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PARTICIPAÇÃO DA MITOCÔNDRIA NA NEUROTOXICIDADE INDUZIDA POR TOXICANTES ENDÓGENOS E AMBIENTAIS EM CÉREBRO DE ROEDORES

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SUMÁRIO

LISTA DE ABREVIATURAS

$\Delta\psi$: Potencial de membrana mitocondrial
$(PhSe)_2$: Difenil Disseleneto
AACR: Aminoácidos de cadeia ramificada
ACCR: α-ceto-ácidos de cadeia ramificada
ADP: Adenosina difosfato
AMP: Adenosina monofosfato
ANT: Translocador de nucleotídeos de adenina
ATP: Adenosina trifosfato
BHE: Barreira hematoencefálica
cit-CK: Creatina cinase isoforma citosólica
CK: Creatina cinase
CoQ: Ubiquinona
CR: Cadeia respiratória
DMEM: Meio Eagle's com modificação de Dubelcco
DMPS: 2,3-Dimercapto-1-propanosulfonato
DMSA: Ácido *Meso*-2,3-dimercaptosuccínico
DMSO: Dimetil sulfóxido
DNA: Ácido desoxirribonucléico
Drp1: Proteína 1 relacionada à dinamina
DXB: Doença do Xarope do Bordo
EDTA: Ácido etilenodiaminotetracético
EGTA: Ácido etilenoglicoltetraacético
EIM: Erros inatos do metabolismo
ERs: Espécies reativas
ERNs: Espécies reativas de nitrogênio
EROs: Espécies reativas de oxigênio
FAD: Flavina adenina dinucleotídeo (forma oxidada)
$FADH_2$: Flavina adenina dinucleotídeo (forma reduzida)
FMN: Flavina mononucleotídeo
GPx: Glutationa peroxidase
GR: Glutationa redutase
GSH: Glutationa
GTP: Guanosina trifosfato
H^1-MR: Espectroscopia de ressonância magnética de prótons
LTP: Potenciação a longo prazo
Mn-SOD: Manganês superóxido dismutase
Mit-CK: Creatina cinase isoforma mitocondrial

MOPS: Ácido 3-(N-morfolino) propanosulfônico
MTT: Brometo de 3-(4,5)-dimetiltialzolil-2,5 difeniltetrazólio
Na_2SeO_3: Selenito de sódio
NAC: *N*-Acetilcisteína
NADH: Nicotinamida adenina dinucleotídeo (forma reduzida)
PCr: Fosfocreatina
Se: Selênio
SFB: Soro fetal bovino
SNC: Sistema nervoso central

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RESUMO

A mitocôndria é a organela responsável pela maior produção líquida de energia na célula. Numerosos estudos já têm demonstrado seu envolvimento na fisiopatologia de vários processos neurodegenerativos, como nas doenças de Alzheimer, Parkinson e Hungtington. Além disso, sabe-se que ela é alvo de toxicantes, tanto exógenos quanto endógenos, como por exemplo, o contaminante ambiental metilmercúrio (MeHg) e as altas concentrações de leucina que acumulam da Doença do Xarope do Bordo (DXB). Sabe-se que o MeHg causa severos danos neurológicos tanto em animais quanto em humanos. A principal forma de intoxicação humana é através da ingesta de peixes contaminados, sendo que o MeHg acumula-se principalmente no sistema nervoso central. A leucina e seu derivado α-cetoácido, α-cetoisocaproato são os principais metabólitos acumulados na DXB, e estes parecem ser responsáveis pelos principais sintomas neurológicos, incluindo o prejuízo cognitivo, que os pacientes com esta patologia apresentam. Desta forma, o objetivo do presente trabalho foi de melhor entender os mecanismos patogênicos responsáveis pela neurotoxicidade induzida pela exposição à toxicantes exógenos e endógenos, principalmente em nível mitocondrial, em cérebro de roedores; visto que existe um grande número de evidências na literatura que demonstra que a gênese dos processos neurodegenerativos está intimamente relacionado com deficiências na produção energética mitocondrial. Observou-se que o MeHg causou estresse oxidativo e diminuiu a atividade dos complexos da cadeia respiratória, além de inibir severamente a enzima creatina cinase, tanto em sistemas *in vivo* como *in vitro*. Desta forma, o MeHg prejudica a produção de ATP no cérebro, podendo ser uma das causas da neurodegeneração desencadeada por este toxicante. Para proteger das alterações causadas pelo MeHg, compostos de selênio tem sido usados, pois sabe-se que possuem alta afinidade por este toxicante. Desta forma, administramos dois compostos contendo selênio para proteger contra os efeitos causados pelo MeHg, o difenil disseleneto ($(PhSe)_2$) e o selenito de sódio (Na_2SeO_3), e verificamos que principalmente o $(PhSe)_2$ foi capaz de proteger contra os efeitos do MeHg *in vivo*. Por outro lado, o Na_2SeO_3 na dose utilizada foi potencialmente tóxico. Os dois compostos foram capazes de reduzir a deposição do mercurial no cérebro, provavelmente pela formação de um complexo HgSe. Para a leucina, observamos que esta altera a função da cadeia respiratória mitocondrial

e impede a formação de memória, este último verificado por análise do LTP no hipocampo de animais injetados intrahipocampalmente com leucina, possivelmente sendo um dos mecanismos responsáveis pelo déficit neurológico em pacientes com a doença da urina de xarope de bordo. Concluindo, podemos observar que tantos toxicantes endógenos como exógenos compartilham de mecanismos que levam ao prejuízo no sistema nervoso central, tendo com um dos alvos a mitocôndria e o metabolismo energético.

ABSTRACT

Mitochondria are responsible for cell energy production. Several works have demonstrated the involvement of this cell organell in the physiopathology of neurodegenerative processes, including Alzheimer, Parkinson and Hungtington diseases. Moreover, mitochondria are also targets of endogenous and exogenous toxicants, *i.e.* the environmental pollutant methylmercury (MeHg), or the high leucine concentrations found in individuals affected by maple syrup urine disease (MSUD), a genetic human disease. It is known that MeHg exposure provokes severe neurologic damage, both in animals and humans. The major form of human contamination is through ingestion of contaminated fish, and it has been demonstrated that MeHg accumulates preferencially in brain mitochondria. On the other hand, leucine and its α-ketoacid, α-ketoisocaproic, are the main metabolites accumulating in MSUD, and these compounds appear to be responsible for the main neurological symptoms of the disease, including the characteristic cognitive impairment of affected patients. Considering that there is a great body of evidences indicating that the ethiopatogeny of neurodegeneratives processes is related to dysfunction of brain energy production, the aim of present study was to better understand the neuropathogenic mechanisms induced by endogenous and exogenous toxicants at the mitochondrial level in rodent brain. We observed that MeHg caused oxidative stress and energy impairment, the latter, by diminishing the mitochondrial enzymes complex activities and by inhibiting creatine kinase activity, *in vitro* and *in vivo*. In this scenario, MeHg compromised brain ATP production, which might be one of the toxic mechanisms involved in the MeHg-induced neurodegeneration. Compounds containing selenium has been proposed as good candidates for preventing MeHg toxicity, mainly because of the high affinity for the mercurial. Therefore, two seleno compounds, diphenyl diselenide ($(PhSe)_2$) and sodium selenite (Na_2SeO_3), were administered in order to protect from the MeHg effects, and it was verified that mainly $(PhSe)_2$ was able to prevent most of the *in vivo* alterations induced by the mercurial, while Na_2SeO_3 resulted potentially toxic. However, both compounds were equally efficient in reducing brain metal deposition, probably by forming a inert and insoluble metabolite, HgSe. Regarding leucine experiments, we observed that this amino acid, when intrahippocampaly administrated, impairs the respiratory chain function,

and inhibits memory consolidation and a complete impairment of LTP generation at supramaximal stimulation, effects possibly related to the cognitive impairment in MSUD. Finally, it is feasible that both endogenous and exogenous toxicants share common mechanisms involving mitochondrial dysfunction, which would lead to brain dysfunction.

1. INTRODUÇÃO

1.1 Metabolismo energético cerebral

A glicólise e a fosforilação oxidativa mitocondrial são particularmente importantes no cérebro para a produção energética, porque a glicose é o principal substrato energético utilizado pelo sistema nervoso central (SNC), e no cérebro a fosforilação oxidativa fornece mais de 95% do ATP sintetizado. Por outro lado, a oxidação de corpos cetônicos ocorre no cérebro de forma efetiva no jejum (Siesjo, 1978).

Em condições de normoglicemia o conteúdo de glicose no cérebro é de aproximadamente 2 – 3 μmol . g^{-1} de tecido (Cunningham et al., 1986, Mason et al., 1992). O transporte de glicose através da barreira hematoencefálica (BHE), bem como através de membranas neuronais e das células gliais é muito rápido. Sendo assim, o metabolismo cerebral da glicose é regulado principalmente pela sua fosforilação mais do que pelo seu transporte (Lund-Andersen, 1979). A reserva energética cerebral, glicogênio, é extremamente pequena em relação a sua elevada atividade metabólica (3–5 μmol . g^{-1} de tecido) (Oz et al., 2007), de modo que a função normal do SNC requer o suprimento contínuo de glicose a partir da circulação (Erecinska et al., 1994). O glicogênio está localizado principalmente nos astrócitos (Cataldo and Broadwell, 1986, Oz et al., 2007). No cérebro mais de 95% da glicose é convertida em CO_2 e água, enquanto que uma pequena fração é convertida em lactato ou segue outras rotas metabólicas (Hawkins et al., 1974, Siesjo, 1978).

Lactato e piruvato podem ser transportados através da BHE por mecanismos específicos saturáveis que envolvem transportadores para ácidos monocarboxílicos, e ambos podem ser prontamente oxidados pelas células cerebrais. Neste contexto, o lactato tem sido identificado como um importante substrato energético durante o período neonatal (Medina, 1985).

Em estados de cetoacidose, os corpos cetônicos, D-β-hidroxibutirato e acetoacetato, podem substituir em parte a glicose, e são oxidados pelo cérebro em quantidades significativas (Owen et al., 1967). Nos recém-nascidos o acetoacetato é metabolizado pelo cérebro com a mesma velocidade que a glicose, enquanto que adultos metabolizam a glicose mais rapidamente (Spitzer, 1973).

Embora o tecido cerebral contenha todas as enzimas envolvidas na oxidação de ácidos graxos, este processo acontece em pequena escala

(Abood and Geiger, 1955). O mesmo acontece para os aminoácidos (Lajtha and Toth, 1961).

Tendo em vista que a fosforilação oxidativa é responsável pela quase totalidade do ATP produzido no SNC, a regulação da respiração mitocondrial se torna essencial para o correto metabolismo energético cerebral (Erecinska et al., 1994).

Outro sistema cerebral para a manutenção dos níveis energéticos é o sistema catalisado pela enzima creatina cinase (CK). O cérebro de mamíferos contém uma reserva energética adicional na forma de sistema fosfocreatina / creatina. O conteúdo total de nucleotídeos de adenina (ATP + ADP + AMP) é de aproximadamente 3 µmol . g^{-1} de tecido. A concentração de ATP excede em 10 vezes a do ADP e em quase 100 vezes a do AMP. Fosfocreatina / creatina totalizam 10 -14 µmol . g^{-1} de tecido e estão presentes na proporção de 1:1 (Erecinska et al., 1994).

1.1.1 Mitocôndria

A mitocôndria é a organela celular responsável pela maior produção líquida de energia. Eugene Kennedy e Albert Lehninger descreveram há mais de 50 anos que a mitocôndria contém proteínas envolvidas com a oxidação de nutrientes bem como com a respiração celular com concomitante geração de energia (Lehninger and Smith, 1949, Kennedy and Lehninger, 1950, 1951). Esta organela tem uma estrutura basicamente membranosa, sendo seu envoltório formado por duas membranas, a membrana externa e a membrana interna, ambas com composição química e estrutural semelhante à plasmalema. A membrana externa é mais permeável que a membrana interna, e entre ambas é determinado um espaço denominado intermembranoso onde ocorrem reações essenciais ao metabolismo celular. A membrana interna é formada por pregas que se expandem no espaço intramitocondrial (matriz mitocondrial) denominadas cristas mitocondriais (Lehninger et al., 2004) (Figura 1).

A maquinaria molecular para a produção energética mitocondrial está constituída por enzimas presentes na matriz mitocondrial (ciclo de Krebs), e por proteínas organizadas de maneira a formar uma assembléia localizada na membrana mitocondrial interna (cadeia transportadora de elétrons ou cadeia respiratória; Figura 1). Os complexos protéicos envolvidos na formação de energia e respiração celular são codificados pelo genoma nuclear e mitocondrial (Di Donato, 2000).

1.1.2 Glicólise

A utilização da glicose para a produção energética está presente em todos os seres vivos, desde a mais antiga e simples bactéria até os complexos organismos multicelulares. A glicólise, também conhecida como via de Ebden-Meyerhof, é a rota metabólica pela qual a glicose é convertida em piruvato com geração de dois moles de ATP / mol de glicose através de dez reações enzimáticas. Em condições de aerobiose o piruvato formado é oxidado a CO_2 e água pelo ciclo dos ácidos tricarboxílicos seguido da fosforilação oxidativa. Entretanto sob condições de anaerobiose, o piruvato é prontamente convertido no produto final reduzido, lactato (Voet and Voet, 1995). Todas as enzimas envolvidas na via glicolítica estão localizadas no citosol (Voet and Voet, 1995).

1.1.3 Ciclo dos ácidos tricarboxílicos – Ciclo de Krebs

Nos organismos aeróbios o piruvato resultante da glicólise entra na mitocôndria e sofre descarboxilação oxidativa pela ação de um complexo enzimático denominado piruvato desidrogenase, formando uma molécula de NADH e uma de acetil-CoA que será oxidada no ciclo de Krebs. A oxidação completa deste substrato originará a formação de GTP, CO_2 e nucleotídeos reduzidos (3 NADH e 1 $FADH_2$). Todas as enzimas envolvidas neste ciclo oxidativo se encontram localizadas na matriz mitocondrial (Voet and Voet, 1995, Lehninger et al., 2004).

1.1.4 Cadeia respiratória e fosforilação oxidativa

A fosforilação oxidativa é um processo que requer a ação orquestrada de cinco complexos enzimáticos distribuídos de forma especial na membrana mitocondrial interna e constituem a denominada cadeia respiratória (CR). Os elétrons oriundos do NADH e do $FADH_2$ provenientes do ciclo de Krebs e de outras reações catalisadas por desidrogenases são transferidos para a CR, tendo o oxigênio molecular como aceptor final. Este processo é acoplado à translocação de prótons através da membrana mitocondrial interna e a síntese endergônica de ATP, empregando como força motriz a energia armazenada como gradiente eletroquímico de prótons (Babcock and Wikstrom, 1992, Voet and Voet, 1995) (Figura 1). Os primeiros dois eventos ligados à respiração, transferência de elétrons e bombeamento de prótons, são realizados pela CR. Os complexos enzimáticos da CR compreendem a

maior parte das proteínas embebidas na membrana mitocondrial interna. Cada complexo é constituído de várias subunidades protéicas que se encontram associados com uma variedade de grupamentos prostéticos com potencial de oxi-redução sucessivamente maiores (Voet and Voet, 1995).

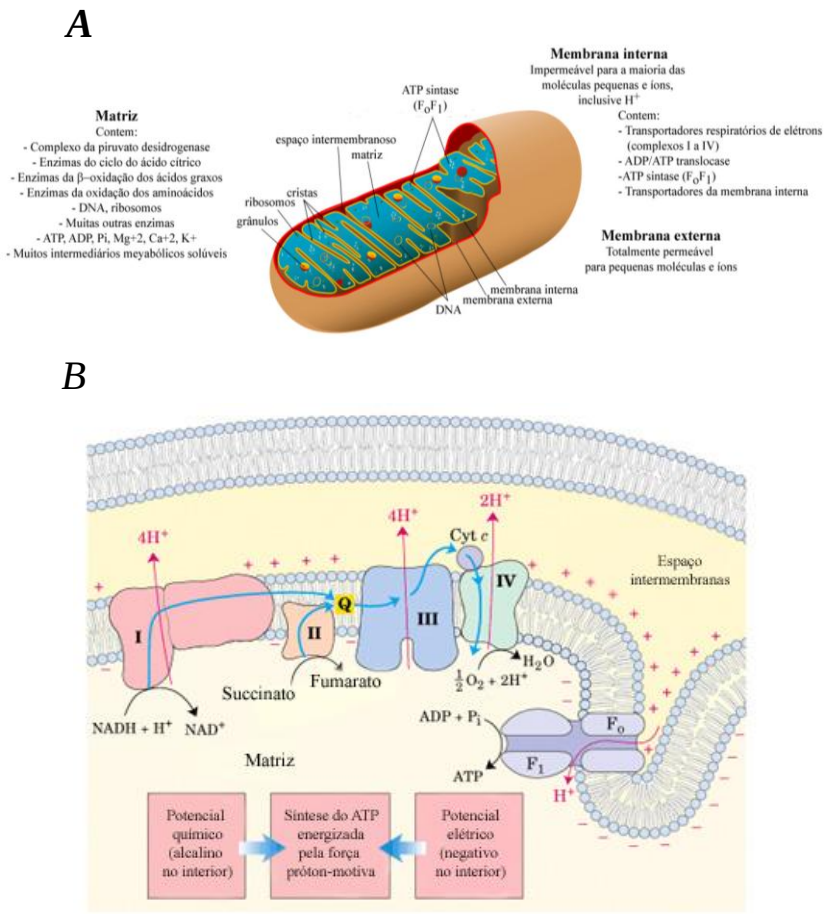

Figura 1. Anatomia bioquímica da mitocôndria (A) e proteínas envolvidas na fosforilação oxidativa (B) (Adaptado de Lehninger et al., 2004).

Complexo I: NADH – Coenzima Q redutase: O complexo I transfere os elétrons do NADH, principalmente formado a partir da glicólise e do ciclo de Krebs, para a coenzima Q, também chamada de ubiquinona (CoQ). Este complexo é o maior componente protéico presente na membrana mitocondrial interna e é formado por sete unidades codificadas pelo DNA mitocondrial e pelo menos por 34 subunidades codificadas pelo DNA nuclear (Voet and Voet, 1995, Di Donato, 2000). Com aproximadamente 850 kD o complexo I contém uma molécula de flavina mononucleotídeo (FMN) como grupamento prostético e de seis a sete centros ferro-enxofre que participam do processo de transferência de elétrons. FMN e CoQ podem admitir três estados de oxidação, embora o NADH possa transferir dois elétrons, FMN e CoQ são capazes de aceitar um ou dois elétrons de cada vez, porque suas formas semiquinonas são estáveis.

Complexo II: Succinato – Coenzima Q redutase: O complexo II é composto por quatro subunidades protéicas, incluindo a enzima dimérica succinato desidrogenase, componente do ciclo de Krebs, todas codificadas pelo DNA nuclear. Este complexo transfere os elétrons do succinato para a CoQ. Este processo envolve a participação de um FAD covalentemente ligado à succinato desidrogenase, dois centros ferro-enxofre e um citocromo b_{560} (Voet and Voet, 1995, Di Donato, 2000).

Complexo III: Coenzima Q – citocromo c redutase: O complexo III transfere os elétrons da CoQ para o carreador móvel de elétrons, o citocromo *c*. O complexo III está arranjado assimetricamente na membrana mitocondrial interna e contém 11 subunidades, onde três delas contém centros redox que são utilizados na formação de energia. Essas três unidades chaves estão representadas pelo citocromo *b* (único codificado pelo genoma mitocondrial), um centro ferro-enxofre e o citocromo c_1 (Saraste, 1990).

Complexo IV: Citocromo *c* oxidase: A citocromo c oxidase é o complexo terminal da cadeia transportadora de elétrons. O complexo IV transfere os elétrons a partir do ferrocitocromo *c* para o oxigênio molecular. O complexo IV consiste de doze ou mais subunidades polipeptídicas (Barrientos et al., 2002). As três maiores subunidades formam o centro catalítico da enzima e são codificadas pelo DNA mitocondrial. A subunidade I contém os grupamentos heme e um dos íons Cu (Cu_B), enquanto que a subunidade II contem um centro de Cu binuclear (Cu_A) (Capaldi, 1990). A subunidade III não apresenta

grupamento prostético e não parece estar envolvida na síntese de ATP, mas favorece a estabilidade estrutural. As demais subunidades, todas codificadas pelo DNA nuclear, parecem não serem essenciais ao mecanismo catalítico básico de redução de oxigênio e à transferência vetorial de prótons (Saraste, 1990, Barrientos et al., 2002). A citocromo *c* oxidase é uma enzima chave na produção energética mitocondrial, uma vez que a reação redox entre o citocromo *c* e o oxigênio molecular é essencialmente irreversível (Poyton and McEwen, 1996). Além disso, sabe-se que a atividade desta enzima é regulada por relações aumentadas de ATP/ADP intramitocondrial, e pelas concentrações do radical óxido nítrico (Cooper and Brown, 2008).

Complexo V: ATP sintase. A síntese de ATP é realizada pelo complexo V. Este complexo é formado por duas subunidades codificadas pelo DNA mitocondrial (ATPase 6 e 8) e pelo menos por doze subunidades codificadas pelo DNA nuclear. Funcionalmente e estruturalmente, o complexo V é formado por um componente catalítico solúvel na matriz mitocondrial (F_1-ATPase) e um componente de membrana hidrofóbico (F_o-ATPase) que contém um canal de prótons (Saraste, 1990).

Os complexos transmembrana I, III e IV além de participar na CR têm a sua atividade vinculada à transferência de prótons da matriz mitocondrial para o espaço intermembranas, contribuindo para a formação de um gradiente eletroquímico. Este gradiente determina uma polarização da membrana mitocondrial interna (potencial de membrana mitocondrial; $\Delta\psi$), que pode ser revertida pelo fluxo desses prótons através do componente F_0 da ATP sintase. O fluxo de prótons leva à condensação do ADP e de fosfato inorgânico em ATP (Saraste, 1990, Wallace, 1999). A ATPsintase é uma enzima funcionalmente reversível que pode catalisar tanto a síntese quanto a hidrólise de ATP (Saraste, 1990).

1.1.5 Creatina cinase (CK)

As CKs (ATP:creatina *N*-fosforibosiltransferase) são uma família de enzimas que catalisam a transferência reversível da um grupamento *N*-fosforibosil entre fosfocreatina (PCr) e ADP, conforme a seguinte reação (Bessman and Carpenter, 1985, Bittl and Ingwall, 1985).

$$\mathbf{PCr + Mg^{2+}\text{-}ADP + H^{+} \leftrightarrow Creatina + Mg^{2+}\text{-}ATP}$$

As CKs tem papel fundamental na transferência de energia nas células que apresentam elevado metabolismo energético, fornecendo um sistema eficaz de tamponamento do ATP (Bessman and Carpenter, 1985). A velocidade de reação excede em magnitude à velocidade de síntese de ATP celular. Esse fenômeno pode explicar a habilidade dos tecidos cardíaco e muscular e dos neurônios em alternar a velocidade de consumo de energia durante os períodos de maior atividade (Bittl and Ingwall, 1985, Saks et al., 1996a, Saks et al., 1996b).

As CKs constituem um grupo de diferentes isoformas que são específicas de cada tecido e que são codificadas por genes diferentes. As isoenzimas citosólicas existem exclusivamente como moléculas diméricas, compostas por dois tipos de subunidades, originando três diferentes isoformas: CK-MM e CK-BB, como homodímeros, e o heterodímero CK-MB. A CK-MM é predominante no tecido muscular esquelético maduro e no miocárdio de mamíferos, a CK-BB está presente no cérebro e tecido nervoso periférico, e a CK-MB é encontrada somente no tecido cardíaco (Wallimann et al., 1992).

As isoenzimas citosólicas (cit-CK) e mitocondriais (mit-CK) são co-expressas na maioria dos tecidos que possuem atividade de CK. Mit-CK está presente principalmente em tecidos com alta demanda energética como no músculo esquelético, coração, cérebro, retina e espermatozóides. Mit-CK sarcomérica (smit-CK) é quase exclusivamente expressa no coração e músculo esquelético, enquanto que a mit-CK ubíqua (umit-CK) é principalmente encontrada nos rins, placenta, intestino e cérebro. Dessa forma, parece que a smit-CK acompanha a distribuição de CK-M, enquanto que umit-CK a CK-B (Wyss et al., 1992). Tem sido demonstrado que as isoformas presentes no cérebro são o homodímero citosólico CK-BB e a isoenzima mitocondrial umit-CK. Ainda, foi observado que a expressão de CK-BB é maior nos astrócitos e oligodendrócitos do que nos neurônios (Molloy et al., 1992).

As isoformas mitocondriais podem apresentar uma conformação dimérica ou octamérica, sendo esta última a estrutura funcional. As mit-CKs estão localizadas no espaço intermembranas mitocondrial (Jacobs et al., 1964) onde os octâmeros ligam-se à membrana mitocondrial externa, interagindo funcionalmente com as proteínas que conformam o poro de transição mitocondrial, o traslocador de nucleotídeos de adenina

(ANT) na membrana mitocondrial interna e à porina da membrana externa (Eppenberger et al., 1967, Brooks and Suelter, 1987, Wyss et al., 1992, Schlattner et al., 1998). As mit-CKs tem acesso preferencial ao ATP gerado a partir da fosforilação oxidativa que é exportado da matriz mitocondrial através do ANT (Saks et al., 1986, Vendelin et al., 2004). Regiões enriquecidas em mit-CK, ANT e porinas são chamados de sítios de contato entre as membranas externa e interna da mitocôndria (Beutner et al., 1996, Beutner et al., 1998).

1.1.6 Mecanismos de disfunção mitocondrial associados à neurodegeneração

O cérebro é um dos órgãos metabolicamente mais ativos, requerendo duas vezes mais energia que o coração em repouso. Este tecido representa 2% da massa corporal do homem adulto e consome em torno de 20% do total de O_2 disponível para o organismo (Dickinson, 1996). Tendo em vista que a fosforilação oxidativa é responsável pela quase totalidade do ATP produzido no SNC, a regulação da respiração mitocondrial se torna essencial para o correto metabolismo energético cerebral (Erecinska et al., 1994). Neste sentido, a disfunção mitocondrial tem sido apontada como o mecanismo-chave na neurodegeneração induzida por estímulos agudos e crônicos (Fiskum et al., 1999, Lin and Beal, 2006).

As doenças neurodegenerativas crônicas podem ser definidas como um grupo de desordens heterogêneas caracterizadas por um início insidioso, de progressão lenta e com características neuropatológicas fortemente associadas a uma área específica do cérebro (Lin and Beal, 2006). Apesar da heterogeneidade destas entidades a resposta adaptativa crônica aos diferentes fatores geradores de estresse e que comprometem a homeostase celular, parece estar relacionada com mudanças específicas na função mitocondrial. Uma complexa rede de sinalização permite que a mitocôndria identifique as mudanças do meio provocando uma alteração nas respostas bioenergéticas, apoptóticas ou oxidativas. Estas alterações no funcionamento da mitocôndria têm sido reconhecidas como um componente-chave não só nesses processos neurodegenerativos crônicos, como também em processos de neurotoxicidade aguda, incluindo as induzidas por toxicantes endógenos como o glutamato nos acidentes cérebro-vasculares, isquemia ou trauma (Choi and Rothman, 1990), erros inatos do metabolismo (Wajner et al.,

2004, Latini et al., 2007), bem como por contaminantes ambientais como mercúrio, metilmercúrio, zinco, alumínio, cobre, etc. (Sharpley and Hirst, 2006, Franco et al., 2009).

Luft e colaboradores (Luft et al., 1962) descreveram o primeiro caso clínico com disfunção mitocondrial, onde o defeito estava representado por um desacople da respiração mitocondrial, o que significa que a transferência de elétrons através da CR não estava em sincronia com a síntese de ATP. Esta primeira documentação clínica permitiu abrir novos horizontes para melhor entender os mecanismos de toxicidade envolvidos em processos neurodegenerativos. Neste contexto, as principais conseqüências da disfunção mitocondrial parecem envolver indução de estresse oxidativo, alteração na homeostase do cálcio, apoptose e falha metabólica. Historicamente, a maior atenção tem sido dada ao estudo da expressão ou funcionamento dos componentes da cadeia respiratória. No entanto, o foco está atualmente sendo dirigido ao estudo dos efeitos do estresse oxidativo sobre a respiração mitocondrial.

O estresse oxidativo é uma condição definida como um desbalanço entre a produção de espécies reativas e as defesas antioxidantes do tecido, favorecendo a primeira (Cadenas and Sies, 1985, Sies and Cadenas, 1985, Halliwell and Gutteridge, 1990). Devido ao fato que a mitocôndria é o sítio celular onde acontece a redução do oxigênio em água, esta organela representa o principal local de produção de espécies reativas do oxigênio (EROs) em condições fisiológicas (Chance et al., 1979, Sipos et al., 2003).

Os principais componentes geradores de EROs da CR são os complexos I e III, sendo que a produção de radicais livres aumenta no caso de um bloqueio na transferência de elétrons (Nicholls and Budd, 2000, Sipos et al., 2003). Pode-se citar a produção de superóxido e a de peróxido de hidrogênio (H_2O_2) como os principais agentes causadores de estresse oxidativo na célula. Para contrabalancear a produção de espécies reativas, a mitocôndria possui sistemas de defesa antioxidantes, como as enzimas manganês superóxido dismutase (Mn-SOD), peroxiredoxinas, o sistema glutationa peroxidase/glutationa redutase, a coenzima Q10 (ubiquinona), creatina e nicotinamida (Okado-Matsumoto and Fridovich, 2001, Droge, 2002, Fernandez-Checa, 2003, James et al., 2004, Kojo, 2004). Ainda, foi recentemente demonstrado que cinases mitocondriais, como a hexoquinase e a CK, possuem um

papel essencial como antioxidantes mitocondriais (Dolder et al., 2003, Santiago et al., 2008). Este efeito parece estar relacionado com a capacidade de modular o $\Delta\psi$; quanto maior o valor do $\Delta\psi$, maior a probabilidade de formar EROs. Sabe-se ainda que a taxa de produção de EROs é inversamente proporcional à disponibilidade de ADP intramitocondrial (Korshunov et al., 1997, Cadenas and Davies, 2000). A produção excessiva de EROs pode também induzir a oxidação de ácidos graxos poliinsaturados de membrana, muito concentrados no SNC, levando a múltiplos produtos tóxicos de peroxidação lipídica (Poli and Schaur, 2000).

EROs e espécies reativas do nitrogênio (ERNs) podem inibir vários complexos da cadeia respiratória, bem como oxidar e fragmentar o DNA mitocondrial (Radi et al., 2002), gerando um círculo vicioso entre o bloqueio da transferência de elétrons e a produção de espécies reativas. Neste sentido, foi também demonstrado que deficiências nas atividades dos complexos da cadeia respiratória são acompanhadas de depleção celular de glutationa (GSH; principal antioxidante natural), e ainda que o grau de inibição da cadeia respiratória é proporcional à magnitude da depleção desse antioxidante (Barker et al., 1996, Bolanos et al., 1996). O déficit energético e o aumento da produção de espécies reativas podem levar secundariamente a uma diminuição na atividade da Na^+, K^+-ATPase com conseqüente despolarização da membrana plasmática, perda da homeostase celular, excitotoxicidade secundária e/ou ativação das cascatas de apoptose (Beal, 2005). Neste cenário, a produção de EROs, juntamente com a liberação de proteínas pró-apoptóticas para o espaço intermembranas, desencadeia a morte apoptótica, uma forma controlada de morte celular, a qual apresenta papel fundamental durante o desenvolvimento embrionário e na manutenção dos tecidos no adulto. Defeitos na regulação desta via tem sido associados com a patogênese de doenças neurodegenerativas (Li et al., 1997, Budihardjo et al., 1999, Allan and Clarke, 2009). Ainda, a abertura do poro de transição mitocondrial, passo essencial para induzir apoptose, parece estar regulada em parte pela atividade da umit-CK (Andrienko et al., 2003, Vyssokikh and Brdiczka, 2003). Portanto, considerando que a mitocôndria ocupa um papel central tanto na sobrevida como nos mecanismos que levam à morte das células neurais, o presente estudo teve como objetivo estudar a participação da disfunção mitocondrial nos mecanismos neurotóxicos envolvidos nas

alterações do SNC em duas situações neurodegenerativas, na intoxicação ambiental por metilmercúrio (MeHg) bem como no acúmulo do aminoácido leucina no SNC, que caracteriza o erro inato do metabolismo denominado doença do xarope do bordo. Este objetivo encontra seu baseamento na hipótese que os mecanismos de neurotoxicidade induzidos por toxicantes tanto endógenos quando exógenos compartilham mecanismos de morte, envolvendo neles a disfunção mitocondrial.

1.2 Neurotoxicidade induzida por toxicante exógeno

1.2.1 Toxicidade induzida por MeHg

O contaminante ambiental MeHg encontra sua origem principalmente em fontes naturais (emissão oceânica, depósitos minerais, vulcões, queimada de florestas e degradação da crosta) e antropogênicas (principalmente através da mineração do ouro) através da liberação de mercúrio elementar (Hg°) conforme mostra a Figura 3 (Clarkson et al., 2003, Pinheiro et al., 2009). O Hg é preferencialmente liberado como vapor de Hg (Hg°) para a atmosfera onde sofre numerosas transformações, incluindo a transformação a Hg na forma iônica, principalmente Hg^{2+}, retornando na superfície terrestre através das chuvas. Este ciclo é mantido pela ecosfera marinha, onde bactérias do ambiente aquático metilam o Hg^{2+} em MeHg que finalmente é bioacumulado através da cadeia trófica aquática atingindo concentrações de 10.000 a 100.000 maiores que as presentes nas águas contaminadas (ATSDR, 1999, Clarkson et al., 2003, Clarkson and Magos, 2006). Desta forma, a ingestão de peixes contaminados torna-se a principal forma de exposição humana ao MeHg (Figura 3) (Veiga et al., 1994, Clarkson et al., 2003). O MeHg na forma livre existe em concentrações baixas nos sistemas biológicos, encontrando-se principalmente ligado a proteínas ou aminoácidos através de grupamentos tiólicos (Clarkson, 1972, Pinheiro et al., 2009).

Aproximadamente 90% do MeHg ingerido é absorvido pelo trato gastrointestinal humano (Ozuah, 2000, Clarkson, 2002). Na circulação o mercurial liga-se à proteínas plasmáticas, principalmente a albumina, aos eritrócitos e a grupos cisteína livres e desta forma é transportado para os tecidos periféricos e para o cérebro, atravessando facilmente a BHE pelo transportador para aminoácidos neutros LAT1 (Yin et al., 2008). Além disso, o MeHg atravessa a placenta, e devido a diferenças

na toxicocinética e toxicodinâmica, ele acumula preferencialmente no cérebro fetal, em concentrações maiores que aquelas encontradas no sangue materno (Inouye et al., 1986, Cernichiari et al., 2007).

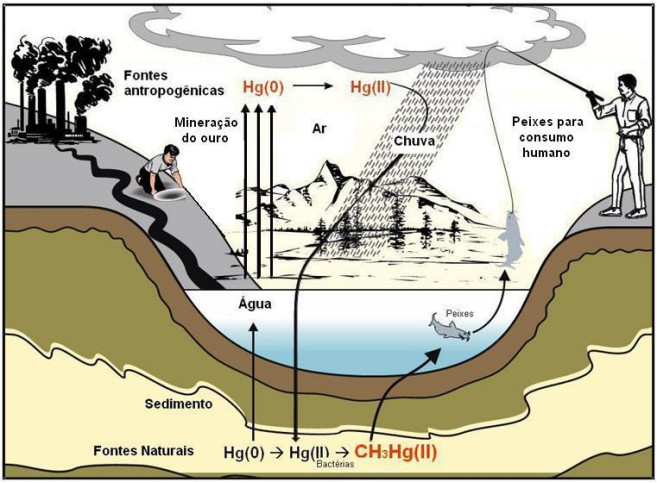

Figura 2. Ciclo do mercúrio na natureza
(Adaptado de Clarkson et al., 2003)

O SNC é extremamente sensível aos danos causado pelo MeHg, e o cérebro fetal pode ser afetado mesmo se a gestante não apresenta sinais de intoxicação (Castoldi et al., 2001). Esta exposição pré-natal que acontece em gestantes intoxicadas causa danos neurais, comportamentais e no desenvolvimento fetal, os quais são observados logo após o nascimento (Myers and Davidson, 2000). Além disso, estudos experimentais demonstram que tanto o Hg quanto o MeHg podem ser excretados no leite materno, tornando-se uma forma de contaminação na fase lactacional (Manfroi et al., 2004, Franco et al., 2006).

Embora todos os órgãos sejam expostos a altos níveis de MeHg após uma intoxicação, o principal local de deposição deste mercurial é no SNC (Clarkson, 2002), sendo cerca de 10% do conteúdo total de

MeHg do organismo retido no cérebro, principalmente no córtex cerebelar e cerebral, além da raiz do gânglio dorsal (Skerfving, 1974).

A exposição ao MeHg causa danos neurológicos severos e irreversíveis tanto em animais quanto em humanos (Choi, 1989, Gilbert and Grant-Webster, 1995, Rice and Barone, 2000, Clarkson et al., 2003).. Os principais conhecimentos sobre a toxicidade do MeHg tem sido obtidos através de episódios catastróficos de contaminação. Os principais ocorreram no Japão em Minamata na década de 1950 e em Niigata na década de 1960 pelo consumo de peixes de águas que estavam severamente poluídas com Hg pelo despejo de efluentes de indústrias locais (WHO, 1976, Harada, 1995, Clarkson, 2002). Outro evento importante de intoxicação pelo MeHg ocorreu no Iraque na década de 1970 quando milhares de pessoas ficaram doentes e centenas morreram por alimentarem-se de pães contendo grãos que haviam sido tratados com um fungicida a base de Hg orgânico (Bakir et al., 1973, Clarkson, 2002).

As principais alterações neurológicas e neuropatológicas induzidas pela exposição ao MeHg incluem desmielinização, disfunção autônoma, atraso na condução nervosa, migração e divisão neuronal anormal (Myers and Davidson, 2000, Stein et al., 2002, Sanfeliu et al., 2003, Spurgeon, 2006). Sintomas de toxicidade crônica incluem parestesia, neuropatia periférica, ataxia cerebelar, acatesia, espasticidade, perda de memória, demência, distúrbios visuais, auditivos, olfáteis e gustativos; disartia, tremores e depressão (Choi, 1989, Gilbert and Grant-Webster, 1995, Rice and Barone, 2000, Clarkson, 2002, Stein et al., 2002, Clarkson et al., 2003, Spurgeon, 2006).

Em contraste com os raros casos relacionados à intoxicação aguda com MeHg, muitas pessoas são expostas cronicamente a níveis de MeHg que, embora consideradas baixas, podem produzir efeitos neurotóxicos, particularmente em lactantes e crianças (Clarkson, 1998, Chapman and Chan, 2000, Counter et al., 2004). Sabe-se que a toxicidade do MeHg exibe um período de latência após a exposição, de tal ordem que quando os sinais e sintomas clínicos aparecem, geralmente é tarde demais para reverter os danos causados pelo metal (Clarkson, 1997).

1.2.2 Mecanismos envolvidos na toxicidade induzida por MeHg

Apesar das severas alterações neurológicas induzidas pela exposição ao MeHg, a sua fisiopatologia ainda não foi completamente definida. No entanto, os principais mecanismos moleculares explorados na neurotoxicidade induzida pelo MeHg envolvem a alteração da homeostase do cálcio intracelular (Sirois and Atchison, 2000), o estresse oxidativo (Ou et al., 1999, Aschner et al., 2007) e a alteração da homeostase glutamatérgica (Aschner et al., 2000, Farina et al., 2003b).

Numerosas evidências sugerem que o principal local de deposição do mercurial no SNC são os astrócitos (Garman et al., 1975, Aschner, 1996, Charleston et al., 1996), provocando inibição da captação de glutamato, cistina e cisteína afetando de forma prejudicial o conteúdo intracelular de glutationa e o estado redox desta célula (Brookes and Kristt, 1989, Dave et al., 1994, Allen et al., 2001a, Allen et al., 2001b, Shanker et al., 2001, Shanker and Aschner, 2001, Shanker et al., 2003)

Todos estes mecanismos parecem envolver alguma forma de disfunção mitocondrial, assim como prejuízo no metabolismo energético do tecido cerebral. Desta forma, tem sido recentemente demonstrado que o MeHg acumula-se preferencialmente na mitocôndrias e que as mitocôndrias de cérebro são mais susceptíveis que as hepáticas ao dano oxidativo induzido pelo toxicante (Mori et al., 2007).

As principais alterações mitocondriais induzidas pelo MeHg descritas estão relacionadas com a redução do potencial transmembrana mitocondrial (Yin et al., 2007), liberação de citocromo *c* no citoplasma (Shenker et al., 2002) seguido por ativação de caspases (Belletti et al., 2002, Shenker et al., 2002) e abertura do poro de transição mitocondrial (Bragadin et al., 2002). Estes efeitos mitotóxicos do MeHg tem sido também relacionados com decréscimos nos níveis de ATP indicando a ocorrência de prejuízo energético (Fonfria et al., 2005).

Embora existam estudos prévios na literatura demonstrando prejuízo na homeostase mitocondrial tanto *in vitro* quanto *in vivo* após a exposição ao MeHg, dados sobre alvos moleculares de vias energéticas cerebrais envolvidos nos efeitos tóxicos deste poluente são escassos. Desta maneira, as limitadas informações sobre o assunto leva-nos a investigar em mais detalhes a participação do metabolismo energético nos efeitos deletérios induzidos pela exposição em longo prazo ao MeHg.

1.2.3 Compostos neuroprotetores contra a toxicidade induzida pelo MeHg

Apesar dos constantes esforços para minimizar os efeitos tóxicos do MeHg, terapias eficazes contra a toxicidade deste mercurial até o presente não foram encontradas. Os principais compostos utilizados até o momento incluem os quelantes ácido meso-2,3-dimercaptosuccínico (DMSA) e o 2,3-dimercapto-1-propanosulfonato (DMPS) (Risher and Amler, 2005). Entretanto, eles possuem além de significativos efeitos secundários, estabilidade limitada em soluções, limitada disponibilidade para o uso em humanos, e uma propensão para mobilizar outros minerais, principalmente cátions divalentes essenciais para as funções fisiológicas normais (Mann and Travers, 1991, Grandjean et al., 1997, Nogueira et al., 2003, Risher and Amler, 2005).

Por outro lado, a *N*-Acetilcisteína (NAC), um antioxidante contendo grupos tióis, também tem sido utilizada para mitigar várias condições de estresse oxidativo induzidas pelo MeHg. Acredita-se que a ação antioxidante do NAC esteja vinculada com a sua capacidade de estimular a síntese de GSH (Moldeus et al., 1986) e de remover EROs (Aruoma et al., 1989). Alguns estudos indicam que NAC também tem atividade quelante em respeito a vários metais pesados (Banner et al., 1986), e aumenta a excreção de MeHg em camundongos (Ballatori et al., 1998).

Nos últimos anos, Nogueira e colaboradores (Nogueira et al., 2004) tem sugerido que os compostos contendo selênio (Se) podem resultar bons candidatos no tratamento das intoxicações com MeHg. A interação entre Hg e selênio foi inicialmente reportada por Parizek e Ostadalova (Parizek and Ostadalova, 1967) que demonstraram o efeito protetor de selenito de sódio (forma inorgânica de Se) contra a toxicidade de Hg inorgânico. Posteriormente, Ganther e colaboradores (Ganther et al., 1972) observaram que este composto diminuía a mortalidade e a perda de peso induzida pelo mercurial.

O Se é um nutriente essencial necessário para a síntese e atividade de aproximadamente vinte e cinco enzimas dependentes de Se, incluindo a glutationa peroxidase (GPx) (Flohe et al., 1973, Forstrom et al., 1978, Islam et al., 2002), a tioredoxina redutase (Holmgren, 1989, Arner and Holmgren, 2000) e muitas outras selenoproteínas que modulam o estado redox e antioxidante das células (Saito et al., 1999, Bianco et al., 2002, Panee et al., 2007). Neste cenário, tem sido sugerido

que o Se protege contra a toxicidade do Hg por regular a expressão e conteúdo protéico destas enzimas antioxidantes. Além disso, a forma reduzida do Se apresenta uma constante de afinidade maior pelo Hg do que por outros compostos que contenham grupamentos tiólicos (Clarkson, 1997, Yoneda and Suzuki, 1997). Assim, a ligação direta entre estes tem sido assumida como um dos mecanismos responsáveis pelo efeito protetor do Se na intoxicação com Hg (WHO, 1976, WHO, 1990, Lee et al., 2004, Clarkson and Magos, 2006, Yang et al., 2007). Neste processo de detoxificação o Se forma um complexo com o Hg, o SeHg, que parece ser metabolicamente inerte (Skerfving, 1978, Raymond and Ralston, 2004).

Além disso, outro composto de selênio, o difenil disseleneto ($(PhSe)_2$), tem se demonstrado eficaz em proteger contra alguns efeitos tóxicos induzidos pelo MeHg (de Freitas et al., 2009). A capacidade antioxidante deste composto já foi demonstrado por alguns pesquisadores (Ghisleni et al., 2003, Burger et al., 2004, Posser et al., 2006, Luchese et al., 2007a, Luchese et al., 2007b, Posser et al., 2008), enquanto outros tem demonstrado que o $(PhSe)_2$ possui efeitos anti-úlcera (Savegnago et al., 2007), antiinflamatório e antinociceptivo (Nogueira, 2003, Zasso, 2005), e hepatoprotetor (Borges et al., 2005, Borges et al., 2006), entre outros.

Assim sendo, o objetivo deste estudo foi também investigar o possível efeito protetor de dois compostos de selênio, difenil disseleneto ($(PhSe)_2$) e selenito de sódio (Na_2SeO_3), sobre a toxicidade induzida pelo MeHg.

1.3 Neurotoxicidade induzida por toxicantes endógenos

1.3.1 Erros inatos do metabolismo

Os erros inatos do metabolismo (EIM) são alterações genéticas que se traduzem na ausência ou na síntese anormal, qualitativa ou quantitativa, de uma proteína, geralmente uma enzima. A ausência ou deficiência severa na atividade enzimática leva a um bloqueio metabólico com repercussão clínica variável no organismo, dependendo principalmente da rota metabólica afetada (Chalmers et al., 1980, Ozand and Gascon, 1991a, b, Gascon et al., 1994, Vilaseca-Busca et al., 2002). O bloqueio da rota metabólica levará ao acúmulo de precursores da reação catalisada pela enzima envolvida com a formação de rotas

metabólicas alternativas, e à deficiência de produtos essenciais ao organismo (Bickel, 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, a incidência cumulativa dos EIM é de aproximadamente um a cada 2.000 recém-nascidos vivos (Baric et al., 2001).

1.3.2 Acidemias orgânicas

Acidemias orgânicas são distúrbios hereditários do metabolismo de aminoácidos, glicídios ou lipídios, causados pela deficiência na atividade de uma enzima e caracterizados bioquimicamente pelo acúmulo de um ou mais ácidos orgânicos e/ou derivados nos tecidos e líquidos biológicos dos indivíduos afetados (Chalmers and Lawson, 1982, Scriver et al., 2001, Cornejo and Raimann, 2003). A freqüência destas doenças na população em geral é pouco conhecida, o que pode ser creditado à falta de laboratórios especializados para o seu diagnóstico e ao desconhecimento médico sobre essas enfermidades. Na Holanda, país considerado como referência para o diagnóstico de EIM, a incidência destas doenças é estimada em 1: 2.200 recém-nascidos vivos (Hoffmann et al., 2004) e na Arábia Saudita, onde a taxa de consangüinidade é elevada, a freqüência aumenta para 1: 740 recém-nascidos vivos (Rashed et al., 1994). No Brasil, tem sido estimada a incidência de algumas patologias isoladas como a da Fenilcetonúria de 1:12.000, da Doença do Xarope do Bordo de 1:43.000 e da deficiência de biotinidase de 1:125.000 em recém-nascidos vivos (Wajner et al., 2002).

Clinicamente, os pacientes afetados por acidemias orgânicas apresentam predominantemente disfunção neurológica em suas mais variadas formas de expressão: regressão neurológica, convulsões, coma, ataxia, hipertonia, irritabilidade, tremores, movimentos coreoatetóticos, tetraparesia espástica, atraso no desenvolvimento psicomotor, retardo mental, etc. As manifestações laboratorias mais frequentes incluem cheiro peculiar na urina e/ou suor, cetose, cetonúria, acidose metabólica, hipo/hiperglicemia, hiperamonemia entre outros (Scriver et al., 2001).

1.3.3 Doença do xarope do Bordo ou cetoacidúria de cadeia ramificada

A doença do xarope do bordo (DXB), acidúria orgânica de herança autossômica recessiva, é um erro inato do catabolismo dos aminoácidos de cadeia ramificada (AACR), leucina, isoleucina e valina, causado pela deficiência dos componentes catalíticos do complexo enzimático da desidrogenase dos α-ceto-ácidos de cadeia ramificada (ACCR) (Figura 3). Como conseqüência deste bloqueio metabólico ocorre o acúmulo dos AACR, bem como dos seus respectivos ACCR, α-cetoisocapróico, α-ceto-β-metilvalérico e α-cetoisovalérico, e dos α-hidroxiácidos de cadeia ramificada, α-hidroxiisocapróico, α-hidroxi-β-metilvalérico e α-hidroxiisovalérico (Chuang et al., 2001).

Os AACR compreendem em torno de 40% dos aminoácidos da dieta, e o principal destino deles é a incorporação em proteínas corporais (Schadewaldt and Wendel, 1997). O catabolismo normal dos AACR inicia com o seu transporte do sangue para a célula através do sistema L. Dentro da célula, os AACR sofrem transaminação reversível originando os ACCR que são posteriormente translocados para dentro da mitocôndria, local onde sofrem descarboxilação oxidativa, reação catalisada pelo complexo enzimático da desidrogenase dos AACR. Os AACR são tanto cetogênicos quanto glicogênicos e servem como precursores para a síntese de ácidos graxos e colesterol ou como substrato para a produção mitocondrial de energia (Chuang et al., 2001).

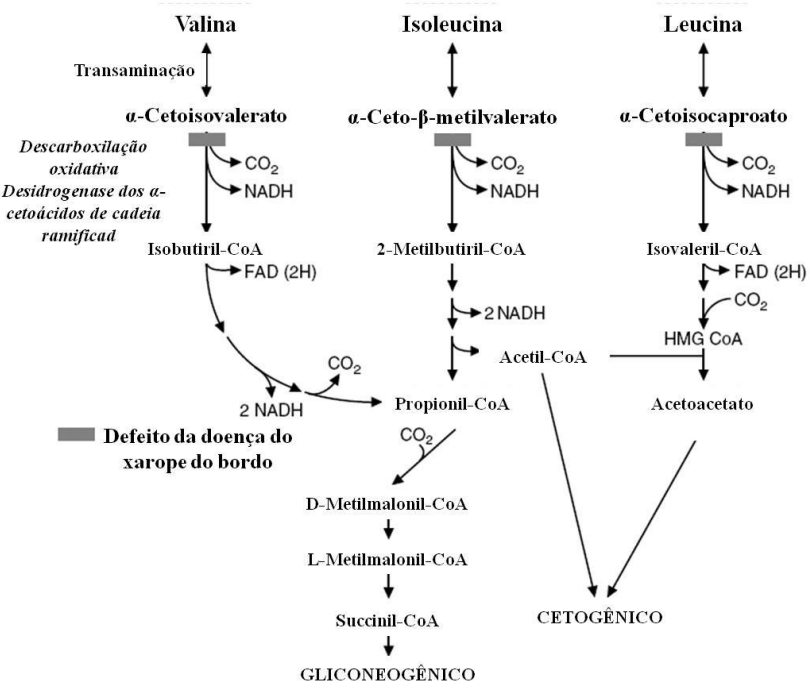

Figura 3. Rota metabólica dos aminoácidos de cadeia ramificada leucina, isoleucina e valina. A seta indica o bloqueio metabólico que ocorre na doença do xarope de bordo (Adaptado de Scriver et al., 2001).

O diagnóstico da DXB é fundamentalmente laboratorial. A identificação de concentrações plasmáticas e urinárias elevadas dos AACR e de seus respectivos ACCR, através de cromatografia para aminoácidos e para ácidos orgânicos, respectivamente, caracteriza a doença. No entanto, a leucina é o principal metabólito acumulado na DXB, atingindo concentrações plasmáticas de até 5 mM, enquanto que os outros AACR não superam 1 mM (Chuang et al., 2001). A confirmação do diagnóstico é feita através da medida da atividade do complexo da desidrogenase dos AACR em cultura de leucócitos periféricos ou de fibroblastos (Peinemann and Danner, 1994). O

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

Os pacientes podem ser classificados em cinco fenótipos diferentes dependendo da apresentação clínica, da tolerância à leucina e da atividade residual do complexo da desidrogenase dos ACCR (Schadewaldt et al., 1998). A forma mais freqüente e também a mais severa está representada pela variante clássica. Esta forma é comumente manifestada no período neonatal, enquanto que as demais formas da doença usualmente ocorrem poucos meses após o nascimento. Ela causa um desenlace fatal em um considerável número de pacientes durante os primeiros meses de vida, se não diagnosticado e tratado prontamente, e os que sobrevivem apresentam um variável grau de retardo metal (Peinemann and Danner, 1994, Chuang et al., 2001). Os recém-nascidos afetados parecem normais ao nascimento e os sintomas começam a se desenvolver entre os 4-7 dias após o nascimento. Letargia e perda do apetite são os primeiros sintomas, seguidos por perda de peso e alteração progressiva dos sinais neurológicos; cetoacidose e odor de açúcar queimado são também característicos (Nyhan, 1984). Os indivíduos afetados apresentam baixa densidade de substância branca, decorrente de hipomielinização/desmielinização e atrofia cerebral (Chuang et al., 2001) e, além disso, geralmente observa-se edema cerebral durante as crises metabólicas agudas (Riviello et al., 1991, McDonald and Schoepp, 1993). O trato piramidal do cordão espinhal e o conteúdo de mielina em torno do núcleo denteado, o corpo caloso e os hemisférios cerebrais são os principais afetados (Chuang et al., 2001).

O tratamento dos pacientes com DXB consiste na restrição dos AACR com o objetivo de normalizar as concentrações plasmáticas destes aminoácidos sem prejudicar o crescimento e desenvolvimento destes. Para tanto, administra-se principalmente um leite especial com concentrações reduzidas em AACR (Snyderman et al., 1964). Na fase aguda, emprega-se um tratamento mais agressivo, pois o aumento dos AACR e ACCR, freqüentemente precipitados por infecções, leva à deterioração das funções cerebrais. Existem três medidas a serem tomadas para o controle das crises metabólicas: remover os toxicantes endógenos, promover suporte nutricional adequado e minimizar o catabolismo e/ou promover o anabolismo (Chuang et al., 2001).

Os mecanismos pelos quais os o acúmulo dos AACR e ACCR resultam tóxicos sobre o sistema nervoso central ainda não estão completamente estabelecidos. A leucina, e seu derivado α-cetoisocapróico, são considerados os principais metabólitos neurotóxicos na DXB (Snyderman et al., 1964, Efron, 1965, Chuang et al., 2001). Pesquisadores brasileiros pioneiros no estudo da fisiopatologia das alterações do sistema nervoso central nas acidemias orgânicas demonstraram que o aumento das concentrações plasmáticas de leucina provoca uma diminuição na captação de aminoácidos essenciais pelo sistema nervoso central, tendo como conseqüência principal a redução na síntese de neurotransmissores (Wajner and Vargas, 1999, Wajner et al., 2000, Araujo et al., 2001). Outros grupos de pesquisa demonstraram que o α-cetoisocapróico aumenta a taxa de oxidação do glutamato com posterior formação de α-cetoglutarato, levando a uma queda de 50% das concentrações deste neurotransmissor (Yudkoff et al., 1994, Yudkoff et al., 1996, Zielke et al., 1997, Yudkoff et al., 2005b).

Por outro lado, estudos em modelos animais da DXB e estudos *in vitro* empregando tecido cerebral têm demonstrado que os AACR, bem como os ACCR e os derivado hidroxilados, induzem estresse oxidativo por aumentar a oxidação dos lipídios e por reduzir as defesas antioxidantes não-enzimáticas (Fontella et al., 2002, Bridi et al., 2003, Bridi et al., 2006). Ainda, estudos em homogeneizado de cérebro de roedores ou cultura de células nervosas expostas ao aminoácido tem demonstrado alterações no metabolismo energético representados por inibição da atividade da enzima creatina cinase, redução da oxidação e do transporte mitocondrial de piruvato, inibição das atividades do complexo piruvato desidrogenase, da enzima α-cetoglutarato desidrogenase e dos complexos da cadeia respiratória (Land et al., 1976, Danner et al., 1989, Pilla et al., 2003a, Pilla et al., 2003b, Sgaravatti et al., 2003, Ribeiro et al., 2008), indução de apoptose neuronal e glial (Jouvet et al., 2000), e mudanças na morfologia dos astrócitos e reorganização do citoesqueleto, levando à morte celular (Funchal et al., 2002, Funchal et al., 2004, Funchal et al., 2006).

Sabe-se que a administração subcutânea e intrahipocampal dos α-cetoácidos acumulados na DXB provocam déficits na aprendizagem em testes comportamentais aversivos e não-aversivos, implicando que eles provavelmente provocam alterações bioquímicas no cérebro, envolvidos

nos processos de aprendizagem (Mello et al., 1999, Vasques Vde et al., 2005). Além disso, as propriedades convulsionantes do ácido α-cetoisovalérico foram demonstrados, sugerindo que este metabólito está provavelmente envolvido na gênese das convulsões características dos pacientes com esta doença (Coitinho et al., 2001).

Como mencionado anteriormente, a dieta restritiva de AACR tem sido o principal alvo para tratar os pacientes acometidos pela DXB. Embora isto tenha contribuído para a sobrevivência dos indivíduos afetados, um número considerável de pacientes "tratados" apresenta um variável grau de retardo mental acompanhado por mudanças crônicas nas estruturas cerebrais, indicando a necessidade do conhecimento mais detalhado da fisiopatologia das alterações neurológicas para que terapias possam ser desenvolvidas.

2. OBJETIVOS

2.1. Objetivo geral

O objetivo geral do presente trabalho visa o melhor entendimento dos mecanismos patogênicos responsáveis da neurotoxicidade induzida pela exposição a toxicantes exógenos e endógenos, principalmente em nível mitocondrial, em cérebro de roedores; visto que existe uma grande evidência na literatura que demonstra que a gênese dos processos neurodegenerativos está intimamente relacionado com deficiências na produção energética mitocondrial.

2.2. Objetivos específicos

Caracterizar o efeito neurotóxico do contaminante ambiental MeHg e de concentrações tóxicas do aminoácido de cadeia ramificada, leucina, na ausência ou presença de compostos de selênio como substâncias potencialmente neuroprotetoras em roedores, através da realização dos seguintes objetivos específicos:

a) Investigar o efeito da administração oral e crônica de diferentes doses de MeHg sobre a atividade dos complexos da cadeia respiratória e sobre a morfologia mitocondrial (microscopia eletrônica) em córtex cerebral de camundongos Swiss adultos.

b) Investigar o efeito da administração oral e crônica de MeHg, bem como da co-administração deste mercurial e de compostos de selênio, $(PhSe)_2$ e Na_2SeO_3, sobre parâmetros de metabolismo energético e estresse oxidativo por técnicas bioquímicas e histológicas em córtex cerebral de camundongos Swiss adultos.

c) Investigar se a co-administração oral e crônica de MeHg e de compostos de selênio, $(PhSe)_2$ e Na_2SeO_3, protege da deposição do mercurial no cérebro.
d) Investigar se a co-administração oral e crônica de MeHg e de compostos de selênio, $(PhSe)_2$ e Na_2SeO_3, protege da neurodegeneração induzida pelo mercurial.
e) Investigar os mecanismos moleculares envolvidos nas eventuais alterações energéticas cerebrais observadas após a intoxicação com MeHg em cultura de células C6 de glioma e homogenatos preparados a partir de córtex cerebral de camundongos Swiss adultos.
h) Investigar o efeito da administração aguda intrahipocampal da leucina sobre a geração de memória através de parâmetros comportamentais e eletrofisiológicos em ratos Wistar adultos.
i) Investigar o efeito da administração aguda intrahipocampal da leucina sobre as atividades dos complexos da cadeia respiratória mitocondrial em ratos Wistar adultos.

3. JUSTIFICATIVA

As doenças neurodegenerativas representam um problema de saúde bastante desafiador para a sociedade, sendo responsável por um grande número de hospitalizações e incapacidades que resultam em prejuízos econômicos e elevados riscos de suicídio. Várias décadas de pesquisa científica permitiram o conhecimento de que algumas doenças mentais resultam de uma combinação de fatores genéticos e ambientais.

Atualmente, graças a estudos interdisciplinares de especialistas em epidemiologia, biologia molecular, neurocientistas, biólogos, bioquímicos, etc, os mecanismos de neurotoxicidade começam a ficar mais claros, no entanto, ainda nos encontramos longe de conseguir instaurar tratamentos eficazes. Neste contexto, o presente estudo pretende contribuir para a geração de conhecimento neste tema a partir da investigação dos mecanismos de neurotoxicidade em dois modelos de doenças neurodegenerativas, na neurotoxicidade induzida pela exposição ao contaminante ambiental MeHg, e no erro inato do metabolismo, a DXB. Assim, o melhor entendimento dos mecanismos moleculares envolvidos na toxicidade neuronal induzida por toxicantes endógenos e exógenos representará um avanço para a descoberta de estratégicas terapêuticas eficazes que consigam prevenir ou atenuar as severas manifestações neurológicas de patologias neurodegenerativas

crônicas bem como de processos de exposição humana e animal aos toxicantes ambientais.

4. MATERIAIS, DESENHO EXPERIMENTAL E MÉTODOS

4.1. Experimentos in vivo com MeHg

4.1.1. Reagentes

Todos os reagentes utilizados foram de grau de pureza PA.

4.1.2. Animais

Foram utilizados camundongos Swiss albinos machos de 60 dias de vida provindos do Biotério Central da Universidade Federal de Santa Catarina. Os animais foram aclimatados no Biotério Setorial do Laboratório de Bioenergética e Estresse Oxidativo (N° cadastro BIO040), com temperatura controlada 23 ± 1º C, com ciclo claro/escuro de 12 horas. Todos os procedimentos foram executados de acordo com o "Guia de Princípios para o uso de Animais em Toxicologia" adotado pela sociedade de toxicologia em Julho de 1989. Todos os experimentos foram aprovados pelo Comitê de Ética para o uso de Animais – CEUA, da Universidade Federal de Santa Catarina (PP00084/CEUA).

4.1.3. Exposição crônica ao MeHg

O modelo experimental de intoxicação com MeHg empregado neste trabalho foi baseado em estudos prévios do nosso grupo de pesquisa que demonstraram que a administração oral e crônica de soluções de MeHg de 20 e 40 mg/L provoca severas alterações comportamentais (coordenação motora) (Farina et al., 2003a, Dietrich et al., 2005). Além disso, o modelo induz a deposição do mercurial no cérebro, atingindo concentrações de 3 – 5 $\mu g \cdot g^{-1}$ tecido (3 – 5 ppm), as que poderiam ser traduzidas em 15 – 30 µM (Franco et al., 2009).

Para a realização deste estudo foram empregados 18 animais divididos e tratados da seguinte forma durante 21 dias:

I- Grupo Controle: os animais beberam água *ad libitum*;

II- Grupo MeHg dose baixa: os animais beberam água *ad libitum* contaminada com MeHg na concentração de 20 mg/L;

III- Grupo MeHg dose alta: os animais beberam água *ad libitum* contaminada com MeHg na concentração de 40 mg/L.

4.1.4. Exposição crônica ao MeHg e compostos antioxidantes de selênio

Para a realização deste protocolo 72 animais foram divididos e tratados da seguinte forma durante 21 dias:

I- Grupo Controle: os animais beberam água *ad libitum* e receberam injeções subcutâneas diárias de salina e dimetil sulfóxido (DMSO; 1mg/Kg);

II- Grupo Na_2SeO_3 (Figura 5): os animais beberam água *ad libitum* e receberam injeções subcutâneas diárias de Na_2SeO_3 (5µmol/kg diluído em salina; (Yamamoto, 1985);

III- Grupo $(PhSe)_2$ (Figura 6): os animais beberam água *ad libitum* e receberam injeções subcutâneas diárias de $(PhSe)_2$ (5µmol/kg diluído em DMSO) (Burger et al., 2006, de Freitas et al., 2009);

IV- Grupo MeHg: os animais beberam água contaminada com MeHg (40 mg/L diluído em água) *ad libitum* e receberam injeções subcutâneas diárias de salina e DMSO;

V- Grupo Na_2SeO_3 + MeHg: os animais beberam água contaminada com MeHg (40 mg/L diluído em água) *ad libitum* e receberam injeções subcutâneas diárias de Na_2SeO_3 (5µmol/kg diluído em salina);

VI- Grupo $(PhSe)_2$ + MeHg: os animais beberam água contaminada com MeHg (40 mg/L diluído em água) *ad libitum* e receberam injeções subcutâneas diárias de $(PhSe)_2$ (5µmol/kg diluído em DMSO).

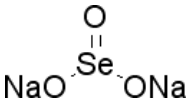

Figura 4. Estrutura química do Na_2SeO

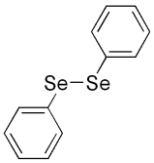

Figura 5. Estrutura química do $(PhSe)_2$

Após aplicados os tratamentos, e 24 horas após a última injeção, os animais foram submetidos à eutanásia e tiveram seu cérebro

removido, e o córtex cerebral foi dissecado para as diferentes análises bioquímicas e histológicas.

4.1.5. Preparação das amostras para análise dos parâmetros bioquímicos

O córtex cerebral foi homogeneizado em cinco volumes de tampão fosfato de sódio 20 mM, pH 7,4, contendo cloreto de potássio 140 mM. Posteriormente, o homogeneizado foi centrifugado a 1.000 x *g* durante 10 minutos a 4 °C. O sobrenadante foi retirado e acondicionado em eppendorfs e utilizado para análises referentes a estresse oxidativo (Latini et al., 2007).

Para a mensuração da atividade dos complexos da cadeia respiratória, o córtex cerebral foi homogeneizado em 20 volumes de tampão fosfato de potássio 5 mM, pH 7,4, contendo sacarose 300 mM, MOPS 5 mM, EGTA 1 mM e albumina sérica bovina 0,1%. Posteriormente, o homogeneizado foi centrifugado a 1.000 x *g* durante 10 minutos a 4 °C. Parte do sobrenadante foi aliquotado para a determinação da atividade da piruvato cinase e o restante foi novamente centrifugado a 15.000 x *g* durante 10 minutos a 4 °C. O sobrenadante foi descartado e o pellet foi suspendido no mesmo tampão usado no processo de homogeneização numa concentração protéica de aproximadamente 20 mg/mL. Esta preparação mitocondrial foi empregada para a determinação das atividades dos complexos da cadeia respiratória. Para a mensuração das atividades da creatina cinase e da adenilato cinase esta fração mitocondrial foi lavada duas vezes com tampão Tris 10 mM, pH 7,5, contendo sacarose 0,25 M e posteriormente suspendida em tampão Tris 100 mM, pH 7,5 contendo $MgSO_4$ 9 mM (Latini et al., 2005).

4.1.6. Preparação do tecido para análise de parâmetros histológicos

Após o término dos diferentes tratamentos *in vivo*, os animais foram perfundidos com solução de para-formaldeído 4%. Posteriormente, o cérebro foi removido, imediatamente imerso nesta solução por 24 horas (processo de fixação), e desidratado em série alcoólica crescente (1 hora em cada solução alcoólica: 70%, 80%, 90% e 100%, este último por duas vezes). Posteriormente, as peças foram imersas em solução alcoólica contendo xilol durante vinte minutos, diafanizadas em xilol e incluídas em parafina em moldes apropriados.

Após solidificação, os blocos de parafina foram removidos dos moldes, aparados e acoplados ao micrótomo rotativo. Os cortes foram realizados na espessura de 6µM.

Para a investigação da deposição do mercurial foi empregado o método da autometalografia (Hfreere and Weibel, 1967, Danscher, 1984, Pedersen et al., 1999). Neste caso, o tecido cerebral foi imerso em solução de Carnoy-sulfeto, ao invés de paraformaldeído 4%, e permaneceram nesta solução por 24 horas. Após, as peças foram colocadas imediatamente em álcool 100% por 48 horas (Santos, 1999), sendo os passos subseqüentes idênticos aos descritos acima.

As análises histológicas foram realizadas em microscópio Olympus modelo BX41 e para as fotografias foi utilizado o sistema de captura de imagens Q-capture Pro 5.1. Para autometalografia, a quantificação das células marcadas foi realizada pelo método de estereologia, em objetiva de imersão (aumento de 1000x), com auxílio da gratícula de Weibel (Weibel Graticule Nº2), em 5-8 campos aleatórios (Hfreere and Weibel, 1967). A análise da marcação imunohistoquímica para dano oxidativo, utilizando anticorpo anti-8-hidroxi-2'-deoxiguanosina (JaICA®, Shizuoka, Japão), também foi realizada em 5-8 campos aleatórios, através da análise de densidade óptica, utilizando-se o software NIH ImageJ, e os dados foram expressos através da média de densidade óptica.

Para a marcação de FluoroJade B (Chemicon International®, Temecula, USA), utilizou-se o microscópio Eclipse modelo 50i com análise de fluorescência em campo escuro (filtro B-2A para FITC, banda de excitação 450-490 nm). As imagens foram realizadas com uma câmera digital (DS-5M-L1; Nikon).

4.1.7. Preparação do tecido para análise da morfologia mitocondrial por microscopia eletrônica

Depois de realizada a perfusão como indicado no item 3.1.6, seções de córtex frontal de 1 x 1 mm foram imersas em solução de glutaraldeído 2,5% e paraformaldeído 2% contendo cacodilato 0,1 M e cloreto de cálcio 0,05%. O material permaneceu nesta solução durante quatro horas a 4 º C e posteriormente foram submetidas a três lavagens de 30 minutos em tampão cacodilato 0,1 M pH 7,4. Em seguida, as peças foram colocadas em tampão cacodilato contendo tetróxido de ósmio 1% por duas horas a 4ºC, e foram novamente lavadas em tampão

cacodilato. O material foi posteriormente desidratado em concentrações crescentes de acetona (30; 50; 10; 90 e 2 vezes em 100% durante 20 minutos) e imerso em solução de acetona e resina Spurr (2:1; 1:1 e 1:2). Finalmente, as peças foram tratadas com resina pura para posterior microtomia, a qual foi realizada em ultramicrótomo na espessura de 60 – 70 nm (Hernandez-Fonseca et al., 2009).

As análises de morfologia mitocondrial foram realizadas em microscópio eletrônico de transmissão JEM-101 (Laboratório Central de Microscopia Eletrônica da UFSC) e para as fotografias foi utilizado o sistema de captura de imagens Gatan Digital Micrograph.

4.2. Experimentos in vitro com MeHg

Os experimentos *in vitro* com MeHg envolveram a exposição de homogenatos corticais de camundongos Swiss machos de 60 dias de vida e células C6 de astroglioma com concentrações crescentes do mercurial (0 – 1,5 mM) durante 15 ou 60 minutos a 37°C (Latini et al., 2005, Funchal et al., 2006). Para isto, quatro a seis animais foram submetidos à eutanásia e tiveram o cérebro removido para posterior dissecação do córtex cerebral.

4.2.1. Preparação dos homogeneizados corticais para determinação de parâmetros bioquímicos

Para a determinação da atividade da enzima creatina cinase total (mitocondrial + citosólica), o córtex cerebral foi homogeneizado em 20 volumes de tampão fosfato de potássio 5 mM, pH 7,4, contendo sacarose 300 mM, MOPS 5 mM, EGTA 1 mM e albumina sérica bovina 0,1 %. Posteriormente, o homogeneizado foi centrifugado a 1.000 x *g* durante 10 minutos a 4°C (Ribeiro et al., 2006).

Para a determinação dos parâmetros de estresse oxidativo, o córtex cerebral foi homogeneizado em cinco volumes de tampão fosfato de sódio 20 mM, pH 7,4, contendo cloreto de potássio 140 mM nas condições acima mencionadas (Latini et al., 2007).

4.2.2. Manutenção da linhagem celular de astroglioma C6

A linhagem celular de astroglioma C6 foi obtida de American Type Culture Collection (Rockville, Maryland, EUA). Estas células adotam características de astrócitos (células 99% GFAP positivas; Figura 6) e tem sido empregada como modelo experimental para o

estudo do efeito *in vitro* de numerosos toxicantes (Haghighat e McCandless, 1997, Haghighat et al., 2000). As células foram semeadas em frascos e cultivadas em meio Eagle's com modificação de Dubelcco (DMEM) contendo 2,5 mg / mL de Fungizone® e 100 U / L de gentamicina, suplementadas com 5 % ($^{V}/_{V}$) de soro fetal bovino (SFB), e mantidas a 37 ° C com um mínimo de 95 % de umidade relativa e em uma atmosfera de ar com 5 % de CO_2. Posteriormente, as células foram tratadas com 0.05 % de tripsina / ácido etileno-diaminotetracético (EDTA) e semeadas em placas de 24 poços (10 x 10^3 células / poço). Após confluência, o meio foi trocado por DMEM livre de SFB e contendo o mercurial nas diferentes concentrações (0 – 1,5 mM).

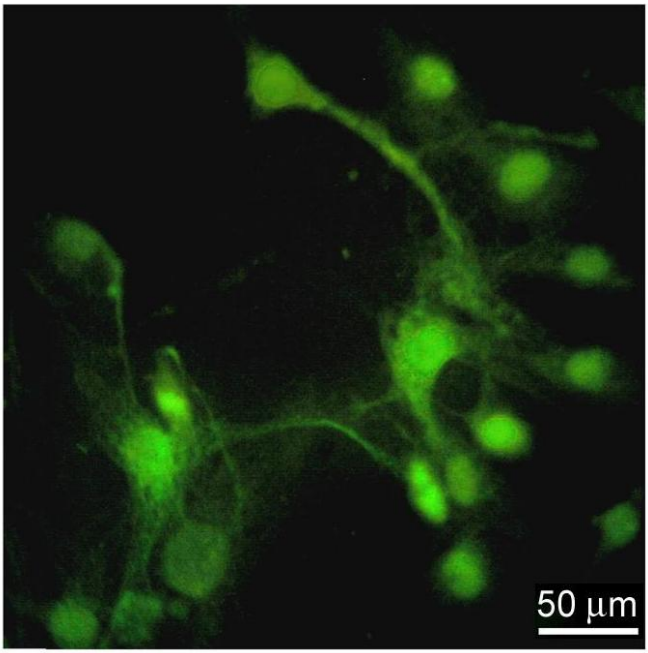

Figura 6. Imagem ilustrativa da morfologia da linhagem de células C6 de glioma, positiva para a proteína ácida fibrilar glial (GFAP). A expressão de GFAP indica que a linhagem utilizada apresenta características de astrócitos.

4.3. Experimentos in vivo com Leucina

4.3.1. Reagentes

Todos os reagentes utilizados foram de grau de pureza PA.

4.3.2. Animais

Foram utilizados 26 ratos Wistar machos de 60 dias de vida pesando entre 270 – 300g provenientes do biotério do departamento de Farmacologia da Faculdade de Ciências Químicas da *Universidad Nacional de Córdoba*, Córdoba, Argentina. Os animais foram mantidos em sala com temperatura controlada 23 ± 1º C e com ciclo claro/escuro de 12 horas, e com livre acesso a água e comida. Os animais foram aclimatados diariamente durante uma semana, antes dos experimentos. Todos os procedimentos foram aprovados pelos comitês de ética para o uso e cuidado de animais na pesquisa da Faculdade de Ciências Químicas da *Universidad Nacional de Córdoba*, Córdoba, Argentina, e da Universidade Federal de Santa Catarina, Florianópolis, Brasil (CEUA; Protocolo N° PP00121).

4.3.3. Estereotaxia – Implantação de cânulas para injeção intrahipocampal de leucina

Os animais foram anestesiados com solução de ketamina (55mg/Kg) e xilazina (11mg/kg) e colocados em um aparato de estereotaxia (Insight Equipamentos®). As cânulas guia foram implantadas bilateralmente na região CA1 do hipocampo, seguindo as coordenadas descritas no atlas de Paxinos (Paxinos and Watson, 1982). As coordenadas relativas ao bregma foram: anterior: −3.6 mm; lateral: ±2.0 mm; vertical: −2.8 mm para região for CA1 do hipocampo. As cânulas guia foram fixadas ao crânio com cimento acrílico dental. Os animais passaram por um período de recuperação da cirurgia de sete dias e posteriormente foram injetados bilateralmente com solução de leucina ou com líquido cérebro-espinhal artificial (animais controle). O volume total injetado por cânula foi de 1 uL (80 nmol de leucina / hipocampo; concentração final aproximada de 1,5 mM no hipocampo). Para as injeções intrahipocampais foi utilizada uma seringa Hamilton de 10 uL conectada a um tubo de polietileno que no seu extremo continha uma agulha com tamanho semelhante ao da cânula guia. Cada infusão foi realizada num período de 1 minuto (Carlini et al., 2007). Para os

estudos comportamentais, eletrofisiológicos e bioquímicos foram empregados animais com cânulas corretamente implantadas (observado através de corte histológico do cérebro).

O modelo de administração intrahipocampal de leucina foi baseado em estudos prévios que demonstraram que a administração do principal metabólito de leucina (α-cetoisocapróico) provoca severas alterações comportamentais e bioquímicas similares aos pacientes afetados pela DXB (Mello et al., 1999, Vasques Vde et al., 2005).

4.3.4. Preparação do tecido hipocampal para o estudo dos parâmetros eletrofisiológicos

Vinte e quatro horas após a administração intrahipocampal de leucina foi realizado o teste comportamental e logo após os animais foram submetidos à eutanásia e tiveram o cérebro removido e o hipocampo dissecado. O hipocampo foi fatiado (400 µm) em fatiador de tecidos (McIlwain, Brinkmann Instruments), conforme descrito por Perez e colaboradores (Perez et al., 2002). Posteriormente, as fatias foram estabilizadas em meio contendo solução de Krebs ($NaHCO_3$ 25,6 mM, glicose 10,4 mM, $CaCl_2$. $2H_2O$ 2,3 mM, NaCl 124,3 mM, KCl 4,9 mM, $MgSO_4$. $7H_2O$ 1,3 mM, KH_2PO_4 1,25 mM) saturada com 95% oxigênio e 5% de dióxido de carbono por aproximadamente três horas.

4.3.5. Preparação do tecido hipocampal para a determinação de parâmetros bioquímicos

Um dia após a administração intrahipocampal de leucina foi realizado o teste comportamental e logo após os animais foram submetidos à eutanásia e tiveram o cérebro removido e o hipocampo dissecado. A preparação dos homogeneizados para a determinação das atividades dos complexos da cadeia respiratória foi realizada como indicado no item *4.1.5.*

5. RESULTADOS

O presente trabalho resultou na confecção de quatro manuscritos, todos submetidos à publicação em periódicos científicos e listados abaixo:

Manuscrito 1: "***Diphenyl diselenide prevents the energy impairment induced by methylmercury poisoning in adult mice brain***", sera submetido à "Toxicological Science".
Manuscrito 2: "***Effects of inorganic selenium administration in methylmercury-induced neurotoxicity in mice***", submetido à "Chemico-Biological Interactions".
Manuscrito 3: "***Oxidative stress-mediated inhibition of brain creatine kinase activity by methylmercury***", submetido à "NeuroToxicology".
Manuscrito 4: "***The intra-hippocampal leucine administration impairs memory consolidation and LTP generation in rats***", submetido à "Cellular and Molecular Neurobiology".

Além disso, resultados adicionais são demonstrados nesta sessão.

Manuscrito 1: "***Diphenyl diselenide prevents the energy impairment induced by methylmercury poisoning in adult mice brain***", será submetido ao periódico "Toxicological Sciences".

Methylmercury-induced brain toxicity is prevented by diphenyl diselenide administration

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Short title: MeHg-induced neurotoxicity is prevented by diphenyl diselenide

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Abstract

Methylmercury (MeHg) is a well-known neurotoxicant and a common form of mercury in the environment. The effect of chronic MeHg exposure on some parameters of energy metabolism in cortical preparations from the mouse brain was investigated. Oral and chronic MeHg administration elicited a marked inhibition of complexes I-III of the respiratory chain in cortical homogenates. This inhibitory effect was further studied in mitochondrial preparations, where the mercurial also inhibited the activity of complexes I-IV. Moreover, the activity of mitochondrial creatine kinase (mCK), an oxidative-stress-susceptible enzyme, was almost abolished after MeHg exposure. In order to prevent these energy impairments, diphenyl diselenide $(PhSe)_2$, a potential neuroprotectant, was subcutaneously and daily administered. $(PhSe)_2$ co-administration significantly counteracted the energy impairment induced by the compound. Some parameters of oxidative stress, namely glutathione peroxidase (GPx) and glutathione reductase (GR) activities and thiobarbituric acid-reactive substances (TBA-RS), as well as, fluorojade B labelling (neurodegeneration), DNA oxidation and metal deposition, were also assessed. Although $(PhSe)_2$ was able to counteract the MeHg-increased TBA-RS levels, the cortical MeHg-induced reduction of GPx and increased GR activities were not modified. Furthermore, MeHg exposure induced neuronal death, DNA oxidation and positive labelled cells for metal brain deposition, which were significantly prevented by $(PhSe)_2$ administration. Together, these data strongly indicate that brain energy metabolism impairment is involved in MeHg neurotoxicity, that mCK is a MeHg-sensitive target and that both mechanisms are probably related to oxidative stress induction, DNA oxidation and to neurodegeneration. Therefore, it is feasible to consider $(PhSe)_2$ as a neuroprotectant in MeHg-induced poisoning.

Keywords: neurotoxicity, respiratory chain complexes, creatine kinase, diphenyl diselenide, methylmercury

Introduction

Methylmercury (MeHg), an organic form of mercury, causes severe and irreversible neurobehavioral and neuropsychological disorders in both humans and animals (Choi, 1989, Gilbert and Grant-Webster, 1995, Rice and Barone, 2000, Clarkson, 2002, Clarkson et al., 2003). MeHg is almost completely absorbed in the human gastrointestinal tract, forms a water-soluble complex, mainly with the sulfur atom of thiol ligands, and easily crosses the blood-brain barrier, complexing L-cysteine (Clarkson, 2002, Yin et al., 2008). Because of differences in toxicokinetics and toxicodynamics, MeHg after being absorbed into the placenta is stored at higher concentrations in the fetal brain than those found in maternal blood (Inouye et al., 1986, Cernichiari et al., 2007). Although all organs are exposed to high levels of MeHg upon intoxication, the most vulnerable target is the central nervous system (Clarkson, 2002). MeHg exposure induces demyelinization, autonomic dysfunction, sensory nerve conduction delay, abnormal neuron migration, and abnormal central nervous system cell division (Sanfeliu et al., 2003, Spurgeon, 2006). Chronic toxicity symptoms include paresthesia; peripheral neuropathy; cerebellar ataxia; akathisia; spasticity; memory loss; dementia; constricted vision; dysarthia; impaired hearing, smell, and taste; tremors; and depression (Choi, 1989, Gilbert and Grant-Webster, 1995, Rice and Barone, 2000, Clarkson, 2002, Clarkson et al., 2003, Spurgeon, 2006). In this context, *postmortem* studies have reported several areas of brain damage from MeHg exposure, mainly represented by pathological changes in the cerebrum and cerebellum (Eto et al., 2007).

The mechanisms currently known to be involved in MeHg-induced neurotoxicity are mainly related to intracellular calcium impairment (Sirois and Atchison, 2000), oxidative stress, and the alteration of glutamate homeostasis (for a review, see (Aschner et al., 2007). All these toxic mechanisms appear to involve mitochondrial dysfunction, as well as, impairment of brain energy metabolism. In this scenario, it has recently been demonstrated that MeHg accumulates inside mitochondria and that brain mitochondria are more susceptible to MeHg-induced oxidative damage than the liver mitochondria (Mori et al., 2007). In addition, apoptosis under mitochondrial control has been shown to have an important role in the neuronal death process (Fiskum et al., 2003), including MeHg-induced neurodegeneration (Nishioku et

al., 2000, Belletti et al., 2002). In line with this, it has been also demonstrated that MeHg poisoning results in reduction of the mitochondrial transmembrane potential (Yin et al., 2007) and release of cytochrome c into the cytoplasm (Shenker et al., 2002) followed by caspase-3 activation (Belletti et al., 2002). It is not surprising that the effects of MeHg on mitochondria have been correlated with decreased ATP levels, suggesting the occurrence of an energy imbalance (Belletti et al., 2002). However, although several studies have reported mitochondrial dyshomeostasis after either *in vitro* (Dreiem et al., 2005, Dreiem and Seegal, 2007) or *in vivo* (Franco et al., 2006, Mori et al., 2007, Franco et al., 2009) exposure to MeHg, data on the molecular targets involved in the mitotoxic effects of this pollutant are scarce. This limited information led us to investigate in more detail the participation of brain energy metabolism in the deleterious effects induced by long-term MeHg exposure, with a particular emphasis on the activities of the respiratory chain complexes I-IV, adenylate kinase (AK), pyruvate kinase (PK), and mitochondrial creatine kinase (mCK) of brain preparations from the mouse cortex. Since the interaction between mercurials and selenium in the biological systems have reported to be extremely important (Parizek, 1978), indicating potential protective effects against mercury toxicity (Farina et al., 2003a, Farina et al., 2003b), these parameters were also studied in MeHg-exposed mice that also received repeated subcutaneous administration of a potential neuroprotective agent, diphenyl diselenide $(PhSe)_2$. In parallel, some parameters of oxidative stress, namely glutathione peroxidase (GPx) and glutathione reductase (GR) activities and thiobarbituric acid-reactive substances (TBA-RS) measurement, were also assessed in the brain of these animals. In order to follow neurodegeneration, fluorojade B cell labelling and DNA oxidation were assessed histologically. Finally, brain metal deposition was also investigated.

Material and methods

Animals and reagents. Male Swiss albino mice of 60 days of life obtained from the Central Animal House of the Centre for Biological Sciences, Universidade Federal de Santa Catarina, Florianópolis - SC, Brazil, were used. The animals were maintained on a 12-h light/dark cycle (lights on 07:00–19:00 h) in a constant temperature (22 °C ± 1 °C) colony room, with free access to water and protein commercial chow

(Nuvital-PR, Brazil). The experimental protocol was approved by the Ethics Committee for Animal Research (PP00084/CEUA) of the Universidade Federal de Santa Catarina, Florianópolis – SC, Brazil. The experiments were carried out in accordance with the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989. All efforts were made to minimize the number of animals used and their suffering.

All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO, USA) except methylmercury (II) chloride which was obtained from Aldrich Chemical Co. (Milwaukee, WI). Diphenyl diselenide $(PhSe)_2$ was prepared and characterized by our group as previously described (Paulmier, 1986). The chemical purity of $(PhSe)_2$ was determined by HPLC (99.9% of purity).

The biochemical measurements were performed in a Varian Cary 50 spectrophotometer (Varian Inc., Palo Alto, CA, USA) with temperature control. For brain tissue preparations an Eppendorf 5415 R (Eppendorf, Hamburg, Germany) centrifuge was used.

Treatments. The first experimental protocol was performed on 18 animas ramdomly divided into three experimental groups as follows: control group; MeHg low dose (20 mg . L^{-1}; 20 ppm); MeHg high dose (40 mg . L^{-1}; 40 ppm). The second protocol included additional treatments which involved the subcutaneus administration of $(PhSe)_2$. Therefore, 24 animals were divided into four experimental groups as follows: controls; MeHg (40 mg . L^{-1}); $(PhSe)_2$ (5 µmol . kg^{-1}) and MeHg plus $(PhSe)_2$.

MeHg was diluted in tap water, and was freely available. The doses administered are known to induced MeHg brain toxic concentrations of 3 – 5 µg . g^{-1} tissue (3 – 5 ppm) (Franco et al., 2006). $(PhSe)_2$ was dissolved in dimethylsulfoxide and subcutaneously administrated (Yamamoto, 1985, Burger et al., 2006). Control animals received vehicle injections (1 mL / kg body weight).

Tissue preparation. Animals were killed by decapitation without anesthesia 24 h after the last subcutaneous administration. The brain was rapidly excised on a Petri dish placed on ice and the cerebral cortex was dissected, weighed and kept chilled until homogenization which was performed using a ground glass type Potter-Elvejhem homogenizer. The maximum period between the tissue preparations and enzyme analysis was always less than a week.

Brain preparations for measuring the respiratory chain complex activities. Mouse cerebral cortex was homogenized in 20 volumes of phosphate buffer pH 7.4, containing 0.3 M sucrose, 5 mM MOPS, 1 mM EGTA and 0.1% bovine serum albumin (homogenization buffer). The homogenates were centrifuged at 1,000 x *g* for 10 min at 4 °C, the pellet was discarded and the supernatants were kept at -70 °C until enzyme activity determination.

Mitochondrial fractions from cerebral cortex were also prepared for the measurements. Briefly, the cerebral cortex was homogenized in 10 volumes of homogenization buffer and centrifuged at 1,500 x g for 10 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 15,000 x *g* in order to separate the mitochondrial fraction, which was finally dissolved in the same buffer (Latini et al., 2005). The supernatant was separate for further determinations.

Brain preparations for measuring the mitochondrial creatine kinase (mCK) and adenylate kinase (AK) activities. The mitochondrial fraction obtained for measuring the respiratory chain complex activities was washed twice with 10 mM Tris isotonic buffer containing 0.25 M sucrose and finally suspended in 100 mM MgSO4–Trizma buffer, pH 7.5.

Brain preparations for measuring the pyruvate kinase (PK) activity. The supernatant resulted after the isolation of the cortical mitochondrial fraction used for measuring the respiratory chain complex activities was used for the assay.

Brain preparations for measuring the oxidative stress parameters. Brain tissue was homogenized in 5 volumes (1:5, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4ºC to discard nuclei and cell debris. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and immediately used for the analyses (Latini et al., 2005).

Measurement of the respiratory chain enzyme activities. Complex I activity was measured by the rate of NADH-dependent ferricyanide reduction at 420 nm (1 mM^{-1} . cm^{-1}) as previously described (Cassina and Radi, 1996). The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate:cytochrome c oxidoreductase (complex II-CoQ-complex III) were determined

according to the method of Fischer et al. (Fischer et al., 1985) and that for cytochrome c oxidase (complex IV) activity according to Rustin et al. (Rustin et al., 1994). The methods described to measure these activities were slightly modified, as detailed in a previous report (Latini et al., 2005). The activities of the respiratory chain complexes were calculated as nmol/min/mg protein.

Measurement of kinases activities. mCK activity was assessed spectrophotometrically based on the creatine formation, which was quantified according to the colorimetric method of Hughes (Hughes, 1962). PK activity was assayed essentially as described by Leong et al. (Leong et al., 1981) and AK activity was measured as described by Dzeja et al. (Dzeja et al., 1999). Enzyme activities were expressed as nmol/min/mg protein.

Measurement of glutathione-related enzymes. GR activity was determined according to Carlberg and Mannervik (Carlberg and Mannervik, 1985). GPx activity was measured according to Wendel (Wendel, 1981). One GPx or GR unit (U) is defined as 1 µmol NADPH consumed per minute. The specific activity was calculated as U/mg protein.

Measurement of thiobarbituric acid-reactive substances (TBA-RS). TBA-RS was determined according to the method of Esterbauer and Cheeseman (Esterbauer and Cheeseman, 1990). 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 levels were calculated as nmol/mg protein.

8-hidroxy-2'-deoxyguanosine immunohistochemistry. Mice were anesthetized with chloral hydrate (400 mg / kg, i.p.) and transcardially perfuse with heparin (1000 U / mL) in physiological saline (NaCl, 0.9%) followed by 4% paraformaldehyde in physiological saline 2 hours after the last injection. The brains were rapidly removed and post-fixed overnight at 4 °C in 4% paraformaldehyde. Subsequently, the brains were sectioned, dehydrated in ethanol, embedded in paraffin, and sectioned in 7 µm slices. The quenching of endogenous peroxidase was carried out using 1.5 % hydrogen peroxide in methanol (v/v) for 20 min. A high temperature antigen retrieval was performed by immersion of the slides in a water bath at 95-98 °C in 10 mM trisodium citrate buffer pH 6.0, for 45 min. Immunohistochemistry was performed to identify the oxidative damage in the cerebral cortex region, using primary

monoclonal antibody anti-8-hidroxy-2'-deoxyguanosine 1:30 (JaICA®), incubated overnight and followed by washes with PBS. After incubation with appropriate biotinylated secondary antibodies and incubated with streptavidin-byotin-peroxidase , the sections were developed with DAB (3,3'-diaminobenzidine) (Dako Cytomation) in chromogen solution and counterstained with Harris's hematoxylin. Control and experimental tissues were placed on the same slide and processed under the same conditions. The immunostaining was assessed in five layers of cortex. Images of stained cortex (I, II, III, IV and V layer) were acquired using a Sight DS-5M-L1 digital camera (Nikon, Melville, NY, USA) connected to an Eclipse 50i light microscope (Nikon) at 100x and 1000x magnification. A threshold for the optical density that better discriminated staining from the background was obtained using the NIH ImageJ 1.36b imaging software (NIH, Bethesda, MD, USA). We captured 5-8 images of per section. For relative quantification of immunoexpression, total pixels intensity was determined and data were expressed as average of optical density (O.D.).

FluoroJade B staining. To determine the extent of neurodegeneration in the mouse cerebral cortex following MeHg chronic administration, dying neurons were stained with FluoroJade B (Chemicon International, Temecula, USA) (Hopkins et al., 2000). Sections were deparafinized, immersed in xylene for 30 min, and then in 100% ethanol for 4 min. This was followed by 5 min in 80% ethanol containing 1% sodium hydroxide, then in 70% ethanol for 2 min and in distilled water for 3 min. Sections were then immersed in 0.06% potassium permanganate ($KMnO_4$) and were gently agitated for 30 min, then placed in distilled water for 2 min. The sections were then transferred to the FluoroJade B solution (0.001% FluoroJade/0.1 acetic acid) and were gently agitated for 30 min. After staining, the sections were rinsed with three 1 min changes of distilled water, dried, cleared in xylene for 1 min (Miltiadous et al., 2009). Finally, sections were mounted with D.P.X.(SERVA, Heidelberg, Germany) and were viewed under a light microscope (Eclipse 50i; Nikon, Melville, NY, EUA) with fluorescence analyze system in dark field (filter B-2A for FITC, 450–490 nm excitation band) for identification of fluorescent neurons positive for FluoroJade B. Images were performed with a digital camera (DS-5M-L1; Nikon).

Brain metal deposition. Brain metal deposition was assessed by light microscopy trough the autometallography (AMG) method (Danscher,

1984), and the sections were counterstained with hematoxylin. After decapitation, one brain hemisphere was immediately immersed in the fixative Carnoy's solution. Afterwards, whole brain tissue was dehydrated in ethanol, embedded in paraffin, and sectioned in 7 µm slices. Metal deposition was visualized by the presence of brown granules, which represents aggregated silver surrounding the deposited metal. To determine the percentage of AMG labeled cells, stereological analysis of brain was performed with an Olympus microscope at 1000x magnification (Olympus, Japan) using a Weibel graticule eyepiece (Weibel Graticule nº2, Tonbridge Kent, England) in twenty random visual fields in each histological section (Hfreere and Weibel, 1967). The measurements were done by an investigator who was blind to the treatment assignments, and it was always carefully taken the same cortical sections for the measurements.

Protein determination. Homogenate and mitochondrial preparation protein content was determined by the method of Bradford et al. (Bradford, 1976) using bovine serum albumin as the standard.

Statistical analysis. Results are presented as mean ± standard deviation, unless stated. Assays were performed in triplicate and the 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. Only significant F values are given in the text. For analysis of dose-dependent effects, linear regression was used. Differences between the groups were rated significant at $P \leq 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

Results

Respiratory chain complex activity in mouse cerebral cortex homogenates after long-term oral methylmercury administration

Figures 1 shows that long-term oral MeHg administration significantly impaired the respiratory chain function in mouse cerebral cortex homogenates. NADH dehydrogenases (including complex I NADH dehydrogenase linked activity; up to 27 %), complexes II (up to 37 %) and II-III (up to 34 %) activities were markedly reduced in MeHg exposed animals [NADH dehydrogenases: $F_{(2,17)}=10.27$; $P < 0.001$; Complex II: $F_{(2,15)}=10.46$; $P < 0.001$; Complex II-III: $F_{(2,14)}=13.62$; $P < 0.001$]. In addition, the inhibition elicited by MeHg exposition on

complexes activities were dose-dependent [complex I: β= -0.705; P<0.01; complex II: complex II: β= -0.734; P<0.001; complex II-III: β= -0.761; P<0.0001].

Complex I-IV activities in mouse cortical mitochondrial preparations after long-term methylmercury plus diphenyl diselenide co-exposition

The inhibition of the respiratory chain complexes were further investigated in brain cortex mitochondrial preparations from mice treated with MeHg (40 ppm) plus $(PhSe)_2$ (5 µmol/kg). Figure 2 shows that the toxicant exposition provoked a significant inhibition of complexes I (up to 45 %, Figure 2A), II (up to 20 %, Figure 2B) II to III (up to 48 %, Figure 2C), and IV (up to 46 %, Figure 2D) of the respiratory chain [complex I: $F_{(3,17)}$=48.70; P < 0.0001; complex II: $F_{(3,16)}$=5.27; P < 0.01; complex II to III: $F_{(3,13)}$=11.30; P < 0.001; complex IV: $F_{(3,12)}$=10.08; P < 0.001;]. Figure 2 (A-D) also depicts that the co-exposition of MeHg plus $(PhSe)_2$ significantly prevented the MeHg-inhibitory effect. However, a mild inhibition of complexes II to III and IV was also observed when $(PhSe)_2$ was administered alone. Finally, enzyme activities of the control group that received just vehicle injections did not differ from untreated animals (data not shown).

Kinase activities in mice cerebral cortex after long-term methylmercury plus diphenyl diselenide co-exposition

The activities of PK, AK and mCK were assessed in cerebral cortex from animals treated with MeHg and the $(PhSe)_2$. Figure 3 shows that the MeHg-treatment did not modify the activities of PK and AK, however, mCK activity was almost abolished (up to 97 %) by the toxicant, and a significant protection was observed after the co-administration of MeHg plus $(PhSe)_2$ [$F_{(3,8)}$=44.29; P < 0.0001].

Oxidative stress parameters in mouse cerebral cortex homogenates after long-term methylmercury plus diphenyl diselenide co-exposition

The activities of the peroxide-removing-related enzymes, GPx and GR, are depicted in Figure 4A and B, respectively. GPx activity was significantly reduced in MeHg-treated mice (up to 26 %) while GR activity was significantly increased (up to 42 %) (GPx activity: [$F_{(3,17)}$=6.76; P < 0.01]; GR activity: [$F_{(3,18)}$=9.39; P < 0.001]). These enzyme activities alterations were not prevented by the use of $(PhSe)_2$. Figure 4 also shows that lipid peroxidation, assessed through the TBA-

RS measurement was significantly induced (up to 70 %) in MeHg-treated mice cerebral cortex homogenates [$F_{(3,18)}$=39.64; P < 0.001]. Furthermore, $(PhSe)_2$ completely prevented the MeHg-induced lipid peroxidation, besides inhibiting the spontaneous lipid oxidation seen in brain control animals (Figure 4C).

DNA oxidation in mouse cerebral cortex after long-term methylmercury plus diphenyl diselenide co-exposition

Figure 5A-G shows that DNA oxidation occurred in cortical brain slices when mice were chronically exposed to MeHg. In addition, it is shown that the seleno compound was able to completely prevent this effect [$F_{(3,8)}$=22.66; P < 0.001].

Neuronal degeneration in mouse cerebral cortex after long-term methylmercury plus diphenyl diselenide co-exposition

Figure 6A-D (representative figure) shows that neuronal degeneration occurred in cortical brain slices when mice were chronically exposed to MeHg. In addition, it is shown that the seleno compound was able to prevent this citotoxic event.

Brain metal deposition in mouse cerebral cortex after long-term methylmercury plus diphenyl diselenide co-exposition

Figure 7A-E shows that metal accumulation was evident in brain from mice exposed chronically to MeHg, which was significantly prevented by $(PhSe)_2$ co-administration [$F_{(3,8)}$=11.13; P < 0.001]. Positive results were also found in brain from only $(PhSe)_2$-treated animals. However, this apparent metal brain deposition was probably due to the diselenide administration, which could chelate zinc (contamination) and also initiate the AMG amplification or by (PhSe)2 cycle to form sulphides, and the technique to identify this (Danscher, 1984).

Discussion

The main mechanisms involved in methylmercury (MeHg) neurotoxicity that have been studied are mostly associated with impairment of intracellular calcium homeostasis (Sirois and Atchison, 2000), alteration of glutamate homeostasis, and induction of oxidative stress (for review see (Aschner et al., 2007). Our study broadens the spectrum of impaired brain systems in MeHg-exposed mice, by providing evidence that energy metabolism, particularly the mitochondrial function, is severely compromised. We also demonstrated that the MeHg poisoning induced DNA oxidation, degeneration of

cortical neurons, effects that could be prevented if the containing selenium compound, $(PhSe)_2$, is co-administered.

The experimental model utilized in the present study was based on previous studies from our group, where it was demonstrated that 21 days of 20-40 ppm MeHg oral exposure of adult mice results in significant neurotoxicity, evaluated by behavioral parameters (motor performance) (Farina et al., 2003a, Dietrich et al., 2005). In addition, this MeHg exposure schedule causes high mercury brain levels of approximately 3 – 5 ppm, which could be translated into 15-30 µM concentration (Franco et al., 2006). Initially, we demonstrated that long-term oral MeHg administration significantly impaired the respiratory chain complex function in mouse cerebral cortex homogenates. NADH dehydrogenases, including that from complex I, complexes II and II-III activities were severely affected by the treatment. These results are in line with those of Yoshino and co-workers (Yoshino et al., 1966) who demonstrated the inhibition of complex II in the visual and motor cortex, cerebellum, and caudate nucleus from rats with MeHg-induced neurological symptoms. In addition, *in vitro* experiments in cultured neurons and astrocytes also demonstrated that free radical generation and oxidative stress is induced when the respiratory chain is challenged with complex III substrate (Yee and Choi, 1996). Moreover, previous reports from our group demonstrated an inhibitory *in vitro* and *in vivo* effect of MeHg on MTT reduction in isolated brain mitochondria (Franco et al., 2007, Franco et al., 2009).

Next, we more thoroughly investigated the 40 ppm MeHg-inhibitory effect on the activity of the respiratory-chain complexes in cerebral cortex mitochondrial-enriched preparations from MeHg-exposed mice. This long-term oral MeHg administration elicited an almost 2-fold inhibition of the respiratory chain complexes in mitochondrial fractions, when compared to cortical homogenates. In addition, a reduction of complex IV activity was also demonstrated in cortical mitochondria, which is in agreement with a previous report demonstrating a similar rate of complex IV inhibition in rat skeletal muscle (Usuki et al., 1998). These results strongly indicate that MeHg exposure disrupts the brain mitochondrial electron transfer function in the cerebral cortex of mice exposed to this toxic compound. It could also be assumed that the electron transfer chain appears to be the cell site where MeHg induces reactive species generation, besides the fact that

MeHg exposure results in preferential mitochondrial accumulation followed by biochemical and ultrastructural changes in the organelle (Yoshino et al., 1966, Mori et al., 2007). This is in line with previous results from our group demonstrating that the powerful mitochondrial antioxidant enzyme, glutathione peroxidase, is markedly inhibited in this MeHg-exposure experimental model, and this inhibition occurs in parallel with the inhibition in MTT reduction (Franco et al., 2009). In addition, MeHg-induced oxidative stress is also consistent with the altered biochemical parameters shown here that clearly indicate impairment of the brain antioxidant capacity, with reduced GPx and increased GR activities. This imbalance in GPx/GR activity might be related to MeHg-induced oxidative stress, mainly by causing lipid peroxidation and GSH depletion as previously described (Yee and Choi, 1996, Stringari et al., 2008, de Freitas et al., 2009, Franco et al., 2009).

On the other hand, we cannot rule out the possibility of a direct MeHg binding/oxidation of mitochondrial complex thiol groups, as previously demonstrated for other thiol-containing enzymes (Hughes, 1957). Thus, impairment of the electron transfer chain could play a critical role in the initiation of neuronal deterioration by limiting energy production and causing oxidative stress. It is also demonstrated here that the mitochondrial isoform of creatine kinase (mCK), a mitochondrial intermembrane space protein, is also a sensitive target of MeHg poisoning. CK catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP, to regenerate ATP. The different known isoenzymes constitute an intricate cellular energy buffering and transport system, connecting sites of energy capture (mitochondria) with sites of energy utilization (cytosol) (Hemmer and Wallimann, 1993, Brdiczka et al., 1994). mCK in conjunction with its tight functional coupling to oxidative phosphorylation (OXPHOS), via the adenine nucleotide translocase, is able to modulate the mitochondrial permeability transition and therefore, the release of cytochrome c into the cytoplasm (Dolder et al., 2003). The lack of equilibrium between these energy systems (OXPHOS and mCK) might potentiate the energy deficit and reactive-species formation in the mitochondria. Considering that energy is essential to maintain the development and regulation of cerebral functions, it has been postulated that alteration in CK activity may be an important step in the toxic mechanisms leading to neurodegeneration (Tomimoto et al., 1993, Wendt et al., 2002), a

condition that was also demonstrated here by using the fluoro-jade B fluorescent probe. Furthermore, the active site of CK contains a cysteinyl residue that is critical for substrate binding (Kenyon, 1996). Consequently, this enzyme is highly susceptible to inactivation by oxidative reactions (Yuan et al., 1992). Therefore, the severe mCK inhibition observed in brain tissue from MeHg-treated animals might be due to the high reactivity of MeHg towards thiol groups. Considering that the MeHg affinity constant for the SH a group is approximately 10^{10-16} (Onyido et al., 2004), any thiol-containing enzyme at physiological pH would be a molecular target of MeHg toxicity. Therefore, it is feasible that the specific mCK inhibition might be linked to the critical SH group located in the cysteine residue of the mCK active site (Kenyon, 1996). Indeed, here we have clearly demonstrated that mCK is a preferential target for MeHg after *in vivo* exposure, but PK or AK is not. Moreover, MeHg-induced GSH depletion would render this critical thiol more vulnerable to the toxicant.

Mitochondrial dysfunction and antioxidant depletion induced by MeHg would also lead to DNA oxidation, and the large DNA damage observed in MeHg-treated cortical slices will also cooperate for inducing neurodegeneration (fluorojabe B).

Considering that there is no effective treatment for MeHg poisoning, we also investigated the *in vivo* effect of the structurally simple organoselenium compound $(PhSe)_2$ in MeHg-orally exposed mice, since it has recently been demonstrated that $(PhSe)_2$ protects from some MeHg-induced toxic effects (Posser et al., 2006, Posser et al., 2008, de Freitas et al., 2009) and dramatically reduces mercury deposition in brain, liver, and kidney (de Freitas et al., 2009). Thus, in this study we selected this organic selenium compound instead of other species of selenium, such as selenomethionine or inorganic selenium, because of the intrinsic antioxidant activities of the compound that positively modulate selenium-containing enzymes. In this context, it is well known that selenium is an essential nutrient involved with the function of major metabolic pathways in the cell, where it is incorporated as selenecysteine at the active site of a wide range of proteins (Hu and Tappel, 1987). In addition, selenium binds mercury with an exceptionally high affinity, and therefore could reduce MeHg-induced toxicity by a simple quenching reaction (Skerfving, 1978, Farina et al., 2003a).

Therefore, we subcutaneously administered $(PhSe)_2$ in order to prevent the toxic effects of the mercurial in brain mitochondria. We observed that long-term administration of $(PhSe)_2$ significantly prevented the reducing MeHg effect on the activities of complexes I to IV of the respiratory chain in cortical mitochondria preparations, as well as partially prevented the marked inhibition of mCK activity (65% prevention). Mechanistically, the $(PhSe)_2$-protective effect could be due to the reactivity of its selenium atom to MeHg or to its previously demonstrated thiol–peroxidase-like activity, based on the ability to form a selenol intermediate (reduced form) which could consequently decompose hydrogen peroxide, peroxynitrite, and lipid peroxides (Nogueira et al., 2004, de Bem et al., 2008, de Freitas et al., 2009). In this scenario, the mild but significant reduction in the activity of complexes II-III and IV elicited by the administration of $(PhSe)_2$ could also be due to the thiol-peroxidase-like activity catalytic cycle, where $(PhSe)_2$ interacts with glutathione or other sources of thiols (including that from proteins) to form the potent nucleophile compound, selenophenol,, leaving this proteins more susceptible to oxidation.

The protective effect induced by $(PhSe)_2$ on the MeHg-induced toxicity that emerged from the experimental model is also in line with the histochemical results (Figures 5-7), where it was demonstrated that $(PhSe)_2$ reduced DNA oxidation, brain MeHg deposition and therefore, was able to prevent from neuronal death. This protection might be related to the ability of the selenium atom to interact with MeHg, resulting in the formation of a stable inert and insoluble complex, HgSe, that is then excreted (Iwata et al., 1982, Bjorkman et al., 1995).

Finally, it is difficult to extrapolate our results to human MeHg poisoning and to correlate the alterations of the electron transfer chain activities and mCK activities in mouse brain cortex with the neurotoxicity caused by MeHg. However, considering the large body of evidence in the literature showing that mitochondrial dysfunction might lead to cell death through reactive-species formation, ATP depletion and DNA oxidation, it is tempting to speculate that this may be one of the underlying pathomechanisms involved with the neurotoxicity induced by MeHg. Therefore, it could be propose $(PhSe)_2$ as a potential neuroprotective agent for preventing MeHg-induced brain poisoning, mainly because of its low toxicity, its capacity to reduces mercury deposition and to slow the neurodegenerative process. Furthermore, the

high *in vivo* MeHg sensitivity of CK activity might make this enzyme a plasma biomarker for MeHg poisoning. Summarizing, we have demonstrated that the electron transfer chain and mCK activities are *in vivo* molecular targets of MeHg neurotoxicity, and the impairment of these key energy enzymes causes cortical DNA oxidation and therefore, neurodegeneration that could be prevented if the organochalcogen $(PhSe)_2$ is co-administered.

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LEGENDS TO FIGURES

Figure 1 Activities of the respiratory chain complexes (RCC) in cortical homogenates from adult mice exposed to MeHg (20mg/L and 40mg/L). Values are mean ± standard deviation from six animals. ** $P \leq 0.01$; *** $P \leq 0.001$, compared to controls (One-way ANOVA followed by the Duncan multiple range test).

Figure 2 Activities of the respiratory chain complexes I (**A**), II (**B**), II-III (**C**) and IV (**D**) in mitochondrial preparations from cerebral cortex from adult mice exposed to methylmercury (MeHg; 40mg/L) and/or diphenyl diselenide ($(PhSe)_2$; 5 µmol . kg^{-1}). Values are mean ± standard deviation from six animals. * $P \leq 0.05$; *** $P \leq 0.001$, compared to controls; $^{\#\#}$ $P \leq 0.01$; $^{\#\#\#}$ $P \leq 0.001$, compared to MeHg (One-way ANOVA followed by the Duncan multiple range test).

Figure 3 Activities of mitochondrial creatine kinase (mCK), adenylate kinase (AK) and pyruvate kinase (PK) in brain from adult mice exposed to methylmercury (MeHg; 40mg/L) and/or diphenyl diselenide ($(PhSe)_2$; 5 µmol . kg^{-1}). Values are mean ± standard deviation from six animals. *** $P \leq 0.001$, compared to controls; $^{\#\#\#}$ $P \leq 0.001$, compared to MeHg (One-way ANOVA followed by the Duncan multiple range test).

Figure 4 Oxidative stress parameters in brain from adult mice exposed to methylmercury (MeHg; 40mg/L) and/or diphenyl diselenide ($(PhSe)_2$; 5 µmol . kg^{-1}). Glutathione peroxidase (GPx; **A**) and glutathione reductase (GR; **B)** activities and measurement thiobarbituric acid-reactive substances (TBA-RS) are expressed as mean ± standard deviation from five to six animals. ** $P \leq 0.01$; *** $P \leq 0.001$, compared to controls; $^{\#\#\#}$ $P \leq 0.001$, compared to MeHg; (One-way ANOVA followed by the Duncan multiple range test).

Figure 5 Immunohistochemistry for 8-hydroxy-2'-deoxyguanosine (DNA oxidation) in cerebral cortex from adult mice exposed to methylmercury (MeHg; 40mg/L) and/or diphenyl diselenide ($(PhSe)_2$; 5 µmol . kg^{-1}). For relative quantification of immunoexpression, total pixels intensity was determined and data were expressed as mean of optical density (O.D.) ± standard deviation (***E***). ***$P \leq 0.001$, compared to controls; $^{\#\#\#}$ $P \leq 0.001$, compared to MeHg; (One-way ANOVA followed by the Duncan multiple range test). Bar represents 200 µm for figures A-D and 20 µm for figures F-G. ***A***: Controls; ***B***: $(PhSe)_2$; ***C***: MeHg; ***D***: MeHg plus $(PhSe)_2$. Arrows indicate 8-hydroxy-2'-deoxyguanosine positive cells.

Figure 6 Representative figure of FluoroJade B staining (neurodegeneration) in cerebral cortex from adult mice exposed to methylmercury (MeHg; 40mg/L) and/or diphenyl diselenide ($(PhSe)_2$; 5 µmol . kg^{-1}). ***A***: Controls; ***B***: $(PhSe)_2$; ***C***: MeHg; ***D***: MeHg plus $(PhSe)_2$.

Figure 7 Brain metal deposition in cerebral cortex from adult mice exposed to methylmercury (MeHg; 40mg/L) and/or diphenyl diselenide ($(PhSe)_2$; 5 µmol . kg^{-1}). Percentage of positive cells for autometallography methodology is expressed as mean ± standard error. *** $P \leq 0.001$, compared to controls; $^{\#\#}$ $P \leq 0.01$, compared to MeHg; (One-way ANOVA followed by the Duncan multiple range test). Bar represents 2.5 µm. Arrowheads indicate the metal deposition.

Figure 1

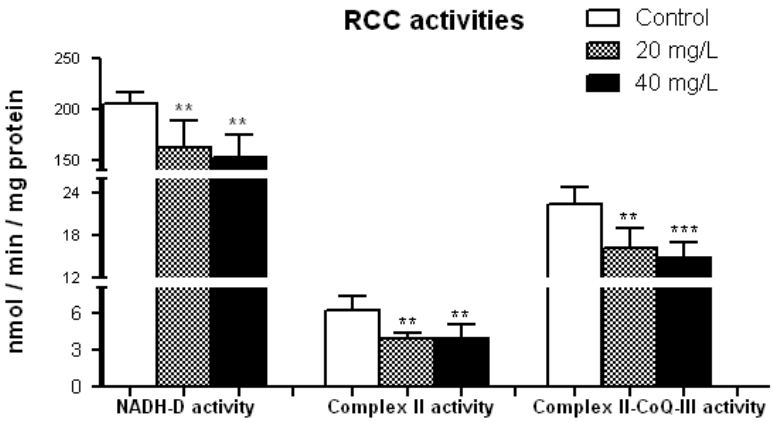

RCC activities
Control
20 mg/L
40 mg/L
nmol / min / mg protein
250
200
150
24
18
12
6
3
0
**
**
**
**
**
***
NADH-D activity
Complex II activity
Complex II-CoQ-III activity

Figure 2

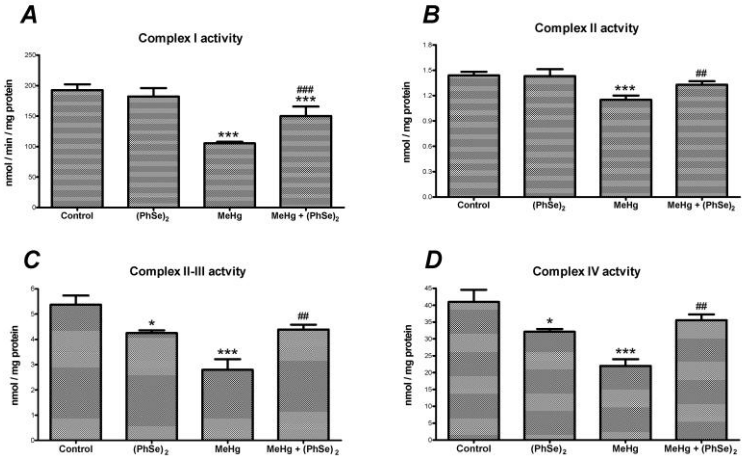

A
Complex I activity
nmol / min / mg protein
Control
(PhSe)2
MeHg
MeHg + (PhSe)2
B
Complex II actvity
nmol / mg protein
C
Complex II-III actvity
nmol / mg protein
D
Complex IV actvity
nmol / mg protein

Figure 3

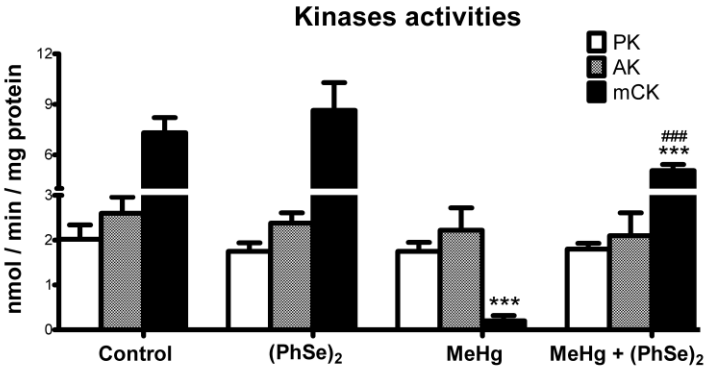

Kinases activities
PK
AK
mCK
nmol / min / mg protein
12
9
6
3
2
1
0
###
***
***
Control
(PhSe)2
MeHg
MeHg + (PhSe)2

Figure 4

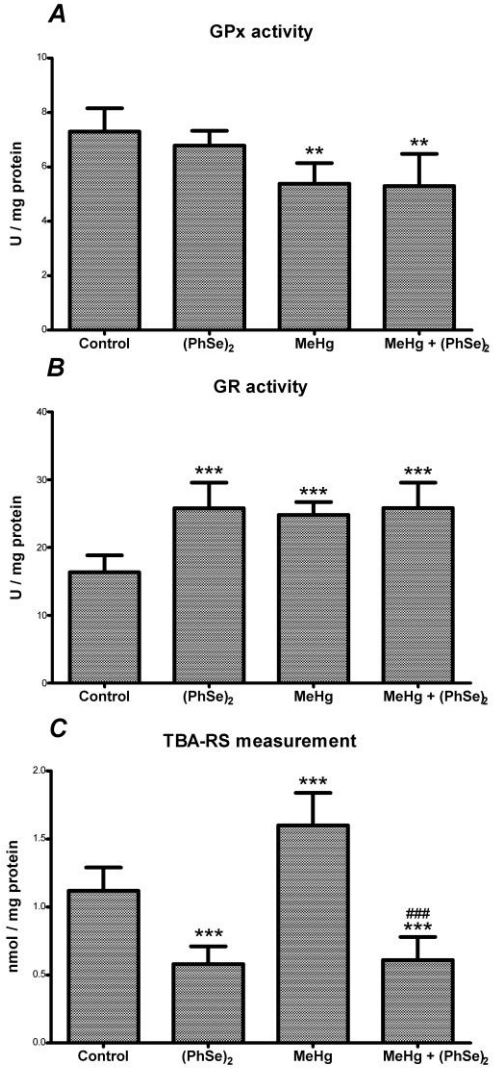

A
GPx activity
U / mg protein
10
8
6
4
2
0
**
**
Control
(PhSe)2
MeHg
MeHg + (PhSe)2
B
GR activity
U / mg protein
40
30
20
10
0
***
***
***
Control
(PhSe)2
MeHg
MeHg + (PhSe)2
C
TBA-RS measurement
nmol / mg protein
2.0
1.5
1.0
0.5
0.0
***
***
###
***
Control
(PhSe)2
MeHg
MeHg + (PhSe)2

Figure 5

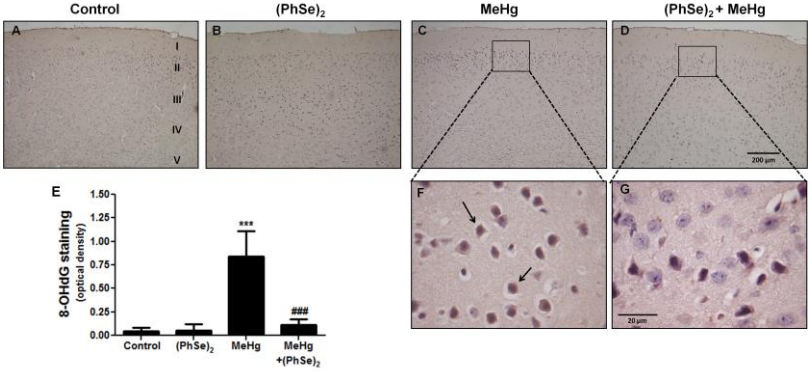

Control
(PhSe)2
MeHg
(PhSe)2 + MeHg
A
B
C
D
I
II
III
IV
V
200 µm
E
8-OHdG staining
(optical density)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
***
###
Control
(PhSe)2
MeHg
MeHg
+(PhSe)2
F
G
20 µm

Figure 6

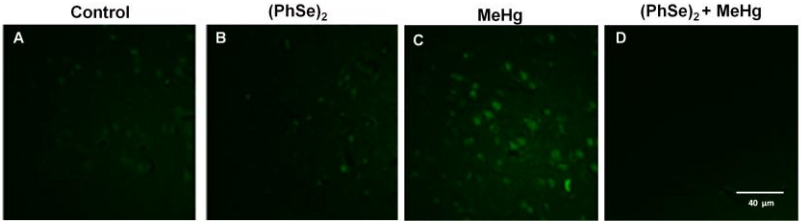
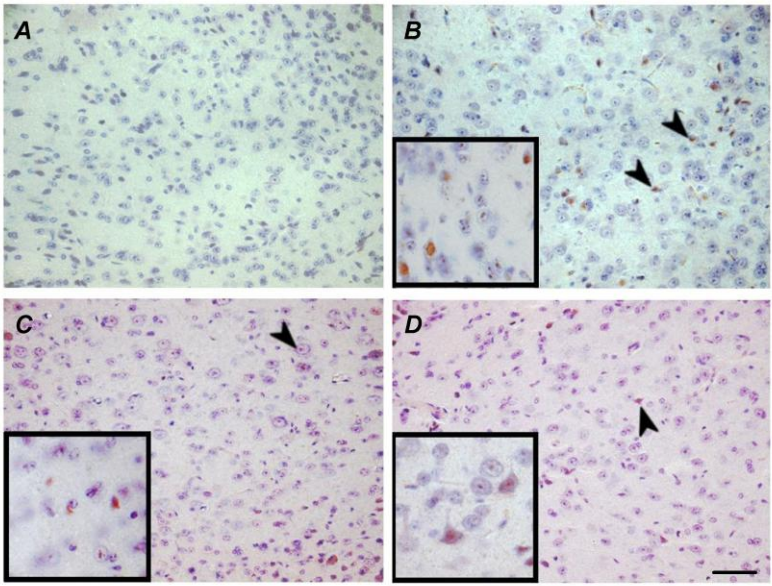
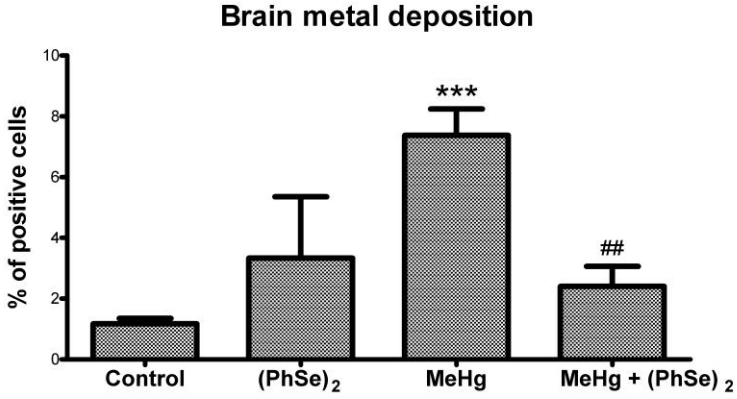


Figure 7


A
B
C
D


Brain metal deposition
% of positive cells
10
8
6
4
2
0
***
##
Control
(PhSe)2
MeHg
MeHg + (PhSe)2

RESULTADOS ADICIONAIS

A fim de verificar a morfologia mitocondrial do tecido cerebral após a intoxicação crônica com MeHg, foi realizada a análise ultra-estrutural através de microscopia eletrônica. A morfologia mitocondrial, estrutura das cristas, integridade das membranas externa e interna, o tamanho e o número de mitocôndrias foram verificados no córtex cerebral de animais intoxicados crônica e oralmentente com MeHg.

MATERIAL E MÉTODOS

Para classificação do desenho experimental, referir-se à seção M&M do manuscrito 1 (página 24). Após a preparação do material, conforme descrito na página 27, sessão *4.1.7*, os cortes foram contrastados com acetato de uranila 5% durante vinte minutos e com citrato de chumbo durante 5 minutos. Após esta etapa, o material foi analisado por microscopia eletrônica de transmissão, e imagens da mitocôndria foram capturadas, para posterior análise morfológica.

RESULTADOS

As figuras apresentadas nesta seção mostram o efeito do MeHg sobre a morfologia mitocondrial analisada por microscopia eletrônica. Pode ser observado nas Figuras 2A, B e C que o MeHg aumentou o número de mitocôndrias em córtex cerebral de animais expostos a este toxicante, quando comparados ao grupo controle (Figuras 1A, 1B e 1C). Além disso, a exposição ao MeHg mostrou mitocôndrias com maior volume mitocondrial (Figuras 2A, B e C), com edema nas cristas mitocondriais (Figuras 3B, 4B e 4C), bem como perda da integridade das cristas (Figuras 3B e 4D) e da membrana mitocondrial (Figuras 3B, 4C e 4F). Ainda, no grupo tratado com MeHg foram observados inclusões na matriz mitocondrial (Figura 3B).

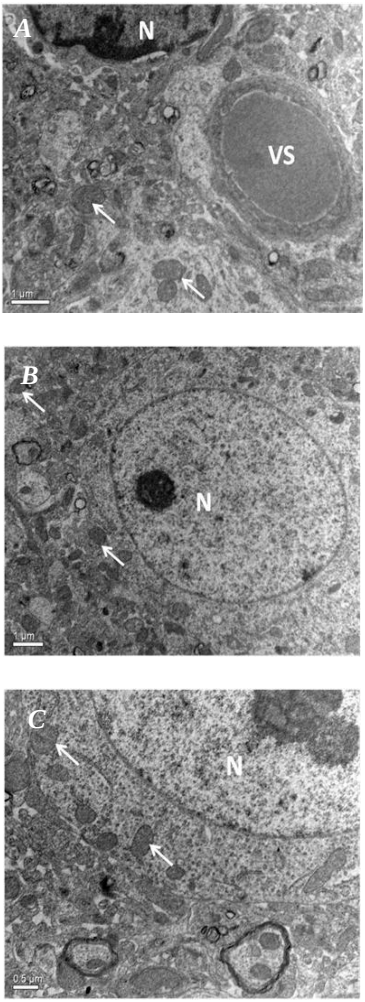


Figura 1. Mitocôndrias preservadas são observadas em córtex cerebral do grupo controle (Magnificações: A = 15.000x; B = 12.000x; C = 20.000x)
(setas: mitocôndrias; N= núcleo; VS= vaso sangüíneo).

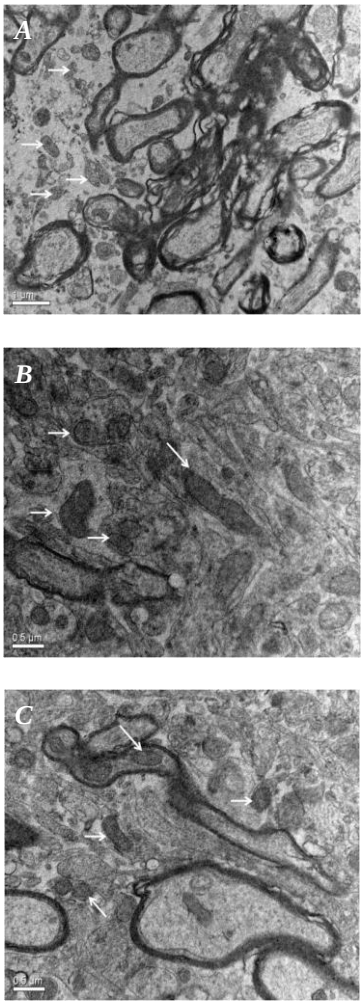


Figura 2. Mitocôndrias de córtex cerebral de animais tratados com MeHg. Observar que estas organelas apresentam-se em maior número e maior tamanho
(Magnificações: A = 15.000x; B,C = 25.000x) (setas: mitocôndrias).

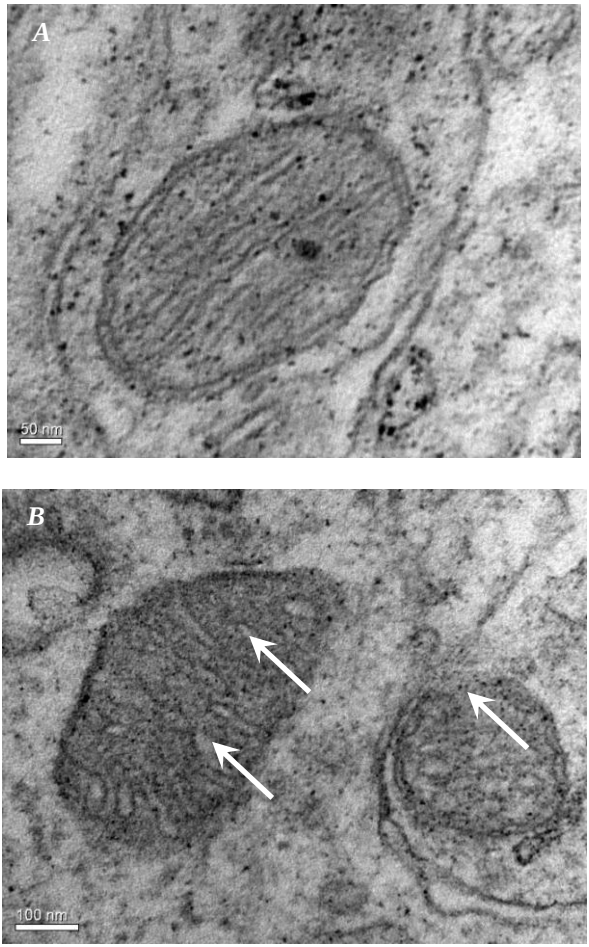


Figura 3. Morfologia mitocondrial em córtex cerebral de animais controle. Abaixo, podemos observar edema nas cristas mitocondriais e inclusões na matriz mitocondrial, além da perda da integridade entre as membranas mitoxondriais em córtex cerebral de animais intoxicados com MeHg (Magnificações: A = 200.000x; B = 150.000x)

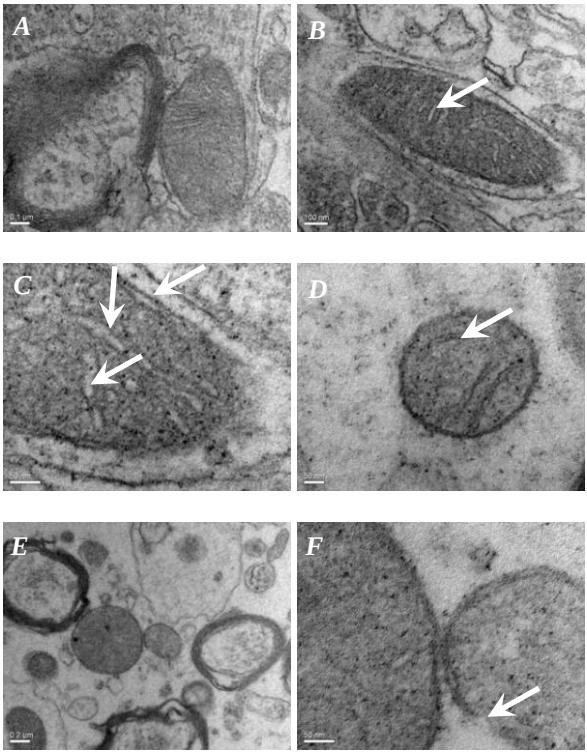


Figura 4. Mitocôndrias de córtex cerebral do grupo tratado com MeHg. Notar as alterações morfológicas indicadas com as setas, como edema nas cristas mitocondriais, perda da integridade das cristas e membranas externa e interna bem como o grande tamanho destas organelas (Magnificações: A = 100.000x; B = 120.000x; C = 300.000x; D = 200.000x; E = 50.000x; F(magnificação de E) = 300.000x).

CONCLUSÕES

Observou-se que as mitocôndrias de córtex cerebral do grupo tratado com MeHg apresentaram-em em maior volume e maior número, sugerindo um remodelamento nos processos de fusão/fissão mitocondrial. Além disso, as mitocôndrias deste grupo possuem edema nas cristas mitocondriais, perda da integridade das cristas e das membranas mitocondriais. Desta forma, o MeHg afeta a função mitocondrial também por alterar sua morfologia.

Manuscrito 2: "***Effects of inorganic selenium administration in methylmercury-induced neurotoxicity in mice***", submetido à "Chemico-Biological Interactions" em 25 de janeiro de 2010.

Effects of inorganic selenium administration in methylmercury-induced neurotoxicity in mice

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Running title: Sodium selenite effects in methylmercury poisoning

Abstract

Selenium can counteract methylmercury (MeHg) neurotoxicity. However, data about the neuroprotective effects of sodium selenite (Na_2SeO_3) on the activity of mitochondrial complexes and creatine kinase (mtCK) are scarce. Therefore, this study investigated the effects of the chronic exposure to Na_2SeO_3 on brain energy metabolism and oxidative stress parameters in MeHg-poisoned mice. Adult male mice were orally treated with MeHg (40 mg . L^{-1} in drinking water, *ad libitum*) during 21 days and simultaneously administrated with daily subcutaneous injections of Na_2SeO_3 (5 µmol . kg^{-1}), a potential neuroprotectant. Mitochondrial complexes I to IV and mtCK activities were measured in cerebral cortex mitochondria. The cerebro-cortical tissue was also used to evaluate the antioxidant enzymes glutathione peroxidase (GPx) and glutathione reductase (GR) activities, as well as lipid peroxidation. Metal deposition was followed autometalographically (AMG). Na_2SeO_3 partially prevented MeHg-induced inhibition of complexes II-III, IV and mtCK activities; however, it was unable to prevent MeHg-induced complex I and II inhibition. MeHg increased lipid peroxidation, GR activity and decreased GPx activity in the cerebral cortex; however, Na_2SeO_3 did not modify such events. Furthermore, Na_2SeO_3 *per se* inhibited complexes I, II-III and IV and mtCK activities and increased GPx and GR activities and lipid peroxidation. These data show that inorganic selenium was ineffective in preventing most of the MeHg-induced brain biochemical alterations. However, the most prominent finding was the selenium-induced reduction of cells labelled for metal deposition, probably by forming a highly insoluble salt of mercury, *i.e.* HgSe that possibly does not react in the AMG method. Although the literature supports the beneficial effects of selenium against mercury toxicity, the toxic effects elicited by Na_2SeO_3 alone or in combination with mercury should be considered when this compound is proposed as a potential protective therapy for MeHg poisoning.

Keywords: methylmercury, sodium selenite, electron transport chain, oxidative stress

Introduction

Mercury is a hazardous metal that is released into the environment from both natural and anthropogenic sources (EPA, 1997, ATSDR, 1999). Once in the aquatic environments, mercury is methylated by widespread sulphate-reducing bacteria into methylmercury (MeHg). As an organic molecule that readily penetrates lipid bilayers, MeHg is assimilated into the foodchain and biomagnifies upwards of 10-million fold through aquatic food chains (EPA, 1997). Thus, the major dietary route of human exposure to MeHg is via the ingestion of seafood for adults and via maternal milk for infants. Dietary MeHg is almost totally absorbed by the human gastrointestinal tract and rapidly enters the bloodstream, easily crossing the blood–brain barrier and the placenta (Clarkson, 1997), and about 10% of the MeHg body content is retained in the brain (Skerfving, 1974). Thus, brain has been ascribed as the most important *in vivo* target of MeHg intoxication (Clarkson, 2002).

MeHg-induced brain damage can be irreparable and characterized by massive neurodegeneration with neuronal phagocytosis and replacement of neurons by glial cells in the cerebral and cerebellar cortices (Verity, 1997, Eto et al., 1999). The neurological sequelae includes cerebellar ataxia, akathisia, spasticity, memory lost, dementia, constricted vision, dysarthria, impaired hearing, smell and taste, tremors, and depression (Choi, 1989).

The mechanisms associated with the enhanced brain sensitivity appear to involved the following major mechanisms: alteration of intracellular Ca^{2+} levels (Sarafian, 1993, Sirois and Atchison, 2000); impairment of glutamatergic system (Atchison and Hare, 1994); induction of oxidative stress (Shanker et al., 2004, Shanker et al., 2005, Kaur et al., 2006, Aschner et al., 2007, Kaur et al., 2007), and interactions with sulfhydryl groups by binding to a variety of enzyme systems eliciting cell injury and cell death (Clarkson, 1972, Schutz and Skerfving, 1975, Rocha et al., 1993, Valentini et al., 2009).

Considering that no effective treatment is available to counteract MeHg toxicity, it has been investigated whether the administration of trace elements with antioxidant properties could protect against or ameliorate the MeHg deleterious effects (Ganther et al., 1972, Skerfving, 1978, Fredriksson et al., 1993, Choi et al., 2008, Weber et al., 2008). In this context, selenium (Se) has been widely recognized as an essential dietary component with numerous beneficial effects on health.

It is known that insufficient Se levels are associated with increased oxidative stress and neurodegeneration (Nishikido et al., 1987, Schweizer et al., 2004a, Schweizer et al., 2004b). In line with this, Se is necessary for the expression of at least twenty-five Se-dependent enzymes, including the powerful antioxidant glutathione peroxidase (GPx), which protects macromolecules from peroxide damage (Flohe et al., 1973, Forstrom et al., 1978, Islam et al., 2002), the thioredoxin reductase (Holmgren, 1989, Arner and Holmgren, 2000) and several other selenoproteins which modulate the cellular redox and antioxidant status (Saito et al., 1999, Bianco et al., 2002, Panee et al., 2007).

On the other hand, several reports in the literature have demonstrated the positive effects of Se as sodium selenite (Na_2SeO_3) in antagonizing the toxicities of various heavy metals (Ganther et al., 1972, Skerfving, 1978, Fredriksson et al., 1993, Choi et al., 2008, Weber et al., 2008). In addition, Na_2SeO_3 supplemented diets (Ganther et al., 1972, Potter and Matrone, 1974) or the simultaneous administration of the seleno compounds plus MeHg in experimental animals (Ganther et al., 1972, Iwata et al., 1973, Skerfving, 1974, Ohi et al., 1975, Skerfving, 1978, Fredriksson et al., 1993, Choi et al., 2008, Orct et al., 2009) have demonstrated protective effects against the mercurial neurotoxicity.

Therefore, we investigated whether the inorganic form of Se, Na_2SeO_3 could prevent MeHg-induced disturbances on energy metabolism and oxidative stress parameters in mice brain. In addition, we also investigated whether Na_2SeO_3 could reduce brain MeHg deposition.

Experimental procedures

Animals and reagents

Male Swiss albino mice of 60 days of life obtained from the Central Animal House of the Centre for Biological Sciences, Universidade Federal de Santa Catarina, Florianópolis - SC, Brazil, were used in the present investigation. The animals were maintained on a 12-h light/dark cycle (lights on 07:00–19:00 h) in a constant temperature (22 °C ± 1 °C) colony room, with free access to water and protein commercial chow (Nuvital-PR, Brazil). The experimental protocol was approved by the Ethics Committee for Animal Research (PP00084/CEUA) of the Universidade Federal de Santa Catarina, Florianópolis – SC, Brazil, and followed the National Institutes of

Health guide for the care and use of laboratory animals (NIH Publications No. 80-23, revised 1978). All efforts were made to minimize the number of animals used and their suffering.

All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO, USA) except methylmercury (II) chloride which was obtained from Aldrich Chemical Co. (Milwaukee, WI).

The biochemical measurements were performed in a Varian Cary 50 spectrophotometer (Varian Inc., Palo Alto, CA, USA) with temperature control. For brain tissue preparations an Eppendorf 5415 R (Eppendorf, Hamburg, Germany) centrifuge was used. The microscopic analyses were performed in an Olympus microscope (Olympus, Japan).

Treatments

The experimental protocol was performed on 24 animals divided into four experimental groups as follows: (i) controls (drinking water *ad libitum* + 1 mL . kg^{-1} daily saline injections); (ii) MeHg (40 mg . L^{-1} diluted in drinking water *ad libitum* + 1 mL . kg^{-1} daily saline injections); (iii) Na_2SeO_3 (daily injections of 5 µmol . kg^{-1} + drinking water *ad libitum*) and (iv) MeHg plus Na_2SeO_3.

MeHg doses administered are known to induced MeHg brain toxic concentrations of 3 – 5 µg . g^{-1} tissue (3 – 5 ppm) (Franco et al., 2009) that provokes alterations in behavioral parameters (motor performance) (Farina et al., 2003a, Dietrich et al., 2005). Na_2SeO_3 was dissolved in saline and subcutaneously administrated (Yamamoto, 1985).

Tissue preparation

Animals were killed by decapitation without anaesthesia 24 h after the last subcutaneous administration. The brain was rapidly excised on a Petri dish placed on ice and the cerebral cortex was dissected, weighed and kept chilled until homogenization which was performed using a ground glass type Potter-Elvejhem homogenizer. The maximum period between the tissue preparations and enzyme analysis was always less than a week.

Brain preparations for measuring the respiratory chain complex activities

Mitochondrial suspensions from cerebral cortex were prepared for the measurements. Briefly, the cerebral cortex (from half hemisphere) was homogenized in 10 volumes (1:10, w/v) of phosphate buffer pH 7.4, containing 0.3 M sucrose, 5 mM MOPS, 1 mM EGTA and 0.1% bovine serum albumin. The homogenates were centrifuged at 1,500 x *g* for 10 min at 4 °C and the pellet was discarded. The supernatant was centrifuged at 15,000 x *g* in order to concentrate mitochondria in the pellet, which was finally dissolved in the same buffer (Latini et al., 2005).

Brain preparations for measuring the mitochondrial creatine kinase (mtCK) activity

The mitochondrial fraction obtained for measuring the respiratory chain complex activities was washed twice with 10 mM Tris isotonic buffer containing 0.25 M sucrose and finally suspended in 100 mM MgSO4–Trizma buffer, pH 7.5.

Brain preparations for measuring oxidative stress parameters

Cerebral cortex (from half hemisphere) was homogenized in 5 volumes (1:5, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 *g* for 10 min at 4ºC to discard nuclei and cell debris (Llesuy et al., 1985, Gonzalez-Flecha and Boveris, 1995). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and immediately used for the analyses.

Mitochondrial enzyme measurements

Measurement of the respiratory chain enzyme activities

Complex I activity was measured by the rate of NADH-dependent ferricyanide reduction as described in (Cassina and Radi, 1996). The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) and succinate:cytochrome c oxidoreductase (complex II-CoQ-complex III) were determined according to the method of Fischer et al. (Fischer et al., 1985) and that for cytochrome c oxidase (complex IV) activity according to Rustin et al. (Rustin et al., 1994). The methods described to measure these activities were slightly modified, as detailed in a previous report (Latini et al., 2005). The activities of the respiratory chain complexes were calculated as nmol . min^{-1}. mg $protein^{-1}$.

Measurement of mtCK activity

mtCK activity was measured using phosphocreatine and ADP as substrates and the creatine formed was estimated according to the colorimetric method of Hughes (Hughes, 1962). Results were calculated and expressed as µmol . min^{-1}. mg $protein^{-1}$.

Oxidative stress parameters

Measurement of glutathione-related enzymes activities: Glutathione reductase (GR) and Glutathione peroxidase (GPx) assays

GR and GPx activities were assessed spectrophotometrically by monitoring the NADPH disappearance at 340 nm by using oxidized glutathione and tert-butylhydroperoxide as substrates, respectively, as previously reported (Wendel, 1981, Carlberg and Mannervik, 1985). The specific activity was calculated as units . mg $protein^{-1}$. One unit of GR or GPx is defined as 1 µmol NADPH consumed . min^{-1}.

Measurement of thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS was determined in an acid-heating reaction containing thiobarbituric acid (Esterbauer and Cheeseman, 1990). After incubation in boiling water, 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. TBA-RS levels were calculated nted as nmol . mg $protein^{-1}$.

Brain metal deposition

Brain metal deposition was assessed by light microscopy trough the autometallography (AMG) method (Danscher, 1984). Cortical sections were counterstained with hematoxylin for better visualization. After decapitation, the brain was immediately immersed in the fixative Carnoy's solution. Afterwards, tissue was dehydrated in ethanol, embedded in paraffin, and sectioned in 7 µm slices. Metal deposition was visualized by the presence of brown granules, which represents aggregated silver surrounding the deposited metal. To determine the percentage of AMG labeled cells, stereological analysis of brain was performed with an Olympus microscope (1000X) using a Weibel graticule eyepiece (Weibel Graticule nº2, Tonbridge Kent, England) in twenty random visual fields in each histological section (Hfreere and

Weibel, 1967). The measurements were done by an investigator who was blind to the treatment assignments, and it was always carefully taken the same cortical sections for the measurements.

Protein determination

Homogenate and mitochondrial preparation protein content was determined by the method of Bradford et al. (Bradford, 1976) using bovine serum albumin as the standard.

Statistical analysis

Results are presented as mean ± standard deviation, unless stated. Assays were performed in triplicate and the mean 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. Only significant F values are given in the text. Differences between the groups were rated significant at $P \leq 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

Results

Energy metabolism parameters in mouse cortical mitochondrial preparations after chronic co-exposition to Na_2SeO_3 plus MeHg

Figure 1A-D shows that MeHg treatment significantly inhibited the activities of the complexes I to IV of the respiratory chain [complex I: $F_{(3,20)}= 85.40$; $P < 0.001$; complex II: $F_{(3,15)}= 19.85$; $P < 0.001$; complex II-III: $F_{(3,14)}= 7.92$; $P < 0.01$; complex IV: $F_{(3,11)}= 14.22$; $P < 0.001$)]. The figure also shows that the inhibitory effect of the toxicant on the activities of complexes II-III and IV was partially prevented by the use of Na_2SeO_3. However, the seleno compound *per se* also elicited a significant inhibition of complex I, II-III and IV activities.

Figure 1E shows that MeHg exposition almost abolished the activity of the key energy metabolism enzyme, mtCK, and this effect was partially prevented by the co-administration of Na_2SeO_3 [$F_{(3,8)}= 97.59$; $P < 0.001$]. However, Na_2SeO_3 alone also provoked a marked reduction on mtCK activity.

Oxidative stress parameters in mouse cortical homogenates after chronic co-administration of Na_2SeO_3 plus MeHg

Figure 2A-C shows the effects of Na_2SeO_3 on the activities of the antioxidant enzymes GPx and GR, and on TBA-RS levels in the cerebral cortex of MeHg-poisoned mice. Figure 2A shows that GPx activity was significantly reduced by MeHg exposure and this phenomenon was not modify by Na_2SeO_3 administration [$F_{(3,17)}$= 5.20; P < 0.01]. In addition, the seleno compound induced a significant increment on this activity. Figure 2B depicts the stimulatory effect of Na_2SeO_3 and MeHg on GR activity. The co-administration of these compounds did not cause additive stimulatory effects on this antioxidant enzyme [$F_{(3,18)}$= 24.33; P < 0.001]. Similar results were observed in TBA-RS measurement (Figure 2C). The increased levels of lipid peroxidation observed after either Na_2SeO_3 or MeHg exposure were not modified by the co-treatment [$F_{(3,16)}$= 27.90; P < 0.001].

Brain metal deposition in mouse cerebral cortex after chronic co-administration of Na_2SeO_3 plus MeHg

Figure 3 shows that cortical cells labelled for metal deposition were significant higher in the cerebral cortex from mice exposed chronically to MeHg, and this was almost completed prevented by Na_2SeO_3 co-administration [$F_{(3,8)}$=57.07; P < 0.001].

Discussion

The present work focused on the effects of Na_2SeO_3 administration on brain biochemical and histological parameters in MeHg poisoned mice. The experimental model utilized was based on previous studies from our group, where it was demonstrated that the oral exposure of adult mice to MeHg (40 ppm in tap water, *ad libitum*) during 21 days of causes significant neurotoxicity, evaluated by behavioral parameters (motor performance) (Farina et al., 2003a, Dietrich et al., 2005). In addition, this MeHg exposure schedule causes high levels of mercury in brain of approximately 3 – 5 ppm, which could be translated into 15-30 µM concentration (Franco et al., 2009). It was observed that the use of the seleno compound partially prevented the marked inhibition induced by MeHg on the activities of the mitochondrial enzymes, complexes II-III and IV and mtCK; however it was unable to protect against MeHg-induced complex I and II inhibition. In addition, the significant changes induced by MeHg poisoning on oxidative stress parameters, increased TBA-RS levels,

reduced GPx activity and increased GR activity, were not modified by the use of Na_2SeO_3. Furthermore, the seleno compound *per se* showed deleterious effects; it inhibited complexes I, II-III and IV and mtCK activities and elicited increased GPx and GR activities and TBA-RS measurement. Although, these results demonstrated that inorganic selenium was not effective in preventing most of the MeHg-induced brain biochemical alterations, the most noteworthy finding was the selenium-induced reduction of cortical cells labelled for metal deposition.

Toxicological studies about human MeHg exposure have demonstrated that the central nervous system is the main target organ of this organic mercurial, and this phenomenon has been associated with the ability of MeHg to easily cross through the blood-brain barrier, and to accumulate in different brain areas as the cerebral cortex, cerebellum, and retina (Mottet et al., 1984, Erie et al., 2005). MeHg neurotoxicity appears to be mediated by triggering oxidative stress (Shanker et al., 2004, Shanker et al., 2005, Kaur et al., 2006, Aschner et al., 2007, Kaur et al., 2007), by altering intracellular calcium (Sarafian, 1993, Sirois and Atchison, 2000) and glutamate homeostasis (Atchison and Hare, 1994, Aschner et al., 2007); and studies from our group (Franco et al., 2007, Franco et al., 2009), others (Belletti et al., 2002, Dreiem et al., 2005, Dreiem and Seegal, 2007, Mori et al., 2007) and from the data presented here demonstrated that MeHg related brain toxicity is also associated with impairment of mitochondrial function.

Considering that there is no effective treatment for MeHg poisoning, several studies have been conducted by using a variety of potential neuroprotective compounds in order to counteract these MeHg-induced brain biochemical alterations. In this scenario, it has been demonstrated that the essential nutrient selenium, including the inorganic form Na_2SeO_3, is able to afford protection against *in vitro* and *in vivo* MeHg-induced toxicity (Ganther et al., 1972, Iwata et al., 1973, Potter and Matrone, 1974, Ohi et al., 1975, Skerfving, 1978, Fredriksson et al., 1993, Frisk et al., 2001, Perottoni et al., 2004, Choi et al., 2008, Orct et al., 2009). The molecular mechanisms responsible for selenium-dependent protective effects are still not completed understood. However, it is known that selenium binds the mercurial with an exceptional high affinity, being capable of reduce the MeHg-induced

toxicity by a simple quenching reaction (Skerfving, 1978, Dyrssen and Wedborg, 1991, Farina et al., 2003a).

In agreement with previous reports in the literature, in the present study it was observed a significant protective effect of Na_2SeO_3 on the brain energy impairment induced by MeHg poisoning. In this context, selenium treatment was able to prevent the inhibition of the activities of the mitochondrial enzymes complexes II-III, IV and mtCK. This effect could be mainly related to the nucleophilicity of selenium metabolites (*i.e.* Se^{-2} or HSe^-) for MeHg, where one compound modifies the pharmacokinetics of the other (Ganther et al., 1972), avoiding the interaction of MeHg with thiol-containing brain energy metabolism enzymes, rather than to the antioxidant behaviour previously demonstrated for Na_2SeO_3 (Ganther et al., 1972, Kasuya, 1976, Frisk et al., 2001, Perottoni et al., 2004). In line with this, mitochondrial complexes and mtCK are sulfhydryl-containing proteins susceptible for oxidation, and the enzymatic impairment could play a critical role in initiating neuronal deterioration by limiting energy production. Particularly, mtCK is a mitochondrial intermembrane space protein that catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP, to regenerate ATP. mtCK contains a cysteinyl residue in the active site that is critical for its activity (Kenyon, 1996) and, as demonstrated here, this key energy enzyme is a highly sensitive target of MeHg poisoning. The possible MeHg-induced inhibition by direct binding/oxidation of mtCK and mitochondrial complex thiol groups is in agreement with previous reports showing the sensitivity of other thiol-containing enzymes to the mercurial exposure (Hughes, 1957, Farina et al., 2003a, Valentini et al., 2009), and whose inhibition was counterbalanced by Na_2SeO_3 administration (Farina et al., 2003a).

Data from the literature regarding Na_2SeO_3 pro-oxidant effects are scarce. El-Demerdash (El-Demerdash, 2001) reported increased lipid peroxidation (increased TBA-RS levels) after similar Na_2SeO_3 concentrations. However, inorganic selenium in combination with mercury partially or totally alleviated the toxic effects of mercury on different studied enzymes. Similarly, Zia and Islam (Zia and Islam, 2000) demonstrated a Na_2SeO_3 dose-dependent increase of lipid peroxidation and thiol oxidation in rat striatum, and this appears to be mediated by superoxide generation (Spallholz, 1997). Therefore, the lack of protection observed in the present study on the oxidative stress

parameters TBA-RS measurement and GPx and GR activities in rat cortical homogenates could be related to inappropriate Na_2SeO_3 / MeHg (Se:Hg) ratios. In this context, it has been stated that the most effective neuroprotection is obtained when selenium is given in equimolar ratios to mercury (Whanger, 1992, Ralston et al., 2007). Consequently, superfluous selenium accumulated in the brain could be more rapidly deleterious than MeHg itself. As previously reported, Na_2SeO_3 metabolism involves the transformation to hydrogen selenide (H_2Se), the central metabolite in the assimilatory and excretory pathways of selenium, via selenodiglutathione with the participation of thiols and NADPH-dependent reductases. In addition, the interaction between selenium and mercury depends on the glutathione-mediated H_2Se formation (Klug et al., 1953, Ganther, 1971, Nogueira et al., 2004). Therefore, it is feasible that more effective and protective effects could be obtained if cysteine or glutathione are Na_2SeO_3-co-administered, as previously reported (Iwata et al., 1982), since the thiol oxidation by Na_2SeO_3 results in a rapid formation of selenide anion, which by redox cycling with oxygen may cause a non-stoichiometric oxidation of thiols (Spallholz, 1997). Alternatively, other investigators have suggested that reduced free and protein thiols may interact with Na_2SeO_3 forming conjugates which also catalytically oxidize thiols (Rhead and Schrauzer, 1974).

Finally, the most prominent finding of our present investigation was the apparent MeHg brain metal deposition elicited by Na_2SeO_3. It has been depicted that inorganic selenium is able to protect adult and developing brain from MeHg-induced toxicity by forming inert complex(es) between selenium and mercury, and that the main inert complex would be represented by HgSe (Iwata et al., 1982, Bjorkman et al., 1995). Although, Newland and co-workers (Newland et al., 2006) demonstrated that selenium administration in MeHg-treated rodents increased the brain concentrations of mercury, the formation of the insoluble and relatively inert salt, HgSe, might afford neuroprotection. However, little is known about the toxicological properties and long-term fate of this insoluble compound. These questions are more complexes for the human species both in view of the extended lifespan and, perhaps, in view of the possible formation of outsized deposits in specific critical brain areas.

On the other hand, the reduced brain mercurial deposition might be also related to the stable Hg-Se complexes formed in the bloodstream as previously reported by Naganuma and collaborators (Naganuma and Imura, 1980, Newland et al., 2006).

Taking together, the aforementioned data support the dual role of inorganic selenium, which should be considered when proposed as an antioxidant therapy for MeHg poisoning.

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LEGENDS TO FIGURES

Figure 1 Activities of the respiratory chain complexes I (***A***), II (***B***), II-III (***C***) and IV (***D***) and creatine kinase (***E***) in cortical mitochondrial preparations from adult mice exposed to methylmercury (MeHg; 40mg/L) and/or sodium selenite (Na_2SeO_3; 5 µmol . kg^{-1}). Values are mean ± standard deviation from three to six animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to controls and $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$, compared to MeHg group (One-way ANOVA followed by the Duncan multiple range test).

Figure 2 Oxidative stress parameters in brain from adult mice exposed to methylmercury (MeHg; 40mg/L) and/or sodium selenite (Na_2SeO_3; 5 µmol . kg^{-1}). Glutathione peroxidase (GPx; **A**) and glutathione reductase (GR; **B)** activities and thiobarbituric acid-reactive substances (TBA-RS) measurement are expressed as mean ± standard deviation from five to six animals. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared to controls; $^{\#}$ $P < 0.05$; $^{\#\#}$ $P < 0.001$; $^{\#\#\#}$ $P < 0.001$, compared to MeHg group; (One-way ANOVA followed by the Duncan multiple range test).

Figure 3 Brain metal deposition in brain from adult mice exposed to methylmercury (MeHg; 40mg/L) and/or sodium selenite (Na_2SeO_3; 5 µmol . kg^{-1}). Percentage of positive cells for autometallography methodology are expressed as mean ± standard error. *** $P < 0.001$, compared to controls; $^{\#\#\#}$ $P < 0.001$, compared to MeHg group; (One-way ANOVA followed by the Duncan multiple range test). Bar represents 2.5 µm. Arrowheads indicates the metal deposition.

Figure 1

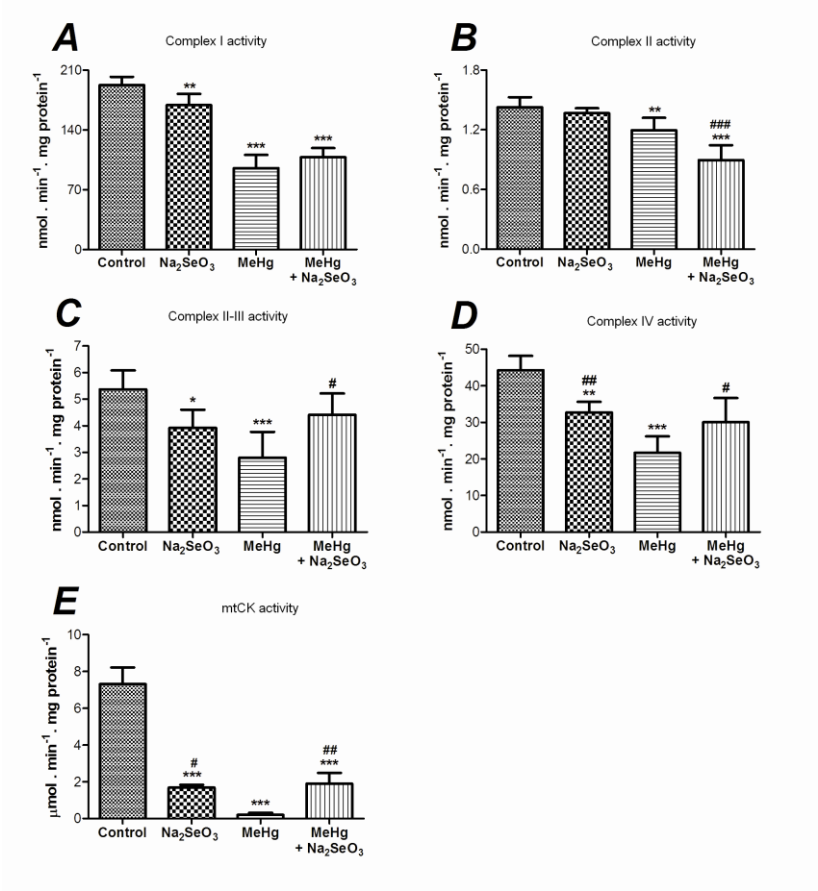

A
Complex I activity
nmol . min-1 . mg protein-1
210
140
70
0
**
***
***
Control
Na2SeO3
MeHg
MeHg + Na2SeO3
B
Complex II activity
nmol . min-1 . mg protein-1
1.8
1.2
0.6
0.0
**
###
***
Control
Na2SeO3
MeHg
MeHg + Na2SeO3
C
Complex II-III activity
nmol . min-1 . mg protein-1
7
6
5
4
3
2
1
0
*
***
#
Control
Na2SeO3
MeHg
MeHg + Na2SeO3
D
Complex IV activity
nmol . min-1 . mg protein-1
50
40
30
20
10
0
##
**
***
#
Control
Na2SeO3
MeHg
MeHg + Na2SeO3
E
mtCK activity
µmol . min-1 . mg protein-1
10
8
6
4
2
0
#
***
***
##
***
Control
Na2SeO3
MeHg
MeHg + Na2SeO3

Figure 2

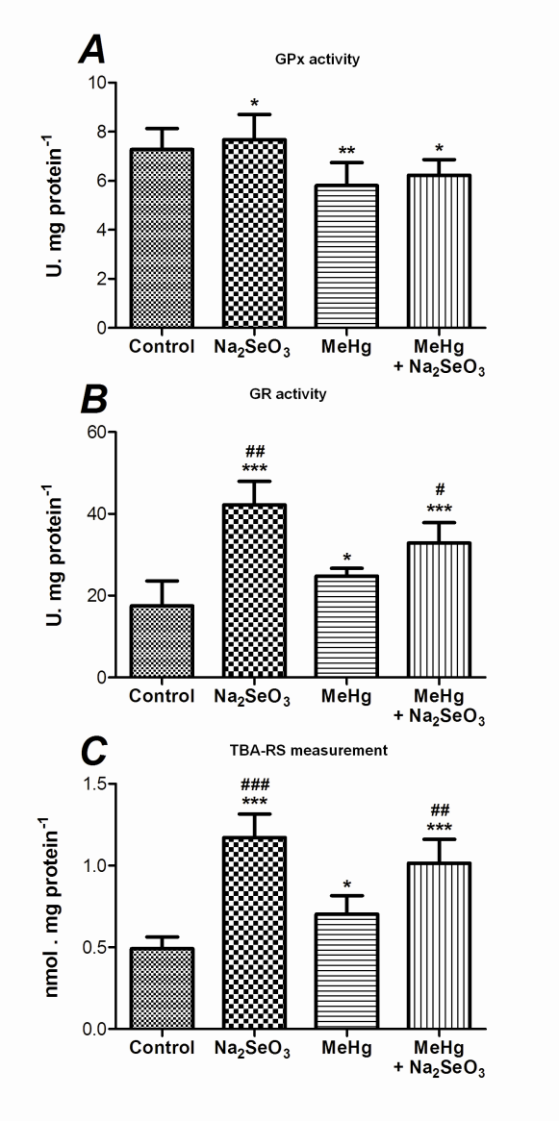
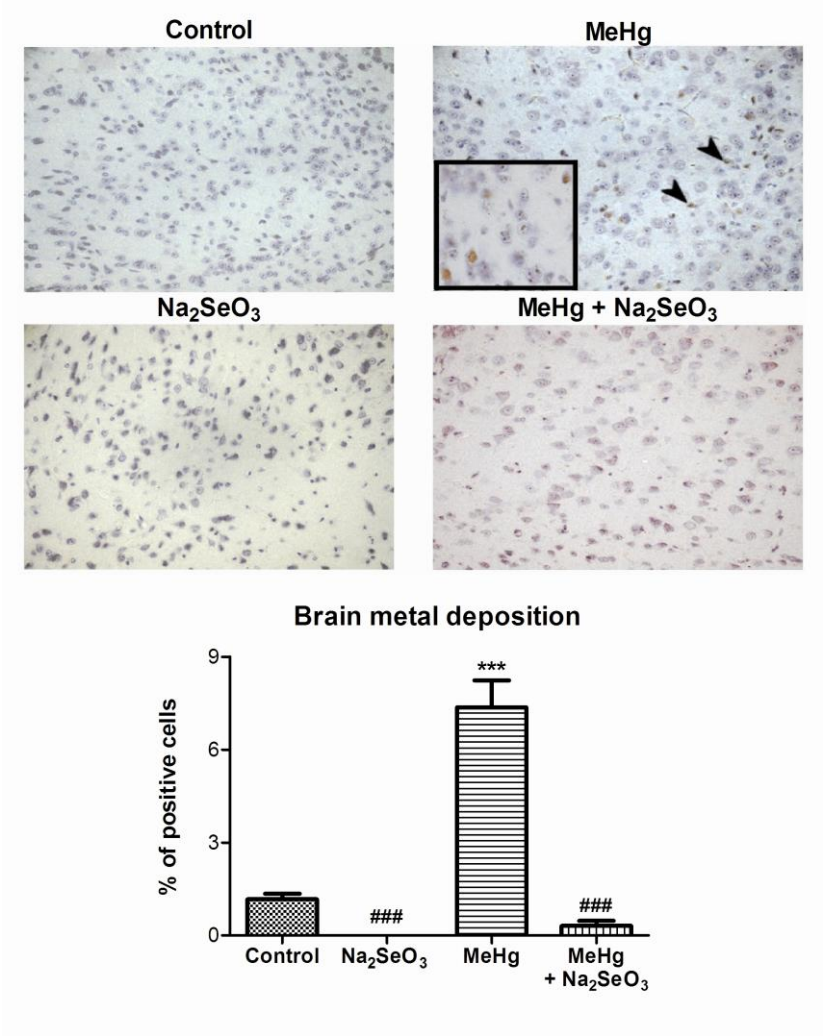

A
GPx activity
U. mg protein-1
10
8
6
4
2
0
*
**
*
Control
Na2SeO3
MeHg
MeHg + Na2SeO3
B
GR activity
U. mg protein-1
60
40
20
0
***
*
***
Control
Na2SeO3
MeHg
MeHg + Na2SeO3
C
TBA-RS measurement
nmol . mg protein-1
1.5
1.0
0.5
0.0
***
*
***
Control
Na2SeO3
MeHg
MeHg + Na2SeO3

Figure 3


Control
MeHg
Na2SeO3
MeHg + Na2SeO3
Brain metal deposition
% of positive cells
9
6
3
0
***
###
###
Control
Na2SeO3
MeHg
MeHg + Na2SeO3

Manuscrito 3: "***Oxidative stress-mediated inhibition of brain creatine kinase activity by methylmercury***", submetido à "NeuroToxicology"em 8 de dezembro de 2009.

Oxidative stress-mediated inhibition of brain creatine kinase activity by methylmercury

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ABSTRACT

Methylmercury (MeHg), a potent neurotoxicant, easily passes through the blood-brain barrier and accumulates in brain causing severe irreversible damage. However, the underlying neurotoxic mechanisms elicited by MeHg are still not completed defined. In this study, we aimed to investigate the *in vitro* toxic effects elicited by crescent concentrations (0-1500 µM) of MeHg on creatine kinase (CK) activity, thiol content (NPSH) and protein carbonyl content (PCC) in mouse brain preparations. In addition, CK activity, MTT reduction and DCFH-DA oxidation (reactive oxygen species (ROS) formation) were also measured in C6 glioma cell linage. CK activity was severely reduced by MeHg treatment in mouse brain preparations. This inhibitory effect was positively correlated to the MeHg-induced reduction of NPSH levels and increment in PCC. Moreover, the positive correlation between brain CK activity and NPSH levels was observed at either 15 min or 60 min of MeHg pre-incubation. In addition, MeHg-treated C6 cells showed also a significant inhibition of CK activity at MeHg concentrations, as low as, 50 µM in parallel to reduced mitochondrial function and increased ROS production. Taking together, these data demonstrate that MeHg severely affects CK activity, an essential enzyme for brain energy buffering to maintain cellular energy homeostasis. This effect appears to be mediated by oxidation of thiol groups that might cause subsequent oxidative stress.

Keywords: creatine kinase, methylmercury, neurotoxicity

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INTRODUCTION

Creatine kinases (CKs, EC 2.7.3.2), a family of enzymes catalyzing the reversible transfer of a phosphoryl group between ATP and creatine [1], plays a key role in the energy metabolism of tissues that have intermittently high and fluctuating energy requirements, such as skeletal and cardiac muscle, and nervous tissue [2]. There are distinct CK isoenzymes, which are compartmentalized specifically in the places where energy is produced (mitochondria) or utilized (cytosol). The cytosolic CK isoforms (Cy-CK) are expressed in a tissue-specific manner, the brain-specific (BB-CK), the skeletal muscle-specific (MM-CK) and the cardiac muscle-specific (MB-CK) isoenzymes [3-5]. The mitochondrial forms of CK (Mi-CK) consist of the muscle-specific sarcomeric isoform Mib-CK and the ubiquitous isoform Mia-CK, which is mainly found in brain tissue mitochondria [3,6-9].

Cy-CK, which in part is associated with specific subcellular compartments or structures [3,10], exists as homo- and heterodimers in the cytosol, and their function is to prevent fluctuations of ATP during periods of high energy demand, such as in cardiac and skeletal muscle contraction, Ca^{2+}-pump activity, photoreceptor-mediated light transduction, and neuronal excitation.

Mi-CK, located in cristae and intermembrane space [11-13], uses ATP supplied by the ANT (adenine nucleotide translocase) to form PCr (phosphocreatine) [14], which is then delivered via the outer membrane VDAC (voltage-dependent anion channel) to the cytosol [15,16]. These contact site complexes (CK/ANT/VDAC) has been pointed out as a functional a structural element of the permeability transition pore (PTP) [17]. The opening of the PTP, possibly regulated by Mi-CK oligomers [18], is considered a key event in the mitochondrial pathways leading to cellular apoptosis [19].

Due to the specific localization of CK isofoms, CK/phosphocreatine-system could in principle provide a spatial ‘‘energy shuttle’’ [12,20,21] or ‘‘energy circuit’’ [3] bridging sites of energy generation with sites of energy consumption [22].

The brain, like other tissues with high and variable rates of ATP metabolism, presents high PCr concentration and CK activity. The importance of creatine and the CK system for normal cell function has been elucidated in transgenic mice lacking the expression of CK [23-26]. These animals showed muscular and neurological dysfunctions and

phenotypes that have some similarities with the clinical symptoms of humans suffering from the so-called ‘‘creatine deficiency syndrome’’ [27]. It is well described that inhibition of CK activity is implicated in the pathogenesis of a number of diseases, especially in the brain [28,29], because of the central role of the PCr/CK system in the regulation of brain ATP concentrations. Therefore, alterations in CK functioning have been proposed in CNS diseases with altered energy metabolism and may represent an important step of a neurodegenerative pathway that leads to neuronal loss in the brain [30,31].

CK isoenzymes are extremely susceptible to damage by reactive species [32-36], and this appears to be mediated by oxidation of a cysteinyl residue (cysteine^{282}) that is critical for substrate binding [37]. It has been demonstrated that the substitution of this cysteine^{282} with a serine results in a 500-fold decrease in enzyme activity [37]. Consequently, CK is highly susceptible to inactivation by oxidative reactions [38]. In this scenario, Mi-CK appears to be more vulnerable than Cy-CK, due to its mitochondrial localization [39]. Most of the reactive species originate directly or indirectly from the activity of the mitochondrial respiratory chain, in particular under conditions of increased oxidative stress like ischemia/reperfusion injury, aging [40-42], as well as, in certain neurodegenerative diseases such as amyotrophic lateral sclerosis, Huntington’s disease, and Alzheimer’s disease [43]. In line with this, it has been demonstrated a compromised CK system in common neurodegenerative diseases [44-46].

On the other hand, environmental pollutants, including the organic form of mercury, methylmercury (MeHg), have been shown to cause severe and irreversible neurobehavioral and neuropsychological disorders in both humans and animals [47-51]. Even though, MeHg-induced neurotoxicity is a widely reported phenomenon, the molecular mechanisms related to its toxicity are not completely understood. The current mechanisms involved in the MeHg-induced neurotoxicity are mainly related to intracellular calcium impairment [52], alteration of glutamate homeostasis and oxidative stress [53]. Indeed, the antioxidant glutathione (GSH) system appears to be an important molecular target of MeHg-induced neurotoxicity [54],, corroborated by decreased GSH levels and activities of GSH-related enzymes in the brain of MeHg-exposed animals. Considering that MeHg is a potent electrophilic molecule that compromise the cellular antioxidant system (oxidizes thiol

groups), in the present investigation we study the *in vitro* effect of MeHg on CK activity, a sensitive thiol-containing enzyme. In addition, we also study the *in vitro* effect of MeHg on the neurochemical parameters, namely non-protein thiol group (NPSH) levels, protein carbonyl content (PCC), DCFH-DA oxidation (reactive oxygen species (ROS) formation) and MTT reduction in mouse brain preparations and in C6 glioma cell linage homogenates.

EXPERIMENTAL PROCEDURES

Animals and reagents

Male *Swiss albino* mice of 60 days of life obtained from the Central Animal House of the Centre for Biological Sciences, Universidade Federal de Santa Catarina, Florianópolis - SC, Brazil, were used. The animals were maintained on a 12-h light/dark cycle (lights on 07:00–19:00 h) in a constant temperature (22 °C ± 1 °C) colony room, with free access to water and protein commercial chow (Nuvital-PR, Brazil). The experimental protocol was approved by the Ethics Committee for Animal Research (PP00084/CEUA) of the Universidade Federal de Santa Catarina, Florianópolis – SC, Brazil, and followed the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989. All efforts were made to minimize the number of animals used and their suffering.

All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO, USA) except methylmercury (II) chloride which was obtained from Aldrich Chemical Co. (Milwaukee, WI). The CK activity, NPSH content, and cell viability assay were performed in a Varian Cary 50 spectrophotometer (Varian Inc., Palo Alto, CA, USA) with temperature control. The rate of oxidation of 2'–7'-dichlorofluorescein (DCFH) was quantified by using a Tecan Austria GmbH M200 (Tecan, Grödig/Salzburg, Austria) fluorescence spectrophotometer. For brain tissue preparations, an Eppendorf 5415 R (Eppendorf, Hamburg, Germany) centrifuge was used. The oxidation of DCFH was also assessed by using a Nikon inverted microscope using the TE-FM Epi-Fluorescence accessory.

Cerebral cortex supernatant preparation

Animals were killed by decapitation without anesthesia, the brain was rapidly excised on a Petri dish placed on ice and the cerebral cortex was dissected, weighed and kept chilled until homogenization which

was performed using a ground glass type Potter-Elvejhem homogenizer. Homogenates were further centrifuged at 1000 x *g* for 10 min at 4 ºC, the pellet was discarded and the supernatants obtained were incubated at 37 ºC for 15 min or 1 hour with MeHg (0-1500 µM). Immediately after incubation, aliquots were taken to determine the biochemical parameters.

Maintenance and treatment of cell line

The C6 astroglioma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and was maintained essentially according to the procedure previously described [55]. The cells were seeded in flasks and cultured in DMEM (pH 7.4) containing 5% fetal bovine serum, 2.5 mg/mL Fungizone® and 100 U/L gentamicin. Cells were kept at 37°C in an atmosphere of 5% CO_2/95% air. Exponentially growing cells were detached from the culture flasks using 0.05% trypsin/ethylene-diaminetetracetic acid and seeded in 24-well plates (10 x 10^3 cells/well). After cells reached confluence, the culture medium was removed by suction and cells were pre-incubated in the presence of MeHg (0-1500 µM) for 15 min or 1 hour, in serum-free DMEM (pH 7.4), at 37°C in an atmosphere of 5% CO2/95% air.

Measurement of creatine kinase (CK) activity

CK activity was measured in a 60 mM Tris-HCl buffer, pH 7.5, containing 7 mM phosphocreatine, 9 mM $MgSO_4$, and approximately 1 µg protein in a final volume of 0.13 mL. After 20 min pre-incubation at 37 °C, the reaction was started by the addition of 0.42 µmol ADP (2.8 mM final concentration). The reaction was stopped after the incubation for 15 minutes by the addition of 1 µmol p-hydroxymercuribenzoic acid (6.25 mM final concentration). The reagent concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure the spontaneous hydrolysis of phosphocreatine. The creatine formed was estimated according by colorimetric measurement [56]. The color was developed by the addition of 0.1 mL 2 % α-naphtol and 0.1 mL 0.05 % diacetyl in a final volume of 1 mL and read after 20 min at 540 nm. Results were expressed as µmol creatine formed/min/mg protein.

Non-protein thiol groups (NPSH) measurement

NPSH groups, whose levels are mainly represented by glutathione (around 90%; [57]), were determined as described previously [58] in a fraction obtained after treating supernatants with 1

volume of 10 % trichloroacetic acid. After centrifugation, an aliquot of supernatant was diluted in 800 mM sodium phosphate buffer, pH 7.4, and 500 µM DTNB (5,5′-dithio*bis*-2-nitrobenzoic acid) were added. Color development resulting from the reaction between DTNB and thiols reaches a maximum in 5 min and is stable for more than 30 min. Absorbance was determined at 412 nm after 10 min. Results were calculated as µmol NPSH/mg protein.

Protein carbonyl content (PCC)

The oxidative damage to protein was measured by the determination of protein carbonyl groups content (PCC), based on the reaction with dinitrophenylhydrazine (DNPH) [59]. MeHg-exposed cortical supernatants were treated with 4 µmol DNPH dissolved in 2.5 N HCl or with 2.5 N HCl (blank control) and left in the dark for 1 h. Samples were then precipitated with 1 volume 20% TCA and centrifuged for 5 min at 10,000 x *g*. The pellet was then washed with 1 mL ethanol:ethyl acetate (1:1, v/v) and re-dissolved in 550 µL 6 M guanidine prepared in 2.5 N HCl. Then, the tubes were incubated at 37 °C for 5 min to assure complete dissolution of the pellet and the resulting sample was determined at 365 nm. The difference between the DNPH-treated and HCl-treated samples was used to calculate the PCC. The results were calculated as nmol of carbonyls groups/mg protein, using the extinction coefficient of 22,000 x 10^6 mM^{-1} cm^{-1} for aliphatic hydrazones.

Measurement of mitochondrial function

Mitochondrial function of C6 glioma cells was assessed by following the MTT (3-[4,5dimethylthiazol-2-yl]-2,5-diphenyltetrazolic bromide) reduction. Active mitochondrial dehydrogenases cleavage and reduce the soluble yellow MTT dye into the insoluble purple formazan [60]. Brain slices or cells were incubated for 1 h with MeHg (0-1500 µM). At the end of the incubation period, MTT test were performed. The formazan formation was spectrophotometrically assayed at 570 nm and 630 nm, and the net $\Delta A_{(570-630)}$) was taken as an index of mitochondrial function. Results were compared to control samples to which 100% activity was attributed.

ROS production measurement through the DCFH-DA oxidation

Intracellular ROS production was detected using the non-fluorescent cell permeating compound, 2'–7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is hydrolyzed by intracellular

esterases to DCFH, which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by action of cellular oxidants. MeHg-exposed C6 cells were treated with DCFH-DA (50 μM) for 30 min at 37°C. Afterwards, the cells were photographed or scraped into PBS with 0.2% Triton X-100. The fluorescence was measured with excitation at 485 nm and emission at 520 nm. Calibration curve was performed with standard DCF (0 - 1 mM) and the level of ROS production was calculated as nmol DCF formed/mg protein [61].

RESULTS

MeHg treatment strongly inhibited CK activity and induced oxidative stress in mouse cerebral cortex homogenates

MeHg *in vitro* effect was first investigated on CK activity in mouse cortical homogenates. Figure 1A shows that 15 or 60 min of pre-incubation with the mercurial elicited a strong inhibition (up to 85%) of CK activity. In addition, the MeHg-inhibitory effect was in a concentration-dependent fashion (15 min pre-incubation= [$F_{(6,28)}$=22.41, $P < 0.001$, $\beta_{(\text{linear regression})}$= -0.64, P<0.001, $R^2_{(\text{best fit non-linear regression})}$= 0.81; 60 min pre-incubation= [$F_{(6,28)}$=20.06, $P < 0.001$, $\beta_{(\text{linear regression})}$= -0.76, $P < 0.001$, $R^2_{(\text{best fit non-linear regression})}$= 0.81].

In parallel, the effect of MeHg on NPSH levels in cortical supernatants was also investigated. Figure 1B shows that 15 min or 60 min MeHg exposure significantly decreased NPSH content (up to 95 %) also in a concentration-dependent manner (15 min pre-incubation: [$F_{(6,28)}$=23.17, $P < 0.001$; $\beta_{(\text{linear regression})}$= -0.84, $P < 0.001$, $R^2_{(\text{best fit non-linear regression})}$= -0.89; 60 min pre-incubation: [$F_{(6,28)}$=9.09, $P < 0.001$, $\beta_{(\text{linear regression})}$= 0.80, $P < 0.001$, $R^2_{(\text{best fit non-linear regression})}$= 0.86].

The IC_{50} (MeHg concentration necessary to reduce 50% of the CK activity) was determined according to Dixon (1964). The IC_{50} values obtained for the CK inhibition induced by MeHg exposition was 189.6 ± 1.18 μM and 87.0 ± 1.15 μM for 60 min and 15 min of pre-incubation, respectively.

Figures 1A and B also show the high sensitivity of this enzyme and of NPSH levels to the pre-incubation conditions, depicted by the reduction in CK activity and thiol content in control samples at 15 min or 1 hour pre-incubation.

Figures 2A and B shows that MeHg-induced inhibition of CK activity was positively correlated with NPSH levels, either with 15 min or 60 min pre-incubation (15 min pre-incubation: $r = 0.85$, $P < 0.001$; 60 min pre-incubation: $r = 0.87$, $P < 0.001$). By using GraphPad software, it is also possible to extrapolate and calculate that at MeHg IC50 for CK inhibition, NPSH levels are slightly reduced (approximately 20 and 10% of reduction for 15 and 60 min pre-incubation, respectively), showing the high enzyme sensitivity to thiol oxidation as a function of time of pre-incubation.

Figure 3 shows that MeHg-exposition significantly induced increased PCC [$F_{(6,14)}=2.85$, $P = 0.05$], and this effect was dependent on the mercurial concentration [$\beta_{(\text{linear regression})}= -0.65$, $P < 0.001$]. In addition, MeHg-induced inhibition of CK activity was slightly but positively correlated with PCC (60 min pre-incubation: $r = 0.53$, $P < 0.05$).

MeHg treatment inhibited creatine kinase activity, disrupted mitochondrial function and increased ROS production in C6 astroglioma cell linage

Figure 4A shows that MeHg-treatment significantly decreased CK activity, mitochondrial function and ROS formation in C6 astroglioma cells. Figure 4A shows a marked inhibition on CK activity in C6 astroglial cells after 15 or 60 min exposure to MeHg [15 min pre-incubation: $F_{(3,8)}=5.90$, $P < 0.05$; 60 min pre-incubation: $F_{(3,8)}=10.65$, $P < 0.01$].

Figure 4B shows that MTT reduction was severely impaired by exposing the cells to MeHg for 15 or 60 min [15 min pre-incubation: $F_{(7,32)}=44.95$, $P < 0.001$; 60 min pre-incubation: $F_{(7,32)}=83.73$, $P < 0.001$], and this effect was in a concentration-dependent manner [15 min pre-incubation: $\beta_{(\text{linear regression})}= -0.21$, $P > 0.05$; $R^2_{(\text{best fit non-linear regression})}= 0.93$; 60 min pre-incubation: $\beta_{(\text{linear regression})}= -0.19$, $P > 0.05$; $R^2_{(\text{best fit non-linear regression})}= 0.96$].

In addition, Figures 5A, B and C, show that ROS production accompanied the MeHg-induced inhibition of C6 cell mitochondrial function [$F_{(2,9)}=87.49$, $P < 0.001$].

DISCUSSION

The MeHg lipophilic nature allows a rapid distribution throughout the body and despite the fact that, all organs are exposed to

high levels of MeHg upon intoxication, the most vulnerable target is the central nervous system [50]. MeHg exposure in humans has been characterized by damaging to several areas of the brain including the cerebral cortices and the cerebellum [62-65], and the physiopathology of these alterations is still not well understood. However, there is strong evidence in the literature pointing to the induction of oxidative stress as one of the main neurotoxic mechanism in MeHg neurotoxicity [53,66-68]. In this context, previous studies have demonstrated that MeHg preferentially interacts with free or protein-bound thiols, leading to a rapid depletion of the cellular antioxidant defenses and consequently to oxidative stress [69-73]. Therefore, it has been hypothesized that the MeHg electrophilic behavior will be decisive in dictating the mercurial-induced neurotoxicity [74]. In this scenario, and considering that *i)* CK activity is essential for brain energy homeostasis [3,75]; *ii)* CK is a thiol-containing enzyme with a critical cysteinyl residue for substrate binding highly susceptible to oxidation [37,38,76]; *iii)* the inactivation of CK (via oxidation of its critical thiol) has been implicated in the toxic mechanisms leading to neurodegeneration [30,34,46], the main objective of this investigation was to study the *in vitro* effect of MeHg on CK activity in brain from adult mice and in C6 astroglioma cells.

Here we showed that CK is a sensitive molecular target of MeHg. By covering a wide range of MeHg concentrations, we demonstrated that in short periods of mercurial exposition, as short as 15 min, (Figure 1A) CK activity was severely inhibited in mouse cortical homogenates. Although, a linear concentration-effect was observed in 15 or 60 min of MeHg pre-incubation (β= 0.64; $P < 0.001$), a stronger concentration-effect relationship was demonstrated when applying the polynomial (non-linear) regression, pointing to the high susceptibility of the single critical thiol (cysteine^{282}; pK_a=5.4; [76] of CK towards the electrophilic activity of MeHg. This inhibitory MeHg-induced effect on CK is in agreement to the MeHg affinity constant for the SH groups, which is approximately 10^{10-16} [77]. Therefore, it could be assumed that any thiol-containing enzyme at physiological pH would be a molecular target of MeHg toxicity, including that of CK. Moreover, we should also consider that a higher selectivity of MeHg toward specific nucleophilic molecules could also be determined by the pKa value. Therefore, the CK thiol group, because of its low pKa 5.4 would be potentially more vulnerable to oxidation than the thiol group of glutathione (GSH; pK_a= 8.7; [78]) at

pH 7.4 and at equimolar concentrations. This is in line with our present results demonstrating that at MeHg concentrations that provoked 50% of CK inhibition (IC_{50} values), NPSH levels were slightly reduced (approximately 20 and 10% of reduction for 15 and 60 min pre-incubation; Figure 1B). It could be also considered that the high GSH levels (up to 12 mM; [57], main contributor to the cellular NPSH content, could initially protect the critical thiol group of CK from MeHg oxidation (by a mass low effect). However, when NPSH concentrations are slightly reduced, CK thiol group became a sensitive target of MeHg to oxidative modification, and this is in line, with the positive relationship observed between CK activity and NPSH levels ($r > 0.85$; Figures 2A and B). In this scenario, it should valuable to measure the IC_{50} on commercial purified CK and comparing it with the values observed in the present investigation.

In addition, it has been demonstrated by our group and others, that MeHg-induced NPSH oxidation is associated with ROS generation, mitochondrial dysfunction and consequently protein oxidation [72,73,79,80]. Indeed, the mitochondrial electron transfer chain, where reactive species are mainly produced has been described as the preferential MeHg accumulation site in the cell, and would contribute to further biochemical and ultra-structural changes in the organelle leading to neurotoxicity [81-84]. Therefore, our next step was to assess whether the MeHg exposition enhances the oxidation of biomolecules through enhanced ROS generation. As shown in Figure 3, we observed significant protein oxidation (increased PCC; Figure 3A) in cortical MeHg-treated homogenates, indicating that apart from CK inhibition and depletion of NPSH, the oxidation of cytosolic proteins (brain homogenates) contribute to perpetuate the MeHg-initiated oxidative stress.

On the other side, CK in conjunction with its tight functional coupling to oxidative phosphorylation (OXPHOS) is able to modulate the mitochondrial function, and it has been demonstrated that the lack of equilibrium between these energy systems (OXPHOS and CK) might potentiate the energy deficit and favours reactive species formation in the mitochondria. This is in line, with our present data and those from Franco *et al*. [73] and Wagner *et al*. [85] demonstrating that MeHg-induced oxidative stress caused a severe mitochondrial dysfunction, as seen by the inhibition of MTT reduction and increased reactive species

content in C6 astroglioma cells (Figures 4A, B and C). In parallel to these alterations, CK activity was markedly inhibited (up to 46 and 60% for 15 and 60 min pre-treatment, respectively) at lower MeHg concentrations (50 μM) than those observed in cortical homogenates, reinforcing the idea that the cytosolic components including proteins and NPSH could initially protect CK activity from the toxicity of MeHg.

Summarizing, the data presented here clearly demonstrate that MeHg severely affects CK activity, an essential enzyme for brain energy buffering to maintain cellular energy homeostasis, and this effect appears to be mediated by oxidation of thiol groups and consequently by inducing oxidative stress.

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LEGENDS TO FIGURES

Figure 1 *In vitro* effect of methylmercury (MeHg) on creatine kinase (CK) activity (***A***) and non-protein thiol group (NPSH) content (***B***) in adult mouse cortical homogenates. Data represents mean ± standard deviation from five independent experiments (animals). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared to controls (One-way ANOVA followed by the Duncan multiple range test). IC_{50}: Concentration of MeHg that provokes 50% of enzyme activity inhibition. $\beta_{(linear\ regression)}$: linear dose-effect relationship; R^2: best fit of non-linear dose-effect relationship. MeHg incubation time: 15 or 60 min.

Figure 2 Scatter-plot of the relationship of creatine kinase (CK) activity and non-protein thiol group (NPSH) content in adult mouse cortical supernatants exposed for 15 min (***A***) or 60 min (***B***) to methylmercury (MeHg). *r*: Pearson´s correlation (significative correlation for 15 min and 60 min of MeHg pre-incubation, $P < 0.001$, two-tailed).

Figure 3 *In vitro* effect of methylmercury (MeHg) on protein carbonyl content in adult mouse cortical homogenates. Data represents mean ± standard deviation from three independent experiments (animals). * $P < 0.05$, compared to controls (One-way ANOVA followed by the Duncan multiple range test). $\beta_{(linear\ regression)}$: linear dose-effect relationship. MeHg incubation time: 60 min.

Figure 4 *In vitro* effect of methylmercury (MeHg) on creatine kinase (CK) activity (***A***) and on mitochondrial activity (MTT reduction) (***B***) in C6 astroglioma cell homogenates. Data represents mean ± standard deviation from three independent experiments. ** $P < 0.01$; *** $P < 0.001$, compared to controls (One-way ANOVA followed by the Duncan multiple range test). $\beta_{(linear\ regression)}$: linear dose-effect relationship. MeHg incubation time: 15 min or 60 min.

Figure 5 *In vitro* effect of methylmercury (MeHg) reactive species generation in C6 astroglioma cells. Data represents mean ± standard deviation from three independent experiments. Panel ***B*** corresponds to the identification of reactive species levels assessed by fluorescence microscopy**.** Panel ***C*** corresponds to the quantification of reactive species by fluorescence microscopy. Panel ***A***: control conditions. *** *P*

< 0.001, compared to controls (One-way ANOVA followed by the Duncan multiple range test). MeHg incubation time: 60 min. The magnification of images is the same (Scale bar = 100 μm).

Figure 1

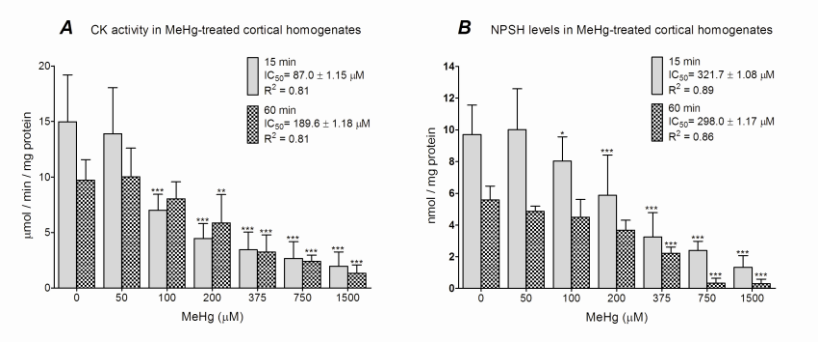
A
CK activity in MeHg-treated cortical homogenates
15 min
IC50= 87.0 ± 1.15 µM
R2 = 0.81
60 min
IC50= 189.6 ± 1.18 µM
R2 = 0.81
µmol / min / mg protein
MeHg (µM)
B
NPSH levels in MeHg-treated cortical homogenates
15 min
IC50= 321.7 ± 1.08 µM
R2 = 0.89
60 min
IC50= 298.0 ± 1.17 µM
R2 = 0.86
nmol / mg protein
MeHg (µM)

Figure 2

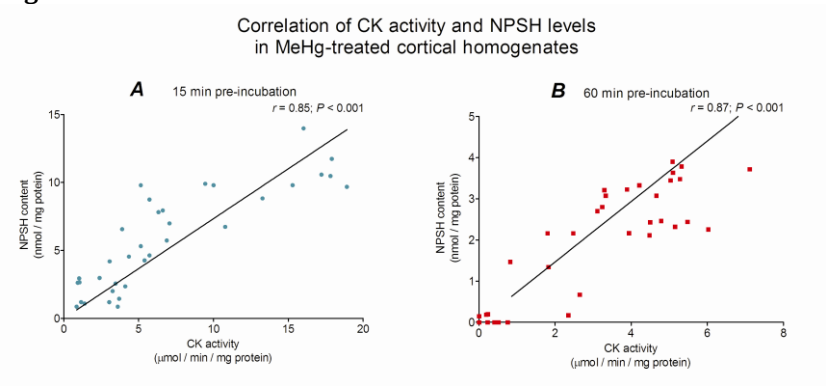
Correlation of CK activity and NPSH levels
in MeHg-treated cortical homogenates
A
15 min pre-incubation
r = 0.85; P < 0.001
NPSH content
(nmol / mg potein)
CK activity
(µmol / min / mg protein)
B
60 min pre-incubation
r = 0.87; P < 0.001
NPSH content
(nmol / mg potein)
CK activity
(µmol / min / mg protein)

Figure 3

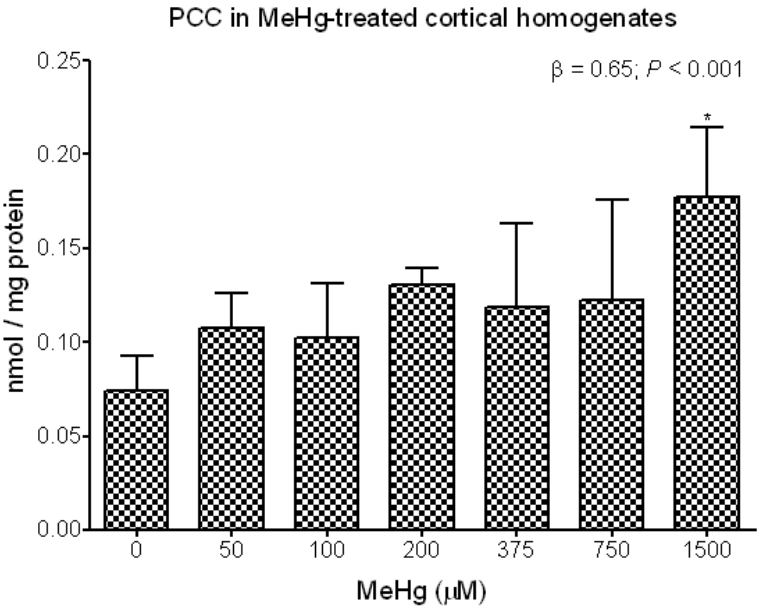
PCC in MeHg-treated cortical homogenates
β = 0.65; P < 0.001
nmol / mg protein
0.25
0.20
0.15
0.10
0.05
0.00
*
0
50
100
200
375
750
1500
MeHg (μM)

Figure 4

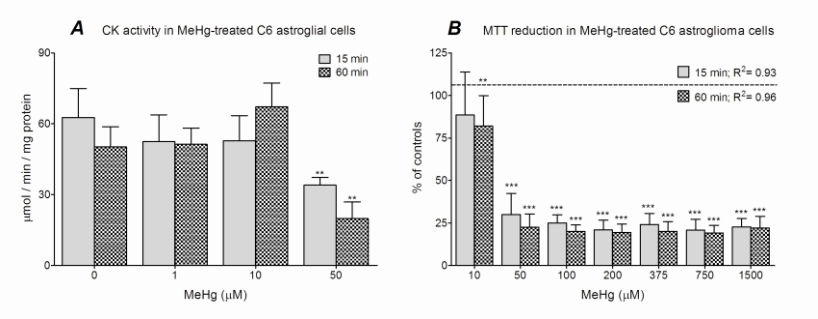
A
CK activity in MeHg-treated C6 astroglial cells
15 min
60 min
µmol / min / mg protein
MeHg (µM)
B
MTT reduction in MeHg-treated C6 astroglioma cells
15 min; R2= 0.93
60 min; R2= 0.96
% of controls
MeHg (µM)

Figure 5


A
B
C Quantitative ROS production in MeHg-treated C6 astroglioma cells
nmol / mg protein
200
150
100
50
0
***
Controls
50 μM MeHg

Manuscrito 4: "***The intra-hippocampal leucine administration impairs memory consolidation and LTP generation in rats***", submetido à "Cellular and Molecular Neurobiology" em 24 de dezembro de 2009.

The intra-hippocampal leucine administration impairs memory consolidation and LTP generation in rats

Short running title: **Leucine impairs memory consolidation and LTP generation**

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Summary

Leucine (LEU) accumulates in fluids and tissues of patients affected by maple syrup urine disease (MSUD), an inherited metabolic disorder, predominantly characterized by neurological dysfunction. Although, a variable degree of cognition/psychomotor delay/mental retardation is found in a considerable number of MSUD individuals, the mechanisms underlying the neuropathology of these alterations are still not defined. Therefore, the aim of this study was to investigate the effect of acute intra-hippocampal LEU administration in the step-down test in rats. In addition, the LEU effects on the electrophysiological parameter, long-term potentiation (LTP) generation, and on the activities of the respiratory chain were also investigated. Male Wistar rats were bilaterally administrated with LEU (80 nmol/hippocampus; 160 nmol/rat) or artificial cerebrospinal fluid (controls) into the hippocampus immediately post-training in the behavioral task. Twenty-four hours after training in the step-down test, the latency time was evaluated and afterwards animals were sacrificed for assessing the *ex-vivo* biochemical measurements. LEU-treated animals showed impairment in memory consolidation and a complete impairment of LTP generation at supramaximal stimulation. In addition, a significant increment in complex IV activity was observed in hippocampus from LEU administered rats. These data strongly indicates that LEU compromise memory consolidation, and that impairment of LTP generation and unbalance of the respiratory chain may be plausible mechanisms underlying the deleterious LEU effect on cognition.

Keywords: leucine, LTP, memory, respiratory chain activity

Introduction

Maple syrup urine disease (MSUD; OMIM 248600) is an autosomal recessive inborn error of metabolism caused by the deficiency of the activity of the enzyme complex branched-chain L-2-ketoacid dehydrogenase. The metabolic blockage results in the accumulation of the branched-chain amino acids leucine (LEU), isoleucine and valine, which undergoes reversible transamination to produce the branched-chain α-ketoacids, α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate, respectively (Chuang *et al.*, 2001). In addition, the hydroxyl derivatives of these branched-chain α-ketoacids produce, α-hydroxyisocaproate, α-hydroxy-β-methylvalerate and α-hydroxyisovalerate, also accumulate in this disorder (Treacy *et al.*, 1992). Blood levels of these metabolites increase rapidly during crises of metabolic decompensation reaching concentrations of the millimolar range (Chuang *et al.*, 2001).

MSUD presents as heterogeneous clinical and molecular phenotypes, ranging from a severe classical form, characterized by severe neonatal encephalopathy including coma and impaired cognitive outcome in later life to mild variants, which is probably due to different residual enzyme activity (Chuang *et al.*, 2001). If untreated by dietary branched-chain amino acid restriction, these patients suffer from seizures, psychomotor delay and deficits in cognitive/language areas. Early start of dietary treatment and careful metabolic control may improve the outcome of patients with classic MSUD (Nyhan *et al.*, 1989; Chuang *et al.*, 2001; Hoffmann *et al.*, 2006). MSUD neuropathological brain changes are cerebral edema, atrophy of the cerebral hemispheres, white matter spongy degeneration and delayed myelinization (Treacy *et al.*, 1992; Chuang *et al.*, 2001).

Although, neurological alterations and neuropathological sequelae are present in most patients, the molecular mechanisms underlying the brain damage is still not completed defined. In this context, a large body of *in vitro* and *in vivo* studies has pointed out LEU accumulation as the main toxic condition in MSUD. In this context, it has been demonstrated that high LEU concentration alters brain energy metabolism (Howell and Lee, 1963; Halestrap *et al.*, 1974; Pilla *et al.*, 2003a,b; Sgaravatti *et al.*, 2003; Ribeiro *et al.*, 2008), glutamatergic neurotransmission system (Tashian, 1961; Tavares *et al.*, 2000), brain uptake of essential amino acids (Araújo *et al.*, 2001) and induces

oxidative stress and apoptosis (Jouvet *et al.*, 2000; Fontella *et al.*, 2002; Bridi *et al.*, 2003; Bridi *et al.*, 2006). In addition, behavioral deficits induced by LEU administration or its cognate α-ketoacid, α-hydroxyisocaproate, have also been reported (Mello *et al.*, 1999; Vasques *et al.*, 2005).

The long-term potentiation (LTP) in hippocampus, and enduring increase in efficacy of glutamatergic synaptic transmission, is accepted as a molecular mechanism for memory storage in the brain (Selden *et al.*, 1991; Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Martin *et al.*, 2000) and it is considered the neurobiological substrate for learning and memory in which contextual cues are relevant (Lømo, 1971; Bliss and Lømo, 1973). It has been demonstrated that the step-down test, a behavioral task that depends on the integrity of hippocampal function, creates a stable memory trace in a single trial test (Whitlock *et al.*, 2006).

Considering that hippocampal LTP is involved in memory consolidation, and that one of the main symptoms in MSUD patients is mental retardation, even with a strict control of LEU plasma levels (Hoffmann *et al.*, 2006), the objective of the present investigation was to study the effect of intra-hippocampal LEU administration in the step-down test performance, LTP generation, and mitochondrial activity in adult male rats.

Material and Methods

Reagents

All reagents were purchased from Sigma Chem. (St. Louis, MO, USA).

LEU was prepared in artificial cerebrospinal fluid (ACSF) at a concentration of 80 mM.

Animals

Adult male Wistar rats weighting between 270–300 g, obtained from the Central Animal House of the Pharmacology Department of School of Chemical Sciences, National University of Cordoba, Córdoba, Argentina, were used in the present investigation. The animals were maintained on a 12-h light/dark cycle (lights on 07:00–19:00 h) in a constant temperature (22 °C ± 1 °C) colony room, with free access to water and food. Rats were handled daily for 7 days before the

experiments. The experimental protocol was approved by the Ethics Committee for Animal Research (PP00121/CEUA) of the Universidade Federal de Santa Catarina, Florianópolis – SC, Brazil and by the Animal Care and Use Committee, School of Chemical Sciences, National University of Cordoba, Córdoba, Argentina. The experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Surgery

The animals were anesthetized with 55 mg/kg ketamine HCl and 11 mg/kg xylazine (both Kensol könig, Argentina) and placed in a stereotaxic apparatus. Then, rats were implanted bilaterally into the CA1 hippocampus area with steel guide cannulae, according to the atlas of Paxinos (Paxinos and Watson, 1986). The coordinates relative to bregma were anterior: −3.6 mm; lateral: ±2.0 mm; vertical: −2.8 mm for CA1 hippocampus. Cannulae were fixed to the skull surface with dental acrylic cement. Animals were allowed to recover from surgery along 7 days and were handled daily to habituate them to the injection procedures. After the recovery period, animals were injected with LEU or ACSF (control animals) immediately after training in the step-down test using a Hamilton syringe connected by Pe-10 polyethylene tubing. Each infusion of 1 μL per side (80 nmol LEU/hippocampus; 160 nmol LEU/rat) was delivered over a 1 min period.

Behavior: step-down test

Rats were subjected to one trial in the step-down test. The training apparatus was a 50 x 25 x 25 cm plastic box with a 2.5 cm high and 7.0 cm wide platform on the left of the training box apparatus. The floor of the apparatus was made of parallel 0.1 cm diameter stainless steel bars spaced 1.0 cm apart from each other. The animals were placed on the platform, and latency to step down by placing the four paws on the grid was measured. In the training session, immediately upon stepping down, the rats received a 0.4 mA, 2s scrambled shock to the feet, and were then immediately removed, administered bilaterally with LEU or ACSF into the CA1 hippocampus, and returned to their home

cages. The retention test was carried out 24 h after training in order to measure long-term memory. This test session was identical to training session, except that no shock was given. A ceiling of 180 sec was imposed on the retention test measures. Latency time was taken as a measure of memory retention. Immediately after test, animals were sacrificed for the electrophysiological experiments and biochemical determination.

Histology

After the behavioral test, rats were sacrificed and had their brains removed for hippocampal dissection for electrophysiology and neurochemical experiments. Cannulae placement was confirmed under scope visualization, and only animals in which the cannulas tip were placed into the hippocampus were used in further experiments. All experiments were performed in each animal, using one side of hippocampus for electrophysiology and the other side for respiratory chain complex activity determinations.

Electrophysiology

Immediately after the step-down test, rats were sacrificed between 11.00 am and noon in order to prevent variations caused by circadian rhythms or nonspecific stressors (Teyler and DiScenna, 1987). The electrophysiological experiments were carried out using an *in vitro* hippocampal slice preparation described elsewhere by Pérez *et al.* (2002). Briefly, hippocampal formation was dissected, and transverse slices of approximately 400 μm thick were placed in a (BSC-BU Harvard Apparatus) recording chamber, perfused with standard Krebs solution (124.3 mM NaCl, 4.9 mM KCl, 1.3 mM $MgSO_4 \cdot 7H_2O$, 1.25 mM H_2KPO_4, 25.6 mM $HNaCO_3$, 10.4 mM glucose, 2.3 mM $CaCl_2 \cdot 2H_2O$) saturated with 95% O_2 and 5% CO_2. The rate of perfusion was 1.6 mL/min, and the bathing solution temperature was kept at 28 °C by the use of a Temperature Controller (TC-202A Harvard Apparatus). A stimulating electrode made of two twisted wires, which were insulated except for the cut ends (diameters 50 μm), was placed in the perforant path (PP). Then, a recording microelectrode was inserted in the dentate granule cell body layer. Only slices showing a stable response were included in this study. Field excitatory post synaptic potentials (fEPSP) that responded to 0.2 Hz stimuli were sampled twice, during four seconds, within a 20-40 min period (baseline). Once no

further changes were observed in the amplitude of fEPSP or in the amplitude of population spike (PS) the stimulation protocol was applied. The tetanization paradigm consisted of three train pulses at 100 Hz (high frequency stimulation; HFS), each of 1 sec duration given at 20 sec intervals. There were delivered to the PP by an A310 Accupulser Pulse Generator (World Precision Instruments Inc.). LTP was considered to have occurred when the amplitude of the fEPSP or the amplitude of the PS recorded after the tetanus at 0.2 Hz, had risen by at least 30% and persisted for 60 min. All collected data were recorded and stored for future analysis.

Respiratory chain complex activity

Immediately after the step-down test, animals were sacrificed and the rat hippocampus was dissected. Tissue was homogenized in 20 volumes of 50 mM phosphate buffer pH 7.4, containing 0.3 M sucrose, 5 mM MOPS, 1 mM EGTA and 0.1% bovine serum albumin. The homogenates were centrifuged at 1000 x *g* for 10 min at 4 °C, the pellet was discarded and the supernatants were kept at -70 °C until enzyme activity determination. The maximal period between homogenate preparation and enzyme activity measurement was always less than 5 days. Homogenate complex I activity (NADH dehydrogenase) was measured by the rate of NADH-dependent ferricyanide reduction at 420 nm (1 mM^{-1}. cm^{-1}) as described in Cassina and Radi (1996). Activity of complex II (succinate-2, 6-dichloroindophenol (DCIP)-oxidoreductase) was determined according to the method of Fischer *et al.* (1985) and complex IV activity (cytochrome *c* oxidase) was assessed according to Rustin *et al.* (1994). The methods described to measure these activities were slightly modified, as detailed in a previous report (Latini *et al.*, 2005). The activities of the respiratory chain complexes were calculated as nmol/min/mg protein.

Protein determination

Homogenate protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Statistics

Since the variables being analyzed from step-down test do not follow a normal distribution and its variance does not fulfil the assumption of homoscedasticity, these data were expressed as medians

(inter-quartile range) and analyzed by the non-parametric test Mann-Whitney U. The data from electrophysiological experiments were expressed as mean ± S.E.M. and analyzed by one-way repeated measures analysis of variance (MANOVA). Respiratory chain activity results were expressed as mean ± SD and analyzed using Student *t*-test for independent samples. Differences between the groups were rated significant at $P \leq 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) and Statistics software.

Results

Intra-hippocampal LEU administration reduced memory retention in adult rats

Initially, it was investigated whether LEU administration into the hippocampus would induce changes on memory consolidation in the step-down test. Figure 1 shows latency time as an index of memory retention after intra-hippocampal LEU administration. The animals injected with LEU 80 nmol/hippocampus, presented significant reduction on memory retention compared to control animals [Median Test Kruscal Walis, $\chi^2_{(1)}= 19.30$; $P < 0.001$ and Mann-Whitney U test, Control vs. LEU 80 nmol/hippocampus = 0.001, $P < 0.001$].

Intra-hippocampal administration of LEU inhibited LTP generation in adult rats

Then, it was investigated if the intra-hippocampal LEU administration would alter hippocampal LTP. Figure 2A shows the position of stimulation and recording electrodes in a hippocampal slice. The figure 2B indicates how fEPSP and PS amplitude were taken. Examples of fEPSP traces for ACSF and LEU groups before and after HFS are sown in figure 2C. Figure 2D shows the percentage of increments observed in fEPSP after HFS, a protocol for LTP induction in hippocampus. Intra-hippocampal administration of LEU completely block LTP generation in hippocampal dentate gyrus, while ACSF administered animals showed LTP generation, that persisted for 60 minutes [$F_{(3,57)}= 3.33$; $P < 0.05$]. Similar results were observed when the amplitude of PS was analyzed (data not shown).

Intra-hippocampal LEU administration increased complex IV activity in adult rats

Finally, this set of experiments was designed to evaluate if the changes on memory consolidation induced by intra-hippocampal LEU administration are associate with changes in the mitochondrial function. Figure 3 shows that intra-hippocampal LEU administration provoked a significant increase in complex IV activity in hippocampus [$t_{(11)}$= 2.36; $P < 0.05$]. It can also be observed that the treatment did not modify complex I and II activities.

Discussion

In the present study, we showed for the first time that a single LEU intra-hippocampal injection in adult rats impairs memory consolidation, LTP generation and modulates mitochondrial function. These results suggest that during crises of metabolic decompensation, as observed in untreated MSDU patients, when brain is exposed to high concentrations of branched-chain amino acids (at millimolar concentrations) and the cognate metabolites, various deleterious mechanisms might be triggered.

Initially, we observed that the bilateral infusion of LEU into the hippocampus (80 nmol LEU/hippocampus; 160 nmol/rat; final concentration of approximately 1.5 mM in hippocampus) disrupted the long-term memory consolidation, which was observed by the significant reduction in the latency to step-down when compared to the control group (ACSF administered; step-down behavioural test). In this context, it is known that the hippocampus has a critical role in several fundamental memory operations (Squire, 1992; Izquierdo and Medina, 1997; Tracy *et al*., 2001). The one-trial step-down test in rodents has long been used as a model for biochemical and pharmacological studies of memory (Izquierdo *et al*., 2006). Therefore, our results from the behavioral experiments, showed memory impairments, suggesting the participation of the hippocampus in the central LEU effects. In addition, it is not likely, that this effect is related to reduced locomotor activity, since it has been previously demonstrated that the intra-hippocampal administration of branched-chain α-hydroxyacids (LEU metabolites) did not affect the animal locomotor activity in the open field test (Vasques *et al*., 2005).

Glutamate mediate most of the excitatory neurotransmission in mammalian central nervous system (CNS), participating in cerebral plasticity, memory and learning, and in the formation of neural networks during development (Erecinska and Silver 1990; Ozawa *et al.*, 1998). Moreover, it has been demonstrated that blockade of downstream pathways triggered by glutamate memory, expression is blocked and amnesia is induced (Jerusalinsky *et al.*, 1992; Bianchin *et al.*, 1993; Izquierdo *et al.*, 1997; Walz *et al.*, 1999, 2000). Therefore, appropriate glutamatergic transmission is essential for normal brain development and function (Meldrum and Garthwaite, 1990; Ozawa *et al.*, 1998).

CNS glutamate synthesis depends of the blood-brain barrier uptake of LEU (Erecinska and Silver 1990; Yudkoff *et al.*, 1993). This branched-chain amino acid is the main nitrogen donor for furnishing the amino group of glutamate (Zielke *et al.*, 1995), and this is essential for neuronal glutamate production, since little glutamine or glutamate crosses into the CNS from the periphery (Grill *et al.*, 1992; Smith, 2000). After LEU-blood uptake by astrocytes, which are in close approximation to brain capillaries, the amino acid is rapidly metabolize into α-hydroxyisocaproate by the mitochondrial branched-chain aminotransferase (BCAT), thereby deriving —NH_2 groups for glutamate and glutamine synthesis (Yudkoff, 1997). α-Hydroxyisocaproate is then release into the intercellular fluid for the future neuronal uptake and re-synthesis of LEU by a cytosolic BCAT, in a process consuming glutamate providing a mechanism for the "buffering" of glutamate if concentrations become excessive (Shank and Aprison, 1977). Therefore, in this scenario, high LEU or α-hydroxyisocaproate concentrations, as those seen in MSUD, which would exceeds 10- to 30-fold normal values (approximately 5 mM in blood and 0.6 mM in CSF; normal values: 0.7 and 0.007 mM; LEU brain tissue concentration is expected to be higher than in fluids; Wajner *et al.*, 2000) might lead to a depletion of glutamate and a consequent reduction in the concentration of brain glutamine, aspartate, alanine, and other amino acids (Wajner *et al.*, 2000; Yudkoff *et al.*, 2005).

On the other hand, it has been demonstrated that the training in the step–down test induces LTP in the hippocampus and that the memory acquisition and consolidation for this task may be modulated by drugs that affect glutamatergic neurotransmission and the LTP generation (Izquierdo *et al.*, 1997; Walz *et al.*, 1999, 2000). In this

context, LTP represents the acquisition and maintenance memories in a synaptic level (Bliss and Collingridge, 1993). Therefore, our results showing reduction on memory retention (reduced latency time) and blockade of LTP generation in hippocampal dentate gyrus, strongly indicate that LEU might interfere with the mechanisms involved in LTP generation and memory consolidation. Moreover, in addition to the known biochemical and electrophysiological mechanisms involved on LTP induction, such as dependence on NMDA receptors in hippocampal dentate gyrus, alternative mechanisms should be considered, including the effect of LEU on associated Ca^{2+} channels to NMDA glutamate receptor, changes on pre – synaptic glutamate release, the action of LEU on the excitability of the membrane of granule cells of dentate gyrus or gabaergic interneurons. All these alternatives may imply that hippocampal increased LEU concentrations would lead to cognitive impairments by modifying synaptic plasticity mechanisms such us LTP generation. In this context, new experiments need to be addressed to understand the mechanisms underlying LEU effects on memory and hippocampal function.

In addition, LEU is known to be involved also in the regulation of brain energy metabolism (Mastorodemos *et al.*, 2005). It has been demonstrated that LEU is a potent allosteric activator of the brain-specific isoform of glutamate dehydrogenase, increasing therefore, the Krebs cycle turn over (Erecinska and Nelson, 1990). Furthermore, previous studies have demonstrated that *in vitro* and *in vivo* brain exposure to increased LEU concentrations reduces the activity of creatine kinase (Pilla *et al.*, 2003a,b) and phosphate-activated glutaminase, respectively (Lellos *et al.*, 1991). Additionally, LEU-induced reduction of brain aspartate concentrations which might further result in an impairment of energy metabolism possibly because of a failure of the malate-aspartate shuttle (Yudkoff *et al.*, 2005). All these mechanisms might interplay and finally lead to brain energy adaptation, which could initiate a compensatory mechanism represented by mitochondrial biogenesis, in order to drive the brain metabolic demands, as previously hypothesized for the anticonvulsant effect of the ketogenic diet (Bough *et al.*, 2006). Indeed, LEU is a source of brain ketone bodies (Hamprecht *et al.*, 1995), and this is in agreement with our results demonstrating increasing hippocampal mitochondrial complex IV activity. In addition, compensatory increased respiratory chain activity

has also been involved with oxidative stress induction (Shigenaga, *et* al., 1994; Witte *et al*., 2009). This is in agreement with the studies performed by Fontella *et al.* (2002) and Bridi *et al*. (2003) demonstrating LEU-induced oxidative stress in rat brain, and with the putative role of ROS in the coordination of the mitochondrial genome and the expression of nuclear encoded mitochondrial genes (Li *et al*., 1995).

Taking together, high LEU hippocampal levels might modulate brain energy metabolism by inhibiting certain key energy enzymes, *i.e*. creatine kinase or the malate-aspartate shuttle related enzymes, and by accelerating the Krebs cycle turnover, which could in turn induce free radical generation thought α-ketoglutarate dehydrogenase, as previously reported (Starkov *et al*., 2004; Tretter and Adam-Vizi 2004). Thus, the LEU-elicited oxidative stress could induce further energy adaptations by increasing the concentration of the respiratory chain mitochondrial enzymes (increased complex IV activity) and augmenting the mitochondrial oxygen consumption with concomitant free radical generation. The probably LEU-induced glutamate depletion and enhanced mitochondrial oxidative stress could, therefore, contribute for interfering in hippocampal synaptic plasticity and consequently, compromising cognition in MSDU patients.

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Figure captions

Figure 1. Effect of leucine (LEU) intra-hippocampal administration on latency time in the step-down test. The animals received LEU (n=13) or artificial cerebrospinal fluid (ACSF; n=10). The latency of time (in seconds) was measured. The results are expressed as median (interquartile range). * $P < 0.05$.

Figure captions

Figure 2. Effect of leucine (LEU) intra-hippocampal administration on LTP generation in dentate gyrus. A) Hippocampal slice cartoon indicating the position of stimulation and recording electrodes. B) Field excitatory post synaptic potentials (fEPSP) example traces showing how measurements of EPSP and population spike amplitude are taken. C) fEPSP sample traces for LEU and ACSF groups before (full line) and after (dotted line) high frequency stimulation (HFS). D) Time course graph showing increments in fEPSP, as % of basal fEPSP, after HFS (100 Hz) in LEU and ACSF groups. Circles represent means ± S.E.M. Black arrow indicates time in which tetanus was delivered. Number of animals for each group is indicated in parenthesis. Significant differences were observed in the LEU group when comparing with the artificial cerebrospinal fluid (ACSF) injected animals (controls; One-way repeated measures analysis of variance; MANOVA).

Figure captions

Figure 3. Effect of leucine (LEU) intra-hippocampal administration on the activities of the respiratory chain complexes I, II and IV. Activities were assessed in hippocampal homogenates. Values are mean ± standard deviation from seven animals. * $P < 0.05$, compared to artificial cerebrospinal fluid (ACSF) injected animals (controls) (Student *t* test for independent samples).

Figure 1

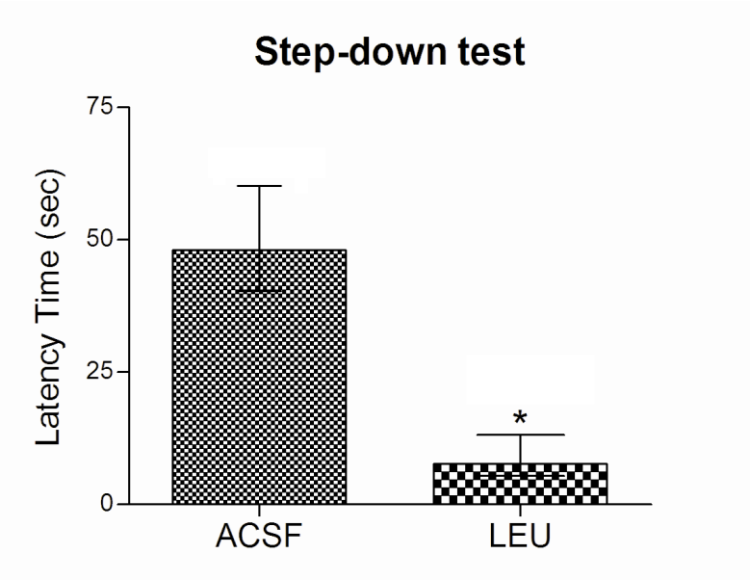


Figure 2

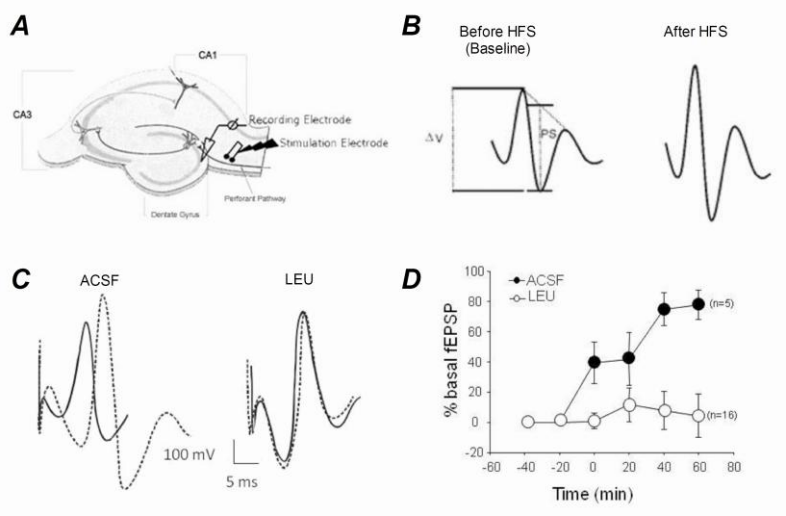
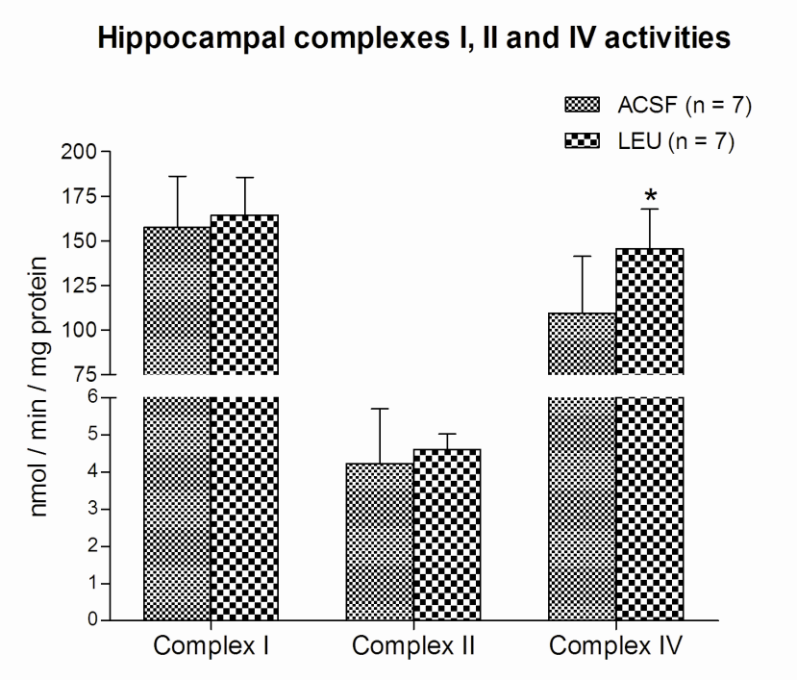

A
CA1
CA3
Recording Electrode
Stimulation Electrode
Perforant Pathway
Dentate Gyrus
B
Before HFS
(Baseline)
After HFS
ΔV
PS
C
ACSF
LEU
100 mV
5 ms
D
ACSF
LEU
(n=5)
(n=16)
% basal fEPSP
100
80
60
40
20
0
-20
-60
-40
-20
0
20
40
60
80
Time (min)

Figure 3


Hippocampal complexes I, II and IV activities
ACSF (n = 7)
LEU (n = 7)
nmol / min / mg protein
200
175
150
125
100
75
6
5
4
3
2
1
0
*
Complex I
Complex II
Complex IV

6. DISCUSSÃO

A existência de eucariontes, organismos pluricelulares, grandes genomas ou da vida, não seria possível sem a presença da mitocôndria (Lane, 2005). A aplicação de princípios de bioenergética no entendimento da fisiologia mitocondrial, bem como no papel fisiopatológico da mitocôndria em numerosas condições humanas encontram-se em crescimento constante. Neste sentido, podemos constatar desde o novo milênio, mais de quarenta e cinco mil publicações relacionando a palavra chave "mitocôndria" com os processos celulares que controlam a sobrevida e a morte celular, na biblioteca virtual www.pubmed.com.

A disfunção desta organela já foi demonstrada na fisiopatologia de processos neurodegenerativos crônicos, por exemplo, nas doenças de Parkinson, Hungtington e de Alzheimer (Fiskum et al., 1999, Lin and Beal, 2006), bem como em processos de neurotoxicidade aguda, incluindo as induzidas por toxicantes endógenos como o glutamato; nos acidentes cérebro-vasculares, isquemia ou trauma (Choi and Rothman, 1990); em erros inatos do metabolismo (Wajner et al., 2004, Latini et al., 2007), e em neurotoxicidade induzida por contaminantes ambientais como Hg, MeHg, zinco, alumínio, cobre, etc. (Sharpley and Hirst, 2006, Franco et al., 2009).

O cérebro é um dos órgãos metabolicamente mais ativos, requerendo duas vezes mais energia que o coração em repouso. Este tecido, rico em mitocôndrias, representa 2% da massa corporal do homem adulto e consome em torno de 20% do total de O_2 disponível para o organismo (Dickinson, 1996). Tendo em vista que a fosforilação oxidativa é responsável pela quase totalidade do ATP produzido no SNC, a regulação da respiração mitocondrial se torna essencial para o correto metabolismo energético cerebral (Erecinska et al., 1994).

Por outro lado, a mitocôndria é a principal fonte geradora de EROs em condições fisiológicas (Chance et al., 1979, Sipos et al., 2003). Esta produção basal de EROs é essencial para diversos processos celulares, incluindo fagocitoses e sinalização celular, entre outros (Del Maestro et al., 1981, Shapiro, 2003).

A produção ou acúmulo de EROs é aumentada quando a função da cadeia respiratória (CR) é prejudicada por mitotoxinas exógenas ou endógenas (Nicholls and Budd, 2000, Sipos et al., 2003, Okayama, 2005, Di Filippo et al., 2006), Neste contexto, o neurotoxicante e

poluente ambiental MeHg (Choi, 1989, Gilbert and Grant-Webster, 1995, Rice and Barone, 2000, Clarkson, 2002, Clarkson et al., 2003) tem como um dos principais alvos intracelulares a mitocôndria, causando alterações bioquímicas e ultraestruturais nesta organela (Dreiem et al., 2005). Além disso, tem sido demonstrado que a exposição ao MeHg induz estresse oxidativo (Yonaha et al., 1983, Farina et al., 2005, Franco et al., 2006, Franco et al., 2007, Stringari et al., 2008, Franco et al., 2009) e que esta condição estaria mediada por geração de EROs e por um grande consumo das concentrações de GSH, provavelmente vinculado ao caráter eletrofílico do mercurial (Aschner and Clarkson, 1988, Shanker et al., 2004, James et al., 2005, Franco et al., 2007, Franco et al., 2009). Desta forma, foi sugerido que a mitocôndria é o local de maior produção de espécies reativas induzida por este toxicante (Yoshino et al., 1966, Denny and Atchison, 1994, Mori et al., 2007).

EROs formado através da cadeia respiratória provocam uma redução da capacidade antioxidante tecidual, gerando um ciclo vicioso de auto-amplificação que pode resultar em danos celulares progressivos com inibição da CR e portanto, de comprometimento da respiração celular e síntese de energia (Turrens and Boveris, 1980, Turrens, 1997). Os principais componentes geradores de EROs na CR são os complexos I e III (Nicholls and Budd, 2000, Sipos et al., 2003). A atividade do complexo I controla o fluxo de elétrons através da CR possuindo desta forma uma função limitante na produção energética do organismo, já que é através deste complexo que os elétrons transportados pela coenzima reduzida, NADH, entram na CR. Ainda, sabe-se que as espécies reativas formadas no complexo I são liberadas para a matriz mitocondrial (Chen et al., 2003), causando dano oxidativo às enzimas mitocondriais, incluindo os complexos da cadeia transportadora de elétrons, enzimas do ciclo de Krebs e outras proteínas sensíveis a oxidação (Zhang et al., 1990, Hausladen and Fridovich, 1994). Desta forma, EROs formados a partir do complexo I poderão provocar inibições em outros complexos da CR, situação que foi observada neste estudo; a exposição ao MeHg também provocou inibição nas atividades dos complexos II, II-CoQ-III e IV.

Além da produção mitocondrial de EROs, outro mecanismo tóxico que pode ser responsável pelas inibições enzimáticas observadas, está relacionado com a estrutura dos complexos mitocondriais, os quais

contém em sua estrutura numerosos grupamentos tiólicos susceptíveis a dano oxidativo (Clementi et al., 1998, Beltran et al., 2000, Taylor et al., 2003, Chen et al., 2007). Por outro lado, não pode ser descartada a possibilidade de uma ligação direta do MeHg aos complexos da cadeia respiratória com conseqüente oxidação, como já foi demonstrado para outras enzimas (Hughes, 1957). Neste cenário, pode se propor que o severo comprometimento da produção energética cerebral pode ser um dos fatores desencadeadores de morte neuronal observados na intoxicação pelo MeHg.

No presente trabalho também foi demonstrado que outra enzima de fundamental importância para o metabolismo energético, a CK, também é inibida pela exposição ao MeHg. A CK é responsável tanto por produzir ATP (a partir de ADP e fosfocreatina), quanto de fornecer ADP (a partir de ATP e creatina), que é substrato para a ATPsintase formar o ATP utilizando o gradiente de prótons ($\Delta\psi$) formado durante a passagem de elétrons pela CR. Assim, a atividade da CK representa um intricado sistema celular de armazenamento e transferência de energia, conectando locais de captura de energia (mitocôndria) com locais de utilização da energia (citosol) (Hemmer and Wallimann, 1993, Brdiczka et al., 1994). A CK possui um resíduo cisteína no sítio catalítico susceptível à oxidação (Yuan et al., 1992). O grupamento tiólico é crítico para a união do substrato e se substituído, por exemplo, por uma serina, provoca uma queda na atividade da enzima em 500% (Kenyon, 1996). A falta de equilíbrio entre os sistemas energéticos, fosforilação oxidativa e CK, deve potencializar o déficit energético e a formação de espécies reativas na mitocôndria. Considerando que a energia é essencial para manter o desenvolvimento e a regulação das funções cerebrais, pode-se postular que alterações na atividade da CK provocadas pelo MeHg deve ser um passo fundamental nos mecanismos tóxicos que levam à neurodegeneração, como já tem sido proposto para outros processos neurotóxicos (Tomimoto et al., 1993, Wendt et al., 2002).

Por outro lado, o estudo *in vitro* em homogeneizados de córtex cerebral de camundongos demonstrou que a inibição da atividade da CK é dependente da concentração do MeHg e que acontece em paralelo com a queda nas concentrações de GSH, demonstrando assim uma forte inter-relação entre o sistema antioxidante e o correto funcionamento dessa enzima. Ainda, comparando as concentrações de MeHg que

provocam 50% de inibição (IC_{50}) da atividade da CK e de oxidação de GSH pode ser observado que a perda da inibição enzimática (50%) acontece a concentrações do mercurial que provocam uma pequena queda nas concentrações de GSH em aproximadamente 15%. Este efeito foi também demonstrado em células astrogliais (células C6), onde também foi evidenciado que a disfunção das desidrogenases mitocondriais (avaliado pelo método da redução do MTT) acontece em concentrações menores que as que induzem formação de EROs e inibição da atividade da CK, o que sugere que a disfunção da CR seria o passo chave na toxicidade do MeHg. Entretanto, não pode ser descartada a possibilidade de que a severa inibição da CK observada no tecido cerebral dos animais tratados com MeHg e nos sistemas *in vitro* seja também devida a alta reatividade do mercurial aos grupos tióis da enzima. Considerando que a constante de afinidade do MeHg pelos grupamentos tiólicos é aproximadamente $10^{10\text{-}16}$ (Onyido et al., 2004), qualquer tiol protéico ou livre, em pH fisiológico poderia ser um alvo molecular do MeHg.

O severo comprometimento no sistema energético cerebral induzido pelo MeHg foi também observado na análise ultra-estrutural através de microscopia eletrônica. A administração oral e crônica do mercurial provocou alterações morfológicas em numerosas mitocôndrias de preparações corticais, tanto em axônios quanto em corpos neuronais. A análise preliminar por microscopia eletrônica demonstrou a presença de grandes mitocôndrias com cristas e membranas internas alteradas, com acúmulo de material eletronicamente denso na matriz mitocondrial e inchaço, além de um maior número de mitocôndrias. Estas alterações são consistentes com uma inibição da respiração, e o maior número desta organela nos córtices cerebrais de animais tratados com MeHg devem refletir um mecanismo compensatório do deficiente metabolismo aeróbio induzido pelo mercurial (Figuras 1-4 de "Resultados adicionais").

O grande tamanho das mitocôndrias sugere que estas organelas poderiam ter sofrido fusão devido ao déficit energético celular e também para prevenir o acúmulo de DNA mitocondrial danificado, como demonstrado em outras condições de neurotoxicidade (Rapaport et al., 1998, Nakada et al., 2001, Ono et al., 2001), Por outro lado, o aumento no número de mitocôndrias também indica que em determinados momentos do processo de neurotoxicidade gatilhado pelo MeHg o

processo de fissão se sobrepõe ao de fusão. Sabe-se que a remodelação mitocondrial envolve a ativação de proteínas-chaves, a proteína 1 relacionada à dinamina (Drp1) e Fis1 responsáveis pela fissão e as mitofusinas (Mfn1 e Mfn2) e OPA1, envolvidas com a fusão de mitocôndrias (Mattson et al., 2008). Pode-se sugerir então que algumas proteínas se encontram temporalmente inibidas favorecendo um desses processos de remodelação mitocondrial. Desta forma, e considerando que o bloqueio da ação de Drp1 inibe a fragmentação mitocondrial, e que previne a perda do potencial de membrana mitocondrial e conseqüente liberação de citocromo c (Frank et al., 2001, Breckenridge et al., 2003, Lee et al., 2004, Cassidy-Stone et al., 2008), o aumento no tamanho (volume) mitocondrial pode ser um mecanismo de proteção prévio à indução de fissão mitocondrial, quando o déficit energético cerebral fica comprometido pela exposição ao mercurial. Entretanto, estudos relacionados à expressão e conteúdo destas proteínas na intoxicação pelo MeHg necessitam ainda serem elucidados, para o melhor entendimento da toxicidade deste toxicante sobre parâmetros de fusão/fissão mitocondrial.

As EROs formadas pela exposição ao MeHg além de oxidarem proteínas, como observado neste trabalho pela mensuração dos níveis de proteínas carboniladas, podem oxidar o DNA (Stohs and Bagchi, 1995, Jin et al., 2008, Grotto et al., 2009), tanto nuclear quanto mitocondrial (DNAmit). O DNAmit é mais susceptível a danos oxidativos, pois este não é protegido por histonas e não possui um sistema de reparo como o DNA nuclear. A significativa oxidação do DNA observada na intoxicação por MeHg (utilização de anticorpo anti-8-hidroxi-2'-deoxiguanosina) é consistente com um aumento na produção de EROs. A oxidação do DNA ocasiona mutações no genoma, podendo levar à diferenças na expressão de proteínas-chave para o funcionamento celular. O DNAmit codifica treze das proteínas da CR, e como este é mais susceptível ao dano devido aos mecanismos descritos acima, a expressão errônea dos complexos da CR também pode ser sugerida como um dos mecanismos que afetaria o metabolismo energético no tecido cerebral.

Além disso, observamos que todos os mecanismos desencadeados pelo MeHg, incluindo inibição da cadeia respiratória e da CK, prejuízo no sistema antioxidante e o dano oxidativo à proteínas e ao DNA, levam

a um processo de neurodegeneração, como verificado pela técnica de FluoroJade B.

Compostos contendo Se têm sido estudados nos últimos anos como terapia neuroprotetora à intoxicação por MeHg (Nogueira et al., 2004), pois o Se é um nutriente essencial necessário para a síntese e atividade de aproximadamente vinte e cinco enzimas dependentes de Se, incluindo a glutationa peroxidase (GPx) (Flohe et al., 1973, Forstrom et al., 1978, Islam et al., 2002), a tioredoxina redutase (Holmgren, 1989, Arner and Holmgren, 2000) e outras selenoproteínas que modulam o estado redox e antioxidante das células (Saito et al., 1999, Bianco et al., 2002, Panee et al., 2007). Desta forma, o potencial efeito protetor de dois compostos contendo Se, Na_2SeO_3 e $(PhSe)_2$, foram investigados neste trabalho. O $(PhSe)_2$, um composto orgânico de Se, demonstrou um potencial efeito protetor contra os efeitos do MeHg relacionados às inibições dos complexos da CR, da CK, da indução de estresse oxidativo e neurodegeneração. O efeito protetor do $(PhSe)_2$ provavelmente está vinculado à reatividade do átomo de selênio pelo MeHg, formando um composto inerte, o HgSe (Iwata et al., 1982, Bjorkman et al., 1995). Além disso, pode ser devido a sua já descrita atividade tiol-peroxidase baseada na habilidade de formar um intermediário selenol que pode conseqüentemente decompor peróxidos inorgânicos e lipídicos (Nogueira et al., 2004, de Bem et al., 2008, Posser et al., 2008, de Freitas et al., 2009).

Por outro lado, o Na_2SeO_3, não se demonstrou eficaz em prevenir a maioria das alterações neuroquímicas induzidas pelo mercurial. Ainda, o Na_2SeO_3 *per se* diminuiu as atividades de todos os complexos da CR mensurados, da CK e provocou estresse oxidativo. Estes efeitos pró-oxidantes do Na_2SeO_3 devem estar relacionados com o seu metabolismo, visto que durante a liberação de Se para posterior incorporação em selenoproteínas o Na_2SeO_3 oxida cataliticamente GSH, provocando estresse oxidativo por perda da capacidade antioxidante celular (Painter, 1941, Ganther, 1968, Seko, 1989, Seko Y, 1989, Farina et al., 2003a, Nogueira et al., 2004, Stringari et al., 2006, Stringari et al., 2008).

Embora os dois compostos de Se não se demonstraram igualmente neuroprotetores, eles tiveram como característica comum a capacidade de reduzir a deposição do mercurial no cérebro (autometalografia, método de Timm), provavelmente devido à alta afinidade de seus grupos selênio com o Hg (10^{45} M) (Dyrssen and

Wedborg, 1991). Sabe-se que o Se conjuga o Hg formando um sal inerte, que é principalmente representado pelo HgSe (Iwata et al., 1982, Bjorkman et al., 1995). Além disso, a redução da deposição do mercurial no cérebro deve estar também relacionada à formação deste complexo HgSe na corrente sangüínea, como previamente demonstrado por Naganuma e Imura (Naganuma and Imura, 1980).

Desta forma, temos demonstrado que a disfunção mitocondrial associada a estresse oxidativo está envolvida nos processos tóxicos induzidos pelo contaminante ambiental MeHg, e que o uso de compostos de Se, principalmente $(PhSe)_2$, previne essas alterações neuroquímicas.

Ainda, neste trabalho foi investigado o efeito da administração aguda intra-hipocampal de altas concentrações de leucina em ratos Wistar adultos. Leucina é um aminoácido ramificado que acumula nos tecidos e nos fluídos biológicos dos indivíduos afetados pelo erro inato do metabolismo denominado de DXB. Os níveis plasmáticos de leucina ou do seu metabólito α-cetoisocaproato, considerados os principais metabólitos neurotóxicos, variam de 0,5 a 5 mM, sendo que os níveis sanguíneos normais da leucina são de 10 a 50 vezes menores (Snyderman et al., 1964, Efron, 1965, Chuang et al., 2001)

A forma clássica da DXB se caracteriza por sintomas severos na primeira semana de vida. Quando não tratada, leva a maioria dos pacientes ao óbito ou então a seqüelas neurológicas irreversíveis. Aqueles que apresentam variantes menos severas da doença (atividade deficiente da enzima entre 2 e 40%) apresentam atraso no desenvolvimento psicomotor e severo retardo mental, porém uma menor incidência de convulsões e outros achados neurológicos (Chuang et al., 2001). O diagnóstico e o tratamento precoces podem resultar na prevenção dessas manifestações neurológicas (Chuang et al., 2001).

Alguns mecanismos têm sido descritos para entender a fisiopatologia dos sintomas neurológicos apresentados por estes pacientes. Encontra-se, na literatura, dados experimentais consideráveis que demonstram que ácidos orgânicos acumulados em várias acidúrias orgânicas induzem a geração de EROs e diminuem as defesas antioxidantes *in vitro*. A maioria destes efeitos ocorre em concentrações próximas daquelas detectadas no plasma ou líquor dos pacientes, enquanto outros são observados somente em concentrações suprafisiológicas (Wajner, 2004). Fontella e colaboradores (Fontella et

al., 2002, Bridi et al., 2003) demonstraram que os metabólitos acumulados na DXB aumentaram a peroxidação lipídica em cérebro de ratos e consomem as defesas antioxidantes celulares. Embora, em grande parte deste tipo de doenças metabólicas, os níveis cerebrais dos metabólitos acumulados não sejam conhecidos, não se exclui a possibilidade de que a concentração cerebral esteja próxima daquelas detectadas no líquor dos pacientes (Hoffmann et al., 2004) Todavia, um estudo demonstrou que as concentrações dos aminoácidos e ACCR em cérebro de pacientes, estimadas através de espectroscopia de ressonância magnética de prótons (H^1-MR) atingem um terço da encontrada no plasma, em torno de 0,9 mM (Heindel et al., 1995).

No presente estudo investigou-se o efeito da administração aguda intra-hipocampal de leucina sobre a atividade da CR e sobre parâmetros comportamentais e de eletrofisiologia em ratos Wistar adultos. A dose utilizada de leucina foi próxima das concentrações detectadas na DXB (80 nmol leucina/hipocampo; 160 nmol/animal; concentração final aproximada de 1,5 mM no hipocampo). Estas concentrações hipocampais de leucina mimetizariam uma das condições bioquímicas (acúmulo tecidual de leucina) que acontece durante uma crise de descompensação metabólica característica dos pacientes com DXB.

Foi observado que este tratamento provoca adaptações rápidas que levam a um aumento da atividade do complexo IV no hipocampo, mas que por outro lado, compromete a consolidação da memória, fenômeno observado através de uma diminuição no tempo de latência no teste comportamental de esquiva inibitória "*step down"* e pela inibição da geração do LTP (parâmetro eletrofisiológico) no hipocampo.

Sabe-se que glutamato e hipocampo apresentam um papel essencial na formação e consolidação da memória (Squire, 1992, Izquierdo and Medina, 1997, Tracy et al., 2001). O glutamato medeia a maioria da neurotransmissão excitatória no SNC de mamíferos que é necessária nos processos de neuroplasticidade, memória e aprendizado, e também na formação de redes neurais durante o desenvolvimento (Erecinska and Silver, 1990, Meldrum and Garthwaite, 1990, Ozawa et al., 1998). Vários investigadores têm demonstrado que o teste comportamental de esquiva inibitória empregado para avaliar a consolidação da memória (*step down*) pode ser modulado pela administração de drogas que afetam a transmissão glutamatérgica e/ou a geração do LTP (Izquierdo and Medina, 1997, Walz et al., 1999, Walz

et al., 2000). Portanto, nossos resultados demonstrando prejuízos na retenção da memória sugerem que altas concentrações de leucina devem ser responsáveis pelas alterações neurológicas vinculadas ao hipocampo, como o característico retardo mental dos indivíduos afetados pela DXB.

A síntese cerebral de glutamato depende da captação cerebral de leucina (Erecinska and Silver, 1990, Yudkoff et al., 1993) pois este é o principal aminoácido ramificado doador de nitrogênio para o grupamento amino do glutamato (Zielke et al., 1996, Yudkoff, 1997) e por pouco glutamato ou glutamina passarem da periferia para o SNC (Grill et al., 1992, Smith, 2000). Após ocorrer a captação da leucina plasmática pelos astrócitos, esta é rapidamente metabolizada a α-hidroxiisocaproato pela enzima mitocondrial aminotransferase de cadeia ramificada, assim o grupamento amino pode ser usado para a síntese de glutamato e glutamina (Yudkoff, 1997). O α-hidroxiisocaproato é então liberado no fluido intercelular, assim podendo ser captado pelos neurônios e utilizado para re-síntese de leucina por uma aminotransferase de cadeia ramificada citosólica, envolvendo a utilização de glutamato provendo assim um mecanismo de tamponamento do glutamato se suas concentrações se tornarem excessivas (Shank and Aprison, 1977). Entretanto, altas concentrações de leucina ou α-hidroxiisocaproato, como observadas na DXB podem levar a uma depleção de glutamato e uma conseqüente redução na concentração de glutamina, aspartato, alanina e outros aminoácidos no cérebro (Wajner et al., 2000, Yudkoff et al., 2005a). Isto pode implicar que concentrações aumentadas de leucina no hipocampo durante uma crise metabólica levariam a deficiências cognitivas por inibir a geração do LTP como uma conseqüência, talvez transitória, de baixas concentrações de glutamato a nível sináptico.

Por outro lado, a leucina parece estar envolvida na regulação do metabolismo energético celular (Mastorodemos et al., 2005). Leucina é um potente ativador alostérico da isoforma da glutamato desidrogenase cerebral, assim aumentando o *turnover* do ciclo de Krebs (Erecinska and Nelson, 1990). Estudos *in vitro* e *in vivo* tem demonstrado que concentrações aumentadas cerebrais de leucina reduzem a atividade da enzima creatina cinase (Pilla et al., 2003a, Pilla et al., 2003b) e da glutaminase (Lellos et al., 1991). Adicionalmente, o aumento da concentração de LEU parece prejudicar a regulação das concentrações de aspartato com conseqüente redução da atividade do sistema de

lançadeira malato-aspartato, prejudicando o metabolismo energético mitocondrial (Yudkoff et al., 2005a). Todos estes mecanismos bioquímicos possivelmente se encontram interligados e cooperam entre si para adaptar ao tecido cerebral a deficiências no metabolismo energético. Esta adaptação poderia estar representada pela indução da biogênese mitocondrial, aumentando o número de mitocôndrias (aumento da atividade do complexo IV) a fim de atender às demandas energéticas, como já hipotetizado para o efeito anticonvulsionante da dieta cetogênica, visto que a leucina é uma fonte de corpos cetônicos (Bough et al., 2006). O mecanismo compensatório de biogênese mitocondrial tem sido também demonstrado em situações de estresse oxidativo (Shigenaga et al., 1994, Witte et al., 2009), o que está de acordo com estudos prévios na literatura onde foi demonstrado que o acúmulo de leucina induz peroxidação lipídica e consumo de GSH (Fontella et al., 2002, Bridi et al., 2003). Ainda, foi postulado que a expressão de genes nucleares que codificam para proteínas da CR estão coordenados pela produção de EROs (Li et al., 1995).

Em conjunto, este grupo de resultados sugere que o acúmulo de leucina no hipocampo deve modular o metabolismo energético, por provocar uma depleção das concentrações de glutamato secundário ao aumento de leucina, que gatilharia por um lado a produção de EROs pela mitocôndria, e que por outro comprometeria a plasticidade hipocampal e portanto a cognição dos pacientes afetados pela DXB.

7. CONCLUSÕES

Quanto à toxicidade *in vivo* do MeHg em córtex cerebral de camundongos Swiss adultos:

- O MeHg causa prejuízo energético celular pois inibe a atividade dos complexos I, II, II-CoQ-III IV da CR, além de inibir a atividade da enzima CK;
- O toxicante MeHg provoca estresse oxidativo por alterar a atividade de enzimas antioxidantes (inibição e aumento das atividades da GPx e GR, respectivamente), aumentando a peroxidação lipídica e o dano oxidativo ao DNA, além de levar à neurodegeneração;
- O MeHg altera os processos de fussão/fissão mitocondrial, além de alterar a morfologia mitocondrial

- O $(PhSe)_2$ foi capaz de proteger contra os danos causados pelo MeHg, relacionados à atividade dos complexos da CR, à atividade da CK, além disso, à indução de peroxidação lipídica, do dano oxidativo ao DNA e à neurodegeneração desencadeados pela administração do MeHg;
- O Na_2SeO_3 parcialmente protegeu contra os efeitos tóxicos do MeHg relacionados às atividades dos complexos II-CoQ-III e IV da cadeia respiratória e da inibição da CK, no entanto, na dose utilizada *per se* aumentou a lipoperoxidação, prejudicou a atividade dos complexos I, II-CoQ-III e IV da cadeia respiratória, inibiu a atividade da enzima CK, e alterou a atividade do sistema GPx/GR;
- O $(PhSe)_2$ e o Na_2SeO_3foram eficazes em evitar a deposição do mercurial no tecido cerebral, observado pelo método da autometalografia;

Quanto à toxicidade do MeHg em sistemas experimentais *in vitro*:

- O MeHg *in vitro* compromete o sistema energético e antioxidante por inibir a atividade da enzima CK e por oxidar o antioxidante GSH e as proteínas celulares (carbonilação protéica) em homogeneizados de córtex cerebral de camundongos Swiss adultos;
- O efeito tóxico *in* vitro do MeHg sobre o metabolismo energético e sobre a produção de ERs foi verificado também em cultura de células C6.

Quanto à toxicidade *in vivo* da leucina em hipocampo de ratos Wistar adultos:

- Altas concentrações do aminoácido ramificado leucina readaptam o sistema energético cerebral por modificar a atividade da cadeia transportadora de elétrons;
- Altas concentrações hipocampais de leucina causam déficit de memória por inibir a formação da potenciação em longo prazo (LTP; eletrofisiologia) e demonstrado pelo por aumentar no tempo de latência no teste comportamental de esquiva inibitória (*step down*).

8. CONCLUSÃO GERAL

A exposição aos toxicantes MeHg e LEU (altas concentrações hipocampais) prejudicam a atividade mitocondrial e geram estresse oxidativo, por prejudicar diretamente a função desta organela ou por diminuir os níveis de defesas antioxidantes no tecido cerebral, comprometendo desta forma os processos celulares de detoxificação de EROs. Assim, podemos considerar que tanto os toxicantes endógenos quanto os ambientais compartilham mecanismos de neurotoxicidade que envolvem a disfunção mitocondrial (Figura 7).

9. PERSPECTIVAS

- Analisar o conteúdo das proteínas caspase-3 e Bcl-2, a fim de investigar a regulação temporal do processo apoptótico durante a exposição crônica ao MeHg (7-28 dias) em cérebro de camundongos Swiss adultos.

- Analisar o conteúdo de GFAP, a fim de investigar gliose reativa em diferentes tempos de exposição crônica *in vivo* a MeHg (7-28 dias) em cérebro de camundongos Swiss adultos.

- Realizar o estudo de morfologia ultraestrutural em mitocôndrias de cérebro de camundongos Swiss adultos expostos cronicamente ao MeHg (7-28 dias).

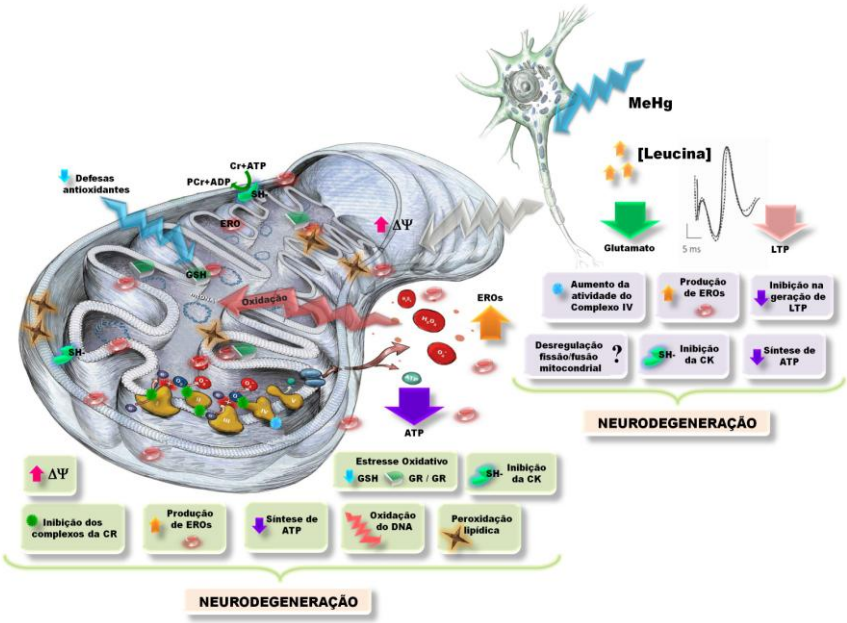


Figura 7. Possíveis mecanismos de toxicidade induzidos pelos toxicantes metilmercúrio (MeHg) e leucina.
MeHg e leucina compartilham mecanismos de neurotoxicidade que envolvem disfunção mitocondrial:
1) Inibição ou estimulação dos complexos da cadeia respiratória (CR)
2) Inibição da atividade da creatina cinase (CK)
3) Inibição da síntese de ATP
4) Aumento ou perda do potencial de membrana mitocondrial (ΔΨ)
5) Aumento da produção de espécies reativas do oxigênio (EROs)
6) Diminuição da capacidade antioxidante celular
7) Peroxidação de lipídeos de membrana
8) Oxidação de DNA mitocondrial e nuclear
9) Desregulação dos processos de fissão / fusão mitocondrial
GSH: glutationa; GPx: glutationa peroxidase; GR: glutationa redutase; Cr: creatina; PCr: fosfocreatina

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