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# EFEITOS IN VITRO DA 3-METILCROTONILGLICINA SOBRE VÁRIOS PARÂMETROS DO METABOLISMO ENERGÉTICO EM CÓRTEX CEREBRAL DE RATOS JOVENS

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**SUMÁRIO**

# PARTE I

## *Introdução e Objetivos*

## RESUMO

A deficiência da 3-metilcrotonil-CoA-carboxilase (3-MCCD) é uma desordem autossômica recessiva do catabolismo da leucina. Os pacientes afetados pela 3-MCCD apresentam acúmulo tecidual de 3-metilcrotonil-CoA, o que leva a um aumento da formação e excreção urinária do ácido 3-hidroxiisovalérico (3-HIVA), 3-metilcrotonilglicina (3-MCG) bem como elevação das concentrações no plasma de 3-hidroxiisovaleril-carnitina (C5-OH). A apresentação clínica é extremamente variável e caracterizada principalmente por uma disfunção neurológica severa com encefalopatia e paralisia cerebral. Os pacientes também apresentam retardo psicomotor, hipotonia muscular, cardiomiopatia e leucodistrofia. Infelizmente os mecanismos responsáveis pelo dano cerebral apresentado por esses pacientes ainda são pouco conhecidos. Considerando a importância do metabolismo energético para o sistema nervoso central e que os pacientes afetados por 3-MCCD acumulam e excretam grandes quantidades de 3-MCG, o presente estudo se propôs a investigar a influência deste metabólito sobre importantes parâmetros do metabolismo energético cerebral em ratos jovens. Inicialmente, observamos que a 3-MCG diminuiu a produção de $CO_2$ a partir de acetato [1-$^{14}$C], sugerindo que houve comprometimento no funcionamento do ciclo do ácido cítrico. Além disso, a 3-MCG diminuiu a atividade do complexo II-III da cadeia transportadora de elétrons, indicando que o fluxo de elétrons através dessa cadeia está prejudicado.Também verificamos que as atividades das enzimas creatina-quinase e $Na^+,K^+$-ATPase foram alteradas pela 3-MCG. Tomados em seu conjunto, esses achados indicam que a produção e transferência de energia estão comprometidas bem como a neurotransmissão que é dependente de uma atividade normal da $Na^+,K^+$-ATPase. Observamos também que antioxidantes foram capazes de atenuar ou prevenir completamente o efeito inibitório da 3-MCG sobre as atividades da creatina-quinase e da $Na^+,K^+$-ATPase, sugerindo o envolvimento de radicais livres nesses efeitos. Essa hipótese foi reforçada pela observação de que a 3-MCG provoca dano oxidativo lipídico (peroxidação lipídica). Nossos resultados sugerem que a 3-MCG prejudica a homeostase mitocondrial e o potencial de membrana que podem estar envolvidos no dano neurológico apresentado pelos pacientes afetados pela deficiência da 3-metilcrotonil-CoA-carboxilase.

## ABSTRACT

Deficiency of 3-methylcrotonyl-CoA-carboxylase (3-MCCD) is an autosomal recessive disorder of leucine catabolism. Affected patients usually present accumulation of 3-methylcrotonyl-CoA in tissues, leading to high synthesis and urinary excretion of 3-hydroisovalerate (3-HIVA), 3-methylcrotonylglycine (3-MCG), as well as increased levels of 3-hydroxyisovaleryl-carnitine (C5-OH) in plasma.The clinical presentation is highly variable and mainly characterized by severe neurological dysfunction with encephalopathy and cerebral paralysis.Patients also present important psychomotor retardation, muscular hypotonia, cardiomyopathy and leukodystrophy. Unfortunately, the underlying mechanisms involved in the cerebral damage of these patients are practically unknown. Considering the importance of energy metabolism to the central nervous system and that patients affected by 3-MCCD accumulate and excrete large amounts of 3-MCG, the present study investigated the influence of this metabolite on important parameters of brain energy metabolism in young rats. Initially, we observed that 3-MCG decreased $CO_2$ production from acetate [1-$^{14}$C], suggesting impairment in the function of the citric acid cycle. Furthermore, 3-MCG decreased the activities of complex II-III of the respiratory chain, indicating that the electron transport chain flow is impaired in the presence of this metabolite. We also verified that the activities of enzymes creatine-kinase and $Na^+$,$K^+$-ATPase from synaptic membrane were altered by 3-MCG.Taken together, these findings indicate that energy production and transfer are compromised.We also observed that antioxidants were able to attenuate or fully prevent the inhibitory effect of 3-MCG on creatine-kinase and synaptic membrane $Na^+$,$K^+$-ATPase activities, suggesting the involvement of free radicals in these effects.This hypothesis was reinforced by the observation that 3-MCG causes oxidative lipid damage (lipid peroxidation). Our results suggest that 3-MCG compromises mitochondrial homeostasis and membrane potential and it may be involved in the neurological damage found in patients affected by 3-MCCD.

## *LISTA DE ABREVIATURAS*

ACR – aminoácido de cadeia ramificada

CAC - ciclo do ácido cítrico

CK – creatina-quinase

cCK – creatina-quinase citosólica

CoA – coenzima A

CTE – cadeia transportadora de elétrons

C5-OH – 3-hidroxiisovaleril-carnitina

EIM – erros inatos do metabolismo

GSH – glutationa

L-NAME – N-(G)- L-arginina metil- éster

mCK – creatina-quinase mitocondrial

MEL – melatonina

TBA-RS – espécies reativas ao ácido tiobarbitúrico

TRO – trolox

3-HIVA – ácido 3-hidroxiisovalérico

3-MCC – 3-metilcrotonil-CoA carboxilase

3-MCCD – deficiência da 3-metilcrotonil-CoA carboxilase

3-MCG – 3-metilcrotonilglicina

## I.1. INTRODUÇÃO

### I.1.1 Erros inatos do metabolismo

Em 1908, Sir Archibald E. Garrod usou o termo erros inatos do metabolismo (EIM) para designar doenças como a alcaptonúria, em que os indivíduos afetados excretam grandes quantidades de ácido homogentísico na urina. Garrod observou uma maior frequência desta doença em indivíduos de uma mesma família e maior incidência de consanguinidade entre os pais dos pacientes. Baseando-se nas leis de Mendel e no fato de que os pais dos indivíduos afetados não apresentavam a doença, Garrod propôs um modelo de herança autossômica recessiva para este distúrbio. Através da observação de que o ácido homogentísico presente em excesso na urina dos pacientes era um metabólito normal da degradação protéica, ele relacionou este acúmulo a um bloqueio na rota de catabolismo da tirosina. Com o surgimento de novos distúrbios relacionados a alterações genéticas e que envolviam o acúmulo de outras substâncias nos líquidos biológicos dos pacientes, postulou-se que estas doenças resultavam da síntese qualitativa ou quantitativamente anormal de uma proteína, enzimática ou não, pertencente ao metabolismo (Scriver et al. 2001). Presumiu-se, então, que como consequência deste bloqueio metabólico pode ocorrer o acúmulo de precursores da reação catalisada pela enzima envolvida, com a formação de rotas metabólicas alternativas e a deficiência de produtos essenciais ao organismo (Bickel et al. 1987).

Até o momento foram descritos mais de 500 EIM, a maioria deles envolvendo processos de síntese, degradação, transporte e armazenamento de moléculas no organismo (Scriver et al. 2001). Embora individualmente raras, essas doenças em seu conjunto afetam aproximadamente 1 a cada 500/2.000 recém nascidos vivos (Baric et al. 2001).

### I.1.2 Aminoácidos de cadeia ramificada

Os aminoácidos de cadeia ramificada (ACR) leucina, valina e isoleucina correspondem a aproximadamente 40% dos aminoácidos essenciais nos indivíduos normais e 35% dos aminoácidos indispensáveis para o tecido muscular. O principal destino metabólico dos ACR é a incorporação em proteínas corporais (Schadewaldt e Wendel 1997).

O passo inicial do catabolismo dos ACR é uma transaminação reversível catalisada pela aminotransferase de cadeia ramificada com formação de $\alpha$-cetoácidos de cadeia ramificada (Figura 1). O segundo passo no catabolismo dos ACR é a descarboxilação oxidativa irreversível dos $\alpha$-cetoácidos ácidos pela desidrogenase dos $\alpha$-cetoácidos de cadeia ramificada, com formação de tioésteres de acil-CoA de cadeia ramificada (Scriver et al. 2001). Posteriormente, as rotas de degradação dos ACR divergem. A leucina é catabolizada até a formação de acetoacetato e acetil-CoA que entra no ciclo do ácido cítrico (CAC). O passo final no catabolismo da isoleucina envolve sua clivagem em acetil-CoA e propionil-CoA, que também entra no CAC através da conversão para succinil-CoA. O aminoácido valina é metabolizado à propionil-CoA (Fernandes et al. 2006).

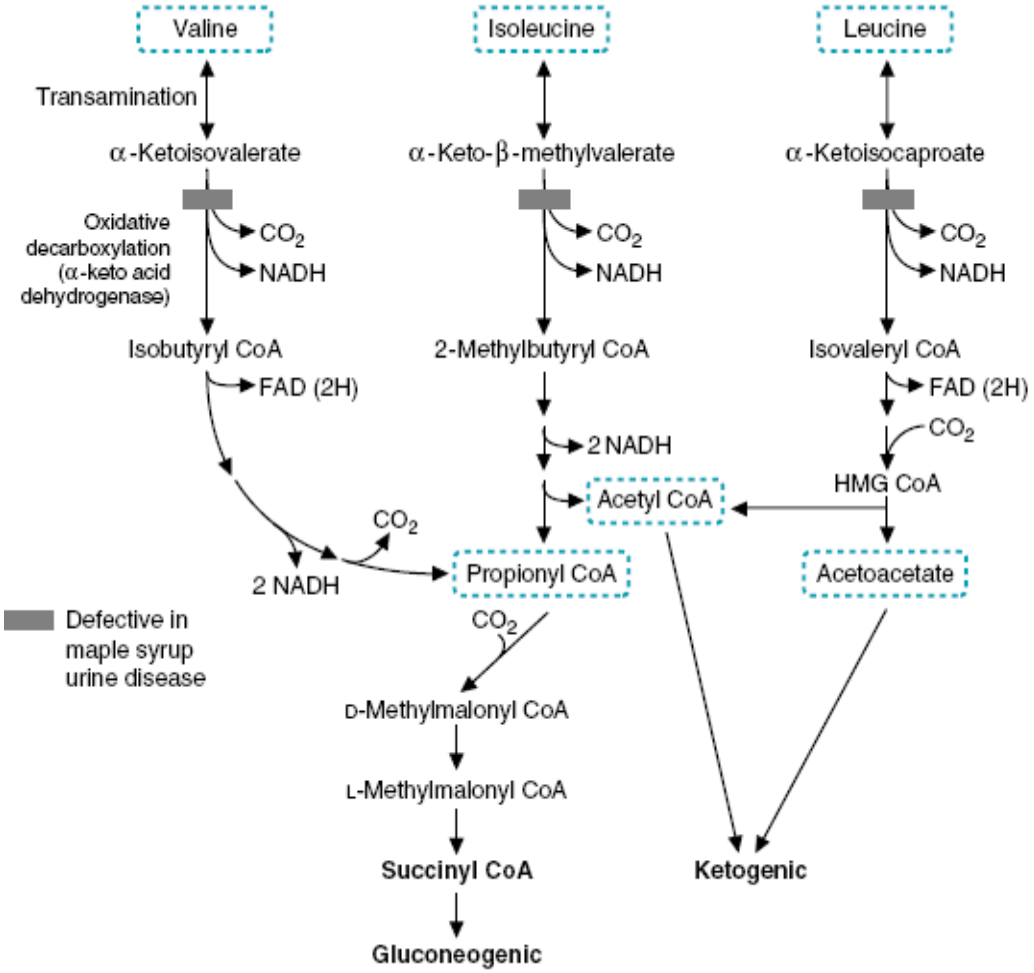


**Figura 1-** Rota catabólica dos aminoácidos de cadeia ramificada leucina, isoleucina e valina. (Adaptado de Lieberman e Marks 2009).

### I.1.3 3-Metilcrotonil- CoA carboxilase

O produto da isovaleril-CoA-desidrogenase, 3-metilcrotonil-CoA, no catabolismo da leucina, é carboxilado no carbono quatro pela 3-metilcrotonil-CoA-carboxilase (3-MCC) para formar 3-metilglutaconil-CoA. A reação é reversível e utiliza ATP e um bicarbonato como fonte do grupo carboxil (Scriver et al. 2001). A enzima 3-MCC tem aproximadamente 835 kDa sendo uma proteína heteromérica. Existem duas subunidades não-idênticas, a MCC-α de 61Da e a MCC-β de 73,5 KDa, que contêm uma ligação covalente com biotina, sendo codificadas por mcca e mccb respectivamente (Scriver et al. 2001).Mutações nesses genes causam a deficiência da 3-metilcrotonil-CoA- carboxilsase (3-MCCD).

A enzima 3-MCC é associada com a membrana mitocondrial interna sendo que fibroblastos e leucócitos humanos normais expressam a atividade da mesma (Scriver et al. 2001).

### I.1.4 Deficiência da atividade da enzima 3-metilcrotonil-CoA carboxilase

A 3-MCCD, também conhecida como 3-metilcrotonilglicinúria é uma desordem do catabolismo da leucina de caráter autossômico recessivo sendo uma das mais freqüentes acidúrias orgânicas detectadas nos Estados Unidos (Gibson et al. 1998; Naylor e Chace 1999; Smith et al. 2000), Europa (Roscher 2000) e Austrália (Wilcken 2000) com uma frequência estimada de 1: 50.000 nascidos vivos (Baumgartner et al. 2001).

Na 3-MCCD o catabolismo da leucina é bloqueado pela deficiência da atividade da 3-MCC. Pacientes com 3-MCCD apresentam acúmulo e elevada

excreção urinária de ácido 3-HIVA e 3-MCG, bem como aumento das concentrações plasmáticas de C5-OH, geralmente combinada com deficiência secundária de L-carnitina (Ficicioglu et al. 2006). Devido ao bloqueio do passo enzimático, os compostos 3-metilcrotonil-CoA e ácido 3-metilcrotônico se acumulam. A maior parte da 3-metilcrotonil-CoA acumulada é conjugada com glicina para formar 3-MCG. As concentrações cerebrais da 3-MCG nos pacientes não são conhecidas. O 3-HIVA é derivado através da ação de uma crotonase sobre o 3-metilcrotonil-CoA e subseqüente hidrólise do éster-CoA. A acilação do 3-HIVA com a L-carnitina leva a formação de C5-OH que é a principal acilcarnitina anormal encontrada no sangue dos pacientes afetados pela 3-MCCD identificada através de técnicas de espectrometria de massa em Tandem (Fernandes et al. 2006).

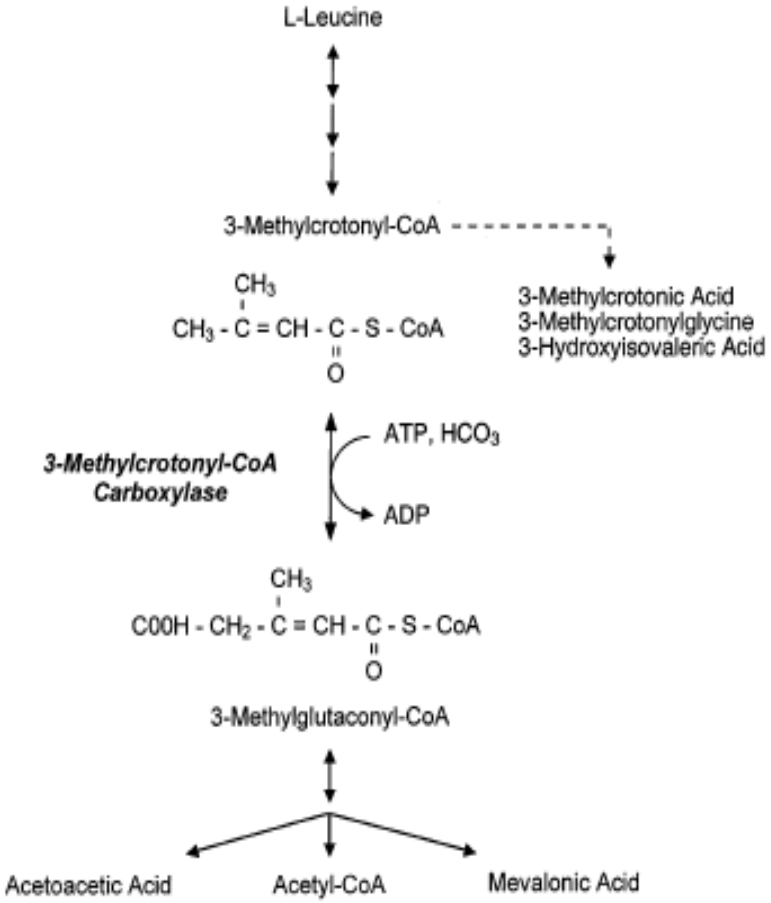


**Figura 2-** Rota de catabolismo da leucina com a formação dos derivados da 3-metilcrotonil-CoA. Em detalhe, a atuação da enzima 3-metilcrotonil-CoA carboxilase (adaptado de Baumgartner et al. 2001).

### I.1.4.1 Achados clínicos

O fenótipo clínico é variável, desde uma apresentação no período neonatal com severo envolvimento neurológico podendo levar a morte até indivíduos adultos assintomáticos (Fernandes et al. 2006). Alguns pacientes apresentam convulsões intratáveis nos primeiros dias de vida, outros possuem dificuldade de se alimentar, retardo do desenvolvimento, hipotonia e cardiomiopatia (Fernandes et al. 2006). Aqueles que sofrem convulsões

recorrentes acabam por apresentar microcefalia e atraso no desenvolvimento. A maioria apresenta a síndrome Reye caracterizada por manifestações neurológicas com hipoglicemia, cetoacidose, hiperamonemia, que é precipitada por doenças intercorrentes (infecções) ou dieta hiperproteica nos dois primeiros anos de vida (entre 2 e 33 meses de idade), bem como diminuição nas concentrações de L-carnitina plasmática (Dirik et pal. 2008). Também são observados nos pacientes com 3-MCCD atrofia cortical progressiva particularmente nas áreas subcorticais dos lobos frontal, temporal e parietal, leucodistrofia, bem como edema cerebral e dilatação ventricular (Baykal et al. 2005; de Kremer et al. 2002; Dirik et al. 2008). Verificou-se a falta de correlação entre o nível de atividade residual da enzima 3-MCC e a apresentação clínica do paciente (Baumgartner 2005).

### I.1.4.2 Fisiopatologia

Pacientes afetados por essa deficiência apresentam acúmulo de 3-MCG e 3-HIVA que potencialmente podem contribuir para a patogênese da doença (Baykal et al. 2005; Dirik et al. 2008*;* Luttikhuis et al. 2005). Um dado interessante é que alguns pacientes apresentam um acúmulo e aumento da excreção de ácido lático (Baykal et al. 2005; Leonard et al. 1981), indicando um comprometimento da função mitocondrial. Neste particular, estudos anteriores mostraram que o 3-HIVA não compromete a bioenergética em ratos jovens (Ribeiro et al. 2007), dessa forma consideramos a hipótese de que a 3-MCG possa ser potencialmente tóxico para o desenvolvimento e

funcionamento do cérebro. Por outro lado, praticamente nada tem sido descrito sobre a patogênese do dano cerebral da 3-MCCD.

### I.1.4.3 Diagnóstico

O diagnóstico é baseado em um perfil característico de ácidos orgânicos encontrados na urina, os quais são quantificados através de cromatografia gasosa / espectrometria de massa. Os pacientes apresentam aumento na excreção de 3-MCG (50 a 4000 mmol/mol de creatinina) (Scriver et al. 2001). Além disso, os níveis de L-carnitina total e livre no plasma são extremamente baixos (Fernandes et al. 2006).

Para o diagnóstico definitivo da 3-MCCD isolada, é necessário confirmar a diminuição específica da atividade enzimática (3-MCC) em leucócitos ou fibroblastos (Fernandes et al. 2006). Heterozigotos para a 3-MCCD não podem ser diagnosticados com segurança pelo ensaio enzimático (Scriver et al. 2001). Uma vez que a encefalopatia aguda apresentada pelos pacientes pode causar um dano permanente, o diagnóstico precoce é fundamental na 3-MCCD.

### I.1.4.4 Tratamento

O tratamento em pacientes sintomáticos baseia-se em uma dieta com diminuição de leucina e com moderada restrição protéica, resultando em uma melhora geral do quadro clínico e redução do número de internações. A dieta é eficaz na redução da excreção anormal dos ácidos orgânicos que, no

entanto, nunca se normalizam (Fernandes et al. 2006). As terapias com suplementação de glicina e L-carnitina para aumentar a excreção de seus conjugados e repor os níveis normais de L-carnitina são complementares (Fernandes et al. 2006).

O efeito da administração de glicina e L-carnitina têm se mostrado variável. Alguns estudos demonstram que a suplementação com glicina leva a um aumento da excreção de 3-metilcrotonil-CoA, embora outros estudos não observem um efeito persistente de excreção (Arnold et al. 2008; Fries et al. 1996; Rutledge et al. 1995).

### I.1.5 Metabolismo energético cerebral

O cérebro é um dos órgãos mais ativos metabolicamente, entretanto possui reservas energéticas extremamente pequenas em relação a sua alta taxa metabólica (Dickinson 1996).

A glicose é o principal substrato energético no cérebro (Erecinska e Silver 1994). Em condições normais o metabolismo energético nos tecidos neurais é mantido, quase que exclusivamente, pelo metabolismo oxidativo da glicose (Sokoloff 1993). A oxidação da glicose no cérebro ocorre mais rapidamente do que em outros órgãos como fígado, coração ou rins. Em contraste com outros tecidos, o cérebro não necessita de insulina para captar e oxidar a glicose. Entretanto, no jejum, corpos cetônicos podem substituir mais de 50% das necessidades energéticas cerebrais (Dickinson 1996).

A oxidação da glicose através da via glicolítica forma piruvato, que é convertido a $CO_2$ e $H_2O$ no ciclo do ácido cítrico e na cadeia transportadora

de elétrons (CTE). O acoplamento entre a CTE e a fosforilação oxidativa gera grande parte do ATP necessário ao cérebro (Erecinska e Silver 1994).

É bem conhecido que no cérebro a fosforilação oxidativa fornece em torno de 95% de todo o ATP sintetizado. O mecanismo que auxilia a manutenção dos níveis cerebrais de ATP é o sistema da creatina-quinase (CK).A CK está presente tanto no citosol quanto ligada às membranas mitocondriais e catalisa a transferência reversível de um fosfato entre a fosfocreatina e o ADP, formando ATP e creatina. O alto fluxo da reação na direção da síntese de ATP, em situações de consumo de ATP, indica que a reação é crucial para a manutenção de concentrações constantes dos substratos energéticos no citosol. O sistema creatina/fosfocreatina/creatina-quinase tem sido associado a algumas funções particularmente importantes para o cérebro: tamponamento energético (através da regeneração do ATP a da manutenção de níveis baixos de ADP) e transferência de ATP de sítios de produção para outros de consumo (Erecinska e Silver 1994).

A enzima $Na^+$-$K^+$ -ATPase é uma enzima transmembrana que cataliza o transporte ativo de sódio e potássio através da membrana celular. Esse transporte é de grande importância já que mais de um terço do ATP consumido é utilizado para bombear tais íons (Aperia 2007). A $Na^+$-$K^+$ -ATPase está presente em praticamente todas as células, incluindo o cérebro e os músculos esquelético e cardíaco (Aperia 2007).

A $Na^+$-$K^+$ -ATPase desempenha um papel fundamental no SNC, sendo responsável pela manutenção dos gradientes iônicos e pela propagação do impulso nervoso.

Considerando a importância da $Na^+$-$K^+$ -ATPase para o funcionamento normal do organismo e do SNC, e seu papel fundamental na manutenção do gradiente elétrico da membrana celular, a inibição dessa enzima tem sido associada a fisiopatologia de diversas doenças, como a isquemia cerebral (Wyse et al. 2000), epilepsia (Grisar 1984), desordens neurodegenerativas (Yu 2003) e doença de Alzheimer (Hattori et al. 1998).

### I.1.6 Metabolismo energético e doenças neurodegenerativas

Numerosas hipóteses têm sido propostas para explicar a fisiopatologia das doenças de Alzheimer, Huntington e Parkinson, sem, no entanto, obter até o momento uma explicação satisfatória para o dano cerebral dessas doenças. Entretanto acredita-se que possíveis mecanismos envolvam deficiência no metabolismo energético, estresse oxidativo e neurotoxicidade mediada por receptores glutamatérgicos do tipo NMDA, ou, possivelmente, um somatório desses fatores (Rose e Henneberry 1994). Uma das hipóteses é de que alterações na CTE seria o evento etiológico primário na maioria dessas doenças (Parker et al. 1990; Swerdlow et al. 1998).

Numerosas evidências relacionam doenças neurodegenerativas a uma diminuição no metabolismo energético. Estudos demonstraram uma diminuição na atividade do complexo I da CTE em cérebros *postmortem* de pacientes portadores de doença de Parkinson (Janetzky et al. 1994; Schapira et al. 1990). Também há relatos de defeitos nos complexos II e III da CTE e na enzima $\alpha$-cetoglutarato desidrogenase, importante enzima do ciclo do ácido cítrico, nessa doença (Mizuno et al. 1990).

Na doença de Alzheimer, a mais comum dentre as doenças neurodegenerativas, é encontrada principalmente uma redução na atividade do complexo IV da CTE (Maurer et al. 2000). Estudos em cérebros postmortem demostraram uma diminuição na atividade do complexo enzimático da piruvato desidrogenase e na atividade da enzima $\alpha$-cetoglutarato desidrogenase na doença de Alzheimer (Gibson et al. 1988; Mastrogiacomo et al. 1993 ; Perry et al. 1980).

### I.1.7 Estresse oxidativo e doenças neurodegenerativas

Na doença de Alzheimer, a mais comum dentre as doenças neurodegenerativas, é possível que o estresse oxidativo tenha um papel chave na morte neuronal. Tem sido proposto que o peptídeo β-amilóide, o formador das chamadas placas senis, tenha a capacidade de gerar radicais livres espontaneamente. Estudos *in vivo* também evidenciaram um dano oxidativo em cérebros humanos *postmortem* com doença de Alzheimer, através da observação de aumento de 8-hidroxi-2'-deoxiguanosina (8-OHdGA), produtos de oxidação de outras bases e de RNA, carbonilas de proteínas, nitrotirosina e marcadores de peroxidação lipídica (Lovell et al. 2000; Markesbery e Carney, 1999; Nourooz-Zadeh et al. 1999; Smith et al. 1991).

Por outro lado, verificou-se um dano oxidativo importante em pacientes portadores da doença de Huntington, particularmente representado pela formação de 3-nitrotirosina nas áreas afetadas (Alexi et al. 2000). Entretanto,

o dano oxidativo observado nessa doença aparentemente tem menor importância do que nas doenças de Parkinson e Alzheimer.

Nos últimos anos, foi também verificado que vários metabólitos acumulados em alguns EIM com comprometimento severo do SNC induzem estresse oxidativo no cérebro de animais experimentais (Feksa et al. 2008; Kessler et al. 2008; Latini et al. 2007; Ribeiro et al. 2007; Zugno et al. 2008) e em seres humanos (Barschak et al. 2008a,b; Deon et al. 2007; Deon et al. 2008) indicando que os compostos acumulados nestas doenças possam causar dano oxidativo.

## I.2 OBJETIVOS

### I.2.1 Objetivo geral

Tendo em vista que os mecanismos do dano cerebral apresentado pelos pacientes portadores da 3-MCCD são pouco conhecidos e que não há conhecimento prévio dos efeitos tóxicos da 3-MCG, que é o principal metabólito acumulado na 3-MCCD, nosso trabalho se propôs a avaliar o efeito *in vitro* da 3-MCG sobre importantes parâmetros bioquímicos de homeostase mitocondrial em córtex cerebral de ratos jovens.

### I.2.2 Objetivos específicos

a. Avaliar o efeito da 3-MCG nas concentrações de 0,1 , 1 e 5mM sobre a produção de $CO_2$ a partir de acetato [1-$^{14}$C] em homogeneizado de córtex cerebral de ratos jovens.

b. Avaliar o efeito da 3-MCG nas concentrações de 0,1, 1 e 5mM sobre a atividade dos complexos enzimáticos I-III, II, II-III e IV da CTE em homogeneizado de córtex cerebral de ratos jovens.

c. Avaliar o efeito da 3-MCG nas concentrações de 0,1, 1 e 5mM sobre a atividade das enzimas do ciclo do ácido cítrico em mitocôndrias isoladas de córtex cerebral de ratos jovens.

d. Avaliar o efeito da 3-MCG nas concentrações de 0,1, 1 e 5mM sobre a atividade da enzima CK, e suas frações citosólica (cCK) e mitocondrial (mCK) em homogeneizado de córtex cerebral de ratos jovens.

e. Avaliar o efeito da 3-MCG nas concentrações de 0,1, 1 e 5mM sobre a atividade da enzima $Na^+$,-$K^+$-ATPase em membrana sináptica de córtex cerebral de ratos jovens.

# PARTE II

## *Artigo Científico*

# Capítulo I

## 3-Methylcrotonylglycine disrupts mitochondrial energy homeostasis and inhibits synaptic $Na^+,K^+$-ATPase activity in brain of young rats

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ORIGINAL RESEARCH

# 3-Methylcrotonylglycine Disrupts Mitochondrial Energy Homeostasis and Inhibits Synaptic $Na^+,K^+$-ATPase Activity in Brain of Young Rats

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**Abstract** Deficiency of 3-methylcrotonyl-CoA carboxylase activity is an inherited metabolic disease biochemically characterized by accumulation and high urinary excretion of 3-methylcrotonylglycine (3MCG), and also of 3-hydroisovalerate in lesser amounts. Affected patients usually have neurologic dysfunction, brain abnormalities and cardiomyopathy, whose pathogenesis is still unknown. The present study investigated the in vitro effects of 3MCG on important parameters of energy metabolism, including $CO_2$ production from labeled acetate, enzyme activities of the citric acid cycle, as well as of the respiratory chain complexes I–IV (oxidative phosphorylation), creatine kinase (intracellular ATP transfer), and synaptic $Na^+,K^+$-ATPase (neurotransmission) in brain cortex of young rats. 3MCG significantly reduced $CO_2$ production, implying that this compound compromises citric acid cycle activity. Furthermore, 3MCG diminished the activities of complex II-III of the respiratory chain, mitochondrial creatine kinase and synaptic membrane $Na^+,K^+$-ATPase. Furthermore, antioxidants were able to attenuate or fully prevent the inhibitory effect of 3MCG on creatine kinase and synaptic membrane $Na^+,K^+$-ATPase activities. We also observed that lipid peroxidation was elicited by 3MCG, suggesting the involvement of free radicals on 3MCG-induced effects. Considering the importance of the citric acid cycle and the electron flow through the respiratory chain for brain energy production, creatine kinase for intracellular energy transfer, and $Na^+,K^+$-ATPase for the maintenance of the cell membrane potential, the present data indicate that 3MCG potentially impairs mitochondrial brain energy homeostasis and neurotransmission. It is presumed that these pathomechanisms may be involved in the neurological damage found in patients affected by 3-methylcrotonyl-CoA carboxylase deficiency.

**Keywords** 3-Methylcrotonyl-CoA carboxylase deficiency · 3-Methylcrotonylglycine · Brain bioenergetics · Reactive species

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## Introduction

3-Methylcrotonyl-coenzyme A (CoA) carboxylase deficiency (3MCCD) is an autosomal recessive disorder of leucine catabolism biochemically characterized by accumulation and high urinary excretion of 3-methylcrotonylglycine (3MCG) and 3-hydroxyisovaleric acid to lesser amounts. It is phenotypically a heterogeneous disorder whose main clinical symptoms are failure to thrive, psychomotor retardation, hypotonia or hypertonia, and cardiomyopathy. Severe metabolic decompensation following acute stress (usually following infections or increased protein load) with acidosis and seizures leading to permanent neurological damage or death is also observed in the affected patients (Eminoglu et al. 2009; Sweetman and Williams 2001). Brain imaging usually shows cerebral edema, ventricular dilatation, leukodystrophy, and cerebral atrophy particularly in subcortical areas of frontal, temporal, and parietal lobes (Baykal et al. 2005; de Kremer et al. 2002; Dirik et al. 2008; Murayama et al. 1997).

Although brain dysfunction and abnormalities are predominant features in 3MCCD, the pathogenesis of the cerebral damage in this disorder is virtually unknown. However, hyperlacticacidemia has been reported in some patients indicating impairment of bioenergetics. To our knowledge there is no previous study reporting the effects of 3MCG, the major metabolite accumulating in 3MCCD, on cell energy homeostasis in the brain. Therefore, in the present study we investigated the effects of 3MCG on the citric acid cycle (CAC) functioning ($CO_2$ production from [1-$^{14}$C] acetate and various activities of the CAC) and of the respiratory chain complexes I–IV, as well as on creatine kinase and $Na^+,K^+$-ATPase activities in cerebral cortex of developing rats in the hopes to evaluate energy production (CAC and oxidative phosphorylation), transfer (creatine kinase activity) and utilization ($Na^+,K^+$-ATPase, which uses approximately 40–60% of the ATP produced intracellularly). We also tested the effect of 3MCG on thiobarbituric acid reactive species (TBA-RS) levels, an index of lipid oxidative damage, and whether antioxidants could prevent the inhibitory action of 3MCG on creatine kinase and $Na^+,K^+$-ATPase activities.

## Experimental Procedures

### Animals and Reagents

We used 91 30-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, UFRGS, in the assays. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1°C) colony room. The "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) were followed in all experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were made to minimize the number of animals used and their suffering.

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for [1-$^{14}$C] acetate which was purchased from Amersham International plc, UK and 3MCG, (99% purity), which was synthesized by Dr. Ernesto Brunet, from Universidad Autonoma Madrid. 3MCG was dissolved on the day of the experiments in the buffer used for each assay with pH adjusted to 7.4.

The biochemical parameters were determined in the presence of various concentrations of 3MCG (0.1–5 mM), whereas control groups did not contain this metabolite in the incubation medium. We always carried out parallel experiments with various blanks (controls) in the presence or absence of 3MCG and also with or without brain preparations in the reaction medium to detect any interference (artifact) of this metabolite on the techniques utilized to measure the biochemical parameters. Finally, in some experiments antioxidants were incubated simultaneously with 5 mM 3MCG, after which the biochemical parameters were determined. The concentrations of the antioxidants used in these assays were chosen according to Viegas et al. (2008; 2009).

### Cerebral Cortex Preparation

The animals were sacrificed by decapitation, the brain was rapidly removed and the cerebral cortex was isolated. For $CO_2$ production, the cerebral cortex was homogenized (1:10, w/v) in Krebs–Ringer bicarbonate buffer, pH 7.4. For the determination of the activities of the respiratory chain complexes I–III, II, II–III, and IV and the CAC enzymes, cerebral cortex was homogenized (1:20, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base, and 50 UI $ml^{-1}$ heparin). The homogenates were centrifuged at 800×*g* for 10 min and the supernatants were kept at −70°C until being used for enzyme activity determination. For total creatine kinase activity determination, the cerebral cortex was homogenized (1:10 w/v) in isosmotic saline solution. For preparation of mitochondrial and cytosolic fractions, the homogenates were centrifuged at 800×*g* for 10 min at 4°C and the pellet discarded (Ramirez and Jimenez 2000). The supernatant was then centrifuged at 27,000×*g* for 30 min at 4°C in a Sorval DC-2B centrifuge. The pellet containing the mitochondria was washed three times with saline solution and used as the mitochondrial fraction for the mitochondrial creatine kinase (mCK) enzymatic assay. The supernatants were further centrifuged at 125,000×*g* for 60 min at 4°C in an OTD-65B Sorval centrifuge, the microsomal pellet discarded, and the cytosol (supernatant) was used for the determination of cytosolic creatine kinase (cCK) activity. The period between tissue preparation and measurement of the various parameters was always less than 5 days, except for mCK, cCK, and $CO_2$ production assays, which were performed in the same day of the preparations. We applied approximately 0.01–1 mg protein in the assays carried out with homogenates.

### Preparation of Synaptic Plasma Membrane from Rat Cerebrum

Cortical cerebral was homogenized in 10 vol of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. Synaptical plasma membranes were prepared afterward according to the method of Jones and Matus (1974) using a discontinuous sucrose density gradient

consisting of successive layers of 0.3, 0.8, and 1.0 mM. After centrifugation at 69,000×*g* for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the membrane enzyme preparation.

### $CO_2$ Production

We employed 11 animals for these experiments. Homogenates prepared in Krebs–Ringer bicarbonate buffer, pH 7.4, were added to small flasks (11 $cm^3$) in a volume of 0.45 ml. Flasks were pre-incubated at 35°C for 30 min in the presence of 3-MCG in a metabolic shaker (90 oscillations $min^{-1}$) with 625 µM *n*-dodecyl-*β*-D-maltoside to permeabilize the mitochondrial membranes. Controls did not contain the metabolite in the incubation medium. After pre-incubation, 0.055 µCi [1-$^{14}$C] acetate plus 1.0 mM of unlabeled acetate were added to the incubation medium. The flasks were gassed with a $O_2$/$CO_2$ (95:5) mixture and sealed with rubber stoppers Parafilm M. Glass center wells containing a folded 6/4 cm piece of Whatman 3 filter paper were hung from the stoppers. After 60 min incubation at 35°C in a metabolic shaker (90 oscillations $min^{-1}$), 0.2 ml of 50% trichloroacetic acid was supplemented to the medium and 0.1 ml of benzethonium hydroxide was added to the center of the wells with needles introduced through the rubber stopper. The flasks were left to stand for 30 min to complete $CO_2$ trapping and then opened. The filter paper were removed and added to vials containing scintillation fluid, and radioactivity was counted (Reis de Assis et al. 2004). Results were calculated as *ρ*mol $CO_2$ $h^{-1}$ g $tissue^{-1}$.

### Spectrophotometric Analyses of the Activities of Citric Acid Cycle (CAC) Enzymes

We employed 28 animals for these experiments. Citrate synthase activity was measured according to Srere (1969), by determining DTNB reduction at $\lambda = 412$ nm. Isocitrate dehydrogenase activity was accessed by the method of Plaut (1969), by following $NAD^+$ reduction at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activity of α-ketoglutarate dehydrogenase complex was evaluated according to Lai and Cooper (1986) and Tretter and Adam-Vizi (2004), with slight modifications (Viegas et al. 2009). The reduction of $NAD^+$ was recorded in a Hitachi F-4500 spectrofluorometer at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activity of succinate dehydrogenase was determined as described by Fischer (1985). Fumarase activity was measured according to O'Hare and Doonan (1985), measuring the increase of absorbance at $\lambda = 250$ nm. Malate dehydrogenase activity was determined according to Kitto (1969) by following the reduction of NADH at wavelengths of excitation and emission of 340 and 466 nm, respectively. The activities of the CAC enzymes were calculated as nmol $min^{-1}$ mg $protein^{-1}$, mmol $min^{-1}$ mg $protein^{-1}$ or µmol $min^{-1}$ mg $protein^{-1}$.

### Spectrophotometric Analysis of the Respiratory Chain Complexes I–IV Activities

We employed 12 animals for these experiments. The activity of NADH:cytochrome *c* oxidoreductase (complex I–III) was assayed in cerebral cortex homogenates according to the method described by Schapira et al. (1990). The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate:cytochrome *c* oxidoreductase (complex II–III) were determined in homogenates from cerebral cortex according to Fischer et al. (1985) and that of cytochrome *c* oxidase (complex IV) according to Rustin et al. (1994). 3MCG was added to the reaction medium at the beginning of the assays, while no metabolite was added to controls.

Complex I–III activity was measured by following the increase in absorbance because of reduction of cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\varepsilon = 19.1$ $mM^{-1}$ cm). The reaction mixture contained 20 mM potassium phosphate buffer, pH 8.0, 2 mM KCN, 10 µM EDTA, 50 µM cytochrome *c*, and 10–20 µg homogenate protein. The reaction was initiated by the addition of 25 µM NADH and was monitored at 25 C for 3 min before addition of 10 µM rotenone, after which the activity was measured for an additional 3 min. Complex I–III activity was the rotenone sensitive NADH: cytochrome *c* reductase activity.

The activity of the respiratory chain enzyme complex succinate: DCIP oxiredutase (complex II) was determined by following the decrease in absorbance because of the reduction of 2,6-DCIP at 600 nm with 700 nm as reference wavelength ($\varepsilon = 19.1$ $mM^{-1}$ cm). The reaction medium consisting of 40 mM potassium phosphate buffer, pH 7.4, 16.0 mM sodium succinate and 8 µM DCIP was preincubated with 40–80 µg homogenate protein at 30 C for 30 min. Subsequently, 4 mM sodium azide and 7 µM rotenone were added, and the reaction was initiated by addition of 40 µM DCIP and was monitored for 5 min. Results were expressed as nmol of reduced DCIP per min per mg protein.

Complex II–III (succinate: cytochrome *c* oxireductase) activity was measured by following the increase in absorbance because of the reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength ($\varepsilon = 19.1$ $mM^{-1}$ cm. The reaction medium consisting of 40 mM potassium phosphate buffer, pH 7.4, and 16 mM sodium succinate was preincubated with 40–80 µg homogenate protein at 30 C for 30 min. Subsequently, 4 mM sodium

azide and 7 µM rotenone were added, and the reaction was initiated by addition of 0.6 µg $ml^{-1}$ cytochrome *c* and monitored for 5 min.

The activity of cytochrome *c* oxidase (complex IV, COX) was measured by following the decrease in absorbance because of the oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\varepsilon = 19.1$ $mM^{-1}$ cm). The reaction mixture contained 10 mM potassium phosphate buffer, pH 7.0, 0.6 mM *n*-dodecyl-*β*-D-maltoside, 2–4 µg homogenate protein and the reaction was initiated with addition of 0.7 µg reduced cytochrome *c*. The activity of complex IV was measured at 25°C for 10 min.

### Spectrophotometric Analysis of Creatine Kinase Activity

We employed 16 animals for these experiments. Creatine kinase (CK) activity was measured in total homogenates according to Hughes (1962) with slight modifications (da Silva et al. 2004). In brief, the reaction mixture consisted of 50 mM Tris buffer, pH 7.5, containing 7.0 mM phosphocreatine, 7.5 mM $MgSO_4$, and cortical homogenates in a final volume of 0.1 ml. 3-MCG was supplemented to the medium and submitted to a pre-incubation at 37°C for 30 min. The reaction was then started by addition of 4.0 mM ADP and stopped after 10 min by addition of 0.02 ml of 50 mM *p*-hydroxy-mercuribenzoic acid. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 ml 20% α-naphtol and 0.1 ml 20% diacetyl in a final volume of 1.0 ml and read after 20 min at $\lambda = 540$ nm. Results were calculated as µmol of creatine $min^{-1}$ mg $protein^{-1}$. In some experiments, the antioxidants reduced glutathione (GSH, 0.2 mM), melatonin (MEL, 0.2 mM), trolox (TRO, soluble α-tocopherol 1.5 µM) a combination of the antioxidant enzymes catalase (CAT, 50 mU/ml) plus superoxide dismutase (SOD, 50 mU/ml) or the nitric oxide synthase inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 1 mM) were co-incubated with 5 mM 3MCG. We used these antioxidants because they are the main antioxidants present in neurons (Dringen et al. 1999; Siu et al. 1998).

### Spectrophotometric Analysis of $Na^+$,$K^+$-ATPase Activity

We employed 19 animals for these experiments. The reaction mixture for the $Na^+$,$K^+$-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, 40 mM Tris–HCl buffer, pH 7.4, and purified synaptic membranes (approximately 3 µg of protein) in a final volume of 200 µl. The enzymatic assay occurred at 37°C during 5 min and started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. The reaction was stopped by the addition of 200 µl of 10% trichloroacetic acid. $Mg^{2+}$-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. $Na^+$, $K^+$-ATPase activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos 1984). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Enzyme-specific activities were calculated as nmol Pi $released^{-1}$ $min^{-1}$ mg $protein^{-1}$. In some experiments, the antioxidants reduced glutathione (GSH, 0.2 mM), melatonin (MEL, 0.2 mM), TRO (water soluble analogue of α-tocopherol, 1.5 µM), a combination of the antioxidant enzymes catalase (CAT, 50 mU $ml^{-1}$) plus superoxide dismutase (SOD, 50 mU $ml^{-1}$) or L-NAME (1 mM) were first co-incubated with 5 mM 3MCG, following by isolation of the membranes and measurement of the enzymatic activity.

### Determination of Thiobarbituric Acid-Reactive Substances (TBA-RS)

We employed six animals for these experiments. TBA-RS was determined according to the method of Esterbauer and Cheeseman (1990). In brief, cerebral cortex homogenates (approximately 0.3 mg protein) were incubated at 37°C for 1 h with 3MCG. Immediately after incubation, 300 µl of cold 10% trichloroacetic acid were added to an aliquot of 150 µl of pre-treated homogenates and centrifuged at 3000×*g* for 10 min. Three hundred µl of the supernatants were transferred to a Pyrex tube and incubated with 300 µl of 0.67% TBA in 7.1% sodium sulfate on a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool on running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. TBA-RS values were calculated as nmol $mg^{-1}$ protein.

### Protein Determination

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

### Statistical Analysis

Unless otherwise stated, results are presented as mean ± standard error of the mean (SEM). Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data was analyzed using one-way analysis of variance (ANOVA) followed by the

post-hoc Duncan multiple range test, when $F$ was significant. Linear regression was also used to detect dose-dependent effects. The Student $t$ test for paired samples was used for comparison of two means. Only significant $F$ and $t$ values are shown in the text. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

## Results

We first investigated the in vitro effects of 3MCG on $^{14}CO_2$ production from [1-$^{14}$C] acetate in cerebral cortex from 30-day-old rats to evaluate whether this compound could affect the citric acid cycle (CAC) function. It can be seen in Fig. 1a that 3MCG significantly inhibited $CO_2$ production (up to 30%, $F_{(3,20)} = 9.804$, $P < 0.001$) in a dose-dependent fashion ($\beta = -0.730$, $t = -5.010$, $P < 0.001$). We also observed that this inhibitory effect did not change when 1.0 mM CoA was added to the medium ($F_{(2,12)} = 23.648$, $P < 0.001$, Fig. 1b), ruling out a shortage of CoA because of a possible competition between 3MCG and acetate for free CoA to generate acetyl-CoA. These data indicate a reduction of citric cycle acid (CAC) function caused by 3MCG possibly because of an inhibitory effect of this compound on one or more enzymatic steps of the CAC or secondary to a blockage of the respiratory chain. The activities of the various CAC evaluated were not altered by 3MCG (data not shown). It should be stressed that aconitase activity could not be measured in the presence of 3MCG because this compound interfered with the assay.

The next set of experiments was performed to evaluate the effect of 3MCG on various respiratory chain complex activities in an attempt to elucidate whether 3MCG could reduce the activity of the electron transfer chain. Figure 2 (panel C) shows that 3MCG strongly inhibited complex II-III activity (up to 35%, $F_{(5,24)} = 39.354$, $P < 0.001$) in a dose-dependent fashion ($\beta = -0.856$, $t = -8.744$, $P < 0.001$), whereas the other activities remained unmodified.

Next we evaluated the effects of 3MCG on total creatine kinase (tCK) activity in homogenates from the rat cerebral cortex. 3MCG inhibited tCK activity (up to 20%, $F_{(3,16)} = 3.239$, $P < 0.05$, Fig. 3a). Furthermore, the mitochondrial CK fraction (mCK) was significantly inhibited by 3MCG (up to 65%, $F_{(3,20)} = 21.810$, $P < 0.001$) in a dose-dependent way ($\beta = -0.873$, $t = -8.386$, $P < 0.001$, Fig. 3b) with no significant alteration of cytosolic CK isoform activity (cCK) (Fig. 3c).

We also tested whether the significant reduction of mCK activity caused by 3MCG was mediated by oxidation of

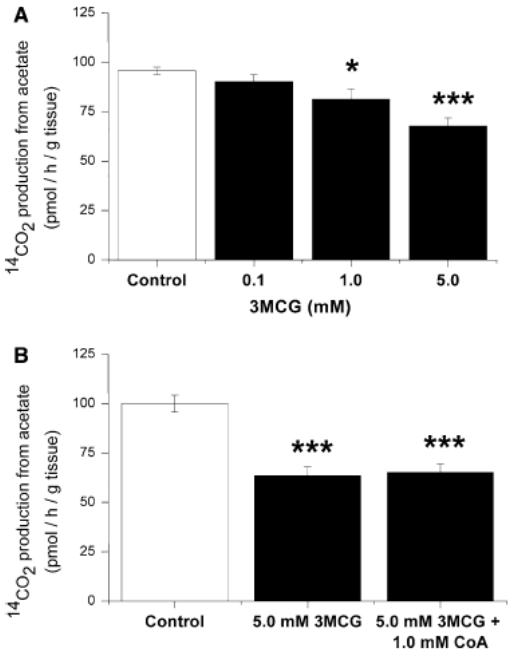


**Fig. 1** Effect of 3MCG on $CO_2$ production from [1-$^{14}$C] acetate in rat cerebral cortex (**a**). In some experiments, $CO_2$ production was measured in the presence of 1 mM CoA (**b**). Values are mean ± SEM for five to six independent experiments (animals) per group and expressed as pmol $CO_2$ $h^{-1}$ g $tissue^{-1}$. *$P < 0.05$ and *** $P < 0.001$ compared to control (ANOVA followed by Duncan multiple range test)

critical groups of the enzyme that are susceptible to free radical attack, by pre-incubating mitochondrial preparations from cerebral cortex in the presence of the antioxidants GSH, MEL, TRO, CAT plus SOD or the nitric oxide synthase inhibitor L-NAME. It can be seen in Fig. 4 that GSH fully prevented 3MCG-induced inhibitory effect on mCK activity ($F_{(6,28)} = 7.657$, $P < 0.001$) suggesting a pro-oxidant effect of 3MCG directed toward sulfhydryl groups of the enzyme which are usually protected by GSH.

Finally, we investigated the effects of 3MCG on synaptic $Na^+$,$K^+$-ATPase activity, a critical enzyme for the maintenance of the membrane potential necessary for neurotransmission. Figure 5a shows that purified synaptic membrane preparations exposed to increasing concentrations of 3MCG resulted in a significant inhibition (up to 45%, $F_{(4,20)} = 5.296$, $P < 0.01$) of $Na^+$,$K^+$-ATPase activity. We also incubated cortical homogenates in the presence of 5 mM 3MCG and measured $Na^+$, $K^+$-ATPase activity in synaptic plasma membranes prepared from these homogenates after incubation. Figure 5b shows that exposition of homogenates to 3MCG also resulted in a

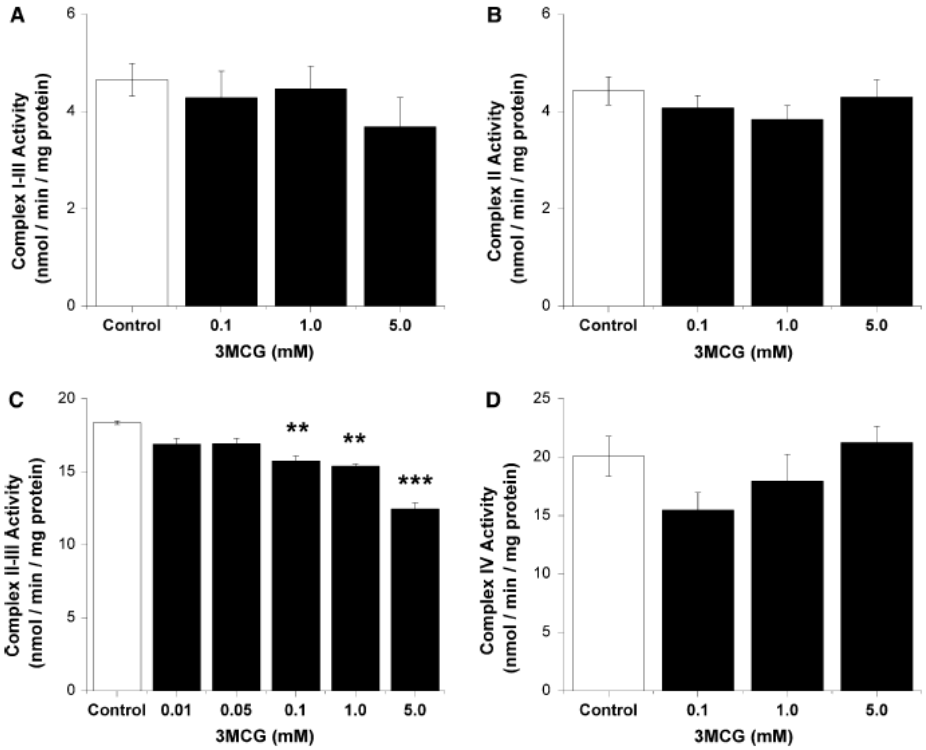


**Fig. 2** Effect of 3MCG on the activities of the respiratory chain complexes I–IV in rat cerebral cortex. Values are mean ± SEM for four to five independent experiments (animals) per group. The activity of complex I–III (**a**) is expressed as nmol cytochrome *c* reduced $min^{-1}$ mg $protein^{-1}$ and of complex II (**b**) as nmol DCIP reduced $min^{-1}$ mg $protein^{-1}$. The activities of complexes II–III (**c**) and IV (**d**) are expressed, respectively, as nmol cytochrome *c* reduced $min^{-1}$ mg $protein^{-1}$ and nmol cytochrome *c* oxidized $min^{-1}$ mg $protein^{-1}$. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control (ANOVA followed by Duncan multiple range test)

significant inhibition (40%) of synaptic $Na^+,K^+$-ATPase activity ($t_{(4)} = 3.544$, $P < 0.05$). Furthermore, to clarify the participation of reactive species in the inhibitory effect elicited by 3MCG on $Na^+,K^+$-ATPase activity, cerebral cortex homogenates were simultaneously co-incubated 5 mM 3MCG with the antioxidants GSH, MEL, TRO, CAT plus SOD or L-NAME. As observed in Fig. 6, MEL, TRO, and L-NAME partially prevented the inhibitory effect caused by 3MCG on $Na^+,K^+$-ATPase activity, whereas GSH had no effect on this inhibition ($F_{(6,27)} = 10.208$, $P < 0.05$).

Considering that antioxidant agents were able to attenuate or fully prevent the 3MCG-elicited inhibition on mCK and $Na^+,K^+$-ATPase activities, we evaluated whether this compound could provoke lipid oxidative damage in cortical homogenates. Figure 7 shows that TBA-RS values were markedly increased by 3MCG (up to 90%, $F_{(4,20)} = 12.783 < 0.001$) in a dose-dependent manner ($\beta = -0.874$, $t = -7.629$, $P < 0.001$).

## Discussion

High tissue and body fluids levels of 3MCG and also of 3-hydroisovaleric acid to a lesser extent, accompanied in some cases by hypoglycemia, hyperlacticacidemia, and hyperammonemia are characteristic of 3MCCD (Sweetman and Williams 2001). Patients affected by this syndrome commonly present progressive neurological dysfunction or acute encephalopathy, accompanied by developmental delay, hypotonia/hypertonia, seizures, and cardiomyopathy (Baykal et al. 2005; Dirik et al. 2008; Oude Luttikhuis et al. 2005). Although progressive cortical brain atrophy with leukodystrophy is usually observed in these patients (Baykal et al. 2005; de Kremer et al. 2002; Dirik et al. 2008; Murayama et al. 1997), the underlying mechanisms involved in the neuropathology of 3MCCD are practically unknown.

Affected patients with severe CNS damage present normal blood glucose levels and worsening of the

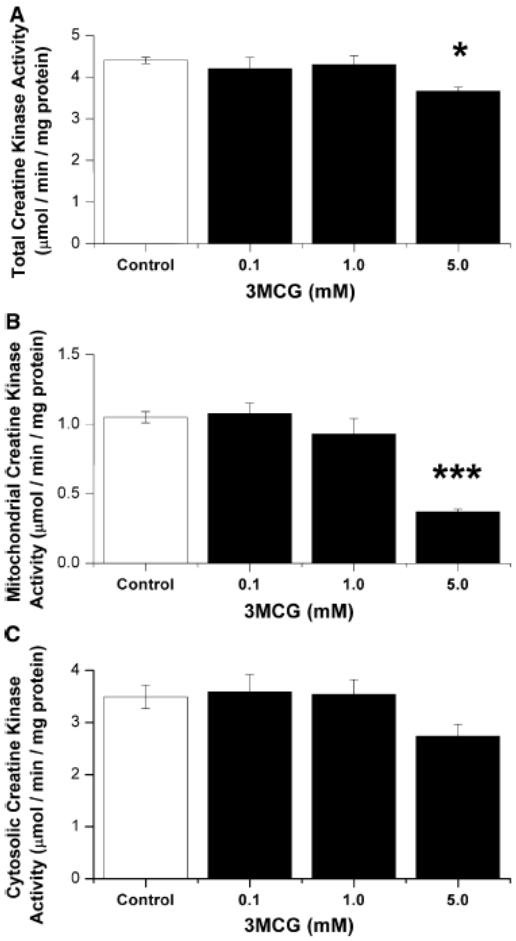


**Fig. 3** Effect of 3MCG on total (**a**), mitochondrial (**b**) and cytosolic (**c**) creatine kinase (CK) activities in cerebral cortex preparations from rats. Values are mean ± SEM for five to six independent experiments (animals) and are expressed as mmol creatine $min^{-1}$ mg protein $^{-1}$. $^{*}P < 0.05$ and $^{***}P < 0.001$, compared to control (ANOVA followed by Duncan multiple range test)

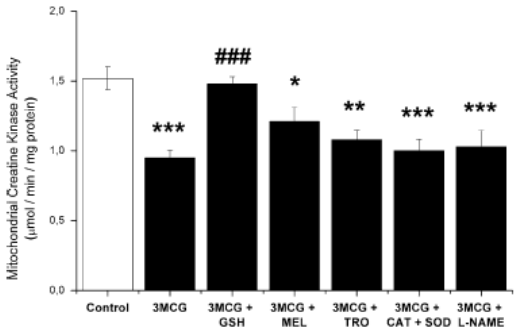


**Fig. 4** Effect of antioxidants on the inhibition of mitochondrial creatine kinase (mCK) activity provoked by 3MCG in cerebral cortex preparations from rats. The antioxidants reduced glutathione (GSH, 0.2 mM) melatonin (MEL, 0.2 mM), trolox (TRO, 1.5 µM), a combination of the antioxidant enzymes catalase (CAT, 50 mU $ml^{-1}$) plus superoxide dismutase (SOD, 50 mU $ml^{-1}$) or the nitric oxide synthase inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 1 mM) were co-incubated with 5 mM 3MCG and the activity of the enzyme measured afterward. Values are mean ± SEM for five independent experiments (animals) and are expressed as mmol creatine $min^{-1}$ mg $protein^{-1}$. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared to control; $^{\#\#\#}P < 0.001$, compared to 3MCG (ANOVA followed by Duncan multiple range test)

neurologic signs do not usually relate to the relapses of hypoglycemia observed in these patients (de Kremer et al. 2002; Dirik et al. 2008), suggesting that the accumulating metabolites in 3MCCD are potentially neurotoxic and might contribute to the pathogenesis of the disease. Furthermore, some individuals with 3MCCD also present accumulation and excretion of high amounts of lactic acid (Baykal et al. 2005; Leonard et al. 1981), indicating an impaired mitochondrial function. Therefore, in the current study we evaluated the in vitro effects of 3MCG, the major compound accumulating in 3MCCD, on important biochemical parameters of mitochondrial homeostasis, particularly those related to bioenergetics in cerebral cortex of young rats to provide mechanistic insights for 3MCCD neuropathology. Interestingly, 3-hydroxyisovaleric acid, which is also accumulated in this disorder, was previously shown not to compromise bioenergetics in brain of young rodents (Ribeiro et al. 2007), reinforcing the need to examine whether 3MCG could affect this important system necessary for brain development and functioning.

We initially verified that acetate oxidation was significantly decreased (up to 30%) by 3MCG and that the addition of CoA to the medium did not change the inhibitory action of 3MCG on $CO_2$ production, implying that there was not a shortage of CoA because of a competition between acetate and 3MCG for binding to this coenzyme. These findings point to a blockage of the CAC that may occur because of inhibition of one or more enzymatic activities of the cycle or, alternatively, secondary to a blockage of the electron transfer flow through the respiratory chain. We found that the activities of the various enzymatic steps of the CAC were not affected by 3MCG at concentrations as high as 5 mM. In contrast, 3MCG significantly inhibited the activity of complex II-III (up to 35%) of the respiratory chain. Considering that the complex II (succinate dehydrogenase) was not inhibited by 3MCG, we presume that complex III activity was inhibited by this compound. Reduction of the electron transport chain flow (impaired oxidative phosphorylation) implies

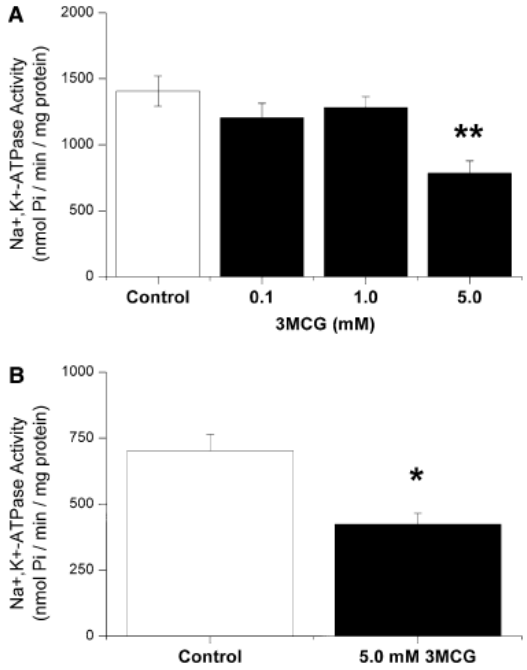


**Fig. 5** Effect of 3MCG on $Na^+,K^+$-ATPase activity from purified synaptic plasma membranes of rat cerebrum (**a**). 3MCG was pre-incubated for 30 min with the synaptic membranes before the addition of ATP to start the reaction. Effect of pre-incubating cerebrum homogenates with 5 mM 3MCG on synaptic membrane $Na^+,K^+$-ATPase activity (**b**). 3MCG was pre-incubated for 30 min with cortical homogenates, after which synaptic membranes were prepared and the enzyme activity measured. Values are mean ± SEM for five independent experiments (animals) and are expressed as nmol Pi $min^{-1}$ mg $protein^{-1}$. ** $P < 0.01$ (ANOVA followed by Duncan multiple range test) and * $P < 0.05$, compared to control (Student *t* test for paired samples)

lower ATP synthesis, which is basically produced in the cell during the electron transport through the respiratory chain. On the other hand, it is conceivable that the inhibition of the electron transport through the respiratory chain leads to a significant increase of reduced equivalents ($NADH/NAD^+$ ratio) with a consequent alosteric inhibition of various critical steps of this cycle catalyzed by dehydrogenases. This may possibly explain the 3MCG-induced reduction of CAC activity, as reflected by the reduction of $CO_2$ formation from acetate. We cannot, however, exclude the possibility that aconitase activity was inhibited by 3MCG given that we did not measure this activity because 3MCG interfered with the enzymatic assay.

Mitochondrial creatine kinase (mCK) activity, which is essential for intracellular energy buffering and transfers, was also inhibited by 3MCG. These observations are important since this enzyme activity is crucial for normal brain cell functioning and has been recognized as an important metabolic regulator during health and disease (Gross et al. 1996; Hamman et al. 1995; Holtzman et al. 1997; Wallimann et al. 1998; Wyss et al. 1992). It should be stressed that mCK activity decreases after brain exposure to agents promoting generation of free radicals probably by oxidation of essential cysteine residues of the enzyme (Arstall et al. 1998; Burmistrov et al. 1992; Konorev et al. 1998; Stachowiak et al. 1998; Wallimann et al. 1998; Wolosker et al. 1996). In this scenario, we found that GSH totally prevented the 3MCG-induced inhibitory effect on mCK activity. It is emphasized that GSH, besides being an effective scavenger of the reactive oxygen species (ROS) hydroxyl, peroxyl, and alcoxyl, as well as of carbonyl, is mainly a protector of protein sulfhydryl groups from oxidizing and cross-linking (Halliwell and Gutteridge 2007) that are part of the catalytic center of CK. Therefore, it is presumed that oxidation of essential sulfhydryl of other groups of the mCK isoform was probably involved in this effect.

So far, the present data indicate that energy production and intracellular transfer is compromised by 3MCG in cerebral cortex of young rats.

3MCG strongly inhibited $Na^+,K^+$-ATPase (up to 45%), an important activity of plasma synaptic membrane that is necessary for neuronal excitability and cellular volume control. This enzyme is present at high concentrations in the brain, consuming about 40–50% of the ATP generated in this tissue, highlighting its importance for normal brain functioning. Alterations of $Na^+,K^+$-ATPase activity may be secondary to free radical attack (Kurella et al. 1997; Lees 1993; Yousef et al. 2002) or to changes in membrane fluidity (Erecinska et al. 2004; Erecinska and Silver 1994; Wheeler et al. 1975). In this context, we found that the antioxidants MEL, TRO, and L-NAME were able to attenuate the synaptic $Na^+,K^+$-ATPase inhibition provoked by 3MCG, when pre-incubated with cortical homogenates, implying an indirect action of this compound possibly generating hydroxyl and peroxyl radicals, as well as reactive nitrogen species that could provoke oxidative damage on vulnerable groups of the enzyme. This was probably the case since we showed here that 3MCG markedly caused lipid peroxidation, as determined by a marked increase (90%) of TBA-RS values. In this scenario, our present results are in accordance with previous data showing that peroxynitrite is an inhibitor of renal $Na^+,K^+$-ATPase activity and that GSH was unable to reverse this inhibition (Reifenberger et al. 2008). Furthermore, inhibition of rat brain $Na^+,K^+$-ATPase activity by guanidinoacetate and arginine in vivo administration was shown to be similarly prevented by L-NAME, but not by GSH (Wyse et al. 2001; Zugno

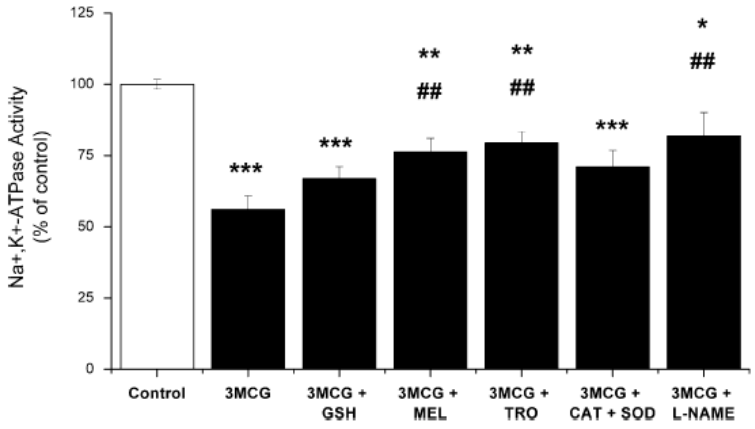


**Fig. 6** Effect of antioxidants on the inhibition of synaptic $Na^+,K^+$-ATPase activity provoked by 3MCG in rat cerebrum. The antioxidants reduced glutathione (GSH, 0.2 mM) melatonin (MEL, 0.2 mM), trolox (TRO, 1.5 μM), a combination of the antioxidant enzymes catalase (CAT, 50 mU $ml^{-1}$) plus superoxide dismutase (SOD, 50 mU $ml^{-1}$) or the nitric oxide synthase inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 1 mM) were co-incubated with 5 mM 3MCG and the enzyme activity measured afterward. Values represent mean ± SEM for four to seven independent experiments (animals) and are expressed as percentage of controls (mean of controls: 1438 Pi $min^{-1}$ mg $protein^{-1}$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared to control; $^{\#\#}P < 0.01$, compared to 3MCG (ANOVA followed by Duncan multiple range test)

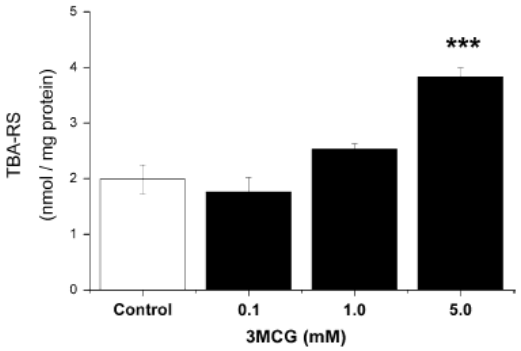


**Fig. 7** Effect of 3MCG on thiobarbituric acid reactive substances (TBA-RS) values in rat cerebral cortex. Data are represented as mean ± SEM for five independent experiments (animals) per group and expressed as nmol mg $protein^{-1}$. $^{***}P < 0.001$ compared to control (ANOVA followed by Duncan's multiple range test)

et al. 2004), indicating the involvement of reactive nitrogen species in this effect.

We cannot rule out the possibility that at least part of the inhibition of synaptic membrane $Na^+,K^+$-ATPase activity by 3MCG could be attributed to peroxidation of membrane lipids leading to alterations of lipid arrangement and/or of interactions between lipids and proteins, interfering with the membrane environment where $Na^+,K^+$-ATPase is embedded (Abeywardena and Charnock 1983; Kimelberg and Papahadjopoulos 1974; Wheeler et al. 1975).

Regarding to the differences observed with the various antioxidants in the prevention or attenuation of the inhibitory effects of 3MCG on creatine kinase and $Na^+,K^+$-ATPase activities, the obtained data suggest that essential cysteine residues (sulfhydryl groups) of the enzyme creatine kinase protected by GSH are particularly vulnerable to oxidative attack, whereas $Na^+,K^+$-ATPase is more susceptible to oxidation by reactive nitrogen species and by hydroxyl and peroxyl radicals.

Relatively to the possible consequences of the significant inhibition of $Na^+,K^+$-ATPase activity provoked by 3MCG to neural cellular function, there is increasing evidence suggesting that this enzyme is critical for normal brain function and reduction of this activity is related to selective neuronal damage in rat and human brain (Cousin et al. 1995; Lees 1993). Furthermore, inhibition of $Na^+,K^+$-ATPase has also been associated with excitotoxicity and epilepsy (Cousin et al. 1995; Lees and Leong 1995).

In conclusion, to the best of our knowledge this is the first report showing that 3MCG markedly disturbs brain bioenergetics and decreases synaptic $Na^+,K^+$-ATPase activity. It is therefore presumed that disruption of energy production and neurotransmission may contribute synergistically with other factors to account for the neurological damage found in patients affected by 3MCCD. Although it is difficult to determine at the present the exact pathophysiological relevance of our in vitro data, in case the present findings can be extrapolated to the in vivo human condition, it is conceivable that 3MCG may be at least in part responsible for the neuropathology of 3MCCD.

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# PARTE III

## *Discussão e Conclusões*

## III.1 DISCUSSÃO

Pacientes afetados por 3-MCCD apresentam predominantemente sintomas neurológicos (Baykal et al. 2005; Dirik et al. 2008; Luttikhuis et al. 2005) com elevação acentuada dos níveis de 3-MCG e também de 3-HIVA em menor quantidade nos tecidos e nos líquidos corporais, acompanhado em alguns casos por hipoglicemia, hiperlacticacidemia, hiperamonemia (Sweetman e Williams 2001). Apesar da atrofia cortical progressiva com leucodistrofia ser observada em muitos desses pacientes (Baykal et al. 2005; de Kremer et al. 2002; Dirik et al. 2008; Murayama et al. 1997), o mecanismo envolvido na neuropatologia da 3-MCCD é praticamente desconhecido.

Visto que esses pacientes apresentam níveis normais de glicose e que o agravamento dos sintomas neurológicos nem sempre está relacionado com um quadro de hipoglicemia (de Kremer et al. 2002; Dirik et al. 2008), é possível que os metabólitos acumulados na 3-MCCD sejam potencialmente tóxicos podendo contribuir para patogênese da doença. Outro dado interessante é que alguns indivíduos com 3-MCCD apresentam acúmulo e excreção de altas quantidades de ácido lático (Baykal et al. 2005; Leonard et al. 1981), indicando comprometimento da função mitocondrial. É fundamental ressaltar que a mitocôndria, além de ser importante na geração de espécies reativas, desempenha papel central na geração de energia para a homeostase dos processos celulares, através da manutenção dos níveis de ATP, além de participar ativamente da manutenção dos níveis intracelulares de cálcio (Nicholls e Akerman 1982) e estar envolvida em diversos processos que levam à morte celular (Liu et al. 1996).

Portanto, no presente estudo avaliamos os efeitos *in vitro* da 3-MCG, o principal composto acumulado na 3-MCCD, sobre importantes parâmetros bioquímicos da homeostase mitocondrial em córtex cerebral de ratos jovens a fim de esclarecer mecanismos envolvidos na neuropatologia da 3-MCCD. Neste cenário, foi demonstrado que 3-HIVA, que também é acumulado nessa desordem, não comprometeu a bioenergética em cérebro de ratos jovens (Ribeiro et al. 2007), reforçando a necessidade de investigar se a 3-MCG pode afetar o funcionamento e desenvolvimento cerebral.

Verificamos inicialmente o funcionamento do CAC e observamos que a oxidação de acetato foi diminuída significativamente (30%) pela presença de 3-MCG. Para demonstrar que não ocorreu falta CoA no ensaio devido uma competição entre o acetato e a 3-MCG para se ligar a essa coenzima foi adicionado CoA (1mM) ao meio de incubação, o que não alterou a ação inibitória da 3-MCG sobre a produção de $CO_2$.Esses achados apontam para um bloqueio no CAC que pode ocorrer devido à inibição de uma ou mais enzimas do ciclo, ou através de inibição secundária por um bloqueio da transferência de elétrons através da CTE. Neste particular, observamos que a atividade de várias enzimas do CAC não foi afetada pela 3-MCG nas mesmas concentrações testadas. Por outro lado, demonstramos que a 3-MCG inibiu significativamente a atividade do complexo II-III da CTE (35%). Considerando que a atividade do complexo II (succinato desidrogenase) não foi inibida pela 3-MCG, poder-se-ia sugerir que a atividade do complexo III foi comprometida por esse metabólito. A redução do fluxo de elétrons através da cadeia respiratória implica em uma diminuição da síntese de ATP, que é basicamente produzido na célula durante o transporte de elétrons na CTE.

Por outro lado, sabe-se que uma inibição do transporte de elétrons na CTE leva a um aumento significante de equivalentes reduzidos (razão $NADH/NAD^+$) com consequente inibição alostérica de vários passos do ciclo catalizados por desidrogenases. Isso pode possivelmente explicar a diminuição da atividade do CAC pela 3-MCG, que foi refletido pela diminuição da produção de $CO_2$ a partir de acetato. Contudo, não podemos excluir a possibilidade da atividade da enzima aconitase estar inibida pela 3-MCG visto que não conseguimos medir sua atividade devido a uma interferência do metabólito no ensaio enzimático. A aconitase é uma enzima altamente suscetível à oxidação, sendo a mesma também considerada um parâmetro de estresse oxidativo (Chepelev et al. 2009; Tretter e Adam-Vizi 2000).

Observamos também que a atividade da enzima mCK, essencial para a transferência e tamponamento energético intracelular, foi inibida pela 3-MCG. Esse resultado é importante uma vez que a CK é crucial para o funcionamento normal cerebral e tem sido considerada como um importante regulador metabólico (Gross et al. 1996; Holtzman et al. 1997; Wallimann et al. 1998; Wyss et al. 1992). Verificamos que a GSH preveniu totalmente o efeito inibitório induzido pela 3-MCG sobre a mCK. Sabe-se que GSH, além de ser um sequestrador dos radicais hidroxil, peroxil, bem como carbonil, é também e um protetor contra processos de oxidação dos grupamentos sulfidrila que fazem parte do centro catalítico da enzima (Halliwell e Gutteridge 2007). Portanto, é possível que a oxidação dos grupamentos sulfidril da mCK pela 3-MCG podem estar envolvidos nesse efeito. Estudos demonstram que a atividade da mCK diminui depois da exposição do cérebro a agentes que promovam a geração de radicais livres provavelmente pela

oxidação de resíduos essenciais de cisteína da enzima (Arstall et al. 1998; Burmistrov et al. 1992; Konorev et al. 1998; Stachowiak et al. 1998; Wallimann et al. 1998; Wolosker et al. 1996)

Esses resultados indicam que a produção e a transferência intracelular de energia estão comprometidas pela 3-MCG em córtex cerebral de ratos jovens.

Também verificamos que a 3-MCG inibiu fortemente a atividade da $Na^+$, $K^+$-ATPase (45%), enzima importante para a excitabilidade neuronal e controle do volume celular. Essa enzima é presente em altas concentrações no cérebro, consumindo cerca de 40–50% do ATP gerado no tecido, sendo importante para o funcionamento normal do cérebro. Tem sido demonstrado que alterações da atividade da $Na^+$, $K^+$-ATPase podem ser secundárias a ataque de radicais livres (Kurella et al. 1997; Lees 1993; Yousef et al. 2002) ou mudanças da fluidez da membrana (Erecinska et al. 2004; Erecinska e Silver 1994; Wheeler et al. 1975). Neste contexto, observamos que os antioxidantes melatonina (MEL), trolox (TRO) e L-arginina metil-éster (L-NAME) foram capazes de atenuar a inibição da $Na^+$,$K^+$-ATPase sináptica provocada pela 3-MCG, quando pré-incubados com homogeneizados de córtex, indicando uma ação indireta desse metabólito possivelmente através dos radicais peroxil e hidroxil, bem como de espécies reativas de nitrogênio que poderiam provocar dano oxidativo nos grupos vulneráveis da enzima. Estudos anteriores mostrando que o peroxinitrito é um inibidor da $Na^+$, $K^+$-ATPase renal corroboram com nossos resultados (Reifenberger et al. 2008). Outra investigação mostrou inibição da atividade da $Na^+$, $K^+$-ATPase em cérebro de ratos pela administração de guanidinoacetato e arginina *in vivo*

que foi prevenida pelo L-NAME ( Wyse et al. 2001; Zugno et al. 2004), indicando o envolvimento de espécies reativas de nitrogênio nesse efeito.

Não podemos, por outro lado, descartar a possibilidade de que a inibição da $Na^+$, $K^+$-ATPase pela 3-MCG pode ser atribuída, pelo menos em parte, a oxidação dos lipídios de membrana levando à alterações na conformação dos lipídios e/ou interações entre lipídios e proteínas, interferindo com a $Na^+$, $K^+$-ATPase que está incorporada na membrana sináptica (Abeywardena e Charnock 1983; Kimelberg e Papahadjopoulos 1974; Wheeler et al. 1975). Neste particular, foi observado que a 3-MCG induziu peroxidação lipídica (90 %) em cérebro de ratos, verificado por um aumento de substâncias reativas ao ácido tiobarbitúrico (TBA-RS) é o principal produto de oxidação lipídica.

Ao analisarmos as diferenças na prevenção ou atenuação com vários antioxidantes sobre os efeitos da 3-MCG nas atividades da CK e da $Na^+$, $K^+$-ATPase de membrana sináptica, os dados obtidos sugerem que os resíduos essenciais de cisteína (grupos sulfidril) da enzima CK protegidos pelo GSH são vulneráveis ao ataque oxidativo, enquanto a $Na^+$, $K^+$-ATPase é mais suscetível à oxidação por espécies reativas de nitrogênio e aos radicais hidroxil e peroxil.

No que diz respeito às possíveis consequencias na inibição da atividade da $Na^+$, $K^+$-ATPase pela ação da 3-MCG à função celular neuronal, evidências prévias demonstram que essa enzima é fundamental para uma função normal do cérebro e uma redução em sua atividade está relacionada com dano neuronal em cérebro de ratos e humanos (Cousin et al. 1995; Lees

1993). A inibição da $Na^+,K^+$-ATPase também está associada com excitotoxicidade e epilepsia (Cousin et al. 1995; Lees e Leong 1995).

Concluindo, esse é o primeiro estudo mostrando que a 3-MCG leva a um distúrbio na bioenergética cerebral bem como uma diminuição da atividade da $Na^+,K^+$-ATPase. Dessa forma podemos presumir que a alteração na produção de energia e neurotransmissão contribuam sinergicamente com outros fatores para o dano neurológico encontrado dos pacientes afetados pela 3-MCCD. Apesar de ser difícil determinar a relevância dos nossos resultados *in vitro*, já que as concentrações cerebrais da 3-MCG nos pacientes não são conhecidas, no caso dos nossos achados serem confirmados na condição *in vivo* em humanos, é provável que a 3-MCG possa ser ao menos em parte responsável pela neuropatologia da 3-MCCD.

## III.2 CONCLUSÕES

- A 3-MCG inibiu a produção de $CO_2$ a partir de [1-$^{14}$C] acetato, sugerindo que houve prejuízo da atividade do ciclo do CAC. A diminuição na produção de $CO_2$ não foi devida à falta de CoA no meio, descartando uma possível competição entre a 3-MCG e o acetato pela CoA.
- A 3-MCG inibiu a atividade do complexo II-III sem alterar a atividade dos demais complexos, o que indica que esse metabólito pode estar comprometendo o fluxo de elétrons pela CTE, levando a uma possível diminuição de ATP.
- A 3-MCG alterou a atividade da enzima mCK, sugerindo que esse metabólito compromete a transferência intracelular de energia. Essa inibição foi prevenida pela adição de GSH ao meio, indicando que um ataque oxidativo causado pela 3-MCG aos grupamentos sulfidrila da isoforma mCK pode estar envolvido nesse efeito.
- A 3-MCG diminuiu a atividade da enzima $Na^+$,$K^+$-ATPase, indicando que esse metabólito pode alterar o potencial de membrana necessário para a manutenção da excitabilidade neuronal e a neurotransmissão. Essa inibição foi atenuada pelos antioxidantes MEL, TRO e L-NAME, indicando uma ação desse metabólito possivelmente através de radicais peroxil e hidroxil, bem como de espécies reativas de nitrogênio que podem provocar dano oxidativo nos grupos vulneráveis da enzima.

- A 3-MCG aumentou os níveis de TBA-RS, indicando que esse metabólito induz a peroxidação lipídica.

## III. 3 PERSPECTIVAS

- Avaliar o efeito *in vitro* da 3-MCG sobre parâmetros do metabolismo energético em coração de ratos;
- Avaliar o efeito *ex vivo* através de injeção em ratos de 7, 15 e 30 dias da 3-MCG e posterior medida dos parâmetros do metabolismo energético e estresse oxidativo em cérebro;
- Avaliar o efeito da 3-MCG sobre parâmetros de metabolismo energético e estresse oxidativo em culturas de astrócitos e neurônios de cérebro de ratos;
- Avaliar o efeito da 3-MCG sobre parâmetros respiratórios obtidos pelo consumo de oxigênio através oximetria em cérebro de ratos.

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