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

EFEITOS *IN VITRO* DA CISTINA SOBRE A ATIVIDADE DA CREATINAQUINASE EM CÓRTEX CEREBRAL DE RATOS JOVENS

DISSERTAÇÃO DE MESTRADO

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PORTO ALEGRE, 2004

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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EFEITOS *IN VITRO* DA CISTINA SOBRE A ATIVIDADE DA CREATINAQUINASE EM CÓRTEX CEREBRAL DE RATOS JOVENS

por

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A todo povo brasileiro que patrocina nossa pesquisa e ao qual dirige-se a mesma.

SUMÁRIO

RESUMO

Cistinose é um erro inato do metabolismo, associado ao acúmulo de cistina nos lisossomos, causado pelo efluxo defeituoso de cistina. O acúmulo de cistina provoca uma série de sintomas, dependendo do tecido envolvido. Atrofia cortical é uma possível conseqüência do acúmulo de cistina no córtex cerebral. Contudo, os mecanismos pelos quais a cistina é tóxica para os tecidos estão longe de serem esclarecidos.

Considerando que a creatinaquinase é uma enzima tiólica crucial para a homeostasia energética, e que dissulfetos como a cistina podem alterar enzimas tiólicas pela troca tiol/dissulfeto, o objetivo do presente estudo foi investigar o efeito da cistina sobre a atividade da creatinaquinase em homogeneizado total e frações citosólica e mitocondrial de córtex cerebral de ratos Wistar de 21 dias de idade.

Nós também fizemos estudos cinéticos e investigamos o efeito da glutationa reduzida, um protetor natural de grupos tiólicos, e cisteamina, a droga usada para o tratamento da cistinose, sobre a atividade da creatinaquinase.

Os resultados mostraram que a cistina inibe a atividade enzimática não-competitivamente de uma maneira tempo e dose dependente. Resultados mostram ainda que a glutationa preveniu e reverteu parcialmente a inibição causada pela cistina, enquanto a cisteamina preveniu e reverteu completamente aquela inibição, sugerindo que a cistina inibe a atividade da creatinaquinase por interação com os grupos sulfidrílicos da enzima.

Devido ao envolvimento da creatinaquinase na homeostasia energética do córtex cerebral, estes resultados sugerem um possível mecanismo para a toxicidade da cistina e também um possível efeito benéfico no uso da cisteamina em pacientes cistinóticos.

ABSTRACT

Cystinosis is a disorder associated with lysosomal cystine accumulation caused by defective cystine efflux. Cystine accumulation provokes a variable degree of symptoms depending on the involved tissues. Cortical atrophy is a possible consequence of cystine accumulation in brain cortex. However, the mechanisms by which cystine is toxic to the tissues are far from be understood.

Considering that creatine kinase is a thiolic enzime crucial for energy homeostasis, and disulfides like cystine may alter thiolic enzymes by thiol/disulfide exchange, the main objective of the present study was to investigate the effect of cystine on creatine kinase activity in total homogenate, cytosolic and mitochondrial fractions of the brain cortex from 22-day-old Wistar rats. We also performed kinetic studies and investigated the effects of reduced gluthatione, a biologically-occurring thiol group protector, and cysteamine, the drug used for cystinosis treatment, on creatine kinase activity.

Cystine inhibited the enzyme activity non-competitively in a dose- and time-dependent way. Results also shown that gluthatione partially prevented and reversed the inhibition caused by cystine, and cysteamine fully prevented and reversed this inhibition, suggesting that this substance inhibits creatine kinase activity by interaction with the sulfhydryl groups of the enzyme.

Considering that creatine kinase is a crucial enzyme for brain cortex energy homeostasis, these results provide a possible mechanism for cystine toxicity and also a possible new beneficial effect for the use of glutathione in cystinotic patients.

LISTA DE ABREVIATURAS

Adenosina difosfato.....	ADP
Adenosina Monofosfato cíclico.....	AMPc
Adenosina trifosfato.....	ATP
Cisteamina.....	CysN
Creatina.....	Cr
Creatinaquinase cerebral.....	B-CK
Creatinaquinase mitocondrial.....	Mi-CK
Creatinaquinase muscular.....	M-CK
Creatinaquinase.....	CK
Fosfocreatina.....	PCr
Glutationa reduzida.....	GSH
Sódio, potássio, adenosina trifosfatase.....	Na^+, K^+-ATPase

APRESENTAÇÃO

A dissertação está estruturada da seguinte forma:

A Introdução é uma revisão da literatura mais ampla do que a que está fazendo parte do artigo submetido, no qual a introdução deve ser necessariamente reduzida.

A parte de Material e Métodos, resultados e discussão apresentados encontram-se no artigo submetido.

As Conclusões apresentadas ao final da dissertação correspondem às do artigo submetido, apenas organizadas e numeradas.

As Perspectivas representam possibilidades de continuidade do presente trabalho, algumas em andamento, outras ainda a serem equacionadas para posterior implementação.

As Referências Adicionais correspondem, em sua quase totalidade, a referências bibliográficas que não constam no artigo submetido.

1. INTRODUÇÃO

Sir Archibald Garrod propôs, em 1908, o termo erros inatos do metabolismo (EIM) 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 uma doença subseqüente (Bickel, 1987).

Já foram descritos mais de 500 erros inatos do metabolismo até o momento (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 à morte neonatal. Os erros inatos do metabolismo graves, geralmente se manifestam na infância, sendo que os sinais e os sintomas encontrados são semelhantes aos de muitas doenças infantis.

Os erros inatos do metabolismo são raros, quando considerados individualmente, mas relativamente freqüentes em seu conjunto, podendo ocorrer aproximadamente um em cada mil recém-nascidos vivos.

1.1.Cistinose e cistina

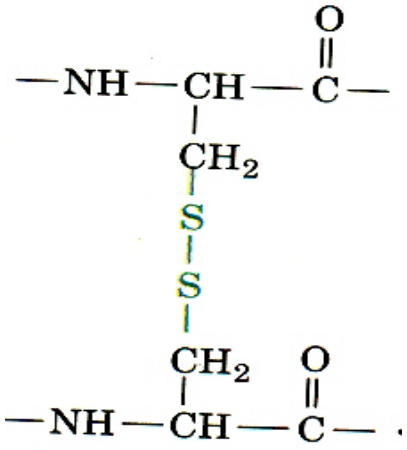
Cistinose é um erro inato do metabolismo, causada por um gene autossômico recessivo (CTNS), com perda de atividade da proteína de membrana cistinosina, responsável pelo efluxo de cistina dos lisossomos. Assim, o aminoácido cistina fica acumulado nos lisosomos de diversos tecidos, entre eles o cérebro, formando cristais(Gahl, 2001).

Schneider et al (1967) encontraram uma concentração de cistina livre seis vezes acima do normal em leucócitos de pais de pacientes com Síndrome de Fanconi, causada pelo acúmulo de cistina nos túbulos renais.

Jonas et al (1982) demonstraram que o efluxo de cistina dos lisossomos é dependente da próton-ATPase e nos lisossomos de células cistinóticas, o efluxo é anormal e não responde à próton-ATPase.

Gahl (1992) estudou lisossomos isolados de leucócitos e verificou um defeito no transporte de cistina através da membrana lisossômica, em pacientes com cistinose.

A cistina é um dissulfeto que provém da cisteína, através de um processo de oxidação.



cistina

A cisteína é um precursor na síntese protéica e um produto da hidrólise protéica. A transulfuração da metionina, que é um aminoácido essencial, gera homocisteína que, combinada com a serina forma cistationina e, através da enzima cistationase, forma cisteína, um precursor da cistina (esquema abaixo):

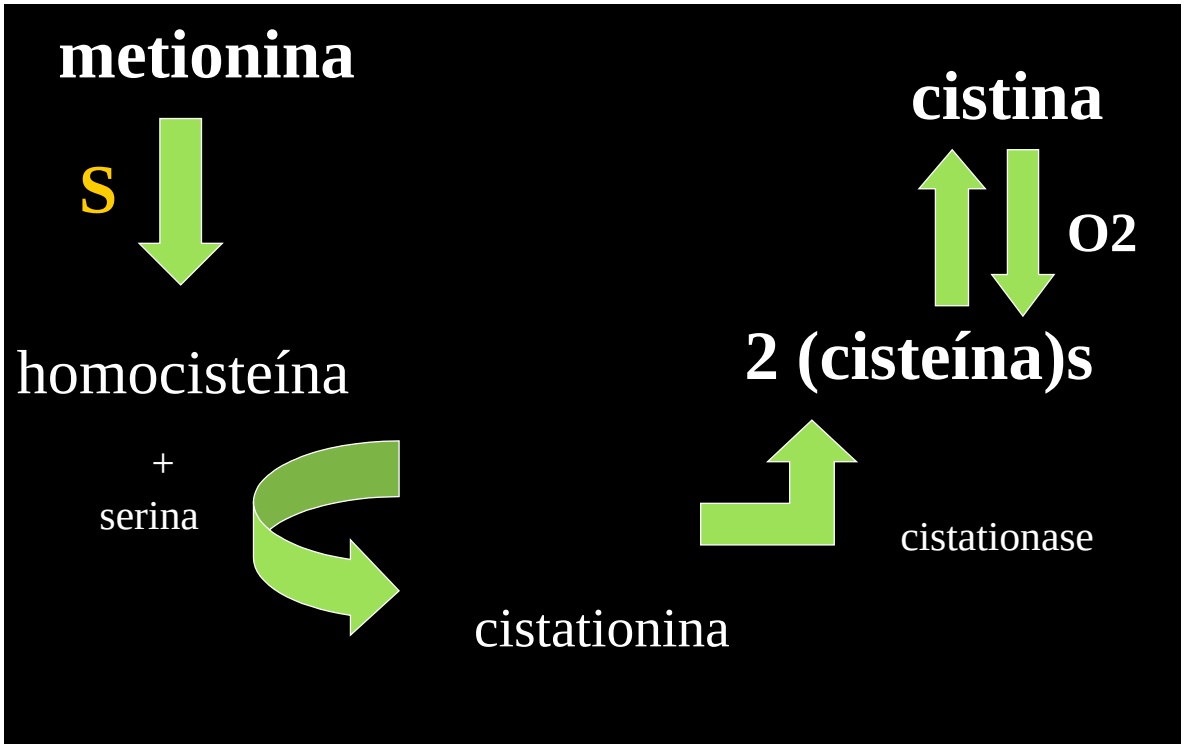


Fig.1. Metabolismo da cistina a partir da metionina.

A cistationase está ausente ou insuficiente na homocistinúria ou cistationinúria, em fetos e neonatos. Nestes pacientes, a cisteína torna-se um aminoácido essencial, já que sua produção não ocorre ou ocorre de maneira deficiente.

A cistina, ao sair dos lisossomos, combina-se com a glutationa do meio citosólico, é reduzida e forma cisteína. O mesmo resultado é obtido com a combinação de cistina e cisteamina dentro dos lisossomos(Gahl, 2001).

1.2. Lisossomos

Os lisossomos são organelas vesiculares, formadas pelo aparelho de Golgi e que formam um sistema digestivo intracelular com enzimas proteolíticas em seu interior. Entre suas diversas funções, armazenam aminoácidos, que provém da hidrólise protéica, tais como cistina, que pode ser usada na síntese *de novo* (Guyton 1992).

Na cistinose, a cistina acumula-se nos lisossomos, formando cristais nos rins, pulmões, pâncreas, fígado, intestino, apêndice, conjuntiva, córnea, retina, baço, células sangüíneas, ossos, tireóide, timo, músculos, placenta, meninges, gengiva. Estes cristais podem acumular-se até 1000 vezes acima do normal e de maneira diferenciada nos

tecidos. A concentração de cistina no plasma e urina é normal, bem como sua absorção pelo intestino (Gahl, 2001).

A cisteamina, um aminotiol, usada no tratamento da cistinose, depleta a cistina da seguinte forma (Kimonis et al, 1995): a cistina fica estocada no lisossomo por causa de um defeito no carreador de cistina na membrana lisossômica. A cisteamina, que tem um grupo amino neutro, precisa adquirir uma carga positiva para entrar no lisossomo. Então ela entra através de um transportador específico. Reage com a cistina, produzindo cisteína e forma um dissulfeto cisteamina-cisteína, que sai do lisossomo através de um transportador de lisina. A cisteína isolada sai do lisossomo por um carreador próprio. A glutationa do citoplasma reduz o complexo cisteamina-cisteína a cisteamina e cisteína. Cada ciclo destes remove 1 mol de meia-cistina por mol de cisteamina (esquema abaixo):

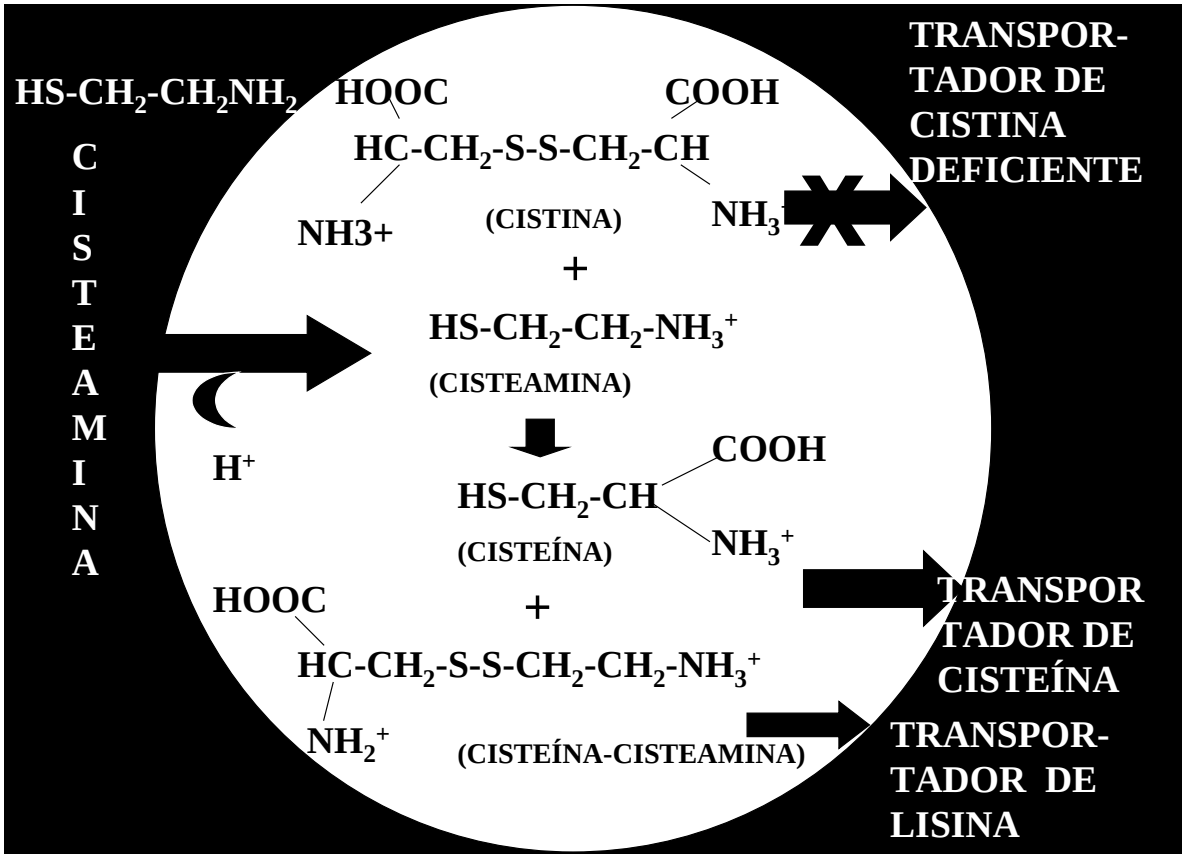


Fig. 2. Remoção da cistina do lisossomo pela cisteamina.

1.3. Genética

Todas as formas de cistinose são autossômicas recessivas; na relação homem/mulher próximo a 1. A incidência da cistinose na população em geral é de 1: 200.000. Na França, a incidência estimada é muito maior na Bretanha (1:26.000) do que no resto da população (1:320.000) (Bois et al,1976). Numa investigação no Centro Nefrológico Pediátrico do Canadá, Gahl et al (1988) observaram que 32 dos 51 pacientes com cistinose eram do Quebec e destes a maioria significativa era franco-canadense. De Braekeleer (1991) registrou a grande freqüência gênica (1:39) para cistinose em franco-canadenses do Quebec. Através de estudos de ligação em cistinóticos franco-canadenses, McGowan-Jordan et al (1999) identificaram o haplotipo presente em aproximadamente metade (21:40) dos cromossomos estudados. Em análise mutacional, foram encontradas duas novas mutações, previamente identificadas em famílias irlandesas (mas não em francesas).

McDowell et al(1995) mapearam o gene da cistinose no braço curto do cromossomo17 pela ligação de marcadores de microsatélite. A localização específica do gene CTNS é 17p 13.

Town et al(1998) encontraram o lócus marcado D17S829 deletado homozigoticamente em 23 dos 70 pacientes com cistinose nefropática e eles mapearam o novo gene CTNS.

Attard et al(1999) verificaram que a cistinosina é a proteína integral de membrana lisossomal, codificada pelo gene CTNS. Eles identificaram novas mutações no gene CTNS, que causavam ruptura nos domínios transmembrana e perda da função protéica.

Forestier et al(1999) descreveram um ensaio de PCR que detecta acuradamente deleções homozigóticas e heterozigóticas.

O gene CTNS, localizado na 4-cM região do cromossomo 17p 13, com 12 éxons, codifica os 367 aminoácidos da proteína cistinosina. Esta proteína tem 7 domínios transmembrana, um dipeptídeo gY próximo do C terminal e 8 resíduos para glicosilação. Várias formas de cistinose são alélicas. Há 11 mutações do gene, produzindo formas variadas para a doença, das quais as formas nefropática e não-nefropática são as principais. A forma nefropática caracteriza-se pela síndrome de Fanconi(falência renal)

já na infância. A forma não-nefropática caracteriza-se por problemas oculares, na fase adulta, sem apresentar problemas renais(Gahl, et al, 2001).

1.4.Aspectos clínicos, diagnóstico e tratamento

Sensenbrenner et al (1974), Hurley e Liu (1977), e Lucky et al (1977) relataram hipoteoidismo, decorrente dos depósitos de cistina na tireóide, como um importante fator no retardo do crescimento na cistinose.

Jonas et al (1987) descreveram pacientes com cistinose com baixa estatura, fotofobia, córneas e conjuntiva carregadas com material refratário e retinopatia; falência ovariana, confusão intermitente, perda da memória de curto prazo e atrofia carebral. Eles também observaram acúmulo de cistina em células pancreáticas, aorta, ovários e cérebro.

Gahl et al (1988) registrarm miopatia com perda muscular generalizada e acúmulo de cistina nas fibras musculares.

Pacientes cistinóticos são aparentemente normais ao nascerem, exceto pela pouca pigmentação na pele e nos cabelos. Aos 6 meses de idade apresentam baixo peso, deficiência de crescimento, pouco apetite, agitação, diurese e ingestão de água excessivas, episódios de acidose e desidratação. Há fotofobia, a função renal é rapidamente perdida (Síndrome de Fanconi), diabete melito, e aos 9 anos em média já necessitam de transplante renal (Gahl, 2001).

Williams et al (1994) verificaram que crianças com cistinose apresentavam QI significantemente abaixo de seus irmãos e pais.

Existe um envolvimento cerebral severo com necrose cística multifocal e vacuolizações que produzem profundos déficits neurológicos. A microscopia eletrônica do cérebro documenta deposição citoplasmática de cristais de cistina dentro de células da substância branca (Vogel, 1990). Outros achados incluem depósitos de cistina no sistema nervoso central, atrofia cerebral, convulsões, tremores, retardo mental, desmielinização da ponte; déficits da memória visual de curta duração, reconhecimento tátil e soletração (Gahl et al,2001). Alterações cerebelares, sinais piramidais, deterioração mental e finalmente, paralisia pseudo-bulbar definem a cistinose encefalopática A outra forma de cistinose engloba convulsões com coma e hemiplegia (Broyer et al, 1996). São ainda descritas hipotonia, dificuldades de deglutição e dificuldades da marcha.

Neuroimagens mostram calcificações e alterações na substância branca (Colombo et al, 1999).

O diagnóstico é feito pela dosagem de cistina em tecidos, células sangüíneas, placenta, ou por exame oftalmológico (Gahl et al, 2001). Schneider et al (1974) mostraram que a cistinose pode ser diagnosticada na 18ª semana de vida num exame de cultura de células do líquido amniótico. Smith et al (1987) registraram diagnóstico de cistinose num feto de 9 semanas pela medida direta de cistina em vilosidades coriônicas.

A terapia é sintomática, com reposição de eletrólitos, hemodiálise ou transplante renal, consumo abundante de água, suplementação vitamínica, medicação apropriada, especialmente com cisteamina(via oral) e colírios à base de cisteamina para dissolver cristais de cistina nas córneas (Gahl, 2001).

Kaiser-Kupfer et al (1987) trataram crianças afetadas com colírio à base de cisteamina e observaram que depois de 4 meses os cristais de cistina na córnea haviam se desfeito. Também Gahl et al (1987) usaram cisteamina via oral em crianças cistinóticas por um período de 73 meses e observaram que a depleção de cistina nos leucócitos foi de 82%.

Conforme Markello et al (1993) concluíram, crianças com cistinose tratadas precocemente (antes dos dois anos de vida) e adequadamente com cisteamina têm suas funções renais aumentadas durante os primeiros cinco anos de vida e que depois declinam para uma taxa normal, enquanto aqueles que não seguem rigorosamente o tratamento, ou o iniciam numa época posterior, não têm resultados tão bons.

Gahl et al (2002) observaram que muitos pacientes passaram da terceira década de vida sem precisar de transplante renal por seguirem o tratamento com cisteamina antes de aparecerem os sintomas.

Kimonis et al (1995) indicam que o tratamento com cisteamina beneficia também a tireóide e que este tratamento previne complicações pós-transplante renal em pacientes com cistinose.

1.5.Creatinaquinase (CK)

A creatinaquinase regenera ATP a partir de ADP e fosfocreatina (PCr) e está

presente em tecidos e células com alta e flutuante demanda energética, tais como músculos, cérebro, coração, retina, espermatozóides, (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).

Há formas citosólicas homodiméricas: (M-CK), encontradas no músculo, e (B-CK) encontrada no cérebro. Elas formam dímeros MM,MB,BB-CK. Há formas mitocondriais homooctaméricas: ubíqua (uMi-CK) e sarcomérica (sMi-CK), que se localizam no espaço intermembrana da mitocôndria (Wyss et al, 1992). Estas isoformas estão acopladas funcionalmente à Na^+, K^+ ATPase (Blum et al, 1991).

Ratos deficientes em B-CK (B-CK-/-) têm uma grande área fibrosa intra e infrapiramidal, diminuição na habituação em campo aberto. No labirinto aquático, ratos adultos B-CK-/- mostraram-se lentos para aprender (Jost 2002).

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 e Wallimann, 1993). O encéfalo tem a capacidade de sintetizar a 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).

O processo de mielinização, bem como as demais atividades do sistema nervoso dependem da maturação funcional da CK durante o desenvolvimento do encéfalo do rato com expressão seqüencial das izoenzimas (Holtzman et al, 1993), da regulação da expressão da B-CK via AMPc nas células gliais (Kuzhikandathil & Molloy, 1994) e da atividade significativa da B-CK nos neurônios, astrócitos e oligodendrócitos (Manos et al, 1991).

Encontramos altos níveis de PCr e Cr em músculos, coração, espermatozóides, células fotorreceptoras da retina. Níveis intermediários são encontrados no cérebro, tecido adiposo marrom, intestino, vesícula seminal e seu fluido.

Creatina e seus precursores têm efeitos antitumorais, antivirais e antidiabéticos e protegem tecidos de hipóxia e isquemia e danos musculares e neurodegenerativos.

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 e Lipton, 1981) e a adição de creatina aumenta os níveis de PCr, reduzindo a queda de ATP, a liberação de Ca^{++} e a morte celular (Carter et al, 1995).

A atividade da enzima CK está severamente reduzida em diversas doenças neurodegenerativas (David et al, 1998; Aksenov et al, 2000).

Como a cistinose está associada a lesões cerebrais, cujos mecanismos são pouco conhecidos, a administração de cistina provoca alterações em alguns parâmetros neuroquímicos associados à homeostasia energética, e o sistema CK/PCr é fundamental para a manutenção das atividades nervosas que se encontram alteradas na cistinose, torna-se importante estudar os efeitos da cistina sobre a atividade da CK.

2. OBJETIVOS

1- Verificar o efeito *in vitro* de diferentes concentrações de cistina sobre a atividade da creatinaquinase, em diferentes tempos de pré-incubação em homogeneizado total e nas frações citosólica e mitocondrial de córtex cerebral de ratos Wistar de 21 dias de idade pós-natal.

2- Verificar a possível prevenção e/ou reversão do efeito da cistina com cisteamina e glutationa reduzida em córtex cerebral de ratos jovens em homogeneizado total, citosol e mitocôndria.

3- Determinar o tipo de inibição enzimática causada pela cistina através de estudos de competição realizados de acordo com Lineweaver e Burk.

3.ARTIGO

ARTIGO

Rochele M. M. Fleck, Valnes Rodrigues Junior, Daiane Parissotto, Juliana Giacomazzi, Carlos S. Dutra Filho, Ângela T. S. Wyse, Moacir Wajner, Clovis M. D. Wanmacher. ***Prevention and reversal by cysteamine of the inhibition of creatine kinase activity caused by cystine in rat brain cortex.*** Neurochemistry International (enviado para publicação).

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Montreal, 18th August, 2004

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Cysteamine prevents and reverses the inhibition of creatine kinase activity caused by cystine in rat brain cortex.

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Running title: cystine inhibits creatine kinase

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Abstract

Cystinosis is a disorder associated with lysosomal cystine accumulation caused by defective cystine efflux. Cystine accumulation provokes a variable degree of symptoms depending on the involved tissues. Adult patients may present brain cortical atrophy. However, the mechanisms by which cystine is toxic to the tissues are not fully understood. Considering that brain damage may be developed by energy deficiency, creatine kinase is a thiolic enzyme crucial for energy homeostasis, and disulfides like cystine may alter thiolic enzymes by thiol/disulfide exchange, the main objective of the present study was to investigate the effect of cystine on creatine kinase activity in total homogenate, cytosolic and mitochondrial fractions of the brain cortex from 21-day-old Wistar rats. We performed kinetic studies and investigated the effects of GSH, a biologically-occurring thiol group protector, and cysteamine, the drug used for cystinosis treatment, to better understand the effect of cystine on creatine kinase activity. Results shown that cystine inhibited the enzyme activity non-competitively in a dose- and time-dependent way. GSH partially prevented and reversed CK inhibition caused by cystine and cysteamine fully prevented and reversed this inhibition, suggesting that cystine inhibits creatine kinase activity by interaction with the sulfhydryl groups of the enzyme. Considering that creatine kinase is a crucial enzyme for brain cortex energy homeostasis, these results provide a possible mechanism for cystine toxicity and also a new possible beneficial effect for the use of cysteamine in cystinotic patients.

Key words: cystinosis, cystine, cysteamine, glutathione, creatine kinase, brain cortex.

1- Introduction

Cystinosis is an autosomal recessive disorder associated with lysosomal cystine accumulation secondary to defective lysosomal cystine efflux (Gahl et al, 2001, Haq et al, 2002). The affected gene (CTNS) encodes cystinosin, the lysosomal membrane protein responsible for cystine transport out of lysosomes (Kalatzis et al, 2001). The nephropathic or infantile type of cystinosis is responsible by renal failure before 12 years of age and other systemic complications in non-treated cystinotic children (Anikster et al, 1999). Patients affected by this disease accumulate cystine in most tissues developing a variable degree of symptoms depending on the involved tissues. The treatment initiated in the first 2 years of age with the aminothiol cisteamina (2-mercaptoethylamine), which causes parenchymal organ cystine depletion (Gahl et al, 1992) allows to delay the evolution towards end stage renal disease (Broyer et al, 2000). Surviving adult patients may present brain cortical atrophy with low cognitive performance (Nichols et al, 1990), intermittent confusion, short-term memory loss (Jonas et al, 1987), and subtle visual processing impairments (Ballantyne et al, 1997). There are severe cerebral involvement with multifocal cystic necrosis, dystrophic calcification, spongy change, and vacuolization that may produce profound neurological deficits (Vogel et al, 1990).

The mechanisms by which cystine is toxic to the tissues are not fully understood. However, it is well known that some enzyme activities may be altered by thiol/disulfide exchange between protein sulfhydryl groups and biologically-occurring disulfides (Gilbert, 1984; Ziegler, 1985). Considering that cystine is a disulfide, it is possible that it may act on intracellular thiol-enzymes, modifying cell function.

Creatine kinase (CK, EC 2.7.3.2) plays a key role in energy metabolism of tissues with intermittently high and fluctuating energy requirements, such as skeletal and cardiac muscle, and neuronal tissues like brain and retina. CK catalyses the reversible transfer of the phosphoryl group from phosphocreatine to ADP, regenerating ATP. There are distinct CK isoenzymes, which are compartmentalized specifically in the places where energy is produced (mitochondria) or utilized (cytosol) (Wallimann et al, 1992; Wallimann et al, 1998; Wyss et al ,1992). CK-BB, sampled on the first day of life, is of limited value in predicting severe brain damage after birth asphyxia (Scotto and Stralin, 1977) because uMtCK expression in neurons is developmentally regulated in post-natal life, constitutively restricted in the adult brain, and regulated by activity in the cortex and hippocampus. This implies that mitochondrial synthesis

of phosphocreatine is restricted to those neurons that express uMtCK and may contribute to protect these cells during periods of increased energy demand (Spear et al, 1989).

There are several reports implicating reduced CK activity in the brain in many diseases affecting brain function. CK BB level is decreased in brain of patients with schizophrenia (Burbaeva et al, 2003). Branched-chain amino acids known to accumulate in maple syrup urine disease inhibit the CK activity in brain cortex of rats (Pilla et al , 2003; Pilla et al , 2003a). Other metabolites known to accumulate in metabolic disorders, such as phenylalanine (phenylketonuria), ethylmalonic acid (short-chain acyl-CoA dehydrogenase deficiency), proline (type II hyperprolinemia), D-2-hydroxyglutaric acid (D-2-hydroxyglutaric aciduria), and L-2-Hydroxyglutaric acid (L-2-hydroxyglutaric aciduria) also inhibit CK activity in brain cortex of rats (Costabeber et al ,2003; Leipnitz et al, 2003; Kessler et al, 2003; da Silva et al, 2003; da Silva et al, 2004).

Therefore, considering that CK is a thiol-enzyme, and cystine is a biologically-occurring disulfide, the main objective of the present study was to investigate the effect of cystine on CK activity in the brain cortex of developing rats. We investigated the effects of reduced glutathione (GSH), a thiol group protector, and cysteamine, on the enzyme activity. In an attempt to better characterize the inhibition cystine caused in CK activity, we also performed kinetic studies.

2. Experimental procedures

2.1 Subjects and reagents

Twenty-one–day-old Wistar rats bred in the Department of Biochemistry, UFRGS, were used in the experiments. Rats were kept with dams until they were sacrificed. The dams had free access to water and to a standard commercial chow (Germani, Porto Alegre, RS, Brazil) containing 20.5% protein (predominantly soybean supplemented with methionine), 54% carbohydrate, 4.5% fiber, 4% lipids, 7% ash and 10% moisture. Temperature was maintained at 24 ± 1 °C, with a 12-12 h light-dark cycle. The “Principles of Laboratory Animal Care” (NIH publication 85-23, revised 1985) were followed in all the experiments, and the experimental protocol was approved by the 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.

2.2 Preparation of brain cortex tissue

The animals were killed by decapitation and the brain cortex was removed and immediately placed on an ice-cooled glass plate. Time elapsed between decapitation and dissection was less than 1 min. After dissection, the brain cortex was washed in SET buffer (0.32 M sucrose / 1 mM EGTA / 10 mM Tris-HCl, pH 7.4), minced finely and homogenized in the same SET buffer (1: 20 (w/ v) with a Potter-Elvehjem glass homogenizer. The homogenate was collected for determination of total homogenate CK activity. A portion of 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.

2.3 Protein determination

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

2.4 Enzyme assay

The reaction mixture contained the following final concentrations: 60 mM Tris-HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM $MgSO_4$, 0.3 % Triton X-100, and approximately 1 μg protein in a final volume of 0.1 mL. After 0, 5, 30 and 60 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 or cysteamine. 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. For the experiments to test the influence of GSH or cysteamine on the effects caused by cystine, GSH or cysteamine was added before or after pre-incubation of the enzymatic material (homogenate, cytosol or mitochondrial fraction) for 0, 30 and 60 min in the presence of cystine. Results were expressed as μmol of creatine formed per min per mg protein.

The competition studies between cystine and the enzyme substrates ADP and phosphocreatine were performed according to Lineweaver-Burk (1934). Phosphocreatine and ADP concentrations were chosen to assure linearity of creatine kinase activity and of cystine inhibition.

2.5.Statistical analysis

Data were analyzed by one-way ANOVA or multivariate analysis followed by the Tukey test when the F values were significant. Linear regression was used to evaluate dose or time response. All data were analyzed through the Statistical Package for Social Sciences inserted in an IBM-compatible PC computer.

3. Results

First, cystine was added 1, 2.5, and 5 mM final concentrations in the creatine kinase assay of total homogenate, cytosolic, and mitochondrial fractions, and preincubated for 5 min. The results showed that cystine inhibited significantly CK activity in all tested concentrations in a dose-dependent way: $F(1,30) = 52.15$; $\beta = -0.80$; $p < 0.0001$ (total homogenate); $F(1,30)=57.63$; $\beta= -0.81$; $p<0.0001$ (cytosolic fraction); $F(1, 30)= 57.37$; $\beta= -0.81$; $p <0.0001$ (mitochondrial fraction (Fig 1).

Then, cystine was added to the incubation medium of the cytosolic or the mitochondrial fraction at 0.1mM, 0.2 mM, or 0.5 mM final concentrations and pre-incubated for 0, 30 and 60 min. The results showed that cystine inhibited CK activity in all tested concentrations in a time-dependent way in cytosolic fraction ($F(1,16)=25.92$; $\beta= -0.78$; $p<0.001$ for cystine 0.1mM; $F(1,16)=35.45$; $\beta= -0.83$; $p<0.0001$ for cystine 0.2mM; $F(1,15)=127.25$; $\beta= -0.94$; $p<0.0001$ for cystine 0.5mM), and in mitochondrial fraction ($F(1,19)=33.88$; $\beta=-0.8$, $p<0.0001$ for cystine 0.1mM; $F(1,19)=37.26$; $\beta= -0.81$, $p<0.0001$ for cystine 0.2mM; $F(1,15)= 51.89$; $\beta= -0.88$; $p<0.0001$ for cystine 0.5mM) (Fig 2).

Considering that CK is a thiol-enzyme, and cystine is a biologically-occurring disulfide, we investigated the effects in vitro of reduced glutathione (GSH), a thiol group protector. We also investigated whether the inhibition caused by cystine on CK activity was reversible or not, by adding GSH 0.5mM on the enzyme activity assay of cytosolic or mitochondrial fractions, without pre-incubation or before or after 30 or 60 min of pre-incubation. Our results showed that cystine inhibited CK activity, whereas GSH partially prevented and reversed the CK inhibition caused by cystine in cytosolic fraction pre-incubated for 30 min ($F(5.30)=74.8$; $p<0.0001$) or 60 min ($F(5.30)=75.7$; $p<0.0001$), and in mitochondrial fraction pre-incubated for 30 min ($F(5.36)=36.43$; $p<0.0001$) or 60 min ($F(5.36)=40.3$; $p<0.0001$) (Fig 3).

Considering that cysteamine is a thiol-compound like GSH, we also investigated its effect on the inhibition of CK activity caused by cystine in cytosolic or mitochondrial fractions, adding cysteamine 1 or 2 mM, before or after pre-incubation for 30 min with cystine 0.2mM. Our results showed that 0.2 mM cystine inhibited CK activity in cytosolic fraction in the experiment with cysteamine 1 mM ($F(5,30)=7.02$; $p<0.01$) or 2 mM ($F(5,30)=8.77$; $p<0.01$). The same inhibition was observed in mitochondrial fraction in the experiment with cysteamine 1 mM ($F(5,30)=8.77$; $p<0.01$) or 2 mM ($F(5,30)=8.97$; $p<0.01$). In all experiments, cysteamine prevented and reversed CK inhibition caused by cystine (Fig 4).

In an attempt to better characterize the inhibition of CK activity caused by cystine, competition studies between cystine and the enzyme substrates ADP and phosphocreatine were performed at 30 min of pre-incubation, according to Lineweaver-Burk. The double-reciprocal plots showed that the inhibition caused by cystine on CK activity was of the noncompetitive type for the two substrates, reinforcing the results obtained with GSH and cysteamine. (Fig 5 and 6).

4. Discussion

Cystinosis is caused by a severe deficiency of CTNS gene, resulting in defective

activity of cystinosin, a protein responsible for the transport of cystine out of lysosomes. Although tissue damage might depend on cystine accumulation in the affected tissues, the mechanisms of cystine toxicity are still obscures. In a study with infantile nephropathic cystinosis underwent cognitive testing (Jonas et al, 1987), ten of 11 patients demonstrated cortical atrophy, like children with nephropathic cystinosis who had renal transplantation (Ehrlich et al, 1991). Visual processing and academic difficulties may reflect a common mechanism of selective cortical damage in children and young adults with infantile nephropathic cystinosis (Ballantyne et al, 1997). Children with cystinosis have visuospatial difficulties, possibly as a result of cystine accumulation in the brain over time (Scarvie et al, 1996) and findings suggest that cystinosis has a differential effect on the two cortical visual processing streams, with spatial functions affected to a greater extent than perceptual functions (Ballantyne et al, 2000).

Cystine is usually considered to be completely isolated from the cytosol within lysosomes. However, the mechanisms by which cystine accumulated in lysosomes causes cell and tissue damage is far from be understood. It is possible that the small cystine crystals may partially disrupt the lysosomal membranes, since positive reaction for acid phosphatase, a lysosomal marker, was found to be localized at the periphery of the cystine crystals only in some of them (Koisumi et al, 1985). Besides, ultrastructural observations in the liver and in the kidney of patients with cystinosis, and in biopsies of renal allografts from patients with cystinosis showed cystine accumulation in the cytoplasm, nucleus and cytoplasmic inclusions of dark cells, and extracellularly, indicating that cystine storage may not be limited to lysosomes (Scotto and Stralin 1977, Spear et al, 1989). On the other hand, renal tubule cells loaded with cystine developed Fanconi's syndrome in rats secondary to a decrease in energy generation (Ben-Nun et al, 1993, Foreman et al 1995). Therefore, it is conceivable that CK activity could be reduced in the tissues of patients affected by cystinosis.

Deficient activities of several thiol enzymes, but not of non-thiol enzymes, in postmortem liver and kidney tissue from patients with nephropathic cystinosis was early reported (Patrick, 1965). These enzymes included glucose-6 phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, hexokinase, succinate dehydrogenase, alcohol dehydrogenase, and delta-aminolevulinate dehydrase, but creatine kinase activity was not investigated. However, it can not be excluded that these enzymes could have normal activities in the intact cells and be inhibited by cystine following lysis of the cells and partial release of

the compartmentalized cystine during the process of tissue preparatiom for enzymes activity determination (Schneider and Schulman, 1983). Considering that lysosome rupture with cell death cannot explain the Fanconi Syndrome in cystinosis, because patiens with acute and chronic renal failure by other causes do not present this syndrome, it is important to investigate other mechanisms for cell dysfunction in cystinosis. Inhibition of thiol enzymes like CK could be one of such mechanisms.

In the present study, we first investigated the effects of different concentrations of cystine at different times of pre-incubation on the activity of CK in cytosolic and mitochondrial fractions of the brain cortex from developing rats. We observed that cystine inhibited the enzyme activity in a dose and time-dependent way in all fractions. Next, we investigated the effect of GSH and cysteamine, a thiol group protector, on the inhibition caused by cystine. We observed that the inhibition cystine caused on CK was partially prevented and reversed by GSH and fully prevented and reversed by cysteamine, suggesting that cystine inhibits CK activity by progressive interaction with the sulfhydryl groups of the enzyme. In order to better characterize the enzyme inhibition, we performed kinetic competition studies between cystine and the two substrates phosphocreatine and ADP. The Lineweaver-Burk plot indicated that the inhibition caused by cystine on CK activity was of the noncompetitive type in relation to phosphocreatine and ADP, reinforcing the hypothesis of progressive action of cystine on the thiol groups of the enzyme.

The CK/ phosphocreatine system exerts several integrated functions in cells, such as temporary energy buffering, metabolic capacity, energy transfer, and metabolic control (Saks et al, 1996). This system is known to be an important metabolic regulator during health and disease (Wallimann et al,1998). Mi-CK is part of a unique temporal and spatial energy buffer system in tissues with high energy requirements, being also important to inhibit the Ca^{+2}-induced opening of the mitochondrial permeability transition pore (PTP) which leads to apoptosis (O'Gorman , 1997; Kroemer et al, 1998; Crompton , 1999; Schlattner and Wallimann 2000).

CK activity decreases after exposure to agents promoting generation of free radicals (Wolosker et al, 1996; Mekhfi et al, 1996; Stachowiak et al, 1998; Arstall et al, 1998; Konorev et al, 1998) and reagents reacting with thiols (Wolosker et al, 1996; Gross et al, 1996), probably by oxidation of the sulfhydryl residues of the enzyme. The active-site Cys-282 determines the sensitivity of the enzyme activity of cytosolic CK towards damage

produced by free radicals (Koufen and Stark, 2000). Mi_b-Ck octamers are fairly resistant structures in the presence of free radicals of water radiolysis, but dissociation of octamers into dimmers, however, permit that Cys-282 become accessible to free radical attack (Koufen et al, 1999). The enzyme protection by thiols are related with the ability of this reagents in to reduce a number of reacting free radicals and to modify the inactivation mechanism in such way that efficient repair of enzyme damage may be achieved (Koufen and Stark, 2000). CK of rat brain homogenates oxidized by Fe/ascorbic acid may be impaired directly by oxygen radicals (Horakova et al, 2002).

We also investigated the influence of GSH on the inhibitory effect caused by cystine and verified that GSH, a naturally occurring thiol-reducing agent, partially prevents and reverses this effect. On the other hand, cysteamine, a thiolic substance, fully prevented and reversed the enzyme inhibition caused by cystine. Considering that the inhibition caused by cystine was of the noncompetitive type, it is possible that the inhibition caused by cystine may be mediated by oxidation of the sulfhydryl groups of the enzyme, or sulfhydryl/disulfide exchange.

Patients affected by cystinosis may accumulate cystine in brain and develop a variable degree of neurologic dysfunction, whose pathophysiology is still unclear (Ballantyne et al, 1997). Our results indicate that cystine strongly inhibits CK activity in the cortex brain of rats. Considering that creatine kinase is a key enzyme for energy homeostasis in brain, in case this effect also occurs in the brain of patients with cystinosis, it is possible to envisage that a diminution of this enzyme activity may potentially impair energy homeostasis, contributing to the brain damage found in some patients affected by this disease. In this context, if CK inhibition also occurs in patients affected by cystinosis, it is conceivable that others important thiol-enzymes might be inhibited by cystine. Considering that cysteamine is used in patients with cystinosis because it causes parenchymal organ cystine depletion (Broyer, 2000), the present data provides a possible new beneficial effect for the use of this drug, since the protective effect of cysteamine could be important in preventing and reversing some metabolic consequences of cystine accumulation on thiol enzymes. Further studies are necessary to evaluate the activity of thiol-enzymes in patients affected by cystinosis.

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Figure 1. Effect of cystine on creatine kinase activity in total homogenate, cytosolic, and mitochondrial fractions from rat brain cortex.

The enzymatic assay was performed after 5 min pre-incubation. Data are mean ± SD of 8 independent experiments performed in triplicate. **$p<0.01$ compared to the other groups (Tukey test).

Figure 2. Effect of cystine on creatine kinase activity in cytosolic (A), and mitochondrial (B) fractions from rat brain cortex pre-incubated at different times.

Data are mean ± SD of 6 independent experiments performed in triplicate. **$p<0.01$ compared to the other groups (Tukey test).

Figure 3. Effect of GSH added before (pre) or after (post) pre-incubation for 30 min (A) or 60 min (B) on the inhibition caused by cystine on creatine kinase activity in cytosolic and mitochondrial fractions from rat brain cortex.

Data are mean ± SD of 6 independent experiments performed in triplicate. **$p<0.01$ compared to the other groups (Tukey test).

Figure 4. Effect of cystemine (CysN) added before (pre) or after (post) pre-incubation for 30 min in cytosolic(A) and mitochondrial (B) fractions on the inhibition caused by cystine on creatine kinase activity in cytosolic and mitochondrial fractions from rat brain cortex.

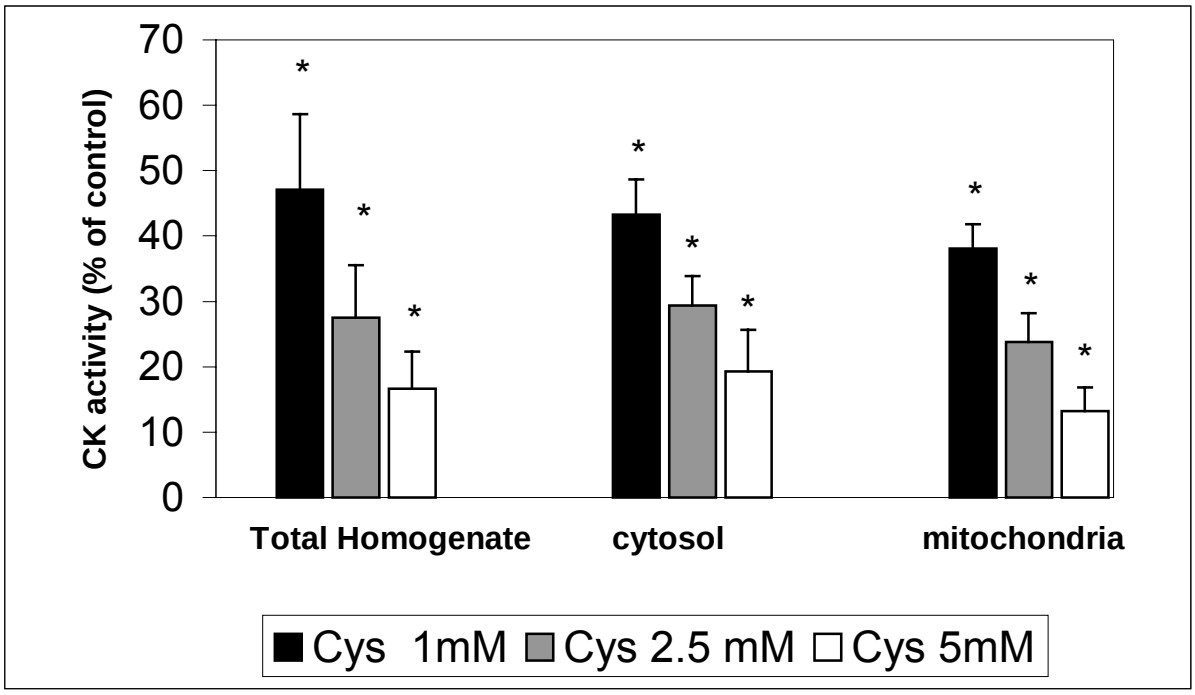
Data are mean ± SD of 6 independent experiments performed in triplicate. **$p<0.01$ compared to the other groups (Tukey test).

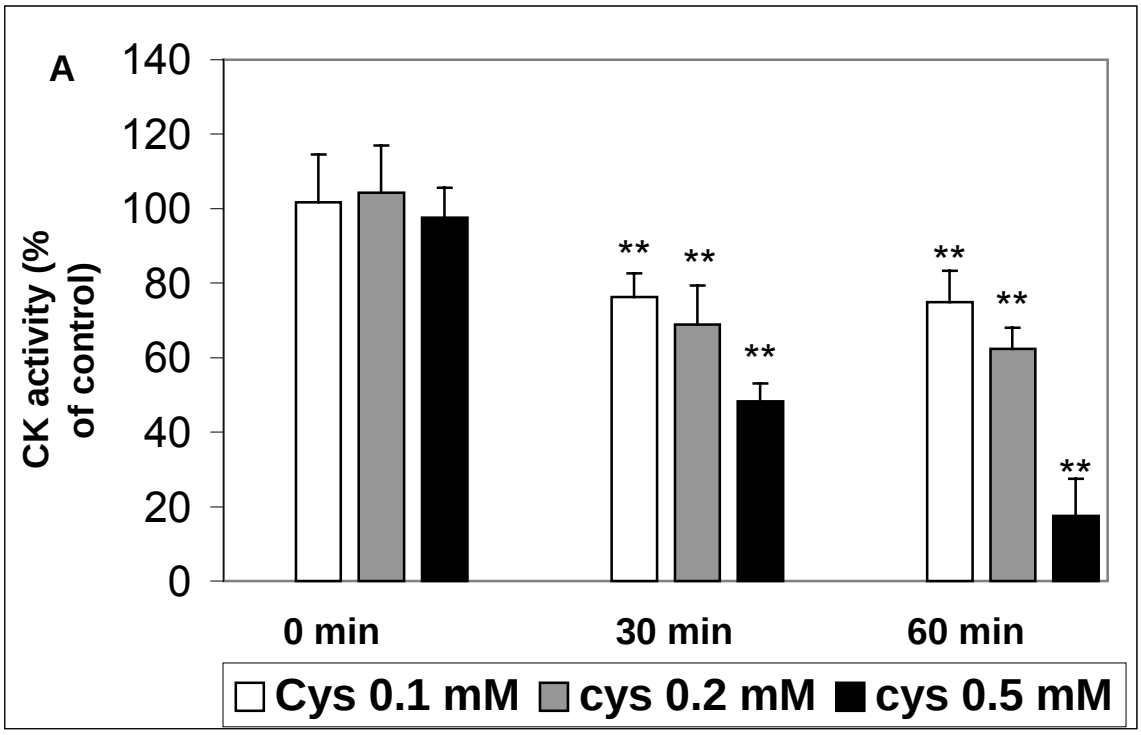
Figure 5. Lineweaver-Burk plot for cytosolic creatine kinase activity with respect to adenosine-5’-diphosphate (ADP) and phosphocreatine (PCr) at various concentrations of cystine.

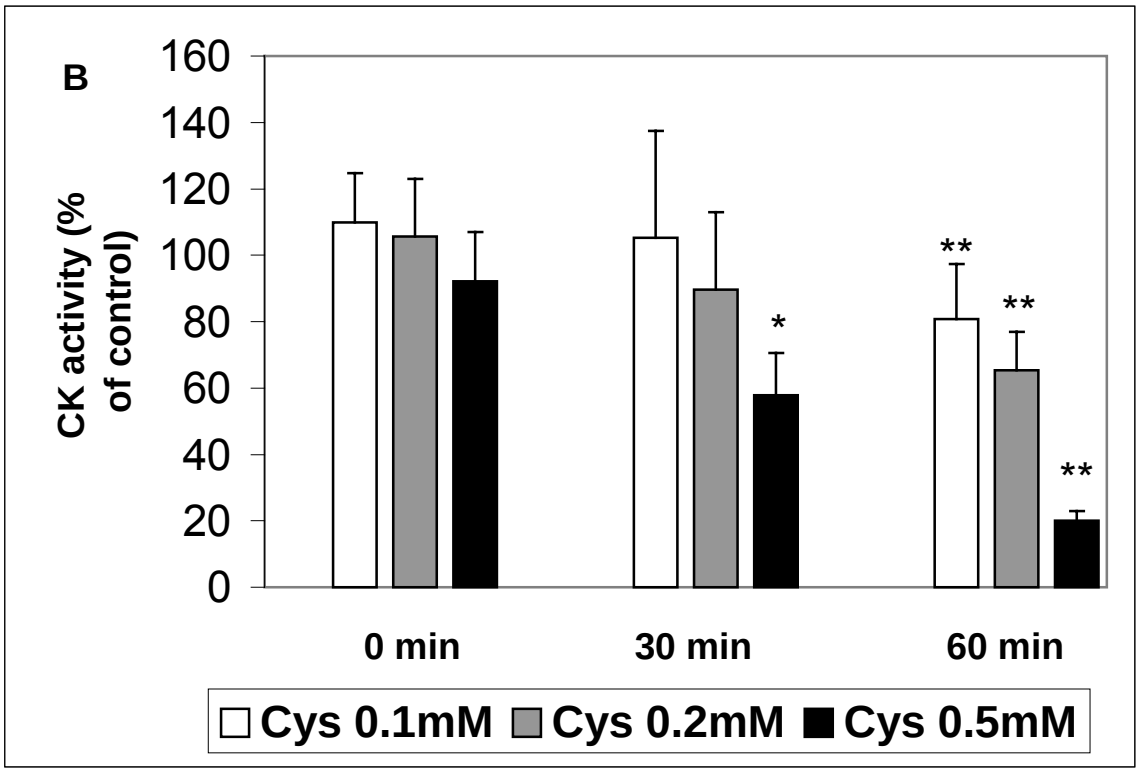
Creatine kinase activity was measured after 30 min of pre-incubation. Data are representative of at least 3 independent experiments performed in triplicate.

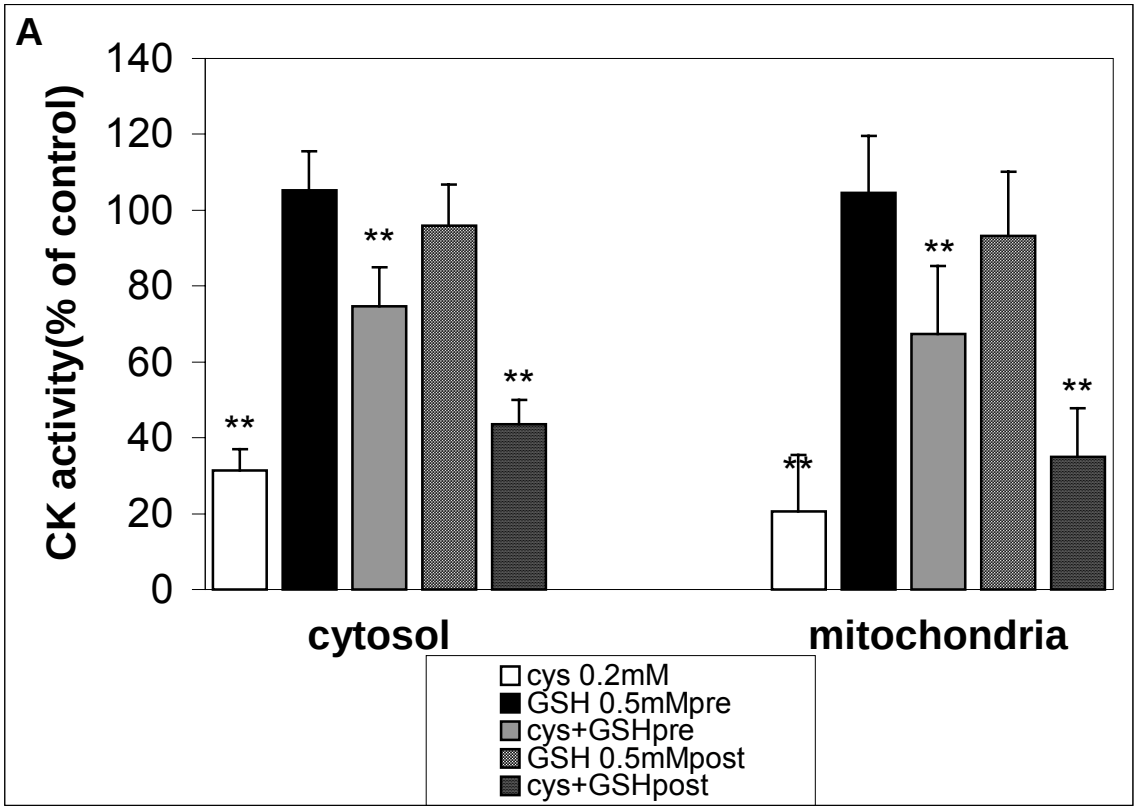
Figure 6. Lineweaver-Burk plot for mitochondrial creatine kinase activity with respect to adenosine-5’-diphosphate (ADP) and phosphocreatine (PCr) at various concentrations of cystine.

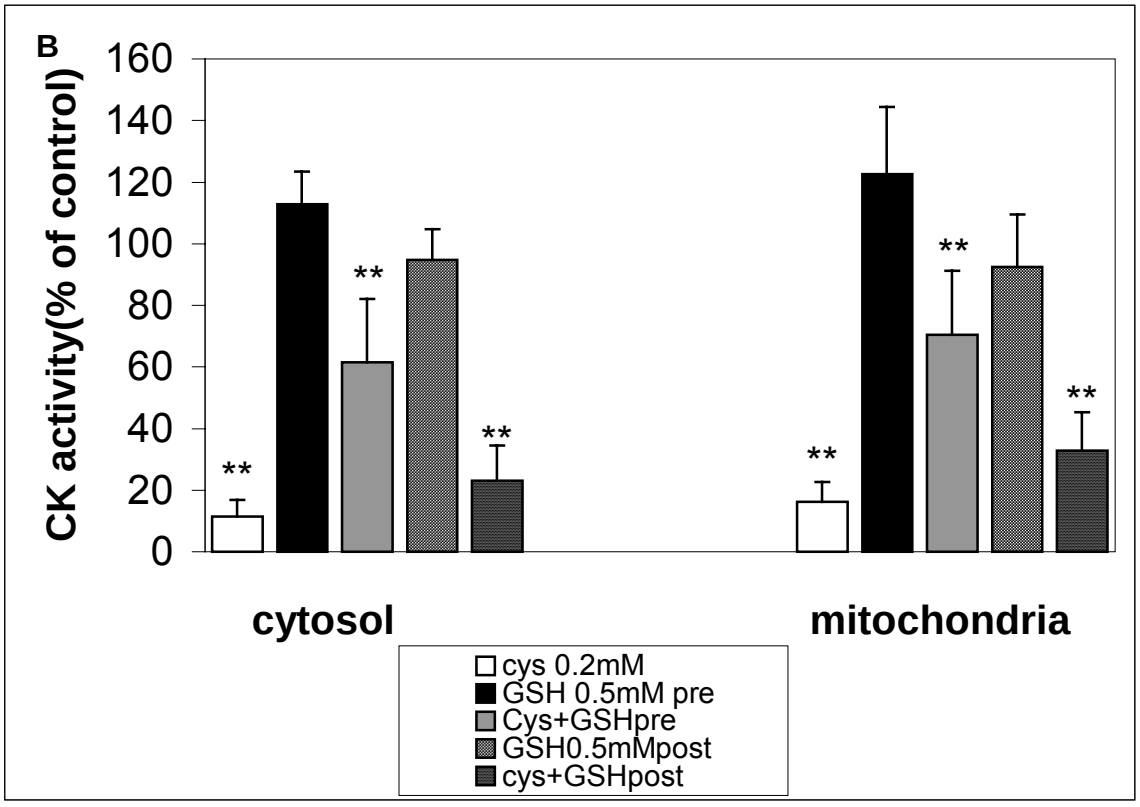
Creatine kinase activity was measured after 30 min of pre-incubation. Data are representative of at least 3 independent experiments performed in triplicate.

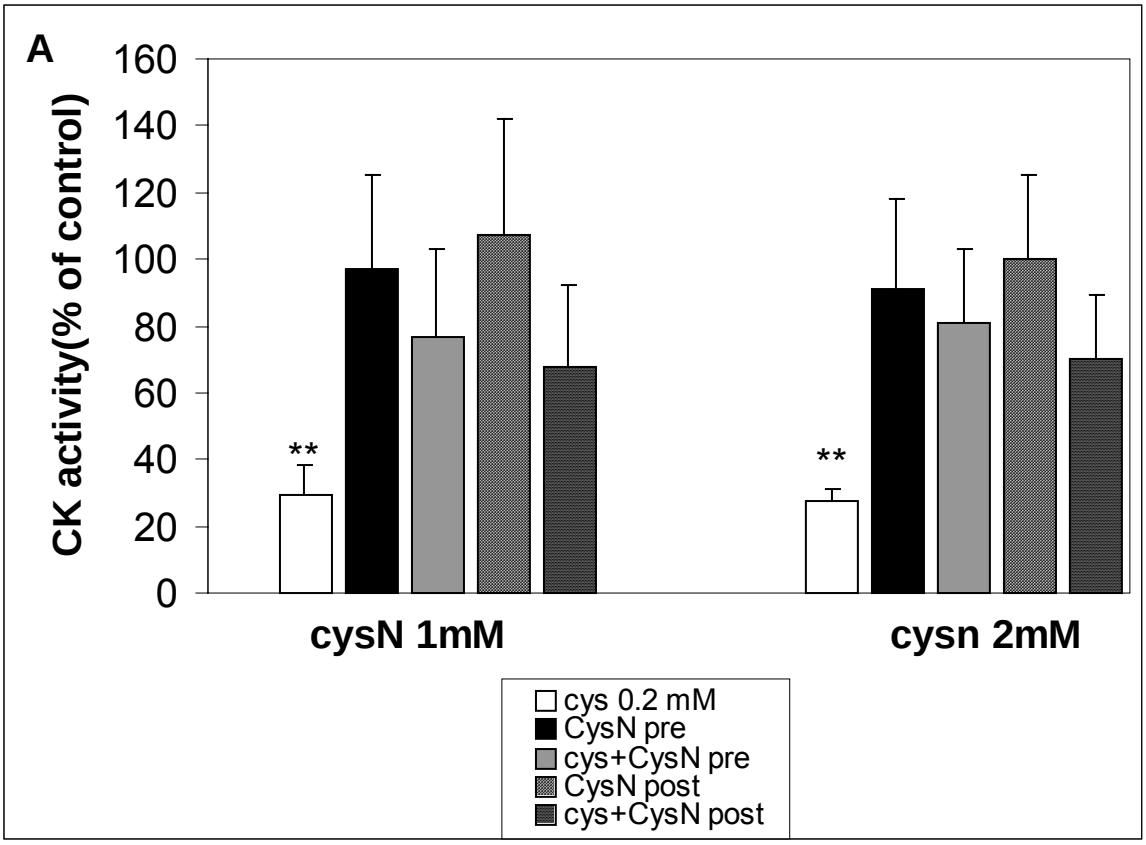

CK activity (% of control)
70
60
50
40
30
20
10
0
*
Total Homogenate
cytosol
mitochondria
Cys 1mM
Cys 2.5 mM
Cys 5mM

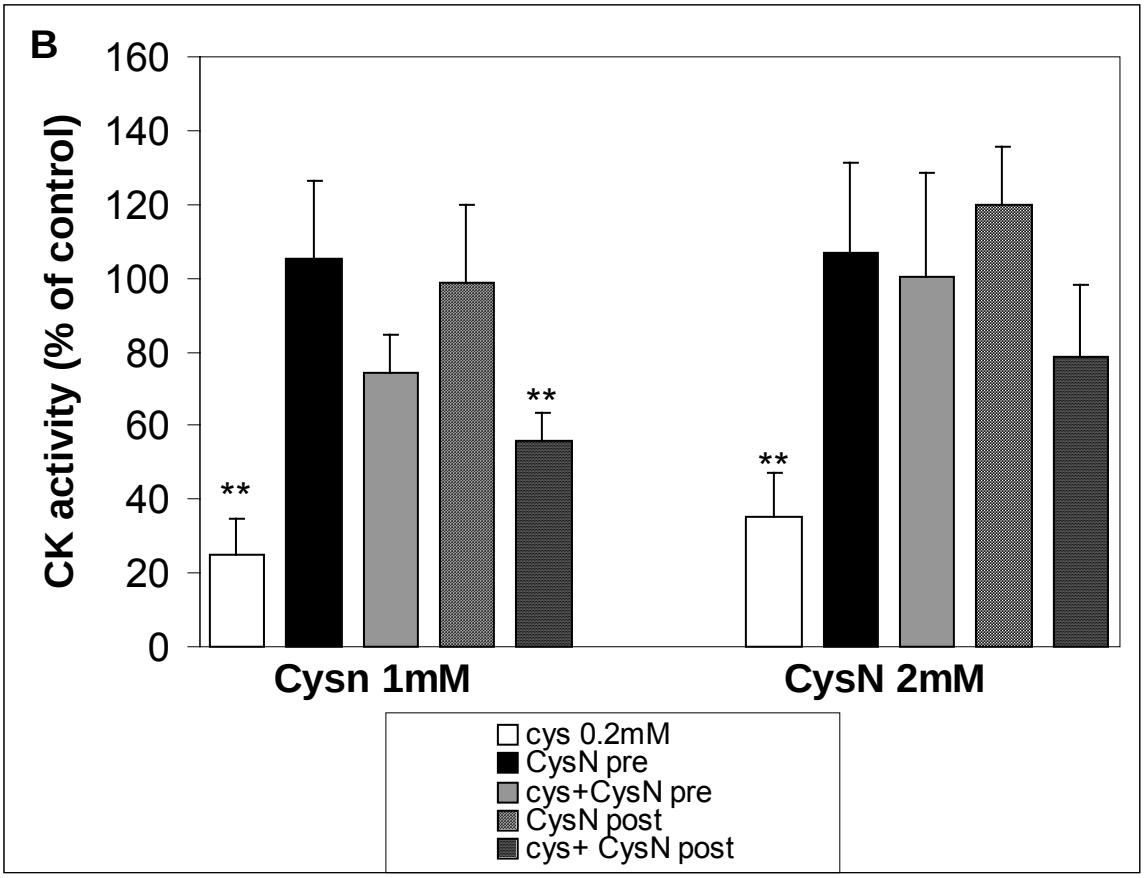

A
CK activity (% of control)
140
120
100
80
60
40
20
0
**
**
**
**
**
**
0 min
30 min
60 min
Cys 0.1 mM
cys 0.2 mM
cys 0.5 mM

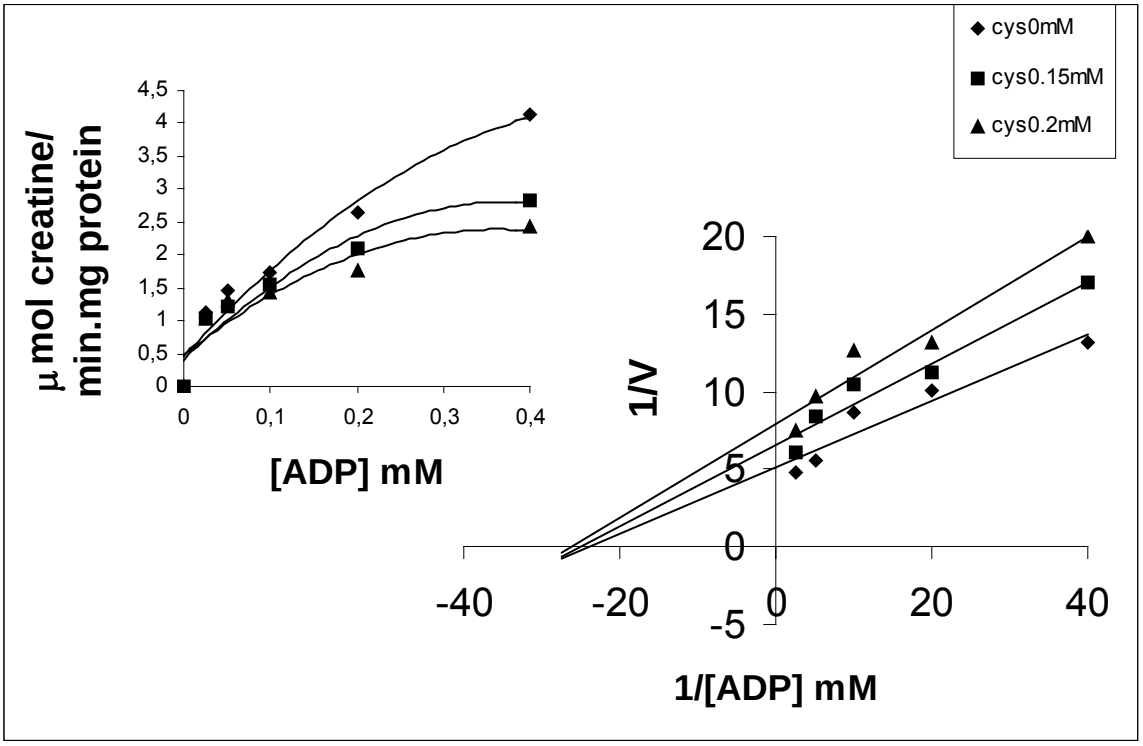

B
CK activity (% of control)
160
140
120
100
80
60
40
20
0
**
*
**
**
0 min
30 min
60 min
Cys 0.1mM
Cys 0.2mM
Cys 0.5mM

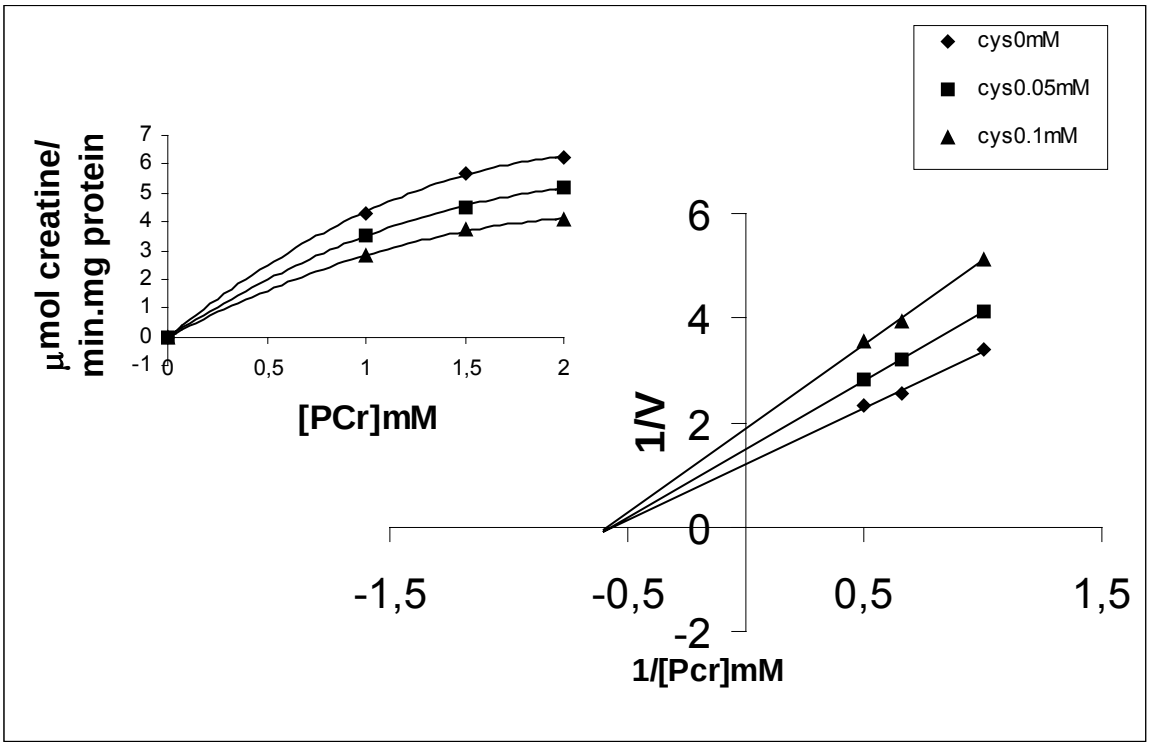

A
CK activity(% of control)
140
120
100
80
60
40
20
0
**
cytosol
mitochondria
cys 0.2mM
GSH 0.5mMpre
cys+GSHpre
GSH 0.5mMpost
cys+GSHpost

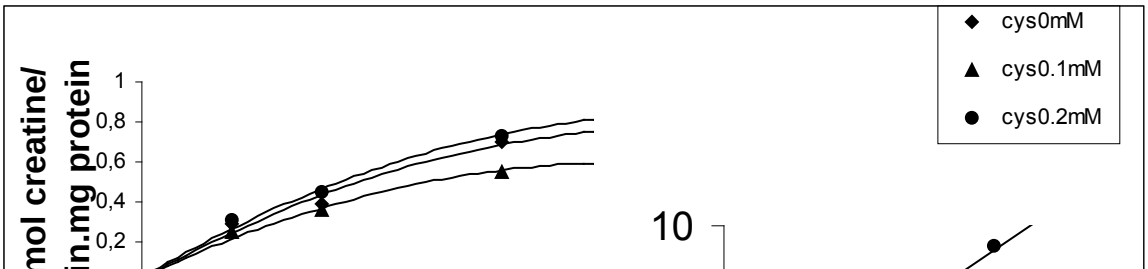

B
CK activity(% of control)
160
140
120
100
80
60
40
20
0
**
cytosol
mitochondria
cys 0.2mM
GSH 0.5mM pre
Cys+GSHpre
GSH0.5mMpost
cys+GSHpost

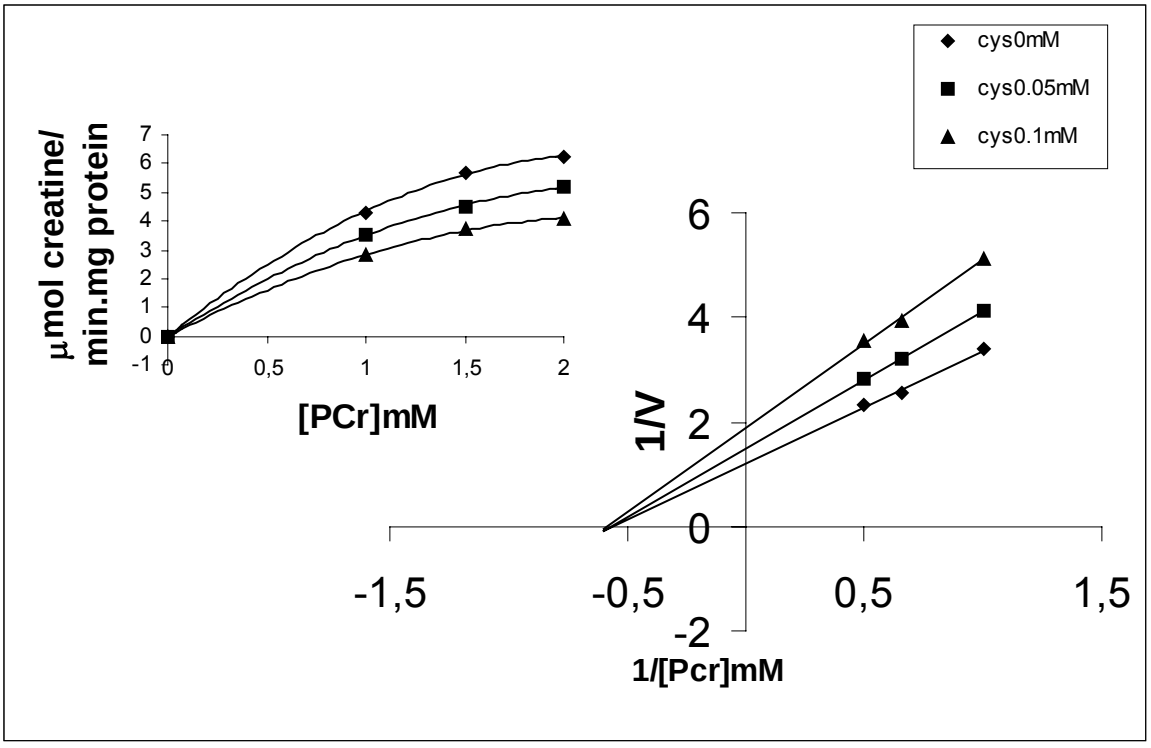

A
CK activity(% of control)
160
140
120
100
80
60
40
20
0
**
**
cysN 1mM
cysn 2mM
cys 0.2 mM
CysN pre
cys+CysN pre
CysN post
cys+CysN post


B
CK activity (% of control)
160
140
120
100
80
60
40
20
0
**
**
**
Cysn 1mM
CysN 2mM
cys 0.2mM
CysN pre
cys+CysN pre
CysN post
cys+ CysN post


cys0mM
cys0.15mM
cys0.2mM
µ mol creatine/ min.mg protein
[ADP] mM
1/V
1/[ADP] mM


cys0mM
cys0.05mM
cys0.1mM
µmol creatine/ min.mg protein
[PCr]mM
1/V
1/[Pcr]mM


cys0mM
cys0.1mM
cys0.2mM



4.CONCLUSÕES E PERSPECTIVAS

4.1 Conclusões

A cistinose é um erro inato do metabolismo dos aminoácidos e que traz, entre diversos sintomas, danos neurológicos, ainda pouco compreendidos. Portanto, é de suma importância a realização de estudos que investiguem a patogênese da doença para que possamos entendê-la melhor e assim buscarmos um tratamento mais adequado para esses indivíduos.

O presente estudo teve por objetivo geral investigar os efeitos da cistina sobre a atividade da creatinaquinase em córtex cerebral de ratos jovens *in vitro* para uma melhor compreensão do déficit neurológico que ocorre nestes pacientes.

Os resultados permitiram as seguintes conclusões:

1- A cistina inibe a atividade da creatinaquinase em homogeneizado total e nas frações citosólica e mitocondrial de maneira dependente da concentração e do tempo de pré-incubação.

2- A glutationa reduzida preveniu e reverteu parcialmente a inibição causada pela cistina, enquanto a cisteamina preveniu e reverteu completamente aquela inibição.

3- A inibição causada pela cistina é do tipo não competitivo em relação aos dois substratos, ADP e fosfocreatina.

Estes resultados sugerem que a cistina possa alterar a transferência de fosfato do ATP para a creatina na mitocôndria, bem como a transferência de fosfato da fosfocreatina para o ADP no citosol, alterando a homeostasia energética do córtex cerebral. Se isto também ocorrer no cérebro dos pacientes cistinóticos, esses dados sugerem que a alteração da homeostasia energética causada pela cistina possa ser um dos mecanismos pelos quais este aminoácido é neurotóxico.

4.2.Perspectivas

Observando os resultados anteriormente citados e considerando que:

-o efeito da cistina sobre a atividade da CK foi testado em homogeneizado total, fração citosólica e mitocondrial de córtex cerebral de ratos *in vitro*,

-a CK é uma enzima tiólica sensível a radicais livres,

-ocorre morte celular em larga escala no tecido nervoso de pacientes cistinóticos, abriu-se a perspectiva da continuação dessa investigação com os seguintes objetivos:

*verificar o efeito da cistina sobre a atividade da CK também *in vivo*;

*verificar parâmetros de estresse oxidativo na cistinose,

*verificar se a cistina provoca morte celular em fatias de cérebro e em cultivos celulares.

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