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MECANISMOS DE CAPTAÇÃO E TOXICIDADE DO METILMERCÚRIO: ENVOLVIMENTO DO SISTEMA GLUTAMATÉRGICO E DO CÁLCIO EM FATIAS E MITOCÔNDRIAS DE RATOS.

TESE DE DOUTORADO

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Santa Maria, RS, Brasil
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por
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"O Homem que tenta descobrir a complexidade do universo, apenas vaga pelo mundo a procura da simplicidade da sua própria essência."

"A certeza é a sabedoria dos tolos
incapazes de imaginar outra forma de pensar".

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica Toxicológica
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Mecanismos de captação e toxicidade do metilmercúrio: Envolvimento do sistema glutamatérgico e do cálcio em fatias e mitocôndrias de ratos.

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LOCAL E DATA DA DEFESA: Santa Maria, Novembro de 2011.

O metilmercúrio (MeHg) é um contaminante ambiental altamente tóxico que pode ser bioacumulado em diferentes tecidos e consequentemente induzir disfunções celulares em diversos órgãos, especialmente no sistema nervoso central (SNC). Os mecanismos pelos quais o mercúrio (Hg) entra nos tecidos, se acumula e causa toxicidade em células e organelas ainda não se encontram totalmente elucidados. No entanto, estudos têm sugerido que a neurotoxicidade mediada pelo MeHg envolve alterações no sistema glutamatérgico e na homeostase do cálcio. Por outro lado, sabe-se que o cálcio (Ca^{2+}) *per se* causa efeitos tóxicos nas células e principalmente em mitocôndrias. Assim o Hg e o Ca^{2+} podem induzir efeitos distintos ou interligados, os quais geralmente culminam com disfunção mitocondrial e morte celular. Com base nestes parâmetros, o presente trabalho visa elucidar os possíveis mecanismos moleculares envolvidos na captação e no acúmulo de Hg em fatias hepáticas expostas a forma livre de $MeHg^{+}$ ou à forma complexada MeHg-Cisteína (MeHg-Cys), bem como, relacionar estes aspectos com a toxicidade celular e mitocondrial causada por ambas as formas de MeHg. Também foi alvo deste estudo a investigação do papel do sistema glutamatérgico na toxicidade mediada pelo MeHg em fatias de córtex cerebral de ratos e os efeitos do cálcio em mitocôndrias isoladas e sustentadas com diferentes substratos energéticos. Com relação à captação de Hg, nossos resultados mostram que fatias expostas ao complexo MeHg-Cys acumulam mais Hg que fatias expostas ao MeHg sozinho. Também foi observado que o pré-tratamento (15 min) com metionina (Met) reduziu significativamente a captação e o acúmulo de Hg nas fatias. Resultados similares foram verificados em mitocôndrias isoladas dessas fatiais. Nos parâmetros bioquímicos: geração de radicais livres, consumo de oxigênio e viabilidade celular/atividade mitocondrial, o complexo MeHg-Cys causou efeitos mais tóxicos quando comparado com o MeHg sozinho. O pré-tratamento com Met foi efetivo em prevenir as alterações mediadas por ambas as formas de mercúrio testadas. Os efeitos neurotóxicos do MeHg em fatias de córtex cerebral foram observados apenas nas maiores concentrações usadas e após 2 ou 5 horas de exposição. A utilização de compostos que possivelmente modulam a homeostase glutamatérgica (MK-801, guanosina e compostos orgânicos de selênio) preveniram os efeitos induzidos por MeHg na geração de radicais livres. Os efeitos do cálcio foram analisados em mitocôndrias isoladas e sustentadas com o substrato energético do complexo mitocondrial I: Malato/Glutamato (Mal/Glu) e do complexo II Succinato (Succ). Os resultados obtidos mostram que o cálcio inibiu a atividade complexo I; mas não alterou a atividade complexo II. Em baixas concentrações o cálcio causou uma pequena diminuição no potencial de membrana ($\Delta\Psi m$) nas mitocôndrias sustentadas com Mal/Glu ou Succ. Por outro lado, uma perda total do potencial foi observada quando as mitocôndrias, sustentadas com ambos os substratos, foram expostas a altas concentrações de cálcio. A exposição ao cálcio causou uma redução no potencial redox (conteúdo NADP(H) e GSH) e no consumo de oxigênio nas mitocôndrias sustentadas com Succ quando comparado com as que receberam Mal/Glu. A geração de radicais livres foi aumentada em mitocôndrias expostas ao cálcio e sustentadas com Succ quando comparadas com as mitocôndrias sustentadas com substrato do complexo I. De forma geral, nossos resultados colaboram para a elucidação dos mecanismos moleculares envolvidos na citotoxicidade induzida por MeHg e por cálcio, bem como para um melhor entendimento sobre o papel destes elementos na indução de disfunção mitocondrial. Além disso, os resultados obtidos poderão contribuir para a descoberta de novos agentes terapêuticos usados para prevenir ou minimizar danos teciduais associados à intoxicação humana por MeHg.

Palavras-chave: Metilmercúrio; sistema glutamatérgico; mitocôndrias; cálcio.

ABSTRACT

Thesis of Doctor's Degree
Federal University of Santa Maria, RS, Brazil

Mechanism of uptake and toxicity of methylmercury: Involvement of glutamatergic system and calcium em fatias e mitocôndrias de ratos.

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DATE AND PLACE OF THE DEFENCE: Santa Maria, November 2011.

Methylmercury (MeHg) is a highly toxic environmental contaminant that can be accumulated on several tissues, inducing cellular dysfunction on various organs, especially in the central nervous system (CNS). The mechanisms involved in the uptake, accumulation and toxicity of mercury (Hg) remain unclear. However, it has been suggested that the neurotoxicity mediated by MeHg induces changes in both glutamatergic system and calcium homeostasis. Indeed, the literature reports that calcium *per se* is able in inducing cellular damage. Thus, mercury and calcium can exert distinct or linked toxic effect that lead to mitochondrial and cellular dysfunction. The purpose of this study was to analyze the mechanisms of uptake and accumulation of mercury on liver slices exposed to MeHg or MeHg-Cysteine complex, as well as to compare the mitochondrial and cellular toxicity caused by both forms of MeHg. Moreover, this work examined the role of glutamatergic system on the toxicity mediated by MeHg in slices of cerebral cortex from rats and the effects of calcium exposure in mitochondria sustained with different energetic substrates. Our results showed that the mercury uptake was higher in the slices exposed to the MeHg-Cys complex that MeHg exposed slices. Indeed, the pretreatment with methionine (Met) (for 15 min.) reduced Hg uptake in liver slices. Likewise, mitochondria isolated of liver slices showed similar effect on Hg uptake. Parameters as free radical generation; oxygen consumption and mitochondrial function/Cell viability were more affected by MeHg-Cys complex than MeHg alone. Met pre-treatment was effective in preventing the MeHg or MeHg-Cys-induced toxicity in both liver slices and mitochondria. In cortical brain slices, the neurotoxicity induced by MeHg was verified only at higher concentration tested and after 2 or 5 hours of exposure. In addition, compounds that potentially modulate glutamatergic system (MK-801, guanosine, organo selenium compounds) were effective in preventing the MeHg-induced free radical generation. In mitochondria isolated from liver, Ca^{2+} caused an inhibition on the complex I activity; however, calcium did not alter the mitochondrial complex II activity. At low concentration, calcium exposure caused a small decrease in the mitochondrial membrane potential ($\Delta\Psi m$) in Malate/Glutamate (Ma/Glu) and Succinate (Succ)-supported mitochondria. On the order hand, higher calcium levels were associated with a total $\Delta\Psi m$ loss in both Mal/Glu and Succ-oxidizing mitochondria. The mitochondrial redox state (NADP(H) and GSH pool) and oxygen consumption were extremely affected by calcium exposure only in Succ-supported mitochondria. Indeed, calcium caused an increase in free radical generation in mitochondria sustained with complex II substrate when compared to Mal/Glu-supported mitochondria. Taken together, our data collaborate to understanding the molecular mechanisms involved on the toxicology of mercury and calcium and consequently provide basis for further investigations on the role of Ca^{2+} and mercury in mitochondrial dysfunctions. In addition, the results obtained here may contribute to the discovery of new therapeutic agents capable of preventing or minimizing the damage induced by MeHg intoxication.

Keywords: Metilmercury, glutamatergic system, mitochondria, calcium.

SUMARIO

1. INTRODUÇÃO

O MeHg é uma forma orgânica de mercúrio encontrada principalmente em ambientes aquáticos, sendo sua origem geralmente antropogênica. A sua concentração no meio aquático é em torno de ng/L, porém como esta forma de Hg pode ser bioacumulada, concentrações relativamente altas (mg/Kg) são encontradas em peixes da extremidade de cadeia alimentar. Estudos têm demonstrado que o MeHg pode ser bioacumulado em diferentes tecidos e consequentemente induzir severas disfunções celulares em muitos órgãos, especialmente no sistema nervoso central (Chang 1980; Clarkson 1997). No entanto, os mecanismos exatos pelos quais o mercúrio entra nos tecidos e se acumula em células e organelas como, por exemplo mitocôndrias, ainda precisam ser elucidados. A similaridade do complexo MeHg-Cys com o aminoácido Met é, em termos de mecanismos, uma das explicações mais especuladas para a entrada de Hg no interior das células e organelas (Roos e cols., 2010). De acordo, estudos preliminares do nosso grupo indicam que a captação e o consequente acúmulo de Hg nos tecidos é facilitado pela formação do complexo MeHg-Cys (mimético ao aminoácido Met) e que a captação deste complexo em nível celular pode ocorrer via transportador de aminoácidos neutros do tipo L (LAT) (Yin e cols., 2008; Roos e cols., 2010). Existem vários subtipos de transportadores de aminoácidos do tipo L (LAT1, LAT2, LAT3), os quais estão distribuídos em vários órgãos e tecidos, como no rim, na placenta, no cérebro, no intestino e no fígado (Palacin e cols., 1998; Kanai e Endou., 2001). Em organelas, os dados da literatura têm relacionado a toxicidade induzida por Hg com a presença de transportadores tipo L nas mitocôndrias (Raymond e cols., 1977). No entanto a função fisiológica e a precisa localização sub-celular destes transportadores ainda não foram bem determinadas. Além da Cys, outro modulador da entrada de mercúrio na célula é o aminoácido Met. Trabalhos na literatura e do nosso grupo sugerem que a Met pode diminuir a captação de MeHg pelas células, principalmente quando este encontra-se complexado com a Cys (MeHg-Cys) (Faro e cols., 2005; Yin e cols., 2008). Em termos de mecanismo(s) molecular(s), é sensato assumir que este efeito da Met esteja associado com a similaridade estrutural ao complexo (MeHg-Cys), e que a Met possa estar atuando como um inibidor competitivo do transporte de (MeHg-Cys) através do sistema L de transporte de aminoácidos (Bridjesand e Zalups., 2005).

Embora os mecanismos pelo qual o MeHg exerça efeitos tóxicos não estejam completamente compreendidos, sugere-se que um aumento na geração de radicais livres como peróxido de hidrogênio, radical superóxido e/ou ainda radical óxido nítrico contribuam

diretamente para os efeitos citotóxicos induzidos pela exposição ao MeHg (Aschner e cols., 2004; 2007; Farina e cols., 2011). Especialmente no sistema nervoso central, estudos têm demonstrado que o aumento do estresse oxidativo induzido pelo MeHg pode envolver alterações na homeostase glutamatérgica (Dalla Corte e cols., 2011), desequilíbrio na homeostase do cálcio (Castoldie e cols., 2001), depleção nos níveis de glutationa (GSH) (Ou e cols., 1999), bem como, disfunção mitocondrial (Seegal e Dreiem 2007). Embora estes eventos representem, muitas vezes, situações distintas parece haver uma relação entre os mesmos. De fato, o aumento de glutamato na fenda sináptica, provavelmente promovido pela diminuição na captação do glutamato pelos astrócitos (Aschner e cols., 1999), causa uma super ativação principalmente dos receptores glutamatérgicos do tipo N-metil D-aspartato (NMDA) e o influxo de Ca^{2+} e Na^{+} intracelular (Choi., 1992), os quais podem levar a uma disfunção mitocondrial.

A citoxicidade oriunda do aumento dos níveis de cálcio está fortemente associada com disfunção mitocondrial, uma vez que as mitocôndrias são aptas a aumentar significativamente a captação de Ca^{2+} quando os níveis intracelulares elevam-se (Sparagna e cols., 1995). O excesso de cálcio mitocondrial pode causar despolarização, aumento na formação de espécies reativas de oxigênio (ERO), inchaço mitocondrial e abertura do poro de transição mitocondrial (PTM) fatores que estão direta ou indiretamente envolvidos com a indução de morte celular (Guitteridge e Halliwell., 2000; Seegal e Dreiem., 2007). Neste contexto, o uso de agentes quelantes de cálcio pode prevenir ou reduzir a morte neuronal, incluindo as induzidas por exposição ao Hg (Marty e Atchison., 1997). Da mesma forma, todos esses eventos isolados ou interligados podem contribuir para o aparecimento dos vários distúrbios teciduais que estão associados à contaminação com MeHg (Clarkson., 2002).

Assim a realização de experimentos que investiguem os efeitos de agentes moduladores sobre a captação, o transporte e efeitos tóxicos do MeHg, quando este se apresenta na forma complexada ou forma livre, bem como, trabalhos que explorem os efeitos do cálcio em termos de mecanismos são de extrema importância para a obtenção de um melhor entendimento acerca da toxicologia do MeHg.

2. DESENVOLVIMENTO

2.1 Mercúrio

O mercúrio desde os tempos da Grécia antiga é manipulado e utilizado para diversos fins industriais, artísticos e medicinais. Por essa razão, este elemento foi estudado por alquimistas dos tempos antigos e por cientistas dos tempos modernos. O mercúrio é um metal líquido a temperatura ambiente obtido através da ustulação de sulfetos e outros minerais (Câmera e cols., 2002). Este metal apresenta uma reatividade relativamente baixa e pode ser encontrado em diferentes compostos químicos.

2.1.1 Formas de mercúrio

2.1.1.1 Mercúrio metálico:

O mercúrio metálico ou elementar (Hg^0) é relativamente estável e pode ser utilizado em diversos processos como, por exemplo, na confecção de termômetros, em amalgamas dentárias, no processo de purificação e extração de ouro e em uma variedade de produtos industriais e domésticos. Apesar da baixa reatividade, o mercúrio metálico apresenta uma alta capacidade de volatilização sendo capaz de formar vapor de mercúrio, o qual pode ser facilmente absorvido via respiração. Uma vez absorvido, essa forma lipossolúvel de Hg pode atravessar facilmente a barreira hematoencefálica, placentária e biomembranas, onde pode ser reduzido à Hg^{2+} e acumulado por vários anos em diversos órgãos tecidos (Braunwald e cols., 2001; Hargreaves e cols., 1988; Takeuchi e cols., 1989). Essas propriedades caracterizam o mercúrio como um elemento bioacumulativo.

2.1.1.2 Sais de mercúrio inorgânico:

Os sais de mercúrio inorgânico podem ser formados naturalmente a partir de formas orgânicas, as quais liberam íons Hg^{2+} ou Hg^{+} (Wood e cols., 1968). Entretanto, a maior parte do mercúrio inorgânico encontrada na natureza é de origem antropogênica e derivada de dejetos industriais (Hargreaves e cols., 1988). Os principais sais de Hg incluem: 1) *Fulminato* ($Hg(CNO)_2$), o qual é utilizado como detonante e seus resíduos são altamente corrosivos e tóxicos; 2) *Cloreto de mercúrio I* (Hg_2Cl_2), forma bastante usada antigamente como purgante, anti-helmíntico e diurético; 3) *Cloreto de mercúrio II* ($HgCl_2$), o qual é empregado como desinfetante e foi o primeiro medicamento eficaz usado contra a sífilis e 4) *Sulfeto de mercúrio* (HgS), utilizado em aparatos elétricos, na ortodontia, etc (Ozuah., 2000).

De uma forma geral os sais inorgânicos de Hg não são voláteis a temperatura ambiente, mas são facilmente absorvidos pela pele ou por ingestão (Clarkson., 2002). As formas iônicas são pouco liposolúveis e não atravessam as membranas biológicas facilmente. Como pouco Hg^{+} entra nas células, os principais sítios de toxicidade dos sais de mercúrio são o meio extracelular e as membranas biológicas (Takeuchi e cols., 1989; Friberg e Mottet., 1989).

2.1.1.3 Mercúrio orgânico:

O metilmercúrio (CH_3Hg) é considerado a principal forma orgânica de mercúrio encontrada na natureza. Esta forma de mercúrio é gerada principalmente através da biometilação do mercúrio metálico proveniente das indústrias mineradoras, as quais utilizam uma grande quantidade de Hg nos processos de purificação do ouro (Hargreaves e cols., 1988). A metilação do Hg ocorre de uma forma relativamente simples. Uma vez presente no meio ambiente, o mercúrio elementar é facilmente incorporado por bactérias e organismos unicelulares, sendo então ionizado através de uma reação que envolve a enzima catalase e o peróxido de hidrogênio. No entanto, como o mercúrio iônico é prejudicial a esses organismos, o mesmo, através da metilação, é transformado em MeHg que é facilmente eliminado por ser lipossolúvel (Wasserman e cols., 2001). A metilação do mercúrio ocorre pela transferência de um ou dois grupos metilcarboânions (CH_3^{-}) ao mercúrio inorgânico, sendo a vitamina B_{12} (metilcobalamina), um derivado do metilcorrinóide, a única coenzima reconhecida como possível doadora do grupo metil para o Hg^{2+} (Wood., 1974).

O etilmercúrio ($C_2H_5Hg^{+}$), uma outra forma de mercúrio orgânico, está presente no Timerosal (Mertiolate®), um medicamento de uso tópico com ação anti-séptica, cuja industrialização e dispensação foram recentemente proibidas. Também pertence à classe orgânica de Hg, o composto dimetilmercúrio ($(CH_3)_2$ Hg). Este é considerado uma forma "supertóxica" de Hg e é encontrado quase que exclusivamente em laboratórios (Braunwald e cols., 2001). De forma geral, essas formas orgânicas de mercúrio atravessam facilmente as membranas celulares, podendo causar o acúmulo de grandes quantidades de mercúrio no interior das células (Clarkson., 2002). Além disso, as formas orgânicas de Hg por serem altamente reativas podem induzir danos em várias biomoléculas, o que caracteriza o mercúrio como um elemento de alto risco para saúde humana e para o ambiente.

2.1.2 Exposição Humana ao Mercúrio:

2.1.2.1 Vapor de mercúrio:

A intoxicação humana por Hg pode ocorrer por inalação acidental do Hg^0 ou por exposição crônica associada a atividades ocupacionais. De acordo com a Organização Mundial de Saúde (OMS), a inalação de ≅ 25 µg/m^3 de mercúrio pode caracterizar uma exposição do tipo ocupacional crônica. Para uma exposição do tipo aguda são considerados níveis de ≅ 500 µg/m^3 de concentração de Hg (OMS., 1991; 2003).

O monitoramento dos níveis de Hg na urina de pessoas expostas a vapor de mercúrio é um procedimento considerado essencial para acessar os riscos de intoxicação humana. A concentração de Hg na urina associada com sintomas sub-clínicos de intoxicação é de 30 µg/g de creatinina e a concentração considerada máxima é de 50 µg/g creatinina. O caso mais comum de exposição ocupacional sub-clínica ao Hg^0 é o de dentistas (Silveira e cols., 2003; Ventura e cols., 2004; 2005; do Canto Pereira e cols., 2005; Rodrigues e cols., 2007; da Costa e cols., 2008).

2.1.2.2. Etilmercúrio:

O uso de medicamentos e vacinas contendo Thimerosal, um composto que contém etilmercúrio, representa outras formas de exposição ao Hg. Em 1999, os órgãos reguladores europeus e os EUA concordaram que os riscos de exposição indicavam a necessidade de remoção do mercado das vacinas de dose única contendo mercúrio. Baseado nos cálculos da União Européia e dos EUA, os níveis acumulados de mercúrio, através das vacinas, em crianças de seis anos de idade excediam a dose de referência estipulada pela Agência de Proteção Ambiental (EPA). Até recentemente, as vacinas DTP e DTaP, hepatite B, Hib e anti-meningite, e algumas vacinas anti-rábicas e anti-pneumonia fabricadas e usadas nos EUA continham Thimerosal (Ball e cols., 2001). Em países subdesenvolvidos algumas vacinas ainda contêm Thimerosal como conservante. Consequentemente, grande parte da população infantil pode ser considerada alvo de exposição ao mercúrio.

2.1.2.3 Metilmercúrio:

A principal forma de exposição humana não ocupacional ao MeHg dá-se através da ingestão de peixes e frutos do mar contaminados. Vegetais e cereais tratados com fungicidas a base de Hg também têm sido associados a episódios de intoxicação humana. Na década de 50 no Japão, estima-se que mais de 900 pessoas morreram devido ao envenenamento por MeHg na baia de Minamata e que cerca de dois milhões de pessoas foram afetadas pela

ingestão de peixes contaminados (Gochfeld., 2003; Robertson e Orrenius., 2000). Na década de 70, no Iraque, no Paquistão, em Gana e na Guatemala ocorreram vários casos de contaminação de agricultores e familiares que utilizavam grãos tratados com fungicidas a base de MeHg na preparação de pão caseiro. Particularmente no Iraque, 6.530 pessoas foram hospitalizadas e 459 mortes foram relacionadas diretamente com a exposição ao MeHg (Watanabe e Satoh., 1996; Oyake e cols., 1966; Bakir e cols., 1973). No Brasil, especificamente na Amazônia, estudos recentes têm evidenciado que várias espécies de peixes carnívoros apresentam altos níveis de MeHg (Malm., 1998; Pinheiro e cols., 2003; 2009) e que as comunidades ribeirinhas localizadas próximas a áreas de garimpo, sofrem exposição crônica a níveis relativamente elevados de MeHg devido a dieta rica em peixes (Pinheiroe cols., 2003; 2009). Como a maioria do Hg liberado no ambiente é metilado e incorporado na base da cadeia alimentar por bactérias metalogênicas, o metilmercúrio se acumula na cadeia alimentar aquática através de um fenômeno chamado bioamplificação, ou seja, a concentração do metal aumenta à medida que ele avança os níveis tróficos (Boening., 2000), podendo permanecer por longos períodos nos tecidos dos organismos. Desta forma, concentrações elevadas de metilmercúrio podem ser encontradas em peixes predadores presentes na extremidade da cadeia alimentar. A tabela abaixo mostra a concentração de mercúrio presente em espécies marinhas da costa brasileira (Kehrig e cols., 2009).

Tabela 1 Concentração média de mercúrio total (Hg_{tot}), em peso seco, nos tecidos

Espécie	Classe	Nome comum	Tecido	Número	$[Hg_{tot}]$ µg g^{-1}
Sotalia guianensis	Cetáceo	Boto-cinza	músculo	21	3,28 ± 1,69
Trichiurus lepturus	Peixe	Peixe espada	músculo	17	1,07 ± 1,06
Paralonchurus brasiliensis	Peixe	Maria-luisa	músculo	11	0,26 ± 0,13
Anchoa filifera	Peixe	Manjubinha	músculo	2	0,25 ± 0,17
Pellona harroweri	Peixe	Sardinha-piaba	músculo	8	0,25 ± 0,11
Isopisthus parvipinnis	Peixe	Pescada-faneca	músculo	12	0,22 ± 0,13
Cynoscion jamaicensis	Peixe	Goete	músculo	9	0,30 ± 0,11
Loligo sanpaulensis	Cefalópode	Lula	manto	5	0,20 ± 0,09

Kehrig e cols., 2009

2.1.3 Transporte e acúmulo de Mercúrio

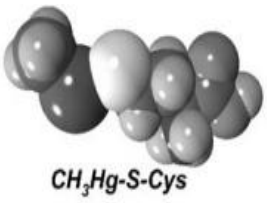
Estudos têm demonstrado que o MeHg pode ser bioacumulado em diferentes tecidos e consequentemente induzir severas disfunções celulares em muitos órgãos, especialmente no sistema nervoso central (Chang., 1980; Clarkson., 1997). No entanto, os mecanismos envolvidos na entrada e acúmulo de mercúrio em células, organelas e tecidos ainda precisam

ser elucidados. O $MeHg^{+}$ praticamente não se encontra na forma catiônica livre nos sistemas biológicos (Hirayama., 1975; Clarkson., 2003) e devido a sua eletrofilicidade, é esperado que o mesmo reaja com nucleófilos como grupamentos sulfidril (-SH) e selenoidril (SeH). Assim, é presumível que nos sistemas biológicos o MeHg encontre-se ligado ao aminoácido Cys, ao peptídeo GSH ou em proteínas contendo resíduos -SH ou SeH, formando complexos que podem ser estabilizados pela forte atração entre o MeHg e o resíduo -SH e/ou SeH dessas moléculas (Clarkson., 2003; Nogueira e cols., 2004; Farina e cols., 2011). Estudos têm demonstrado que essas formas conjugadas de mercúrio circulam facilmente pela corrente sanguínea e em alguns casos permitem uma melhor absorção e captação do mercúrio pelos tecidos (Hirayama., 1975; Hirayama., 1980; Hirayama., 1985).

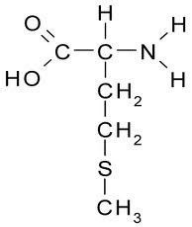
Recentes trabalhos têm relacionado a interação entre vários tióis de baixo peso molecular e diferentes metais pesados, entre eles o Hg, com um fenômeno conhecido como "mimetismo molecular". Ligados a esses tióis, os metais pesados passam a ter maior facilidade para entrar em vários tipos celulares, via mimetismo molecular (Bridges e Zalups., 2005; Yin e cols., 2008). "O mimetismo molecular" refere-se ao fenômeno pelo qual a ligação dos íons metálicos com os grupos nucleofílicos de certas biomoléculas que resulta na formação de complexos organo-metalicos os quais se comportam ou servem como estruturas homólogas de algumas biomoléculas endógenas" (Bridges e Zalups., 2005). Tendo como base esse fenômeno, alguns estudos sugerem que o complexo metilmercurio-L-cisteína (MeHg-Cys) é um mimético do aminoácido Met e que consequentemente pode atravessar as biomembranas via transportador de aminoácidos neutros (sistema L) (Aschner e Clarkson., 1988; Mokrzan e cols., 1995; Roos e cols., 2010). A figura abaixo ilustra a similaridade estrutural entre o complexo MeHg-Cys e o aminoácido Met:

A)

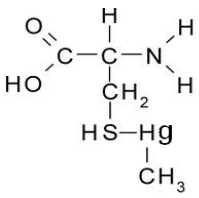
B)



C)



D)



Existem vários subtipos de transportadores de aminoácidos do tipo L (LAT1, LAT2, LAT3), os quais estão distribuídos em vários órgãos e tecidos incluindo rim, placenta, cérebro, intestino e fígado (Palacin e cols., 1998; Kanai e Endou., 2001). Com relação à distribuição em organelas, dados da literatura têm relacionado a toxicidade induzida por Hg com a presença de transportadores tipo L em mitocôndrias (Raymond e cols., 1977). No entanto, a função fisiológica, bem como, a precisa localização sub-celular destes transportadores ainda não foram determinadas. Da mesma forma, existem poucos trabalhos que investigam a distribuição e o acúmulo de Hg via complexo MeHg-Cys em organelas de diferentes tecidos. De forma geral, sabe-se pouco acerca da possível modulação do complexo MeHg-Cys sobre os diferentes subtipos de transportadores LAT nos diferentes órgãos, tecidos e/ou organelas.

2.1.4 Toxicidade do Metilmercúrio:

O MeHg é considerado um dos principais poluentes ambientais e, como mencionado anteriormente, já esteve envolvido em vários casos de desastres ecológicos durante a história da humanidade. O MeHg é bem reconhecido por induzir danos no sistema nervoso central, sendo que fetos e recém nascidos exibem uma maior sensibilidade aos efeitos neurotóxicos do MeHg (Afonso e Alvarez., 1960). Estudos recentes indicam que a exposição ao MeHg afeta o desenvolvimento cerebral mesmo em níveis considerados baixos e que o dano causado no sistema nervoso central, bem como a sua gravidade, são dependentes da quantidade de mercúrio que entra no organismo (ATSDR 1999). Muitos dos sintomas da intoxicação são similares aos observados na paralisia cerebral, sendo a intoxicação por MeHg uma das possíveis causas de uma forma de paralisia cerebral (ATSDR 1999). Além do sistema nervoso, o MeHg pode causar danos em outros órgãos e tecidos como por exemplo: células pulmonares de humanos e animais (Reichl e cols., 2001), monócitos e linfócitos de humanos (Insug e cols., 1997; Shenker e cols., 1997), células β pancreáticas (Chen e cols., 2006), tecido renal e hepático (Freitas e cols., 2009). O exato mecanismo pelo qual o MeHg causa toxicidade ainda não está completamente esclarecido, mas acredita-se que seus efeitos tóxicos em nível celular envolvam alterações nos sistemas antioxidantes, no sistema glutamatérgico e na homeostase do cálcio.

2.1.4.1 Envolvimento do Sistema Antioxidante na toxicidade do MeHg:

Como mencionado previamente, a alta afinidade do mercúrio por proteínas-SH e SH não proteicos tem sido sugerida como um dos fenômenos críticos na toxicidade mediada pelo MeHg (Strycks e Kolthoff., 1953). De uma particular importância é o papel da GSH na

toxicidade do MeHg em condições *in vivo* e *in vitro*. A GSH é um tri-peptídio que contém Cys, um aminoácido que possui grupo sulfidrílico, onde provavelmente o MeHg se liga (Rooney., 2007). A GSH é considerada o maior antioxidante endógeno em mamíferos, e seu papel como antioxidante está vinculado às enzimas que catalisam a síntese da GSH e as reações de interação da GSH com exógenos e moléculas eletrofilícas xenobióticas (Zhu e cols., 2006). As isoformas da enzima glutationa peroxidase (GPx) utilizam a GSH reduzida para detoxificar hidroperóxidos orgânicos, prevenindo assim o dano peroxidativo em biomoléculas (Flohe., 1988). Na reação tiol-peroxidase a GSH é oxidada a glutationa disulfeto (GSSG), a qual é reduzida novamente pela enzima glutationa redutase (GR) às custas de NADPH (Gul e cols., 2000). Baseado na possibilidade de interação química direta do MeHg com a GSH, o que leva a diminuição dos níveis de GSH com o aumento de espécies reativas de oxigênio, trabalhos têm correlacionado os efeitos tóxicos causados pelo MeHg com a geração de estresse oxidativo (Franco e cols., 2006). Entretanto, é importante ressaltar que a concentração de GSH celular é bem maior (faixa mM) quando comparada às concentrações de mercúrio intracelular (faixa de nM) (Franco e cols., 2006; Stringari e cols., 2008). Baseando-se unicamente em uma relação equimolar de MeHg e GSH torna-se difícil determinar como concentrações tão baixas de mercúrio podem induzir significativa depleção de GSH. Esse intrigante fenômeno tem sido explicado pelo fato de que a simples interação MeHg-GSH não é a única causa molecular da oxidação da GSH. De fato, tem sido mostrado que o MeHg pode induzir a formação de espécies reativas de oxigênio (ERO) por interagir com grupos nucleofílicos tiólicos responsáveis pela produção/detoxificação de ERO (Franco e cols., 2007). As ERO por sua vez, podem ser detoxificadas pelo sistema GSH levando à oxidação/depleção da GSH (Franco e cols., 2007). Nesse contexto as mitocôndrias tornam-se um importante alvo dos efeitos do MeHg, uma vez que o mercúrio afeta a transferência de elétrons na cadeia respiratória (principalmente complexo II e III) e induz a produção ERO tais como ânion superóxido e o peróxido de hidrogênio (Mori e cols., 2007). Assim, o desequilíbrio de sistemas antioxidantes tem sido amplamente reconhecido como um dos prováveis mecanismos envolvidos nos danos celulares causados pela exposição ao MeHg.

2.1.4.2 Envolvimento do Sistema Glutamatérgico na Toxicidade do MeHg:

O aminoácido Glutamato (Glu) é considerado o mais importante neurotransmissor excitatório do sistema nervoso central e vários trabalhos destacam o seu envolvimento em importantes processos cerebrais como desenvolvimento, aprendizado, memória e resposta a injúrias (Fornnum., 1984; Ozawa e cols., 1988). Com relação ao envolvimento glutamatérgico na toxicidade mediada por mercúrio, estudos *in vitro* têm evidenciado que a

exposição ao MeHg inibe efetivamente a captação de Glu em culturas de astrócitos, em vesículas sinápticas e em fatias de córtex cerebral (Brookes e Kristt., 1989; Aschner e cols., 2000; Porciuncula e cols., 2003; Moretto e cols., 2005). Esses achados corroboram dados *in vivo* que mostram que o MeHg induz mudanças na captação de Glu em ratos na fase adulta ou durante o período pós natal (Farina e cols., 2003; Manfroi e cols., 2004).

Em termos de mecanismos, tem sido proposto que a neurotoxicidade induzida por MeHg está associada com o aumento dos níveis de cálcio intracelular via super estimulação de receptores glutamatéricos, principalmente os do tipo NMDA (Choi., 1992). Uma das possíveis formas do MeHg causar um aumento nos níveis de Glu na fenda sináptica é via sua interação com os transportadores de Glu (Aschner e cols., 2000; Juarez e cols., 2002). No entanto, o exato mecanismo pelo qual o MeHg interage com os transportadores de Glu ainda não encontra-se elucidado, principalmente por existirem vários tipos de transportadores conhecidos (GLAST (EAAT1), GLT1 (EAAT2), EAAC1 (EAAT3), EAAT4, e EAAT5). Além disso, a distribuição, a expressão e os mecanismos de ação desses transportadores, principalmente durante o desenvolvimento, não são totalmente conhecidos. Uma das hipóteses que vêm sendo bastante especulada neste sentido é a de que o MeHg é capaz de oxidar grupos sulfidrílicos presentes nestes transportadores e consequentemente prejudicar o transporte de Glu da fenda sináptica para os astrócitos (Aschner e cols., 2000; Juarez e cols., 2002). Dessa forma, a disfunção na homeostase glutamatérgica induzida pelo MeHg vem sendo associada com os problemas de desenvolvimento cerebral e os déficits cognitivos e motores observados em indivíduos expostos a esse neurotoxicante (Fornnum., 1984; Ozawa e cols., 1988).

2.1.4.3 Envolvimento do Cálcio na Toxicidade do MeHg:

Como descrito acima, um aumento na concentração de Glu pode causar uma super estimulação dos receptores NMDA e consequentemente um aumento no influxo de Na^{+} e Ca^{2+} (Choi., 1992). Este aumento do cálcio intracelular está associado à geração de estresse oxidativo e neurotoxicidade (Dykens., 1994; Reynolds e Hastings., 1995; Dugan e cols., 1995). Neste contexto, estudos *in vitro* têm demonstrado que a indução de danos neuronais mediada pela exposição ao MeHg está ligada a perda da homeostase do cálcio intracelular (Tobi e cols., 2003). Em neuroblastomas, foi observado que o MeHg causa um aumento nos níveis de cálcio de forma bifásica. O primeiro aumento nos níveis de cálcio (primeira fase) parece ser oriundo da liberação do cálcio dos estoques intracelulares; enquanto um segundo aumento nos níveis de cálcio (segunda fase) é proveniente do influxo de cálcio extracelular, possivelmente via estimulação dos receptores NMDA (Hare e cols., 1993; Mary e Atchison.,

1997; Tobi e cols., 2003). Dessa forma, a toxicidade mediada por MeHg pode estar fortemente ligada à disfunção mitocondrial (Seegal e Dreiem., 2007; Wagner e cols., 2010), uma vez que, o aumento do cálcio mitocondrial está envolvido com os mecanismos de indução de morte celular.

2.1.4.4 MeHg e Mitocôndrias:

Nas mitocôndrias o MeHg pode ter diversos alvos moleculares: i) O MeHg pode interagir com grupamentos tióis dos complexos respiratórios, diminuindo o fluxo de elétrons na cadeia respiratória e dessa forma reduzindo a síntese de ATP e consequentemente causando uma perda energética na célula (Mori e cols., 2007); ii) O MeHg pode oxidar tióis de proteínas de membrana e ocasionar a formação de poros inespecíficos, favorecendo assim a perda do $\Delta\Psi m$ via escape de prótons do espaço intermembranas e também a perda do estado energético celular (Polunas e cols., 2011); iii) O MeHg pode diminuir o potencial redox mitocondrial (pool de NAD(P)H) via oxidação da GSH como descrito anteriormente (Franco e cols., 2007); iv) O MeHg pode, via oxidação de tióis, favorecer a abertura do poro de transição mitocondrial (PTM), fazendo com que ocorra a liberação de compostos de baixo peso molecular da mitocôndria (Bragadin e cols., 2002), bem como do citocromo *c*, induzindo assim a ativação de vias de cascatas apoptóticas (Nori e cols., 2011).

É importante salientar, que a ocorrência desses fenômenos induzidos por MeHg está fortemente associada a alterações na homeostase do cálcio. Neste contexto, um recente trabalho do nosso grupo demonstrou que baixas concentrações de MeHg não alteram os níveis de ERO em mitocôndrias isoladas de fígado de ratos, mas que na presença de cálcio induzem um aumento significativo na formação de ERO (Wagner e cols., 2010). De acordo com tais constatações, trabalhos de outros grupos mostram que quelantes de cálcio intracelular ou bloqueadores de canais de cálcio previnem a toxicidade induzida por MeHg em grânulos cerebelares (Tobi e cols., 2003). De uma forma geral, os dados da literatura suportam a hipótese de que os efeitos tóxicos exibidos pelo MeHg a nível mitocondrial estão vinculados à concentração de cálcio no meio.

2.2 Disfunções Mitocondriais Induzidas por Cálcio:

Em níveis fisiológicos, o cálcio mitocondrial é de extrema importância para processos como: manutenção do gradiente iônico e elétrico, transcrição gênica, controle da atividade de várias enzimas, apoptose, etc. No entanto, uma pequena variação na concentração do cálcio intracelular pode induzir a ativação de eventos que culminam com danos irreversíveis à célula. Como descrito anteriormente, as mitocôndrias são consideradas alvo de toxicidade do

cálcio, principalmente por serem capazes de aumentar a captação deste íon quando os níveis intracelulares elevam-se (Sparagna e cols., 1995). A captação de Ca^{2+} em excesso pelas mitocôndrias pode ocasionar disfunções como despolarização, inchaço mitocondrial e elevação dos níveis de EROs. Esses fatores contribuem para induzir morte mitocondrial e consequentemente morte celular (Seegal e Dreiem 2007).

2.2.1 Cálcio e Complexo Mitocondrial I:

Sabe-se que a mitocôndria pode modular a captação de Ca^{2+} dependendo do substrato energético (Garcia e cols., 2005). Um trabalho recente mostra que quando mitocôndrias são suplementadas com o substrato energético do complexo I (Mal/Glu) sua capacidade de captar cálcio é diminuída quando comparada com mitocôndrias suplementadas com substrato do complexo II, o (Succ) (Garciae cols., 2005). Esse fato tem sido atribuído a uma diminuição no conteúdo de nicotinamida adenina dinucleotídeo (NADH) e a perda do potencial de membrana; fatores que contribuem para a captação iônica nessa organela (Vinogradov e cols., 1972). Como mostrado por Sadek e cols., em 2004, o cálcio pode diminuir a atividade do complexo I em mitocôndrias isoladas de coração de ratos. O complexo I da cadeia respiratória catalisa o primeiro passo para a utilização dos elétrons a partir do NADH. Assim, o complexo I tem um papel fundamental na manutenção do gradiente de prótons e produção de ATP. Uma diminuição na atividade desse complexo pode ocasionar efeitos severos sobre a função mitocondrial e celular, os quais são observados em diversos modelos animais de isquemia/reperfusão e também na intoxicação por MeHg (Rouslin., 1983; Hardy e cols., 1991; Lucas e Szweda., 1999; Sadek e cols., 2002; Mori e cols., 2007).

2.2.2 Cálcio e abertura de poro de transição mitocondrial (PTM):

Outro alvo do cálcio é o poro de transição mitocondrial (PTM). Vários estudos em mitocôndrias isoladas mostram uma complexa interação existente entre os níveis de cálcio e a abertura do PTM (Castilho e cols., 1995; Green e Reed., 1998; Kowaltowski e cols., 1998; Turrens., 2003). Várias enzimas reguladoras estão envolvidas na abertura do PTM, dentre elas se destacam: a hexoquinase (HK); um canal aniônico dependente de voltagem (VDAC); a creatina kinase (CK); uma proteína translocadora de adenina nucleotídeo (ANT); e a ciclofilina D (Cyp-D). Em resumo, a abertura do PTM primeiramente envolve a interação do cálcio com a Cyp-D na matriz mitocondrial, a qual então se liga com a ANT, uma proteína de membrana interna mitocondrial. Essa ligação via oxidação de dois grupos tióis, altera a conformação da ANT, permitindo uma interação da mesma com a porina VDAC (proteína de membrana externa mitocondrial) e conectando as duas membranas mitocondriais através da

formação de um complexo protéico, de modo a formar um poro. A HK e a CK estabilizam a ligação entre ANT e VDAC, a HK, por ser uma proteína citosólica, estabiliza a conformação da VDAC e a CK, por ser uma proteína encontrada no espaço intra-membranas, estabiliza a ligação ANT-VDAC. A formação do PTM permite a liberação de substâncias de baixo peso molecular de até 32 kDa. Vários trabalhos correlacionam a formação de PTM com a geração de ERO em resposta ao cálcio (Castilho e cols., 1995; Green e Reed., 1998; Kowaltowski e cols., 1998; Turrens., 2003). No entanto, o que ainda não está claramente elucidado é se a geração de ERO é uma consequência da abertura do poro ou uma causa proeminente desse processo, o qual causa a liberação de citocromo *c* e conseqüentemente ativa a cascata de apoptose e/ou morte celular (Green e Reed., 1998; Turrens., 2003).

De uma forma geral, sabe-se que a exposição ao MeHg e a perda da homeostase do cálcio podem culminar com morte celular. No entanto, estudos que esclareçam e correlacionem os mecanismos envolvidos nos efeitos tóxicos desses elementos são escassos na literatura. Assim, torna-se importante o desenvolvimento de trabalhos que avaliem a distribuição e a captação de Hg por tecidos e organelas e que relacionem a citotoxicidade induzida por MeHg com estes parâmetros, bem como, investiguem o(s) mecanismo(s) de ação do mercúrio e do cálcio envolvidos na disfunção mitocondrial. Estas pesquisas contribuirão de forma efetiva para o conhecimento dos possíveis mecanismos e alvos toxicológicos desses agentes e consequentemente para a descoberta de novas estratégias terapêuticas usadas em patologias que causem disfunção mitocondrial.

3. OBJETIVOS:

3.1. Objetivos gerais:

Este trabalho teve como objetivo geral investigar os mecanismos envolvidos na captação e acúmulo de MeHg em fatias e organelas, bem como, o papel do sistema glutamatérgico na neurotoxicidade induzida por MeHg. Além disso, foram investigados os alvos moleculares e os mecanismos de toxicidade do cálcio envolvidos na disfunção mitocondrial.

3.1.1 Objetivos Específicos do Artigo Científico I:

◙ Investigar os possíveis mecanismos de transporte e acúmulo de mercúrio em fatias hepáticas e em mitocôndrias isoladas dessas fatias.

◙ Verificar os efeitos do MeHg e do complexo MeHg-Cys na geração de radicais livre nas fatias e nas mitocôndrias isoladas das fatias.

◙ Examinar se a exposição ao MeHg ou MeHg-Cys causa prejuízo no consumo de oxigênio das fatias de fígado.

◙ Averiguar se a exposição ao MeHg e complexo MeHg-Cys interferem na viabilidade/atividade celular e mitocondrial.

◙ Propor um mecanismo de captação e acúmulo de mercúrio em hepatócitos de rato.

3.1.2 Objetivos Específicos do Artigo Científico II:

◙ Avaliar o envolvimento do sistema glutamatérgico sobre a geração de radicais livres induzida pelo MeHg em fatias de córtex cerebral de ratos.

◙ Verificar os efeitos do antagonista de receptores NMDA (MK-801), na toxicidade induzida pelo MeHg.

◙ Examinar os efeitos da Guanosina na neurotoxicidade mediada pelo MeHg.

◙ Propor um mecanismo de envolvimento do sistema glutamatérgico na toxicidade induzida pelo MeHg.

3.1.3 Objetivos Específicos do Manuscrito I:

◙ Avaliar os efeitos do cálcio sobre a atividade dos complexos mitocondriais I e II.

◙ Identificar os possíveis alvos toxicológicos do cálcio em mitocôndrias sustentadas com substrato energético do complexo I ou II.

◙ Analisar o efeito do cálcio sobre diferentes parâmetros associados à funcionalidade mitocondrial em mitocôndrias sustentadas com os substratos Mal/Glu ou Succ.

◙ Propor um possível mecanismo de citotoxicidade do cálcio em mitocôndrias suportadas com os diferentes substratos energéticos avaliados.

4. ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta Tese estão apresentados sob a forma de dois artigos científicos e um manuscrito. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos. Os artigos estão expostos conforme foram publicados nas revistas científicas, e o manuscrito conforme será submetido.

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Modulation of methylmercury uptake by methionine: Prevention of mitochondrial dysfunction in rat liver slices by a mimicry mechanism

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Liver

ABSTRACT

Methylmercury (MeHg) is an ubiquitous environmental pollutant which is transported into the mammalian cells when present as the methylmercury-cysteine conjugate (MeHg–Cys). With special emphasis on hepatic cells, due to their particular propensity to accumulate an appreciable amount of Hg after exposure to MeHg, this study was performed to evaluate the effects of methionine (Met) on Hg uptake, reactive species (RS) formation, oxygen consumption and mitochondrial function/cellular viability in both liver slices and mitochondria isolated from these slices, after exposure to MeHg or the MeHg–Cys complex. The liver slices were pre-treated with Met (250 μM) 15 min before being exposed to MeHg (25 μM) or MeHg–Cys (25 μM each) for 30 min at 37 °C. The treatment with MeHg caused a significant increase in the Hg concentration in both liver slices and mitochondria isolated from liver slices. Moreover, the Hg uptake was higher in the group exposed to the MeHg–Cys complex. In the DCF (dichlorofluorescein) assay, the exposure to MeHg and MeHg–Cys produced a significant increase in DFC reactive species (DFC-RS) formation only in the mitochondria isolated from liver slices. As observed with Hg uptake, DFC-RS levels were significantly higher in the mitochondria treated with the MeHg–Cys complex compared to MeHg alone. MeHg exposure also caused a marked decrease in the oxygen consumption of liver slices when compared to the control group, and this effect was more pronounced in the liver slices treated with the MeHg–Cys complex. Similarly, the loss of mitochondrial activity/cell viability was greater in liver slices exposed to the MeHg–Cys complex when compared to slices treated only with MeHg. In all studied parameters, Met pre-treatment was effective in preventing the MeHg- and/or MeHg–Cys-induced toxicity in both liver slices and mitochondria. Part of the protection afforded by Met against MeHg may be related to a direct interaction with MeHg or to the competition of Met with the complex formed between MeHg and endogenous cysteine. In summary, our results show that Met pre-treatment produces pronounced protection against the toxic effects induced by MeHg and/or the MeHg–Cys complex on mitochondrial function and cell viability. Consequently, this amino acid offers considerable promise as a potential agent for treating acute MeHg exposure.

Introduction

Exposure to methylmercury (MeHg), the most toxic form of mercury (Hg) in the environment, is well recognized as the cause of a series of cellular disorders in several systems, especially in the central nervous system (CNS) (Choi 1991; Sakamoto et al., 1998; Clarkson et al., 2003; Sakaue et al., 2006). However, the exact molecular mechanisms underlying MeHg-induced toxicity in the developing and adult CNS, as well as in other tissues, remain unclear. The methyl mercuric ion (CH_3Hg^+) does not exist in biological systems as a free, unbound cation (Hughes, 1957), but rather, is found conjugated to thiol-containing biomolecules, such as glutathione (GSH), cysteine (Cys) and homocysteine (Hcy) (Clarkson, 1993). Thus, many of the mechanisms proposed to explain the rapid diffusion of MeHg across membranes and, consequently, the cellular damage induced by MeHg is largely based upon its high affinity for −SH groups. Corroborating

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these notions, several studies have demonstrated that the absorption and cellular uptake of MeHg are significantly increased when it is present as Cys– or Homocysteine–MeHg conjugates (Ballatori, 2002; Roos et al., 2010). Additionally, experimental evidence supports the idea that the neutral amino acid transport system L is a significant route for MeHg–Cys transmembrane movement (Yin et al., 2008; Roos et al., 2010), since MeHg–Cys complexes are thought to mimic structurally methionine (Met), a substrate for amino acid carriers such as the L-type large neutral amino acid transporters (LATs). The major LATs subtypes (LAT1, LAT2 and LAT3) are widely expressed in organs and tissues of the kidney, placenta, brain and intestinal wall (Palacín et al., 1998; Kanai and Endou, 2001). In the liver, amino acid transporters with system L transport activity have been identified mainly in human hepatoblastoma cell line HepG2 (Sarkar et al., 1999). However, the physiological function as well as the precise subcellular localization of these transporters in normal hepatic cells has yet to be determined (Bode, 2001; Babu et al., 2003; Fukuhara et al., 2007; Wagner et al., 2010).

A number of particular cellular mechanisms and molecules are the primary targets of MeHg cytotoxicity. Disruption of calcium homeostasis and free radicals generation are among the detrimental effects associated with MeHg-induced toxicity (Limke et al., 2003; Ikeda et al., 1999). In this scenario, mitochondria play a crucial role, as these organelles can act as a buffer against cytosolic calcium and can mediate (RS) formation in cells (Norenberg and Rao, 2007; Chacko et al., 2009). It has been shown that mitochondrial dysfunctions induced by MeHg include the failure of energy metabolism, the disruption of calcium homeostasis and the dissipation of the mitochondrial membrane potential, effects which lead to a mitochondrial burst of reactive oxygen species (ROS) production (Kim and Sharma 2003; Kang et al., 2006; Dreiem and Seegal, 2007). ROS are important mediators of damage to cell structures, including lipids and membranes, as well as proteins and nucleic acids (Poli et al., 2004). The detrimental effects of ROS are balanced by the antioxidant action of non-enzymatic antioxidants in addition to antioxidant enzymes (Poli et al., 2004). However, *in vivo* and *in vitro* experimental observations have shown that the toxic effects of MeHg are accompanied by a significant deficit of antioxidant defenses, such as the depletion of GSH and the inhibition of GSH peroxidase activity (Farina et al., 2004; Chang and Tsai, 2008; Stringari et al., 2008; Farina et al., 2009). Thus, oxidative stress has been implicated in a number of events involved in MeHg-induced cytotoxicity (Roos et al., 2009).

Based on the evidence presented above, it is reasonable to assume that Met, acting as competitive inhibitor of MeHg–Cys transport through system L could prevent or reduce MeHg-induced cytotoxicity. To date, there have been no studies on the efficacy of Met to attenuate mitochondrial MeHg uptake and mitochondrial function. The experimental model employed, namely hepatic cells, possess a particular propensity to accumulate appreciable quantities of Hg after exposure to MeHg (de Freitas et al., 2009). Specifically, we have examined, for the first time, the effects of Met pre-treatment on Hg uptake, RS formation, oxygen consumption and cellular viability in both liver slices and mitochondria isolated from these slices, after exposure to MeHg or the MeHg–Cys complex.

Materials and experimental procedures

Chemicals. MeHgCl and L-Cysteine chloride were obtained from Aldrich (St. Louis, MO). All other chemicals were of analytical reagent grade and were purchased from Merck (Rio de Janeiro, Brazil).

Animals. Adult male *Wistar* rats from our own breeding colony (200–250 g) were maintained in Plexiglas cages with food and water *ad libitum*, in a temperature-controlled room (22–25 °C) and on a 12 h-light/dark cycle with lights on at 7:00 a.m. Animals were handled and treated according to the guidelines set forth by the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

Preparation of liver slices. Animals were killed by decapitation, and the whole liver was quickly removed and placed on ice. Afterward, the liver was cut into transverse slices 300 μm thick using a McIlwain tissue chopper (Campbell Instruments; The Mickle Laboratory Engineering Co). The slices were placed in Krebs–Ringer buffer (10 mM D-glucose, 129 mM NaCl, 1.25 mM $NaHPO_4$, 22 mM $NaHCO_3$, KCl 3 mM, $CaCl_2$ 1.8 mM, $MgSO_4$ 1.8 mM, Hepes 5 mM, pH 7.4), which was previously bubbled with O_2 95% and CO_2 5% for 30 min. Sixty slices (per group) were carefully selected, weighted (30 ± 2 μg each) and randomly placed in buffer (2 mL) for the respective treatments. In the final step of each experiment the total protein content was determined (Peterson, 1977).

Treatment of liver slices. The slices were subdivided to the following groups: (1) control; (2) MeHg (25 μM); (3) Cysteine (25 μM); (4) MeHg–Cys complex (25 μM each); (5) Methionine (250 μM); (6) Met (250 μM) + MeHg (25 μM); and (7) Met (250 μM) + MeHg–Cys complex (25 μM each). The slices were exposed to the different treatments for 30 min at 37 °C, in the presence of O_2 (95%) and CO_2 (5%). The molar ratio of cysteine to MeHg was 1, and the stoicheometric reaction between cysteine and MeHg was confirmed by Ellman's reagent (Ellman, 1959). The Methionine groups (250 μM) were pre-treated for 15 min with methionine before being exposed to MeHg or the MeHg–Cys complex. All reagents were dissolved in Krebs–Ringer buffer.

Mitochondrial preparation. Liver mitochondria were isolated as previously described by Brustovetsky and Dubinsky (2000a, 2000b), with some modifications. After treatment, the liver slices were washed three times and manually homogenized in cold buffer I (manitol 225 mM, sucrose 75 mM, K^+ EGTA 1 mM, bovine serum albumin (BSA) 0.1% and K^+-HEPES 10 mM pH 7.2), using a potter glass (length: 10 cm; diameter: 1 cm). Next, the homogenized slices were centrifuged at $2000 \times g$ for 7 min at 4 °C. The pellet was discarded and the supernatant was centrifuged again at $12{,}000 \times g$ for 10 min at 4 °C. Then, the resultant supernatant was discarded, and the pellet was re-suspended in buffer II (manitol 225 mM, sucrose 75 mM, K^+ EGTA 1 mM and K^+-HEPES 10 mM pH 7.2) and re-centrifuged at $12{,}000 \times g$ for 10 min at 4 °C. Finally, the last supernatant was discarded, and the pellet was re-suspended and maintained in buffer III (sucrose 100 mM, KCl 65 mM, K^+-HEPES 10 mM and EGTA 50 μM pH 7.2) for subsequent analyses.

Mercury quantification. Both the aliquot of the homogenate of liver slices and the mitochondrial suspension isolated from liver slices were subjected to Hg analysis, which was carried out by Cold Vapor-Atomic Fluorescence Spectrometry according to the method described by Bergdahl et al., 1998. The total Hg content was determined after acid digestion with HNO_3, H_2O_2, H_2SO_4 and perchloric acid (Bergdahl et al., 1998).

Evaluation of Reactive Species (RS) formation with DCH (dichlorofluorescein-reactive species, DCH-RS). RS levels were measured using the oxidant sensing fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCHF–DA) (Hempel et al., 1999). The oxidation (DCHF–DA) to fluorescent dichlorofluorescein (DCF) was determined at 488 nm for excitation and 525 nm for emission. After being exposed to the reagents, the liver slices were homogenized in buffer I (1 mL), and an aliquot of 10 μL (50 μg/protein, Peterson, 1977) of both the homogenate of the

Table 1
Effects of MeHg or MeHg–Cys complex exposure on respiratory rates of rat liver slices.

	Rate 1	Rate 2	Rate 3
	Respiration of slices (nmol O_2/mL/min)	Respiration with succ. (nmol O_2/mL/min)	Respiration with MeHg or MeHg–Cys (nmol O_2/mL/min)
Control	2.27 ± 0.22	6.59 ± 0.37	5.32 ± 0.47
MeHg	2.26 ± 0.47	6.09 ± 0.32	3.49 ± 0.52[a,b]
MeHg–Cys	2.10 ± 0.38	5.97 ± 0.18	2.34 ± 0.05[a,b,c]
With methionine			
Control	2.42 ± 0.27	6.17 ± 0.98	5.57 ± 0.75
MeHg	2.38 ± 0.10	6.55 ± 0.53	5.45 ± 0.35[d]
MeHg–Cys	2.37 ± 0.18	6.10 ± 0.34	3.43 ± 0.26[a,b,d]

Slices were pre-treated for 15 min with Met (250 μM) and after exposed for 30 min to MeHg (25 μM) or MeHg–Cys complex (25 μM each).
[a] Indicates $p<0.05$, rate 3 compared to 2.
[b] Indicates $p<0.05$ from control.
[c] Indicates $p<0.05$ from MeHg.
[d] Indicates $p<0.05$ from MeHg–Cys complex, n = 5 mean ± S.E).

liver slices and the homogenate of the isolated mitochondria was added to 3 mL of buffer III (containing 5 mM glutamate and 5 mM succinate). After 10 s, 10 μM (DCHF–DA) (prepared in ethanol) was added to the mixture; and the fluorescence intensity from DCF was measured for 300 s and expressed as a percentage of the untreated control group.

Oxygen consumption of liver slices. The oxygen consumption of the liver slices was measured using an oxymeter (Hansatech model with a Clark-type electrode) at 30 °C. Two slices, weighting approximately 30 μg (30 ± 2 μg) each, were selected and placed in 2 mL Krebs–Ringer buffer. Fifteen minutes after methionine addition, glutamate/succinate (5 mM each) was placed in the medium to increase the respiratory state. After 30 min, either the MeHg solution or the MeHg–Cys complex solution was added. The respiratory ratio and oxygen consumption were determined and compared among groups.

Cell viability/mitochondrial activity. Cell viability and mitochondrial activity were measured by dehydrogenase activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann, 1983). After 30, 60 and 120 min of exposure to the respective treatment, four liver slices were selected and incubated with MTT (5 μg/mL) for 20 min. The MTT reduction reaction was stopped by the addition of 1.5 mL of dimethylsulfoxide (DMSO). The formazan color and the colorimetric intensity were determined by the difference in absorbance readings at 570–630 nm, using an UV 2450 Shimadzu spectrophotometer. The ratio values were standardized to protein content and expressed as a percentage of the untreated control group.

Protein determination. All experiments were standardized to protein concentrations (Peterson, 1977), and, when appropriate, were expressed as a percentage of untreated control values.

Statistical analysis. Data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate. The significance between the respiratory rates (Table 1) was analyzed statistically by *t*-test. Differences between groups were considered to be significant when $P<0.05$.

Results

Hg levels in liver slices and mitochondria isolated from liver slices

The first set of experiments was designated to analyze the Hg content in liver slices and mitochondria isolated from liver slices. The Fig. 1 shows that treatment with MeHg alone caused a significant increase in the Hg concentration in both liver slices (A) and in mitochondria isolated from liver slices (B) and that the content of Hg was further increased in the group exposed to the MeHg–Cys complex when compared to the group treated with MeHg alone (Figs. 1A and B). The data in Fig. 1 also reveal that pre-treatment with Met was effective in reducing the Hg levels of the slices exposed to MeHg or the MeHg–Cys complex (Fig. 1A). However, in mitochondria isolated from these liver slices, the Met pre-treatment effect was observed only in the MeHg–Cys complex group (Fig. 1B).

DFC-RS (Diflouroscein-Reactive Species) formation

The second set of experiments was performed to analyze the effect of Met treatment on RS production caused by MeHg in liver slices and mitochondria isolated from liver slices. Fig. 2 illustrates the levels of DFC-RS production in liver slices (A) and mitochondria isolated from liver slices (B) after 45 min of exposure to Met (50–250 μM). The data show that Met pre-treatment, at all concentrations tested, did not cause any effect on DFC-RS production when compared to control values (Figs. 2A and B).

Fig. 3 shows the effects of exposure to MeHg or the MeHg–Cys complex on DFC-RS generation in liver slices (A) and mitochondria isolated from liver slices (B). In liver slices, the levels of DFC-RS production were slightly enhanced by exposure to MeHg or the MeHg–Cys complex. However, this difference was not statistically significant (Fig. 3A). In contrast, in the mitochondria isolated from these liver slices, MeHg exposure produced a significant increase on DFC-RS production when compared to levels found in the control group (Fig. 3B). Furthermore, the DFC-RS production levels were significantly higher in the mitochondria isolated from liver slices that were treated with the MeHg–Cys complex, when compared to mitochondria isolated from slices exposed to MeHg alone (Fig. 3B). Notably, Met pre-treatment was effective in reducing DFC-RS production only in the mitochondria isolated from slices treated with the MeHg–Cys complex (Fig. 4).

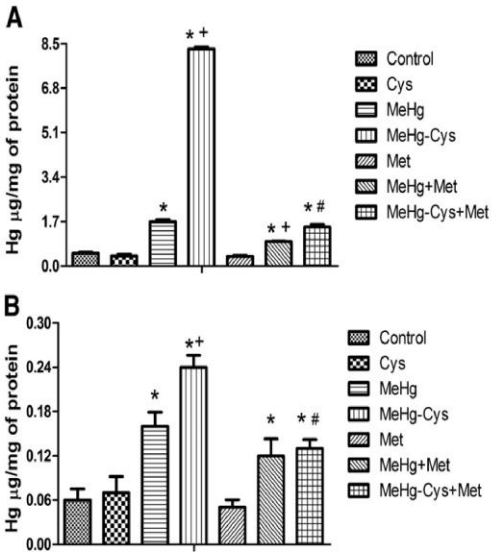

Fig. 1. Effects of Met pre-treatment on Hg uptake in rat liver slices (A) and mitochondria (B) exposed to MeHg or the MeHg–Cys complex. Slices were pre-treated for 15 min with Met (250 μM) and then exposed for 30 min to MeHg (25 μM) or the MeHg–Cys complex (25 μM each); (*Indicates $p<0.05$ from control; +Indicates $p<0.05$ from MeHg; #Indicates $p<0.05$ from the MeHg–Cys complex; n = 6 mean ± S.E.).

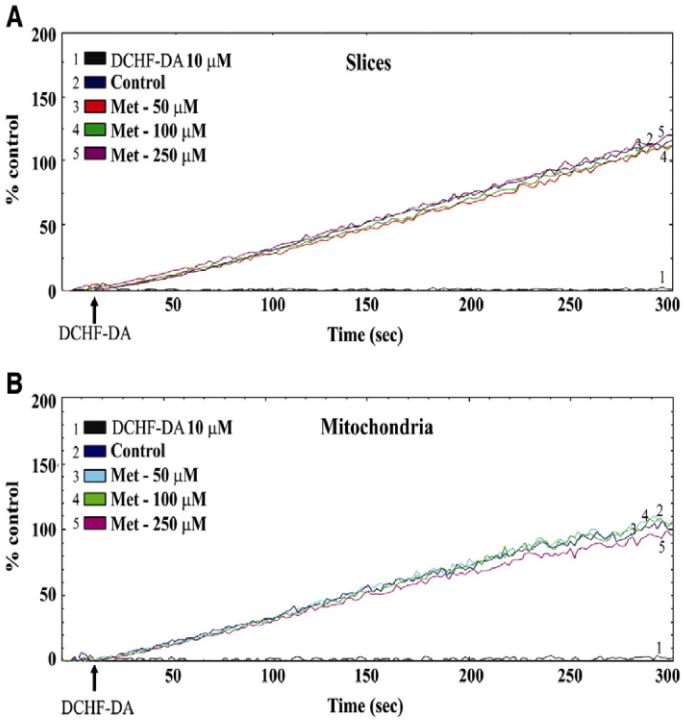

Fig. 2. Effects of Met pre-treatment on DFC-RS production in rat liver slices (A) and mitochondria (B). Slices were pre-treated for 45 min with Met (50, 100 and 250 μM). The tracings of figure are representative lines of 3 independent experiments.

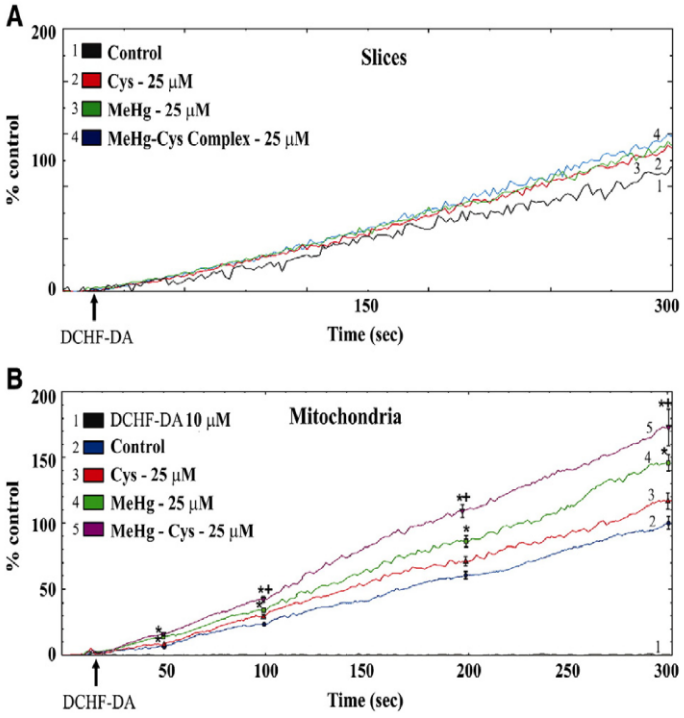

Fig. 3. Effects of exposure to MeHg or the MeHg–Cys complex on DFC-RS production in rat liver slices (A) and mitochondria (B). Slices were exposed for 30 min to MeHg (25 μM) or the MeHg–Cys complex (25 μM each). (*Indicates $p<0.05$ from control; $^{+}$Indicates $p<0.05$ from MeHg; $n=5$ mean ± S.E.). The tracings of Figs. 3A and B are representative and averaged lines respectively.

Oxygen consumption

The third set of experiments was designed to verify mitochondrial viability by determining the oxygen consumption by the liver slices. Fig. 5A shows that MeHg exposure significantly decreased the oxygen consumption of liver slices as compared to the control group, and that this effect was more pronounced in the liver slices treated with the MeHg–Cys complex. Interestingly, Met pre-treatment effectively prevented the reduction of oxygen consumption in both slices treated with MeHg and slices treated with the MeHg–Cys complex (Fig. 5B) when compared to control slices (Fig. 5A). A synopsis of MeHg, MeHg–Cys and Met modulation of mitochondria respiration is depicted in Table 1.

Cell viability/mitochondrial activity

The final set of experiments was performed to evaluate the cell viability/mitochondria activity in liver slices. Fig. 6 shows that treatment with MeHg alone caused a significant decrease in mitochondrial activity at all tested times (30, 60 and 120 min. Figs. 6A, B and C, respectively) when compared to the control group. At 30 and 60 min, the loss of mitochondrial activity was higher in liver slices exposed to the MeHg–Cys complex when compared to those treated only with MeHg (Figs. 6A and B, respectively). At all times tested, Met pre-treatment prevented mitochondrial dysfunction induced by both MeHg and MeHg–Cys complex exposure (Figs. 6A, B and C).

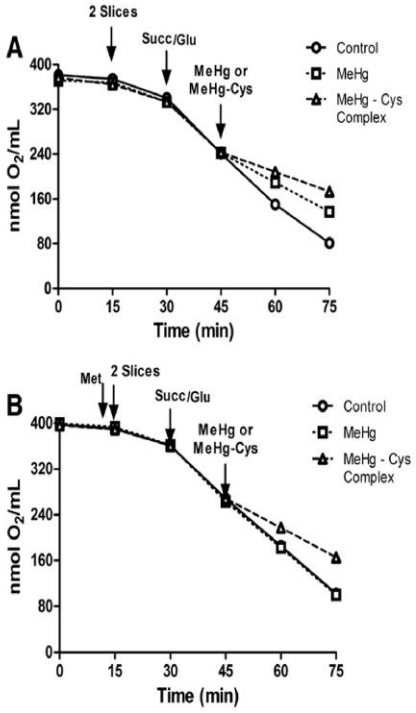

Fig. 5. Effects of exposure to MeHg or the MeHg–Cys complex on oxygen consumption in rat liver slices (A). Effects of Met pre-treatment on oxygen consumption in rat liver slices (B) exposed to MeHg or the MeHg–Cys complex. Slices were pre-treated for 15 min with Met (250 μM) and then exposed for 30 min to MeHg (25 μM) or the MeHg–Cys complex (25 μM each); (n = 5 mean ± S.E.).

Discussion

It has been postulated that MeHg is transported as a MeHg–Cys complex by the ubiquitous L-type large neutral amino acid transporters (LATs) and that transport of this conjugate represents the main pathway through which MeHg exerts its toxicity in many tissues (Kerper et al., 1992; Kajiwara et al., 1996; Simmons-Willis et al., 2002; Adachi 2006; Yin et al., 2008). Corroborating this hypothesis, our group recently reported that mice chronically treated with the MeHg–Cys complex show enhanced Hg uptake, especially in the liver, when compared to other organs, such as the brain and kidney (Roos et al., 2010). These results are most likely due to the fact that the liver is a central organ of protein metabolism and receives amino acids absorbed at the intestinal levels as well as those derived from other organs and systems (Duarte, 2003). Although hepatic cells contain some of the same carriers that have been implicated in the transport of Hg in other organs, the precise mechanisms underlying the MeHg uptake across the membrane into normal hepatocytes as well as the influence of the MeHg–Cys complex on Hg uptake and hepatotoxicity have not previously been well defined. Consequently, our study was primarily designed to investigate the Hg content in hepatic cells, at both cytosolic and mitochondrial levels after exposure to MeHg or the MeHg–Cys complex. Several previous studies have investigated and reported on the toxicology of MeHg, but, to date, only chelating agents have been employed to facilitate the removal of Hg from the body (Pingree et al., 2001; Carvalho et al., 2007). However, these drugs are of limited use because of their adverse side effects. In the present study, we have tested the possible use of Met as an efficacious agent capable of protecting against the deleterious effects of MeHg. We observed that the Hg concentration in liver slices and in the mitochondria isolated from liver slices was higher after exposure to the MeHg–Cys complex (Fig. 1).

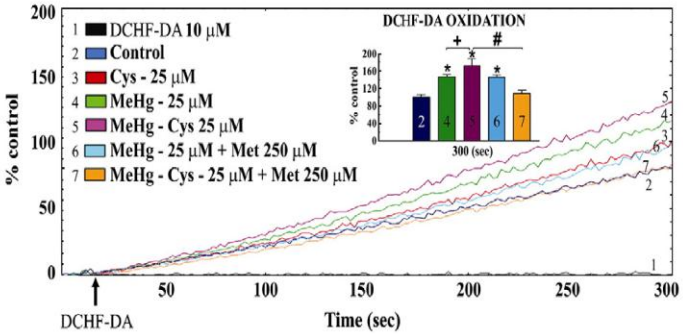

Fig. 4. Effects of Met pre-treatment on DFC-RS production in mitochondria exposed to MeHg or the MeHg–Cys complex. Slices were pre-treated for 15 min with Met (250 μM) and then exposed for 30 min to MeHg (25 μM) or the MeHg–Cys complex (25 μM each). Insets in Fig. 4 represent statistical analysis. (*Indicates $p<0.05$ from control; +Indicates $p<0.05$ from MeHg; #Indicates $p<0.05$ from MeHg–Cys complex; n = 6 mean ± S.E.). The tracings of Fig. 4 are representative lines.

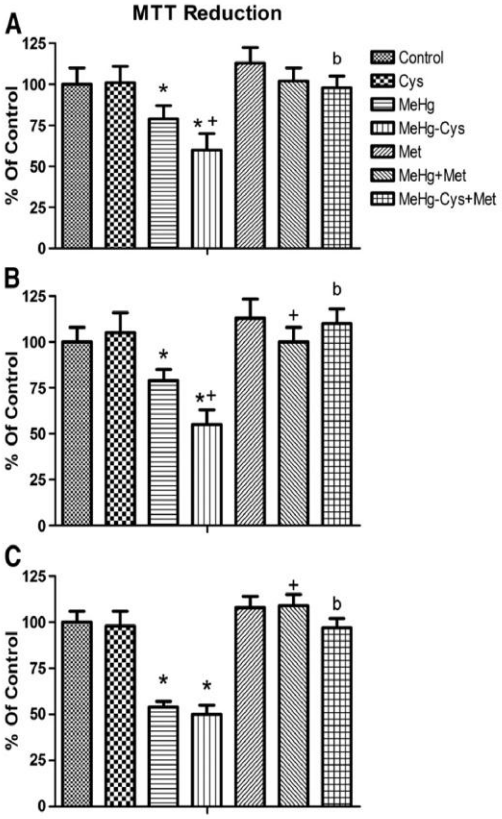

Fig. 6. Effects of Met pre-treatment on mitochondrial function of cells exposed to MeHg or the MeHg–Cys complex. Slices were pre-treated for 15 min with Met (250 μM) and then exposed for 30, 60 or 120 min to MeHg (25 μM) or the MeHg–Cys complex (25 μM each) (Figs. 6A, B, and C respectively). (*Indicates $p<0.05$ from control; $^{+}$Indicates $p<0.05$ from MeHg; $^{\#}$Indicates $p<0.05$ from MeHg–Cys complex; $n=6$ mean ± S.E).

Notably, we observed that Met decreased MeHg uptake by liver slices (Fig. 1). These results are different from those reported by Adachi (2006) after exposure of mice to MeHg. Adachi reported that Met can increase the hepatic deposition of Hg 2 h after intravenously administration of MeHg and/or methionine. Since we have used only a single time-point of exposure of liver slices to MeHg (30 min) and/or Met (45 min), we cannot disregard the possibility that uptake of MeHg could be increased in the presence of Met. Alternatively, the decrease in Hg uptake in the slices by Met may be, at least in part, related to the relatively high concentration of Met in the medium and, consequently, to direct interaction between MeHg and Met, thus lowering the effective free concentration of MeHg. Accordingly, we can posit that the effect observed in the presence of Met may be related to a direct interaction of the sulfur atom and/or amino end of Met with MeHg (Rabenstein and Fairhurst 1975). Alternatively, Met may be reducing the uptake of MeHg complexed with endogenous cysteine in liver slices. In addition, here we have worked with an *in vitro* system derived from rats. It is feasible that results obtained after i*n vivo* exposure may be modified by changes in the amino acids and/or MeHg–Cys complex distribution and metabolism in other animal species.

Thus, our results corroborate that (1) the MeHg–Cys complex is a substrate for the neutral amino acid carrier L-type in the liver and (2) Met prevents the hepatoxicity induced by MeHg, reflecting its ability to reduce MeHg uptake as well as cytotoxicity in liver slices and mitochondria isolated from liver slices treated with the MeHg–Cys complex. Regarding the mechanisms which underlie the MeHg-mediated hepatoxicity, we found that exposure to MeHg or the MeHg–Cys complex increased DFC-RS formation, particularly in mitochondria isolated from liver slices. These results are consistent with previous reports from our group, which have shown that MeHg increases ROS production in cortical brain slices only at high concentrations (100 μM) and after long-term exposure (2 h) (Roos et al., 2009; Wagner et al., 2010). These data also suggest that mitochondria are more sensitive to low MeHg concentrations. In agreement with the present data, it has been previously reported that MeHg, at a concentration of 5 μM, increases ROS levels in mitochondria isolated from rat brain slices (Dreiem and Seegal, 2007; Wagner et al., 2010,). It is noteworthy that in our experimental protocol, MeHg and/or the MeHg–Cys complex reduced mitochondrial activity. These effects are likely related, since ROS can react rapidly with cellular macromolecules and induce mitochondrial damage (Puntel et al., 2010; Colquhoun, 2010; Forkink et al., 2010). Furthermore, because MeHg can cause a pronounced disruption of calcium homeostasis (Stavrovskaya and Kristal, 2010), it is plausible that alterations in Ca^{2+} homeostasis could lead to the collapse of the inner mitochondrial membrane potential, as well as the opening of the mitochondrial permeability pore, events that ultimately result in the loss of mitochondrial function, ROS formation and cell death (Puntel et al., 2010; Colquhoun, 2010; Forkink et al., 2010). Thus, it is reasonable to assume that mitochondria are the primary molecular target for MeHg- and MeHg–Cys-induced cytotoxicity. In addition, we assessed mitochondrial function by analyzing the oxygen consumption of liver slices treated with MeHg or the MeHg–Cys complex. We observed that MeHg exposure attenuated mitochondrial respiration and that this effect was greater in the slices treated with the MeHg–Cys complex. This is in agreement with a recent study, which has demonstrated that dietary MeHg causes a significant decrease in both state 3 of mitochondrial respiration and cytochrome *c* oxidase activity in mitochondria from contaminated zebrafish muscle fibers (Cambier et al., 2009); and inhibits the activity of the mitochondrial complexes II–III, IV, as well as mitochondrial creatine kinase (Glaser et al., 2010). Furthermore, this work has shown that MeHg exposure induces a decoupling of mitochondrial oxidative phosphorylation in the skeletal muscles of the zebrafish (Cambier et al., 2009).

Interestingly, we observed the ability of Met to afford protection against the deleterious effects of MeHg and/or the MeHg–Cys complex. In fact, Met decreased DFC-RS production and prevented the inhibition of mitochondrial respiration and cell viability induced by exposure to MeHg and/or the MeHg–Cys complex. These data show, for the first time, Met's effectiveness in both reducing the bioavailability of MeHg in hepatocytes, as well as its modulation of mitochondrial function. In terms of molecular mechanisms, it is reasonable to assume that the protective effects of Met are linked to its structural similarities with the MeHg–Cys complex. This idea is in agreement with the existence of a mitochondrial neutral amino acid transport (Raymond et al., 1977), which is likely responsible for the uptake of MeHg (as MeHg–Cys complex) into mitochondria. Based on our results, it is possible to state that LAT is not only important for the transport of MeHg into the cell, but also for the transport of MeHg within cellular organelles, allowing for the occurrence of mitochondrial toxicity probably due to the direct effects of MeHg in mitochondrial proteins.

In summary, the results obtained in this study demonstrate that Met prevents the toxic effects of MeHg and the MeHg–Cys conjugate on mitochondrial function and cell viability. Furthermore, the results suggest the possible use of this amino acid as a therapeutic agent for treating acute MeHg exposure. Additional studies to determine the efficacy of Met in reducing the

gastrointestinal absorption of MeHg as well as its ability to accelerate MeHg excretion in animal models of MeHg exposure are well warranted.

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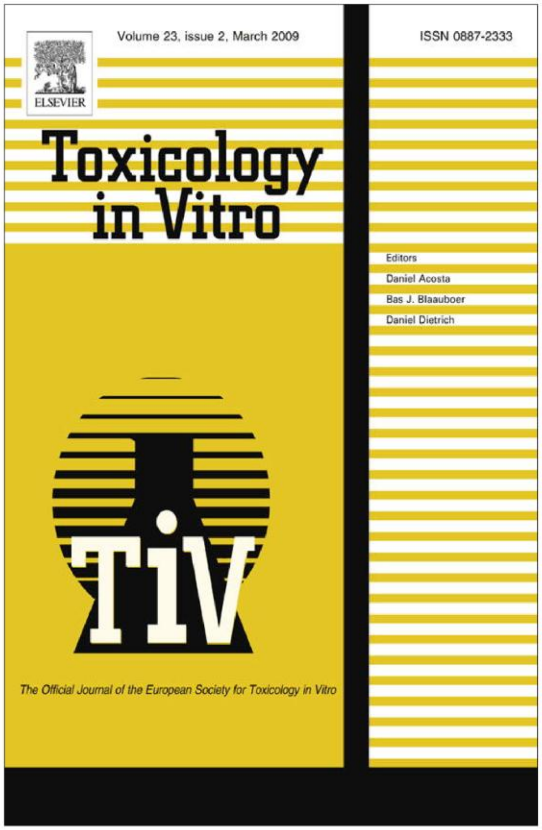

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Guanosine and synthetic organoselenium compounds modulate methylmercury-induced oxidative stress in rat brain cortical slices: Involvement of oxidative stress and glutamatergic system

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ABSTRACT

Excessive formation of reactive oxygen species (ROS) and disruption of glutamate uptake have been pointed as two key mechanisms in methylmercury-toxicity. Thus, here we investigate the involvement of glutamatergic system in methylmercury (MeHg) neurotoxicity and whether diphenyl diselenide, ebselen and guanosine could protect cortical rat brain slices from MeHg-induced ROS generation. MeHg (100 and 200 μM) increased 2′,7′-dichlorodihydrofluorescin (DCFH) oxidation after 2 h of exposure. At 50 μM, MeHg increased DCFH oxidation only after 5 h of exposure. Guanosine (1 and 5 μM) did not caused any effect per se; however, it blocked the increase in DCFH caused by 200 or 50 μM MeHg. Ebselen (5 and 10 μM) decreased significantly the DCFH oxidation after 2 and 5 h of exposure to MeHg. Diphenyl diselenide (5 μM) did not change the basal DCFH oxidation, but abolished the pro-oxidant effect of MeHg. MK-801 also abolished the pro-oxidant effect of MeHg. These results demonstrate for the first time the potential antioxidant properties of organoseleniun compounds and guanosine against MeHg-induced ROS generation after short-term exposure in a simple *in vitro* model. In conclusion, endogenous purine (guanosine) and two synthetic organoselenium compounds can modulate the pro-oxidant effect of MeHg in cortical brain slices.

1. Introduction

Methylmercury (MeHg) is a well-known toxicant that affects a variety of cellular functions, leading to damage in a number of organs and particularly the central nervous system (Chang, 1980; Clarkson, 1997; Crespo-López et al., 2007; Pinheiro et al., 2008). However, the mechanisms by which MeHg exerts its neurotoxic effects are not fully understood and seem to involve a variety of cellular and molecular targets (Allen et al., 2001; Aschner et al., 1999) MeHg has been reported to cause microglial cells death, which can be accompanied by DNA fragmentation and activation of caspase-3-like protease (Nishioku et al., 2000) The generation of reactive oxygen species (ROS), such as H_2O_2, O_2^- and possibly $ONOO^-$, has been proposed as a causal factor in the neurotoxicity induced by MeHg (Aschner et al., 2004). MeHg-induced ROS production is linked to disruption of intracellular calcium homeostasis (Castoldi et al., 2001; Johansson et al., 2007; Kaur et al., 2008), depletion of glutathione (GSH) (Ou et al., 1999; Stringari et al., 2008) and interference with membrane transport (Freitas et al., 1996). Inhibition of glutamate uptake by astrocytes (Aschner et al., 1999; Juarez et al., 2002) and the ensuing increase in the extracellular glutamate levels are likely associated with impaired by ROS production (Juarez et al., 2002).

At high concentrations synaptic glutamate leads to excitotoxicity, a process associated with glutamate receptors over-stimulation and neuronal damage (Danbolt, 2001; Maragakis and Rothstein, 2001). In vivo experiments have also indicated a central role for glutamatergic neurotransmission in MeHg neurotoxicity, particularly the N-methyl D-aspartate (NMDA) receptor. In line with this, MK-801, a noncompetitive NMDA receptor antagonist, can afford protection against MeHg-induced neurotoxicity in neonatal and adult rats (Park et al., 1996; Juarez et al., 2005) and this compound effectively attenuated MeHg toxicity in vitro (Juarez et al., 2005).

Ebselen and diphenyl diselenide, two organochalcogenides, have antioxidant properties that have been connected to their

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glutathione peroxidase-like (Nogueira et al., 2004; Farina et al., 2003; Arteel and Sies, 2001; Moretto et al., 2005, 2007) and thioredoxin-like activities (Zhao and Holmgren, 2002; Zhao et al., 2002b). Furthermore, ebselen has been shown to reverse the in vivo effects of MeHg on glutamate movements in the central nervous system of suckling rats and adult mice (Farina et al., 2004), possibly due to its antioxidant activity. Diphenyl diselenide and ebselen share common chemical and biological properties (Nogueira et al., 2004), thus it is reasonable to suggest that diphenyl diselenide, the simplest of aryl diselenides, could afford some protection against MeHg. In line with this, we have recently demonstrated that diphenyl diselenide can modulate MeHg toxicity after *in vivo* exposure in adult mice (Freitas et al., 2009).

Guanosine exhibits neuroprotective effects in a variety of in vitro and in vivo models of neurotoxicity that involve the over-activation of glutamate receptors, particularly the NMDA-subtype (Frizzo et al., 2001). The exact molecular mechanism(s) involved in the neuroprotection afforded by guanosine is still unknown, but seems to be related to stimulation of glutamate uptake by astrocytes and by cortical slices from young rats (Frizzo et al., 2001, 2003).

Although literature data have supported a role for an over-activation of glutamatergic system in MeHg neurotoxicity (Aschner et al., 2007; Carratu et al., 2006), the possible protective effect of guanosine (an endogenous neuromodulator) and of synthetic antioxidant seleno-organo compounds has not yet been investigated. Thus, the aim of this study was to examine the potential protective effect of organoselenium compounds, MK-801 and guanosine in MeHg-induced neurotoxicity in slices from cortex of rat brain.

2. Experimental procedures

2.1. Animals

Adult male Wistar rats from our own breeding colony (250–350 g) were maintained in an air-conditioned room (22–25 °C) under natural lighting conditions, with water and food (Guabi, RS, Brasil) and ad libitum. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil.

2.2. Slices preparation

Animals were anesthetized with ether and killed by decapitation. The whole brain was quickly removed, placed on ice; the brain cortex was removed and cut into transverse slices of 300 μm thickness using a McIlwain tissue chopper. The slices were placed in PBS buffer containing 10 mM D-glucose, 124 mM NaCl, 10 mM Na_2HPO_4, 5 mM NaH_2PO_4, 5 mM KH_2PO_4, pH 7.4, for approximately 10 min. The PBS buffer was previously bubbled with O_2 95% and CO_2 5% for 30 min. Two slices were selected and placed in the some buffer (PBS 1 ml) for subsequent exposure to reagents.

2.3. Methylmercury exposure

The slices were exposed to MeHg (50–200 μM) for 2 h or (5–50 μM) for 5 h at 37 °C, in the presence of O_2 (95%) and CO_2 (5%). MeHg was dissolved in 25 mM sodium bicarbonate. In some groups, various concentrations of buthionine-l-sulfoxane (BSO), MK-801, guanosine, diphenyl diselenide and ebselen were added during the incubation with MeHg. BSO, MK-801 and guanosine were dissolved in distilled water and organoselenium compounds were dissolved in ethanol. The final concentration of ethanol was 0.025% and this concentration did not alter the control values.

2.4. Evaluation of ROS formation

Formation of intracellular peroxides was detected using an oxidant sensing fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH2–DA) (Hempel et al., 1999). Fluorescence was determined at 488 nm for excitation and 525 nm for emission. After 2 or 5 h of exposure to MeHg, an aliquot (500 μl) was added to 1.5 ml of PBS buffer and 10 μM DCFH2–DA prepared in ethanol, and incubated in the dark for 1 h. The intensity of fluorescence was standardized to protein concentrations (Peterson, 1977) and expressed as percentage of untreated control values.

2.5. Cell viability

The membrane integrity/cell viability was assessed by lactate dehydrogenase (LDH) release in the slices media. The enzyme activity was measured by determining the amount of NADH formed at room temperature at 340 nm (Hewitt et al, 1999). After 2 h of incubation with MeHg the total supernatant of slices (1 ml) was removed and mixed with reaction medium containing (glycine-KOH buffer 100 mM (pH 10), Lactate 50 mM and/or NAD^+ 1 mM. Since thiol oxidizing agents can inhibit LDH (Kade et al., 2009; Zheng et al., 2003), dithiothreitol (DTT 1 mM) was used to restore the LDH activity that was inhibited by MeHg. LDH leakage was expressed as percentage of LDH in the medium over control. Each experiment was performed in duplicate and repeated three times; data were expressed as mean ±S.E.

2.6. Statistical analysis

Data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range test when appropriate. Differences between groups were considered to be significant when $P < 0.05$.

3. Results

3.1. Cell viability

LDH activity was measured to test membrane integrity/cell viability during the insult with MeHg. In the absence of DTT, MeHg caused a marked inhibition of LDH activity from 36% to 55% (50–200 μM, respectively), when compared to control. Addition of DTT 1 mM caused a non-significant decrease in LDH activity. Moreover, when LDH activity was restored with DTT 1 mM, MeHg did not change the LDH activity, thus it suggests that MeHg did not cause significantly LDH leakage from the slices (data not show).

3.2. Effect of BSO on ROS formation

BSO is a classical and specific inhibitor of GSH synthesis (Griffith and Meister, 1979); however, it had no pro-oxidant activity when added to the slices for 2 h (data not show). The lack of a pro-oxidant effect of BSO is possibly related to the short incubation period.

3.3. Effect of MK-801 on ROS formation

Since no literature data was found about the potential effect of the competitive NMDA receptor antagonist on oxidative stress production in cortical brain slices, we have performed a detailed curve of MK-801 in this *in vitro* system. Data depicted in Fig. 1, indicate that MK-801 (25–200 μM) did not modify ROS production, but at

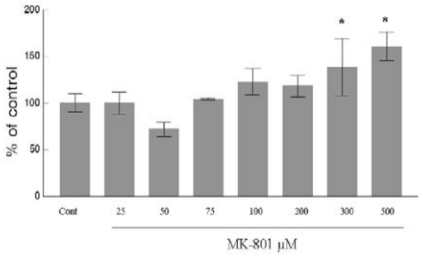

Fig. 1. Effects of MK-801 on DCFH oxidation. Rat brain slices were treated with MK-801 for 2 h. ([a]Indicates $p < 0.05$ from control; Duncan's multiple range test $n = 3–8$; mean ± S.E).

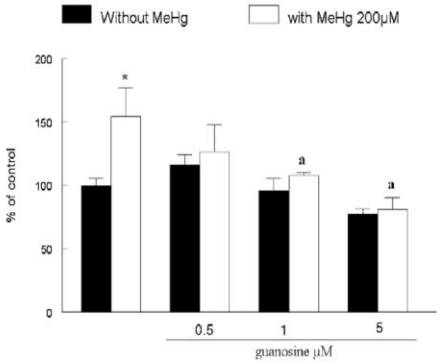

Fig. 3. Effects of guanosine on DCFH oxidation, induced by MeHg (200 μM). Rat brain cortical slices were treated with guanosine for 2 h. ([*]Indicates $p < 0.05$ from control; p [a] < 0.05 from MeHg, by Duncan's multiple range test $n = 3$; mean ± S.E).

higher concentrations (300–500 μM) it caused a significant increase in oxidative stress in rat cortical slices.

3.4. Effects of MeHg on ROS formation

Exposure of cortical brain slices for 2 h to MeHg (100 and 200 μM) produced a significant increase in the rate of DCFH oxidation (Fig. 2A). In addition, DCFH oxidation was stimulated by 50 μM MeHg when incubations were carried out for 5 h (Fig. 2B).

3.5. Effects of guanosine on MeHg-induced ROS formation

ROS generation induced by MeHg (200 μM) was reduced to control levels by guanosine (1–5 μM). However, guanosine did not significantly modify the DCFH oxidation per se (Fig. 3).

3.6. Effect of ebselen and diphenyl diselenide on MeHg-induced ROS formation

After 5 h of incubation, ebselen (5–10 μM) significantly inhibited DCFH oxidation (58% and 53% respectively), when compared to control (Fig. 4A). Moreover, ebselen decreased the pro-oxidant effect of MeHg (50 μM) to basal levels (Fig. 4A). After 2 h of incubation, diphenyl diselenide did not have any significant effect per se on basal DCFH oxidation (Fig. 4B); however, diphenyl diselenide (5 μM) significantly reduced the MeHg (200 μM) – induced DCFH oxidation in cortical slices exposed to MeHg (Fig. 4B).

3.7. Interaction of MK-801 and dipheny diselenide on MeHg-induced ROS formation

Diphenyl diselenide (5 μM) did not change the basal oxidation of DCFH, but abolished the pro-oxidant effect of MeHg (200 μM) after 2 of exposure (Figs. 4B and 5). MK-801 also abolished the pro-oxidant effect of MeHg (200 μM). However, simultaneous exposure to MK-801 and diphenyl diselenide provided no additive antioxidant activity against MeHg (Fig. 5). A similar effect was observed after 5 h of exposure to MeHg (50 μM), (data not shown).

3.8. Interaction between ebselen and guanosine on MeHg-induced ROS formation

Ebselen (5 μM) caused a significant reduction in DCFH oxidation under basal condition, both after 2 (Fig. 6A) or 5 h (Fig. 6B) of incubation. Similarly, ebselen significantly reduced the 200 μM MeHg-induced DCFH oxidation (2 h of exposure) and abolished the pro-oxidant effect caused by 50 μM MeHg (5 h of exposure). Guanosine (5 μM) had no effect on DCFH oxidation under basal condition, but caused a significant decrease in MeHg-induced DCFH oxidation after 2 or 5 h of exposure to MeHg. Simultaneous addition of ebselen and guanosine did not produce additive antioxidant effects (Fig. 6).

4. Discussion

The present results indicate that MeHg caused ROS formation in cortical slices from rats after relatively short periods of exposure (2–5 h). The pro-oxidant activity of MeHg in this in vitro model is apparently related to over-stimulation of NMDA receptors, given that the classical NMDA receptor antagonist, MK-801, blocked the pro-oxidant activity of MeHg.

In this regard, previous studies have reported the protective effects of NMDA receptors antagonists against MeHg-induced neurotoxicity under both in vivo and in vitro conditions (Juarez et al., 2005; Miyamoto et al., 2001). Recently, literature data have demonstrated that MeHg can also increase the expression of NMDA

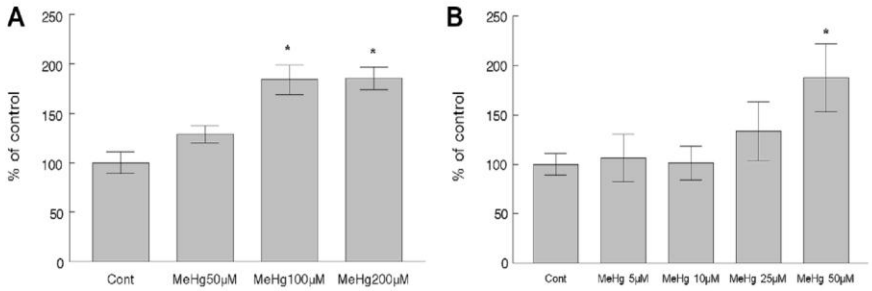

Fig. 2. MeHg-Induced DCFH oxidation in rat brain cortical slices. Slices were incubated for 2 (A) or 5 h (B) with various concentrations of MeHg. MeHg caused an increase in DCFH oxidation ([*] Indicates $p < 0.05$ from control by Duncan's multiple range test $n = 4–6$; mean ± S.E).

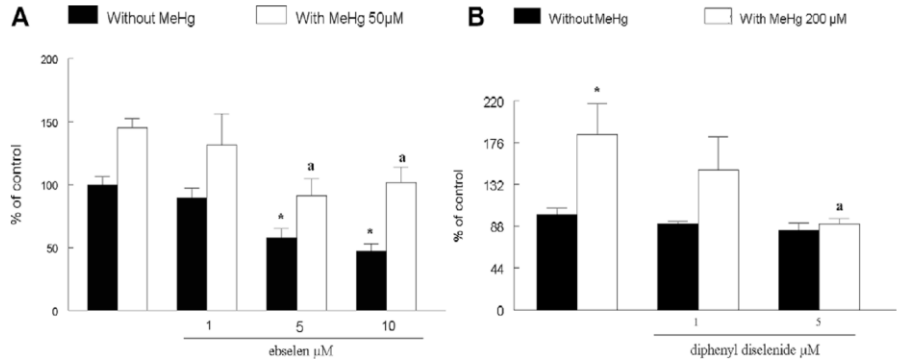

Fig. 4. Effects of ebselen and diphenyl diselenide on MeHg-induced DCFH oxidation. Rat brain cortical slices were treated with ebselen and/or MeHg (50 µM) for 5 h (A). Rat brain cortical slices were treated with diphenyl diselenide and/or MeHg (200 µM) for 2 h (B). (*Indicates $p < 0.05$ from control; [a]Indicates $p < 0.05$ from MeHg, by Duncan's multiple range test $n = 3$; mean ± S.E).

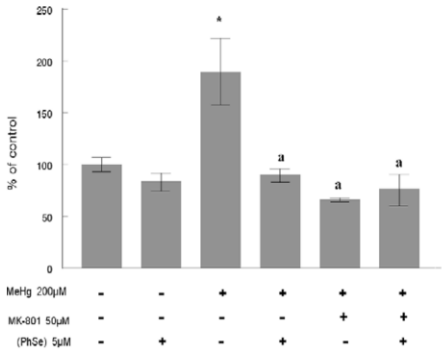

Fig. 5. Effect of (PhSe)2 and MK-801 on DCFH oxidation induced by MeHg. Rat brain cortical slices were incubated with MeHg (200 µM) for 2 h; (*Indicates $p < 0.05$ from control; [a]Indicates $p < 0.05$ from MeHg by Duncan's multiple range test $n = 3–8$; mean ± S.E).

receptors in SH-5Y5Y neoroblastoma, which can enhance further its neurotoxicity (Ndountse and Chan, 2008). In addition, increased extracellular glutamate levels have also been reported after MeHg exposure and this phenomenon appears to be related to glutamate uptake inhibition in astrocytes (Aschner et al., 1999; Juarez et al., 2002) and to an increase in glutamate release from neural cells (Carratu et al., 2006). Furthermore, MeHg activates cytosolic phospholipase A2 (cPLA2) and arachidonic acid (AA) release from the astrocytes (Shanker et al., 2004), which can inhibit glutamate uptake (Saitoh et al., 2004). Based on our results and on the aforementioned studies, one could assume that NMDA overstimulation is a crucial phenomenon related to MeHg-induced ROS formation in cerebral cortical slices.

GSH is an important modulator of MeHg toxicity in astrocytes (Shanker et al., 2005) and exposure to agents that deplete GSH, such as BSO (Griffith and Meister, 1979) increases MeHg neurotoxicity (Shanker et al., 2005). Here we investigated the possible pro-oxidant activity of BSO in cortical slices. However, it had no pro-oxidant effect, likely due to the sort-term period of incubation. Further support for the participation of glutamate in the neurotoxicity of MeHg was given by the protective effect of guanosine. Recently, we and other laboratories have demonstrated that guanosine can act as a neuroprotective agent in a variety of experimental models of toxic injury (Burgos et al., 1998).The mechanism involved in guanosine neuroprotection has been attributed to its ability to stimulate glutamate uptake in brain slices and in astrocytes (Danbolt, 2001; Maragakis and Rothstein, 2001). It is well-known that MeHg inhibits glutamate uptake in astrocytes (Aschner et al., 1999; Juarez et al., 2002) and that these cells are critically involved in modulating extracellular glutamate concentrations in the central nervous system (Aschner et al., 1999). It is possible that guanosine counteracted the pro-oxidant effects of MeHg by lowering the MeHg-induced increase in glutamate. Thus, this is the first demonstration that the endogenous neuromodulator guanosine had a

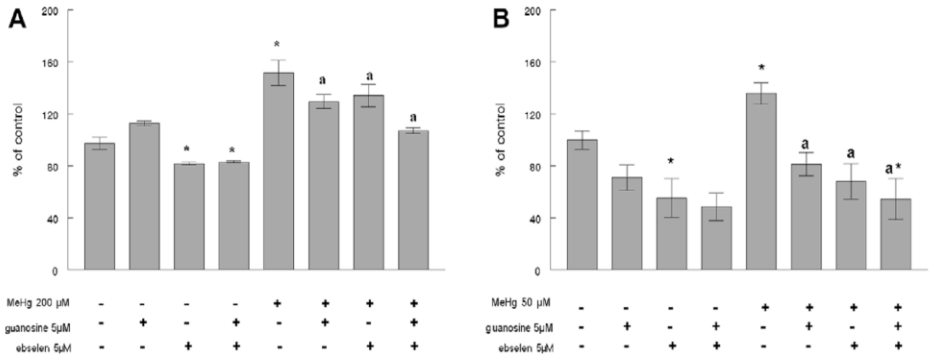

Fig. 6. Effect of ebselen and guanosine on DCFH oxidation induced by MeHg (200 µM) after 2 h of incubation in rat brain cortical slices (A); or after 5 h of incubation with 50 µM MeHg (B); (indicates $p^* < 0.05$ from control; [a]indicates $p < 0.05$ from MeHg by Duncan's multiple range test $n = 4–7$; mean ± S.E).

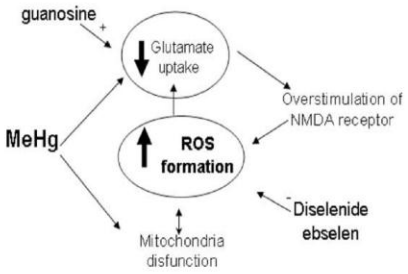

Fig. 7. Scheme to explain the anti-oxidant effects of chalcogens and the involvement of glutamatergic system in the neurotoxicity caused by MeHg.

neuroprotective effect against MeHg-induced neurotoxicity in cerebral cortical slices. Ebselen and diphenyl diselenide, two organic forms of selenium, have been demonstrated to exhibit antioxidant properties and neuroprotective effects in a variety of experimental models of injury (Nogueira et al., 2004; Zhao and Holmgren, 2002). In fact, antioxidants are now been considered potential therapeutic agents by preventing the formation of free radicals and deleterious actions of the ROS oxygen species (ROS) secondary to glutamatergic excitotoxicity (Santamaria et al., 2003; Perez-Severiano et al., 2004; Sutherland et al., 2005, 2006). In the same vein, here we demonstrated for the first time that both antioxidant compounds were effectively in attenuating the prooxidant activity of MeHg in cerebral cortical slices. Since hydrogen peroxide has been proposed as an important mediator of MeHg toxicity (Allen et al., 2002; Farina et al., 2003), the beneficial effects of ebselen and diphenyl diselenide can be related to the maintenance of hydrogen peroxide status at low physiological levels in MeHg-exposed systems (Farina et al., 2003). In addition, an increase in ROS has been observed in (a) mitochondria isolated from MeHg-injected rat brains (Yee and Choi, 1996; Suñol et al., 2008); (b) mitochondria isolated in vitro from rat brain and then exposed to MeHg (Myhre and Fonnum, 2001); (c) mitochondria from Hg and glutamate exposed astrocytes and neurons (Dugan et al., 1995; Brawer et al., 1998).

In agreement with the literature, we showed that MeHg increased hydrogen peroxide formation (measured by DCFH-oxidation) and both organoselenium compounds abolished this phenomenon. The mechanism(s) involved in their antioxidant effects is (are) most likely related to their glutathione peroxidase-like activity (Sies and Arteel, 2000). Furthermore, ebselen, may exert its antioxidant properties by acting as a mimetic of thioredoxin reductase (Zhao et al., 2002). However, a possible direct modulation of the NMDA receptor redox site (Nogueira et al., 2004) by these two organochalcogenes may presently not be ruled out.

Based on the present results and on literature data, we propose a scheme to explain the antioxidant effects of chalcogens and the involvement of glutamatergic system in the neurotoxicity caused by MeHg (Fig. 7). In fact, ebselen and diphenyl diselenide are supposed to act blocking oxidative stress secondary to NMDA overstimulation, whereas guanosine is supposed to decrease oxidative stress by reducing extracellular glutamate.

In conclusion, we suggest that the initial event in MeHg prooxidant activity involves inhibition of glutamate transporters (Aschner et al., 1999; Juarez et al., 2002). This promotes an increase in extracellular glutamate levels that trigger oxidative stress via over-stimulation of NMDA receptor. The antioxidant activity of guanosine is possibly indirect and related to its ability to stimulate glutamate uptake, whereas ebselen and diphenyl diselenide antioxidant properties are supposed to be mediated directly downstream to NMDA receptor stimulation via a blockade of ROS production.

Acknowledgement

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Role of NAD and FAD-Linked Substrates on Mitochondrial Dysfunctions Triggered By Calcium.

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Abstract

The mitochondrial toxicity induced by Ca^{2+} overload may be modulated by many variables, including energetic substrates. In this context, the aim of this study was to compare the events triggered by Ca^{2+} in liver mitochondria oxidizing on NAD malate/glutamate or FAD succinate-linked substrates. Ca^{2+} (60 μM) caused an inhibition of the complex I, but did not alter the activity of complex II in mitochondrial membrane. In malate/glutamate (Mal/Glu) oxidizing mitochondria, the first addition of Ca^{2+} (≈ 15 μM) caused a continuous and gradual decrease in mitochondrial potential (ΔΨm). Different, this effect was rapid and transient in the presence of succinate (succ) as substrate. The second addition of Ca^{2+} (≈ 15 μM) promoted a rapid loss of mitochondrial ΔΨm in both Mal/Glu and succ oxidizing mitochondria. Ciclosporine (CsA) did not modify the effect of the first addition of Ca^{2+} on ΔΨm, but abolished the collapse of ΔΨm caused by subsequent addition of Ca^{2+} in both Mal/Glu and succ oxidizing mitochondria. Ca^{2+} (≈ 60 μM) caused a significant decrease on oxygen consumption in succ-oxidizing mitochondria when compared to Mal/Glu. The mitochondrial redox state, determined by NAD(P)H and GSH pool, was preferentially affected by Ca^{2+} in Succ-oxidizing mitochondria. Furthermore, Ca^{2+} promoted an increase in free radical formation in the presence of succ as substrate when compared to Mal/Glu. These effects were prevented by CsA and ruthenium red (RR). In summary, our data suggest that calcium may induce mitochondrial dysfunction by distinct routes depending on the energetic substrates supporting respiration. However, considering that these pathways could lead to mitochondrial transition pore opening, additional studies are needed to evaluate the role of oxidizable substrates on other parameters of mitochondrial damage induced by Ca^{2+}.

Keywords: Mitochondria; Calcium; Malate/Glutamate; Succinate

1- Introduction

Mitochondria can oxidize a variety of substrates to maintaining the energy-dependent cellular processes; however, numerous factors can affect mitochondrial function causing energy metabolism deficits and cell death (Lai 1992; Nicholls and Ferguson 1992). Of note, it is the role of calcium in modulating mitochondrial function and dysfunction. At physiological concentration, calcium is a key element to intra-mitochondrial metabolic reactions control. On the order hand, perturbations in calcium-handling properties of mitochondria can cause cell death via different pathways (Nicholls and Budd 2000; Villalobo and Lehninger 1980; Dykens 1994; Kowaltowski *et al.*, 1996). In this way, there are a growing number of data showing that mitochondrial calcium accumulation leads to enhanced generation of reactive oxygen species (ROS), which triggers cellular events involved in cell death (Brookes *et al*., 2004; Feisner *et al*., 2009; Komari *et al*., 2010; Adam-Vizi and Starkov 2010).

It has been shown that calcium-induced ROS formation can be associated with thiol oxidation and membrane transition pore (MTP) opening (Brookes and Darley-Usmar 2004), which leads to osmotic swelling of the mitochondrial matrix, dissipation of the $\Delta\Psi m$ (Petrosillo *et al*., 2004), release of NADH intra-mitochondrial and cessation of the ATP synthesis (Kowaltowski *et al*., 2001). Indeed, the MTP opening seems to be strongly dependent on the redox state of the mitochondria since mitochondrial thiol oxidation induced by calcium overload accelerates the oxidation of pyridine nucleotides (Lehninger *et al*., 1978; Constantini *et al*., 1996). To note, the reduction in mitochondrial NAD(P)H and GSH pools induced by calcium can also limit the activity of glutathione peroxidase, contributing further to ROS formation and MPT induction and consequently to maintenance of a vicious cycle (Kowaltowski *et al*., 2001).

Although, there is a general consensus that mitochondrial calcium overload can lead to cell death via stimulation of ROS production, data from *in vitro* studies are quite controversial, since mitochondrial ROS generation in response to a calcium challenge can be modulated by many variables, including calcium concentration, mitochondria source, metabolic state, energetic substrate, etc (Brookes *et al.*, 2004; Komari *et al.*, 2008). Consequently, the precise molecular mechanisms by which calcium can elicit mitochondrial dysfunctions are yet to be elucidated. Regarding the energetic substrates supporting respiration, there are few reports comparing the events triggered by Ca^{2+} in mitochondria respiring on NAD (malate/glutamate) or FAD (succinate)-linked substrates. Regarding to ROS production and MTP opening, there are points of evidence indicating that the rate of H_2O_2 generation induced by calcium can varies depending on the substrate (succinate and/or

glutamate+malate) oxidized by mitochondria and that the sensitivity of the permeability transition pore also depends on the type of oxidizable substrate (Chavez et al., 2002; Komari et al., 2008; Tretter and Adam-Vizi 2007).

Considering the scarcity of experiments investigating the influence of complex I and II substrates on the mitochondrial events elicited by calcium, the present study was performed to evaluate the effects of calcium in mitochondria respiring on NAD or FAD-linked substrates. Specifically, we have investigated the potential toxicity of Ca^{2+} in liver mitochondria sustained by Succ and/or Mal/Glu. Particularly, we have determined the effects of Ca^{2+} on $\Delta\Psi m$ collapse, and/or complex I inhibition; the effect of Ca^{2+} on electron flux in the mitochondrial chain as well as the ability of this ion in changing mitochondrial thiol redox state and consequently NAD(H) pool. Furthermore, the role of substrates on the mitochondrial ROS generation calcium-induced was evaluated.

2 - Materials and Methods:

2.1 Chemicals

Chemicals, including NADH, mannitol, rotenone, succinic acid, malonate, ruthenium red and HEPES were obtained from Sigma Chemical Company (St Louis, MO, USA). All other reagents were commercial products of the highest purity grade available.

2.2 Animals:

Adult male *Wistar* rats from our own breeding colony (250-350g) were maintained in Plexiglas cages with food and water *and libitum*, in a temperature-controlled room (22-25ºC) and on a 12 h-light/dark cycle with lights on at 7:00 a.m. Animals were handled and treated according to the guidelines set forth by the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

2.3 Mitochondrial Preparation:

Mitochondria were isolated from liver of rat as previously described by Navarro et al., 2002, with some modifications. Liver was manually homogenized in cold buffer I [(225 mM mannitol, 75 mM sucrose, K^+ EGTA 1 mM, bovine serum albumin (BSA) 0.1% and 10 mM HEPES (pH 7.2) (KOH)]. The homogenate was centrifuged at 2.000 *g* for 7 min at 4ºC, and the supernatant was centrifuged at 12.000 *g* for 10 min at 4ºC. Then, the resultant supernatant was discarded, and the pellet was re-suspended in buffer II (manitol 225 mM, sucrose 75 mM, K^+ EGTA 1 mM and K^+- HEPES 10 mM pH 7.2) and re-centrifuged at 12.000 x *g* for 10 min at 4ºC. Finally, the last supernatant was discarded, and the pellet was re-suspended and

maintained in buffer III (sucrose 100 mM, KCl 65 mM, K^+-HEPES 10 mM and EGTA 50 µM pH 7.2) for subsequent analyses.

2.4 Mitochondrial Complex I and II Assay:

Rat liver mitochondria were adjusted about 20 mg/mL of protein in buffer III or TFK 100 mM, pH 7.2 and the samples were immediately frozen tree cycles of free-thawing using dry ice/acetone at ≈ -40 °C (Navarro et al., 2002; 2004). The activity of complex I (NADH:ubiquinone oxidoreductase) in mitochondrial membranes (0.5 mg/ml) was measured after Ca^{2+} (60 µM) exposure by addition of NADH 100 µM. The enzymatic activity was determined following the decrease in fluorescence (indicative of NADH oxidation) at 365 nm of excitation and 463 of emission slit 5 at 30 °C, during 180 sec. The complex I activity was expressed in nmol/mL of NADH oxidation.

The activity of complex II (succinate:ubiquinone oxidoreductase) was measured after Ca^{2+} (≈ 60 µM) exposure. Mitochondrial membranes (0.5 mg/mL) in buffer III were supplemented with succinate 5 mM as substrate and incubated with Ca^{2+} (≈ 60 µM) or malonate 8 mM in presence of MTT 50 µg/mL at 30 °C for 10 min. The reaction was stopped by addition of DMSO (1 mL) and the absorbance was determined at 570 - 630 nm. The complex II activity was expressed as % of control.

2.5 *Measurements of mitochondrial transmembrane electrical potential (ΔΨm).*

Mitochondrial ΔΨm was estimated by fluorescence changes in Safranine (2 µM) and record in a RF-5301 Shimadzu spectrofluorometer (Kyoto, Japan) operating at excitation and emission wavelengths of 495 and 586 nm respectively, with slit widths of 3 and 5 nm respectively at 30 °C (Tretter et al., 2007). Mitochondria (1 mg/mL) in buffer III were supplemented with Mal/Glu (2.5 mM each) or succinate (5 mM) as substrate and challenge twice with Ca^{2+} (≈ 15 µM per addition). Data of mitochondrial ΔΨm are presented as arbitrary fluorescence units per second (AAU/s).

2.6 Oxygen uptake measurements.

Oxygen uptake was measured in an oxymeter fitted with a water-jacket Clark-type electrode (Yellow Springs Instruments Co., Model 5300 or Oxytherm, Hansatech). The isolated rat liver mitochondria (0.250 mg/mL) were incubated with standard respiration buffer III in the presence of Mal/Glu (2.5 mM each) or succinate (5 mM) as substrate at 30 °C and challenge with Ca^{2+} ≈ 60 µM. In order to verify if the mitochondria were intact and

responding to substrate (state II) and ADP/Pi (state III), the mitochondria were exposed to ADP/Pi and oligomicine 2 µM.

2.7 Total SH content determination.

The mitochondrial total SH content was measured by Ellman reaction (Ellman 1959) with some modifications. The mitochondria (0.250 mg/mL) were incubated in buffer III in presence of complex I or II substrates for 200 and 900 sec at 30 °C. This medium was diluted 1:1 in Tris-HCl 1 M (pH 7) and 200 µL of the mixture was placed on Elisa plate in presence of DTNB (200 µM). The absorbance was measured at 405 nm (record by Elisa PT reader Thermo Plate) and the results were expressed as percent of control.

2.9 Determination of NAD(P) redox state.

The oxidation or reduction of pyridine nucleotides in the intact mitochondrial suspension was determined in a RF5301 Shimadzu spectrofluorometer (Kyoto, Japan) operating at excitation and emission wavelengths of 365 (slit 3 nm) and 463 nm respectively (slit 5 nm) (Votyakova and Reynolds 2005). The intact mitochondria samples (0.250 mg/mL) in buffer III were supplemented with Mal/Glu (2.5 mM each) or succinate (5 mM) as substrate and challenge with Ca^{2+} (≈ 60 µM) during 900 sec at 30 °C. The values were expressed in nmol of NAD(P)H/mL.

2.8 Estimation of reactive oxygen species (ROS) production.

The mitochondrial generation of ROS was determined spectrofluorimetrically using the membrane permeable fluorescent dye DCHF-DA (5 µM) (Garcia-Ruiz et al., 1997). Fluorescence was determined at 488 nm for excitation and 525 nm for emission, with slit widths of 1.5 nm. Mitochondria (50 µg/mL) in buffer III were supplemented with Mal/Glu (2.5 mM each) or succ (5 mM) as substrate and challenge by Ca^{2+} ≈ 60 µM. The values are expressed as percentage of control with substrate.

2.9. Protein determination

All experiments were standardized to protein concentrations (Peterson 1977), and when appropriate were expressed as percentage of control values.

2.10. Statistical analysis

Data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate. The significance between the respiratory rates (Table 1) was

analyzed statistically by *t*-test. Differences between groups were considered to be significant when $P < 0.05$.

3 - Results:

3.1. Effects of Ca^{2+} on mitochondrial complex I and II activity.

The effect of Ca^{2+} on mitochondrial complex I activity was monitored in disrupted mitochondria on the presence of 300 µM ADP, 1 mm Pi and 0.6 mM $MgCl_2$ 600 µM. Ca^{2+} at 60 µM inhibited approximately 40% of NADH oxidation (Figure 1). Rotenone (3 µM) inhibited the activity of complex I more than 85%. In contrast to complex I, Ca^{2+} (≈ 60 µM) did not alter the mitochondrial complex II when compared with the marked inhibition induced by malonate (8 mM) (data not shown).

3.2. Effects of substrates of complex I and II on Ca^{2+}- induced mitochondrial (ΔΨm) uncoupling.

This set of experiment was used to assess the capacity of mitochondrial complex I or II in maintaining ΔΨm when challenge with Ca^{2+}. In succ oxidizing mitochondria, the first addition of Ca^{2+} (≈ 15 µM) caused a rapid and transient decrease in ΔΨm (Figure 2A, trace 1). However, after the second addition of Ca^{2+} (≈ 15 µM) there was a complete collapse of ΔΨm. Trace 2 shows the effect of CsA (2 µM) on mitochondrial ΔΨm. The addition of CsA did not prevent the collapse of ΔΨm caused by the first addition of Ca^{2+} (final Ca^{2+}concentration of ≈ 15 µM); but prevented the mitochondrial depolarization caused by the second addition of Ca^{2+} (final Ca^{2+}concentration of ≈ 30 µM). The inclusion of RR in the incubation medium prevented completely the Ca^{2+}-induced ΔΨm collapse (Trace 3).

The first addition of Ca^{2+} (≈ 15 µM) to Mal/Glu oxidizing mitochondria caused a continuous and gradual decrease in ΔΨm. In contrast, the second addition of Ca^{2+} (≈ 15 µM) promoted a rapid loss of mitochondrial ΔΨm. The addition of CsA (2 µM) did not modify the effect of the first addition of Ca^{2+} (final Ca^{2+}concentration of ≈ 15 µM) on ΔΨm. On the order hand, the mitochondrial depolarization induced by the second addition of Ca^{2+} (final Ca^{2+} concentration of ≈ 30 µM) was abolished in the presence of CsA. RR caused a complete protection against the effects of Ca^{2+} on ΔΨm collapse (Trace 3).

3.3. Effects of Ca^{2+} in mitochondria in the presence of substrates of complex I or II on oxygen consumption

Oligomicine reduced the oxygen consumption confirming mitochondrial coupling (state IV); and in the presence of uncoupler 2.4 DNP mitochondria returned to basal respiration.

In the presence of Mal/Glu as mitochondrial substrates, Ca^{2+} (≈ 60 μM) caused a significant decrease on oxygen consumption when compared to oxygen consumption determined in mitochondria on state II and III and/or succ-oxidizing mitochondria (Figure 3). Differently, the oxygen consumption in succ oxidizing mitochondria was not modified after Ca^{2+} addition.

3.4. NAD(P)H oxidation in the presence of Ca^{2+} *and substrates of complex I and II*

In Succ-oxidizing mitochondria, Ca^{2+} caused a rapid and transient increase in the NAD(P)H content, which was followed by an abrupt decrease in NAD(P)H pool (Figure 4A, line 1). Different, in the presence of Mal/Glu, Ca^{2+} caused a rapid increase in the NAD(P)H content that was followed by a gradual decrease in NAD(P)H pool (Figure 4A, line 2).
CsA did not modify the transient increase of NAD(P)H pool caused by Ca^{2+}; however, CsA blunted the subsequent Ca^{2+}-induced NAD(P)H oxidation both in the presence of Mal/Glu and Succ as mitochondrial substrates (Figure 4C, line 2). RR abolished the rapid increase in NAD(P)H content stimulated by Ca^{2+} and reduced considerably the subsequent oxidation of NAD(P)H caused by Ca^{2+}, regardless of the substrate used (Figure 4C, line 3). Rotenone (3 μM, used as positive control on NADH oxidation) did not modify the NADH levels in mitochondria sustained with Succ; but it promoted an increase on NADH content in Mal/Glu oxidizing mitochondria.

3.5. Reactive Oxygen Species formation in the presence of Ca^{2+} *and substrates of complex I or II*

ROS formation in liver mitochondria was stimulated by Mal/Glu. Ca^{2+} decreased Mal/Glu-induced ROS formation (Figure 5A, compare line 3 and 4), and this decrease was not reverted by CsA and RR addition (Figure 5B). Similarly to Ca^{2+}, rotenone (3 μM) caused a reduction on ROS generation in Mal/Glu oxidizing mitochondria (Figure 5A, compare line 3 and 5).

Succ caused an increase in ROS generation when compared to control (without substrate). In contrast to that observed with Mal/Glu, Ca^{2+} promoted an increase in ROS formation in succ respiring mitochondria when compared to Succ alone (Figure 5C: compare line 2 and 3). In the presence of Succ as a mitochondrial substrate, CsA blunted Ca^{2+}-induced

ROS formation (Figure 5D). Similarly to CsA, RR (5 µM) prevented the Ca^{2+}-induced ROS formation in mitochondria oxidizing with succ (Figure 5D).

3.6. Effects of Ca^{2+} in mitochondria oxidizing with substrates of complex I and II on total thiol redox state

Data on Table I show that after 900 sec of exposure, Ca^{2+} (≈ 60 µM) caused a significant increase on total thiol oxidation rate in mitochondria oxidizing with both Mal/Glu and Succ when compared to substrates alone. These results are in agreement with the data obtained on NADH oxidation (see Figure 4A and 4B).

CsA (2 µM) was effective in blocking thiol oxidation induced by Ca^{2+} only in mitochondria oxidizing with Mal/Glu. Different, RR (5 µM) prevented thiol oxidation Ca^{2+}-induced in both Mal/Glu and Succ oxidizing mitochondria (Table I).

4 - Discussion:

In view of the complexity of mitochondria in terms of metabolism and function, it is expected that the mitochondrial events triggered by Ca^{2+} can be modulated in the presence of different classes of respiratory substrates. With this in mind, this study was delineated to evaluate specifically the effects of Ca^{2+} on Mal/Glut and Succ oxidizing mitochondria and consequently to obtain more details on the role of Ca^{2+} in mitochondrial dysfunctions. In this work, we have shown that Ca^{2+} affected the activity of complex I, which catalyzes the transfer of electrons from NADH to the ubiquinone pool with an electron flux that is coupled to proton-pumping and is inhibited by rotenone (Sadek et al., 2004). This substantial reduction on complex I activity induced by Ca^{2+} could have been mediated by its direct action on mitochondrial complex I since the conformations and/or states of the enzymatic complex are differentially sensitive to this ion (Gavrikova and Vinogradov 1999; Grivennikova et al., 2001). Thus, an abrupt Ca^{2+} overload could be affecting kinetic parameters of NADH dehydrogenase complex (Kotlyar et al., 1992; Vinogradov 1998). In this way, an interesting study demonstrated that concentration relatively low of Ca^{2+} (10-20 µM) inhibit the complex I in cardiac mitochondrial membranes of rats by changing the oxidative state of protein-sulfhydryl (Sadeke et al., 2004). On the order hand, our data show that Ca^{2+} also decreased significantly the NAD(P)H pool in intact mitochondria. This fact, via substrate depletion, could reflect an indirect effect of Ca^{2+} on the activity of complex I (Lehninger 1978; Kowaltowski et al., 2001). Different from results obtained to complex I, we verify that Ca^{2+} did not modify the complex II activity in mitochondrial membranes. This lack of Ca^{2+} effect

on complex II may be linked to the fact that FAD reduction/oxidation processes occur into Succinate dehydrogenase (SDH) enzyme. Corroborating this hypothesis that the complex II is less vulnerable to Ca^{2+} than complex I, literature data show that ROS like $O_2^{\cdot-}$ inactivates iron-sulfur proteins as NADH dehydrogenase; but presents a mild efficiency toward SDH (Lê-Quôc et al., 1981).

Regarding to ΔΨm, our results show that in Mal/Glu-oxidizing mitochondria the lower Ca^{2+} concentration caused a small but continuous decrease in ΔΨm. However, the higher Ca^{2+} concentration was associated with a rapid ΔΨm loss. Furthermore, in mitochondria sustained with Succ, the lower Ca^{2+} concentration induced a transient reduction in ΔΨm, which was recovered back to the control approximately after 1 min; and the second addition of Ca^{2+} was marked by a total loss of ΔΨm. Taken together, these data indicate that Ca^{2+} exposure promoted two distinct phases in mitochondrial depolarization that were dependents on the concentration and respiratory substrate. Here, we assume the idea that mitochondrial depolarization induced by lower concentration of Ca^{2+} (first phase on mitochondrial depolarization), may be due to a decrease in mitochondria complex I activity, since it was not prevented by CsA. Different, the mitochondrial depolarization caused by higher concentration of Ca^{2+} (subsequent rapid phase on mitochondrial depolarization) seems be linked with the classical MTP opening, because it was blocked by CsA addition. In agreement with the present data, several studies conducted on isolated mitochondria have shown that the MPT pore is triggered by Ca^{+2} overload and that MPT pore opening results in mitochondrial dysfunctions including mitochondrial membrane potential dissipation (Starkov et al., 2002; Chavez et al., 2002; Petrosillo et al., 2004).

It has been postulated that the ΔΨm maintenance and ATP syntheses processes are directly linked to redox state of NAD(P) mitochondrial (Lehninger 1978; Kowaltowski et al., 2001). Moreover, mitochondrial ROS production can be modulated by NAD(P)H content. In fact, NAD(P)H may induce a reduction on mitochondrial ROS formation since is involved in GSH pool maintenance via glutathione peroxidase and thioredoxin reductase activities (Hoek and Rydström 1998). In line with this, a recent study demonstrated that the brain mitochondria respiring on NAD-linked substrates are able in removing high rate of exogenous H_2O_2 (Zoccaroto et al., 2004). Similarly, under our experimental conditions, an abrupt decrease in the NAD(P)H levels was verified when mitochondria were sustained with Succ and challenged with Ca^{2+}. Of note, this effect was also accompanied by a rapid GSH oxidation and ROS overproduction. On the order hand, when Mal/Glu oxidizing mitochondria were exposed to Ca^{2+}, the levels of NAD(P)H decreased gradually with the time. Likewise, the rate of GSH oxidation was slow (900 seconds) and ROS formation was reduced when compared

to the values found in Succ oxidizing mitochondria. It is possible that, under this condition, these events occurred as a consequence of mitochondrial NADH levels (Watabe et al., 1997), which probably were maintained by Ca^{2+}- induced factors as reduced activity of complex I (Figure 1); increased activity of the Krebs Cycle enzymes (Hansford 1985) and/or maintenance of Malate/Glutamate dehydrogenises activity by substrate. Taken together, these data support the idea that a decrease in the thiol redox state (Table I) is linked to increased rate of NAD(P)H oxidation (Figure 4) and that this fact probably modulates free radical formation (Figure 5) in mitochondria respiring on different substrates.

In summary, our data suggest that calcium may induce mitochondrial dysfunction by distinct routes depending on the energetic substrates supporting respiration (scheme). In mitochondria sustained with Mal/Glu, calcium exposure inhibited the activity of complex I causing mitochondrial $\Delta\Psi m$ loss and decrease in oxygen consumption, possibly leading to energetic deficits. However, even under conditions the mitochondria were able in maintaining a positive redox state via a slow NAD(P)H oxidation, GSH oxidation and free radical formation. In mitochondria sustained with Succ and exposed to calcium, the complex II activity, $\Delta\Psi m$ and oxygen consumption were maintained and possibly keeping the energetic state. However, the mitochondrial redox balance was extremely affected as verified by rapid decrease in NAD(P)H and GSH content and increase in free radical formation. Considering that these distinct pathways could lead to mitochondrial transition pore opening, additional studies are needed to evaluate the role of oxidizable substrates on other parameters of mitochondrial damage induced by Ca^{2+}.

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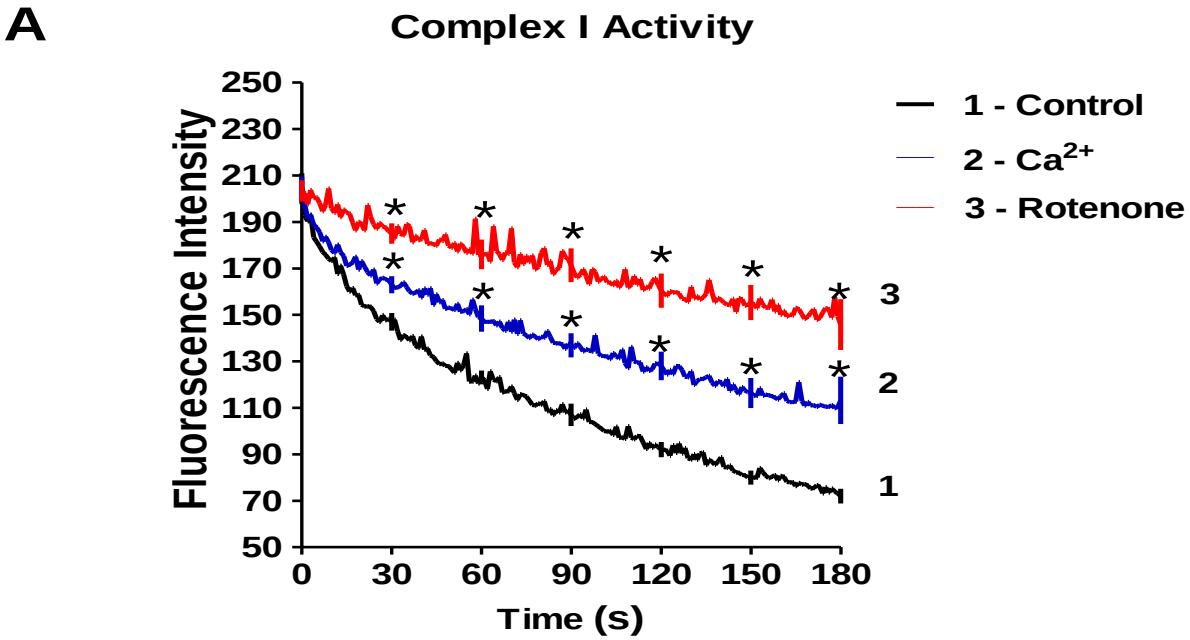


Fig. 1 Effect of Ca^{2+} 60 µM on mitochondrial complex I activity. (*Indicates $p < 0.05$ from control; n = 6 mean ± S.E.M)

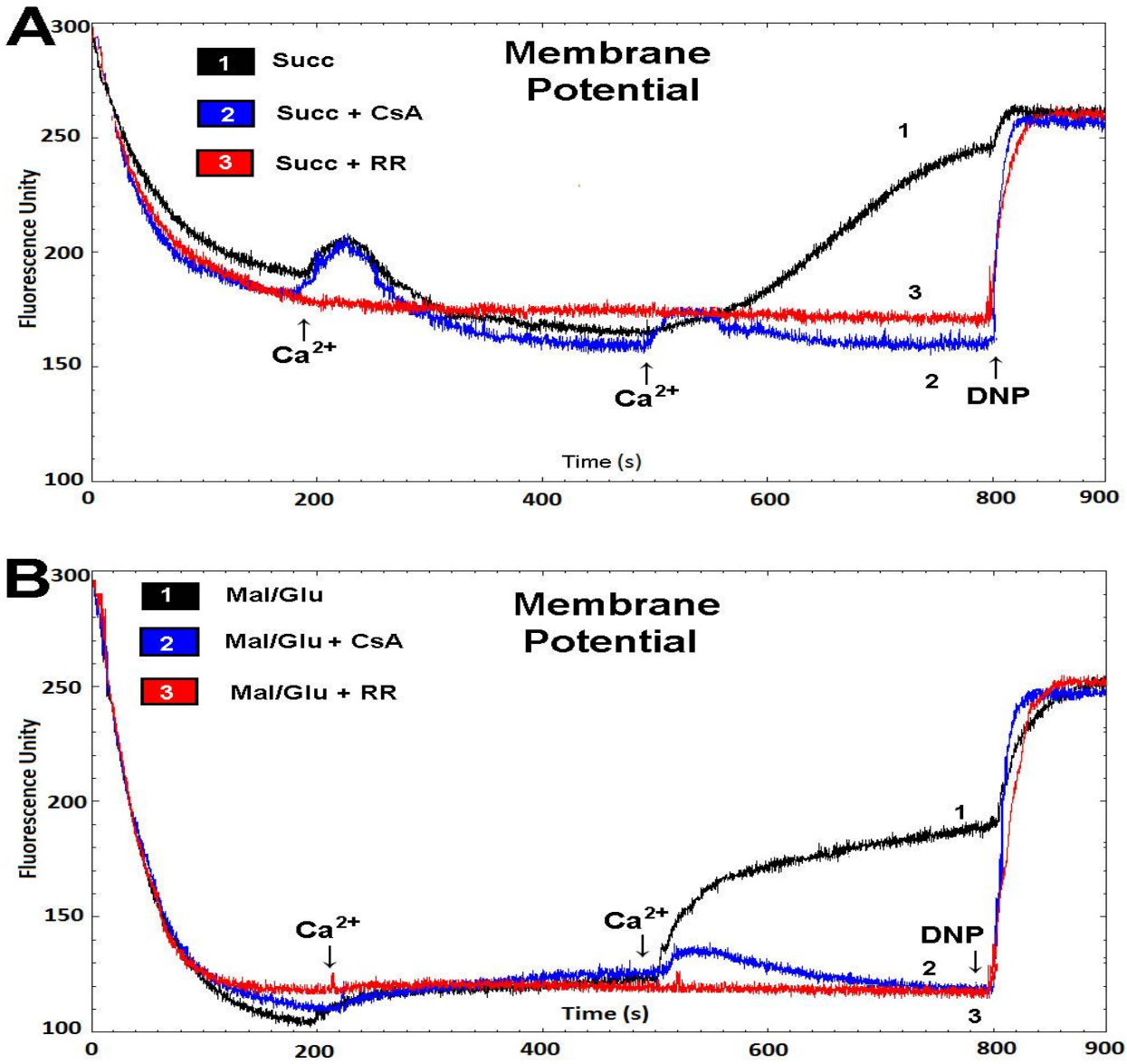


Fig. 2 Effect of Ca^{2+} on Membrane Potential in mitochondria supplemented by substrates of complex II (A) or I (B). Arrow indicates the addition of $Ca^{2+} \approx 15$ µM or DNP 5 µM. When appropriated the experiments were started with CsA 2 µM, RR 5 µM or Rotenone 3 µM. The traces of figure are representative lines of 5 independent experiments.

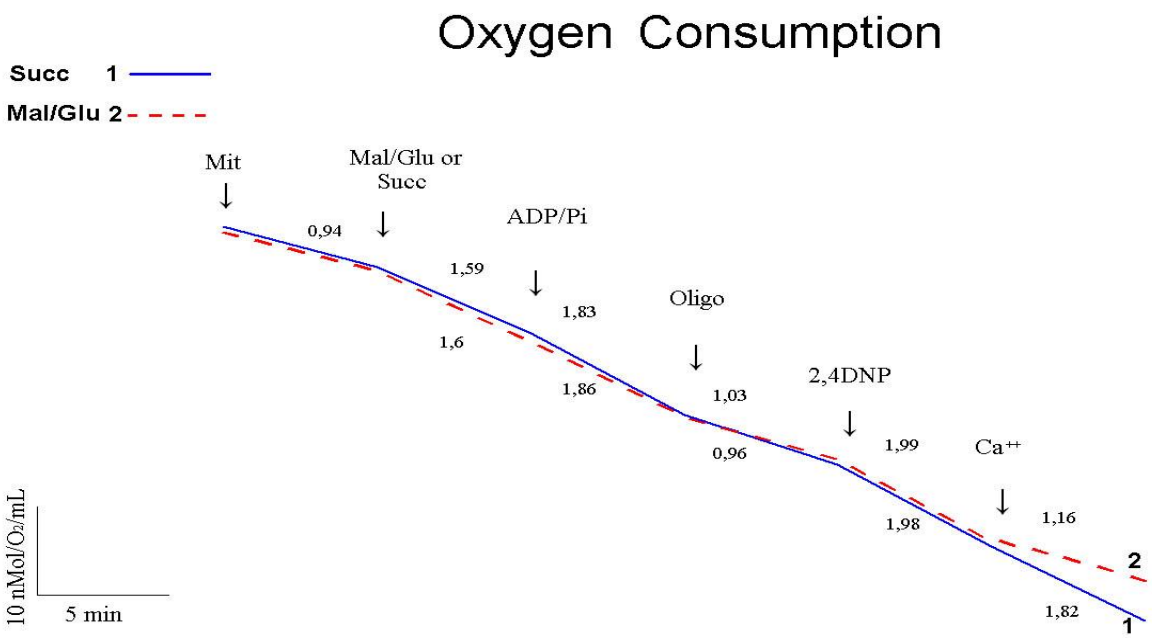


Fig. 3 Effect of Ca^{2+} on oxygen consumption in mitochondria supplemented by substrates of complex I or II. Arrow indicates the addition of (Mal/Glu 2.5 mM each) or (Succ 5 mM); ADP/Pi 300 µM/1 mM; oligomicine 2 µM; 2.4 DNP 5 µM; DNP 1 µM and $Ca^{2+} \approx 60$ µM. The traces of figure are representative lines of 5 independent experiments.

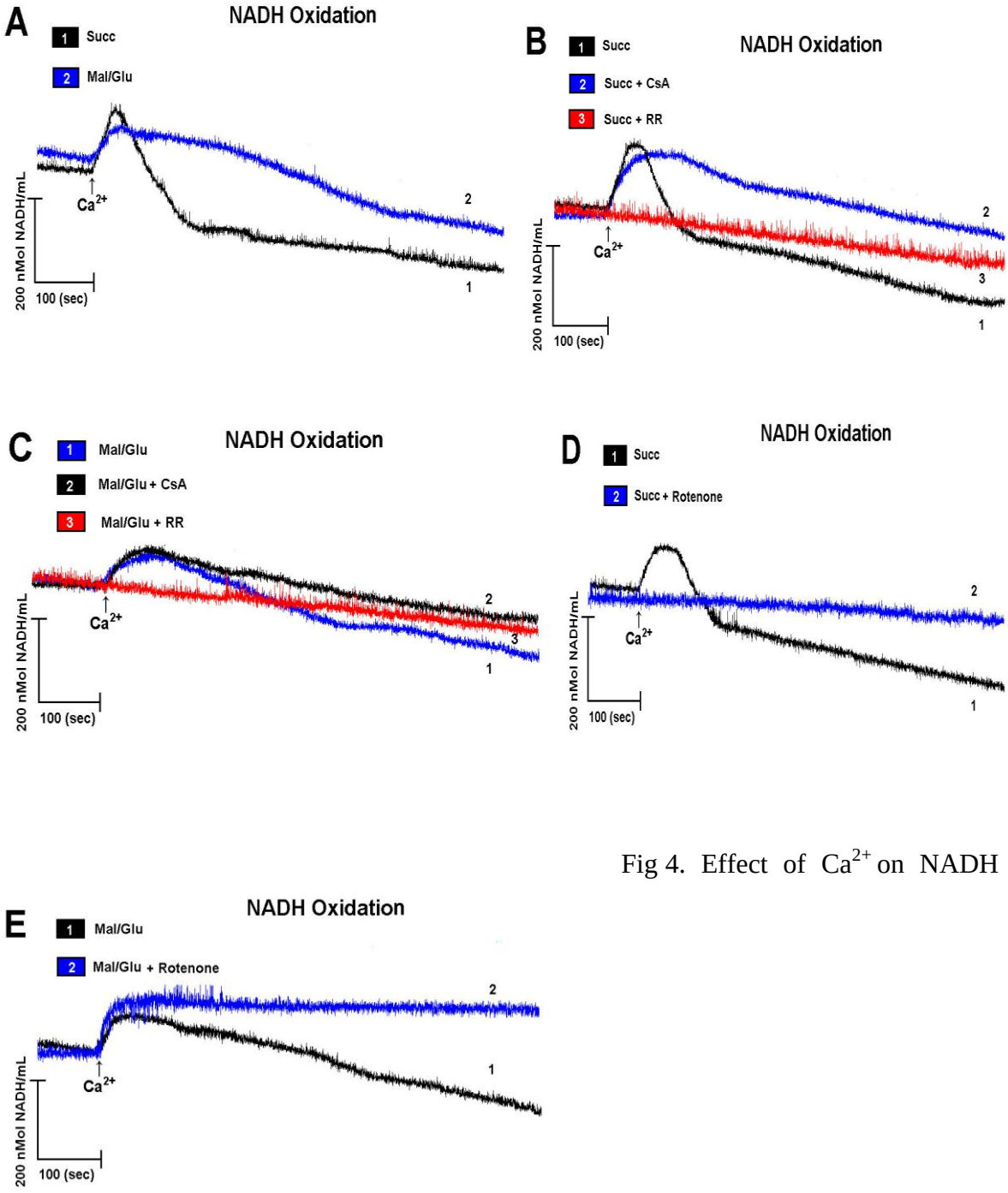
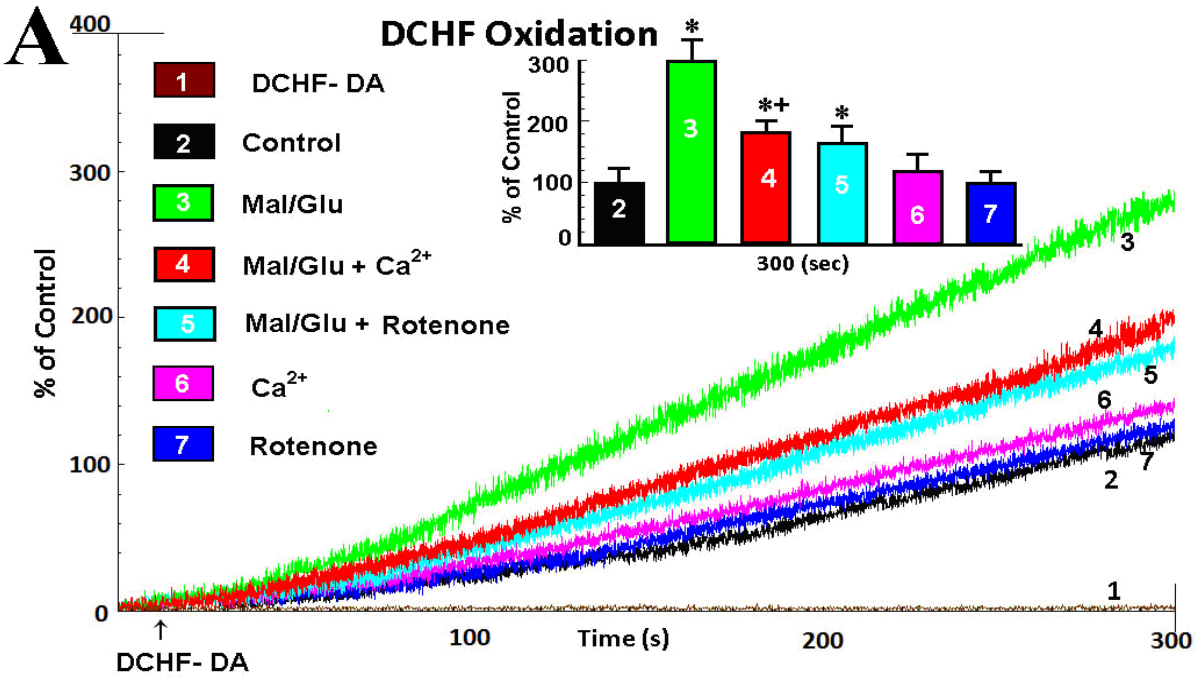
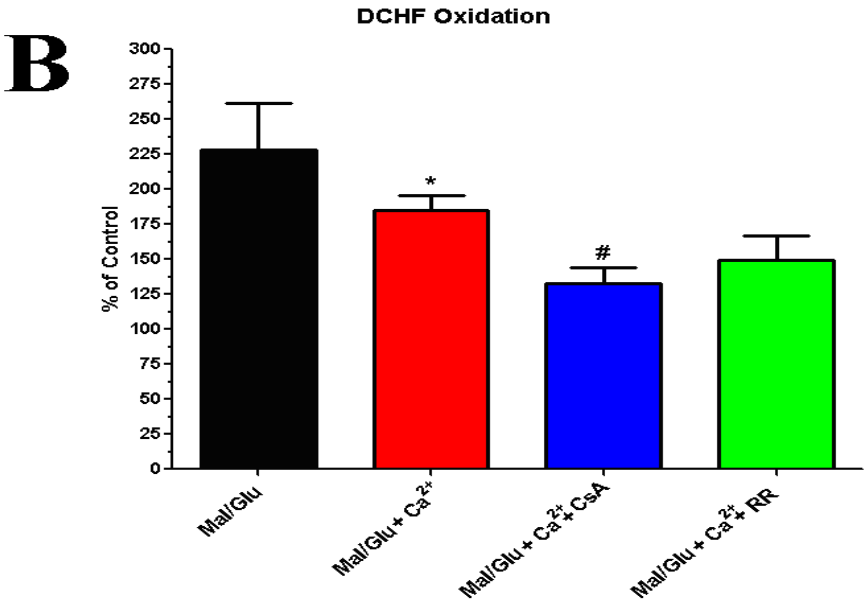


Fig 4. Effect of Ca^{2+} on NADH


A
DCHF Oxidation
1 DCHF- DA
2 Control
3 Mal/Glu
4 Mal/Glu + Ca2+
5 Mal/Glu + Rotenone
6 Ca2+
7 Rotenone
% of Control
Time (s)
DCHF- DA
300 (sec)


B
DCHF Oxidation
% of Control
Mal/Glu
Mal/Glu + Ca2+
Mal/Glu + Ca2+ + CsA
Mal/Glu + Ca2+ + RR

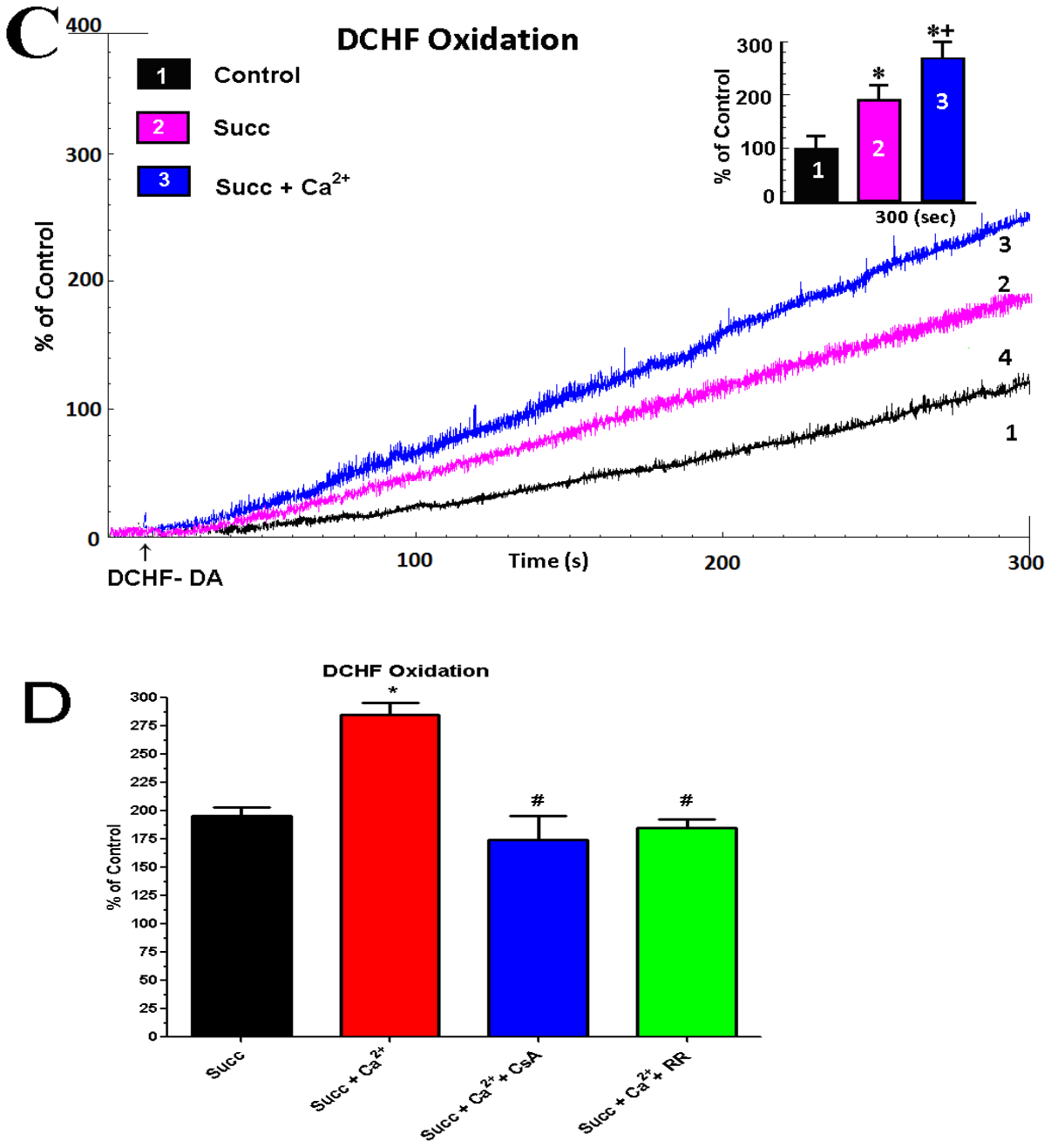


Fig. 2 Effect of Ca^{2+} on reactive species formation in mitochondria supplemented by substrate of complex I (A,D) or II (B,C). The experiments were started with CsA 2 µM or RR 5 µM when appropriated (B) or (D). (*Indicates $p < 0.05$ from control without Ca^{2+}; + Indicates $p < 0.05$ from Ca^{2+} , mean ± S.E.M). The traces of figures are representative lines of 3-5 independent experiments.

	Time 0 Total Thiol % of Control	Time 200 (s) Total Thiol % of Control	Time 900 (s) Total Thiol % of Control
Mal/Glu	100 ± 1.86	97.69 ± 2.54	98.69 ± 2.72
Succ	100 ± 3.77	92.21 ± 4.10	93.09 ± 2.06
	With Ca^{2+}		
Mal/Glu	100 ± 5.3	91.2 ± 5.1	71.80 ± 5.2*
Succ	100 ± 5	81.14 ± 6.86	74.4 ± 5.53*
	With Ca^{2+} and CsA		
Mal/Glu	100 ± 3.31	97.97 ± 1.91	98.23 ± 1.81
Succ	100 ± 2.65	57.65 ± 2.5*	56.12 ± 1.61*
	With Ca^{2+} and RR		
Mal/Glu	100 ±1.45	96.6 ± 3.21	91.9 ± 7.78
Succ	100 ± 2.65	84.65 ± 1.21	82.72 ± 1.79

Table1. Effect of $Ca^{2+} \approx 60$ µM on total thiol oxidation in mitochondria supplemented with substrates of complex I or II. When appropriated the experiments were started with CsA 2 µM or RR 5 µM. (* indicate $P < 0,05$ from control without Ca^{2+}; n = 4-6, mean ± SEM).

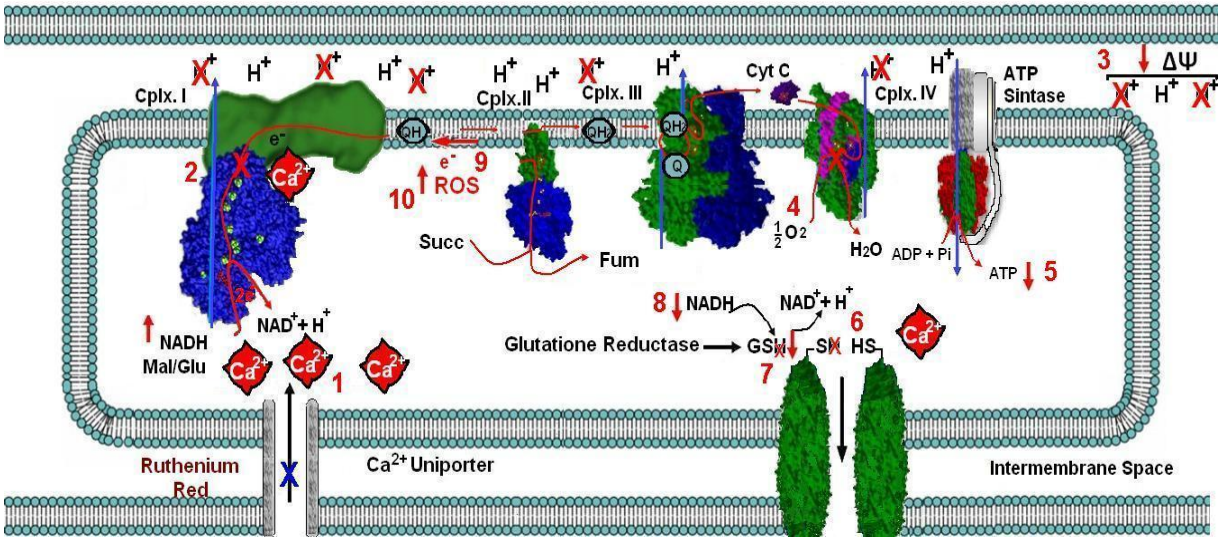


Fig 6. Scheme to explain the possible mechanisms involved in the mitochondrial toxicity calcium-induced. (1) Calcium overload; (2) inhibition of complex I; (3) membrane potential loss; (4) decrease on oxygen consumption; (5) inhibition of ATP synthesis; (6) MTP opening; (7) decrease on total thiol; (9) decrease on NADH content and (10) increase of free radical formation.

5. DISCUSSÃO

O MeHg é a forma mais comum de mercúrio orgânico pela qual humanos e outros animais são expostos. No meio ambiente o MeHg é predominantemente formado pela metilação de íons de mercúrio inorgânico por microorganismos presentes no solo e na água (Zalups., 2000; Clarkson e Magos., 2006; Rooney., 2007). Clinicamente os efeitos mais relevantes oriundos de uma intoxicação por MeHg são em nível de SNC (WHO., 2003). Os íons de MeHg tem uma alta afinidade por vários nucleófilos, especialmente por grupos sulfidril, os quais estão presentes em biomoléculas tais como: Cys, GSH, homocisteína (Hcy), N-acetilcisteína (NAC) e albumina. Devido a essa alta afinidade praticamente não existe MeHg iônico livre em meios biológicos (Hughes., 1957). O sistema L de transporte de aminoácido neutros (LAT) é capaz de transportar uma ampla variedade de substratos (Betz e Goldstein., 1978). Assim, é possível que esses transportadores utilizem o complexo MeHg-Cys como substrato (Kerper e cols., 1992). Nossos resultados do artigo I mostraram que o MeHg complexado a Cys é mais facilmente captado pelas fatias hepáticas e pelas mitocôndrias isoladas dessas fatias do que o MeHg sozinho. Também verificamos neste trabalho que o pré-tratamento com Met foi capaz de diminuir essa captação de MeHg pelas fatias e mitocôndrias isoladas dessas fatias. Esses dados sugerem que a captação e acúmulo de MeHg é via sistema L de transporte de aminoácidos. Uma das possíveis formas de prevenir a captação de mercúrio é através de uma inibição do tipo competitiva. De fato, sabe-se que o complexo MeHg-Cys é um mimético da Met (Lander., 1971; Jernelov., 1973), e que esse aminoácido é transportado para as células hepáticas principalmente via sistema (LAT) (Betz e Goldstein., 1978). Deste modo, um aumento na concentração de Met dificultaria a entrada do complexo MeHg-Cys por estes transportadores, evitando o acúmulo de Hg nas células. Nesse trabalho também foi observado que para todos os parâmetros avaliados (geração de radicais livres; consumo de oxigênio e viabilidade celular/atividade mitocondrial), o complexo MeHg-Cys apresentou uma maior toxicidade quando comparado ao MeHg sozinho. Além disso, a Met foi capaz de prevenir os efeitos tóxicos de ambas as formas de mercúrio nas fatias e nas mitocôndrias isoladas dessas fatias nesses parâmetros. Com base em tais constatações, sugere-se que a Met é capaz de atenuar os efeitos tóxicos do MeHg por estar modulando o processo de captação do mesmo. O epitélio que reveste a barreira hemato-encefálica apresenta transportadores do tipo LAT, principalmente os do tipo LAT1 e LAT2, (Betz e Goldstein., 1978). Desta maneira, o tecido cerebral torna-se um importante alvo de acúmulo de mercúrio. Dados da literatura mostram que o cérebro é um

dos principais órgãos que acumulam Hg e também o mais sensível à sua toxicidade (Aschner e cols., 1990; 1991; Roos e cols., 2010).

Existem várias hipóteses que explicam a toxicidade do MeHg no SNC e uma delas enfatiza que a neurotoxicidade mediada por MeHg envolve um desequilíbrio da homeostase glutamatérgica (Aschner e cols., 1999; Juarez e cols., 2002; Dalla Corte e cols., 2011). Neste contexto, os resultados do artigo II indicam que o MeHg leva à formação de radicais livres em fatias de córtex cerebral de ratos possivelmente via alteração no sistema glutamatérico. Os resultados deste trabalho revelam que o MK-801, um antagonista de receptores do tipo NMDA, bloqueia a atividade pró-oxidante do MeHg, sugerindo uma forte ligação entre o super estímulo desses receptores glutamatérgicos com a neurotoxicidade induzida pelo MeHg. De acordo com tais observações, estudos prévios também têm evidenciado o efeito protetor de antagonistas NMDA na toxicidade mediada por MeHg tanto *in vitro* quanto *in vivo* (Miyamoto e cols., 2001; Juarez e cols., 2005). Além disso, um aumento dos níveis extracelulares de glutamato também tem sido constatado após a exposição ao MeHg. Este fenômeno parece estar ligado a uma inibição da recaptação do Glu pelos astrócitos (Aschner e cols., 1999; Juarez e cols., 2002) e/ou ao aumento da liberação de Glu pelos neurônios na fenda sináptica (Carratu e cols., 2006). Os dados do artigo II condizem com esses dados da literatura, uma vez que, a guanosina foi capaz de reduzir a geração de radicais livres induzida pelo MeHg. A guanosina tem sido descrita como uma molécula neuroprotetora contra vários fatores que causam injúria central via excesso de Glu na fenda sináptica (Danbolt e cols., 2001; Maragakis e Rothsten., 2001; Burgos e cols., 2007). O possível papel neuroprotetor da guanosina tem sido amplamente atribuído à sua capacidade de estimular a captação de Glu pelos astrócitos (Danbolt e cols., 2001; Maragakis e Rothsten., 2001). Em nosso estudo também foi verificado a ação neuroprotetora de dois compostos orgânicos de selênio, o ebselen e o disseleneto de difenila. Esses efeitos provavelmente estejam relacionados à atividade antioxidante dos mesmos, a qual já foi demonstrada em vários modelos de estresse oxidativo (Yee e Choi., 1996; Mihre e Fonnum., 2001; Suñol e cols., 2008; Nogueira & Rocha, 2010). Além disso, estes compostos orgânicos de selênio são capazes de modular os receptores NMDA, possivelmente via redução/oxidação de sítios específicos (Nogueira e cols., 2004). De forma geral, os resultados do artigo II sugerem que após a captação de mercúrio, é possível que esse neurotoxicante promova uma disfunção na homeostase glutamatérgica disparando eventos que aumentem a geração de radicais livres.

A geração de radicais livres mediada pela disfunção glutamatérgica está fortemente ligada à toxicidade do cálcio via disfunção mitocondrial (Choi, 1992; Coyle e Puttfarcken., 1993; White e Reynolds., 1996). De fato, sabe-se que o aumento de Glu na fenda sináptica

causa uma super estimulação dos receptores NMDA que regulam a entrada de cálcio nos neurônios pós-sinápticos. Apesar da toxicidade mitocondrial induzida por MeHg poder ocorrer de modo independente da citotoxicidade induzida pelo excesso de cálcio, vários estudos mostram que há uma forte ligação entre esses eventos (Mori e cols., 2007; Farina e cols., 2011). No manuscrito I, nós avaliamos os efeitos do cálcio em termos de mecanismos mitocondriais sem a presença de mercúrio, com o intuito de melhor compreender as respostas mitocondriais frente ao cálcio. Tem sido demonstrado que os alvos moleculares e os efeitos tóxicos do Ca^{2+} via mitocôndria, podem sofrer influência dos diferentes substratos respiratórios (Zoccaroto e cols., 2004; Doczi e cols., 2011); no entanto, os mecanismos de ação do cálcio com relação a esses parâmetros não estão completamente elucidados. Neste sentido, os resultados do manuscrito I sugerem que o cálcio induz dano mitocondrial por vias distintas, mas interligadas. Em membranas mitocondrias o cálcio diminuiu a atividade do complexo I. Esses dados corroboram com achados de estudos os quais demonstram que concentrações relativamente baixas de cálcio (10-20 µM) inibem a atividade do complexo I em mitocôndrias isoladas de coração, provavelmente por mudar o estado oxidativo de grupos SH presentes neste complexo protéico (Sadek e cols., 2004). A inibição do complexo I pode estar ligada com a perda de potencial em mitocôndrias sustentadas com Mal/Glu, como observado no nosso trabalho. Além disso, um bloqueio do complexo I da mitocôndria pode alterar o fluxo de elétrons na cadeia respiratória, causando uma diminuição no consumo de oxigênio, como verificado nas mitocôndrias sustentados com Ma/Glu. Esses efeitos do cálcio observados em mitocôndria sustentadas com substrato do complexo I, podem também culminar com dano mitocondrial e morte celular via diminuição do estado energético. Por outro lado, nós observamos que quando as mitocôndrias foram sustentadas com Mal/Glu, o potencial redox mitocondrial foi pouco prejudicado, uma vez que, houve uma diminuição lenta do conteúdo de NAD(P)H e GSH reduzida. Estes fatos poderiam explicar, pelo menos em parte, a baixa geração de radicais livres observada nessa situação. Dados da literatura suportam a idéia que substratos do complexo I podem auxiliar na remoção de H_2O_2 exógeno em mitocôndrias (Zoccarato e cols., 2004). De fato, substratos respiratórios que aumentam a formação de NADH podem auxiliar na redução da GSH via sistema tioredoxina e/ou glutationa peroxidase (Zoccarato e cols., 2004) atenuando assim a geração de radicais livres exógenos ou gerados pela toxicidade do cálcio. Contudo, em membranas mitocondriais o cálcio não causou uma diminuição significativa na atividade do complexo II. De acordo, dados da literatura reportam que a succinato desidrogenase em geral é menos vulnerável à perda de atividade quando comparada ao complexo I, provavelmente pelo fato da oxidação/redução do FAD ocorrer no interior da enzima (Lê-Quôc e cols., 1981). Por

conseqüência, nosso trabalho mostra que o cálcio provocou apenas um efeito transitório na perda ΔΨm e não alterou o consumo de oxigênio em mitocôndrias sustentadas com Succ. Porém, o estado redox mitocondrial (oxidação de NAD(P)H e GSH) foi extremamente afetado pela exposição ao cálcio. Assim, o aumento na geração de radicais livres induzido por cálcio em mitocôndrias sustentadas por este substrato pode ser considerado uma provável conseqüência desses eventos.

A abertura de PTM parece ser um evento ocasionado de forma independente do substrato mitocondrial, porém dependente da concentração de cálcio. De fato, as mitocôndrias sustentadas com ambos os substratos apresentaram abertura de PTM apenas quando expostas a altas concentrações de cálcio. No entanto, estudos adicionais são necessários para um melhor entendimento sobre o papel dos substratos energéticos na modulação dos efeitos do cálcio nas mitocôndrias.

De forma geral, os resultados obtidos no presente trabalho colaboram para a caracterização dos possíveis mecanismos envolvidos na captação e acúmulo tecidual de Hg, bem como, somam evidências de que a neurotoxicidade induzida por MeHg pode ser mediada, em termos de mecanismos, por alterações no sistema glutamatérgico. Além disso, através deste estudo obteve-se conhecimentos acerca dos efeitos do cálcio sobre parâmetros de disfunção mitocondrial, os quais provavelmente estão associados à ação tóxica do MeHg.

6. CONCLUSÕES

De acordo com os resultados apresentados nesta tese, podemos sugerir que:

Artigo Científico I

- O complexo MeHg-Cys é preferencialmente captado em fatias e mitocôndrias de fígado.
- A Met previne a captação de mercúrio em fatias e mitocôndrias de fígado.
- A exposição ao complexo MeHg-Cys causa mais dano que a exposição ao MeHg em células e mitocôndrias.
- A Met previne os efeitos tóxicos induzido por MeHg-Cys e MeHg em hepatócitos e mitocôndrias isoladas.
- O mecanismo de captação de mercúrio em hepatócitos envolve o transportador LAT e que o efeito da Met em diminuir a entrada de mercúrio e conseqüente a toxicidade é provavelmente via inibição competitiva.

Artigo Científico II

- O MeHg causa geração de radicais livres apenas em altas concentrações após 2 ou 5 horas de exposição em fatias de córtex cerebral de ratos.
- Antagonistas dos receptores NMDA reduzem a neurotoxicidade induzida pelo MeHg.
- A guanosina diminui a geração de radicais livres induzida pelo MeHg.
- Compostos orgânicos de selênio, com comprovada capacidade antioxidante, podem diminuir a toxicidade do MeHg.
- Compostos que modulem a homeostase glutamatérgica, bem como, o influxo de cálcio, podem reduzir a neurotoxicidade do MeHg em fatias de córtex cerebral de ratos.

Manuscrito I

- O cálcio causa uma diminuição da atividade do complexo mitocondrial I.
- A diminuição do potencial de membrana induzida por cálcio pode envolver sua ação direta ou indireta sobre a atividade do complexo I ou sobre a abertura de poro mitocondrial e que estes efeitos dependem da concentração de cálcio, bem como, do substrato respiratório.
- O cálcio diminui o fluxo de elétrons da cadeia respiratória e consequentemente o consumo de oxigênio em mitocôndrias sustentadas com Mal/Glu.
- O cálcio diminui preferencialmente o estado redox em mitocôndrias sustentadas com Succ.

- A geração de radicais livres induzida pelo cálcio é aumentada apenas em mitocôndrias sustentadas com substrato do complexo II.
- Os efeitos tóxicos do cálcio em mitocôndrias de fígado podem ocorrer por vias distintas, mas interligadas dependendo do substrato respiratório e da concentração de cálcio.

7. PERSPECTIVAS

Os dados apresentados nesta Tese podem servir de base para a realização de trabalhos complementares acerca dos mecanismos moleculares envolvidos na toxicologia do MeHg e do Cálcio. Assim, temos como perspectivas realizar estudos adicionais que visem:

- Identificar o destino do complexo MeHg-Cys (análogo Met) após a entrada nos tecidos.
- Investigar como esse complexo é metabolizado.
- Investigar se existe uma formação "liquida" de MeHg livre a partir do complexo (MeHg-Cys) que possa reagir com outros tióis ou selenóis intracelulares.
- Avaliar se moduladores da captação de (MeHg-Cys) como por exemplo, a Met/e ou a Selenometionina poderiam modular os efeitos neurotóxicos do mercúrio.
- Verificar se moduladores da entrada de cálcio poderiam atenuar a toxicidade do MeHg em células e mitocôndrias.
- Investigar se o substrato energético modula a toxicidade do mercúrio em mitocôndrias isoladas de diferentes tecidos.

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