UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

EFEITOS *IN VITRO* E *IN VIVO* DA PROLINA SOBRE A ATIVIDADE DA CREATINAQUINASE EM ENCÉFALO DE RATOS JOVENS

Dissertação de Mestrado

Adriana Kessler

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por

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Dissertação submetida como requisito para a obtenção do grau de MESTRE EM BIOQUÍMICA

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RESUMO

Hiperprolinemia tipo II é um erro inato do metabolismo dos aminoácidos causado pela deficiência de Δ^1 pirrolino-5-carboxilato desidrogenase, levando ao acúmulo de prolina no plasma e nos tecidos. Embora sintomas neurológicos ocorram em diversos pacientes, a neurotoxicidade da prolina ainda é controversa. O principal objetivo do presente estudo foi investigar o efeito das administrações aguda e crônica de prolina na atividade da creatinaquinase em córtex, cerebelo e encéfalo médio de ratos Wistar. No tratamento agudo, uma única injeção subcutânea de prolina foi administrada em ratos de 22 dias de vida. No tratamento crônico, a prolina foi administrada quatro vezes ao dia, do 6^o ao 21^o dia de vida. Os resultados mostraram que a atividade da creatinaquinase foi inibida significativamente nas três estruturas dos ratos submetidos à administração aguda de prolina. Por outro lado, a atividade da enzima aumentou nos animais submetidos à administração crônica. Também foi investigado o efeito *in vitro* da prolina sobre a atividade da creatinaquinase nas mesmas estruturas encefálicas em ratos de 22 dias não tratados. A prolina inibiu significativamente a atividade da creatinaquinase. Considerando a importância da creatinaquinase na manutenção da homeostase energética encefálica, é possível que a alteração da atividade dessa enzima no encéfalo possa ser um dos mecanismos pelo qual a prolina deva ser neurotóxica.

ABSTRACT

Type II Hyperprolinemia is an amino acid metabolism disorder caused by a deficiency of Δ^1-pyrroline-5-carboxilic acid dehydrogenase, whose biochemical hallmark is proline accumulation in plasma and tissues. Although neurologic symptoms occur in most patients, the neurotoxicity of proline is still controversial. The main objective of the present study was to investigate the effect of acute and chronic administration of proline on creatine kinase activity of brain cortex, cerebellum and midbrain of Wistar rats. Acute treatment was performed by subcutaneous administration of one injection of proline to 22-day-old rats. For chronic treatment, proline was administered four times a day from the 6th to the 21st postpartum day. The results showed that creatine kinase activity was significantly inhibited in the three structures of rats subjected to acute proline administration. In contrast, this activity was increased in animals subjected to chronic administration. We also measured the *in vitro* effect of proline on creatine kinase activity in the same brain structures of 22-day-old non-treated rats. Proline significantly inhibited creatine kinase activity. Considering the importance of creatine kinase for the maintenance of energy homeostasis in the brain, it is conceivable that an alteration of this enzyme activity in the brain may be one of the mechanisms by which proline might be neurotoxic.

1. INTRODUÇÃO

O termo erros inatos do metabolismo (EIM) foi proposto em 1908 por Sir Archibald Garrod, referindo-se a quatro doenças: alcaptonúria, cistinúria, pentosúria e albinismo. Esses distúrbios bioquímicos humanos são alterações genéticas que se manifestam pela diminuição ou mesmo ausência da função de uma proteína, geralmente uma enzima. As alterações resultam em bloqueio de rotas metabólicas, podendo ocorrer tanto o acúmulo de metabólitos tóxicos como a falta de produtos essenciais, acarretando em doença subseqüente (Bickel, 1987).

De acordo com Stambury et al. (1983), todos os processos bioquímicos no organismo estão sob controle gênico e estão sujeitos a serem realizados de forma deficiente sempre que uma mutação gênica alterar a função de uma proteína.

Os EIM são distúrbios monogênicos de expressão fenotípica variável. Em alguns, como na histidinemia e na hiperprolinemia, a maioria dos afetados não apresenta um fenótipo de doença; em outros, como na fenilcetonúria e nas acidemias metilmalônica e propiônica, a sintomatologia clínica é expressa de forma clara em quase todos os afetados.

Até o momento foram descritos mais de 500 erros inatos do metabolismo (Scriver et al., 2001), a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Benson & Fenson, 1985), causando um grande número de defeitos com quadros clínicos variados, podendo ser desde assintomáticos até tão graves que levam a morte neonatal. Os erros inatos do metabolismo são graves e geralmente se manifestam na infância, sendo que os sinais e os sintomas encontrados são semelhantes aos de muitas doenças infantis.

Apesar de raros quando considerados individualmente, os EIM são relativamente freqüentes em seu conjunto, podendo ocorrer aproximadamente um em cada mil recém-nascidos vivos.

As técnicas de investigação bioquímica têm colaborado para a descoberta de novos erros inatos do metabolismo, esclarecendo cada vez mais os já conhecidos.

1.1 Sinais Clínicos e Laboratoriais

Embora a sintomatologia dos EIM seja bastante variável, há alguns sinais e sintomas que aparecem com maior freqüência nesses distúrbios (Burton,1987).

As principais manifestações clínicas são: deficiência de crescimento, dificuldade alimentar, vômitos, diarréia, letargia ou coma, hipotonicidade ou hipertonicidade, convulsões, dificuldade respiratória e apnéia, icterícia,

hepatomegalia, fascies grosseira, dismorfias, odor anormal na pele e na urina, anormalidades oculares, cabelos anormais e macroglossia.

Entre os achados laboratoriais mais comuns no período neonatal estão: acidose metabólica, hipoglicemia, hiperamonemia, transaminases elevadas, substâncias redutoras na urina, teste de cloreto férrico positivo, cetonúria, neutropenia, trombocitopenia, anemia e linfócitos vacuolados em esfregaço periférico.

1.2 Tratamento

O tratamento dessas doenças será tanto mais bem sucedido quanto mais precoce for o seu diagnóstico e pode ser conduzido de diversas maneiras:

- Limitando a entrada do precursor (como na fenilcetonúria, evitando a ingestão de fenilalanina);
- Suplementando o metabólito ausente (como no hipotireoidismo, administrando a tiroxina);
- Inibindo a formação da substância acumulada (como na gota, administrando alopurinol para inibir a xantina oxidase);
- Inibindo o acúmulo de determinada substância (como na Doença de Wilson, impedindo a formação de cobre com o uso de drogas);
- Controlando fatores desencadeantes (evitando substâncias como fármacos);

- Aumentando a atividade enzimática (como na homocistinúria, aumentando as doses do cofator, a piroxina);

- Suplementando a proteína não enzimática deficiente (como na hemofilia, administrando o fator VIII);

- Suplementando a enzima deficiente.

1.3 Hiperprolinemias

As hiperprolinemias são erros inatos do metabolismo dos aminoácidos causados pela deficiência de enzimas envolvidas na rota de degradação da prolina, levando ao acúmulo deste aminoácido no plasma e nos tecidos.

1.3.1 Prolina

A L-prolina (Pro) é um aminoácido não essencial em crianças e adultos e condicionalmente essencial em prematuros. A D-prolina não ocorre naturalmente no metabolismo humano.

Os aminoácidos são conhecidos como α-aminoácidos porque possuem em sua estrutura química um grupo amino primário ($-NH_2$) e um grupo carboxílico (-COOH) ligados a um mesmo átomo de carbono (α). Segundo Lenhinger et al. (2000), eles diferem uns dos outros através de suas cadeias laterais, que variam em tamanho, estrutura e carga elétrica.

Na estrutura química da prolina existe um grupo amino secundário (-NH_2), formando uma base de Schiff (imino). (Fig. 1) Devido a esta reação bioquímica entre uma amina e um aldeído, a prolina é considerada um iminoácido (Voet & Voet,1999).

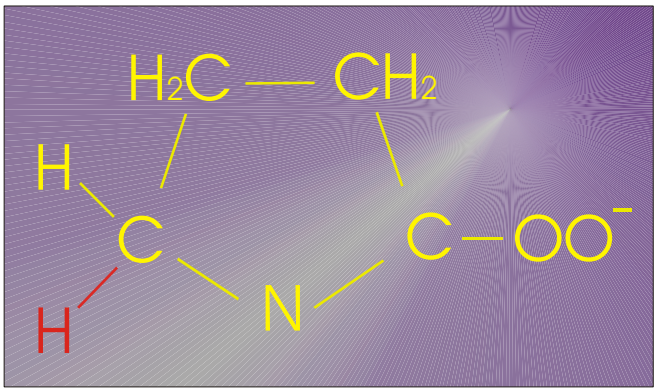

Figura 1 – *Estrutura da prolina.*

A prolina pode ser sintetizada a partir da ornitina ou do glutamato podendo também ser degradada a glutamato, tendo glutamato-γ-semialdeído e pirrolino-5-carboxilato (P5C) como intermediários comuns (Voet & Voet, 1999). O glutamato-γ-semialdeído participa tanto da biossíntese quanto da degradação da prolina, formando uma base de Schiff interna com a P5C, catalisada na biossíntese pela P5C redutase e na degradação por Δ^1 pirrolino-5-desidrogenase (Lehninger et al., 2000).

É importante salientar que a transformação de P5C em glutamato-γ-semialdeído é espontânea, reversível e diretamente relacionada com o momento funcional da célula. A regulação do metabolismo da prolina é complexa devido à presença de intermediários comuns entre o anabolismo e o catabolismo (Fig.2).

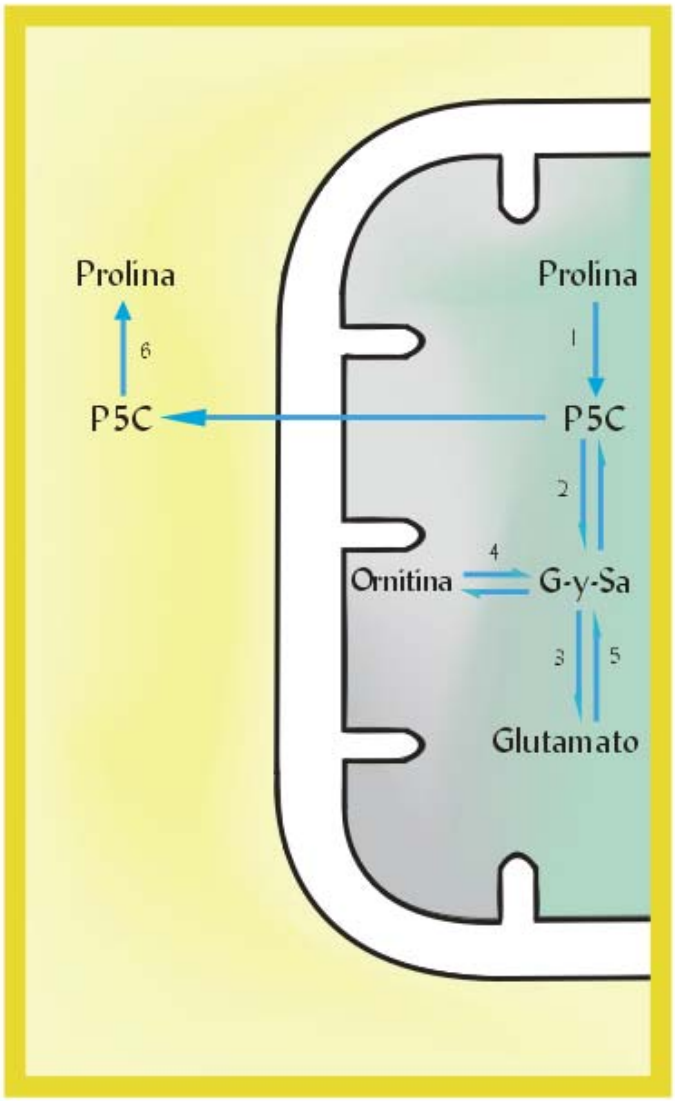

Figura 2 – *Biossíntese e degradação da prolina* (G-y-Sa: Glutamato - γ - semi-aldeído; 1: prolina oxidase; 2: reação não enzimática; 3: Δ^1 pirrolino-5-carboxilato desidrogenase; 4: ornitina-aminotransferase; 5: P5C sintase; 6: P5C redutase).

A enzima P5C sintase catalisa a produção de P5C a partir do glutamato. Esta enzima é ATP e NADPH dependente e tem alta atividade na mucosa do intestino delgado, cólon, pâncreas, timo, e cérebro. A enzima P5C redutase catalisa a

conversão de P5C em prolina tendo como cofator NADH ou NADPH e é encontrada em todos os tecidos (Phang et al., 2001).

As rotas que envolvem a interconversão entre glutamato, ornitina e prolina têm como função formar prolina para a síntese protéica, para a gliconeogênese hepática e para a síntese de ornitina (pela ação da ornitina-δ-aminotransferase) e de arginina (ciclo da uréia), tendo como intermediário a P5C.

Além de ser um intermediário no metabolismo da prolina, a P5C é um constituinte do plasma humano. Seus níveis variam ao longo do dia, apresentando picos associados às refeições.

Em adultos, a concentração normal de prolina no plasma é de 100 a 450 μM, sendo estes valores menores em crianças. No líquor, os valores variam de 1 a 4 μM. Iminoglicinúria neonatal e prolinúria pós-natal são achados freqüentes em crianças normais e ocorrem devido à imaturidade do sistema de reabsorção tubular de prolina.

1.3.2 Classificação

Segundo Selkoe (1969), as hiperprolinemias podem ser classificadas em tipo I e tipo II. Esta distinção é feita em razão de encontrarmos dois pontos distintos na rota metabólica da prolina onde ocorrem os bloqueios.

A hiperprolinemia tipo I (HPI) é comumente associada a níveis menos elevados de prolina, sendo causada pela deficiência da enzima prolina oxidase,

responsável pelo primeiro passo na via de degradação da prolina. Não existem evidências entre a deficiência desta enzima e manifestações clínicas. O diagnóstico é feito por exclusão, isto é, os casos de hiperprolinemias não devidos à deficiência da P5C desidrogenase são considerados do tipo I. Não existe demonstração direta da deficiência da prolina oxidase, visto que essa enzima não apresenta atividade em leucócitos ou em cultura de fibroblastos (Phang et al., 2001).

A hiperprolinemia tipo II (HPII) é causada pela deficiência da enzima responsável pelo segundo passo da rota de degradação de prolina, P5C desidrogenase, que catalisa a transformação de P5C em glutamato. Embora esta desordem seja considerada benigna, há uma associação causal com manifestações neurológicas na infância (Phang et al., 2001).

Visto que alguns pacientes possuem disfunções neurológicas e que poucos são os trabalhos que estudam os prováveis mecanismos destas disfunções, o presente estudo focou sua investigação na HPII.

1.3.3 Hiperprolinemia tipo II

1.3.3.1 Conceito

A hiperprolinemia tipo II (HPII) é um erro inato do metabolismo da prolina, de característica autossômica recessiva, onde ocorre ausência ou deficiência na

atividade da enzima P5C desidrogenase (Fig. 3), levando a um acúmulo de prolina no plasma e nos tecidos. (Phang et al., 2001).

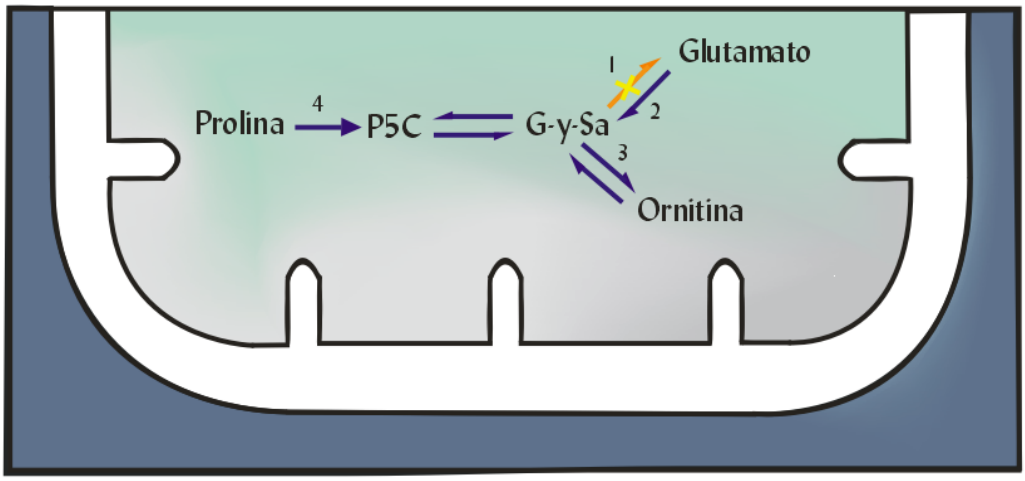

Figura 3 – *Local do bloqueio na rota da prolina responsável pela hiperprolinemia tipo II.* (G-y-Sa: Glutamato - γ - semi-aldeído; 1: P5C desidrogenase; 2: P5C sintase; 3: ornitina aminotransferase; 4: prolina oxidase).

1.3.3.2 Diagnóstico

Segundo Phang et al.(2001), o diagnóstico da doença é baseado em níveis séricos aumentados de prolina, em torno de 2000 μM (10 a 15 vezes superiores aos valores normais: 100 a 450 μM) e em níveis aumentados de P5C plasmático e urinário (10 a 40 vezes superiores aos valores normais).

Os níveis de prolina e glutamato estão elevados no líquor, provavelmente devido à competição entre prolina e glutamato (Van Herreveld & Fifkova, 1973; Rohads et al., 1983; Phang et al., 2001), podendo haver presença de iminoglicinúria, devido à competição da prolina com outros aminoácidos que

compartilham um sistema de transporte renal; e aumento da excreção urinária de P5C (Applegarth et al., 1974; Goodman et al.,1974).

O P5C pode ser identificado qualitativamente na urina quando reage com aminobenzoaldeído e ácido tricloroacético (TCA) em álcool produzindo uma cor amarela; e de acordo com Mixson et al. (1991) e Fleming et al. (1984), quantativamente medido na urina e no plasma por testes específicos. A deficiência da P5C desidrogenase pode ser determinada em cultura de fibroblastos ou em leucócitos circulantes (Phang et al., 2001).

1.3.3.3 Manifestações Clínicas

As conseqüências dos altos níveis de prolina e P5C plasmáticos sobre a função do sistema nervoso central (SNC) são incertas. Muitos pacientes hiperprolinêmicos apresentam alterações neurológicas, convulsões e retardo mental, enquanto outros são clinicamente normais. Segundo Flynn et al. (1989), estes achados indicam que a manutenção de altas concentrações plasmáticas de prolina não é suficiente para causar dano neurológico, mas pode predispor a convulsões.

Entretanto, a prolina exerce um papel aparente no SNC, ativando receptores glutamatégicos (Ault, 1987; Henzi, 1992; Martin, 1992; Pace, 1992; Nistri & Moreli, 1978), apresentando transportadores próprios expressos em neurônios glutamatérgicos (Fremeau, 1992; Fremeau, 1996; Shafqat, 1995; Velaz-Fairclooth, 1995; Fremeau, 1992; Nadler, 1992; Nadler & Cohen, 1995) e

modulando a neurotransmissão glutamatérgica. Assim sendo, é possível que elevadas concentrações de prolina tenham uma ação excitotóxica que pode contribuir no aparecimento das alterações neurológicas de indivíduos com HPII.

Os mecanismos responsáveis pelas alterações neurológicas ainda não estão elucidados (Pavone et al., 1975). Nesse contexto, Moreira et al. (1989), desenvolveram um modelo químico experimental de hiperprolinemia tipo II e observaram que ratos submetidos a este modelo apresentaram deficiência nos processos de aprendizagem/memória. Outros estudos realizados no Laboratório de Erros Inatos do Metabolismo do Departamento de Bioquímica da UFRGS demonstraram redução na concentração de neurofilamentos pesados (200 kDa) do citoesqueleto neuronal cortical (Rubin et al., 1991), redução da atividade da Na+,K+-ATPase (Pontes et al., 1999) e redução da atividade cerebral da acetilcolinesterase através da produção de radicais livres (Delwing et al. 2002). Por outro lado, altos níveis de prolina podem levar a alterações no potencial redox celular e acúmulo de NADH (Phang et al., 2001).

1.3.3.4 Tratamento

As hiperprolinemias são distúrbios considerados benignos, pois não causam doença em muitas crianças afetadas. Na HPII, as convulsões ocorrem geralmente na infância, sendo que os adultos são assintomáticos. Até o presente momento, não existe indicação de tratamento dietético para este distúrbio (Phang et al., 2001).

Em 1995, Benson e Fenson defenderam a idéia da redução precoce dos níveis de prolina nos indivíduos afetados através de restrição dietética. No entanto, como a prolina é um aminoácido não essencial, ela é sintetizada através de outros aminoácidos. Portanto, a maioria das proteínas contém resíduos de prolina, tornando a restrição dietética desse iminoácido muito difícil, resultando em um modesto controle plasmático e, conseqüentemente, não causando impacto nas manifestações clínicas (Phang et al., 2001).

1.4 Creatinaquinase

O metabolismo energético encefálico é caracterizado por níveis altos e flutuantes de síntese e de utilização de ATP. O encéfalo contém altos níveis de creatinaquinase (CK) EC 2.7.3.2, uma enzima que regenera ATP a partir de ADP e de fosfocreatina (PCr), uma forma de reserva energética (Wallimann & Hemmer, 1994). O intercâmbio do grupo fosforil entre PCr e ADP é extremamente importante para a homeostasia energética do tecido nervoso (Wallimann et al., 1992).

O sistema creatinaquinase / fosfocreatina exerce várias funções integradas nas células do encéfalo, tais como tampão energético temporário, capacidade metabólica, transferência de energia e controle metabólico (Saks et al., 1996). Este sistema é reconhecido como um importante regulador metabólico tanto na saúde quanto na doença (Wallimann et al., 1998).

A CK possui isoenzimas localizadas especificamente em locais de demanda e de produção de energia, sendo ligadas pelo circuito creatina / creatinafosfato (Fig. 4). Elas estão compartimentalizadas subcelularmente e ligadas em sítios de produção e de consumo de energia. Segundo Wyss et al. (1992), no tecido nervoso, a CK encontra-se em duas isoformas: uma mitocondrial, octamérica (Mi_a-CK) e outra citosólica, dimérica (B-CK), acoplada funcionalmente à Na^+,K^+-ATPase (Blum et al., 1991). A atividade da B-CK é maior na substância branca do que na substância cinzenta do encéfalo de porcos (Holtzman et al., 1996) e maior na substância branca cerebelar do que no córtex cerebelar de ratos (Kato et al., 1986). As atividades da Mi_a-CK e da B-CK são equivalentes na substância cinza, enquanto a atividade da B-CK predomina na substância branca (Hemmer & Wallimann, 1993). O encéfalo tem a capacidade de sintetizar parte da creatina de que necessita (Defalco & Davies, 1961), sendo o restante captado através de um sistema de co-transporte Na^+/ creatina (Guimbal & Kilimann, 1993).

A maturação funcional da CK durante o desenvolvimento do encéfalo do rato com expressão seqüencial das isoenzimas (Holtzman et al., 1993), a regulação da expressão da B-CK via AMPc nas células gliais (Kuzhikandathil & Molloy, 1994) e a atividade significativa da B-CK nos neurônios, astrócitos e oligodendrócitos (Manos et al., 1991) são compatíveis com um papel importante exercido pelo sistema CK, principalmente pela B-CK, no processo de mielinização e nas diversas atividades do sistema nervoso.

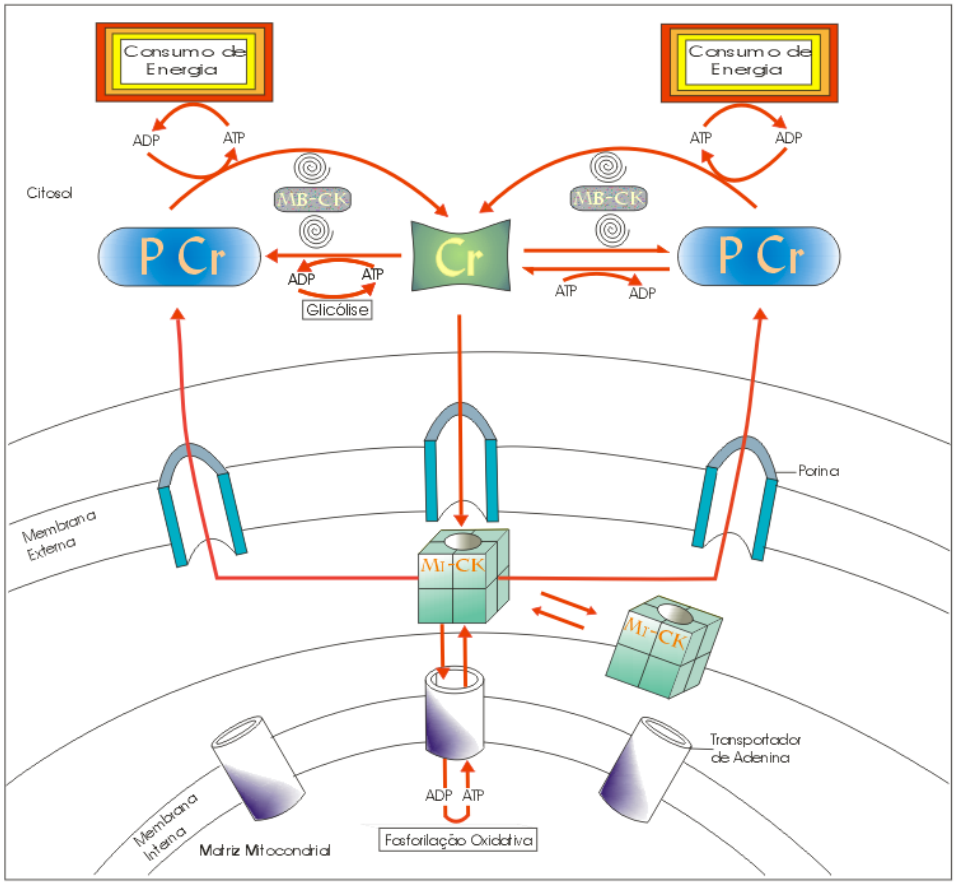

Figura 4 – *Modelo do circuito creatina (Cr) / fosfocreatina (PCr) na homeostase energética intracelular*. São mostradas duas isoformas da creatinaquinase, uma mitocondrial (Mi-CK) e outra citosólica (MB-CK). A Mi-CK está ligada funcionalmente a fosforilação oxidativa, enquanto a MB-CK pode estar associada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

Como a energia é necessária para manter o desenvolvimento e a regulação das funções encefálicas, tem sido postulado que danos na função da CK podem ser um passo importante da rota neurodegenerativa no encéfalo (Tomimoto et al., 1993). Estudos recentes têm reforçado esta hipótese, mostrando que a atividade da enzima é severamente reduzida em diversas doenças neurodegenerativas (David et al., 1998; Aksenov et al., 2000).

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

Na expectativa de podermos contribuir para um melhor conhecimento da patogênese do dano encefálico na HPII e considerando que: 1- esse distúrbio está associado a lesões cerebrais cujos mecanismos são pouco conhecidos; 2- a administração de Pro provoca alterações em alguns parâmetros neuroquímicos associados à homeostasia energética; 3- o sistema CK/PCr é fundamental para a manutenção das atividades nervosas que se encontram alteradas na HPII e que defeitos nesse sistema produzem alterações clínicas similares às encontradas em hiperprolinêmicos, tornou-se importante estudar os efeitos da Pro sobre a atividade da CK.

2. OBJETIVOS

2.1 – Investigar os efeitos da prolina sobre a atividade da creatinaquinase em córtex cerebral de ratos jovens.

Consistiu na determinação do efeito das administrações aguda e crônica de prolina no homogeneizado total e nas frações citosólica e mitocondrial sobre a atividade da creatinaquinase, assim como na determinação do efeito *in vitro* da prolina sobre a atividade da mesma enzima em córtex de ratos não tratados.

2.2 - Investigar os efeitos da prolina sobre a atividade da creatinaquinase em cerebelo e em encéfalo médio de ratos jovens.

Consistiu na determinação do efeito das administrações aguda e crônica de prolina no homogeneizado total sobre a atividade da creatinaquinase, assim como na determinação do efeito *in vitro* da prolina sobre a atividade desta enzima em cerebelo e em encéfalo médio de ratos não tratados.

3. RESULTADOS

OBJETIVO 1

Investigar os efeitos da prolina sobre a atividade da creatinaquinase em córtex cerebral de ratos jovens.

ARTIGO 1

Adriana Kessler, Elisa Costabeber, Carlos S. Dutra-Filho, Ângela T. S. Wyse, Moacir Wajner, Clovis M. D. Wannmacher. ***Proline reduces creatine kinase activity in the brain cortex of rats.*** Aceito para publicação no Neurochemical Research.

OBJETIVO 2

Investigar os efeitos da prolina sobre a atividade da creatinaquinase em cerebelo e encéfalo médio de ratos jovens.

ARTIGO 2

Adriana Kessler, Elisa Costabeber, Carlos S. Dutra-Filho, Ângela T. S. Wyse, Moacir Wajner, Clovis M. D. Wannmacher. ***Effect of proline on creatine kinase activity in rat brain.*** Aceito para publicação no Metabolic Brain Disease.

Proline Reduces Creatine Kinase Activity in the Brain Cortex of Rats

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Running title: **Proline and creatine kinase**

Abstract

Type II Hyperprolinemia is an inherited disorder caused by a deficiency of Δ^1-pyrroline-5-carboxilic acid dehydrogenase, whose biochemical hallmark is proline accumulation in plasma and tissues. Although neurologic symptoms occur in most patients, the neurotoxicity of proline is still controversial. The main objective of the present study was to investigate the effect of acute and chronic administration of proline on creatine kinase activity of brain cortex of Wistar rats. Acute treatment was performed by subcutaneous administration of one injection of proline to 22-day-old rats. For chronic treatment, proline was administered twice a day from the 6th to the 21st postpartum day. The results showed that creatine kinase activity was significantly inhibited in the brain cortex of rats subjected to acute proline administration. In contrast, this activity was increased in animals subjected to chronic administration. We also measured the *in vitro* effect of proline on creatine kinase activity in cerebral cortex of 22-day-old non-treated rats. Proline significantly inhibited creatine kinase activity. Considering the importance of creatine kinase for the maintenance of energy homeostasis in the brain, it is conceivable that an alteration of this enzyme activity in the brain may be one of the mechanisms by which proline might be neurotoxic.

Key words: proline, hyperprolinemia, creatine kinase, brain energy

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1. INTRODUCTION

Type II Hyperprolinemia (HPII) is an autosomal recessive disorder of amino acid metabolism caused by the deficiency of Δ^1-pyrroline-5-carboxilic acid dehydrogenase activity which results in proline accumulation in plasma and tissues of the affected individuals (1). Although asymptomatic hyperprolinemic siblings have been identified in various pedigrees (2), a considerable number of HPII patients so far detected show neurological manifestations including seizures and mental retardation. In this context, a relationship between high concentration of proline and neurological symptoms has been demonstrated in patients with HPII (3). However, the mechanisms underlying these symptoms are far from be understood. We have previously reported that rats subjected to chronic administration of proline presented a significant impairment of learning/memory (4) and decreased Na^+,K^+-ATPase activity in synaptic plasma membranes from cerebral cortex (5). It has also been reported that high proline levels can lead to alterations in the cell redox giving rise to a decrease in the oxygen consumption necessary to oxidize the NADH formed by the cell, decreasing energy production (6). Therefore, it seems that high proline levels may be neurotoxic or at least may predispose to brain damage.

Creatine kinase (CK) plays a key role in energy metabolism of tissues with intermittently high and fluctuating energy requirements, such as skeletal and cardiac muscle, neuronal tissues like brain and retina, or spermatozoa and electrocytes. CK catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP, to regenerate ATP. There are distinct CK isoenzymes,

which are compartmentalized specifically in the places where energy is produced or utilized (7). Besides, different cells may contain a number of different creatine kinase isoforms. Two isoforms, M-CK and ubiquitous B-CK, are cytosolic, and two other isoforms, Mi b-CK and ubiquitous Mi a-CK are mitochondrial (8). Because energy is necessary to maintain the development and regulation of cerebral functions, it has been postulated that alteration in CK activity may represent an important step of a neurodegenerative pathway that leads to neuronal loss in the brain (9). Recent findings have reinforced this hypothesis, showing that CK activity is severely reduced in several neurodegenerative diseases (10,11).

Therefore, the main objective of the present study was to investigate the effects of acute and chronic administration of proline on CK activity from rat cerebral cortex. We also investigated the *in vitro* effects of proline on CK activity in the same cerebral structure from non-treated rats.

2. MATERIALS AND METHODS

***Animals and chemicals*:** Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, Porto Alegre, RS, Brazil were used in the experiments. Rats were kept with dams while receiving the drugs until they were sacrificed. The dams had free access to water and to a standard commercial chow (Germani, Porto Alegre, RS, Brazil) containing 20.5% protein (predominantly soybean supplemented with methionine), 54% carbohydrate, 4.5% fiber, 4% lipids, 7% ash and 10% moisture. Temperature was maintained at 22 ± 1 °C, with a 12 -12 h light-dark cycle. The "Principles of Laboratory Animal Care" (NIH

publication 85-23, revised 1985) were followed in all the experiments and the experimental protocol was approved by the Ethics Committee For Animal Research of the Federal University of Rio Grande do Sul. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

***Chronic treatment*:** Hyperprolinemia (HP) was induced by daily subcutaneous administration of proline from the 6^{th} to the 21^{st} day of life. Proline was dissolved in 0.85% saline and buffered to pH 7.4 with NaOH. The animals were randomly assigned to two groups as previously reported (4): 1 – HP group: rats received subcutaneous administration of proline four times a day at 4 h intervals, in doses calculated from pharmacokinetic parameters to achieve plasma proline levels between 1.2 and 1.5 mM and brain proline concentrations between 0.2 and 0.5 mM, 60 min after the injection. During the first 8 days of treatment the rats received 12.8 μmol of proline / g body weight, from day 14 to 17 they received 14.6 μmol of proline / g body weight and from day 18 to 21 they received 16.4 μmol of proline / g body weight; 2 – Control group: rats received 0.85% NaCl, four times a day at 4 h intervals, in the same volumes as those applied to proline-treated animals. Twelve hours after the last injection, the animals were killed by decapitation without anesthesia and the brain cortex was separated for creatine kinase activity and protein determinations. At this time, proline levels were normal in the plasma (60 - 100 μM) and in the brain (30 - 50 μM).

***Acute treatment*:** Twenty two-day-old rats were randomly assigned to 2 groups and treated with one single subcutaneous injection: 1 – HP group: rats

received 16.4 μmol proline / g body weight; 2- Control group: rats received the same volumes of 0.85% NaCl. Doses were calculated according to body weight and prepared as stated above. One hour after the subcutaneous administration the animals were killed by decapitation without anesthesia and the brain cortex was separated for creatine kinase activity and protein determinations.

For the ***in vitro*** experiments, non-treated 22-day-old rats were used. Proline was dissolved in 100 mM MgSO4-Trizma buffer, pH 7.5, and added to the assay at 0.5 – 5.0 mM final concentrations.

Preparation of brain tissue*:* The animals were sacrificed by decapitation, the brain rapidly removed and the cerebral cortex was isolated and homogenized with a Teflon-glass homogenizer in 20 volumes of ice-cold TRIS-sucrose buffer, pH 7.5 (250 mM sucrose, 10 mM Trizma, 1 mM EGTA), pH 7.5. The homogenate was centrifuged at 800 x g for 10 min, the pellet was discarded and the supernatant was centrifuged at 10 000 x g for 15 min. The supernatant of the second centrifugation, containing cytosol, and other cellular components as endoplasmic reticulum was collected for determination of cytosolic CK activity. The pellet, containing mitochondria, myelin, synaptosomes, and membrane fragments, was washed twice with the same TRIS-sacarose isotonic buffer, resuspended in 100 mM MgSO4-Trizma buffer, pH 7.5, for determination of mitochondrial CK activity. Homogenate, cytosolic and mitochondrial fractions were stored at -70^0 C when the assay was not carried out immediately. The mitochondrial fraction was frozen and thawed three times for the enzymatic assay.

Protein determination: The protein content of cerebral cortex homogenates was determined by the method of Lowry et al. (12) using serum bovine albumin as the standard.

Creatine kinase activity assay*:* The reaction mixture contained the following final concentrations: 60 mM Tris-HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, and approximately 1 μg protein in a final volume of 0.1 mL. After 5 minutes of pre-incubation at 37°C, the reaction was started by the addition of 0.3 μmol ADP plus 0.08 μmol reduced glutathione. The reaction was stopped after 10 minutes by the addition of 1 μmol p-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL 2 % α-naphtol and 0.1 mL 0.05 % diacetyl in a final volume of 1 mL and read after 20 minutes at 540 nm. Appropriate controls were carried out because proline interferes with color development. Results were expressed as μmol of creatine formed per min per mg protein.

Statistical analysis: Data from the acute and chronic treatments were analyzed by the Student's t test for independent samples. Data from the *in vitro* experiments were analyzed by one-way ANOVA followed by the Tukey test when the F value was significant. Dose-dependent effect was analyzed by linear regression. All data were analyzed by the Statistical Package for the Social Sciences software in a PC computer.

3. RESULTS

First, we measured CK activity in total homogenates (Fig 1A), as well as in the cytosolic (Fig 1B) and mitochondrial (Fig 1C) preparations of brain cortex from 22-day-old rats subjected to acute or chronic administration of proline. CK activity was reduced in the cortical homogenates [$t(12) = 4.49$; $p< 0.01$] and also in the cytosolic [$t(12) = 7.71$; $p< 0.001$], and mitochondrial [$t(12) = 2.24$; $p< 0.05$] fractions from animals that received one acute injection of proline. The ratio between mitochondrial and cytosolic activities of acutely proline-treated animals did not differ from those of saline-treated rats [$t(12) = 1.71$; $p> 0.10$], indicating that proline effect on the isozymes of the two fractions was of the same magnitude (Fig 1D). Furthermore, chronic proline administration significantly reduced CK activity in the cytosolic fraction [$t(14) = 2.79$; $p< 0.01$] (Fig 1B), whereas this activity was increased in the total homogenates [$t(14) = 2.65$; $p< 0.01$] (Fig 1A) and in the mitochondrial fraction [$t(14) = 3.15$; $p< 0.01$] (Fig 1A). The ratio between the mitochondrial and the cytosolic activities in chronically proline-treated rats was increased when compared to control rats [$t(14) = 5.77$; $p < 0.001$] (Fig 1D), reinforcing that the increased CK activity of total homogenates was due to the mitochondrial fraction.

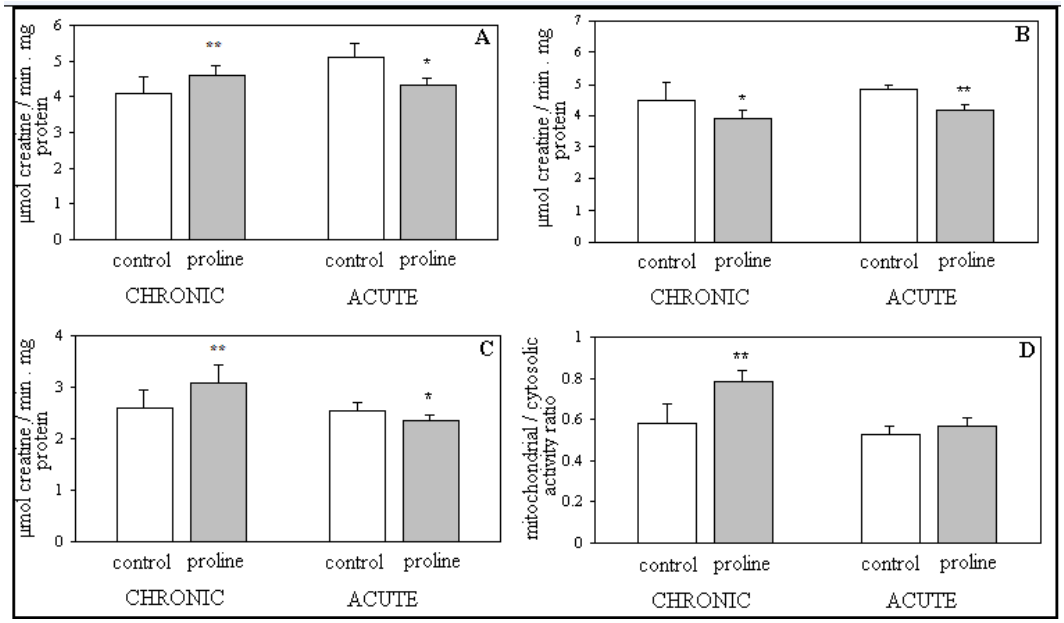

Fig. 1: Effect of acute and chronic proline administration on creatine kinase activity in total homogenates (A), cytosolic fraction (B), mitochondrial fraction (C), and mitochondrial/cytosolic ratio (D) from brain cortex of young rats.

Data are mean ± SD for 7-8 independent experiments performed in triplicate. Different from control, *p<0.05; **p<0.01 (Student's t test).

Next, we studied the in vitro effect of various concentrations (0.5-5 mM) of proline on CK activity in total homogenates (Fig 2A), as well as in the cytosolic (Fig 2B) and mitochondrial (Fig 2C) preparations of the brain cortex from 22-day-old non-treated rats. Proline caused an inhibition of CK activity in all preparations (total homogenates [$F(1,28) = 10.18$; $\beta = -0.51$; $p < 0.01$], cytosolic fraction [$F(1,28) = 21.25$; $\beta = -0.66$; $p < 0.001$], and mitochondrial fraction [$F(1,28) = 11.56$; $\beta = -0.54$; $p < 0.01$]). The ratio between mitochondrial and cytosolic activities did not differ from those of the controls in all

concentrations tested [F(4,25) = 0.78; p> 0.53] (Fig 2D), indicating that proline effect on the isozymes of the two fractions was of the same magnitude.

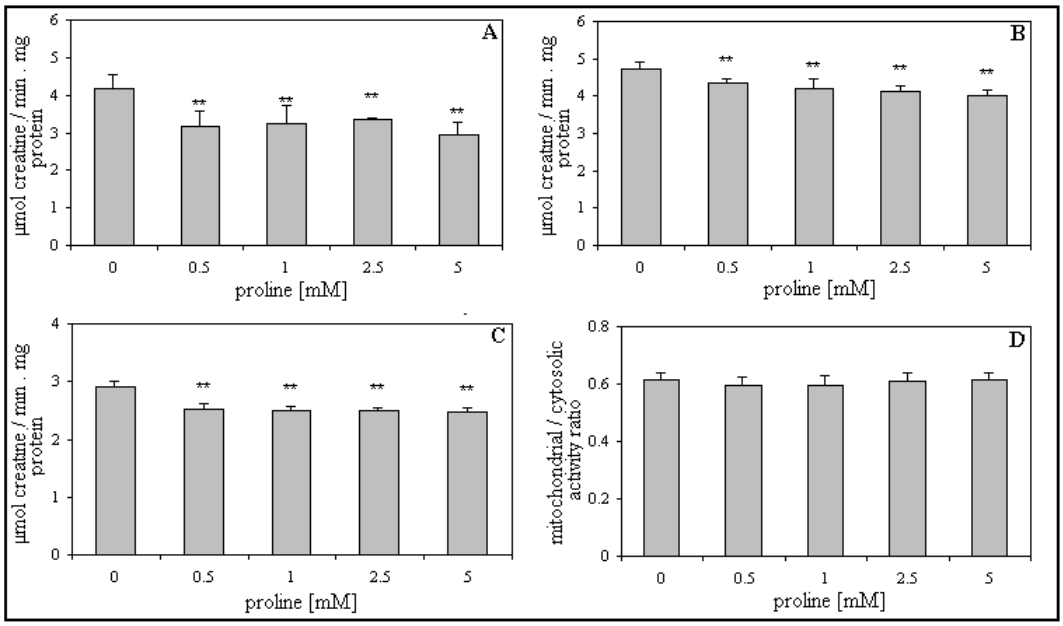

Fig.2 - *In vitro* effect of proline on creatine kinase activity in total homogenates (A), cytosolic fraction (B), mitochondrial fraction (C), and mitochondrial/cytosolic ratio (D) from brain cortex of young rats.

Data are mean ± SD for 6 independent experiments performed in triplicate. Different from control, ** $p<0.01$ (Tukey test).

Since in theory proline could be used for glutamate synthesis in brain, we investigated the in vitro effect of glutamate on CK activity, in order to evaluate whether glutamate could be responsible for the effects attributed to proline. Our results indicated that glutamate did not alter CK activity: (control = 3.9 ± 0.3; glutamate 1 mM = 3.8 ± 0.4; glutamate 5 mM = 3.9 ± 0.3 µmol/min . mg protein for 7 animals; F(2,18) = 0.28; p> 0.75).

4. DISCUSSION

We have previously demonstrated that sustained plasma proline levels, similar to those found in the plasma of HPII patients, induce behavior deficits (4) and reduction of Na+,K+-ATPase activity (5) in cerebral cortex of developing rats. In the present study, we demonstrated that acute administration of proline reduces total, cytosolic, and mitochondrial CK activities in brain cortex from rats. In addition, proline concentrations similar to those found in the plasma and brain of proline- treated rats (1 mM and 0.5 mM, respectively), inhibited the *in vitro* CK activity of total homogenates, cytosolic, and mitochondrial fractions. Since proline has a direct inhibitory action on CK activity, this effect observed *in vitro*, probably also occurs *in vivo.* However, the CK activity was not measured directly in vivo, but in an *in vitro* assay of cerebral cortex preparations from acutely proline-treated animals. In this case, the inhibition caused by the presence of this amino acid in the assay is unlikely to occur, because the preparations from brain were diluted 10,000 times for the incubation. Therefore, considering that the maximal concentration of proline in the brain at the time of rat sacrifice was around 0.5 mM, the final proline concentration in the assay medium was as low as 0.05 μM, one thousand times lower than the normal brain levels. Therefore, the diminution of CK activity observed in the brain preparations from acutely proline-treated animals might be caused by down-regulation of the expression or by posttranslational modification of existing enzyme molecules. This later mechanism was proposed by other investigators who found that the reduced level

of CK, observed in several neurodegenerative disorders was the result of posttranslational modifications of the enzyme (14,15).

Chronic hyperprolinemia also reduced the cytosolic CK activity, but, in contrast, significantly increased the mitochondrial activity. The overall effect of chronic hyperprolinemia, including mitochondrial and cytosolic CK activities, was an increase in total homogenate activity. In respect to the observed increase of the mitochondrial CK activity in the brain cortex of rats subjected to chronic hyperprolinemia, it is possible that the amino acid causes an initial reduction of CK activity of both cytosolic and mitochondrial isozymes, and the mechanisms of recuperation of the mitochondrial enzyme activity to overcome the enzyme inhibition are more active than those of the cytosolic activity, a hypothesis that must be confirmed. In this case, mitochondrial CK activity would be elevated when assayed in the absence of significant amounts of proline. This biphasic response (an initial decreased activity reflecting inactivation of the enzyme, followed by an increased activity corresponding to a response of the organism to maintain the enzyme function) was previously observed in Na^+,K^+-ATPase activity in synaptosomal membranes of brain cortex of rats subjected to phenylalanine administration (16). Taken together, the results of proline administration, and those of the in vitro effect of proline suggest that highly sustained proline levels may decrease CK activity in the cerebral cortex. However, it is not possible to assure that the observed decrease in creatine kinase activity provoked by proline (up to 20%) is relevant to the pathology of hyperprolinemia. Nevertheless, considering that the Km (Michaelis constant) of CK for ADP as

substrate (0.3 ± 0.1 mM) indicates that the enzyme is far from be saturated at normal brain ADP levels (02-04 mM) (17), we may presume that a 20% reduction on CK activity may lead to increase in ADP and decrease in ATP concentrations.

Proline effect on CK can not be attributed to glutamate, because glutamate synthesis from proline do not occur in most brain regions (18). Besides, glutamate has no effect on the in vitro CK activity.

The CK / phosphocreatine system exerts several integrated functions in brain cells, such as temporary energy buffering, metabolic capacity, energy transfer, and metabolic control (19). This system is now recognized as an important metabolic regulator during health and disease (20). Furthermore, it has been postulated that CK and the creatine-phosphocreatine energy shuttle may play a role in brain development that is associated with oligodendrocyte function and/or myelogenesis (21).

A decrease of CK activity is considered one of the biochemical markers of brain cell damage in age-related neurodegenerative diseases, including Alzheimer's disease (14). The decrease of CK activity in the brain correlates well with the neurodegeneration parameters in severely affected brain regions in Alzheimer's disease (22). Therefore, impairment of CK function may play an important role in the neurodegenerative pathway that leads to brain damage (9). These findings are reinforced by the observation that creatine and phosphocreatine have neuroprotective effects against energy deprivation and glutamate

excitoxicity, attributable to an enhancement of the cytosolic high-energy phosphate stores (23).

On the other hand, considering the existence of a brain specific high affinity of the Na^+/Cl^--dependent proline transporter (24-27) which is expressed exclusively in a subset of glutamatergic neurons (28,29), it has been proposed that proline might serve as a modulator of glutamate neurotransmission. The finding of high levels of proline and glutamate found in the cerebrospinal fluid of patients with HPll is in agreement with this hypothesis (6,30,31). Taken together these observations and the reduction of brain CK activity found in the present study, which may potentially induce excitotoxicity, it is possible that high concentrations of proline may induce excitotoxicity.

In summary, our results indicate that proline alters CK activity in the cerebral cortex of rats. Considering that creatine kinase is a key enzyme for energy homeostasis in the brain, in case this effect also occurs in the brain of HPII patients, it is possible to envisage that an alteration of this enzyme activity may potentially impair brain energy metabolism, contributing to the brain damage found in many patients affected by this disease. Finally, considering that the current diet therapy is ineffective in HPII (1), that creatine easily crosses the blood-brain barrier (32), and that creatine administration results in significant improvement in patients with mitochondrial encephalopathy and other neurological diseases (33), it may be interesting to test whether creatine supplementation would benefit HPII patients.

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Effect of Proline on Creatine Kinase Activity in Rat Brain

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Running title: **Proline and creatine kinase**

Abstract

Type II Hyperprolinemia is an inherited disorder caused by a deficiency of Δ^1-pyrroline-5-carboxilic acid dehydrogenase, whose biochemical hallmark is proline accumulation in plasma and tissues. Although neurologic symptoms occur in most patients, the neurotoxicity of proline is still controversial. The main objective of the present study was to investigate the effect of acute and chronic administration of proline on creatine kinase activity in the homogenates of cerebellum and midbrain from Wistar rats. Acute treatment was performed by subcutaneous administration of one injection of proline to 22-day-old rats. For chronic treatment, proline was administered four times a day from the 6th to the 21st postpartum day. The results showed that creatine kinase activity was significantly inhibited in the cerebellum and midbrain of rats subjected to acute proline administration. In contrast, this activity was increased in animals subjected to chronic administration. We also measured the *in vitro* effect of proline on creatine kinase activity in the same brain structures of 22-day-old non-treated rats. Proline significantly inhibited creatine kinase activity. Considering the importance of creatine kinase for the maintenance of energy homeostasis in the brain, it is conceivable that an alteration of this enzyme activity in the brain may be one of the mechanisms by which proline might be neurotoxic.

Key words: proline, hyperprolinemia, creatine kinase, brain energy

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1. INTRODUCTION

Type II Hyperprolinemia (HPII) is an autosomal recessive disorder of amino acid metabolism caused by the deficiency of Δ^1-pyrroline-5-carboxilic acid dehydrogenase activity which results in proline accumulation in plasma and tissues of the affected individuals (Phang and Valle, 2001). Although asymptomatic hyperprolinemic siblings have been identified in various pedigrees (Pavone et al, 1975), a considerable number of HPII patients so far detected show neurological manifestations including seizures and mental retardation. In this context, a relationship between high concentration of proline and neurological symptoms has been demonstrated in patients with HPII (Flynn et al, 1989). However, the mechanisms underlying these symptoms are far from be understood. We have previously reported that rats subjected to chronic administration of proline (Pro) presented a significant impairment of learning/memory (Moreira et al, 1989) and decreased Na^+,K^+-ATPase activity in synaptic plasma membranes from cerebral cortex (Pontes et al, 1999). It has also been reported that high proline levels decrease the oxygen consumption and energy production (Phang et al, 1995). Therefore, it seems that high Pro levels may be neurotoxic or at least may predispose to brain damage.

Creatine kinase (CK) catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP, to regenerate ATP. This enzyme activity plays a key role in energy metabolism of tissues with intermittently high and fluctuating energy requirements, such as skeletal, cardiac, and neuronal tissues like brain and retina (Wallimann et al, 1992). There are distinct CK isoenzymes,

which are compartmentalized specifically in the places where energy is produced or utilized (Wallimann et al, 1998). Because energy is necessary to maintain the development and regulation of brain functions, it has been postulated that alteration in CK activity may participate of a neurodegenerative pathway leading to neuronal loss in the brain (Tomimoto et al, 1993). Recent findings have reinforced this hypothesis, showing that CK activity is severely reduced in several neurodegenerative diseases (Aksenov et al, 1997; David et al, 1998; Aksenova et al, 1999; Aksenov et al, 2000).

Therefore, the main objective of the present study was to investigate the effects of acute and chronic administration of proline on CK activity from rat cerebellum and midbrain. We also investigated the *in vitro* effects of proline on CK activity in the same brain structures from non-treated rats.

2. MATERIALS AND METHODS

Animals and chemicals: Twenty four Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, Porto Alegre, RS, Brazil were used in the experiments. Rats were kept with dams while receiving the drugs until they were sacrificed. The dams had free access to water and to a standard commercial chow (Germani, Porto Alegre, RS, Brazil) containing 20.5% protein (predominantly soybean supplemented with methionine), 54% carbohydrate, 4.5% fiber, 4% lipids, 7% ash and 10% moisture. Temperature was maintained at 22 ± 1 °C, with a 12 -12 h light-dark cycle. The "Principles of Laboratory Animal Care" (NIH

publication 85-23, revised 1985) were followed in all the experiments and the experimental protocol was approved by the Ethics Committee For Animal Research of the Federal University of Rio Grande do Sul. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

***Chronic treatment*:** Hyperprolinemia (HP) was induced by daily subcutaneous administration of proline from the 6^{th} to the 21^{st} day of life. At the 6^{th} postpartum day, rat brain development is equivalent to the brain development of a human child at birth. Proline was dissolved in 0.85% saline and adjusted to pH 7.4 with NaOH addition. The animals were randomly assigned to two groups as previously reported (Moreira et al, 1989): 1 – HP group: rats received subcutaneous administration of proline four times a day, at 4h intervals, in doses calculated from pharmacokinetic parameters, to achieve plasma proline levels between 1.2 and 1.5 mM and brain proline concentrations between 0.2 and 0.5 mM, 60 min after the injection. During the first 8 days of treatment, the animals received 12.8 μmol of proline / g body weight; from day 14 to 17 they received 14.6 μmol of proline / g body weight, and from day 18 to 21 they received 16.4 μmol of proline / g body weight ; 2 – Control group: rats received 0.85% NaCl four times a day in the same volumes as those applied to proline-treated animals. Twelve hours after the last injection, the animals were killed by decapitation without anesthesia. Cerebellum and midbrain (brain minus cortex, olphatory bulbs, cerebellum and brain stem) were separated for creatine kinase activity and protein determinations. At the time of sacrifice, proline levels did not differ between saline and proline-treated rats in plasma (saline-treated: 81 ± 10 μM

(n = 4); proline-treated: 88 ± 15 μM (n = 4); t(6) = 0.77; p > 0.5) and in brain (saline-treated: 43 ± 10 μM (n = 4); proline-treated: 41±4 μM (n = 4); t(6) = 0.37; p > 0.5).

***Acute treatment*:** Twenty two-day-old rats were randomly assigned to 2 groups and treated with one single subcutaneous injection: 1 – HP group: rats received 16.4 μmol proline / g body weight; 2 – Control group: rats received the same volumes of 0.85% NaCl. Proline solution was prepared as stated above. One hour after the subcutaneous administration, the animals were killed by decapitation without anesthesia and the cerebellum and midbrain were separated for creatine kinase activity and protein determinations.

For the ***in vitro*** experiments, non-treated twenty two-day-old rats were used. Proline was dissolved in 100 mM MgSO4-Trizma buffer, pH 7.5, and added to the assay at 0.5 – 2.5 mM final concentrations.

Preparation of brain tissue*:* cerebellum and midbrain were isolated and homogenized with a Teflon-glass homogenizer in 20 volumes of ice-cold TRIS-sucrose buffer (250 mM sucrose, 10 mM Trizma, 1mM EGTA), pH 7.5. Time elapsed between animal decapitation and tissue homogenization was less than 1 min. The homogenate was stored at -70^0 C when the assay was not carried out immediately.

Protein determination: The protein content of cerebellum and midbrain homogenates was determined by the method of Lowry et al, (1951) using serum bovine albumin as the standard.

Creatine kinase activity assay: The reaction mixture contained the following final concentrations: 60 mM Tris-HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, 0.02% triton X-100, and approximately 1 μg protein in a final volume of 0.1 mL. After 5 minutes of pre-incubation at 37°C, the reaction was started by the addition of 0.3 μmol ADP plus 0.08 μmol reduced glutathione. The reaction was stopped after 10 minutes by the addition of 1 μmol p-hydroxymercuribenzoic acid. The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL 2 % α-naphtol and 0.1 mL 0.05 % diacetyl in a final volume of 1 mL and read after 20 minutes at 540 nm. Because proline interferes with color development, we carried out, for each CK activity measure, a control containing all reagents but not the brain homogenate. The difference between the two assays was taken as the actual proline effect. Results were expressed as μmol of creatine formed per min per mg protein**.**

Statistical analysis: Data from the acute and chronic treatments were analyzed by the Student t test for independent samples. Data from the *in vitro* experiments were analyzed by one-way ANOVA followed by the Tukey test when the F value was significant. All data were analyzed by the Statistical Package for the Social Sciences software with an IBM compatible PC computer.

3. RESULTS

First, we measured CK activity in total homogenates of cerebellum (Fig 1) and midbrain (Fig 2) from 22-day-old rats subjected to acute or chronic administration of proline. CK activity was reduced in the homogenates of cerebellum [$t(12) = 13.6$; $p < 0.01$] and midbrain [$t(12) = 2.75$; $p<0,05$] from animals that received one acute injection of proline. Furthermore, chronic proline administration significantly increased CK activity in the total homogenates of cerebellum [$t(14) = 3.65$; $p < 0.01$] and midbrain [$t(14) = 4.42$; $p < 0.01$].

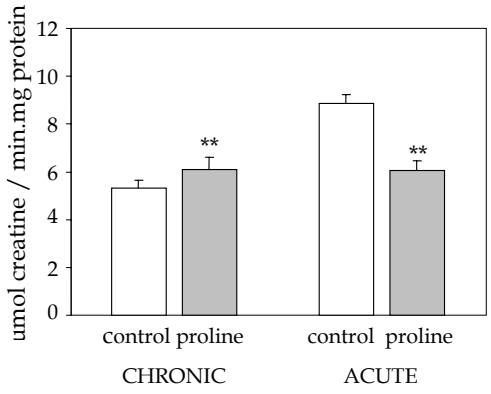

Fig. 1: Effect of acute and chronic proline administration on creatine kinase activity in rat cerebellum homogenates.

Data are mean ± SD for 7-8 independent experiments performed in triplicate. Different from control, **p<0.01 (Student t test).

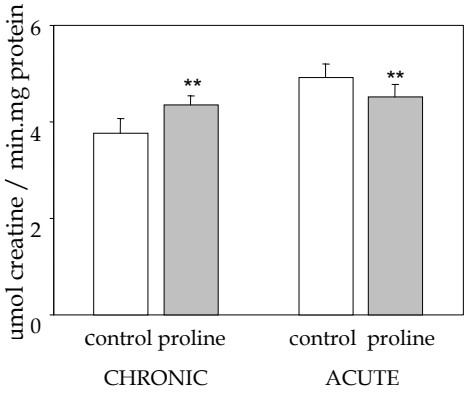

Fig. 2: Effect of acute and chronic proline administration on creatine kinase activity in rat midbrain homogenates.

Data are mean ± SD for 7-8 independent experiments performed in triplicate. Different from control, **p<0.01 (Student t test).

It is possible that handling of the animals in chronic treatment may reduce brain CK activity, since this activity is lower in control animals subjected to chronic than to acute treatment.

Next, we studied the in vitro effect of various concentrations (0.5-2.5 mM) of proline on CK activity in total homogenates of cerebellum (Fig 3) and midbrain (Fig 4) from 22-day-old non-treated rats. Proline significantly inhibited CK activity in the cerebellum [$F(3,20) = 5.18$; $p<0.05$] and midbrain [$F(3,20) = 12.67$; $p< 0.001$].

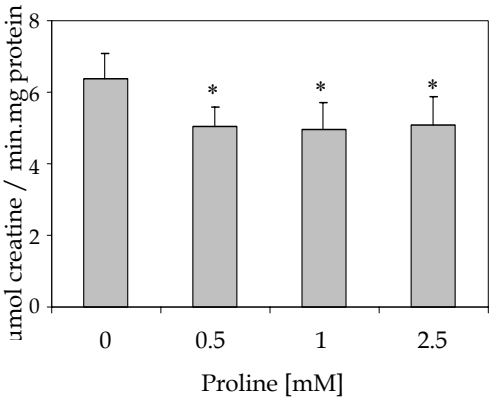

Fig. 3: *In vitro* effect of proline on creatine kinase activity in rat cerebellum homogenates.
Data are mean ± SD for 6 independent experiments performed in triplicate. Different from control, *p<0.05 (Tukey test).

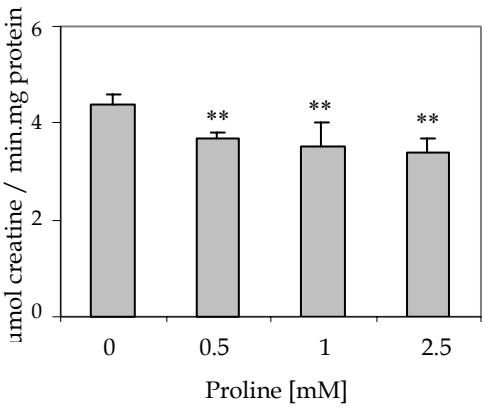

Fig. 4: *In vitro* effect of proline on creatine kinase activity in rat midbrain homogenates.
Data are mean ± SD for 6 independent experiments performed in triplicate. Different from control, **p<0.01 (Tukey test).

In order to evaluate whether the enzyme inhibition could be due to glutamate synthesized from proline, we tested the in vitro effect of glutamate on CK activity. Our results indicated that glutamate did not alter CK activity in midbrain (control = 3.4 ± 0.2; glutamate 1 mM = 3.3 ± 0.1; glutamate 5 mM = 3.5 ± 0.1

μmol/min.mg protein for 7 animals; $F(2,18) = 1.21$; $p > 0.3$) or in cerebellum (control = 4.3 ± 0.3; glutamate 1 mM = 4.3 ± 0.2; glutamate 5 mM = 4.2 ± 0.3 μmol/min.mg protein for 7 animals; $F(2,18) = 0.28$; $p > 0.7$).

4. DISCUSSION

We have previously demonstrated that sustained plasma proline levels, similar to those found in the plasma of HPII patients, induce behavior deficits (Moreira et al, 1989) and reduction of Na^+,K^+-ATPase activity (Pontes et al, 1999) in cerebral cortex of developing rats. We have also demonstrated that proline inhibits the *in vivo* and *in vitro* CK activity in rat cerebral cortex (Kessler et al, 2003). In the present study, we demonstrated that acute administration of proline reduces CK activity in the cerebellum and midbrain homogenates from young rats. In addition, proline concentrations similar to those found in the plasma and in the brain of proline- treated rats (1 mM and 0.5 mM, respectively), inhibited the *in vitro* CK activity in cerebellum and midbrain homogenates. Since proline has a direct inhibitory action on CK activity, this effect observed *in vitro*, probably also occurs *in vivo.* However, the CK activity was not measured directly in vivo, but in an *in vitro* assay of brain tissue preparations from acutely proline-treated animals. In this case, the inhibition is not caused by the presence of this amino acid in the assay, because the brain tissues were diluted 10 000 times for the incubation. Therefore, considering that the maximal concentration of proline in the brain at the time of rat sacrifice was around 0.5 mM, the final proline concentration in the

assay medium was as low as 0.05 μM, one thousand times lower than the normal proline brain levels. Therefore, the diminution of CK activity observed in the brain preparations from acutely proline-treated animals might be caused by down-regulation of the expression or by posttranslational modification of existing enzyme molecules. This later mechanism was proposed by other investigators who found that the reduced level of CK, observed in several neurodegenerative disorders, was the result of posttranslational modifications of the enzyme (Aksenov et al, 1997; Aksenova et al, 1999).

In contrast, chronic hyperprolinemia significantly increased CK activity in both brain structures. It is possible that the amino acid causes an initial reduction of CK activity, followed by mechanisms of recuperation of the enzyme activity to overcome the enzyme inhibition. In this case, CK activity would be elevated when assayed in the absence of significant amounts of proline. This biphasic response (an initial decreased activity reflecting inactivation of the enzyme, followed by an increased activity corresponding to a response of the organism to maintain the enzyme function) was previously observed in Na^+,K^+-ATPase activity in synaptosomal membranes of brain cortex of rats subjected to phenylalanine administration (Wyse et al, 1995). Taken together, the results of proline administration, and those of the in vitro effect of proline, suggest that highly sustained proline levels may decrease CK activity in the cerebellum and midbrain. However, it is not possible to assure that the observed decrease in creatine kinase activity provoked by proline (up to 20%) is relevant to the pathology of hyperprolinemia. Nevertheless, considering that the Km (Michaelis constant) of

CK for ADP as substrate (0.3 ± 0.1 mM) indicates that the enzyme is far from be saturated at normal brain ADP levels (02-04 mM) (Plaschke et al, 1999), we may presume that a 30% reduction on CK activity may lead to increase in ADP and decrease in ATP concentrations. Taken together these observations, and the reduction of brain CK activity found in the present study, it is possible that proline at high concentrations may induce excitotoxicity.

Proline effect on CK cannot be attributed to glutamate, because glutamate synthesis from proline does not occur in most brain regions (Thompson et al, 1985). Besides, glutamate has no effect on the in vitro CK activity.

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

A decrease of CK activity is considered one of the biochemical markers of brain cell damage in age-related neurodegenerative diseases (Aksenov et al, 1997). The decrease of CK activity in the brain correlates well with the neurodegeneration parameters in severely affected brain regions in Alzheimer's disease (Hensley et al, 1995). Therefore, impairment of CK function may play an important role in the neurodegenerative pathway that leads to brain damage

(Tomimoto et al, 1993). These findings are in agreement with the observation that creatine and phosphocreatine have neuroprotective effects against energy deprivation and glutamate excitoxicity, by enhancing the cytosolic high-energy phosphate stores (Brustovetsky et al, 2001).

There is a growing evidence about the existence of a brain specific high affinity of the Na^+/Cl^--dependent proline transporter (Fremeau et al, 1992; Fremeau et al, 1996; Shafqat et al, 1995; Velaz-Faircloth et al, 1995) which is expressed exclusively in a subset of glutamatergic neurons (Nadler et al, 1992), suggesting that proline might serve as a modulator of glutamate neurotransmission. On the other hand, proline has been considered toxic toward rat hippocampal neurons (Nadler et al, 1988), a hypothesis reinforced by the observations that this amino acid induces depolarization mediated by NMDA-receptors (Martin et al (1992), and potentiates glutamate transmission (Cohen and Nadler, 1997). In this context, the finding of high levels of proline and glutamate in the cerebrospinal fluid of patients with HPll is in agreement with this hypothesis (Van Herreveld and Fifkova, 1973; Rohads et al, 1983; Phang et al, 1995). Taken together these observations and the reduction of brain CK activity found in the present study, which may potentially induce excitotoxicity, it is possible that high concentrations of proline may induce excitotoxicity.

In summary, our results indicate that proline alters CK activity in the cerebellum and midbrain of young rats. Considering that proline also alters CK activity in rat cerebral cortex and that creatine kinase is a key enzyme for energy

homeostasis in the brain, in case this effect also occurs in the brain of HPII patients, it is possible to envisage that an alteration of this enzyme activity may potentially impair brain energy metabolism, contributing to the brain damage found in many patients affected by this disease. Finally, considering that the current diet therapy is ineffective in HPII (Phang et al, 2001), that creatine easily crosses the blood-brain barrier (Hemmer and Wallimann, 1993), and that creatine administration results in significant improvement in patients with mitochondrial encephalopathy and other neurological diseases (Tarnopolsky and Beal, 2001), it may be interesting to test whether creatine supplementation would benefit HPII patients.

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4. CONCLUSÕES E PERSPECTIVAS

4.1 Conclusões

A hiperprolinemia tipo II é um erro inato do metabolismo dos aminoácidos ainda pouco estudado, portanto não sabemos porque somente alguns portadores desse distúrbio apresentam manifestações neurológicas, e tão pouco quais são os mecanismos que levam a estas manifestações. Assim sendo, é de grande importância a realização de estudos que investiguem a patogênese da HPII para que possamos melhor entendê-la e, conseqüentemente, buscarmos um tratamento mais adequado para esses indivíduos.

Neste sentido, o presente estudo teve o objetivo de verificar os efeitos da Pro sobre a atividade da CK em diferentes regiões encefálicas, no intuito de colaborar com um melhor conhecimento da patogênese das crises convulsivas e do retardo mental presentes em alguns hiperprolinêmicos.

Os resultados desse estudo evidenciaram que, em homogeneizado total de córtex, cerebelo e encéfalo médio de ratos Wistar, uma única administração de Pro reduziu a atividade da CK e que *in vitro* houve inibição da enzima. Este efeito inibitório *in vitro* parece ser específico, visto que o glutamato não alterou a

atividade da CK, reforçando que o efeito da prolina pode ocorrer em indivíduos portadores de HPII. Por sua vez, o tratamento crônico aumentou a atividade da CK, provavelmente por efeito rebote, ou seja, aumento do número de moléculas ativas para compensar as moléculas enzimáticas inibidas pela prolina. No córtex também houve redução da atividade enzimática nas frações citosólica e mitocondrial, indicando que a Pro altera tanto a transferência de fosfato do ATP para a creatina na mitocôndria, quanto a transferência de fosfato da fosfocreatina para o ADP no citosol, indicando que a redução da atividade da CK pela prolina altera a homeostasia energética encefálica.

Estes dados indicam que a alteração da homeostasia energética causada pela Pro pode ser um dos mecanismos pelos quais esse aminoácido é neurotóxico.

4.2 Perspectivas

Observando os resultados anteriormente citados e considerando que: 1- o efeito da prolina foi estudado em frações citosólica e mitocondrial não purificada de córtex cerebral; 2- a CK é uma enzima tiólica sensível a radicais livres; 3- a Pro produz radicais livres, abriu-se a perspectiva da continuação dessa investigação com os seguintes objetivos:

- Verificar os efeitos da hiperprolinemia quimicamente induzida, bem como os efeitos *in vitro* da Pro sobre a atividade da CK nas frações citosólica e mitocondrial purificadas em córtex cerebral, encéfalo médio e cerebelo de ratos jovens.

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

5. REFERÊNCIAS BIBLIOGRÁFICAS ADICIONAIS

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