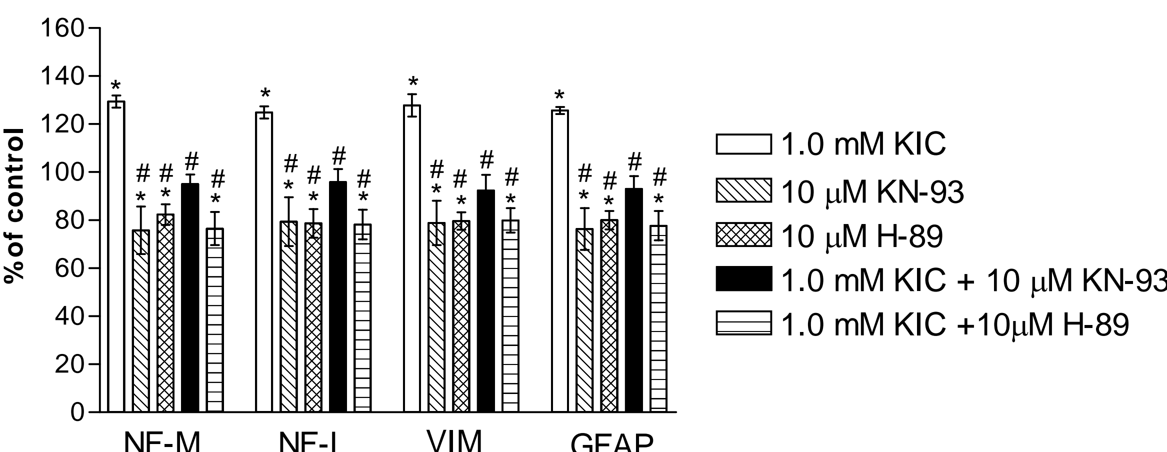

Figure 4

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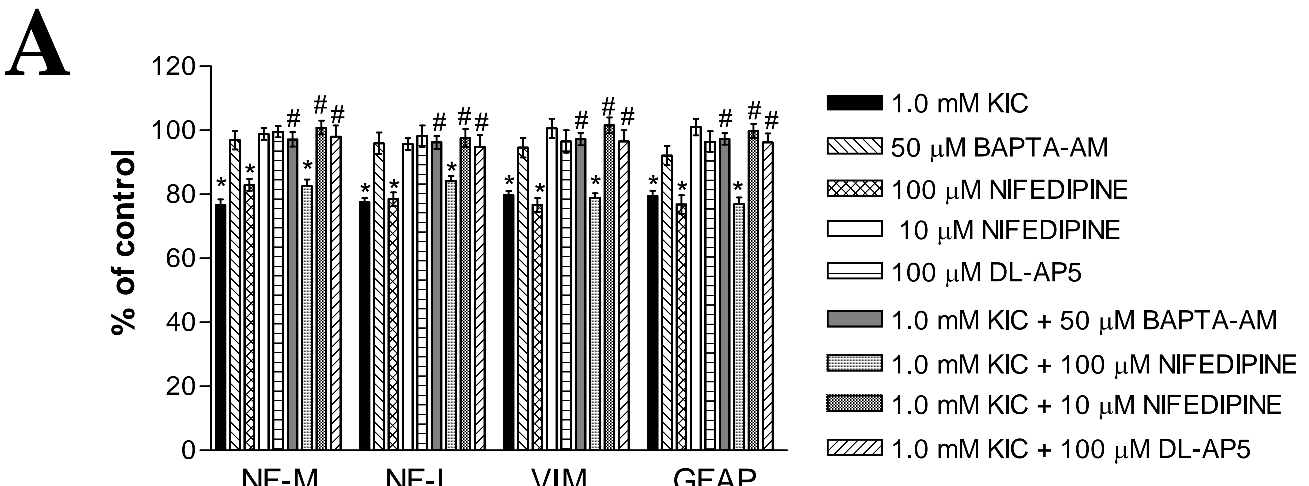

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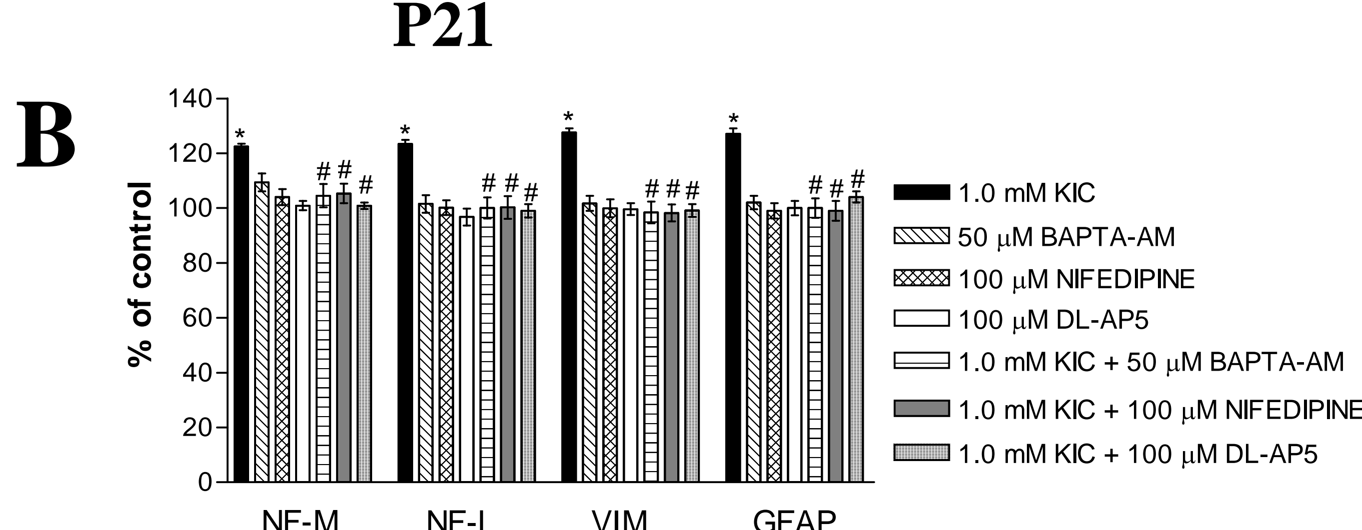
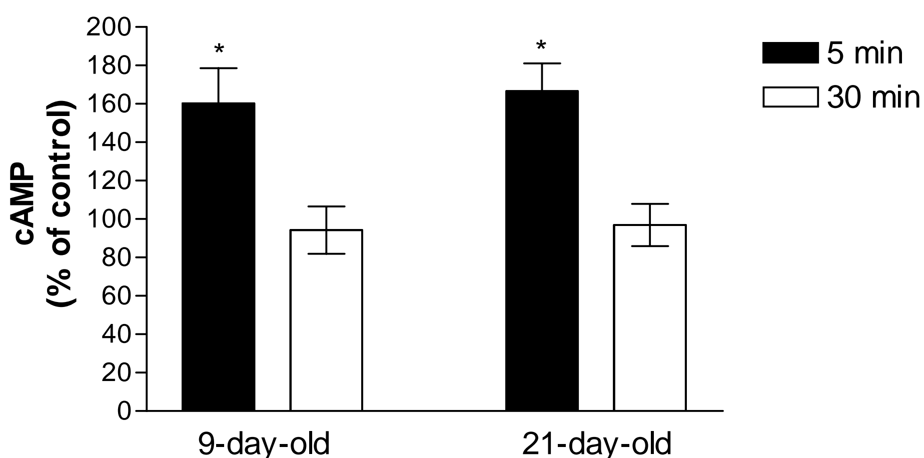


Figure 5



IV - ANEXO 2

Artigo publicado no periódico **Journal of the Neurological Sciences**

Título: α-Keto-β-methylvaleric acid increases the in vitro phosphorylation of intermediate filaments in cerebral cortex of young rats through the gabaergic system

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α-Keto-β-methylvaleric acid increases the in vitro phosphorylation of intermediate filaments in cerebral cortex of young rats through the gabaergic system

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Abstract

In this study we investigated the effects of α-ketoisovaleric (KIV) and α-keto-β-methylvaleric acids (KMV), metabolites accumulating in the inherited neurometabolic disorder maple syrup urine disease (MSUD), on the in vitro incorporation of ^{32}P into intermediate filament (IF) proteins from cerebral cortex of young rats during development (9–21 days of age) We observed that KMV significantly increased the in vitro incorporation of ^{32}P into the IF proteins studied in cortical slices of 12-day-old rats through the PKA and PKCaMII, with no alteration at the other ages. In contrast, KIV was ineffective in altering the phosphorylating system associated with IF proteins at all ages examined. A similar effect on IF phosphorylation was achieved by incubating cortical slices with γ-aminobutiric acid (GABA). Furthermore, by using specific GABA antagonists, we verified that KMV induced a stimulatory effect on IF phosphorylation of tissue slices from 12-day-old rats mediated by $GABA_A$ and $GABA_B$ receptors. In conclusion, our results indicate the involvement of the GABAergic system in the alterations of IF phosphorylation caused by KMV, one of the branched-chain keto acids accumulating in MSUD.
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Keywords: Maple syrup urine disease; Intermediate filaments; Phosphorylation; Gabaergic system; α-Ketoisovaleric acid; α-Keto-β-methylvaleric acid

1. Introduction

Intermediate filaments (IFs) are major components of the cytoskeleton and nuclear envelope in most types of eukaryotic cells. They are expressed in cell-type-specific patterns and play an important structural or tension-bearing role in the cell. Evidence is now emerging that IF also acts as an important framework for the modulation and control of essential cell processes, in particular, signal transduction events [1]. The neuronal cytoskeleton comprises a protein network formed mainly by microtubules (MT) and neurofilaments (NF), the IFs of neurons Neurofilaments are composed of three different polypeptides whose approximate molecular masses are 200, 160, and 68 kDa, and are commonly referred to as heavy (NF-H), medium (NF-M) and light (NF-L) neurofilament subunits [2] Glial fibrillary acidic protein (GFAP) is the intermediate filament of mature astrocytes [3] and vimentin is the IF of cells of mesenchimal origin [4].

The amino and the carboxy-terminal tail domains of NF subunits are potential phosphorylation sites [5]. The phosphorylation sites located on the amino terminal domain of the neurofilament subunits are phosphorylated by second messenger-dependent protein kinases including protein kinase C, cyclic AMP-(PKA) and Ca^{2+}/calmodulin-dependent (CaMK II) protein kinases [6]. The functional role of neurofilament phosphorylation is to date not completely clear. However, the regulation of IF polymerization by amino-terminal phosphorylation is well described in the literature. Vimentin filaments reconstituted in vitro undergo complete disassembly when phosphorylated by purified protein kinase A or protein kinase C [7]. A similar in vitro

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disassembly induced by phosphorylation has been noted for almost all major IF proteins, such as glial fibrillary acidic protein (GFAP) [8], desmin [9], keratin [10], α-internexin [11], NF-L [12] and lamin [13]. On the other hand, the carboxy terminal side arm domains of NF-H and NF-M subunits are extensively phosphorylated by several protein kinases, such as glycogen synthetic kinase (GSK) 3, extracellular signal-regulated kinase (ERK), stress activated protein kinase, protein kinase K, protein kinase C and Cdk-5 [14–17]. It has been demonstrated that in vitro phosphorylation of carboxy-terminal domains of NF-H and NF-M straightens individual neurofilaments and promotes their alignment into bundles [18], whereas in vivo phosphorylation of these proteins is associated with an increased interneurofilament spacing [19]. As a consequence, NF-H and NF-M carboxy-terminal side arms extend and form crossbridges among neurofilaments and other cytoskeletal elements [20].

Maple syrup urine disease (MSUD) is a genetic disorder caused by a defect on the branched chain keto acid dehydrogenase complex [21]. This defect leads to accumulation of millimolar concentrations of α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV), α-ketoisovaleric acid (KIV) and their precursor amino acids leucine, isoleucine and valine in body tissues of affected patients [22,23]. The clinical features of MSUD include keto acidosis, poor feeding, vomiting, apnea, seizures, coma, psychomotor delay and mental retardation [24,21]. Although neurological deterioration and convulsions are the most prominent symptoms, specially during the first days of life, the mechanisms underlying the neurotoxicity of this disorder remain unclear and have been poorly studied.

We have previously showed that KIC, the main keto acid accumulating in MSUD decreased the in vitro incorporation of ^{32}P into the proteins studied up to day 12 and increased this phosphorylation in cortical slices of 17- and 21-day-old rats. We also found a similar effect on IF phosphorylation during development by incubating cortical slices with glutamate, suggesting that the effect of KIC in cerebral cortex of 9- and 12-day-old rats was mediated by the glutamate ionotropic receptors NMDA, AMPA and kainate [25].

In the present investigation we studied the effects of α-ketoisovaleric (KIV) and α-keto-β-methylvaleric acids (KMV), metabolites accumulating in MSUD, on the in vitro incorporation of ^{32}P into IF proteins from cerebral cortex of young rats during development (9–21 days of age). We also evaluated the role of GABAergic receptors on the effects elicited by KMV on this phosphorylation in cortical slices of 12-day-old rats, since growing evidence in the literature suggest a role for altered GABAergic function in neurological and psychiatric disorders of humans including, Huntington's disease [26], epilepsy [27], sleep disorders [28], Parkinson's disease [29] and mental retardation [30]. Despite these evidences, little is known about the effects of GABA on the phosphorylating system associated to the cytoskeleton. We also examined the role of protein kinases on the effects exerted by KMV on cytoskeletal protein phosphorylation.

2. Material and methods

2.1. Radiochemicals and compounds

[^{32}P]Na_2HPO_4 was purchased from CNEN, São Paulo, Brazil, α-keto-β-methylvaleric acid, α-ketoisovaleric acid, gamma-aminobutyric acid (GABA), bicuculline methiodide, benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide and bis-acrylamide were obtained from Sigma (St. Louis, MO, USA). Phaclofen and picrotoxin were purchased from Tocris Neuramin (Bristol, UK). KN-93 and H-89 were obtained from Calbiochem (La Jolla, CA, USA).

2.2. Animals

Wistar rats (9, 12, 17 and 21 days of age) were obtained from our breeding stock. Rats were maintained on a 12-h light/12-h dark cycle in a constant temperature (22 °C) colony room. On the day of birth the litter size was culled to eight pups. Litters smaller than eight pups were not included in the experiments. Water and a 20% (w/w) protein commercial chow were provided ad libitum.

2.3. Preparation and labelling of slices

Rats were killed by decapitation, the cerebral cortex was dissected onto Petri dishes placed on ice and cut into 400 μm thick slices with a McIlwain chopper.

2.4. Preincubation

Tissue slices were initially preincubated at 30 °C for 10 min in 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES (pH 7.4), 12 mM glucose, 1mM $CaCl_2$, and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin, 0.7 μM chymostatin. In the experiments using GABA receptor antagonists, 50 μM bicuculline methiodide, 50 μM picrotoxin or 50 μM phaclofen was added to the medium during preincubation.

2.5. Incubation

After preincubation, the medium was changed and incubation was carried out at 30 °C with 100 μl of the basic medium containing 80 μCi of [^{32}P] ortho-phosphate with or without addition of the different drugs. When indicated, 1 mM GABA, 50 μM bicuculline methiodide, 50 μM picrotoxin, 50 μM phaclofen, 0.5, 0.75 or 1.0 mM α-keto-β-methylvaleric acid, 0.5, 0.75 or 1.0 α-ketoisovaleric acid was added to the incubation medium. In some experiments,

GABA plus α-keto-β-methylvaleric acid were added to the incubation medium. In the experiments using the GABA receptor antagonists, they were added to the medium during preincubation and incubation in the presence or absence of α-keto-β-methylvaleric acid. The labelling reaction was normally allowed to proceed for 30 min at 30 °C and stopped with 1 ml of cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, Tris–HCl 50 mM, pH 6.5, and the protease inhibitors described above). Slices were then washed twice by decantation with stop buffer to remove excess radioactivity.

2.6. Kinase activity assays

Tissue slices of 12 days old rats were initially preincubated at 30 °C for 20 min with 10 μM KN-93, a Ca^{2+}/calmodulin dependent protein kinase (PKCaMII) inhibitor or 10 μM H-89, a PKA inhibitor. After preincubation, the medium was changed and incubation was carried out at 30 °C with 100 μl of the basic medium containing 80 μCi of [^{32}P] ortho-phosphate, with or without addition of 1.0 mM KMV in the presence or absence of one of the kinase inhibitors described above.

2.7. Preparation of the high salt-Triton insoluble cytoskeletal fraction from slices of cerebral cortex

After treatment, preparations of total IF were obtained from cerebral cortex of 9-, 12-, 17- and 21-day-old rats as described by Paz et al. [31]. Briefly, after the labelling reaction, slices were homogenized in 400 μl of ice-cold high salt buffer containing 5 mM KH_2PO_4, (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1% Triton X-100 and the protease inhibitors described above. The homogenate was centrifuged at $15{,}800 \times g$ for 10 min at 4 °C, in an Eppendorf centrifuge, the supernatant discarded and the pellet homogenized with the same volume of the high salt medium. The resuspended homogenate was centrifuged as described and the supernatant was discarded. The Triton-insoluble intermediate filament-enriched pellet, containing neurofilament subunits, vimentin and GFAP, was dissolved in 1% SDS and protein concentration was determined by the method of Lowry et al. [32].

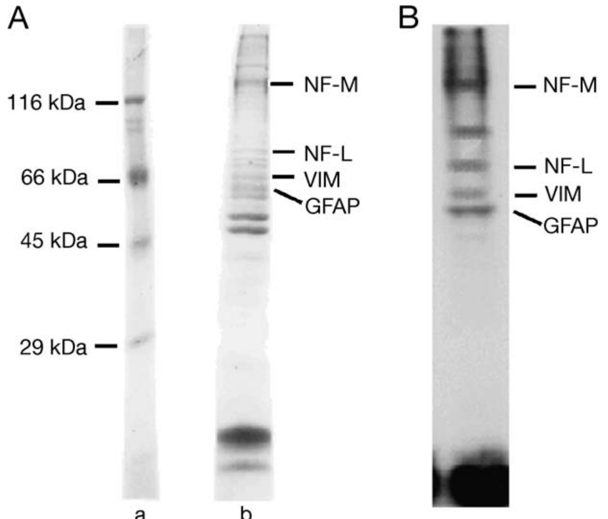

Fig. 1. Polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiograph of the cytoskeletal fraction. (A) Lane a, SDS-PAGE of molecular weight standards (kDa). Lane b, SDS-PAGE of the cytoskeletal fraction of cerebral cortex of 17-day-old rats. The gel was stained with Coomassie blue R-250. NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit; Vim, vimentin, GFAP, glial fibrillary acidic protein. (B) Autoradiograph of the Triton-insoluble intermediate filament enriched cytoskeletal fraction from a 21-day-old rat.

Table 1
Effect of cortical slices exposed to KIV on the in vitro incorporation of ^{32}P into intermediate filaments proteins

	KIV (mM)	NF-M	NF-L	Vim	GFAP
P9	0.5	94.9 ± 3.85	98.1 ± 2.78	101.3 ± 3.36	103.3 ± 1.75
	0.75	96.1 ± 2.81	97.2 ± 3.80	99.4 ± 2.98	104.1 ± 2.43
	1.0	93.4 ± 1.95	91.7 ± 2.10	94.7 ± 2.88	96.6 ± 3.15
P12	0.5	97.4 ± 3.27	101.6 ± 4.28	105.3 ± 1.64	101.7 ± 2.66
	0.75	97.9 ± 1.26	101.0 ± 4.01	102.5 ± 4.06	100.2 ± 4.12
	1.0	98.5 ± 2.05	101.2 ± 4.49	100.3 ± 4.21	100.1 ± 3.38
P17	0.5	101.3 ± 1.2	102.2 ± 4.00	100.4 ± 2.54	100.9 ± 4.14
	0.75	99.8 ± 4.50	105.2 ± 4.71	108.1 ± 2.33	106.3 ± 3.55
	1.0	100.2 ± 1.34	106.6 ± 2.85	105.7 ± 3.61	101.2 ± 3.75
P21	0.5	105.1 ± 4.62	100.1 ± 2.24	101.2 ± 3.77	101.4 ± 3.47
	0.75	100.4 ± 4.12	100.3 ± 2.81	101.6 ± 2.60	97.9 ± 2.76
	1.0	102.1 ± 5.49	97.2 ± 3.73	102.5 ± 3.66	102.1 ± 3.51

Data represent ^{32}P incorporation into intermediate filament proteins, NF-M, NF-L, Vim, GFAP, and are reported as means ± S.E.M. of six to eight animals expressed as percentage of controls. Tissue slices were incubated with 0.5, 0.75 and 1.0 mM KIV and the cytoskeletal fraction was prepared as indicated under Material and methods. Comparison between means was determined by ANOVA.

2.8. Polyacrylamide gel electrophoresis (SDS-PAGE)

The cytoskeletal fraction was prepared as described above. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli [33]. After drying, the gels were exposed to X-ray films (X-Omat XK1) at − 70 °C with intensifying screens and finally the autoradiograph was obtained. Cytoskeletal proteins were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studied proteins.

2.9. Statistical analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple

comparisons test when the F-test was significant. All analyses were performed using the SPSS software program on an IBM-PC compatible computer.

3. Results

Fig. 1A shows a Coomassie blue stained SDS-PAGE of the IF-enriched cytoskeletal fraction extracted from cerebral cortex slices of 21-day-old rats. The autoradiograph of a similar IF-enriched cytoskeletal fraction is shown in Fig. 1B. These intermediate filament proteins have been previously identified by us using specific monoclonal antibodies [34]. When tissue slices from cerebral cortex of 9-, 12-, 17- and 21-day-old rats were incubated with different concentrations of KIV (0.5, 0.75 and 1.0 mM), we did not observe any alteration in the in vitro ^{32}P incorporation into the intermediate filament subunits studied (Table 1). However, incubation of cortical slices with ^{32}P-orthophosphate in the presence of the same concentrations of KMV provoked an altered ^{32}P incorporation pattern into the cytoskeletal proteins studied during development (Fig. 2A–D). It can be seen a small but not significant increase on the in vitro phosphorylation of these proteins in very young rats (day 9) (Fig. 2A). This increase became statistically significant at day 12 (Fig. 2B), whereas at older ages (17 and 21 days) KMV caused no alteration on the phosphorylation of cortical slices of IF proteins (Fig. 2C and D). We also investigated whether PKA and PKCaMII were involved in the activating effect of KMV on the phosphorylating system associated to IF proteins extracted in high-salt Triton containing buffer in the cerebral cortex of 12-day-old rats. We added the specific protein kinase inhibitors H-89 and KN-93 of PKA and PKCaMII, respectively, to the incubation system in the presence of KMV. Results showed that both inhibitors not only prevented but also decreased this phosphorylation, as compared to controls (Fig. 3A and B). These results suggest that KMV activating effect on the phosphorylation of the cytoskeletal proteins from cerebral cortex of 12-day-old rats is mediated by PKA and PKCaMII.

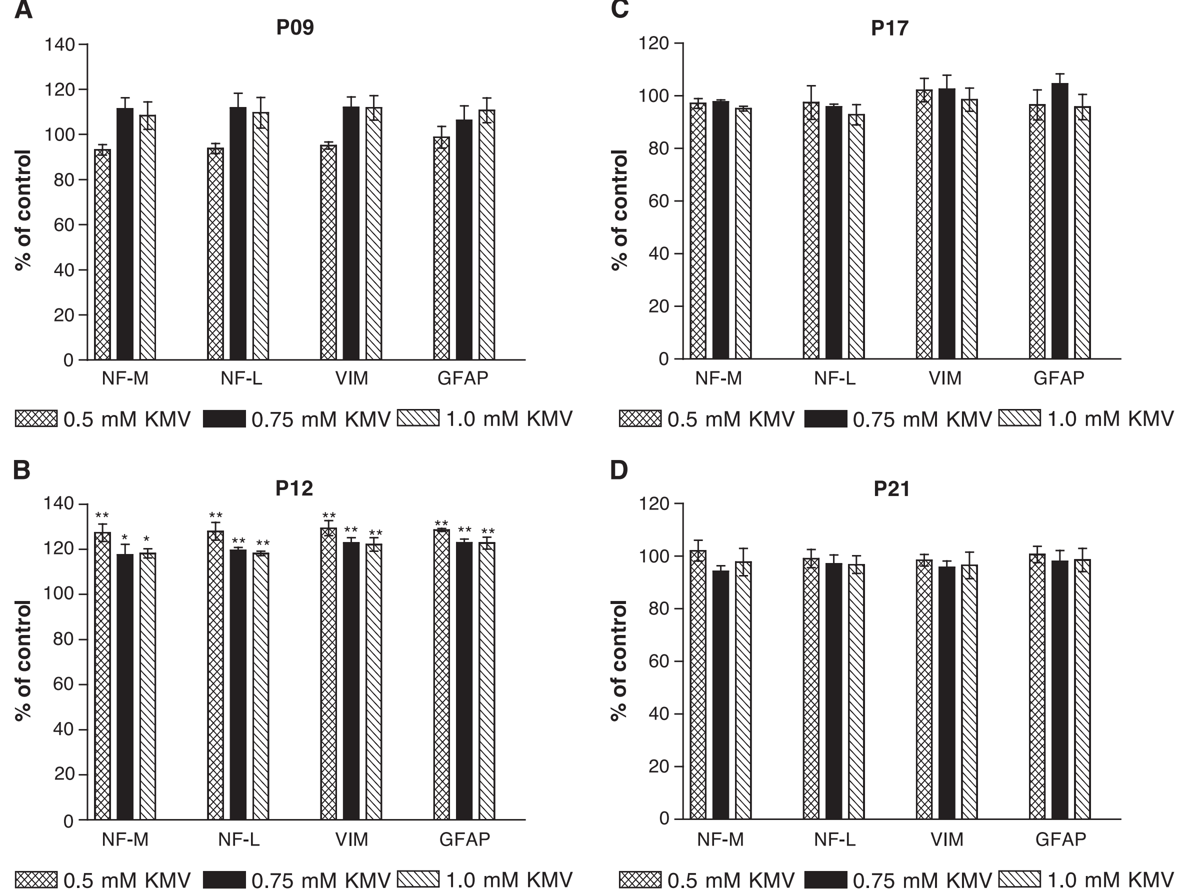

Fig. 2. Effect of KMV on the phosphorylation of intermediate filament subunits of rat cerebral cortex during development. Slices of cerebral cortex of (A) 9-, (B) 12-, (C) 17- and (D) 21-day-old rats were incubated with 0.5, 0.75 and 1.0 mM KMV in the presence of ^{32}P-orthophosphate. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into intermediate filament subunits was measured as described in Material and methods. NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit; Vim, vimentin; GFAP, glial fibrillary acidic protein. Data are reported as means ± S.E.M. of six to eight animals expressed as percentage of controls. Statistically significant differences from controls, as determined by ANOVA followed by Tukey-Kramer multiple comparison test are indicated: $^{**}P<0.001$, $^{*}P<0.01$.

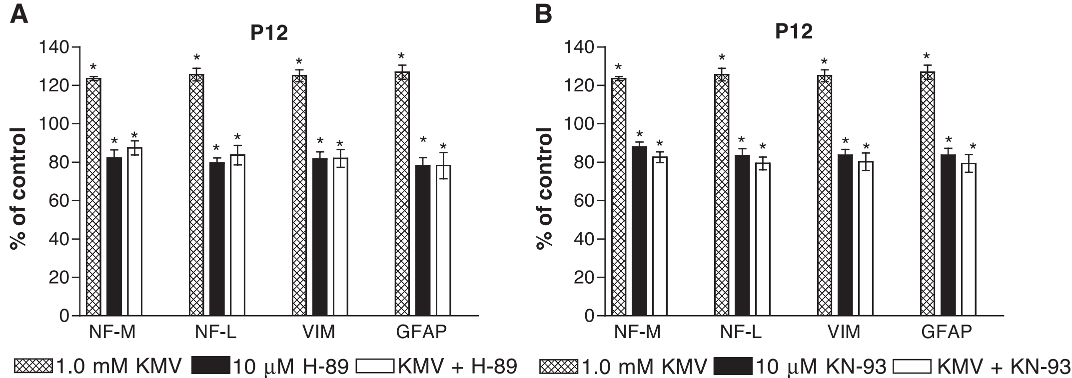

Fig. 3. Effect of treatment of tissue slices with 1.0 mM KMV on cAMP- or Ca^{2+}/calmodulin-dependent in vitro incorporation of ^{32}P into IFs in the cytoskeletal fraction of cerebral cortex of 12-day-old rats. Slices of cerebral cortex were preincubated and incubated with 1.0 mM KMV and ^{32}P-orthophosphate, in the presence or absence of 10 μM H-89 (A) or 10 μM KN-93 (B), as described in Material and methods. The cytoskeletal fraction was extracted and the radioactivity incorporated into middle molecular weight neurofilament subunit (NF-M), low molecular weight neurofilament subunit (NF-L), vimentin (Vim) and glial fibrillary acidic protein (GFAP) was measured. Data are reported as means ± S.E.M. of eight animals in each group and expressed as percent of controls. Statistically significant differences from controls as determined by one-way ANOVA followed by Tukey-Kramer multiple comparison test are indicated: $^{*}P<0.001$.

In order to verify whether the stimulatory effect of KMV on the phosphorylation of intermediate filament subunits was mediated by GABA receptors, tissue slices of 12-day-old rats were incubated with ^{32}P-orthophosphate in the presence of 1.0 mM GABA. We verified that GABA was able to mimic the effect of 1.0 mM KMV. Moreover, we did not observe an additional stimulatory effect when slices were incubated with KMV in the presence of GABA, suggesting that GABA and KMV act through the same mechanism (Fig. 4). Next we tested the effect of 1.0 mM KMV in the presence of GABAergic receptor antagonists. When tissue slices of 12-day-old rats were incubated with KMV following preincubation with the specific $GABA_A$

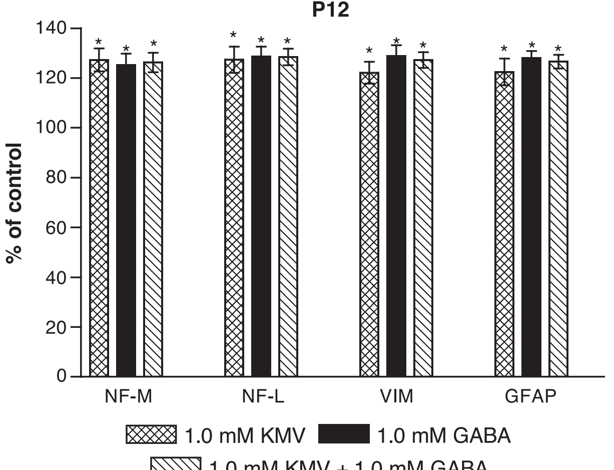

Fig. 4. Effect of GABA on the phosphorylation of intermediate filament subunits of cerebral cortex of 12-day-old rats. Slices of cerebral cortex of 12-day-old rats were incubated with 1.0 mM KMV and/or 1.0 mM GABA in the presence of ^{32}P-orthophosphate. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into intermediate filament subunits was measured as described in Material and methods. NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit; Vim, vimentin; GFAP, glial fibrillary acidic protein. Data are reported as means ± S.E.M. of six to eight animals expressed as percentage of controls. Statistically significant differences from controls as determined by ANOVA followed by Tukey-Kramer multiple comparison test are indicated: $^{*}P<0.001$.

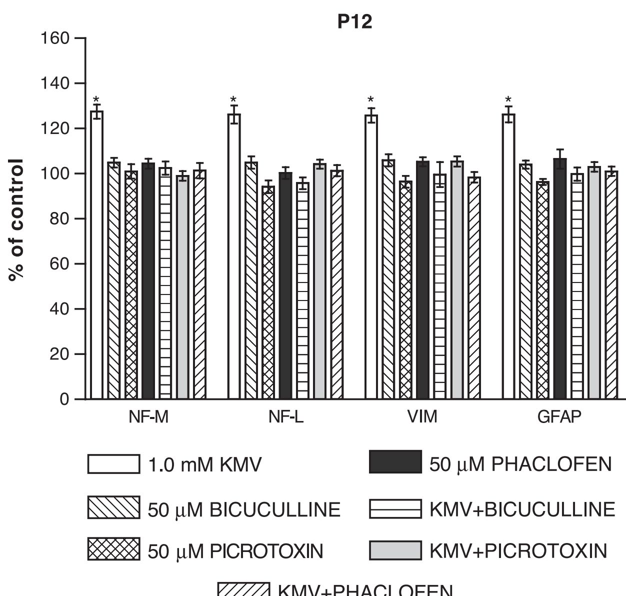

Fig. 5. Effect of the GABA A and GABA B antagonists on the phosphorylation of intermediate filament subunits of cerebral cortex of 12-day-old rats. Slices of cerebral cortex of 12-day-old rats were incubated with 1.0 mM KMV and/or 50 μM bicuculline, 50 μM picrotoxin, 50 μM Phaclofen in the presence of ^{32}P-orthophosphate. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into intermediate filament subunits was measured as described in Material and methods. NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit; Vim, vimentin; GFAP, glial fibrillary acidic protein. Data are reported as means ± S.E.M. of six to eight animals expressed as percentage of controls. Statistically significant differences from controls as determined by ANOVA followed by Tukey-Kramer test multiple comparison test are indicated: $^{*}P<0.001$.

antagonists bicuculline and picrotoxin or the $GABA_B$ antagonist phaclofen, the stimulatory effect of KMV on the in vitro phosphorylation of the various cytoskeletal proteins was prevented (Fig. 5). Taken together, these results suggest that KMV increased the in vitro phosphorylation of intermediate filament subunits in a developmentally regulated manner and that this effect was mediated by $GABA_A$ and $GABA_B$ receptors.

4. Discussion

In the present report we investigated the effect of KMV and KIV, metabolites accumulating in MSUD, on the in vitro phosphorylation of IF proteins from cerebral cortex slices of 9-, 12-, 17- and 21-day-old rats. We observed that KIV was ineffective in altering the in vitro ^{32}P incorporation into neurofilament subunits, vimentin and GFAP, in contrast to KMV, which increased the in vitro phosphorylation of these proteins in cortices from 12-day-old rats, but not from other ages. The differential effect of both metabolites indicates a specific action for KMV. We demonstrated that the KMV-induced stimulatory effect on the phosphorylating system associated to these cytoskeletal proteins in rat probably occurs via the protein kinases PKA and PKCaMII. Our conclusions are based on the experiments using the protein kinase inhibitors H-89, a cell-permeable, selective and potent inhibitor of PKA [35] and KN-93, a specific PKCaMII inhibitor [36], in the presence of KMV. Results showed that pretreatment of tissue slices with 10 μM H-89 or KN-93 totally prevented the effect of the acid on the in vitro phosphorylation of the protein studied. We have previously identified that these kinases are associated to the cytoskeletal fraction in our experimental conditions [37]. We also showed that the effect of KMV was mimicked by GABA. In order to verify and better understand the involvement of the GABA receptors in such effect, we used the $GABA_A$ antagonists bicuculline and picrotoxin and the $GABA_B$ antagonist phaclofen in the next set of experiments. The convulsant bicuculline is the classical $GABA_A$-receptor antagonist, which, by competing with $GABA_A$ for binding to one or both sites on the $GABA_A$ receptor [38] reduces current by decreasing the opening frequency and mean open time of the channel [39,40]. On the other hand, the convulsant compound picrotoxin is a channel blocker, which causes a decrease in mean channel open time, picrotoxin works by preferentially shifting opening channels to the briefest open state (1 ms) [38]. We showed that both bicuculline and picrotoxin fully prevented the effect of KMV on the in vitro phosphorylation of the IF proteins studied, strongly suggesting the participation of $GABA_A$ receptors in KMV action. We also observed that the potent selective $GABA_B$ receptor antagonist phaclofen prevented the stimulatory effect of KMV on the in vitro phosphorylation of IF proteins, suggesting the participation of $GABA_B$, in addition to $GABA_A$ receptors, in the effect induced by KMV.

Taken together, it can be concluded that KMV induces an stimulation of the in vitro phosphorylation of the proteins studied mediated by the GABAergic system. Growing evidence in the literature demonstrate an interaction between $GABA_A$ receptors and the cytoskeleton, which is presumably needed for receptor trafficking, anchoring, and/or synaptic clustering [41]. On the other hand, it has been described that microtubules participate in the maintenance of normal subcellular distribution of $GABA_A$ receptors in neurons and that the organization of microfilaments may play a role in modulating the gene expression of $GABA_A$ receptor subunits [42].

The involvement of $GABA_A$ receptors in neurodegenerative diseases has been largely described. Glass et al. [26] have demonstrated an increase in $GABA_A$ receptor binding to the globus pallidus, in Huntington's disease. In addition, Yu et al. [29] described that the density of $GABA_A$ receptors increased in an animal model of parkinsonism. However little is known about the involvement of GABAergic mechanisms mediating the altered phosphorylation of cytoskeletal proteins in neurodegenerative diseases. Our results, showing that KMV treatment increased both the astrocytic (GFAP) and neuronal (neurofilaments) IF proteins phosphorylation, via GABAergic mechanisms, are supported by previous reports demonstrating that astrocytes also express a large variety of receptors for neurotransmitters and neuropeptides [43]. Runquist and Alonso [44] suggested that GFAP expression and the morphology of adult astrocytes are affected by GABAergic signaling. Our present results showing that the stimulatory effects of KMV on GFAP phosphorylation is prevented by GABA antagonists is in line with these findings indicating that astrocytes express high levels of GABA receptors [45].

Our present results with KMV differ from those achieved with KIC, another metabolite accumulating in MSUD, which, as previously demonstrated, decreases the in vitro ^{32}P incorporation into IF proteins from cerebral cortex of very young rats (9- and 12-day-old rats) via glutamate receptors [25]. The exact mechanisms underlying the involvement of the GABAergic system mediating the effect of KMV on the phosphorylating system associated with IF proteins in a developmentally regulated manner are still unknown. However, it may be related to the expression pattern of the synapses in the CNS, which change during development, being initially GABAergic, turning to a progressively increased glutamate synapse expression. The developmental change of the receptor expression is probably responsible for important processes that take place primarily or exclusively in the developing brain including cell division, differentiation and migration, synapse formation and elimination, programmed cell death and the formation of brain structures [46]. To perform these functions, several receptors, ionic channels, second messengers, growth factors and signaling molecules have unique features during development. Regarding the ionic channels, it is well established that $GABA_A$ and $GABA_B$ receptors are involved in the induction and mainte-

nance of intracellular calcium concentrations [47,48]. In addition, previous works related $GABA_A$ receptors to brain cytoskeletal proteins [41,42] and neudegeneration [26,29].

It is difficult to directly extrapolate our present findings to the human condition and to correlate the alterations of the phosphorylating system provoked by KMV to the cerebral injury of MSUD children. Although we cannot establish at the present whether the degree of alteration on the cytoskeletal phosphorylation (20–30%) provoked by KMV would be able to kill neural cells, since phosphorylation of brain proteins represents an important regulatory mechanism for various essential functions in the brain and alterations of cytoskeletal protein phosphorylation disassemble cell organization, it may be presumed that our present results are indicative of cell dysfunction. Furthermore considering the great body of evidence in the literature showing that alterations of cytoskeletal proteins may lead to disorganization of cellular structure and alterations of protein phosphorylation can be involved in brain damage [49] it is tempting to speculate that this may be at least one of the factors associated with the neurodegeneration and cerebral atrophy characteristic of MSUD patients. At this point, it should be emphasized that MSUD children present severe neurological findings (coma and convulsions) soon after birth, which reflects a severe injury to the immature CNS in early stages of development.

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V - ANEXO 3

Artigo publicado no periódico **Neurochemical Research**

Título: Reduction of glutamate uptake into cerebral cortex of developing rats by the branched-chain alpha-keto acids accumulating in maple syrup urine disease

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Reduction of Glutamate Uptake into Cerebral Cortex of Developing Rats by the Branched-Chain Alpha-Keto Acids Accumulating in Maple Syrup Urine Disease

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In the current study we investigated the effect of the branched-chain alpha-keto acids (BCKA) α-ketoisocaproic (KIC), α-keto-β-methylvaleric (KMV), and α-ketoisovaleric (KIV) acids, which accumulate in maple syrup urine disease (MSUD), on the *in vitro* uptake of [^{3}H]glutamate by cerebral cortical slices from rats aged 9, 21, and 60 days of life. We initially observed that glutamate uptake into cerebral cortex of 9- and 21-day-old rats was significantly higher, as compared to that of 60-day-old rats. Furthermore, KIC inhibited this uptake by tissue slices at all ages studied, whereas KMV and KIV produced the same effect only in cortical slices of 21- and 60-day-old rats. Kinetic assays showed that KIC significantly inhibited glutamate uptake in the presence of high glutamate concentrations (50 μM and greater). We also verified that the reduction of glutamate uptake was not due to cellular death, as evidenced by tetrazolium salt and lactate dehydrogenase viability tests of cortical slices in the presence of the BCKA. It is therefore presumed that the reduced glutamate uptake caused by the BCKA accumulating in MSUD may lead to higher extracellular glutamate levels and potentially to excitotoxicity, which may contribute to the neurological dysfunction of the affected individuals.

KEY WORDS: Maple syrup urine disease; glutamate uptake; cerebral cortex; α-ketoisocaproic acid; α-keto-β-methylvaleric acid; α-ketoisovaleric acid.

INTRODUCTION

Maple syrup urine disease (MSUD) is an autosomal recessive disorder of the metabolism caused by severe deficiency in the activity of the branched-chain α-keto acid dehydrogenase complex (1,2). As a consequence of the defect, the branched-chain keto acids (BCKA) α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV), and α-ketoisovaleric acid (KIV) and their corresponding amino acids leucine, isoleucine, and valine accumulate in tissues of the affected patients. The characteristic features of MSUD include ketoacidosis, poor feeding, vomiting, apnea, seizures, coma, psychomotor delay, mental retardation, cerebral edema, and atrophy (3). Although neurological deterioration and convulsions are common symptoms, the mechanisms underlying the brain damage of this disorder remain unclear and have been poorly studied. However, leucine and its keto acid KIC, the metabolites that most accumulate in MSUD,

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achieving plasma levels of 5 mM during crises, have been considered the main neurotoxins in this disorder because their rapid accumulation is associated with the appearance of neurological symptoms. On the other hand, plasma levels of KMV and KIV have been reported to range from 0.2 to 1.5 mM (2,4). Although the concentrations of the BCKA within the neural cells are unknown, it has been postulated that during crises of metabolic decompensation they may be even higher than those of plasma (5).

The amino acid L-glutamate is considered to be the main excitatory neurotransmitter in the mammalian central nervous system (CNS). Glutamate is involved in synaptic plasticity, being essential for normal brain function, including memory and learning (6–10). Glutamate also plays a major role in crucial steps of CNS development, participating in synapse induction and elimination, cell migration, and differentiation (11). Glutamate exerts its signaling role by acting on glutamate receptors located on the neural cell surface in such a way that glutamate concentration in the surrounding extracellular fluid usually determines the extent of receptor stimulation. The amount of glutamate in the synaptic cleft depends on the balance between its release by presynaptic neurons and its uptake that occurs fundamentally through system localized in the astrocyte membrane and also in the presynaptic neuronal terminal because no extracellular enzyme capable of significantly metabolizing glutamate is known (11,12). When present at high concentrations in the synaptic cleft, glutamate may lead to excitotoxicity, a process corresponding to overstimulation of glutamate receptors leading subsequently to neuronal damage (13). Indeed, excitotoxicity has been related to the neuropathology of various acute and chronic neurodegenerative disorders (14–19).

The uptake of glutamate is mainly accomplished by Na^+-dependent high-affinity systems mediated by a family of proteins known as amino acid transporters (20,21). It is becoming increasingly clear that glutamate transporter dysfunction plays a major role in some neurological diseases, such as stroke, epilepsy, amyotrophic lateral sclerosis, and Alzheimer disease (13).

Therefore, considering the mounting evidence that alterations of the glutamatergic neurotransmission occur in some neurodegenerative conditions and the poorly known mechanisms responsible for the neurological dysfunction of patients affected by MSUD, the purpose of our research was to investigate whether the BCKA accumulating in this disease (KIC, KIV, and KMV) could alter glutamate uptake by cerebral cortex of rats during development. We also studied the kinetics of glutamate uptake and the influence of KIC on this kinetics.

EXPERIMENTAL PROCEDURE

Materials. L-[^{3}H]Glutamate was purchased from Amersham International (UK). α-Ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV), and α-ketoisovaleric acid (KIV) and the other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Animals. Wistar rats 9, 21, and 60 days of age were obtained from our breeding stock. Rats were maintained on a 12-h light/12-h dark cycle in a constant temperature (22°C) colony room. Free water and 20% (w/w) protein commercial chow were provided. On the day of birth the litter size was culled to eight pups, and litters smaller were not included in the experiments.

Glutamate Uptake. The animals were decapitated, and the brain was immediately removed and submerged in Hank's balanced salt solution (HBSS) containing 137 mM NaCl, 0.63 mM $Na_2HPO_4{\cdot}7H_2O$, 4.17 mM $NaHCO_3$, 5.36 mM KCl, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2{\cdot}2H_2O$, 0.41 mM $MgSO_4{\cdot}7H_2O$, 0.49 mM $MgCl_2{\cdot}6H_2O$, and 1.11 mM glucose, adjusted to pH 7.2. Cerebral cortex was dissected, and cortical slices (400 μm) were obtained using a McIlwain chopper. The slices were washed with HBSS, and the sections were finally separated with the help of a magnifying glass. Glutamate uptake was performed according to Frizzo et al. (22). Slices from 9-, 21-, and 60-day-old rats were used in these experiments. Briefly, slices were preincubated at 35°C for 23 min in the presence or absence of 1 or 5 mM KIC, 1 mM KMV, or 1 mM KIV. In some experiments 0.01, 0.1, 1, and 5 mM KIC was used to better evaluate the effect of this BCKA on glutamate uptake into cortical slices. Incubation was carried out at 35°C by adding 12.2 MBq/L L-[^{3}H]glutamate and 100 μM unlabeled glutamate in HBSS to the incubation system. The reaction was stopped after 7 min by two ice-cold washes with 1 ml HBSS, immediately followed by addition of 0.5 M NaOH, which was kept overnight. Sodium independent uptake (nonspecific uptake) was determined by using *N*-methyl-D-glucamine instead of sodium chloride. Sodium dependent uptake was calculated as the difference between the uptake measured in a medium containing sodium and the uptake measured in a similar medium in the absence of sodium. Radioactivity incorporated was determined with a Wallac scintillation spectrometer. Protein concentration was measured by the method of Peterson (23). All experiments were performed in triplicate, and the mean was used for the statistical calculations.

Kinetics of Glutamate Uptake. The kinetics of glutamate uptake was performed with various concentrations of glutamate ranging from 0.3 to 100 μM. Incubations were carried out as described above. To determine the effect of the BCKA on the kinetics of glutamate uptake, we added 1 mM KIC to the assays and varied glutamate concentrations (0.3–100 μM).

Viability Assays

Tetrazolium Salt Method (MTT Assay). The viability assay was performed by the colorimetric 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Slices of 21-day-old rats were preincubated at 35°C for 30 min in HBSS in the presence or absence of 1 or 5 mM KIC, 1 mM KMV, or 1 mM KIV. Immediately after preincubation, 0.5 mg/ml of MTT was added to the medium containing the slices, followed by an incubation at 37°C for 45 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide (DMSO) and measured at 490 and 630 nm. Only viable slices are able to reduce MTT.

Lactate Dehydrogenase Assay (LDH Assay). The viability was assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase into the medium. Cortical slices from 21-day-old rats were incubated at 35°C for 30 min in HBSS in the presence or absence of 1 or 5 mM KIC, 1 mM KMV, or 1 mM KIV. LDH measurement was carried out in 25 μl aliquots using the LDH kit from Doles reagents.

Statistical Analysis. Data are presented as mean ± SEM and were analyzed statistically by one-way ANOVA followed by the Tukey test when the F-test was significant. All analyses were performed using the SPSS software program on an IBM-PC compatible computer. A $P < .05$ was considered to be significant.

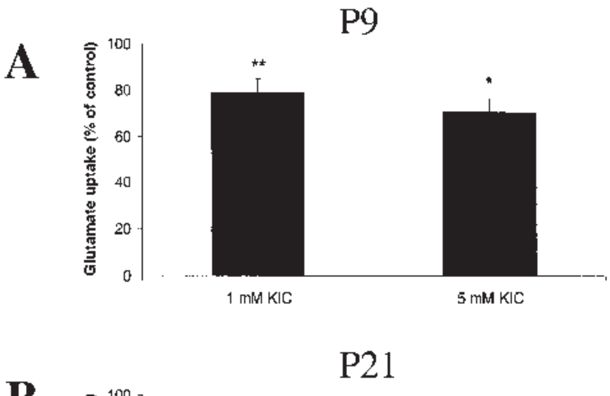
RESULTS

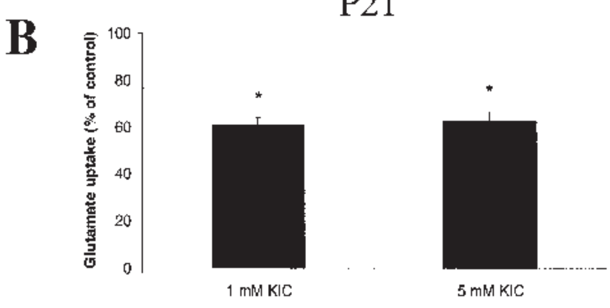
Table I shows the basal levels of glutamate uptake into slices of rat cerebral cortex during development. It can be seen that glutamate uptake into slices of 9- and 21-day-old rats was similar and significantly higher than that of cortical slices from 60-day-old rats. Furthermore, when slices from cerebral cortex of rats of 9, 21, and 60 days of age were incubated with 1.0 and 5.0 mM KIC, a significantly decreased glutamate uptake was observed in slices of 9- and 21-day-old animals with these doses of the metabolite (Fig. 1A and B). However, in cortical slices of 60-day-old rats, only 5 mM KIC was effective in inhibiting glutamate uptake (Fig. 1C). On the other hand, KIV and KMV provoked a distinct pattern from that of KIC. It can be seen in Figure 2A and B that, at 1.0 mM concentration, KIV and KMV were able to significantly inhibit glutamate uptake into cortical slices from 21- and 60-day-old rats, but not from slices of rats with 9 days of age. The kinetics of glutamate uptake into cortical slices from 21-day-old rats was also determined by using increasing glutamate concentrations. Results show that glutamate uptake followed a sigmoidal curve, showing two patterns of uptake, which is consistent with cooperative substrate binding (Fig. 3). To verify the effect of the BCKA on glutamate uptake kinetics we incubated various concentrations of glutamate in the presence of 1 mM KIC (Fig. 3). We have chosen 1 mM KIC on the basis of the concentration curve shown in the insert in Figure 3. It can be seen that KIC was able to decrease glutamate uptake only at high glutamate concentrations (50 μM and higher), but not at low concentrations of the neurotransmitter in the incubation medium (Fig. 3).

Table I. Basal Levels of Glutamate Uptake into Slices of Cerebral Cortex of Rats During Development

Age (days)	9	21	60
Glutamate uptake (nmol/mg/min)	0.940 ± 0.07	0.950 ± 0.11	0.4* ± 0.05

Note: Results are expressed as mean ± SEM of 10 animals in each group. Statistically significant difference determined by one-way ANOVA followed by the Tukey test is indicated (*$P < .001$).





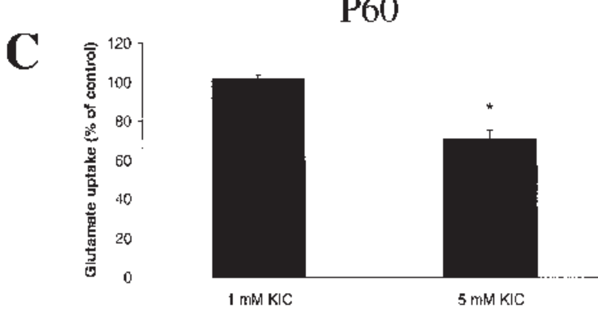
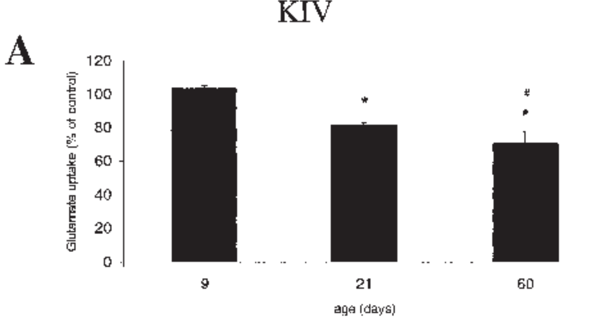


Fig. 1. Effect of 1.0 and 5.0 mM KIC on glutamate uptake into slices of cerebral cortex of 9- (A), 21- (B), and 60-day-old (C) rats. Results are reported as mean ± SEM of 6 or 7 animals in each group and expressed as percentage of controls. Statistically significant differences from controls, as determined by one-way ANOVA followed by the Tukey test are indicated (*$P < .001$, **$P < .01$).

We also investigated whether the reduction of glutamate uptake caused by the BCKA could be ascribed to cellular death and consequently to a reduced number of transporters. For this purpose, we used cortical slices from 21-day-old rats and two different viability assays, the MTT method and the release of LDH. Table II shows that the BCKA, at the concentrations used in the first set of experiments, did not reduce cellular viability in our experimental conditions, implying that the effects observed were due to impairment of glutamate uptake rather to cell death.



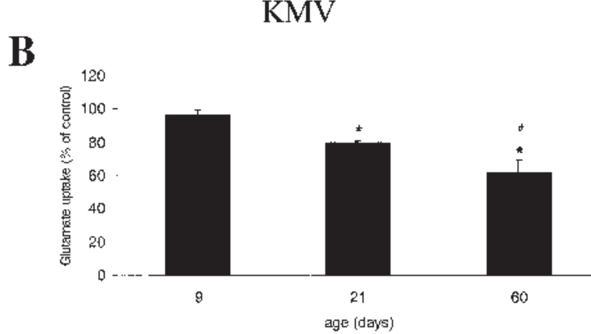


Fig. 2. Effect of 1.0 mM KIV (A) and 1.0 mM KMV (B) on glutamate uptake into slices of cerebral cortex 9-, 21-, and 60-day-old rats. Results are reported as mean ± SEM of six animals in each group and expressed as percentage of controls. Statistically significant differences as determined by one-way ANOVA followed by the Tukey test is indicated. Control: $^{*}P < .001$; 21-day-old rats: $\#P < .005$.

DISCUSSION

There is some evidence that excitotoxicity may play a relevant role in the neurological dysfunction observed in MSUD patients, who characteristically present generalized convulsions and coma during metabolic crises, when the concentrations of the BCKA increase dramatically. In this context, we have previously verified that KIC, the α-keto acid accumulating at highest concentrations in MSUD, alters the *in vitro* ^{32}P incorporation into cytoskeletal proteins via the glutamate ionotropic receptors *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate in cortical slices of rats (24). Furthermore, it has been previously demonstrated that intrastriatal injection of KIV provoked convulsions in rats through overstimulation of NMDA receptors because they were prevented by previous infusion of the NMDA antagonist MK-801 (25). In addition, it has been demonstrated that KIC and KMV, but not KIV, inhibit glutamate uptake into synaptic vesicles of rat brain, potentially leading to an imbalance in the glutamate-glutamine cycle (26).

The present study was therefore designed to investigate whether glutamate uptake by cerebral cortex slices during rat development could be influenced by the BCKA accumulating in MSUD in an attempt to clarify the mechanisms of neurotoxicity induced by these metabolites. Tissue slices were used in the experiments because they contain both neuronal and glial cells and probably constitute a good physiological model to study glutamate uptake. In this context, it should be mentioned that the excitatory effects of glutamate are terminated by the action of high-affinity transporters located on neurons and particularly on glial cells that are able to rapidly remove glutamate and other excitatory amino acids from the synaptic cleft (27).

We initially observed that glutamate uptake by cerebral cortex was higher during the phase of greater brain development, that is, cerebral cortex from 9- and 21-day-old animals uptake more glutamate compared to that of older animals (60-day-old rats). We presume that the high uptake of this excitatory neurotransmitter at early ages may reflect the greater activity of the glutamatergic system during brain development. In addition, we found that all BCKA were able to significantly inhibit glutamate uptake into cortical slices with a distinct pattern during development. KIC provoked a decrease of glutamate uptake into slices from 9- and 21-day-old rats at 1 and 5 mM concentrations, whereas only 5 mM KIC inhibited glutamate uptake in cortical slices of 60-day-old animals. In contrast, KIV and KMV, at 1 mM concentration, did not alter glutamate uptake by cerebral cortex of very young rats (9-day-old), but were capable of decreasing this uptake into cortical slices of 21- and 60-day-old animals. Therefore KIC produced its effects mainly in very young rats, whereas KIV and KMV acted preferentially in older animals. At present we do not know the exact explanation for these findings. However, it is possible that these keto acids may have acted on distinct glutamate transporters according to their differential expression along development (28,29).

We also verified that the kinetics of glutamate uptake corresponded to a sigmoidal curve, characterized by a small slope at low concentrations of glutamate, followed by a sharp increase of this uptake between 40 and 50 μM glutamate, and finally attaining saturation at concentrations of the neurotransmitter higher than 50 μM. More importantly, KIC significantly reduced glutamate uptake only at high glutamate concentrations, which are known to be related to excitotoxicity.

In regard to the possible consequences of the reduced glutamate transport into cortical neural cells caused by the BCKA, it should be stressed the compelling evidence indicating that impairment or reversal of this transport leads to increased extracellular concentrations of glutamate and

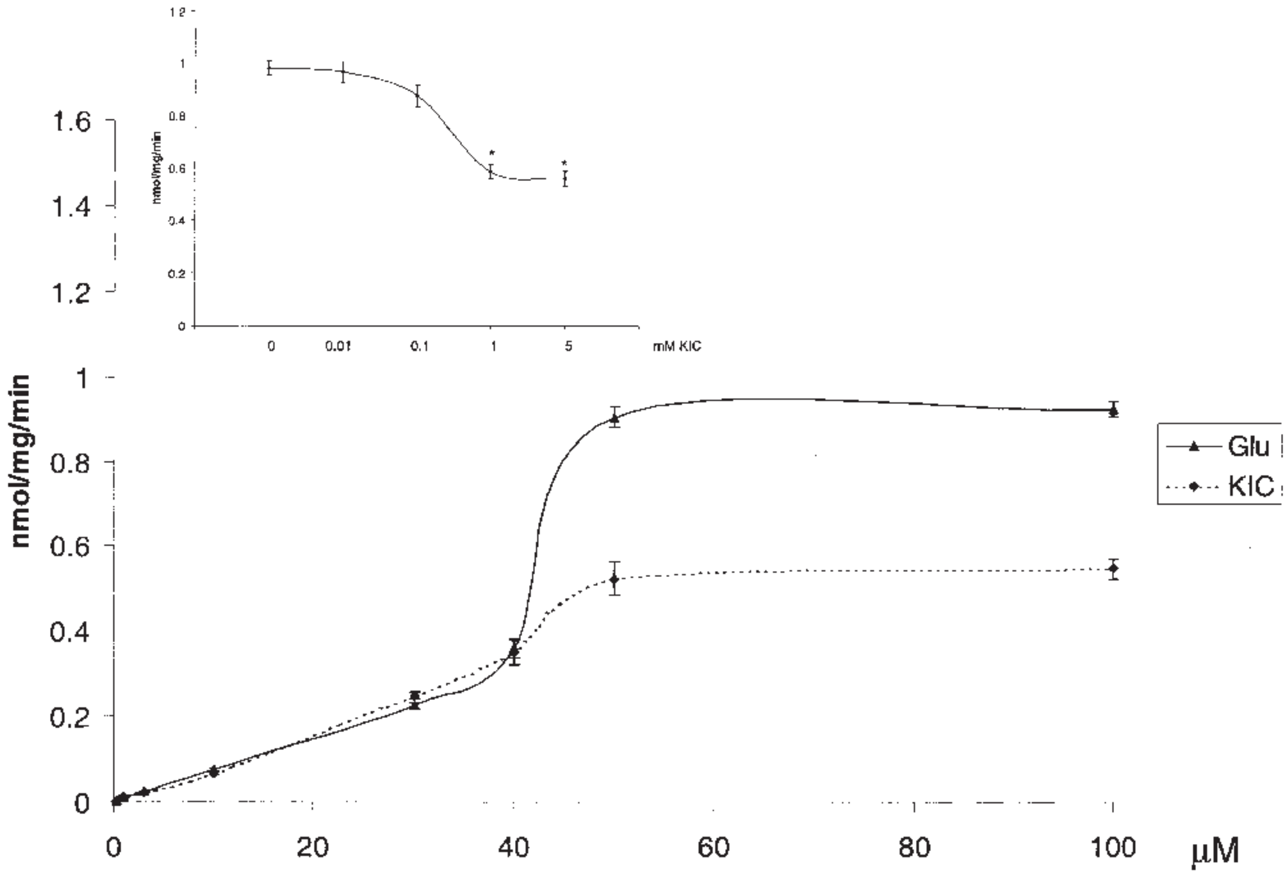


Fig. 3. Kinetics of glutamate uptake into slices of cerebral cortex of 21-day-old rats. Effect of KIC on the kinetics of glutamate uptake. The kinetics of glutamate uptake was determined using 0.3, 1, 3, 10, 30, 40, 50, and 100 µM glutamate as described in Experimental Procedure. The effect of 1 mM KIC on glutamate uptake was determined in the presence of the different glutamate concentrations. The KIC concentration (1 mM) was chosen on the basis of the concentration curve shown in the insert. Results are expressed as mean ± SEM of 4–6 animals in each group.

the development of acute or progressive neurodegenerative pathologies (14,16,17,30) and that alterations in the expression or functional properties of glutamate transporters commonly produce seizures (31). Therefore we are tempting to presume that these keto acids may provoke excitotoxicity by interfering with glutamate uptake by the neural cells, possibly leading more glutamate in the synaptic cleft. This inhibition of glutamate uptake caused by BCKA, as shown here, may therefore reflect a possible mechanism by which these BCKA induce neurotoxic effects.

We do not know the mechanisms by which the keto acids accumulating in MSUD provoke reduction of glutamate uptake. A tentative explanation could be due to the fact that these metabolites inhibit energy homeostasis (5,32–35). In this context, it is known that impairment of energy production in the brain (ATP depletion) leads to failure of ATP-dependent ion pumps and channels,

Table II. Effect of the Branched-Chain α-Keto Acids KIV, KMV, and KIC on the Viability of Cortical Slices from 21-day-old Rats as Determined by the MTT Method and by LDH Release

	1 mM KIV	1 mM KMV	1 mM KIC	5 mM KIC
MTT	103.3 ± 4.75	95.2 ± 8.98	101.7 ± 4.63	99.6 ± 2.60
LDH	90.1 ± 4.01	92.65 ± 3.94	98.86 ± 7.29	94.80 ± 10.95

Note: Results are reported as mean ± SEM of eight animals in each group and expressed as percentage of controls. No significant differences from controls were found (one-way ANOVA).

resulting in less glutamate transport, the consequent accumulation of the neurotransmitter in the synaptic cleft, finally resulting in cell depolarization (36). Therefore it might be presumed that the BCKA-induced reduction of glutamate uptake may have occurred because of the energy deficit caused by these metabolites in the cortical slices.

We cannot ascribe the reduction of glutamate uptake by the BCKA to death of neural cells because two methods that measure cell viability, namely the cytosolic release of the enzyme LDH in the incubation medium and the MTT determination that measures the activity of mitochondrial dehydrogenases, revealed similar results in the presence or absence (controls) of the BCKA with cortical slices from 21-day-old rats.

Our present results of reduced overall glutamate uptake into cortical slices, allied to previous findings showing reduced uptake of this neurotransmitter into synaptic vesicles provoked by the BCKA (26), likely may lead to a disarrangement of glutamate metabolism in the brain because the coordinated action of both transporters from plasma and vesicular membranes results in the effective storage of glutamate in the synaptic vesicles, decreasing its concentration in the synaptic cleft and allowing its release upon depolarization (13).

CONCLUSION

Although the consequences of the effects detected in the present study are still obscure, in case our *in vitro* findings can be extrapolated to human MSUD, it is temping to speculate that inhibition of glutamate uptake into cerebral cortex by the major metabolites (BCKA) accumulating in this disorder may represent a mechanism associated with the neurodegeneration, convulsions, and the cerebral edema and atrophy characteristic of MSUD patients.

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VI - ANEXO 4

Artigo publicado no periódico **GLIA**

Título: Evidence that the branched-chain alpha-keto acids accumulating in maple syrup urine disease induce morphological alterations and death in cultured astrocytes from rat cerebral cortex

Autores: Cláudia Funchal, Carmem Gottfried, Lúcia Maria Vieira de Almeida, Moacir Wajner e Regina Pessoa-Pureur

GLIA 48:230–240 (2004)

Evidence That the Branched-Chain α-Keto Acids Accumulating in Maple Syrup Urine Disease Induce Morphological Alterations and Death in Cultured Astrocytes From Rat Cerebral Cortex

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KEY WORDS cytoskeleton; actin; branched-chain keto acids; lysophosphatidic acid; creatine

ABSTRACT Severe neurological symptoms, cerebral edema, and atrophy are common features of the inherited metabolic disorder maple syrup urine disease (MSUD). However, the pathomechanisms involved in the neuropathology of this disease are not well established. In this study, we investigated the effects of the branched-chain keto acids (BCKA) α-ketoisocaproic (KIC), α-ketoisovaleric (KIV), and α-keto–β–methylvaleric (KMV), which accumulate in MSUD, on astrocyte morphology and cytoskeleton reorganization. Cultured astrocytes from cerebral cortex of neonatal rats were exposed to various concentrations of the BCKA and cell morphology was studied. We observed that these cells changed their usual polygonal morphology when exposed to BCKA, leading to the appearance of fusiform or process-bearing cells. Furthermore, longer exposures to the BCKA elicited cell death at all concentrations studied, attaining massive death at the highest concentrations. Immunocytochemistry with anti-actin or anti-GFAP antibodies revealed that the BCKA induced reorganization of actin and GFAP cytoskeleton. In addition, astrocytes treated with lysophosphatidic acid, an upstream activator of the RhoA GTPase pathway, totally prevented the morphological alterations and cytoskeletal reorganization induced by KIV, indicating that this effect could be mediated by the RhoA signaling pathway. Furthermore, the effects of BCKA on astrocyte morphology were prevented by creatine. In addition, creatine kinase activity was inhibited by KIC and KIV; this inhibition was prevented by creatine, indicating that these keto acids compromise brain energy metabolism. Considering that astroglial cells are critical to brain development and functioning, it is conceivable that alterations of the actin network by BCKA may have important implications in astrocytic function and possibly in the pathogenesis of the neurological dysfunction and brain damage of MSUD patients.
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INTRODUCTION

Astrocytes play a crucial role in CNS organization, development, and normal function (Fedoroff and Vernadakis, 1986). They provide structural and nutritional support for neurons and are involved in many functions, including stimulation of neurite outgrowth and guidance of migrating neurons, synaptogenesis, and synaptic plasticity (Ullian et al., 2001). They also have specific roles in genetic and neoplastic diseases as well as in CNS infections, degeneration, and trauma (Bignami and Dahl, 1976; Vijayan et al., 1991; Kindy et al., 1992).

Intermediate filaments (IFs) constitute a family of cytoskeletal proteins organized into a three-dimensional scaffold in the cytoplasm. Three cytoskeletal proteins have been identified in astrocytes: glial fibrillary acidic protein (GFAP), vimentin, and nestin. GFAP is present in mature astrocytes, whereas nestin appears in immature cells and vimentin occurs in both immature and in certain mature astrocyte subtypes (Galou et al., 1996; Colucci-Guyon et al., 1999). IFs are expressed in a cell type-specific manner and play an important structural or tension-bearing role in the cell (Steinert and Roop, 1988). However, little is known about the function of IF proteins in astrocytes.

The actin cytoskeleton is the major determinant of cell morphology, being involved in cell motility, phagocytosis, migration, adhesion, cytokinesis, and endocytosis (Zigmond, 1996). Actin also plays specific roles in astrocytes. In this context, there is considerable evidence that toxins such as cytochalasin D, which prevent actin polymerization, are able to alter various astrocytic functions, including calcium uptake, release, and signaling, as well as Cl^- conductance, glutamate uptake, and hormone modulation of cell growth (Cotrina et al., 1998; Lascola et al., 1998; Duan et al., 1999; Sergeeva et al., 2000). In contrast, the rearrangement of actin cytoskeleton represents an important biological response to several extracellular stimuli, which are mediated by the Rho family of small GTPases.

Rho GTPases in the GTP-bound active state can interact with a number of effectors to transduce signals leading to diverse biological responses, including actin cytoskeletal rearrangements, cell cycle regulation, control of apoptosis, and membrane trafficking (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998; Bishop and Hall, 2000). When the bound GTP is hydrolyzed to GDP, Rho proteins return to the inactive basal state. The Rho GTPases include many distinct family members in mammalian cells (Rac1-3, Cdc42, TC10, RhoA-E, G, H, and Rnd1 and 2), but the best characterized are the Cdc42, Rac1, and RhoA. In fibroblasts, Cdc42 triggers the formation of filopodia, whereas Rac1 regulates the formation of lamellipodia and RhoA triggers the assembly of focal contacts and stress fibers (Aspenstrom, 1999). Furthermore, growth factors and lysophosphatidic acid (LPA) are upstream regulators of RhoA. Moreover, it has been shown that LPA and RhoA are involved in the regulation of astrocyte morphology (Suidan et al., 1997; Manning et al., 1998).

Maple syrup urine disease (MSUD) is an inherited metabolic disorder caused by a severe deficiency of the branched-chain keto acid dehydrogenase complex, which leads to tissue accumulation of the branched-chain amino acids (BCAA) leucine (Leu), isoleucine (Iso), and valine (Val) and the corresponding branched-chain keto acids (BCKA) α-ketoisocaproic (KIC), α-keto-β-methylvaleric (KMV), and α-ketoisovaleric (KIV) (Chuang and Shih, 2001).

The characteristic features of MSUD include keto acidosis, poor feeding, vomiting, apnea, seizures, coma, psychomotor delay, mental retardation, cerebral edema, and atrophy (Nyhan, 1984). Although neurological deterioration and convulsions are the most prominent symptoms, the underlying etiopathogenic mechanisms leading to neurological dysfunction in this disease are not well understood. However, increased plasma concentrations of Leu and and/or its keto acid KIC have been associated with the appearance of neurological symptoms and are probably the main neurotoxic metabolites in MSUD (Snyderman et al., 1964; Chuang and Shih, 2001).

We have recently reported that the BCKA altered phosphorylation of IF proteins (neurofilaments, vimentin, and GFAP) in cerebral cortex of rats (Funchal et al., 2002). Therefore, taking into account that phosphorylation is an important mechanism regulating cytoskeletal dynamics and that cytoskeleton is essential for normal cell morphology, in the present study we investigated the effects of exposing cultured astrocytes to the BCKA accumulating in MSUD on cell morphology and viability. We also studied whether the RhoA signaling pathway was involved in the effects elicited by these compounds and the possibility that these effects were induced by an energetic deficit.

MATERIALS AND METHODS

Materials

α-Ketoisocaproic acid, α-keto-β-methylvaleric acid, α-ketoisovaleric acid, Dulbecco's modified Eagle's medium (DMEM), LPA, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), creatine, polyclonal anti-actin antibody, diaminobenzidine, and material for cell culture were purchased from Sigma (St. Louis MO), polyclonal anti-GFAP was from DAKO. Fetal calf serum (FCS) was purchased from Cultilab (Campinas, SP, Brazil), peroxidase-conjugated IgG was from Amersham Pharmacia Biotech (Brazil) and 4′,6′-diamidino-2-phenylindole (DAPI) was from Calbiochem (La Jolla, CA).

Astrocyte Cultures

Primary cerebral cortex astrocyte cultures were prepared as described previously (Gottfried et al., 1999).

Briefly, cerebral cortex of newborn Wistar rats (P1–2) was dissected and placed in a Ca^{2+}- and Mg^{2+}-free balanced salt solution (CMF-BSS) pH 7.4 containing (mM): NaCl, 137; KCl, 5.36; Na_2HPO_4, 0.27; KH_2PO_4, 1.1; glucose, 6.1. The tissue was then cleaned of meninges and dissociated by sequential passage through a Pasteur pipette. The cell suspension was allowed to settle and the supernatant transferred to another tube. The sedimented cells were resuspended in CMF-BSS and dissociated again. The two supernatants were combined and the resulting cell suspension centrifuged at 150*g* for 5 min. The supernatant was discarded, the pellet resuspended in culture medium (DMEM, pH 7.6), supplemented with 8.39 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% fungisone; 0.032% garamicine, and 10% FCS. The cells were plated at a density of 5×10^4 cells/well onto 96-well plates pre-treated with poly-L-lysine. Cultures were maintained in 5% CO_2/95% air at 37°C and allowed to grow to near confluence (2 weeks). Medium was changed every 3–4 days. Immediately before medium changes, plates were shaken manually to remove nonadherent cells. Western blotting with an antibody to neuron-specific β-tubulin III excluded contamination with neurons.

Morphological Studies

After cells reached confluence, the culture medium was removed by suction and the cells were incubated for 6, 24, or 30 h at 37°C in an atmosphere of 5% CO_2/95% air in DMEM (pH 7.4) containing 1% FCS in the presence or absence (controls) of the BCKA at 0.1–10 mM concentration. Morphological studies were performed using phase-contrast optics and cells were photographed or fixed for immunocytochemistry.

Immunocytochemistry

Pre-confluent cells were fixed for 20 min with 4% paraformaldehyde in phosphate-buffered saline (PBS) (in mM): KH_2PO4, 2.9; $Na_2HPO_47H_2O$, 38; NaCl, 130; KCl, 1.2, rinsed with PBS and permeabilized for 10 min in PBS containing 0.2% Triton X-100. Fixed cells were then blocked for 60 min with PBS containing 0.5% bovine serum albumin (BSA) and incubated overnight with polyclonal anti-GFAP (1:200) or polyclonal anti-actin (1:200), followed by peroxidase-conjugated IgG (1:1000) for 2 h. Finally the cells were treated with 0.05% diaminobenzidine containing 0.01% hydrogen peroxide for 10 min.

Propidium Iodide

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake. Cells were treated during 6 h with 7.5 μM PI, at 37°C in an atmosphere of 5% CO_2/95% air in DMEM + 1% FCS, after which they were analyzed and photographed with a Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory. Optical density was determined with the Optiquant version 02.00 software (Packard Instrument). Density values obtained were expressed as density light unit (DLU).

Nuclear Morphology Assay

Astrocytes were cultured on circular glass coverslips and treated with 10 mM KIC, KMV, or KIV for 6 h. Cell cultures were fixed for 20 min with 4% paraformaldehyde in phosphate buffer (PBS), stained with 0.2 μg/ml of 4′,6′-diamidino-2-phenylindole (DAPI) for 1 h and visualized under a fluorescent microscope (Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory). Apoptotic cells were morphologically defined by nuclear shrinkage and chromatin condensation or fragmentation.

Creatine Kinase (CK) Activity Assay

Cultured cells were homogenized with a 0.9% saline solution and an aliquot containing ~1 μg protein was pre-incubated for 15 min at 37°C in a mixture containing the following final concentrations: 60 mM Tris-HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$ in a final volume of 0.1 ml. Incubation was started by the addition of 3.2 mM ADP plus 0.8 mM reduced glutathione. The reaction was stopped after 10 min by the addition of 10 mmol p-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to ensure 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 20% α-naphthol and 0.1 ml 20% diacetyl in a final volume of 1 ml and read after 20 min at 540 nm. BCKA did not interfere with spectrometric readings or color development. Results were obtained as μmol of creatine formed per min per mg protein.

Protein Determination

The protein content of astrocyte homogenates was determined by the method of Peterson (1977), using BSA as the standard.

Statistical Analysis

Data from the experiments are presented as mean ±SD and were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey test when the F-test was significant. Values of $P < 0.05$ were considered significant. All analyses were carried

out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

RESULTS

Effects of the BCKA on Astrocyte Morphology

In our control culture conditions, astrocytes are polygonal and flat. In order to evaluate the effect of the BCKA accumulating in MSUD on the morphology of astrocytes in culture, cells were treated with different concentrations (0.1, 1, 5, and 10 mM) of KIC, KIV, and KMV and morphologically analyzed by phase-contrast microscopy after different exposure times (6, 24, and 30 h) (Fig. 1). Our results show that all the BCKA induced differential morphological alterations in the protoplasmic astrocyte cells, which were dependent on the concentration of the BCKA, as well as on exposure time, and led to a progressively increased cellular death. Morphologically altered cells were already observed at 6-h exposure and alterations varied from fusiform to process-bearing cells, depending on the concentration and on the BCKA used. KIV-treated astrocytes presented the most dramatic morphological alterations, which was reflected by the appearance of process-bearing cells at very low concentrations as compared with the other BCKA (Fig. 1C). In contrast, 6-h cell exposure to KMV induced fusiform morphology with few process-bearing cells only at 10 mM concentration (Fig. 1B). In contrast, cultured astrocytes treated with 10 mM KIC presented a massive transformation from polygonal to stellate shape at 6 h exposure (Fig. 1A). Finally, cell death occurred when cultures were exposed for longer periods (24 and 30 h) to 1, 5, and 10 mM of the three keto acids studied. In this case, refringent cell aggregates were viewed in phase-contrast microscopy images (Fig. 1).

We also tested the effect of methylmalonic acid, which accumulates in the inherited neurometabolic disorder methylmalonic academia. Results showed that this organic acid did not alter astrocyte morphology, indicating that the alterations observed for the BCKA were not nonspecific effects due to acidic compounds (results not shown).

Effect of the BCKA on Astrocyte Viability

Cell viability was evaluated by the propidium iodide (PI) method (Pringle et al., 1996), which measures the incorporation of PI by the astrocytes. Astrocytes were incubated for 6 h in the presence of 0.1, 1, and 5 mM KIC, KMV, and KIV and 7.5 μM PI. Figure 2A,B shows that the exposure to increasing concentrations of the BCKA led to cell death in a concentration dependent manner, which is consistent with the results showing alterations of cell morphology observed by visual inspection prior to PI uptake. Exposure to longer periods of time was not carried out because cell death was too high at 24 and 30 h.

Effect of the BCKA on Nuclear Morphology

To better characterize the damage induced by the metabolites on the cultured astrocytes, we used the DAPI staining method to visualize nuclear integrity. When cells were treated with 10 mM KIC, KMV, or KIV for 6 h, we observed that KIC was the only BCKA that provokes condensed and fragmented chromatin. In contrast, KMV and KIV did not cause any significant alteration in nuclear morphology (Fig. 2C). Taken together, the results obtained with PI and DAPI methods appear to show that KIC was the only BCKA provoking morphological features characteristic of apoptosis. Therefore, it may be presumed that KMV and KIV induced astrocytic death via necrosis.

Immunocytochemistry of the BCKA-Treated Astrocytes

Immunocytochemical analysis using anti-actin or anti-GFAP antibodies was also carried out to test the involvement of cytoskeletal actin and IF reorganization in the cell morphology alterations induced by the BCKA (Fig. 3). Under basal conditions, cells presented intense cytoplasmic immunolabeling to GFAP and actin. In addition, nearly all cultured cells (>98%) stained positively to GFAP, attesting to their astrocyte phenotype, and GFAP stained filaments formed a meshwork extending across the cytoplasm, whereas actin filaments formed typical stress fiber bundles. When cells were exposed to 0.1, 1.0, and 5.0 mM KIC, KMV, and KIV for 6 h, we observed a readily apparent disorganization of stress fiber bundles, accompanied by rearrangement of the GFAP filament network that was more distributed along the cytoplasm and processes.

Effect of Lysophosphatidic Acid on the Morphological Astrocytic Alterations Induced by KIV

To evaluate whether the effects of the BCKA on the rearrangement of actin fibers in astrocytes was mediated by a Rho signaling pathway, cultured astrocytes were treated with 2 μM LPA, which is a specific upstream regulator of Rho A, in the absence (controls) or presence of 10 mM KIV for 6 h. It should be emphasized that the organization and dynamics of actin cytoskeleton are regulated by the small Rho GTPases, linking the extracellular stimuli to the organization of the actin cytoskeleton (Guasch et al., 2003). Initially, we verified that cells treated with LPA with no acid supplemented (control) presented polygonal and flattened morphology, as occurs in basal cultures (Fig. 4A). Immunocytochemistry also revealed that LPA treatment did not disturb stress fiber organization of actin fila-

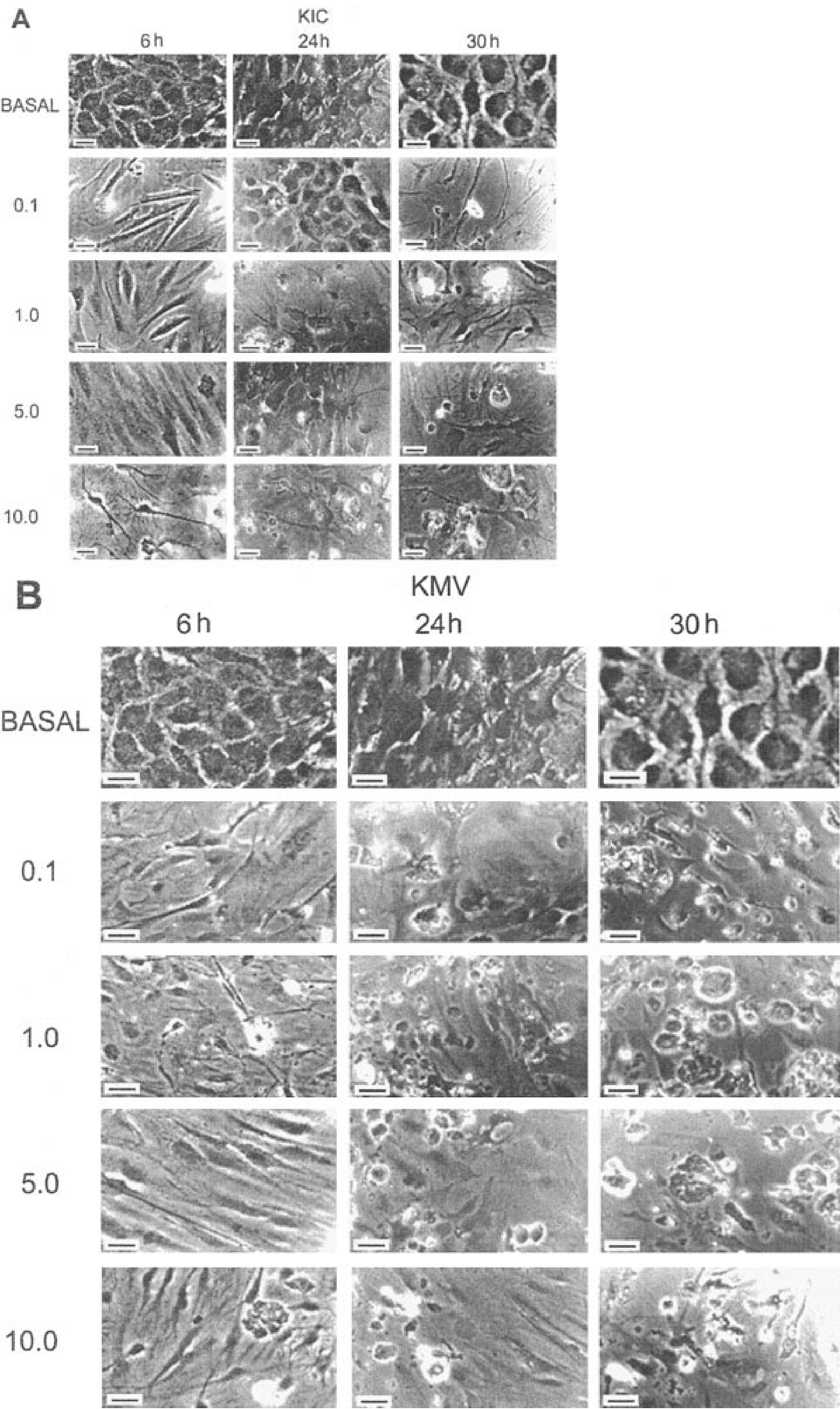

Fig. 1. Effect of branched-chain keto acids (BCKA) on cortical astrocyte morphology. Cells were cultured to confluence in DMEM + 10% fetal calf serum (FCS). The medium was then changed to DMEM + 1% FCS and cells incubated for 6, 24, or 30 h in the presence or absence of the BCKA at different concentrations (0.1, 1, 5, and 10 mM). **A:** ketoisocaproic (KIC) acid. **B:** ketomethylvaleric (KMV) acid. **C:** ketoisovaleric (KIV) acid. After incubation, cells were fixed and phase-contrast images were recorded as described in Materials and Methods. Original images were adjusted by increasing contrast. Scale bar = 50 μm.

ments (Fig. 4C) or GFAP cytoplasmic network (Fig. 4E). However, the most interesting finding was that the presence of LPA ensured a normal astrocyte morphology in cells treated with a high concentration of KIV (10 mM), which indicates that the morphological alterations induced by KIV are modulated via RhoA path-

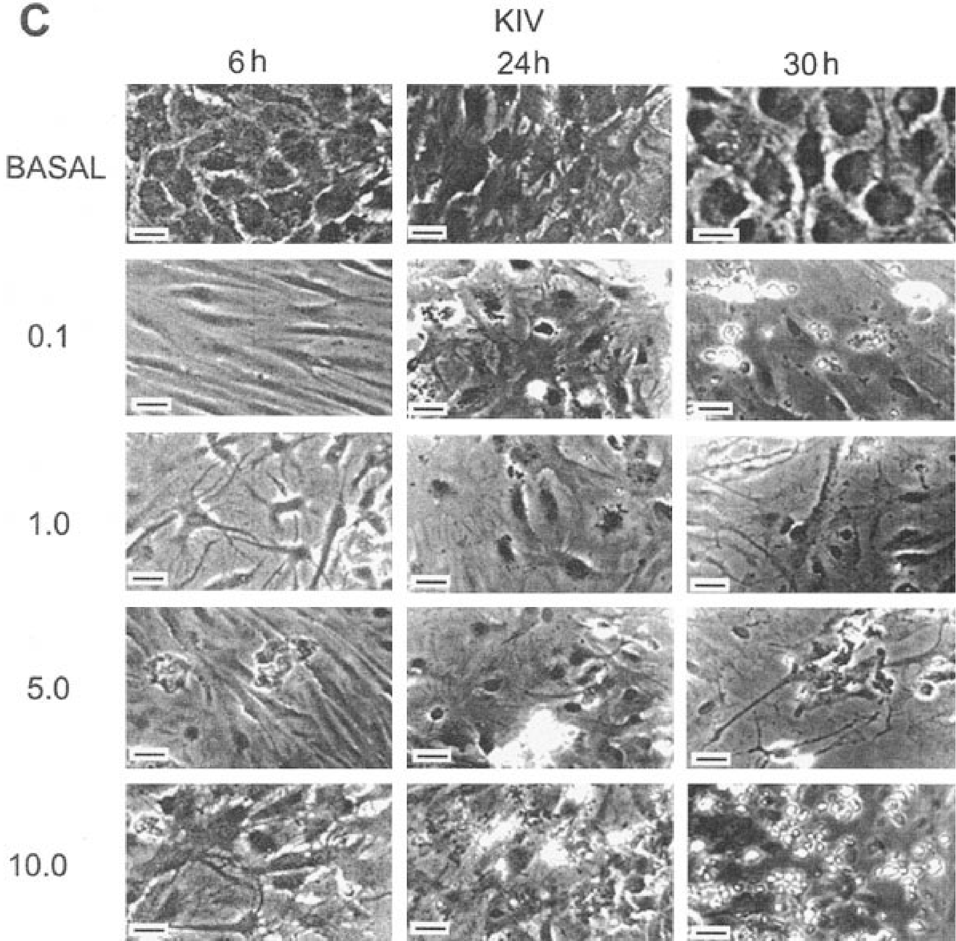

Figure 1. (Continued)

way (Fig. 4B). Finally, by using anti-GFAP and anti-actin antibodies, we confirmed that GFAP organization across the cytoplasm (Fig. 4D) and actin stress fiber arrangement was maintained in KIV-treated cells when LPA was present in the incubation medium (Fig. 4F).

Effect of Creatine on the Morphological Astrocytic Alterations Induced by the BCKA

Considering previous findings showing that the BCKA impair cell energy metabolism (Pilla et al., 2003; Royes et al., 2003) we investigated the possibility that the morphological alterations induced by the BCKA in cultured astrocytes could be due to alterations in the energy homeostasis of these cells. Therefore, astrocyte cultures were supplemented with 5 mM creatine in the presence or absence of 10 mM KIC, KMV, or KIV. We first observed that the presence of creatine for 6 h in the cultures prevented the astrocytic morphological alterations induced by KIV, but not by KIC and KMV (Fig. 5). However, after 24-h exposure, astrocytes incubated with creatine and KIC or creatine and KMV reversed to their polygonal and flattened characteristic morphology, with little cell death detected by the trypan blue exclusion assay (results not shown).

Effects of the BCKA on Creatine Kinase Activity

To evaluate the mechanisms by which the BCKA altered astrocyte morphology, especially whether the cell energy metabolism was compromised by these keto acids, CK activity, a key enzyme of energy homeostasis, was measured in cultured astrocytes. Cells were treated for 6 h with 10 mM KIC, KMV, or KIV; 5 mM creatine; and creatine plus each BCKA, 2 μM LPA, and KIV plus LPA. Cultured cells were then homogenized and the activity of CK was measured. We verified that KIC and KIV significantly reduced CK activity, in contrast to KMV, which had no effect on this activity. Furthermore, co-incubation of creatine with the BCKA revealed that creatine restores normal CK activity, indicating that lack of creatine or phosphocreatine is probably involved in the effect caused by the BCKA. In contrast, co-incubation of LPA with KIV did not prevent CK inhibition caused by this keto acid, suggesting that LPA does not interfere with energy metabolism (Table 1).

DISCUSSION

We have previously demonstrated that the BCKA accumulating in MSUD altered the in vitro ^{32}P incor-

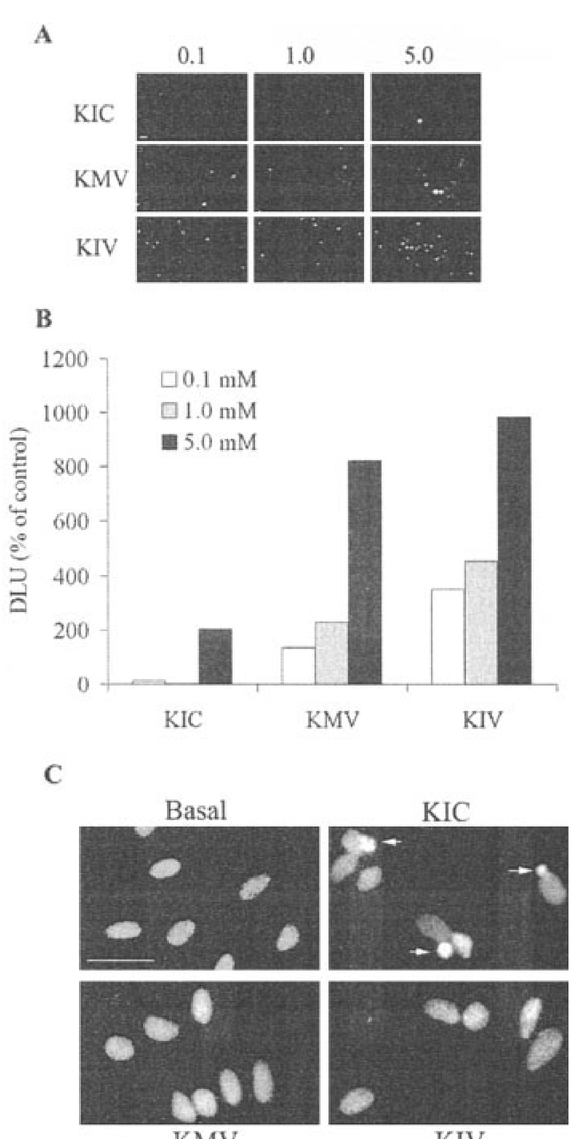

Fig. 2. Cellular damage induced by branched-chain keto acids (BCKA). Cortical astrocytes were cultured to confluence in DMEM + 10% fetal calf serum (FCS). **A:** Representative images of three separate experiments with propidium iodide method. Cells were transferred to DMEM + 1% FCS containing 7.5 μM propidium iodide in the absence or presence of 0.1, 1, 5 mM BCKA and the incubation continued for 6 h. **B:** Optical density values obtained from images shown in A, expressed as density light unit (DLU % of control). **C:** Representative images of two separate experiments with DAPI method for nuclei morphology. Cells were transferred to DMEM + 1% FCS in the absence or presence of 10 mM BCKA and the incubation continued for 6 h. Cells were fixed and DAPI staining was carried out as described in Materials and Methods. Apoptotic nuclei are indicated by arrows. Cells were analyzed and photographed as described in Material and Methods. Scale bar = 50 μm.

poration into the neurofilament subunits, vimentin and GFAP, from cerebral cortex of young rats (Funchal et al., 2002). Because aberrant cytoskeletal phosphorylation/dephosphorylation may have serious consequences for cellular function and structure, and one of these mechanisms may be responsible for the neuronal damage in various neurodegenerative pathologies, in the present study we investigated whether these BCKA could affect cytoskeletal organization, as well as astrocyte shape and viability.

We demonstrated that KIC, KIV, and KMV provoked marked morphological alterations in astrocytes from rat cerebral cortex at doses as low as 0.1 mM, which was followed by cell death when these cells were exposed to higher concentrations of these substances or/and to longer exposure time. Immunocytochemistry showed that the alterations identified in cell shape involved rearrangement of actin and GFAP filaments, with loss of actin stress fibers, which are typical of protoplasmic astrocytes in culture. Therefore, exposure of cultured astrocytes to BCKA concentrations similar to those encountered in blood and other tissues of MSUD patients profoundly altered actin organization in these cells. We observed by immunocytochemical staining with actin antibody that 6 h after addition of 0.1 mM KIC, KIV, or KMV stress fibers start to reorganize, leading to a fusiform cellular shape. By using GFAP antibody, we also evidenced an altered organization of GFAP filaments supporting therefore the reorganization of actin cytoskeleton. Furthermore, actin alterations were more prominent at 6-h exposure to higher concentrations (1, 5, and 10 mM) of these organic acids, leading to the appearance of processes-bearing cells and to a differential reduction of cell viability, pending on the acid used. However, longer exposures to the BCKA (24 and 30 h) elicited progressive cell death at all concentrations studied, attaining massive death at 10 mM. Our observations also indicate that KIV was the metabolite that induced the most dramatic effect on cell shape, since it elicited contraction of cell body and process formation already at 6 h exposure to 1 mM concentration, while similar effects were observed only at higher concentrations for 10 mM KIC and KMV. Our data indicate that the alteration of cell morphology was similar in cultures supplemented by each of the BCKA, turning from a fusiform shape towards process-bearing and finally cell death. The effects elicited by the BCKA were not nonspecific due to acidic compounds, since 5 mM methylmalonic acid caused no alteration of cell morphology.

We also verified that LPA, a specific RhoA activator, prevented the effects of the BCKA on astrocyte morphology, implying that Rho GTPase signaling pathway probably mediated these effects. In this context, it has been established that RhoA is the best-characterized member of the Rho family of low molecular weight GTPases, which are mediators of cell growth and actin cytoskeletal rearrangement in mammalian cells. The role of RhoA in actomyosin-based contractility and in stress fiber formation has already been demonstrated in several cell lines, including S3T3 fibroblasts (Ridley and Hall, 1992) and astrocytes (Guasch et al., 2003). It has been shown that RhoA action is dependent on LPA, one of the factors present in serum and known to act through a G protein coupled receptor (GPCR), eliciting stress fiber formation and focal adhesion complex as-

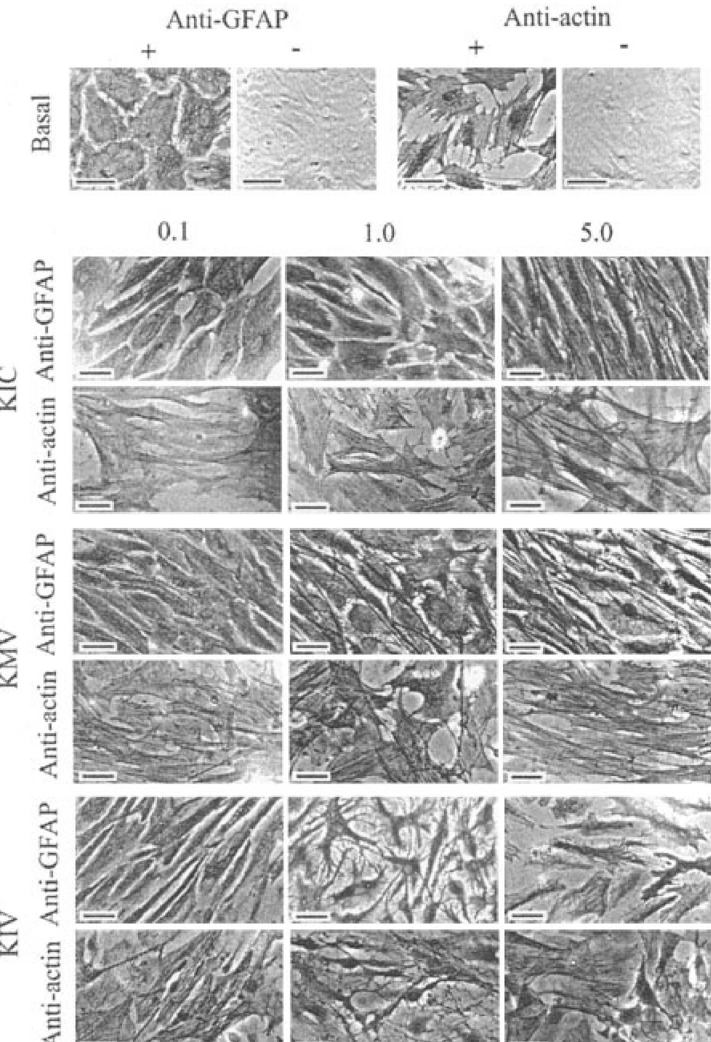

Fig. 3. Immunostaining of cortical astrocytes exposed to branched-chain keto acids (BCKA). Cells were cultured to confluence in DMEM + 10% fetal calf serum (FCS). The medium was then changed to DMEM + 1% FCS in the presence or absence of the BCKA (0.1, 1, and 5 mM) for 6 h after which they were fixed and immunostained with anti-actin or anti-GFAP as described in Materials and Methods. Original images were adjusted by increasing contrast. GFAP, glial fibrillary acidic protein. Scale bar = 50 μm.

sembly in a RhoA-dependent manner (Fukushima et al., 1998).

Our results are in line with previous studies showing that RhoA and LPA are involved in astrocyte morphology (Guasch et al., 2003). The exact explanation for our results showing the involvement of the RhoA signaling pathway in the morphological and actin alterations provoked by the BCKA accumulating in MSUD is unknown. However, we presume that these metabolites may somehow interfere with RhoA signaling pathway, which is a complex process that requires activation of Rho GTPases and their interaction with different molecules affecting the GDP/GTP cycle, mainly at the plasma membrane (Guasch et al., 2003). The BCKA accumulating in MSUD might therefore interfere with one or more steps during this process.

In contrast, the RhoA signaling pathway inducing actomyosin contraction and generating tension through the formation of stress fibers and focal adhesions (Ridley and Hall, 1994; Nobes and Hall, 1995; Hall, 1998) is an active and energy-consuming process depending on the availability of normal ATP concentrations (George et al., 1988; Schmidt and Hall, 1998). Since the BCKA might impair energy production in the brain (Howell and Lee, 1963; Halestrap et al., 1974), it is conceivable that the effect of the BCKA on stress fiber inhibition may be a consequence of the energetic deficit interfering with the cell signaling pathway. To test this hypothesis, we supplemented creatine to the cultures and observed that the morphological alterations and death induced by the BCKA were totally prevented by this compound, strongly indicating that impairment of energy homeostasis is involved in the BCKA action. In order to further evaluate whether these keto acids could compromise energy metabolism, we investigated the effect of the BCKA on CK activity, a crucial enzyme for brain energy metabolism which is closely related to oxidative phosphorylation and therefore dependent on high energy phosphate levels (Saks et al., 1985). The system phosphocreatine/CK represents an important mechanism to maintain cellular energy homeostasis of cells with high and fluctuating energy demand like the neural cells (Wallimann et al., 1992). The CK isoenzymes are specifically located at

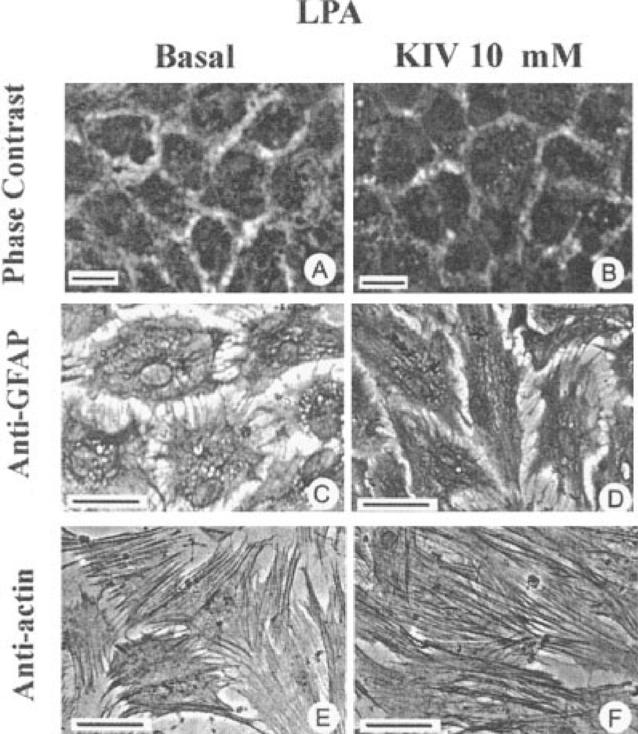

Fig. 4. Effect of lysophosphatidic acid (LPA) on ketoisovaleric (KIV) acid-induced alterations in cortical astrocyte morphology. Cells were cultured to confluence in DMEM + 10% fetal calf serum (FCS), after which cells were transferred to DMEM + 1% FCS with the addition of 2 μM LPA in the absence or presence of 10 mM KIV and the incubation continued for 6 h . Cells were fixed and immunostained with anti-GFAP or anti-actin as described in Materials and Methods. Original images were adjusted by increasing contrast. GFAP, glial fibrillary acidic protein. Scale bar = 50 μm.

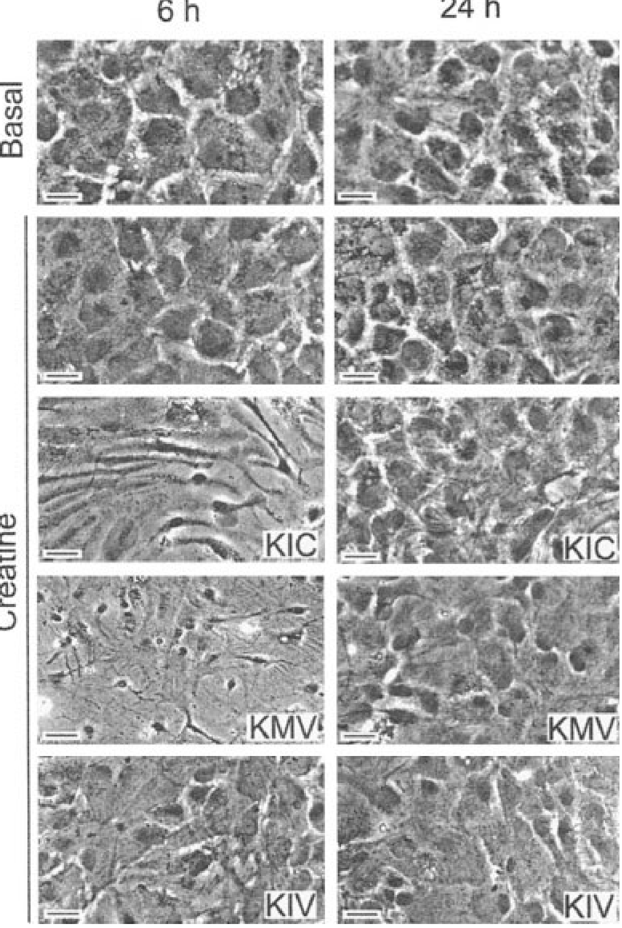

Fig. 5. Effect of creatine on branched-chain keto acids (BCKA)-induced alterations in astrocyte morphology. Cells were cultured to confluence in DMEM + 10% fetal calf serum (FCS) and then transferred to DMEM + 1% FCS with the addition of 5 mM creatine in the absence or presence of 10 mM BCKA. After 6- and 24-h incubation, cells were fixed and phase-contrast images were recorded as described in Materials and Methods. Original images were adjusted by increasing contrast. Scale bar = 50 μm.

places of high energy demand and production, linking energy production and energy utilization by the creatine/phosphocreatine circuit (Wyss et al., 1992). Our results showed that KIC and KIV, but not KMV, inhibited CK activity. Furthermore, creatine supplementation to the cultures prevented this inhibition, suggesting that the inhibitory effect of these keto acids on CK activity was probably due to a reduction of cellular energy charge. Interestingly, we have recently found that the BCKA markedly inhibit the respiratory chain (Sgaravatti et al., 2003), and this may be related to our findings of reduced CK activity due to low availability of ATP. Alternatively, considering that the BCKA are able to induce oxidative stress in the brain (Fontella et al., 2002; Bridi et al., 2003) and that CK is highly vulnerable to free radical attack, especially on critical cysteine residues (Stachowiak et al., 1998), we cannot rule out that CK activity may have been inhibited by the BCKA via free radical generation. We also observed that LPA was not able to prevent the inhibitory effect of KIV on CK activity, indicating that LPA was able to prevent the morphological alterations induced by this keto acid via other mechanisms than energy depletion. Therefore, at present we cannot, establish a direct relationship between energy depletion induced by these BCKA and disrupted RhoA signaling pathway. In contrast, it is also conceivable that BCKA may exert the cytotoxic action towards astrocytes via these two mechanisms. Further experiments will be necessary to clarify this point.

As regards the consequences of alterations of the actin cytoskeleton network, it may have important implications on various critical astrocyte functions, such calcium signaling and uptake (Cotrina et al., 1998; Sergeeva et al., 2000), glutamate transport (Duan et al., 1999), Cl^- conductance (Lascola et al., 1998), endo- and exocytosis of vesicles and protein trafficking (Ridley, 2001). It is also established that one or more of these factors acting together may ultimately compromise astrocytic viability. Accordingly, our experimental results showed a correlation between the BCKA induced actin reorganization and cell death. To test cell viability, we used the PI staining method, which estimates the number of nonviable and viable cells (Cima-

TABLE 1. Effect of α-Ketoisocaproic Acid (KIC), α-Keto-β-Methylvaleric Acid (KMV) and α-Keto-Isovaleric Acid (KIV) on Creatine Kinase Activity From Astrocytes in Culture[†]

	Creatine kinase activity (% of control)			
	10 mM KIC	10 mM KMV	10 mM KIV	
	63.88 ± 2.79*	98.46 ± 4.53	65.03 ± 1.15*	—
5 mM creatine	93.95 ± 3.31	97.02 ± 2.29	95.73 ± 3.6	95.03 ± 2.00
2 μM LPA	—	—	67.76 ± 3.71*	96.91 ± 1.68

LPA, lysophosphatidic acid.
[†]Results are expressed as percentage of control ±SD (control: 5.82 ± 0.80 μmol of creatine/min/mg protein) of three independent experiments. Statistically significant difference determined by one-way ANOVA followed by the Tukey test is indicated: $*P < 0.01$.

rosti et al., 2001) and is based on the fact that viable cells do not allow PI uptake, whereas in nonviable cells, because of the loss of membrane integrity, the stain crosses the plasma membrane and binds to DNA, becoming highly fluorescent.

Cell death may occur via apoptosis or necrosis (Ellis et al., 1991). Necrotic death is usually the consequence of physical injury, whereas apoptosis is the result of highly regulated active process, controlled by complex signal-transduction pathways (McConkey and Orrenius, 1994; Steller, 1995). Apoptosis can be distinguished from necrosis on the basis of several morphological and biochemical parameters. Therefore, to investigate the mechanisms of cell death induced by the BCKA, we used the DAPI staining method. The DAPI is a vital dye that is rapidly taken up into cellular DNA yielding highly fluorescent nuclei and no detectable cytoplasmic fluorescence in cultured cells. The dye binds selectively to DNA and forms strongly fluorescent DNA-DAPI complexes with high specificity, identifying nuclear shrinkage and chromatin condensation or fragmentation, an important parameter of the apoptotic process (Barile et al., 1978). We observed that KIC induced nuclei fragmentation and chromatin condensation, in contrast to the other BCKA, which did not cause this phenomenon. These results are indicative that KIC elicited morphological features characteristic of apoptosis. Considering that KMV, and especially KIV, also induced astrocytic death, it may be presumed that these BCKA provoked cell death probably via necrosis. Interestingly, it has been demonstrated that a Rho-dependent actin reorganization from stress fibers to actin rings is formed in cells destined for apoptosis (Rosenblatt et al., 2001). Furthermore, a previous study showed that the BCKA and their respective branched-chain amino acids accumulating in MSUD decrease neuronal and astrocytic viability in cell cultures by inducing apoptosis, with KIC the most toxic metabolite (Jouvet et al., 2000). These investigators showed that KIC-treated cells were rounded and contained condensed nuclei with chromatin crescents. Cytoplasm was shrunken, and organelles, including mitochondria, were tightly packed into the remaining cytosol. Changes in mitochondrial morphology, in particular reduction in mitochondrial volume, was also consistent with cell death by apoptosis. Besides the mitochondrial changes at the ultrastructural level, the KIC-treated cells presented reduction in cell respiration (Jouvet et al., 2000).

In summary, our results demonstrate that KIC, KIV, and KMV at concentrations found in plasma and tissues of MSUD patients disturb actin cytoskeleton organization probably via the RhoA signaling pathway. It is possible that the interference of these compounds with this pathway might impair astrocyte ability to maintain their actin cytoskeleton organization as stress fibers, perhaps leading to cell death. Since actin cytoskeleton participates in major astrocytic functions, actin reorganization induced by the BCKA might have important consequences, including alterations in protein trafficking, uptake, and release of neurotransmitters, and calcium control. Finally, it is feasible that our results may contribute to the understanding of the pathogenesis of the severe neurological dysfunction and neural loss (cortical atrophy) characteristic of MSUD patients. We should also emphasize that other organic acids accumulating in various inherited neurometabolic disorders, including glutaric acidemia type I (Silva et al., 2000), D-2-hydroxyglutaric acidemia (Kolker et al., 2002; Silva et al., 2004), and methylmalonic acidemia (Wajner and Coelho, 1997; Okun et al., 2002), provoked neural damage and energy depletion.

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VII - ANEXO 5

Manuscrito em fase de preparação para ser submetido ao periódico **Metabolic Brain Disease**

Título: Branched chain α-keto acids accumulating in maple syrup urine disease induce reorganization of phosphorykated GFAP in C6 glioma cells

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BRANCHED-CHAIN α-KETO ACIDS ACCUMULATING IN MAPLE SYRUP URINE DISEASE INDUCE REORGANIZATION OF PHOSPHORYLATED GFAP IN C6 GLIOMA CELLS

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RUNNING TITLE: GFAP hyperphosphorylation and cytoskeletal reorganization

KEYWORDS: Maple syrup urine disease, GFAP, phosphorylation, immunocytochemistry

ABSTRACT

In this study we investigate the effects of the brached-chain keto acids (BCKA) α-ketoisocaproic (KIC), α-ketoisovaleric (KIV) and α-ketoβ-methylvaleric (KMV) acids, metabolites accumulating in maple syrup urine disease (MSUD), on the in vitro phosphorylation of glial fibrillary acidic protein (GFAP) and cytoskeletal reorganization in C6 glioma cells. We observed that after 3 h treatment with KIC, KIV or KMV cells showed retracted cytoplasm with bipolar processes containing packed GFAP filaments as revealed by immunocytochemistry. Western blot analysis by anti GFAP monoclonal antibody demonstrated that BCKA were not able to alter GFAP immunocontent in total cell homogenate, but the immunocontent as well as the in vitro ^{32}P incorporation into GFAP recovered into the high salt Triton-insoluble cytoskeletal fraction were significantly increased. Western blot using monoclonal anti phosphoserine antibody showed that BCKA induced increased immunocontent of phosphoserine-containing amino acids in several proteins in total cell homogenate. In addition, the immunocontent of phosphoserine-containing amino acids was also greatly increased in GFAP recovered in the high-salt Triton insoluble cytoskeletal fraction, corresponding to the polymerized IF proteins present in the cell. In conclusion, our results indicate that KIC, KIV or KMV increased the serine/threonine in vitro phosphorylation of GFAP leading to increased Triton-insoluble GFAP immunocontent and cytoskeletal reorganization. Considering that intermediate filament (IF) networks can be regulated by phosphorylation of polypeptide subunits leading to reorganization of the IF filamentous structure, we could suppose that GFAP hyperphosphorylation and disorganization of cellular structure could be involved in the brain damage characteristic of MSUD patients.

INTRODUCTION

Glial fibrillary acidic protein (GFAP) is the major intermediate filament (IF) protein in mature astrocytes. The high abundance of GFAP in astrocytes and its strong conservation among vertebrates suggests that it plays a critical function in the central nervous system (CNS). Although the exact functions of GFAP in astrocytes remain to be elucidated, the onset of GFAP expression is observed in radial glial cells of primates (Levitt and Rakic, 1980) or during astrocyte differentiation in rats (Dahl, 1981). GFAP is also dramatically upregulated in gliosis along with astrocyte hypertrophy (Eng and Lee, 1995). These features support the hypothesis that GFAP determines to a large extent the complex astrocyte morphology that includes multiple processes that contact blood vessel walls, ensheate neuronal synapses, abut nodes of Ranvier, and interdigitate with one another at the pial surface to form the glial limitans (Messing and Brenner, 2003).

The C6-glioma cell line was originally derived from rat brain tumors induced with N-nitrosomethylurea (Benda et al, 1968). This cell line has oligodendrocytic, astrocytic and neuronal properties (Parker et al, 1980), constituting a useful model in studies of glial cell differentiation (Mangoura et al, 1989) and glioma cell migration (Goldberg et al, 1992).In addition, C6-glioma cells have been considered a model to study the in vitro effects of neurotoxicants, such as ammonium chloride, affecting the oxidative metabolism (Haghighat et al, 2000), since these cells have a greater rate of oxidative metabolism than astrocytes (Haghighat and McCandless, 1997). Previous studies have demonstrated that GFAP is expressed in both malignant and benign astrocytomas (Jacque et al, 1979) and that the overexpression of GFAP in rat astrocytoma C6 cells altered the morphological phenotype and supressed cell growth (Toda et al, 1994).

The amino and the carboxy-terminal tail domains of GFAP subunits are potential phosphorylation sites. Thereafter, it has become increasingly evident that site-specific phosphorylation of IF proteins alters filament structure. In this context, phosphorylation and dephosphorylation of specific amino acid residues in the amino-terminal tail of GFAP are involved in the regulation of GFAP assembly (Inagaki et al, 1994). Interestingly, abnormal phosphorylation of cytoskeletal proteins has been related with neurotoxicity in several neurodegenerative diseases such as Alzheimer's disease and related tauopathies (Iqbal et al, 2002).

Maple syrup urine disease (MSUD), or branched-chain keto aciduria, is an inborn error of metabolism caused by severe deficiency of the branched chain α-keto acid dehydrogenase complex (BCKAD, E.C. 1.2.4.4) activity (Chuang and Shih, 2001). The inability of this enzyme complex to oxidize the branched-chain keto acids (BCKA) α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV) leads to tissue accumulation of these metabolites and their precursor amino acids leucine, isoleucine and valine, respectively, in the affected individuals. Patients with MSUD present poor feeding, apnea, ketoacidosis, convulsions, coma and psychomotor delay. CNS imaging reveals low density of white matter corresponding to hypomyelination/demyelination and cerebral atrophy. The disease is severe enough to cause a fatal outcome in a significant number of patients if not diagnosed and treated promptly. Those who survive present a variable degree of mental retardation (Chuang and Shih, 2001). Although neurological deterioration and convulsions are the most prominent symptoms, the underlying etiopathogenic mechanisms leading to neurological dysfunction in this disease are not well understood. However, increased plasma concentrations of

leucine and and/or its keto acid KIC have been associated with the appearance of neurological symptoms and are probably the main neurotoxic metabolites in MSUD (Snyderman et al, 1964; Chuang and Shih, 2001).

Considering that we have previously demonstrated that KIC, KIV and KMV were able to induce morphological alterations in astrocytes in culture (Funchal et al, 2004a), and that KIC and KMV altered the in vitro incorporation of ^{32}P into IF proteins in slices of cerebral cortex of rats (Funchal et al, 2002;Funchal et al, 2004b), in the present study we used C6 cells to examine the effect of these metabolites on cell morphology as well as analyze the possible involvement of GFAP in vitro phosphorylation on the morphological alterations observed.

METHODS

Radiochemicals and compounds- [^{32}P]orthophosphate was purchased from CNEN, São Paulo, Brazil, α-keto-β-methylvaleric acid, α-ketoisovaleric acid, benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide, bis-acrylamide, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), anti glial fibrillary acidic protein - GFAP (clone G-A-5), anti phosphoserine (clone PSR-45) and material for cell culture were purchased from Sigma (St. Louis MO, USA). Dulbecco´s modified Eagle´s medium (DMEM) was from Gibco BRL (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Cultilab (Campinas, SP, Brazil).

Maintenance of cell line: The C6 rat glioma cell line was obtained from American Type Culture Collection (Rockville, Maryland, USA). The cells were grown and maintained in

DMEM (pH 7.4) containing 2.5 mg/ml Fungizone® and 100 U/l gentamicin, and supplemented with 5 % FBS. Cells were kept at a temperature of 37° C, a minimum relative humidity of 95 %, and an atmosphere of 5 % CO_2 in air.

Morphological studies: After cells reached confluence, the culture medium was removed by suction and the cells were incubated for 3 h at 37°C in an atmosphere of 5% CO_2 / 95% air in DMEM (pH 7.4) containing 0% FBS in the presence or absence (controls) of the BCKA at 1 to 10 mM concentration. Morphological studies were performed using phase contrast optics and cells were fixed for immunocytochemistry.

Immunocytochemistry: C6 cells were cultured on circular glass coverlips, treated with BCKA for 3 h and fixed for 20 minutes with 4% paraformaldehyde in phosphate buffer saline (PBS) (in mM): KH_2PO4, 2.9; $Na_2HPO_47H_2O$, 38; NaCl, 130; KCl, 1.2, rinsed with PBS and permeabilized for 10 min in PBS containing 0.2 % Triton X-100. To reduce nonspecific background staining the fixed cells were then blocked for 60 min with PBS containing 0.5% bovine serum albumin and incubated overnight with polyclonal anti-GFAP (1:200) followed by peroxidase-conjugated IgG (1:200) for 2 h. Finally the cells were treated with 0.05% diaminobenzidine containing 0.01% hydrogen peroxide for 10 min.

In vitro phosphorylation- C6 cells were preincubated for 3h in the presence of BCKA, then the medium was changed and incubation was carried out at 30 ^{o}C with 100 μl of a medium containing 124 mM NaCl, 4mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES (pH 7.4), 12 mM glucose, 1mM $CaCl_2$, the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin, 0.7 μM chymostatin, and 80 μCi of [^{32}P] orthophosphate with or without addition of the BCKA. The labelling reaction was normally allowed to proceed for 1 h at 30 ^{o}C and stopped with 1 ml of cold stop buffer (150

mM NaF, 5 mM, EDTA, 5 mM EGTA, Tris-HCl 50 mM, pH 6.5, and the protease inhibitors described above). Cells were then washed twice by decantation with stop buffer to remove excess radioactivity.

Preparation of the high-salt Triton insoluble cytoskeletal fraction from C6 cells- After treatment, preparations of total IF were obtained from C6 cells as described by Funchal et al (2003). Briefly, after the labelling reaction, cells were homogenized in 200 µl of ice-cold high salt buffer containing 5 mM KH_2PO_4, (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1 % Triton X-100 and the protease inhibitors described above. The homogenate was centrifuged at 15800 x g for 10 min at 4 °C, in an Eppendorf centrifuge, the supernatant discarded and the pellet homogenized with the same volume of the high-salt medium. The resuspended homogenate was centrifuged as described and the supernatant was discarded. The Triton-insoluble intermediate filament-enriched pellet, containing GFAP, was dissolved in 1% SDS and protein concentration was determined by the method of Lowry et al (1951).

Total protein homogenate- C6 cells were homogenized in 200 µl of a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, 4% SDS. For electrophoresis analysis, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 3 min.

Immunoblotting Analysis- Cytoskeletal fractions or total protein homogenate were separated by 10% SDS-PAGE (40 µg/lane of total protein) and transferred (Trans-blot SD semi-dry transfer cell, BioRad) to nitrocellulose membranes for 1 hr at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5),

followed by 2 h incubation in blocking solution (TBS plus 5% defatted dry milk). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05% Tween-20 (T-TBS) and then incubated overnight at 4°C in blocking solution containing one of the following monoclonal antibodies: anti glial fibrillary acidic protein - GFAP (clone G-A-5) diluted 1:400, and anti phosphoserine (clone PSR-45) diluted 1:500 (Sigma). The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated rabbit anti mouse IgG diluted 1:2000. The blot was again washed twice for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario).

Polyacrylamide gel electrophoresis (SDS-PAGE) - The cytoskeletal fraction and the total protein homogenate were prepared as described above. Equal protein concentrations were loaded onto 10 % polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). After drying, the gels were exposed to X-ray films (X-Omat XK1) at -70 ^{o}C with intensifying screens and finally the autoradiograph was obtained. Proteins were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studied proteins.

Statistical analysis- Data were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey test when the F-test was significant. All analyses were performed using the SPSS software program on an IBM-PC compatible computer.

RESULTS

In our control conditions C6 glioma cells were roundedl and flat, with abundant cytoplasm containing an evident GFAP filaments array, as visualized by immunocytochemistry using monoclonal anti GFAP antibody. To assess the effect of the BCKA on the reorganization of GFAP filaments and cell morphology, C6 cells were treated with 1, 5 or 10 mM KIC, KIV or KMV for 3 hours and cells were analyzed by immunocytochemistry. After 3 h treatment with 1 or 5 mM KIC, KIV or KMV most of the cells presented retracted cytoplasm with bipolar processes containing packed GFAP filaments. We also observed that this morphological alteration was somewhat dependent on the concentration of the BCKA used, since in the presence of 10 mM KIC, KIV or KMV cells continued to present retracted cytoplasm with packed GFAP filaments, however bipolar processes were shortened (Figure 1). Western blot analysis of total cell homogenate by anti GFAP monoclonal antibody revealed that exposure to 1 mM of each BCKA for 3 h was not able to alter GFAP immunocontent (Figure 2A). Conversely, the immunocontent of GFAP recovered into the high-salt Triton-insoluble cytoskeletal fraction was significantly increased in the same experimental conditions, suggesting that exposure to 1 mM KIC, KIV or KMV was able to interfere with the dynamic polimerization of these IF proteins (Figure 2B). It is largely described in the literature that phosphorylation regulates the assembly/disassembly ability of IF proteins (Inada et al, 2000). Thus we verified whether exposure of C6 cells to 1 mM BCKA for 3 h could alter the in vitro incorporation of ^{32}P into GFAP protein. Results showed that BCKA treatment significantly increased the in vitro phosphorylation of this protein as compared to controls (Figure 3). Otherwise, it has become increasingly evident that site-specific phosphorylation of IF proteins alters filament

structure (Inada et al, 2000). Western blot using monoclonal anti phosphoserine antibody showed that total homogenates of cells treated with 1 mM of each BCKA for 3 h presented an increased immunocontent of phosphoserine-containing amino acids in several proteins at a molecular range from 50 to 200 kDa, including a 55 kDa protein corresponding to GFAP (Figure 4A). In addition, the immunocontent of phosphoserine was also greatly increased in GFAP obtained in the high-salt Triton insoluble cytoskeletal fraction, corresponding to the polymerized IF proteins present in the cell (Funchal et al, 2003) (Figure 4 B).

DISCUSSION

We have previously reported that KIC and KMV were able to increase the in vitro phosphorylation of IF proteins in slices of cerebral cortex of rats and the activity of serine/threonine kinases such as cyclicAMP- and Ca^{++}/calmodulin-dependent protein kinases (Branco et al, 2000, Funchal et al, 2004b). In addition KIC, KIV and KMV altered the morphology of cultured astrocytes by interferring with cytoskeletal organization (Funchal et al, 2004a). However, at the present we have no evidence regarding the consequences of altered phosphorylation of IF proteins and reorganization of IF cytoskeleton in neural cells exposed to these BCKA. Because aberrant cytoskeletal phosphorylation/dephosphorylation may have serious consequences for cellular function and structure, and since these mechanisms may be responsible for the neuronal damage in various neurodegenerative pathologies, in the present study we investigated whether the alteration of IF phosphorylation induced by these BCKA is related to IF reorganization in cultured neural cells. To address this issue, we used the astrocyte-derived C6 glioma cells due to their high amount of GFAP expression (Toda et al, 1994) and compeling evidences

pointing this cell line as a useful model to study in vitro cytoskeletal alterations (Ryken et al 1987).

We demonstrated that KIC, KIV and KMV at concentrations found in blood and other tissues of patients with MSUD (1, 5 or 10 mM), were able to induce an increased serine phosphorylation level, as well as an increased GFAP immunocontent in the Triton insoluble cytoskeletal fraction, and that these alterations were acompanied by GFAP reorganization, probably contributing to the altered cell morphology observed in C6 glioma cells.

Immunocytochemical staining with anti GFAP antibody showed a rearrangement of GFAP filaments in C6 glioma cells after 3 h exposure to 1, 5 or 10 mM of each of the BCKA used (KIC, KIV or KMV). It is conceivable that this GFAP reorganization was responsible for the altered cell morphology observed, turning from polygonal with abundant cytoplasm towards compacted cytoplasm with bipolar process-bearing cells. The requirement of GFAP for the cytoskeletal reorganization as observed in the present study, is supported by the evidence that the expression of GFAP has dramatic effects on cell morphology in C6 cells (Toda et al, 1994). Moreover, the involvement of GFAP in the formation of astrocytic processes was demonstrated by using antisense inhibition of GFAP expression (Weinstein et al, 1991).

Results of GFAP immunocontent in C6 cell extract revealed that the reorganization of GFAP filaments observed after 3 h exposure to the BCKA was not due to an effect on GFAP expression. Then we investigated the effect of BCKA on the polymerization/depolymerization equilibrium of GFAP subunits, based on the increasing evidence that the structural organization of the IF network is regulated spatially and temporally by phosphorylation (Inagaki et al, 1996). Taking into account these evidences

and since the high-salt Triton insoluble cytoskeletal fraction is enriched in polymerized or aggregated forms of IF proteins (Funchal et al, 2003), we tested the effect of BCKA treatment on GFAP immunocontent and on in vitro phosphorylation in the high-salt Triton insoluble cytoskeletal fraction. Results indicated that BCKA treatment lead to an accumulation of the high-salt Triton insoluble GFAP, probably because of an alteration on the polymerization/depolymerization equilibrium of this IF protein towards polymerized forms. In addition, assays of in vitro ^{32}P incorporation showed an increased 50% in vitro phosphorylation of GFAP proteins recovered in the cytoskeletal fraction. Taken together, these results suggest that after 1 mM KIC, KIV or KMV exposure the phosphorylated form of GFAP was either in a filamentous organization or in irregular aggregates. These results are in line with previously reported effects of KMV leading to an increased immunoreactivity of phosphorylated high molecular weight neurofilament subunit (NF-H) in slices of cerebral cortex of rats (Pessoa-Pureur et al, 2002).

Moreover, extracts of C6 glioma cells exposed to BCKA presented increased immunoreactivity to antiphosphoserine antibody in polypeptides ranging from 30 to 200 kDa, indicating that the increased phosphorylation levels observed after exposure of C6 cells to 1 mM BCKA could be ascribed to activated endogenous serine/threonine kinases. Interestingly this phosphoserine immunoreactivity was also increased in GFAP recovered in the cytoskeletal fraction, suggesting that BCKA treatment of C6 cells induced an increased polymerization of GFAP phosphorylated in serine residues. These findings may be correlated with our previous evidences using protein kinase inhibitors indicating that KIC increased the in vitro phosphorylation of neurofilament subunits, vimentin and GFAP recovered in the high-salt Triton insoluble cytoskeletal fraction in slices of cerebral cortex of rats by the serine/threonine Ca^{2+}/calmodulin- and cAMP-dependent protein kinases

(Branco et al, 2000). On the other hand it has been described that when amino acid residues in the N-terminal region are phosphorylated, GFAP filaments disassemble into a soluble form (Inagaki et al, 1990).

The phosphorylation sites of second messenger-dependent protein kinases have been identified in the N-terminal region of GFAP (Inagaki et al, 1994) and are frequently related to disassembly rather than assembly of the IF proteins (Inagaki et al, 1990; Inagaki et al, 1994). Then, an interesting question to be considered is how the BCKA-induced serine phosphorylation of GFAP affect the dynamic equilibrium of polimerization of this protein. In this context, our findings are supported by the observations that receptor agonists that increase cyclic AMP levels in cultured astroglia have been shown to increase ^{32}P-labeling of the IF proteins GFAP and vimentin in these cells, inducing a morhplogical change from polygonal to process-bearing cells whithin 20-30 min of drug exposure (Pollenz and McCarthy, 1986). In addition, microinjection of purified catalytic subunit of the cAMP-dependent protein kinase into living rat embryo fibroblasts led to dramatic changes in viment organization, involving the collapse of the filaments into tight bundles. Metabolic pulse labeling of injected cells revealed that accompanying these changes in IF organization was a dramatic increase in the cAMP-dependent phosphorylation which appeared maximal when the IF were fully rearranged (Lamb et al, 1989). However, further experiments will be necessary to verify whether the cAMP-dependent protein kinase activity is related to GFAP reorganization in our experimental model.

The exact consequences of the effects of KIC, KIV or KMV on the in vitro phosphorylation and reorganization of GFAP are still obscure. However, hyperphosphorylated cytoskeletal proteins have been related with cytoskeletal reorganization and neurotoxicity in other systems, such as the formation of paired helical

filaments (PHF)/ neurofibrillary tangles by hyperphosphorylated tau in Alzheimer disease and related tauopathies (Iqbal et al, 2002). On the other hand, deregulation of the phosphorylating system related to key proteins closely linked to cell structure and function will ultimately give rise to brain damage.

MSUD is an inherited neurodegenerative metabolic disorder characterized by severe impairment of CNS function (Chuang and Shih, 2001). Although some neurochemical effects of the metabolites appearing in high concentrations in MSUD have been reported (Land et al, 1976; Tashian, 1961), the mechanisms of neurotoxicity of the disorder are so far not well understood. Our present findings show that KIC, KIV and KMV alter the phosphorylating level leading to reorganization of an important brain protein related to cellular structure and function. It is difficult to extrapolate our results to the human condition and to correlate the alterations of the phosphorylating system provoked by the BCKA in the brain to the cerebral injury of MSUD children. However, considering the great body of evidence in the literature showing that alterations of cytoskeletal proteins may lead to disorganization of cellular structure, it is tempting to speculate that this may be at least one of the factors associated with the neurodegeneration and cerebral atrophy characteristic of MSUD patients.

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Figure 1. Immunostaining of C6 glioma cells exposed to branched-chain keto acids (BCKA). Cells were cultured to confluence in DMEM + 5% fetal calf serum (FCS). The medium was then changed to DMEM + 0% FCS in the presence or absence of the BCKA (1, 5, or 10 mM) for 3 hours, after which they were fixed and immunostained with anti-GFAP as described in Material and Methods. Original images were adjusted by increasing contrast . Scale bar = 50 μM.

Figure 2 .Immunoblotting of GFAP from C6 glioma cells treated with KIC, KMV or KIV. C6 glioma cells were treated for 3 hours with 1 mM KIC, KMV or KIV and the immunocontent of GFAP in cell homogente (A) or in the high-salt Triton-insoluble cytoskeletal fraction (B) was measured. All lanes received equivalent amount (40 μg) of total protein from cell extract or high-salt Triton-insoluble fraction. Replicas were reacted with anti GFAP antibody diluted 1:400. The blots were developed using an ECL kit. Data are reported as means ± S.E.M. of three diferent experiments expressed as percentage of controls. Statistically significant differences from controls as determined by ANOVA followed by Tukey test are indicated: *P<0,01. Inserts indicate representatives immunological reactions.

Figure 3. Effect of KIC, KMV or KIV on the in vitro phosphorylation of GFAP in C6 glioma cells. Cell cultures were preincubated with 1 mM KIC, KMV or KIV for 3 hours and incubated with 1 mM KIC, KMV or KIV in the presence of ^{32}P orthophosphate for 1 hour. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into GFAP was measured as described in Material and Methods.

Data are reported as means ±S.E.M. of three diferent experiments expressed as percentage of controls. Statistically significant differences from controls as determined by ANOVA followed by Tukey test are indicated: *P <0.01. Inserts indicate representatives immunological reactions.

Figure 4. Immunoblotting of GFAP and phosphoserine from C6 glioma cells treated with KIC, KMV or KIV. C6 glioma cells were treated for 3 hours with 1 mM KIC, KMV or KIV and the immunocontent of GFAP and phosphoserine in cell homogente (A) or in the high- salt Triton-insoluble cytoskeletal fraction (B) were measured. The arrow indicates the molecular weight corresponding to GFAP. All lanes received equivalent amount (40 µg) of total protein from cell extract or high-salt Triton-insoluble fraction. Replicas were reacted with anti GFAP antibody diluted 1:400 or anti phosphoserine diluted 1:500. The blots were developed using an ECL kit. Data are reported as means ± S.E.M. of three diferent experiments expressed as percentage of controls. Statistically significant differences from controls as determined by ANOVA followed by Tukey test are indicated: *P<0.01. Inserts represent representatives immunological reactions.

Figure 1

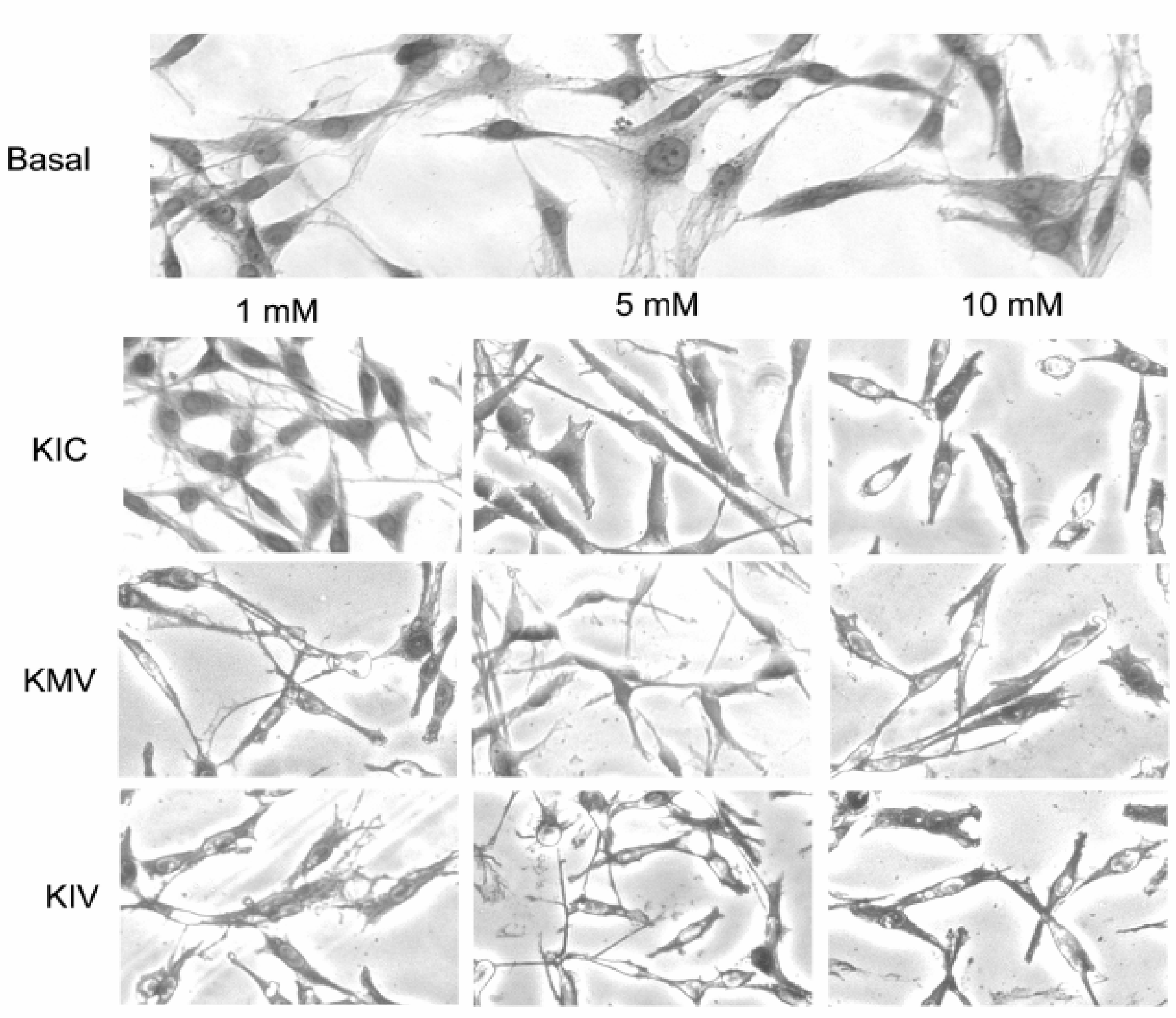


Figure 2

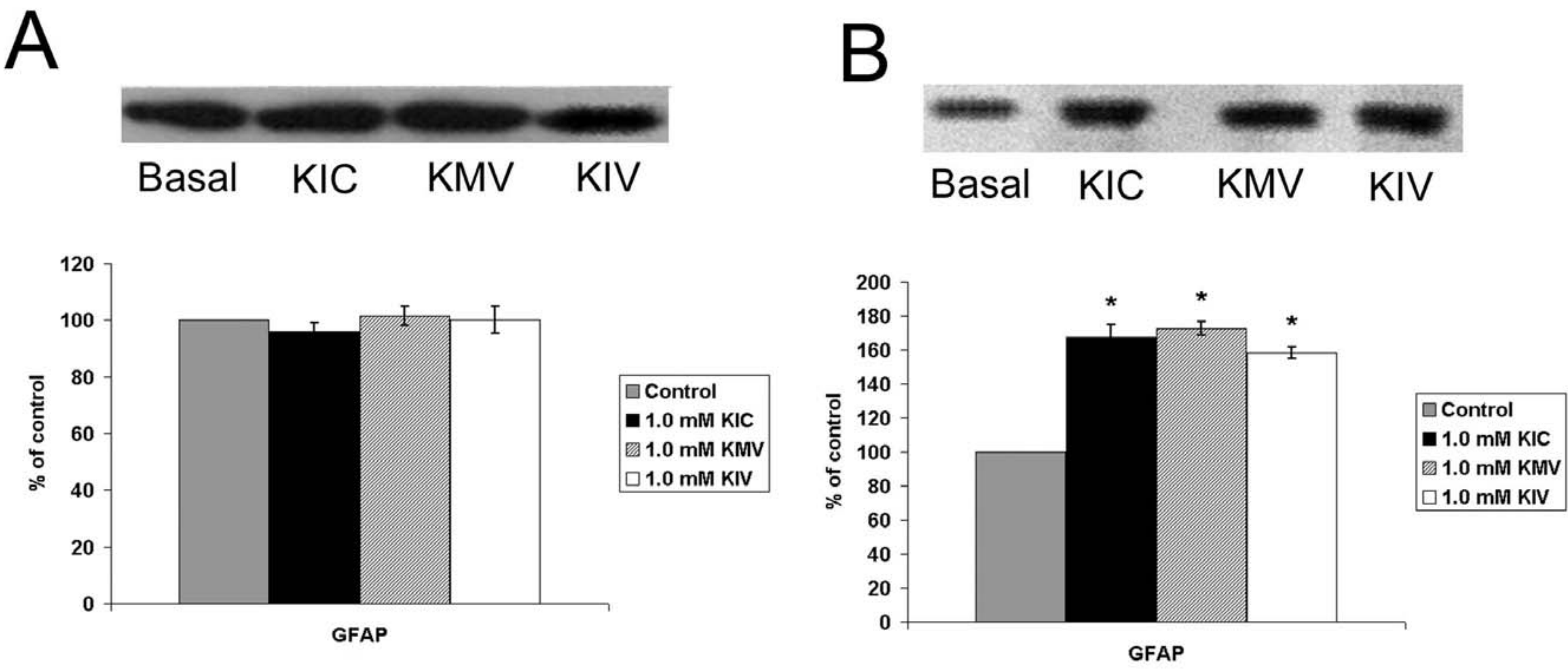


Figure 3

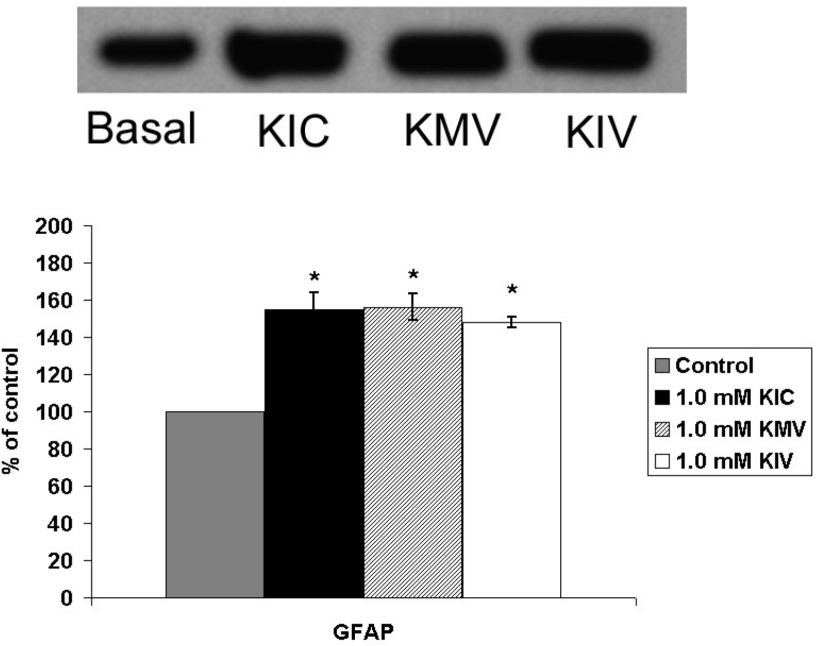
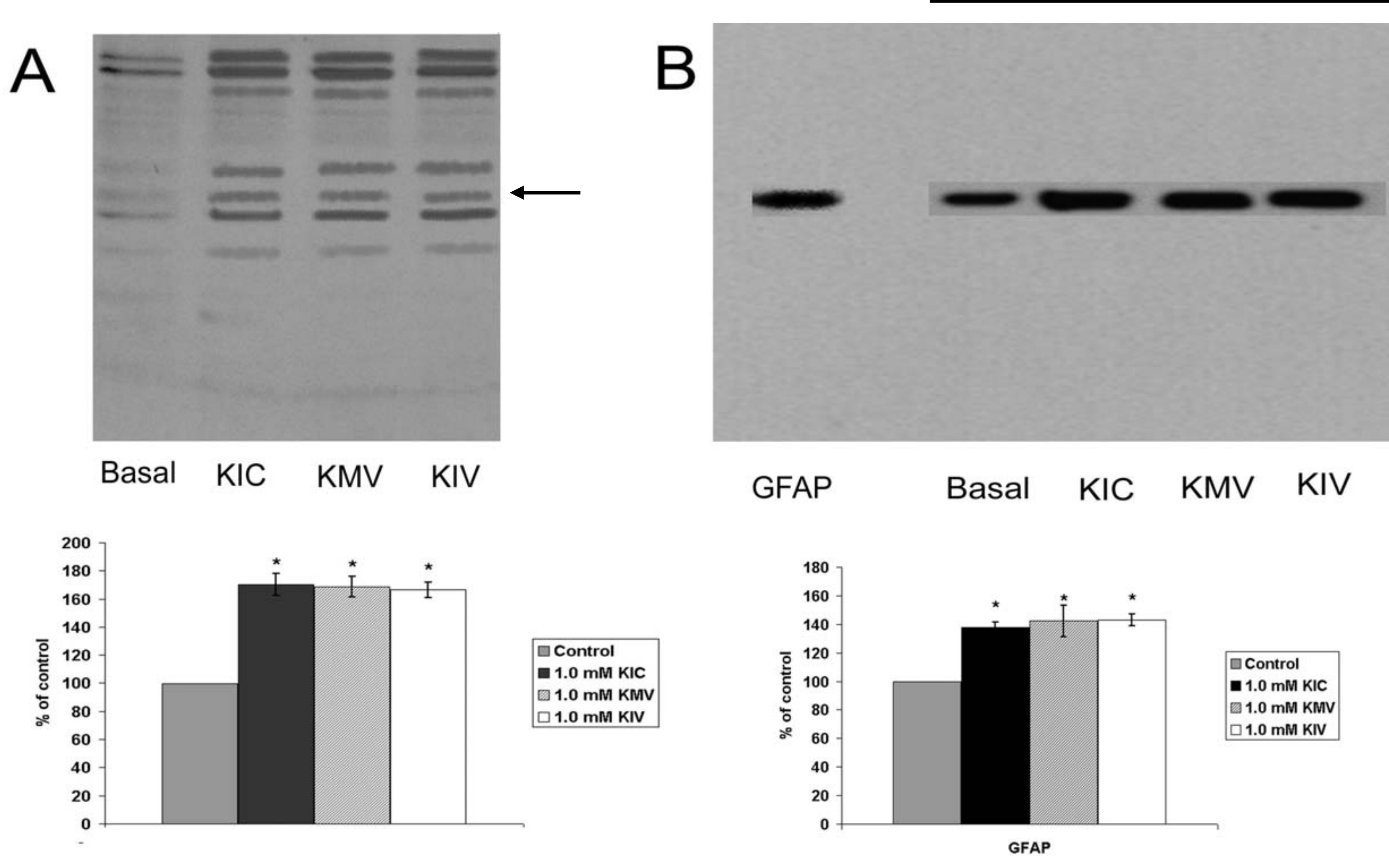


Figure 4



VII- DISCUSSÃO

Os mecanismos responsáveis pela disfunção neurológica dos pacientes portadores de DXB são complexos e pouco conhecidos, entretanto parece que muitos fatores agem em conjunto envolvendo várias alterações bioquímicas que poderiam ser responsáveis pela neuropatologia dessa desordem. Nos últimos anos muitos mecanismos de dano cerebral em EIM foram descritos, tais como estresse oxidativo (Bridi et al, 2003; Latini et al, 2003, Wajner et al, 2004), falência energética (Sgaravati et al, 2003; Schuck et al, 2004) e excitotoxicidade (Brusque et al, 2001; Kolker et al, 2002).

Considerando que é bastante descrita na literatura a relação entre alterações no citoesqueleto de células neurais e mecanismos de neurodegeneração (Julien e Mushinski, 1998; Fitzpatrick et al, 1998; Lariviere e Julien, 2004) e considerando também a escassez de dados referentes aos efeitos dos metabólitos que se acumulam na DXB sobre o citoesqueleto de células neurais, neste trabalho utilizamos um modelo experimental de DXB com o objetivo de verificar os efeitos dos CACR que se acumulam nessa desordem neurodegenerativa sobre o citoesqueleto de células neurais de ratos.

Inicialmente demonstramos que os CACR que se acumulam na DXB, CIC, CMV e CIV, inibiram a captação de glutamato em fatias de córtex cerebral de ratos durante o desenvolvimento, provavelmente fazendo com que se acumule glutamato na fenda sináptica. É descrito que o glutamato exerce um importante papel como sinalizador extracelular, agindo em receptores presentes nas membranas dos terminais neurais de uma forma dependente de sua concentração no espaço extracelular (Ozawa et al, 1998). Sabe-se também que quando a concentração de glutamato na fenda sináptica é alta ocorre estimulação excessiva dos receptores glutamatérgicos, o que pode causar excitotoxicidade, levando subseqüentemente ao dano neural (Danboldt, 2001). A excitotoxicidade tem sido descrita em várias patologias neurodegenerativas (Greenamyre e Young, 1989; Gilad et al, 1990; Rothestein et al, 1992; Lipton e Rosemberg, 1994; Price, 1999; Maragaskis e Rothstein, 2001). O fato dos CACR diminuírem a captação de

glutamato em nosso modelo experimental sugere que esses metabólitos possam agir aumentando a excitotoxicidade glutamatérgica.

A ligação dos neurotransmissores aos seus receptores específicos causa uma alteração na concentração de vários segundos mensageiros intracelulares, tais como Ca^{2+} e AMPc, promovendo a ativação de várias enzimas, entre elas proteínas quinases e fosfatases, que levam a uma resposta celular ao sinal externo (Cooper, 2001). O aumento destes segundos mensageiros é capaz de ativar proteínas quinases e fosfatases associadas FI, alterando o nível de fosforilação e consequentemente o equilíbrio dinâmico das estruturas do citoesqueleto (Walaas e Greengard, 1991; Rodnight e Wofchuk, 1992; Nestler e Greengard, 1999).

Nossos dados mostraram que o CIC alterou a fosforilação das proteínas estudadas e que este efeito foi mediado por receptores glutamatérgicos ionotrópicos de maneira dependente do desenvolvimento. Em animais de 9 dias de idade o CIC diminuiu a fosforilação dos FI, enquanto que em animais de 21 dias este metabólito causou um aumento na fosforilação dessas proteínas. Além disso, observamos que em animais de 9 dias de idade o efeito do CIC foi mediado pelas proteínas fosfatases PP2A e PP2B, enquanto que em animais de 21 dias de idade o efeito deste metabólito foi mediado pelas proteínas quinases PKA e PKCaMII. Verificamos também que o CIC promoveu um aumento dos níveis de importantes segundos mensageiros celulares, como o AMPc e o Ca^{2+}. O aumento do Ca^{2+} intracelular provocado pelo CIC foi demonstrado pelo uso de bloqueadores específicos de canais de cálcio dependentes de voltagem tipo L (nifedipina), canais de cálcio dependentes de ligantes (NMDA) e de quelantes de cálcio intracelular (BAPTA-AM).

Diferentemente do CIC, o CMV aumentou a fosforilação dos FI somente em ratos de 12 dias de idade, sendo esse efeito mediado por receptores GABAérgicos do tipo A e B, desencadeando a ativação das proteínas quinases PKA e PKCaMII. É importante salientar que o CIV não alterou a atividade do sistema fosforilante em nenhuma das idades estudadas. O conjunto destes resultados mostra que os diferentes metabólitos que se acumulam na DXB são capazes de atuar

diferentemente sobre o citoesqueleto de fatias de córtex cerebral de ratos, o que evidencia a complexidade dos mecanismos de ação dos mesmos sobre o SNC.

Além dos efeitos causados pelos CACR que se acumulam na DXB sobre a atividade do sistema fosforilante associado aos FI em fatias de córtex cerebral de ratos, demonstramos que esses metabólitos foram capazes de alterar a fosforilação da GFAP na linhagem de glioma C6. O aumento de fosforilação da GFAP causou uma reorganização do citoesqueleto das células C6 e aumentou o imunoconteúdo da GFAP na fração citoesquelética, significando uma provável modificação do equilíbrio de polimerização dessa proteína, favorecendo a forma insolúvel, constituída de estruturas filamentosas ou agregados de proteínas. Também observamos nas culturas de células C6 uma relação entre o nível de fosforilação da GFAP e a reorganização do citoesqueleto. Esses dados confirmam evidências da literatura enfatizando a importância da fosforilação de proteínas de FI como mecanismo regulatório da organização do citoesqueleto e conseqüentemente de suas funções (Pollenz e McCarty, 1986; Lamb et al, 1989).

Alterações nos níveis de fosforilação de proteínas do citoesqueleto têm sido descritas em várias doenças neurodegenerativas como a Doença de Alzheimer (Schmidt et al, 1996) e a Doença de Parkinson (Laviere e Julien, 2004). Com base nos nossos resultados, podemos sugerir que a neurotoxicidade dos CACR, possa estar relacionada, entre outros fatores, com as alterações do citoesqueleto evidenciadas neste modelo experimental. No entanto, mais experimentos serão necessários para esclarecer esta possibilidade.

É descrito na literatura que respostas celulares a sinais externos freqüentemente envolvem mudanças no movimento e na forma celular, aspectos de comportamento celular que são controlados principalmente pelo citoesqueleto de actina (Cooper et al, 2001). No presente trabalho demonstramos que o CIC, o CMV e o CIV, em concentrações encontradas em pacientes portadores de DXB, levaram a uma reorganização dos filamentos de GFAP e uma desorganização das fibras de estresse. Além disso, observamos uma morte celular progressiva quando os astrócitos foram expostos por várias horas aos metabólitos, reforçando a neurotoxicidade dos cetoácidos nas concentrações utilizadas.

Verificamos também que os efeitos do CIC, CIV e CMV sobre a morfologia dos astrócitos foram desencadeados por mecanismos de membrana que diminuíram a atividade da RhoA, uma GTPase monomérica. Esse mecanismo foi evidenciado utilizando-se LPA, um ativador específico da RhoA, o qual preveniu a desorganização das fibras de estresse e a reorganização dos filamentos de GFAP, mantendo a morfologia normal das, indicando que a via de sinalização da RhoA provavelmente estaria envolvida nos efeitos causados pelos metabólitos. É descrito que membros da subfamília Rho de pequenas GTPases desempenham papéis centrais regulando a estabilidade do citoesqueleto de actina (Hall, 1998). Um importante exemplo da via de sinalização da RhoA é a promoção da interação dos filamentos de actina e miosina, resultando na formação de fibras de estresse e adesões focais, importantes processos de adesão e locomoção celular (Ridley e Hall, 1994; Nobles e Hall, 1995). Esse processo é dependente de energia e da disponibilidade de ATP (Schmidt e Hall, 1998).

Outro aspecto a ser considerado com respeito aos efeitos dos CACR sobre o citoesqueleto das células neurais em nosso modelo experimental é o fato de que os CACR são descritos como sendo inibidores metabólicos, causando uma diminuição nos mecanismos de obtenção de energia, levando à falência energética da célula (Howell e Lee, 1963; Halestrap et al, 1974; Sgaravati et al, 2003). Esta falência energética poderia causar uma diminuição nos níveis intracelulares de ATP, causando uma disfunção dos transportadores dos neurotransmissores e conseqüentemente um colapso do gradiente de sódio e potássio (Lipton e Rosemberg, 1994).

É possível que o efeito desses metabólitos sobre a reorganização do citoesqueleto de actina e GFAP em astrócitos em cultura possa ser conseqüência do déficit energético causado por eles. Esta possibilidade foi confirmada ao observarmos que as alterações morfológicas e a morte celular induzidas pelos CACR em culturas de astrócitos foram totalmente evitadas com a suplementação de creatina às culturas, indicando que a homeostase energética provavelmente estaria envolvida nos efeitos causados pelos CACR. Além disso, para verificar o comprometimento do metabolismo energético das células por estes metabólitos

investigamos o efeito dos CACR sobre a atividade da creatina quinase (CQ). A CQ é uma enzima crucial para o metabolismo energético, especialmente em tecidos de grande consumo energético, como o cérebro (Wallimann et al, 1992). A atividade da CQ está relacionada com a fosforilação oxidativa sendo, portanto, dependente de níveis altos de fosfato (Saks et al, 1985). Demonstramos que os CACR inibiram a atividade da CQ e que a suplementação de creatina às culturas de astrócitos preveniu esta inibição, sugerindo que o efeito destes metabólitos em cultura de astrócitos provavelmente se deva a uma redução de energia da célula.

Também observamos que o LPA não foi capaz de prevenir o efeito inibitório causado pelo CIV sobre a atividade da CQ, indicando que o LPA provavelmente previne as alterações morfológicas induzidas pelos CACR por outros mecanismos que deverão ser esclarecidos futuramente. No momento não podemos estabelecer uma relação direta entre o déficit energético causado pelos CACR e a via de sinalização mediada pela RhoA, embora nossos resultados sugiram que esses dois mecanismos estejam envolvidos na ação excitotóxica desses metabólitos, evidenciando mais uma vez a complexidade dos mecanismos de ação dos CACR.

Resumindo, os dados obtidos neste trabalho nos levaram a propor uma hipótese, representada na figura 9 para explicar o conjunto de alterações observadas. Os CACR diminuem a captação de glutamato, provavelmente fazendo com que este neurotransmissor se acumule na fenda sináptica. O acúmulo de neurotransmissores na fenda sináptica faria com que ocorresse a abertura de canais iônicos e o influxo de íons para o meio intracelular, os quais aumentariam os níveis de segundos mensageiros (AMPc e Ca^{2+}) e estimulariam cascatas de sinalização intracelular as quais ativariam proteínas quinases e fosfatases fosforilando/desfosforilando as proteínas do citoesqueleto. A modificação no estado de fosforilação destas proteínas poderia levar a uma reorganização do citoesqueleto fazendo com que as células alterassem a sua morfologia.

Considerando que as células neurais são de fundamental importância para o desenvolvimento e funcionamento do cérebro, é provável que a diminuição na captação de glutamato, as modificações nos níveis de segundos mensageiros, no

estado de fosforilação de proteínas do citoesqueleto evidenciadas principalmente em fatias de córtex cerebral e as alterações morfológicas das células em cultura causadas pelos CACR em nosso modelo experimental possam contribuir para a melhor compreensão das disfunções neurológicas características dos pacientes portadores de DXB.

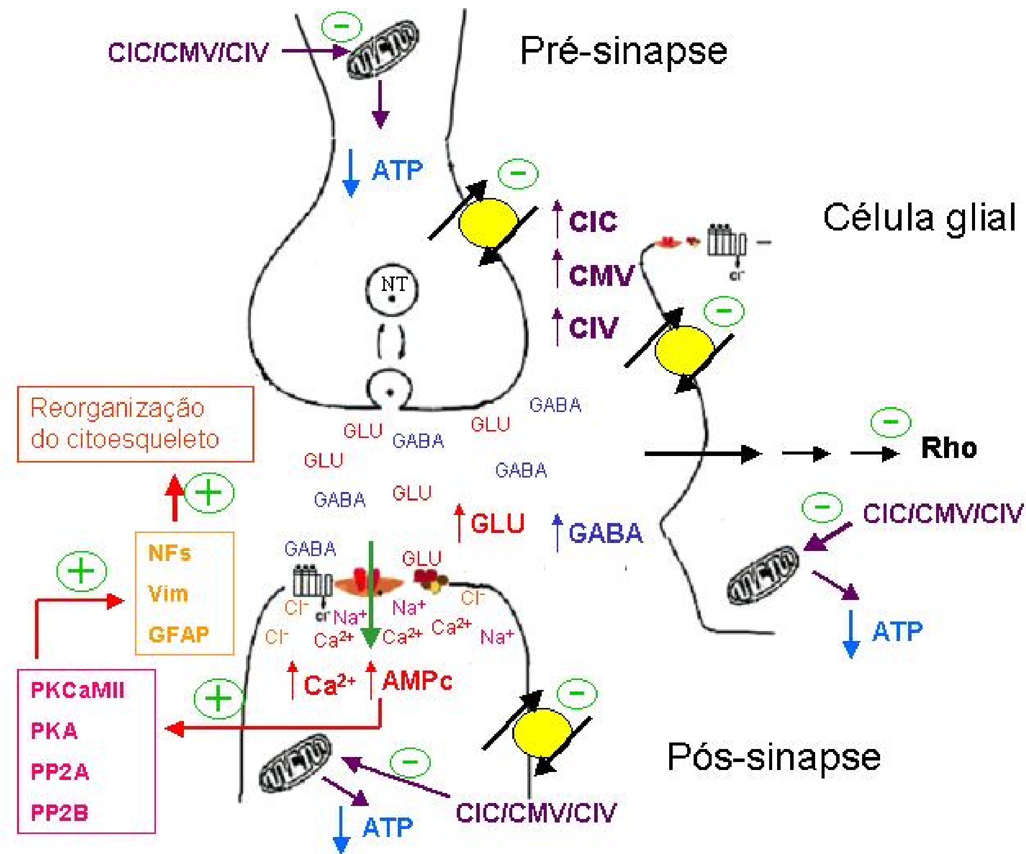


Figura 9. Hipótese do mecanismo de ação através do qual os α-cetoácidos de cadeia ramificada alteram as proteínas do citoesqueleto.

= transportador de neurotransmissor

= receptor glutamatérgico inotrópico

= receptor glutamatérgico metabotrópico

= receptor GABAérgico

IX - CONCLUSÕES

CONCLUSÃO GERAL

Os α-cetoácidos de cadeia ramificada, α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico, que se acumulam na Doença do Xarope do Bordo alteram a captação de glutamato e a fosforilação de proteínas do citoesqueleto através de mecanismos de sinalização celular em fatias de córtex cerebral de ratos. Além disso, esses metabólitos alteram a organização do citoesqueleto de astrócitos e células C6 em cultura.

CONCLUSÕES ESPECÍFICAS

1. O efeito do ácido α-cetoisocapróico sobre a incorporação *in vitro* de ^{32}P em proteínas de filamento intermediário - NF-M, NF-L, vimentina e GFAP – em fatias de córtex cerebral de ratos de 9 dias de idade é mediado pelas proteínas fosfatases PP2A e PP2B, enquanto o efeito deste metabólito sobre a fosforilação *in vitro* das mesmas proteínas é mediado pelas proteínas quinases PKA e PKCaMII em animais de 21 dias de idade.

2. Os efeitos do ácido α-cetoisocapróico sobre a incorporação *in vitro* de ^{32}P em proteínas de filamento intermediário - NF-M, NF-L, vimentina e GFAP – em fatias de córtex cerebral de ratos de 9 e 21 dias de idade são dependentes de cálcio, como demonstrado pelo uso de bloqueadores específicos de canais de cálcio dependentes de voltagem (tipo L), canais de cálcio dependentes de ligantes (NMDA) e de quelantes de cálcio intracelular (BAPTA-AM).

3. O ácido α-cetoisovalérico não altera a fosforilção *in vitro* de filamentos intermediários em fatias de córtex cerebral de ratos durante o desenvolvimento. No entanto, o ácido α-ceto-β-metilvalérico aumenta a incorporação *in vitro* de ^{32}P

nas subunidades NF-M, NF-L, vimentina e GFAP em fatias de córtex cerebral somente em ratos de 12 dias de idade.

4. O efeito do ácido α-ceto-β-metilvalérico sobre a incorporação *in vitro* de ^{32}P em proteínas de filamento intermediário - NF-M, NF-L, vimentina e GFAP – em fatias de córtex cerebral de ratos de 12 dias de vida é mediado por receptores GABAérgicos do tipo A e B, desencadeando a ativação das proteínas quinases PKA e PKCaMII.

5. O ácido α-cetoisocapróico inibiu a captação de glutamato em fatias de córtex cerebral de ratos de 9, 21 e 60 dias de idade. Os ácidos α-ceto-β-metilvalérico e α-cetoisovalérico inibiram a captação de glutamato em fatias de córtex cerebral de ratos de 21 e 60 dias.

6. Os ácidos α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico induzem a reorganização do citoesqueleto, alterando a morfologia de astrócitos em cultura após 6h de incubação. Além disso, quando os astrócitos foram expostos por várias horas aos metabólitos observou-se um aumento progressivo da morte celular.

7. Os efeitos dos ácidos α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico sobre a reorganização do citoesqueleto de astrócitos em cultura são dependentes da proteína Rho monomérica. Além disso, a reorganização do citoesqueleto também é causada pelo déficit energético provocado pelos metabólitos.

8. Os ácidos α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico aumentam a fosforilação *in vitro* da proteína glial fibrilar ácida, bem como o imunoconteúdo da proteína glial fibrilar ácida na fração citoesquelética e induzem a reorganização desses filamentos em células C6.

X – PERSPECTIVAS

Este trabalho abre inúmeras oportunidades de novos estudos no modelo experimental da Doença do Xarope do Bordo. As principais metas a serem seguidas são:

1- Estudar os efeitos dos α-cetoácidos de cadeia ramificada α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico sobre a morfologia de neurônios em cultura.

2- Estudar os efeitos dos α-cetoácidos de cadeia ramificada α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico sobre a interação neurônio-glia em co-cultura.

3- Estudar as vias de transdução de sinal envolvidas nos efeitos destes α-cetoácidos de cadeia ramificada em culturas de neurônios e astrócitos.

4- Utilizar neuroprotetores (antioxidantes, antagonistas glutamatérgicos, etc) para prevenir os efeitos causados pelos α-cetoácidos de cadeia ramificada em culturas de neurônios e astrócitos.

5- Dosar S-100 B em culturas de astrócitos tratadas com os α-cetoácidos de cadeia ramificada.

XI- OUTROS TRABALHOS REALIZADOS DURANTE O DOUTORADO

1- In vitro phosphorylation of cytoskeletal proteins from cerebral cortex of rats. Funchal, C., Vieira de Almeida, L.M., Oliveira Loureiro, S., Vivian, L.; de Lima Pelaez, P., Dall Bello Pessutto, F., Rosa, A.M., Wajner, M., Pessoa-Pureur, R.
   Artigo publicado no periódico Brain Research Protocols 11: 111-118, 2003.

2- Effect of propionic and methylmalonic acids on the in vitro phosphorylation of intermediate filaments from cerebral cortex of rats during development. Vieira de Almeida, L.M., Funchal, C., de Lima Pelaez, P., Dall Bello Pessutto, F., Oliveira Loureiro, S., Vivian, L.; Wajner, M., Pessoa-Pureur, R. Artigo publicado no periódico Metabolic Brain Disease 18: 207-219, 2003.

3- Selenium compounds prevent the effects of methylmercury on the in vitro phosphorylation of cytoskeletal proteins in cerebral cortex of young rats. Moretto, M.B., Funchal, C., Zeni, G., Pessoa-Pureur, R, Rocha, J.B.T.
   Artigo aceito para publicação no periódico Toxicological Sciences, 2004.

4- Involvement of calcium-dependent mechanisms on the action of T_3 on the in vitro phosphorylation of vimentin on immature rat testis. Zamoner, A., Corbelini, P.F., Funchal, C., Menegaz, D., Silva, F.R.M.B., Pessoa-Pureur, R.
   Artigo submetido ao periódico Life Sciences.

5- Morphological alterations and cell death provoked by the branched-chain α-amino acids accumulating in maple syrup urine disease in astrocytes from rat cerebral cortex. Funchal, C., Gottfried, C., Vieira de Almeida, L.M., Santos, A.Q., Wajner, M., Pessoa-Pureur, R.
   Artigo submetido ao periódico Cellular and Molecular Neurobiology.

6- *In vitro* effect of 3-hidroxyglutaric acid on energy metabolism in brain of young rats. Latini, A., Rodriguez, M., Rosa, R.B., Cassina, A., Scissiato, K., Leipnitz, G., Assis, D.R., Ferreira, G.C., Funchal, C., Jacques-Silva, M.C., Buzin, L., Giuliani, R., Radi, R., Wajner, M.
   Artigo submetido ao periódico Molecular Genetics and Metabolism.

7- Organoselenium compounds prevent hyperphosphorylation of cytoskeletal proteins induced by the neurotoxic agent diphenyl ditelluride in cerebral cortex of young rats. Moretto, M.B., Funchal, C., Zeni, G., Pessoa-Pureur, R, Rocha, J.B.T.
   Artigo submetido no periódico Toxicology.

8- Branched-chain α-ketoacids accumulating in maple syrup urine disease induce morphological alterations and oxidative stress in C6 glioma cells. Funchal, C., Jacques-Silva, M.C., Latini, A., Santos, A.Q., Buzin, L., Gottfried, C., Wajner, M., Pessoa-Pureur, R.
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