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**EFEITOS *IN VITRO* DO ÁCIDO 3-HIDROXIISOBUTÍRICO SOBRE**  
**PARÂMETROS BIOQUÍMICOS DO METABOLISMO ENERGÉTICO EM**  
**CÉREBRO DE RATOS JOVENS**

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**Porto Alegre, 2008.**

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*À minha mãe por todas as abdições para  
que eu pudesse chegar até aqui.*

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## RESUMO

A acidúria 3-hidróxiisobutírica é uma doença metabólica hereditária causada pela deficiência da atividade da 3-hidroxiisobutiril-Coenzima A desidrogenase. Acúmulo tecidual e alta excreção urinária do ácido 3-hidroxiisobutírico são as características bioquímicas desta desordem. As manifestações clínicas são heterogêneas, geralmente incluindo achados dismórficos, atraso no desenvolvimento motor, retardo mental severo e encefalopatia aguda. A acidemia láctica também ocorre nos pacientes afetados, indicando que uma disfunção mitocondrial pode estar envolvida na fisiopatogenia desta doença. Portanto, o objetivo do presente trabalho foi investigar o efeito *in vitro* do ácido 3-hidroxiisobutírico (0,1; 0,5 e 1,0 mM) sobre a atividade de enzimas essenciais para o metabolismo energético, tais como os complexos I-V da cadeia respiratória, creatina quinase total, citosólica e mitocondrial e Na<sup>+</sup>, K<sup>+</sup>-ATPase em homogeneizados de córtex cerebral de ratos de 30 dias de idade. Também medimos o consumo de oxigênio em preparações mitocondriais de cérebro total na presença de ácido 3-hidroxiisobutírico. O ácido 3-hidroxiisobutírico inibiu significativamente o complexo I-III (20%), sem afetar as outras atividades da cadeia respiratória. Além disso, o ácido 3-hidroxiisobutírico não alterou o estado III, estado IV e o índice de controle respiratório na presença de glutamato/malato ou succinato, sugerindo que seu efeito sobre a respiração celular foi de pequena intensidade. Por outro lado as atividades da creatina quinase total e mitocondrial, mas não citosólica, foram inibidas (30%) pelo ácido 3-hidroxiisobutírico. Também verificamos que a inibição provocada por esse ácido sobre a atividade mitocondrial da creatina quinase foi completamente prevenida por pré-incubação dos homogeneizados com glutatona reduzida,  $\alpha$ -tocoferol ou a combinação de superóxido desmutase com catalase, indicando que esta inibição foi mediada por oxidações de grupos tióis essenciais para a enzima e provavelmente pelos radicais superóxido, peróxido de hidrogênio e/ou peroxilas. Também demonstramos que a atividade da Na<sup>+</sup>, K<sup>+</sup>-ATPase da membrana plasmática sináptica foi marcadamente reduzida (37%) pelo ácido 3-hidroxiisobutírico e que esse efeito foi prevenido pela co-incubação com  $\alpha$ -tocoferol, sugerindo que radicais peroxilas estavam provavelmente envolvidos nesta ação. Considerando a importância das atividades enzimáticas afetadas pelo ácido 3-hidroxiisobutírico para o metabolismo cerebral e para a neurotransmissão, sugerimos que os aumentos teciduais das concentrações desse ácido possam contribuir para a neurodegeneração dos pacientes afetados pela acidúria 3-hidroxiisobutírica, e possivelmente expliquem achados prévios de elevada produção e excreção de lactato.

**Palavras-chave:** Acidúria 3-hidróxiisobutírica, ácido 3-hidroxiisobutírico, metabolismo energético, córtex cerebral.



## ABSTRACT

3-Hydroxyisobutyric aciduria is an inherited metabolic disease caused by 3-hydroxyisobutyryl-CoA dehydrogenase deficiency. Tissue accumulation and high urinary excretion of 3-hydroxyisobutyric acid is the biochemical hallmark of this disorder. Clinical phenotype is heterogeneous and generally includes dysmorphic features, delayed motor development, profound mental impairment, and acute encephalopathy. Lactic acidemia is also found in the affected patients, indicating that mitochondrial dysfunction may be involved in the pathophysiology of this disorder. Therefore, the aim of the present work was to investigate the *in vitro* effect of 3-hydroxyisobutyric acid (0.1, 0.5 and 1mM) on essential enzymes of energy metabolism, namely the activities of the respiratory chain complexes I-V, total, cytosolic and mitochondrial creatine kinase and Na<sup>+</sup>, K<sup>+</sup>-ATPase in cerebral cortex homogenates of 30-day-old rats. We also measured the rate of oxygen consumption in brain mitochondrial preparations in the presence of 3-hydroxyisobutyric acid. 3-Hydroxyisobutyric acid significantly reduced complex I-III (20%), without affecting the other activities of the electron transport chain. Furthermore, 3-hydroxyisobutyric acid did not change state III, state IV and the respiratory control ratio in the presence of glutamate/malate or succinate, suggesting that its effect on cellular respiration was weak. On the other hand, the activities of total and mitochondrial creatine kinase, but not cytosolic creatine kinase, were inhibited (30%) by 3-hydroxyisobutyric acid. We also observed that 3-hydroxyisobutyric acid-induced inhibition of mitochondrial creatine kinase activity was fully prevented by pre-incubation of the homogenates with reduced glutathione,  $\alpha$ -tocopherol or the combination of superoxide dismutase plus catalase, suggesting that this inhibition was mediated by oxidation of essential thiol groups of the enzyme probably by superoxide, hydrogen peroxide and/or peroxy radicals. It was also demonstrated that Na<sup>+</sup>, K<sup>+</sup>-ATPase activity from synaptic plasma membranes was markedly suppressed (37 %) by 3-hydroxyisobutyric acid and that this effect was prevented by  $\alpha$ -tocopherol co-incubation implying that peroxy radicals were probably involved in this action. Considering the importance of the affected enzyme activities for brain metabolism homeostasis and neurotransmission, it is suggested that increased tissue levels of 3-hydroxyisobutyric acid may contribute to the neurodegeneration of patients affected by 3-hydroxyisobutyric aciduria and possibly explain previous reports describing elevated production and excretion of lactate.

**Keywords:** 3-Hydroxyisobutyric aciduria, 3-hydroxyisobutyric acid, energy metabolism, and cerebral cortex.

## LISTA DE ABREVIATURAS

3-HiB	ácido 3-hidroxiisobutírico
3-HiBúria	acidúria 3-hidroxiisobutírica
CAT	catalase
Cy-CK	fração citosólica da creatina quinase
GSH	glutathiona reduzida
Mi-CK	fração mitocondrial da creatina quinase
Pi	fosfato inorgânico
RCR	índice de controle respiratório
SNC	sistema nervoso central
SOD	superóxido desmutase

# 1 INTRODUÇÃO

## 1.1 ACIDÚRIAS ORGÂNICAS

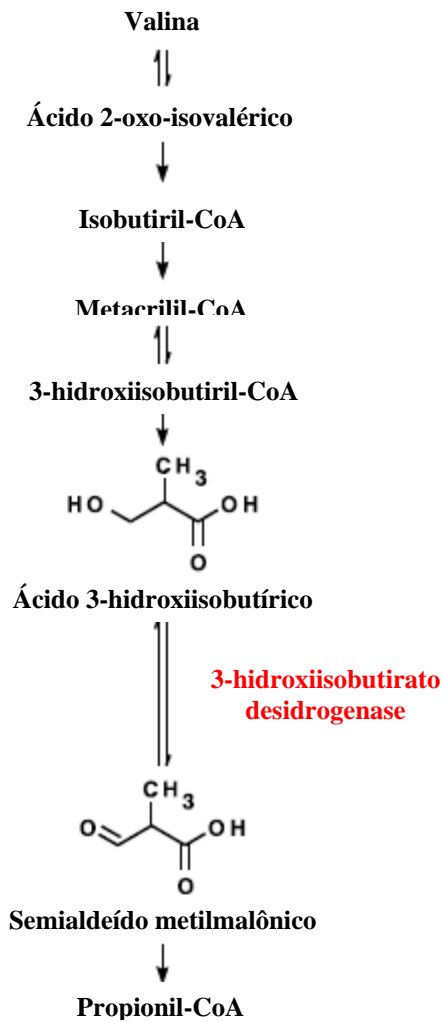
As acidúrias ou acidemias orgânicas são erros inatos do metabolismo nos quais um ou mais ácidos orgânicos acumulam-se nos tecidos dos pacientes afetados devido à deficiência da atividade de uma enzima do metabolismo de aminoácidos, lipídeos ou carboidratos (CHALMERS e LAWSON, 1982; SCRIVER *et al.*, 2001). Vários ácidos orgânicos estão presentes no sangue e na urina de indivíduos normais, porém em concentrações reduzidas. Nos pacientes com estes distúrbios, estes ácidos encontram-se em altas concentrações no sangue e, principalmente, na urina. Clinicamente os pacientes afetados apresentam como sintomatologia mais comum, disfunção neurológica em suas diversas formas de expressão: regressão neurológica, convulsões, coma, ataxia, hipotonia, hipertonia, irritabilidade, tremores, movimentos coreatetóticos, tetraparesia espástica, atraso no desenvolvimento psicomotor e outros. As mais freqüentes manifestações laboratoriais são cetose, cetonúria, neutropenia, trombocitopenia, acidose metabólica, baixos níveis de bicarbonato, hiperglicemia, hiperglicinúria, hiperamonemia, hipo/hiperglicemia, acidemia láctica, aumento dos níveis séricos de ácidos graxos livres, bem como cheiro peculiar na urina e/ou suor e outros (SCRIVER *et al.*, 2001). Com a utilização da tomografia computadorizada e ressonância magnética nuclear, foram verificadas freqüentemente alterações de

substância branca (hipomielização e/ou desmielização), atrofia cerebral generalizada ou de gânglios da base (necrose ou calcificação), megaencefalia, atrofia frontotemporal e atrofia cerebelar em pacientes afetados por estas doenças (MAYATEPEK *et al.*, 1996).

### **1.1.1 Acidúria 3-hidroxiisobutírica**

A acidúria 3-hidroxiisobutírica (3-HiBúria - OMIM 236795) é uma acidúria orgânica que envolve o metabolismo da valina (Figura 1). O acúmulo tecidual e a excreção urinária aumentada do ácido 3-hidroxiisobutírico (3-HiB) são os marcadores bioquímicos desta desordem metabólica. Até o momento doze pacientes foram descritos com excreção aumentada do ácido 3-hidroxiisobutírico, sugerindo uma deficiência enzimática da 3-hidroxiisobutirato desidrogenase. A variação fenotípica desta desordem é bastante ampla sendo que alguns pacientes apresentam manifestações clínicas moderadas, enquanto outros apresentam sintomas severos (KO *et al.*, 1991; CHITAYAT *et al.*, 1992; SASAKI *et al.*, 2001; SHIELD *et al.*, 2001; LOUPATTY *et al.*, 2006; SASAKI *et al.*, 2006).

Figura 1



**FIGURA 1:** Rota catabólica do aminoácido L-valina.

#### 1.1.1.1 Aspectos Clínicos

As manifestações clínicas dos pacientes com 3-HiBúria são variáveis, podendo apresentar vômitos com desenvolvimento cognitivo e cerebral normal, atraso no desenvolvimento motor, retardo mental severo, ou crises encefalopáticas com acidose metabólica e mesmo morte prematura (SWEETMAN e WILLIAMS, 2001).

#### **1.1.1.2 Aspectos bioquímicos**

Todos os pacientes com 3-HiBuria excretam quantidades aumentadas de 3-HiB, variando de 60 a 390 mmol/mol de creatinina podendo chegar a concentrações de até 10000 mmol/mol de creatinina durante episódios agudos (KO *et al.*, 1991). Os pacientes podem também apresentar ácido láctico elevado ou aumento na excreção de outros 3-hidroxiácidos (SWEETMAN e WILLIAMS, 2001).

#### **1.1.1.3 Aspectos genéticos**

O padrão de herança desta desordem não foi determinado até o momento. Dentre os pacientes descritos com 3-HiBuria, nove eram do sexo masculino, sendo que os outros três pacientes o sexo não foi relatado (SWEETMAN e WILLIAMS, 2001; SASAKI *et al.*, 2006).

#### **1.1.1.4 Diagnóstico**

O diagnóstico precoce da 3-HiBuria é extremamente importante já que um tratamento na fase inicial da doença pode potencialmente retardar ou até mesmo evitar as manifestações neurológicas irreversíveis desta doença. O diagnóstico da 3-HiBuria é feito pela análise de ácidos orgânicos na urina que mostra uma excreção elevada de 3-HiB (KO *et al.*, 1991). Assim, a excreção elevada de 3-HiB de 60 a 600 mmol/mol de creatinina é sugestivo desta desordem quando o paciente não está em estado cetótico, visto que durante a cetonemia os níveis desse ácido podem aumentar (LANDAAS, 1975; SWEETMAN e WILLIAMS, 2001).

O diagnóstico pré-natal é possível, pois o 3-HiB está elevado no líquido amniótico dos pacientes, chegando a 7  $\mu\text{M}$  (normal: 0,9 a 2,4  $\mu\text{M}$ ) (CHITAYAT *et al.*, 1992).

#### **1.1.1.5 Tratamento**

O tratamento dos pacientes afetados pela 3-HiBuria durante os episódios agudos é praticamente o mesmo dos afetados por outras acidúrias orgânicas e consta fundamentalmente de hidratação, infusão de glicose para prover calorias e reduzir o catabolismo protéico endógeno (TANAKA *et al.*, 1980). O tratamento durante a recuperação e remissão geralmente consiste na restrição protéica (entre 0,75 e 2,0 g/Kg por dia) e suplementação com L-carnitina (100 mg/Kg), que resulta em uma redução marcante na freqüência e severidade dos episódios de acidose (SWEETMAN e WILLIANS, 2001).

#### **1.1.1.6 Fisiopatogenia**

Até o momento a fisiopatogenia do dano cerebral nos pacientes afetados por essa doença é desconhecida, porém a acidemia e a acidúria láctica encontrada nos pacientes são indicativos de disfunção mitocondrial. Neste contexto, biópsia muscular realizada em um paciente afetado pela 3-HiBuria mostrou uma redução na atividade dos complexos I e IV da cadeia respiratória (NADH desidrogenase e citocromo *c* oxidase, respectivamente). Também, foi observada, através de histoquímica enzimática (biópsia muscular), uma proliferação mitocondrial anormal e fibras vermelhas rompidas, sugerindo um defeito na cadeia respiratória (LOUPATTY *et al.*, 2006).

## 1.2 METABOLISMO ENERGÉTICO CEREBRAL

O cérebro possui uma intensa atividade metabólica, porém suas reservas energéticas são extremamente pequenas em relação à sua demanda. Assim, há a necessidade de substratos energéticos para o cérebro de mamíferos, sendo a glicose o principal deles, onde, em contraste com outros tecidos, não necessita de insulina para ser captada e oxidada (DICKINSON, 1996). No entanto, o padrão de utilização deste nutriente varia conforme a etapa de desenvolvimento do sistema nervoso central (SNC), o estado nutricional do indivíduo e o destino de sua cadeia de átomos de carbono (SMITH *et al.*, 2004). Situações de jejum prolongado fazem com que o SNC passe a utilizar corpos cetônicos para obtenção de energia para poupar o organismo de um catabolismo protéico exacerbado resultante da necessidade da manutenção da glicemia via gliconeogênese (SMITH *et al.*, 2004). A glicose captada pelo cérebro é, no entanto, fonte de carbono para a síntese de diversas outras biomoléculas essenciais (por exemplo, neurotransmissores), o que reforça a idéia de que a utilização de glicose não está atrelada somente à produção de energia (NICHOLLS e FERGUSON, 2001).

Mitocôndrias de mamíferos são organelas intracelulares ubíquas, responsáveis pela produção de ATP através do metabolismo aeróbico. Tecidos com alta atividade aeróbica tais como cérebro, músculos esquelético e cardíaco, apresentam altas concentrações de mitocôndrias. A mitocôndria desempenha uma série de funções intracelulares além da produção de ATP, tendo um papel crítico



no processo de apoptose e servindo como um tampão de cálcio (ORTH e SCHAPIRA, 2001).

O ciclo do ácido cítrico é a via comum de oxidação dos glicídios, aminoácidos e ácidos graxos (aproximadamente 95% do ATP sintetizado). O metabolismo energético cerebral é essencialmente aeróbico, sendo a glicose o principal substrato utilizado (CLARK *et al.*, 1993), entrando no ciclo sob a forma de acetil-CoA, que é então oxidada completamente a CO<sub>2</sub>. Quando não há hipóxia, a fosforilação oxidativa é dependente da concentração de ATP, ADP e fosfato inorgânico (Pi) e da razão mitocondrial de NADH/NAD<sup>+</sup>, que é determinada pela atividade da cadeia transportadora de elétrons e pela transferência de elétrons provenientes de reações catalisadas por enzimas mitocondriais. A cadeia transportadora de elétrons oxida o NADH e bombeia prótons para o espaço intermembrana da mitocôndria formando assim um gradiente de prótons que através da passagem pela ATP-sintase, produz ATP na fosforilação oxidativa (NICHOLLS e FERGUSON, 2001).

Outro importante sistema que auxilia a manutenção dos níveis cerebrais de ATP é o da creatina quinase, que está presente tanto no citosol quanto ligada às membranas mitocondriais e catalisa a transferência reversível de um grupamento fosfato entre a fosfocreatina e o ATP. Esse sistema tem sido associado a algumas funções particularmente importantes para o cérebro, como tamponamento energético (através da regeneração do ATP e 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<sup>+</sup>,K<sup>+</sup>-ATPase é uma proteína transmembrana cuja função é

translocar  $\text{Na}^+$  (muito mais concentrado fora do que dentro da célula) e  $\text{K}^+$  (muito mais concentrado dentro do que fora da célula), através da membrana plasmática, contra seus gradientes de concentração utilizando energia (ATP). A enzima transporta simultaneamente três  $\text{Na}^+$  para fora e dois  $\text{K}^+$  para dentro da célula. A saída de  $\text{Na}^+$  capacita as células animais a controlar osmoticamente seu conteúdo de água. Como três cargas positivas são transportadas para o meio extracelular e apenas duas para o meio intracelular, o fluxo de íons  $\text{Na}^+$  e  $\text{K}^+$  produz um gradiente eletroquímico através da membrana celular (LINGREL e KUNTZWEILER, 1994), que é usado como fonte de energia para a despolarização e repolarização do potencial de membrana, manutenção e regulação do volume celular, transporte ativo, transporte dependente de  $\text{Na}^+$ , de glicose, de aminoácidos e de neurotransmissores e cotransporte/antiporte de outros íons (GEERING, 1990). Enfatiza-se que todas as células eucarióticas superiores consomem grandes quantidades do ATP por elas produzido para a manutenção das concentrações citosólicas de  $\text{Na}^+$  e  $\text{K}^+$ , sendo que o consumo chega a ser de 40 a 60% nas células neuronais (WHITTAN, 1962).

Alterações nos mecanismos que mantêm o equilíbrio entre a taxa de sódio e potássio intra e extraneuronal podem ter conseqüências graves para as células do SNC (ERECINSKA e SILVER, 1994), sendo associada com despolarização excessiva, instabilidade da membrana e descargas paroxísticas (DONALDSON *et al.*, 1971).

### 1.2.1 Metabolismo Energético nas Doenças Neurodegenerativas

Numerosas hipóteses têm sido propostas para explicar a fisiopatologia das doenças neurodegenerativas Alzheimer, Huntington e Parkinson, bem como algumas acidúrias orgânicas, sem, no entanto, se obter até o momento uma explicação satisfatória para o dano cerebral dessas doenças. No entanto, 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 cadeia transportadora de elétrons seria o evento etiológico primário na maioria dessas doenças (PARKER *et al.*, 1990, 1994; SWERDLOW *et al.*, 1996, 1997).

O cérebro é altamente dependente de energia para seu funcionamento normal (ROSSEN *et al.*, 1943) e a mitocôndria é a estrutura intracelular que mantém os suprimentos de energia para este tecido. Uma alteração funcional nessa estrutura pode levar, portanto, a alterações patológicas nos neurônios e astrócitos (BEAL *et al.*, 1993; BEAL, 1995; BOWLING e BEAL, 1995; DAVIS *et al.*, 1995). Mutações no DNA mitocondrial e reações envolvendo geração de espécies reativas podem danificar a mitocôndria e diminuir a atividade dos complexos da cadeia respiratória. Um prejuízo no transporte de elétrons, além de causar um prejuízo na produção de ATP, leva a uma dispersão dos elétrons na forma de radicais livres potencialmente danosos à célula. Visto que os sistemas de defesa antioxidantes, como as enzimas superóxido dismutase, glutatona peroxidase,

glutathione redutase e catalase, são relativamente deficientes no cérebro (MARKLUND *et al.*, 1982; MARTILLA *et al.*, 1988), um ciclo vicioso pode ocorrer, aumentando o dano oxidativo a neurônios e levando à morte neuronal característica de doenças neurodegenerativas. Um prejuízo na fosforilação oxidativa também reduz o bombeamento de prótons através da membrana mitocondrial, diminuindo o potencial da membrana e, conseqüentemente, levando à abertura do poro de transição da permeabilidade mitocondrial. Evidências sugerem que uma diminuição no potencial de membrana mitocondrial seja um evento primário na iniciação do processo apoptótico (WADIA *et al.*, 1998).

Numerosas evidências relacionam doenças neurodegenerativas a uma diminuição no metabolismo energético. Estudos *postmortem* demonstraram uma diminuição na atividade do complexo I da cadeia respiratória em cérebros de pacientes portadores de doença de Parkinson (SCHAPIRA *et al.*, 1989, 1990a,b; JANETZKY *et al.*, 1994; GU *et al.*, 1998). Também há relatos de defeitos nos complexos II e III da cadeia respiratória e na enzima  $\alpha$ -cetoglutarato desidrogenase, importante enzima do ciclo do ácido cítrico, nessa doença (MIZUNO *et al.*, 1990, 1994).

Na doença de Alzheimer, a mais comum dentre as doenças neurodegenerativas, é encontrada principalmente uma redução na atividade do complexo IV da cadeia respiratória (MAURER *et al.*, 2000). Estudos de imagem *in vivo* demonstraram uma redução no metabolismo da glicose em pacientes portadores da doença de Alzheimer, e essa diminuição aparece precocemente, precedendo os sintomas clínicos, e se acentua com a severidade da doença

(JAGUST *et al.*, 1988; SMITH *et al.*, 1992; KENNEDY *et al.*, 1995; SMALL *et al.*, 1995). Estudos *postmortem* em cérebros demonstraram uma diminuição na atividade do complexo enzimático da piruvato desidrogenase e na atividade da enzima  $\alpha$ -cetoglutarato desidrogenase na doença de Alzheimer (PERRY *et al.*, 1980; GIBSON *et al.*, 1988; MASTROGIACOMO *et al.*, 1993).

Por outro lado, vários estudos têm demonstrado uma diminuição na utilização de glicose em estriado e córtex cerebral de pacientes portadores da doença de Huntington (KUHL *et al.*, 1982; HAYDEN *et al.*, 1986; YOUNG *et al.*, 1986; GRAFTON *et al.*, 1992; KUWERT *et al.*, 1993), além de um aumento nos níveis cerebrais de lactato (JENKINS *et al.*, 1993). Também se observou uma redução na utilização de oxigênio, bem como na atividade dos complexos II, III e IV da cadeia respiratória no núcleo caudato de pacientes portadores dessa doença (BRENNAN *et al.*, 1985; GU *et al.*, 1996).

No que se refere às acidúrias orgânicas, um número considerável de achados indicam que vários ácidos orgânicos acumulados nestas doenças provocam disfunção energética cerebral, por alterar o funcionamento da cadeia respiratória (STRAUSS e MORTON, 2003; KÖLKER *et al.*, 2004; FERREIRA *et al.*, 2007), ciclo de Krebs (OKUN *et al.*, 2002) e enzimas cruciais para o tamponamento energético cerebral (creatina quinase) (BARSCHAK *et al.*, 2006) e neurotransmissão ( $\text{Na}^+$ ,  $\text{K}^+$ -ATPase) (de ASSIS *et al.*, 2006).

## 2 OBJETIVOS

### 2.1 OBJETIVO GERAL

Este trabalho teve por objetivo avaliar o efeito *in vitro* do ácido 3-hidroxiisobutírico (3-HiB) sobre o metabolismo energético em cérebro de ratos jovens e os mecanismos envolvidos em uma possível disfunção energética induzida por este ácido.

### 2.2 OBJETIVOS ESPECÍFICOS

- Avaliar o efeito *in vitro* do 3-HiB sobre a atividade dos complexos I, II, III, IV e V da cadeia respiratória em homogeneizado de córtex cerebral de ratos de 30 dias de vida.
- Avaliar o efeito *in vitro* do 3-HiB sobre as atividades das isoformas da enzima creatina quinase em córtex cerebral de ratos de 30 dias de vida.
- Avaliar o efeito *in vitro* do 3-HiB sobre a atividade da enzima  $\text{Na}^+, \text{K}^+$ ATPase em córtex cerebral de ratos de 30 dias de vida.
- Avaliar o efeito *in vitro* do 3-HiB sobre os parâmetros respiratórios medidos por oximetria em preparações mitocondriais de cérebro de ratos de 30 dias de vida.
- Estudar os mecanismos envolvidos nas possíveis alterações provocadas pelo 3-HiB sobre estas atividades.

### **3 MATERIAL, MÉTODOS E RESULTADOS**

Material, Métodos e os Resultados desta dissertação foram organizados na forma de um artigo científico que foi aceito para publicação na revista *International Journal of Developmental Neuroscience*.

## Evidence that 3-hydroxyisobutyric acid inhibits key enzymes of energy metabolism in cerebral cortex of young rats

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### Abstract

3-Hydroxyisobutyric aciduria is an inherited metabolic disease caused by 3-hydroxyisobutyryl-CoA dehydrogenase deficiency. Tissue accumulation and high urinary excretion of 3-hydroxyisobutyric acid is the biochemical hallmark of this disorder. Clinical phenotype is heterogeneous and generally includes dysmorphic features, delayed motor development, profound mental impairment, and acute encephalopathy. Lactic acidemia is also found in the affected patients, indicating that mitochondrial dysfunction may be involved in the pathophysiology of this disorder. Therefore, the aim of the present work was to investigate the *in vitro* effect of 3-hydroxyisobutyric acid (0.1, 0.5 and 1 mM) on essential enzymes of energy metabolism, namely the activities of the respiratory chain complexes I–V, total, cytosolic and mitochondrial creatine kinase and Na<sup>+</sup>, K<sup>+</sup>-ATPase in cerebral cortex homogenates of 30-day-old rats. We also measured the rate of oxygen consumption in brain mitochondrial preparations in the presence of 3-hydroxyisobutyric acid. 3-Hydroxyisobutyric acid significantly reduced complex I–III (20%), without affecting the other activities of the electron transport chain. Furthermore, 3-hydroxyisobutyric acid did not change state III, state IV and the respiratory control ratio in the presence of glutamate/malate or succinate, suggesting that its effect on cellular respiration was weak. On the other hand, the activities of total and mitochondrial creatine kinase, but not cytosolic creatine kinase, were inhibited (30%) by 3-hydroxyisobutyric acid. We also observed that 3-hydroxyisobutyric acid-induced inhibition of mitochondrial creatine kinase activity was fully prevented by pre-incubation of the homogenates with reduced glutathione,  $\alpha$ -tocopherol or the combination of superoxide dismutase plus catalase, suggesting that this inhibition was mediated by oxidation of essential thiol groups of the enzyme probably by superoxide, hydrogen peroxide and/or peroxy radicals. It was also demonstrated that Na<sup>+</sup>, K<sup>+</sup>-ATPase activity from synaptic plasma membranes was markedly suppressed (37%) by 3-hydroxyisobutyric acid and that this effect was prevented by  $\alpha$ -tocopherol co-incubation implying that peroxy radicals were probably involved in this action. Considering the importance of the affected enzyme activities for brain metabolism homeostasis and neurotransmission, it is suggested that increased tissue levels of 3-hydroxyisobutyric acid may contribute to the neurodegeneration of patients affected by 3-hydroxyisobutyric aciduria and possibly explain previous reports describing elevated production and excretion of lactate.

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**Keywords:** 3-Hydroxyisobutyric aciduria; 3-Hydroxyisobutyric acid; Energy metabolism; Respiratory chain; Creatine kinase; Na<sup>+</sup>; K<sup>+</sup>-ATPase; Cerebral cortex

### 1. Introduction

3-Hydroxyisobutyric aciduria (3-HiB) (OMIM 236795) is an organic aciduria of valine metabolism and so far only 12

patients have been reported (Sweetman and Williams, 2001). The phenotypic variation of this disorder is wide. Some patients show a mild clinical course, while others have a poor prognosis. They usually present dysmorphic features with microcephaly, congenital brain malformations, especially migration disorders, basal ganglia lesions with delayed motor development, profound mental retardation, vomiting and early death (Sasaki et al., 2006). The characteristic laboratory finding is elevated amounts of 3-hydroxyisobutyric acid (3-HiB) in urine (60–390 mmol/mol of creatinine), which may increase 100-fold during acute

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ketoacidotic episodes (Ko et al., 1991). A further common biochemical feature of these patients, especially during episodes of ketoacidosis, is elevated lactate levels (Ko et al., 1991; Chitayat et al., 1992; Sasaki et al., 2001; Shield et al., 2001; Loupatty et al., 2006), indicating an impairment of mitochondrial function.

The biochemical and molecular basis of 3-HiBuria is poorly known, as it is the pathomechanisms leading to the central nervous system (CNS) damage observed in the affected patients. Investigation on the role of 3-HiB on the CNS function and development, particularly directed towards the mitochondrial function, will eventually lead to a better understanding of the relationship between the clinical features and the biochemical abnormalities (enhanced production and excretion of 3-HiB and lactate), as well as the pathophysiology of this disorder. In this context, since 3-HiB is produced in the mitochondria matrix, it seems appropriate to evaluate the role of this compound on cellular energy metabolism, which basically occurs in this organelle. Thus the present study was undertaken to investigate the *in vitro* influence of 3-HiB on important parameters of energy metabolism, including the activities of the respiratory chain complexes I–V, creatine kinase and Na<sup>+</sup>, K<sup>+</sup>-ATPase, as well as on respiratory parameters measured by the rate of oxygen consumption in brain preparations of young rats in order to clarify the mechanisms involved in the neuropathology of 3-HiBuria. The effect of antioxidants on the inhibition caused by 3-HiB on creatine kinase and Na<sup>+</sup>, K<sup>+</sup>-ATPase activities was also investigated.

## 2. Experimental procedures

### 2.1. Reagents

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA, except for 3-HiB, which was purchased from Dr. Ernesto Brunet, Madrid, Spain with 99% purity. 3-HiB was dissolved on the day of the experiments in the incubation medium used for each technique with pH adjusted to 7.4. The final concentrations of the acid in the medium ranged from 0.1 to 1.0 mM.

### 2.2. Animals

Thirty-day-old Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, ICBS, UFRGS, were used 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 85-23, revised 1985) 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.

### 2.3. Cerebral cortex preparation

The animals were sacrificed by decapitation, the brain was rapidly removed and the cerebral cortex was isolated. For the determination of the activities of the respiratory chain complexes I–III, II, II–III and IV, 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<sup>-1</sup> 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 the determination of the activities of complexes I and V and the mitochondrial respiratory parameters, mitochondrial fractions were prepared according to Cassina and Radi (1996). For total creatine kinase activity determination, the cerebral cortex was homogenized (1:1000, w/v)

in isosmotic saline solution. For the 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 Jiménez, 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 enzymatic assay. The supernatants were further centrifuged at 125,000 × g for 60 min at 4 °C in a OTD-65B Sorval centrifuge, the microsomal pellet discarded, and the cytosol (supernatant) was used for the cytosolic creatine kinase enzymatic assay. The period between tissue preparation and measurement of the various parameters was always less than 5 days, except for mitochondrial and cytosolic and creatine kinase enzymatic assays, as well as oxygen consumption experiments, which were performed within the same day of the experiments.

The biochemical parameters were determined in the presence of various concentrations of 3-HiB (0.1–1.0 mM), whereas control groups did not contain the metabolite in the incubation medium. Some experiments were performed in the presence of glutathione (GSH; 0.2 mM), melatonin (MEL; 0.2 mM), soluble α-tocopherol (trolox; 0.05 mM) and the combination of superoxide dismutase (SOD; 50 mU mL<sup>-1</sup>) plus catalase (CAT; 50 mU mL<sup>-1</sup>).

### 2.4. Preparation of synaptic plasma membrane from rat cerebral cortex

Cerebral cortex was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. The homogenate was pre-incubated at 37 °C for 1 h in the presence or absence of 0.1–1.0 mM 3-HiB. Membranes were prepared afterwards 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. In some experiments, 3-HiB (0.1–1.0 mM) was incubated at 37 °C for 30 min with purified synaptic membranes.

### 2.5. Spectrophotometric analysis of the respiratory chain complexes I–V activities

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). 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) and that of cytochrome *c* oxidase (complex IV) according to Rustin et al. (1994). The methods described to measure these activities were slightly modified, as described in details in a previous report (da Silva et al., 2002). NADH dehydrogenase (complex I) and ATP synthase (complex V) activities were measured in mitochondrial preparations from cerebral cortex. Complex I was determined by the rate of NADH-dependent ferricyanide reduction at λ = 420 nm (ε = 1 mM<sup>-1</sup> cm<sup>-1</sup>) according to Cassina and Radi (1996) and complex V activity was assayed by coupling the reaction of pyruvate kinase with lactate dehydrogenase and following NADH oxidation at λ = 340 nm (ε = 6.22 mM<sup>-1</sup> cm<sup>-1</sup>) (Rustin et al., 1994). 3-HiB (0.1–1.0 mM) was added to the reaction medium at the beginning of the assays, while no 3-HiB was added to controls. The activities of the respiratory chain complexes were calculated as nmol min<sup>-1</sup> mg protein<sup>-1</sup> or mmol min<sup>-1</sup> mg protein<sup>-1</sup>.

### 2.6. Mitochondrial respiratory parameters

The rate of oxygen consumption was measured polarographically using a Clark-type electrode in a thermostatically controlled (37 °C) and magnetically stirred incubation chamber of 1.0 mL capacity (Cassina and Radi, 1996). 3-HiB (1.0 mM) was added to the reaction medium at the beginning of the assay in the test tubes. The assay was performed in purified mitochondrial preparations (1.0 mg mL protein<sup>-1</sup>) incubated in the same buffer used for mitochondrial isolation in the presence of NAD-linked (glutamate/malate: 2.5 mM each) or FAD-linked (5.0 mM succinate) substrates. The rate of oxygen consumption measured under these conditions corresponded to state IV mitochondrial respiration. State III was initiated by adding 1 mmol ADP. The whole procedure

lasted approximately 4 min. The respiratory control ratio (RCR; state III/state IV) was then calculated. State IV and state III were expressed as nmol O<sub>2</sub> consumed min<sup>-1</sup> mg protein<sup>-1</sup>.

### 2.7. Spectrophotometric analysis of creatine kinase activity

Creatine kinase (CK) activity was measured in total homogenates as well as in the cytosolic and mitochondrial preparations according to Hughes (1962) with slight modifications (Schuck et al., 2002). Briefly, the reaction mixture consisted of 50 mM Tris buffer, pH 7.5, containing 7.0 mM phosphocreatine, 7.5 mM MgSO<sub>4</sub>, and 0.5–1.0 μg protein in a final volume of 0.1 mL. 3-HiB (0.1–1.0 mM) 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 colour 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 λ = 540 nm. Results were calculated as μmol of creatine min<sup>-1</sup> mg protein<sup>-1</sup> and expressed as percentage of controls.

### 2.8. Spectrophotometric analysis of Na<sup>+</sup>, K<sup>+</sup>-ATPase activity

The reaction mixture for the Na<sup>+</sup>, K<sup>+</sup>-ATPase assay contained 5 mM MgCl<sub>2</sub>, 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<sup>2+</sup>-ATPase ouabain-insensitive was assayed under the same conditions with the addition of 1 mM ouabain. Na<sup>+</sup>, K<sup>+</sup>-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<sup>-1</sup> min<sup>-1</sup> mg protein and expressed as percentage of controls.

### 2.9. Protein determination

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

### 2.10. Statistical analysis

Unless otherwise stated, results are presented as mean ± standard error of the mean. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc Duncan multiple range test when *F* was significant. For analysis of dose-dependent effects, linear regression was used. The Student's *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.

Table 1

In vitro effect of 3-HiB on the activities of the respiratory chain complexes I, II, II-III, IV and V in cerebral cortex homogenates of 30-old-day rats

	Complex I	Complex II	Complex II-III	Complex IV	Complex V
Control	1.03 ± 0.09	5.46 ± 0.17	31.25 ± 1.32	316 ± 24.9	476 ± 31.2
3HiB 0.1 mM	–	5.55 ± 0.24	31.02 ± 0.98	307 ± 11.8	–
3HiB 0.5 mM	–	5.80 ± 0.16	31.35 ± 1.63	241 ± 26.7	–
3HiB 1.0 mM	0.96 ± 0.22	5.38 ± 0.27	30.17 ± 0.39	309 ± 19.4	486 ± 22.3

Values are mean ± standard error for four to six independent experiments (animals) per group. The activity of complex I is expressed as mmol NADH-dependent reduction min<sup>-1</sup> mg protein<sup>-1</sup>. The activities of complexes II, II-III, IV and V are expressed as nmol DCIP reduction min<sup>-1</sup> mg protein<sup>-1</sup>, nmol cytochrome *c* reduction min<sup>-1</sup> mg protein<sup>-1</sup>, nmol cytochrome *c* oxidation min<sup>-1</sup> mg protein<sup>-1</sup> and nmol NADH oxidation min<sup>-1</sup> mg protein<sup>-1</sup>, respectively. No significant differences were detected (One-way ANOVA or Student's *t*-test for paired samples).

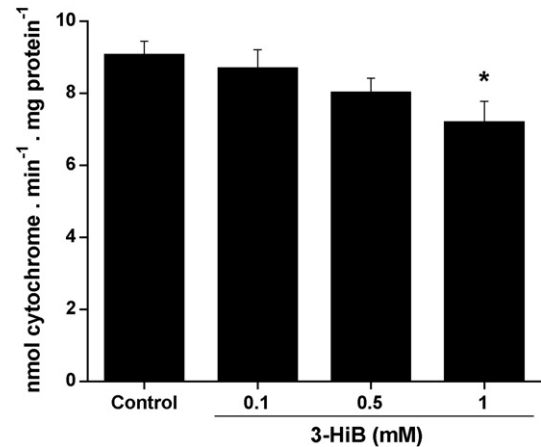


Fig. 1. *In vitro* effect of 3-hydroxyisobutyric acid (3-HiB) on the activity of complex I-III of the respiratory chain in rat cortical homogenates. Data are expressed as mean ± S.E.M. for six independent experiments performed in duplicate expressed as nmol reduced cytochrome min<sup>-1</sup> mg protein<sup>-1</sup>. \**p* < 0.05, compared to control (Duncan multiple range test).

## 3. Results

### 3.1. 3-HiB inhibits complex I-III activity of the respiratory chain in rat cerebral cortex homogenates

First we investigated the effect of 3-HiB (0.1–1.0 mM) on the various respiratory chain enzyme activities in an attempt to elucidate whether the respiratory chain activity could be altered by the major metabolite accumulating in 3-HiBuria. For these experiments, six rats were used. We found that complex I-III activity was significantly inhibited by 3-HiB (up to 21%) [*F*<sub>(3,20)</sub> = 3.163, *p* < 0.05] in a dose-dependent manner [*β* = -0.550; *p* < 0.005] (Fig. 1), with no significant alteration of the activities of the respiratory chain complexes II, II-III and IV in homogenates and complexes I and V in mitochondrial preparations (Table 1).

### 3.2. 3-HiB does not change the respiratory parameters measured by oxygen consumption in mitochondrial enriched fractions from cerebrum

In order to assess the overall consequences of 3-HiB on complex I-III inhibitory effect on mitochondrial respiration, we also investigated whether the respiratory state IV (NAD and FAD-linked substrates), state III (ADP stimulated respiration)

Table 2

Effect of 3-hydroxyisobutyric acid (3-HiB) on respiratory parameters in mitochondrial preparations from rat cerebrum

	Glutamate/malate		Succinate	
	Control	1 mM 3-HiB	Control	1 mM 3-HiB
State IV	5.90 ± 0.85	5.90 ± 0.53	16.4 ± 1.31	18.5 ± 1.25
State III	32.8 ± 1.28	34.1 ± 1.08	58.6 ± 5.58	62.0 ± 5.74
RCR	5.74 ± 0.66	5.88 ± 0.58	3.56 ± 0.08	3.37 ± 0.32

RCR: respiratory control ratio. Values are mean ± standard error for three to four independent (animals) experiments per group. State IV and state III are expressed as nmol O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup>. No significant differences were detected (Student's *t*-test for paired samples).

and respiratory control ratio parameters were altered in rat cerebrum mitochondria exposed to 1.0 mM 3-HiB. For these experiments a total of four rats were used. Table 2 shows that the respiring state IV, state III and the RCR values were not modified by 1.0 mM 3-HiB when mitochondria were stimulated with NAD- or FAD-linked substrates.

### 3.3. 3-HiB inhibits mitochondrial creatine kinase activity from rat cerebral cortex

We also examined the effect of 3-HiB, at concentrations varying from 0.1 to 1.0 mM, on CK activity in total homogenates (tCK) from rat cerebral cortex. Eighteen animals were used in these experiments. 3-HiB significantly inhibited tCK activity (up to 32%) ( $F_{(3,24)} = 4.957, p < 0.01$ ) (Fig. 2A). We then investigated the effect of 3-HiB (1.0 mM) on CK activity on mitochondrial (Mi-CK) and cytosolic (Cy-CK) fractions of rat cerebral cortex. It can be seen that the metabolite significantly inhibited Mi-CK activity (up to 30%) [ $t(5) = 3.495, p < 0.05$ ] (Fig. 2B), but had no effect on Cy-CK activity (Fig. 2C).

In order to test whether the significant reduction of cerebral cortex Mi-CK activity caused by 3-HiB was mediated by oxidation of critical thiol groups of the enzyme, we pre-incubated mitochondrial preparations from cerebral cortex in the presence of the antioxidant GSH or the free radical scavengers MEL, SOD plus CAT and trolox ( $\alpha$ -tocopherol). Twelve animals were used in these experiments. We verified that these antioxidants per se did not alter Mi-CK activity. However, GSH, SOD plus CAT and trolox fully prevented, whereas melatonin did not affect 3-HiB-induced inhibitory effect on Mi-CK activity [ $F_{(9,50)} = 2.701; p < 0.01$ ] (Fig. 3).

### 3.4. 3-HiB inhibits mitochondrial Na<sup>+</sup>, K<sup>+</sup>-ATPase activity from synaptic plasma membranes of rat cerebral cortex

The next set of experiments was carried out to test the influence of 3-HiB, at 0.1–1.0 mM concentrations, on Na<sup>+</sup>, K<sup>+</sup>-ATPase activity in synaptic plasma membranes prepared from cortical homogenates. Initially, cortical homogenates were pre-incubated with the metabolite, and the synaptic plasma membranes isolated afterwards. Fig. 4A shows that 3-HiB

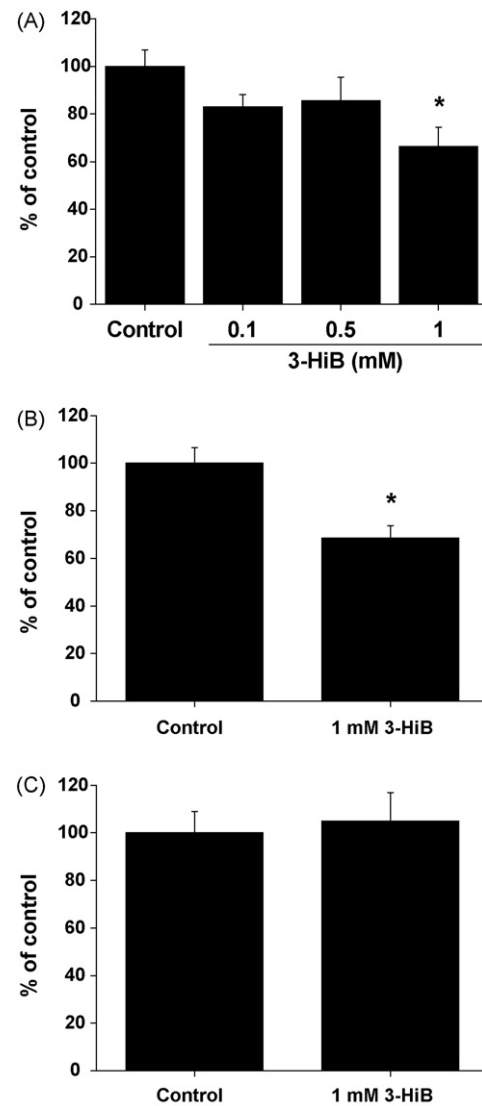


Fig. 2. *In vitro* effect of 3-hydroxyisobutyric acid (3-HiB) on the total (tCK) (A), mitochondrial (Mi-CK) (B) and cytosolic (Cy-CK) (C) creatine kinase activity in rat cortical homogenates. Values are mean ± S.E.M. of six independent experiments (animals) performed in triplicate expressed as percentage of controls (tCK: 10.71 ± 0.75 μmol creatine min<sup>-1</sup> mg protein<sup>-1</sup>; Mi-CK: 11.91 ± 0.76 μmol creatine min<sup>-1</sup> mg protein<sup>-1</sup>; Cy-CK: 12.25 ± 1.11 μmol creatine min<sup>-1</sup> mg protein<sup>-1</sup>). \* $p < 0.05$ , compared to control (Duncan multiple range test).

exposition to homogenates provoked a significant inhibition of Na<sup>+</sup>, K<sup>+</sup>-ATPase activity (36%) [ $F_{(3,20)} = 5.682; p < 0.01$ ]. We also investigated the effect of 3-HiB on Na<sup>+</sup>, K<sup>+</sup>-ATPase activity on purified synaptic membrane preparations. We observed that 3-HiB significantly inhibited (32%) Na<sup>+</sup>, K<sup>+</sup>-ATPase activity from purified synaptic membranes [ $F_{(3,20)} = 4.709; p < 0.05$ ] (Fig. 4B). Since Na<sup>+</sup>, K<sup>+</sup>-ATPase activity is susceptible to free radical attack, we tested whether pre-treatment of cortical homogenates with antioxidants. We observed that GSH (0.2 mM) was not able to prevent the 3-HiB-induced inhibition of Na<sup>+</sup>, K<sup>+</sup>-ATPase activity caused by 3-HiB, whereas trolox (0.05 mM) prevented this inhibition [ $F_{(5,30)} = 3.438; p < 0.05$ ] (Fig. 5).

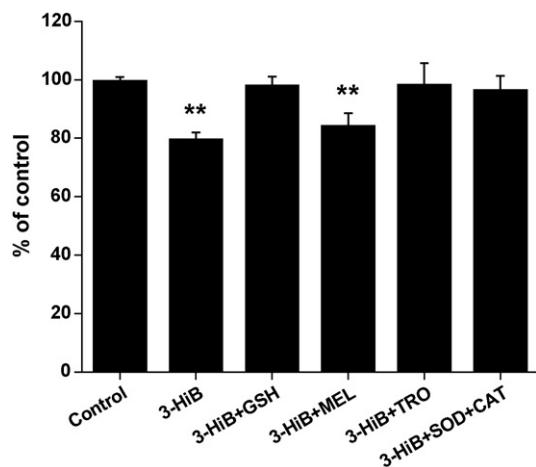


Fig. 3. *In vitro* effect of the antioxidants reduced glutathione (GSH; 0.2 mM), melatonin (MEL; 0.2 mM), trolox (TRO; 0.05 mM) and the combination of superoxide dismutase (SOD; 50 mU mL<sup>-1</sup>) plus catalase (CAT; 50 mU mL<sup>-1</sup>) on the inhibition induced by of 1.0 mM 3-hydroxyisobutyric acid (3-HiB) on mitochondrial creatine kinase activity from rat cerebral cortex. Data are mean  $\pm$  S.E.M. for six independent experiments (animals) performed in triplicate expressed as percentage of controls ( $10.1 \pm 0.12 \mu\text{mol creatine min}^{-1} \text{mg protein}^{-1}$ ). \*\* $p < 0.01$ , compared to control (Duncan multiple range test).

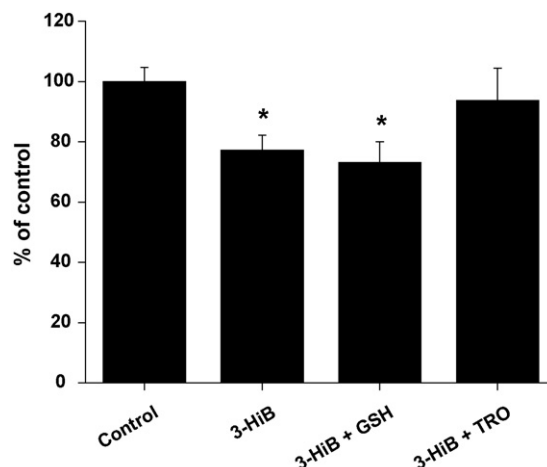


Fig. 5. *In vitro* effect of the antioxidants glutathione (0.2 mM) and trolox (0.05 mM) on the inhibition induced by of 1.0 mM 3-hydroxyisobutyric acid (3-HiB) on Na<sup>+</sup>, K<sup>+</sup>-ATPase activity from synaptic plasma membrane of cerebral cortex of young rats. Cortical homogenates were pre-treated with the antioxidants during 1 h and synaptic plasma membranes were isolated afterwards. Values are mean  $\pm$  S.E.M. of six independent experiments (animals) expressed as percentage of controls ( $1181 \pm 158 \text{ nmol Pi released min}^{-1}$ ). \* $p < 0.05$ , compared to controls (Duncan multiple range test).

#### 4. Discussion

Patients with 3-HiBuria present a heterogeneous clinical phenotype, with severe neurological abnormalities, complicated by crises of ketoacidosis. However, some affected individuals may show a mild clinical course. The biochemical hallmark of this disorder is the accumulation and excretion of 3-

HiB, whose concentrations may increase a 100-fold in urine and possibly in tissues during episodes of acute metabolic decompensation. These episodes are also accompanied by high urinary excretion of lactic acid, indicating mitochondrial dysfunction. Thus, in the present study we investigated whether 3-HiB, the major accumulating metabolite in this disorder, could compromise energy metabolism in cerebral cortex from young rats. This approach seems valid since this metabolite is basically generated inside the mitochondria, which is the organelle mainly responsible for cellular energy production.

We first verified that 3-HiB selectively inhibited (20%) complex I–III, without affecting the other activities of the respiratory chain. In order to assess the overall consequences of 3-HiB inhibitory effect on mitochondrial respiration, we tested the effect of 1.0 mM 3-HiB on the rate of mitochondrial oxygen consumption in the presence of glutamate/malate and succinate as substrates. 3-HiB did not alter the respiratory parameters state III, state IV and RCR, implying that the influence of this organic acid on energy production is rather mild. We also observed that creatine kinase activity, a crucial enzyme activity involved in intracellular ATP transfer and buffering, was significantly inhibited by 3-HiB. It was seen that the activity of the mitochondrial isoenzyme (Mi-CK), but not of the cytosolic isoform (Cy-CK), was inhibited by this organic acid. Furthermore, the naturally occurring antioxidant GSH and the free radical scavengers SOD plus CAT and trolox ( $\alpha$ -tocopherol) fully prevented, whereas melatonin did not affect 3-HiB-induced inhibitory effect on Mi-CK activity, indicating that these effects were probably mediated by oxidation of sulfhydryl groups of the enzyme via free radical production, possibly superoxide/hydrogen peroxide and/or peroxy radicals. Taken together these observations, it can be presumed that 3-HiB impairs energy transfer in rat cerebral cortex.

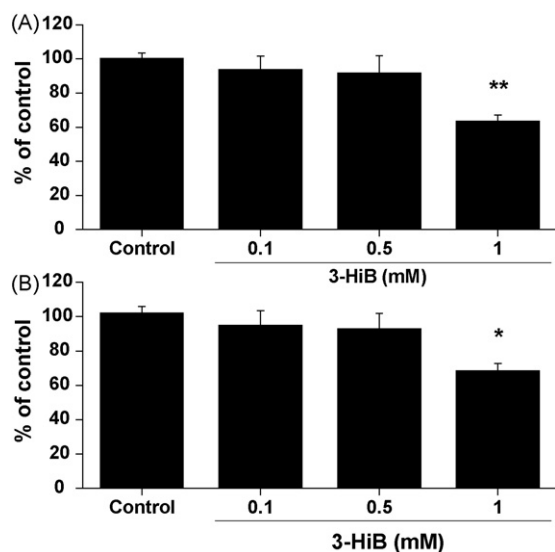


Fig. 4. *In vitro* effect of 3-hydroxyisobutyric acid (3-HiB) on the Na<sup>+</sup>, K<sup>+</sup>-ATPase activity in synaptic plasma membranes of rat cerebral cortex. (A) 3-HiB was pre-incubated at 37 °C for 1 h with cortical homogenates, after which the synaptic membranes were isolated and the activity measured afterwards. (B) 3-HiB was pre-incubated at 37 °C for 30 min with purified synaptic plasma membranes and the activity measured afterwards. Values are mean  $\pm$  S.E.M. for six independent experiments (animals) performed in duplicate expressed as percentage of controls ( $951 \pm 76 \text{ nmol Pi released min}^{-1}$ ). \* $p < 0.05$ , \*\* $p < 0.01$  compared to control (Duncan multiple range test).

$\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity from synaptic plasma membranes was also significantly inhibited by 3-HiB when cerebral cortex homogenates from young rats were pre-incubated for 1 h with the acid and the synaptic membranes prepared afterwards. As regards to the mechanisms underlying the inhibitory effect of 3-HiB on  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity, increasing evidence is emerging demonstrating that  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase is highly vulnerable to free radical attack (Lees, 1993; Kurella et al., 1997; Yousef et al., 2002). In this context, pre-treatment of homogenates with trolox ( $\alpha$ -tocopherol) totally prevented the inhibition caused by 3-HiB, indicating that peroxide radicals were mainly responsible for 3-HiB effect by acting on critical groups of the enzyme. We cannot however rule out that lipid peroxidation of the synaptic membrane in which the enzyme is embedded leading to reduction of its activity may have occurred because  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase stability is highly dependent on the surrounding membrane lipids. On the other hand, pre-incubation of cortical homogenates with GSH did not alter the inhibition of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase provoked by the acid, suggesting that modifications of sulfhydryl groups of the enzyme were not involved in the 3-HiB inhibitory effect. Further experiments revealed that direct exposition of 3-HiB to purified plasma synaptic membranes also provoked a decrease of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity, indicating that other mechanisms besides free radicals were implicated in this effect. In this context, potent inhibitory activity of various substances on  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase are thought to be due to their property of penetrating into the hydrophobic interior of the membrane disturbing the interactions of ionic and hydrophobic groups on the enzyme necessary for its normal function (Kimmelberg and Pahadjopoulos, 1974; Abeywardena et al., 1983; Carfagna and Muhoberac, 1993). Thus, it might be presumed that 3-HiB may be incorporated into the membrane lipid bilayer, thereafter provoking its inhibitory effect on  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase.

Our data may be of pathophysiological relevance, since the phosphocreatine/creatine/creatine kinase system is fundamental for normal energy homeostasis (Khuchua et al., 1998; Schlattner and Wallimann, 2000) and impairment of this system has been observed in neurodegenerative and mental diseases, such as Alzheimer's disease and schizophrenia (Burbaeva et al., 1999). Furthermore, there is increasing evidence suggesting that reduction of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity may be a link between common neurotoxic mechanisms in neurons (Swadner, 1979; Lees, 1991; Satoh and Nakazato, 1992; Lees, 1993). Inhibition of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase has also been associated with excitotoxicity and epilepsy (Grisar, 1984; Ben-Ari, 1985; Choi and Rothman, 1990; Cousin et al., 1995; Lees and Leong, 1995).

In conclusion, to our knowledge this is the first study reporting neurotoxic effects of 3-HiB, the major metabolite accumulating in 3-HiBuria. We found that 3-HiB inhibits crucial enzymes responsible for the energy buffering system and for maintaining the basal potential membrane necessary for a normal neurotransmission, probably through oxidation of essential groups of the enzymes mainly responsible for these processes. In case our *in vitro* results could be extrapolated to the human condition, it is feasible that they might contribute, at

least in part, to the brain injury especially during encephalopathic crises following infections or other stress situations characterized by catabolism, in which 3-HiB concentrations dramatically increase (Ko et al., 1991).

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## 4 DISCUSSÃO

O cérebro depende de níveis adequados de energia na forma de ATP. Situações como hipóxia e hipoglicemia podem levar a dano irreversível com morte neuronal pela incapacidade das células neurais de manter um gradiente iônico adequado para a neurotransmissão que é dependente de bombas que utilizam energia para a sua atividade (por exemplo,  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase). A energia necessária para manutenção das funções cerebrais bem como do gradiente iônico é proveniente principalmente da glicólise e da fosforilação oxidativa que, em condições normais, atuam sinergicamente (SILVER, DEAS e ERECINSKA, 1997). Neste particular, a atividade dos complexos da cadeia respiratória é essencial para o funcionamento normal do cérebro. Pacientes afetados por doenças neurodegenerativas comuns podem apresentar disfunção do metabolismo energético cerebral (SCHAPIRA *et al.*, 1990a, 1998). Além disso, algumas doenças genéticas, incluindo várias acidemias orgânicas, em que ocorre inibição da fosforilação oxidativa geram um acúmulo de NADH que por sua vez inibe o ciclo de Krebs favorecendo a conversão de piruvato em lactato, o qual se encontra aumentado no plasma dos indivíduos afetados por essas desordens (SCHAPIRA, 1995).

O acúmulo tecidual do ácido 3-HiB é característico da 3-HiBúria, uma doença metabólica genética caracterizada clinicamente por um quadro neurológico. No entanto, o fenótipo deste distúrbio é bastante variável, sendo que alguns pacientes apresentam manifestações clínicas moderadas, enquanto outros

apresentam sintomas severos (LOUPATY *et al*, 2006; SASAKI *et al.*, 2006). O defeito bioquímico primário da 3-HiBúria e os mecanismos responsáveis pelo dano do sistema nervoso central nos pacientes afetados são até o momento desconhecidos. Entretanto, o fato dos pacientes apresentarem durante os episódios de agudização da doença níveis elevados de ácido láctico indica um comprometimento da função mitocondrial. Tendo em vista que o 3-HiB é produzido dentro da mitocôndria, no presente trabalho investigamos a ação *in vitro* deste ácido sobre o metabolismo energético mitocondrial em cérebro de ratos jovens.

Ao examinarmos o efeito do 3-HiB sobre as atividades da cadeia respiratória em homogeneizado de córtex cerebral, verificamos uma inibição seletiva provocada pelo mesmo (20 %) sobre o complexo I-III, sem afetar os outros complexos medidos. Com objetivo de melhor avaliarmos as conseqüências do efeito inibitório do 3-HiB sobre a respiração mitocondrial, testamos o efeito do 3-HiB na concentração de 1,0 mM sobre os parâmetros respiratórios estado III, estado IV e índice de controle respiratório (RCR) medidos através do consumo de oxigênio em preparações mitocondriais. Não encontramos nenhum efeito significativo deste ácido sobre estes parâmetros, sugerindo que a inibição da cadeia respiratória por este ácido orgânico não foi suficiente para alterar de modo significativo a respiração celular.

Observamos ainda que a atividade da creatina quinase, uma enzima crucial envolvida na transferência e no tamponamento de ATP, foi inibida significativamente pelo 3-HiB na concentração de 1 mM. Neste particular, demonstramos que a isoenzima afetada foi a da fração mitocondrial (Mi-CK), mas não a isoforma citosólica (Cy-CK).



O passo seguinte foi investigar o mecanismo de inibição da Mi-CK causada pelo 3-HiB. Recentemente, foi demonstrado que a CK é susceptível à inativação por espécies ativas e que a mesma se deve à alteração de seus grupamentos tióis (sulfidrilas) causada pela ação de agentes oxidantes (STACHOWIAK *et al.*, 1998). Considerando, portanto, que a CK é vulnerável à ação de radicais livres, testamos se antioxidantes poderiam prevenir esta inibição. Observamos que a glutathiona reduzida (GSH), o antioxidante presente em maior concentração no cérebro, bem como os seqüestradores de radicais livres trolox ( $\alpha$ -tocoferol) e a enzima superóxido desmutase (SOD) associada à catalase (CAT) preveniram totalmente o efeito inibitório induzido pelo 3-HiB sobre a atividade da Mi-CK, enquanto a melatonina, não alterou este efeito. Tais observações indicam que o efeito inibitório sobre a Mi-CK foi provavelmente secundário à ação de espécies reativas de oxigênio induzidas pelo 3-HiB. Considerando que a Mi-CK apresenta em seu centro catalítico grupos sulfidrilas essenciais para a sua função (WYSS e KADDURAH-DAOUK, 2000) e que o GSH impediu o efeito inibitório do ácido sobre essa enzima, presumimos que a modificação (oxidação) de grupos sulfidrilas ou de outros grupamentos essenciais à atividade da enzima foi um dos mecanismos pelo qual o 3-HiB afetou essa atividade. Também podemos concluir que produção de espécies reativas, possivelmente superóxido/peróxido de hidrogênio e/ou radicais peroxila, estiveram envolvidos nesta ação, visto que SOD e CAT e o trolox, seqüestradores destas espécies ativas preveniram o efeito do 3-HiB. Uma vez que a atividade da Mi-CK é fundamental para o tamponamento energético intracelular através da transferência de energia entre a matriz

mitocondrial e o citosol (KALDIS *et al.*, 1996; WYSS e KADDURAH-DAOUK, 2000), nossos resultados indicam que o 3-HiB prejudica a transferência de energia intracelular em córtex cerebral.

A seguir avaliamos a atividade da  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase de membranas plasmáticas sinápticas, uma enzima que tem papel vital para diversas funções celulares e observamos que esta atividade foi inibida pelo 3-HiB quando homogeneizados de córtex cerebral de ratos jovens foram pré-incubados por 1h com o 3-HiB e as membranas plasmáticas sinápticas isoladas posteriormente.

No que se refere aos mecanismos envolvidos no efeito inibitório do 3-HiB sobre a atividade da  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, existem evidências concretas, demonstrando que a  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase é muito vulnerável ao ataque de radicais livres (LEES, 1993; KURELLA *et al.*, 1997; YOUSEF *et al.*, 2002). Portanto, co-incubamos o 3-HiB com antioxidantes em homogeneizado de córtex cerebral por 1h para avaliar o papel do dano oxidativo sobre a inibição da enzima por este ácido. Verificamos que o trolox ( $\alpha$ -tocopherol) foi capaz de prevenir totalmente a inibição causada pelo 3-HiB, indicando que radicais peróxido podem estar envolvidos neste efeito inibitório provavelmente por modificar grupos críticos para a função da enzima. Não podemos, no entanto, excluir que a lipoperoxidação da membrana sináptica induzida pelo 3-HiB poderia levar a redução de sua atividade, já que a estabilidade da  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase é dependente dos componentes lipídicos da membrana na qual a enzima está inserida. Por outro lado, a co-incubação do homogeneizado de córtex cerebral com GSH não preveniu a inibição da  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase provocada pelo ácido, sugerindo que modificações nos grupamentos sulfidrilas da enzima não estão envolvidos no efeito inibitório do 3-HiB.

Demonstramos ainda que a exposição das membranas plasmáticas sinápticas purificadas de córtex cerebral de ratos ao 3-HiB também resultou em uma diminuição significativa da atividade da  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, sugerindo uma ação direta deste ácido orgânico sobre a enzima ou sobre componentes da membrana plasmática em que ela se ancora.

Quanto à relevância fisiopatológica de nossos achados, devemos considerar que o sistema fosfocreatina/creatina/creatina quinase é fundamental para a homeostasia energética (KHUCHUA, *et al.*, 1998; SCHLATTNER e WALLIMANN, 2000). Assim, considerando que a diminuição da atividade da CK tem sido encontrada em pacientes com doenças neurodegenerativas em que o dano oxidativo parece estar relacionado à neurodegeneração (AKSENOV *et al.*, 2000; BURKLEN *et al.*, 2006), é possível presumir que nossos resultados possam ter relevância para explicar a disfunção neurológica da 3-HiBburia.

No que diz respeito às conseqüências da inibição da atividade da  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase pelo 3-HiB ao funcionamento do sistema nervoso central, evidências têm sido demonstradas associando alterações na atividade da  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase com neurotoxicidade (SWEADNER, 1979; LEES *et al.*, 1990; SATOH e NAKAZATO, 1992; LEES, 1993), bem como com apoptose e necrose de células neurais (XIAO *et al.*, 2002; WANG *et al.*, 2003). Além disso, uma redução da atividade da  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase foi associada com o estado epiléptico e com a leucoencefalopatia no córtex cerebral de um neonato (RENKAWEK *et al.*, 1992). Inibição da  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase tem também sido associada à excitotoxicidade e epilepsia (GRISAR, 1984; BEN-ARI, 1985; CHOI e ROTHMAN, 1990; COUSIN *et al.*, 1995; LEES e LEONG, 1995). Nossos resultados poderiam, portanto, explicar ao menos em parte as

crises encefalopáticas que ocorrem nos pacientes afetados pela acidúria 3-hidroxiisobutírica após infecções ou outras situações de estresse caracterizadas por catabolismo em que os níveis plasmáticos do 3-HiB durante esses episódios agudos aumentam significativamente (KO *et al.*, 1991), sendo similares aos que provocaram inibição significativa da atividade da  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase em nossa investigação.

Concluindo, este é o primeiro estudo relatando efeitos neurotóxicos do 3-HiB, o principal metabólito que se acumula na 3-HiBuria. Demonstramos que o 3-HiB inibe atividades enzimáticas essenciais para o sistema de tamponamento energético e a manutenção do potencial de membrana basal necessário para a neurotransmissão normal provavelmente através da oxidação de grupamentos essenciais das enzimas responsáveis por estes processos. Caso nossos resultados *in vitro* possam ser extrapolados para a condição humana, é possível que eles contribuam, ao menos em parte, para explicar os achados neurológicos, especialmente durante as crises encefalopáticas que os pacientes afetados pela 3-HiBuria apresentam durante situações com catabolismo acelerado nas quais as concentrações de 3-HiB aumentam drasticamente (KO *et al.*, 1991).

## 5 CONCLUSÕES

- O ácido 3-HiB, na concentração de 1 mM, inibiu em cerca de 20% a atividade do complexo I-III, sem alterar as atividades dos complexos II, II-III e IV da cadeia respiratória em homogeneizado de córtex cerebral de ratos jovens.
- As atividades dos complexos I e V da cadeia respiratória não foram alteradas pelo 3-HiB nas concentração de 1 mM em preparações mitocondriais de córtex cerebral de ratos jovens.
- O 3-HiB, na concentração de 1 mM, não alterou os parâmetros respiratórios estado III, estado IV e índice de controle respiratório, medidos através do consumo de oxigênio em preparações mitocondriais de cérebro total de ratos jovens.
- O 3-HiB, na concentração de 1 mM, inibiu cerca de 30% a atividade da enzima creatina quinase total em homogeneizado de córtex cerebral de ratos jovens.
- O 3-HiB, na concentração de 1 mM, inibiu a fração mitocondrial da enzima creatina quinase (Mi-CK) de córtex cerebral de ratos jovens, sem alterar a fração citosólica desta enzima (Cy-CK).

- O efeito inibitório do 3-HiB sobre a atividade da Mi-CK de córtex cerebral de ratos jovens foi totalmente prevenido pelos antioxidantes GSH (0,2 mM),  $\alpha$ -tocopherol (trolox) (0,05 mM) ou a combinação de SOD (50 mU . mL<sup>-1</sup>) e CAT(50 mU . mL<sup>-1</sup>), mas não pelo antioxidante melatonina (0,2mM).
- O 3-HiB, na concentração de 1 mM, quando pré-incubado durante 1 hora a 37°C com homogeneizados de córtex cerebral, inibiu em torno de 36% a atividade da enzima Na<sup>+</sup>,K<sup>+</sup>-ATPase em membranas sinápticas plasmáticas obtidas de homogeneizados de córtex cerebral de ratos jovens.
- O efeito inibitório do 3-HiB sobre a atividade da enzima Na<sup>+</sup>,K<sup>+</sup>-ATPase em membranas sinápticas plasmáticas de córtex cerebral de ratos jovens foi totalmente prevenido pela pré-incubação dos homogeneizados com o antioxidante  $\alpha$ -tocopherol (trolox) (0,05 mM), mas não por GSH (0,2mM).
- O 3-HiB, na concentração de 1 mM, inibiu em torno de 32% a atividade da enzima Na<sup>+</sup>,K<sup>+</sup>-ATPase, quando exposto diretamente a membranas sinápticas plasmáticas obtidas de homogeneizados de córtex cerebral de ratos jovens.

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