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Tese de Doutorado

MODULAÇÃO DO SISTEMA GLUTAMATÉRGICO EM PLAQUETAS: EFEITO DE METAIS PESADOS E ORGANOCALCOGÊNIOS

Vanessa Corralo Borges

Santa Maria, RS, Brasil

2007

MODULAÇÃO DO SISTEMA GLUTAMATÉRGICO EM PLAQUETAS: EFEITO DE METAIS PESADOS E ORGANOCALCOGÊNIOS

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por

Vanessa Corralo Borges

Tese apresentada ao Programa de Pós-Graduação em Bioquímica Toxicológica, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
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MODULAÇÃO DO SISTEMA GLUTAMATÉRGICO EM PLAQUETAS: EFEITO DE METAIS PESADOS E ORGANOCALCOGÊNIOS

Elaborada por **Vanessa Corralo Borges** como requisito parcial para a obtenção do grau de **Doutor em Bioquímica Toxicológica**

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

MODULAÇÃO DO SISTEMA GLUTAMATÉRGICO EM PLAQUETAS: EFEITO DE METAIS PESADOS E ORGANOCALCOGÊNIOS

AUTORA: Vanessa Corralo Borges
ORIENTADORA: Cristina Wayne Nogueira
DATA E LOCAL DA DEFESA: Santa Maria, janeiro de 2007.

Recentes pesquisas têm sido desenvolvidas para caracterizar biomarcadores em tecidos periféricos que possam ser utilizados em casos de dano, disfunção ou interações envolvendo alvos de agentes tóxicos. As plaquetas têm sido amplamente sugeridas como um excelente modelo do aparato sináptico e, ainda tem sido demonstrado que estas células acumulam glutamato de maneira similar a sinaptossomas de cérebro de ratos. De fato, as células sanguíneas são ideais para monitorar os efeitos neurotóxicos causados por agentes químicos, por serem de fácil acesso e devido a estas apresentarem funções similares às encontradas em neurônios do sistema nervoso central. O aumentado número de agentes químicos presentes no meio ambiente tem elevado o risco de intoxicação ocupacional. Os metais pesados são alguns dos principais contaminantes encontrados no meio ambiente. Estes metais podem interagir com grupos -SH, os quais são necessários para a função de muitas enzimas, proteínas estruturais e receptores. Os compostos de organoselênio (disseleneto de difenila e ebselen) e de organotelúrio (ditelureto de difenila) também foram alvo deste estudo, visto que também possuem a capacidade de oxidar grupos –SH de moléculas biologicamente ativas. Assim como os metais pesados, os mecanismos pelos quais os organocalcogênios desencadeiam ações tóxicas não são bem descritos. Levando em consideração que grupos –SH estão presentes em receptores e transportadores, investigou-se os efeitos dos metais pesados (Hg^{2+}, Pb^{2+} e Cd^{2+}), bem como do disseleneto de difenila, ditelureto de difenila e ebselen sobre o sistema glutamatérgico em plaquetas humanas. Os resultados obtidos demonstraram que o Hg^{2+}, o Cd^{2+} e o Pb^{2+} inibem a captação de [^{3}H]-glutamato em plaquetas. O Hg^{2+} inibiu a união específica de [^{3}H]-glutamato, enquanto o Cd^{2+} e o Pb^{2+} estimularam a união específica de [^{3}H]-glutamato em plaquetas. Os metais pesados testados causaram um aumento na peroxidação lipídica e nos níveis de espécies reativas de oxigênio em plaquetas, sugerindo que o estresse oxidativo, pelo menos em parte, pode estar envolvido na neurotoxicidade induzida por estes metais. O 2,3-dimercaptopropanol (BAL), um agente quelante ditiólico usado terapeuticamente no tratamento de intoxicações por metais pesados, foi capaz de proteger e restaurar os efeitos causados por Pb^{2+}, Cd^{2+} e Hg^{2+} sobre a união específica de [^{3}H]-glutamato. Outro agente quelante ditiólico testado, o ácido 2,3-dimercapto-1-propanosulfônico (DMPS), foi capaz de proteger e restaurar os efeitos causados pelos metais pesados sobre a união específica de [^{3}H]-glutamato em plaquetas. Resultados similares foram obtidos com o ditiotreitol (DTT), um agente utilizado como redutor de grupos -SH. Os agentes ditiólicos sozinhos não alteraram a união específica de [^{3}H]-glutamato. Em contraste, a glutationa reduzida (GSH), um agente redutor monotiol, causou inibição sobre a união específica de [^{3}H]-glutamato em todas as concentrações testadas. Esse composto não foi capaz de proteger do efeito causado pelos metais. Esses resultados sugerem que os

agentes ditiólicos (BAL, DMPS e DTT) podem atuar quelando os metais pesados e/ou como redutores de grupos –SH dos receptores glutamatérgicos. O disseleneto de difenila e ditelureto de difenila inibiram a união específica de [^{3}H]-glutamato, mas o ebselen não interferiu nesse parâmetro. Além disso, todos os organocalcogênios testados inibiram a captação de [^{3}H]-glutamato em plaquetas. A inibição causada por estes compostos sobre a captação e união específica de [^{3}H]-glutamato, não foi revertida pela adição de agentes redutores (DTT e GSH), indicando que os efeitos tóxicos dos organocalcogênios não está exclusivamente relacionado com à oxidação de grupos –SH. Considerando os resultados obtidos neste estudo, é possível sugerir que as plaquetas podem servir como marcadores periféricos em casos de exposição por agentes tóxicos e, ainda representam um bom modelo para investigar os mecanismos envolvidos nas alterações causadas por estes compostos.

Palavras-chave: Plaquetas, sistema glutamatérgico, metais pesados, selênio, telúrio e agentes quelantes.

ABSTRACT

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

MODULATION OF GLUTAMATERGIC SYSTEM IN PLATELETS: EFFECT OF HEAVY METALS AND ORGANOCHALCOGENS

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ADVISOR: Cristina Wayne Nogueira
DATE AND PLACE OF THE DEFENSE: Santa Maria, 2007

Research strategies have been developed to characterize parameters in peripheral tissues that might easily be measured in humans as surrogate markers of damage, dysfunction or interactions involving neural targets of toxicants. Human platelets have been repeatedly suggested as an excellent model for various aspects of the synaptic apparatus and these cells have been shown to accumulate glutamate in a manner similar to that in as synaptosomal preparations. In fact, blood cells such as platelets are ideally suited for monitoring a chemical's neurotoxic effects because of their easy accessibility and because they share a number of functions similar to those of central nervous system neurons. The increasing number of chemicals present in the habitational and occupational environment elevates the risk of toxic human exposure. Heavy metals have become one of many contaminants found in our environment. These metals may react with free protein cysteine thiol groups, which are involved in the function of many enzymes, structural proteins and receptors. Organoselenium (diphenyl diselenide and ebselen) and organotellurium (diphenyl ditelluride) are known to disturb cellular functions by oxidizing -SH groups of biomolecules. Considering that -SH groups are important constituents of receptors and transporters, the effects of heavy metals (Hg^{2+}, $Pb^{2+}$and Cd^{2+}) as well as diphenyl diselenide, diphenyl ditelluride and ebselen on glutamatergic system in human platelets were examined. The results clearly demonstrated that Hg^{2+}, Cd^{2+} and Pb^{2+} inhibited [^{3}H]-glutamate uptake in human platelets. Hg^{2+} inhibited [^{3}H]-glutamate binding, while Cd^{2+} and Pb^{2+} stimulated [^{3}H]-glutamate binding in human platelets. Heavy metals caused an increase on lipid peroxidation and reactive oxygen species measurement in platelets, suggesting that oxidative stress, at least in part, could be one of the mechanisms involved in heavy metals-induced neurotoxicity. The use of 2,3-dimercaptopropanol (BAL), a dithiol chelating agent therapeutically used for the treatment of heavy metals poisoning, was able to protect and restoring [^{3}H]-glutamate binding against effects caused by Pb^{2+}, Cd^{2+} and Hg^{2+}. 2,3 dimercaptopropane-1-sulfonic acid (DMPS), another dithiol chelating agent, was able protecting and restoring against the effects caused by heavy metals in [^{3}H]-glutamate binding in platelets. Similar results were obtained with ditiothreitol (DTT), a sulfhydryl agent commonly used as a -SH groups reductor. The dithiol agents alone did not alter [^{3}H]-glutamate binding. In contrast, reduced glutathione (GSH), a monothiol reducing agent, inhibited [^{3}H]-glutamate binding in all tested concentrations. In addition, this compound did not protect the effect caused by heavy metals. These results suggest that dithiol agents (BAL, DMPS and DTT) could act by chelating heavy metals or/and by reducing –SH groups of glutamatergic receptors. Diphenyl diselenide and diphenyl ditelluride inhibited [^{3}H]-glutamate binding, but ebselen did not alter this parameter. All organochalcogens tested inhibited [^{3}H]-glutamate uptake in human platelets. The inhibition caused by organochalcogens in glutamatergic system, was not recovered by the addition of reducing agents (DTT and GSH),

indicating that toxicological properties of these compounds an not exclusively related to oxidation of –SH groups. Considering the results obtained, could be suggested that platelets to be a suitable biomarker for exposure by toxic compounds and represented a good model for investigated the mechanism involved in changes caused by these compounds.

Key words: Platelets, glutamatergic system, heavy metals, selenium, tellurium, chelating agents.

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LISTA DE ABREVIATURAS

AMPA- ácido α-amino-3-hidroxi-5-metil-4-isoxazol-propiônico

ANOVA- análise de variância

BAL- 2,3-dimercaptopropanol, dimercaprol

DL_{50}- dose letal 50

DMPS- ácido 2,3-dimercaptopropano 1-sulfônico

DMSA- ácido meso-2,3-dimercaptosuccínico

DMSO- dimetilsulfóxido

DTT- DL-ditiotreitol

EAAT- transportador de aminoácidos excitatórios

EDTA- ácido etilenodiaminotetracético

EROs- espécies reativas de oxigênio

GABA- ácido γ-aminobutírico

GPx- glutationa peroxidase

GSH- glutationa reduzida

KA- ácido caínico

MDA- malondialdeído

MeHg- Metil-mercúrio

mGluR- receptores glutamatérgicos metabotrópicos

Mk-801- (+)-5-metil-10,11-diidro-5H-dibenzo[a,d]ciclohepten-5,10-imina ou dizolcilpina

MT- metalotioneina

NAC- N-acetilcisteína

NMDA- N-metil-D-aspartato

PhSeSePh ou $(PhSe)_2$- disseleneto de difenila

PhTeTePh ou $(PhTe)_2$- ditelureto de difenila

SNC- sistema nervoso central

TBARS- espécies reativas ao ácido tiobarbitúrico

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APRESENTAÇÃO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos, os quais encontram-se no ítem **ARTIGOS CIENTÍFICOS**. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos e representam à íntegra deste estudo.

Os itens, **DISCUSSÃO E CONCLUSÕES**, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre todos os artigos científicos contidos neste trabalho.

No item **PERSPECTIVAS** estão expostos os possíveis estudos para continuação do estudo do autor, referente a esse assunto.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO**, **REVISÃO BIBLIOGRÁFICA, DISCUSSÃO e CONCLUSÕES** desta tese.

No item **ANEXO** encontram-se os demais trabalhos desenvolvidos durante o Curso de Doutorado.

1- INTRODUÇÃO

O estudo das interações entre agentes químicos e neurotransmissores cerebrais, bem como receptores e sistemas de segundos mensageiros, pode levar à identificação de alvos moleculares e celulares os quais estão presentes não somente no sistema nervoso central (SNC), mas também em sítios mais específicos, como as células sanguíneas (Manzo et al., 1995). Conseqüentemente, recentes pesquisas estão sendo desenvolvidas na tentativa de caracterizar biomarcadores em tecidos periféricos que possam ser utilizados em casos de dano, disfunção ou interações envolvendo alvos de agentes tóxicos. De fato, as plaquetas têm sido amplamente sugeridas como um excelente modelo do aparato sináptico (de Gaetano & Garattini, 1978), e ainda apresentam vantagens por serem facilmente obtidas de forma pouca invasiva.

A exposição das populações humanas a uma variedade de metais tóxicos encontrados no meio ambiente é um problema de saúde pública (Goyer et al., 1996). Devido ao amplo emprego industrial destes compostos, a exposição ocupacional constitui uma das principais formas de intoxicação (Salgado, 1996). Dentre os metais de maior preocupação estão o chumbo, o mercúrio, o arsênio e o cádmio (Klaassen, 1996). Os metais pesados podem causar toxicidade em vários órgãos como fígado, rim, pulmão, ovários, testículos e SNC.

Exposições prolongadas a agentes tóxicos ambientais, como os citados anteriormente, podem levar a uma alteração nos sistemas de neurotransmissão e, conseqüente neurotoxicidade.

Os organocalcogênios são reagentes muito utilizados em laboratórios de química como intermediários em reações de síntese orgânica (Paulmier, 1986; Braga et al., 1996; 1997). Recentemente, em virtude da descoberta de suas propriedades biológicas (Parnham & Graf, 1991; Kanda et al., 1999; Nogueira et al., 2004), os organocalcogênios têm sido alvo de estudos em laboratórios de farmacologia. Conseqüentemente, o risco de contaminação ocupacional por organocalcogênios motiva estudos toxicológicos.

Os compostos orgânicos de selênio e telúrio afetam um grande número de processos neurais, entre eles a modulação do sistema glutamatérgico (Nogueira et al., 2001c; 2002). Baseado nas considerações acima, torna-se importante a avaliação

dos efeitos neurotóxicos dos metais pesados e organocalcogênios, utilizando um modelo periférico. Além disso, a descoberta de biomarcadores periféricos que possam estar alterados precocemente frente à exposição a agentes tóxicos motiva ainda mais nossos estudos.

2- REVISÃO BIBLIOGRÁFICA

2.1. Plaquetas

As plaquetas (Figura 1) são células sanguíneas, sintetizadas pela medula óssea, como resultado da fragmentação do megacariócito, seu antecedente medular. O megacariócito é uma célula grande e multinucleada e as plaquetas nada mais são do que fragmentos do seu citoplasma. A fragmentação do megacariócito pode dar origem a até mil plaquetas. As plaquetas são células pequenas, anucleadas, cujo diâmetro varia de 2 a 4 μM e perfazem na corrente sanguínea em torno de 130 a 400 mil/mm^3 em um adulto normal. Sua produção é estimulada pelo hormônio trombopoietina, formado no fígado. Quando não estimuladas, possuem vários tipos de grânulos: lisossomais (que contêm ácido hidrólico), densos (contendo ADP, ATP, serotonina e cálcio) e alfa (contendo fibrinogênio, Fator V e Fator de von Willebrand). A plaqueta em média circula no sangue de 9 - 10 dias. Depois disso, ela é sequestrada pelo baço e destruída pelo mesmo. Essas células têm como função atuar no controle da coagulação sanguínea e homeostasia (Pontual et al., 2004).

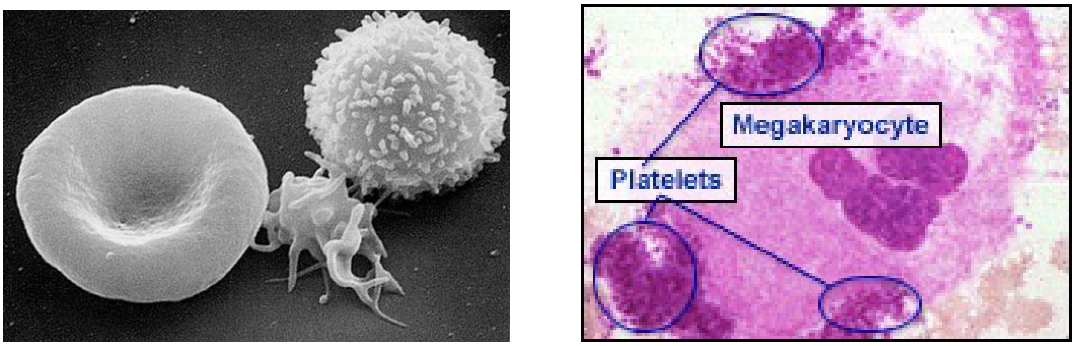
As plaquetas têm sido amplamente sugeridas como um excelente modelo do aparato sináptico (de Gaetano & Garattini, 1978) e foi demonstrado que estas células parecem acumular glutamato de maneira similar à preparação de sinaptossomas (Mangano & Schwarcz, 1981 b). De fato, os defeitos que ocorrem na função sináptica central podem ser demonstrados em plaquetas. Diversos autores utilizam esse modelo para investigar inúmeras desordens neuropsiquiátricas, como doença de Alzheimer (Ferrarese et al., 2000), esclerose amiotrófica lateral (Ferrarese et al., 2001), doença de Huntington (Mangano & Schwarcz, 1981, a), doença de Parkinson (Ferrarese et al., 1999), transtorno bipolar (Nascimento et al., 2006), entre outras.

Os transportadores de glutamato expressos no SNC apresentam um grau de homologia relativamente elevado com os transportadores de aminoácidos expressos em tecidos periféricos (Ariza et al., 1993). A existência dos transportadores de glutamato dependentes de Na^+ em plaquetas foi descrita a cerca de duas décadas por Mangano & Schwarcz (1981 a, b), e esses apresentam propriedades cinéticas bastante similares àquelas encontradas em sinaptossomas de cérebro de ratos, embora a expressão deste sistema seja muito maior no cérebro do que em plaquetas.

De fato, Zoia e colaboradores (2004) demonstraram que as plaquetas apresentam os três maiores subtipos de transportadores de glutamato, conhecidos como EAAT1, EAAT2 e EAAT3 (transportadores de aminoácidos excitatórios). Além disso, Tremolizzo e colaboradores (2006) relataram a presença de transportadores vesiculares de glutamato (VGLUT1 e VGLUT2).

As plaquetas expressam uma variedade de receptores em sua superfície, muitos dos quais são similares estruturalmente aos do SNC (Pletscher et al., 1988). Os receptores NMDA foram descritos em plaquetas, sugerindo sua importância em regular os processos de agregação e ativação plaquetária (Franconi et al., 1998). Apesar de existirem evidências demonstrando a similaridade do sistema glutamatérgico nas plaquetas e no SNC, o papel do glutamato nestas células permanece desconhecido. Entretanto, Tremolizzo e colaboradores (2006) postularam algumas hipóteses para explicar o papel do glutamato nas plaquetas. O glutamato poderia estar envolvido na regulação da agregação e ativação plaquetária, já que elas possuem os receptores NMDA os quais apresentam papel anti-agregante. Além disso, o glutamato poderia modular as funções dos leucócitos (Kuo et al., 2001), e desse modo ter um papel importante na interação entre essas células e as plaquetas durante uma injúria tecidual.

Além dos receptores e transportadores de glutamato, essas células também expressam receptores α-adrenérgicos, GABAérgicos, sistemas enzimáticos (monoamina oxidase e Na^+,K^+-ATPase) e sistemas de captação (serotonina e GABA) (Manzo et al., 1995), os quais estão sendo intensamente estudados.



A **B**

Figura 1- Microscopia eletrônica de eritrócito, plaqueta e leucócito (esquerda para direita) (A). Distenção sanguínea de medula óssea (B).

2.2. Glutamato

As principais vias excitatórias do sistema nervoso central utilizam glutamato como neurotransmissor (Ozawa et al., 1998; Meldrum et al, 1999). Na década de 50, Curtis e colaboradores demonstraram pela primeira vez que o L-glutamato e outros aminoácidos, de ocorrência natural, estavam envolvidos na excitação neuronal em cérebro de mamíferos (Collingridge & Lester, 1989; Bennett & Balcar, 1999).

O glutamato é encontrado em altas concentrações (10 mM) no cérebro de mamíferos e está envolvido em uma variedade de processos fisiológicos, tais como aprendizado, memória e formação de redes neuronais durante o desenvolvimento (Ozawa et al., 1998). Recentemente, estudos têm relacionado alguns distúrbios psiquiátricos (Esquizofrenia, Depressão Maior e Transtorno Bipolar) e doenças neurodegenerativas (Alzheimer, Esclerose Amiotrófica Lateral e Parkinson) com alterações periféricas e centrais na expressão e sensibilidade dos receptores glutamatérgicos ao glutamato (Ferrarese et al., 2000, 2001a, b; Berk et al., 2000).

Este aminoácido é sintetizado nos terminais pré-sinápticos, predominantemente a partir de glutamina através da ação da enzima glutaminase, mas pode provir do α-cetoglutarato, via glutamato desidrogenase e α-cetoglutarato aminotransferases (Kvamme et al., 1998). Um aumento nas quantidades de glutamato na fenda sináptica pode levar à estimulação excessiva dos receptores glutamatérgicos (excitotoxicidade) com conseqüente morte neuronal (Lipton & Rosenberg, 1994). Entretanto, a ação excitatória do glutamato é finalizada através de

sua captação pelas células gliais ou pelos neurônios pré-sinápticos, onde é armazenado nas vesículas sinápticas.

A captação do glutamato da fenda sináptica envolve dois sistemas de transporte: um sistema de alta afinidade e dependente de Na^+, localizado nas membranas pré-sinápticas e gliais (Robinson & Dowd, 1997) e outro com baixa afinidade e independente de Na^+, nas membranas das vesículas sinápticas. Esse fenômeno ocorre similarmente em células periféricas de mamíferos (Mangano e Schwarcz, 1981a; b). A captação de glutamato apresenta uma função vital na manutenção de altos níveis de precursores de glutamato e baixas concentrações extracelulares deste neurotransmissor (Dichter & Wilcox, 1997).

O glutamato, uma vez armazenado, poderá ser liberado na fenda, desde que as membranas pré-sinápticas sejam despolarizadas. Após sua liberação, o glutamato exerce suas funções fisiológicas ativando os receptores localizados nas membranas pré e pós-sinápticas, bem como nas membranas das células gliais (Meldrum et al., 1999).

Os receptores glutamatérgicos podem ser classificados de acordo com estudos farmacológicos e moleculares, em dois grandes grupos: receptores ionotrópicos e metabotrópicos (Dichter & Wilcox, 1997; Ozawa et al., 1998) (Figura 2). Os receptores ionotrópicos são canais iônicos que permeiam cátions através da membrana neuronal, desencadeando uma resposta excitatória. Estes receptores são subdivididos em N-metil-D-aspartato (NMDA); ácido α-amino-3-hidroxi-5-metil-4-isoxazol-propiônico (AMPA) e ácido caínico (KA), com base na sua sensibilidade a agonistas específicos.

Os receptores NMDA medeiam a transmissão excitatória lenta, são canais com grande permeabilidade ao Ca^{2+} e baixa permeabilidade ao Na^+ e K^+ (Lipton & Rosemberg, 1994; Ozawa et al., 1998). Esse receptor apresenta diversos sítios para ligantes que regulam a abertura do canal: um sítio para o glutamato ou NMDA, um sítio para o co-agonista endógeno glicina (insensível à estricnina), um sítio para a união de bloqueadores (MK-801, PCP) e sítios modulatórios, tais como um sítio para o zinco, outro para as poliaminas, um sensível à modulação redox (modulado por agentes oxidantes ou redutores) e um sensível a prótons (Gozlan & Bem-Ari, 1995; Piggott et al., 1992; Euler & Liu, 1993; Ozawa et al., 1998) (Figura 3). Estudos demonstraram que este receptor está presente em plaquetas, sugerindo sua

importância em regular os processos de agregação e ativação plaquetária (Franconi et al., 1998).

Os receptores AMPA medeiam a neurotransmissão excitatória rápida e são canais com grande permeabilidade a cátions monovalentes (Na^+ e K^+) e com baixa permeabilidade ao Ca^{2+} (Dichter & Wilcox, 1997). Os receptores do KA diferem da maioria dos receptores AMPA por serem relativamente permeáveis aos íons Ca^{2+} (Ozawa et al., 1998) e estão concentrados em poucas áreas cerebrais, ao contrário dos AMPA, que apresentam ampla distribuição no SNC (Scatton, 1993).

Os receptores metabotrópicos (mGluRs) estão associados a sistemas de segundos mensageiros intracelulares (Conn & Pinn, 1997). Esses receptores são acoplados a proteínas G e modulam a atividade de efetores intracelulares, tais como adenilato ciclase e fosfolipase C, responsáveis pela produção de segundos mensageiros (Schoepp & Conn, 1993; Cotmann et al., 1995). Os mGluRs estão envolvidos na participação da indução da plasticidade neuronal (Bliss & Collinbridge, 1993). Entretanto, possuem papel importante na indução de convulsões e morte neuronal (Tizzano et al., 1995; Nicoletti et al., 1996). Sua ativação pode promover efeitos excitatórios e inibitórios (Ozawa et al., 1998).

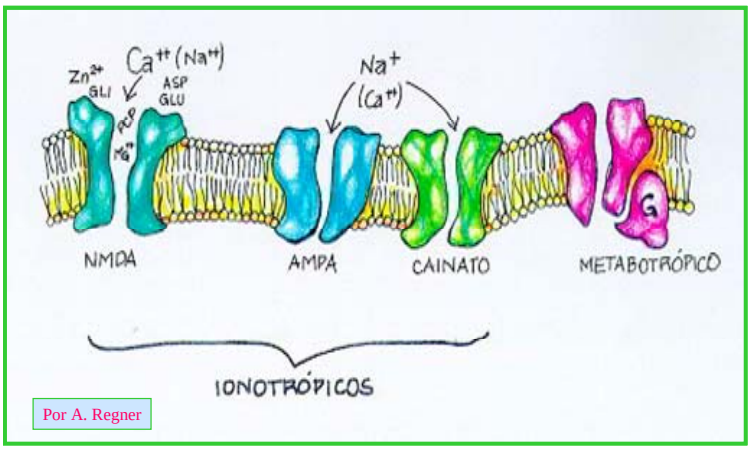


Figura 2- Representação esquemática dos receptores glutamatérgicos ionotrópicos e metabotrópicos.

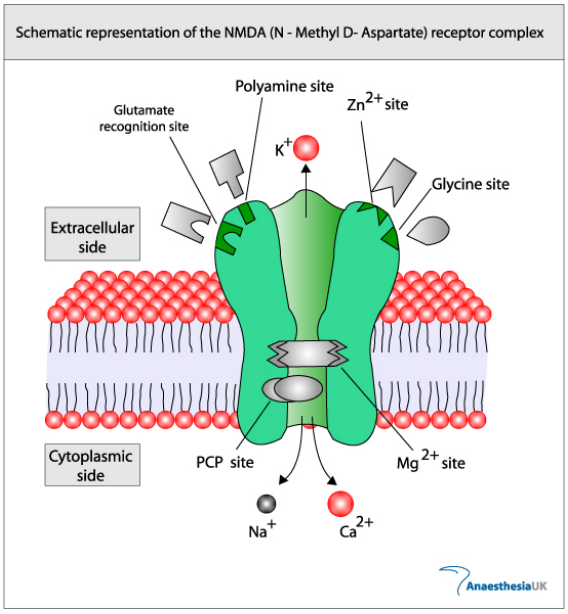


Figura 3- Representação esquemática do receptor N-metil-D-aspartato (NMDA).

2.3. Metais Pesados

Os metais pesados são alguns dos principais contaminantes encontrados no meio ambiente. Estes metais têm amplo emprego industrial, constituindo uma das principais formas de intoxicação ocupacional (Salgado, 1996). A longa meia-vida biológica contribui para o seu acúmulo no organismo, causando diversos efeitos adversos (Aschner et al., 1990; Minami et al., 2001). Tais metais exercem seus efeitos tóxicos combinando-se a grupos reativos, como os grupos sulfidrilas (-SH), de moléculas biologicamente ativas. Dentre os metais de maior preocupação estão o chumbo, o mercúrio, o arsênio e o cádmio (Klaassen, 1996).

2.3.1. Chumbo

O chumbo (Pb) é um metal onipresente no meio ambiente, como resultado de sua ocorrência natural e sua utilização industrial. Esse metal permanece associado a patologias em todo o mundo, principalmente em países em desenvolvimento. O chumbo era utilizado desde a cunhagem de moedas, produção de tubulações e

soldas, em antifúngicos, antidetonante, até a produção de baterias acumuladoras de energia, atualmente (Needleman et al., 2000; Vahter et al., 2002).

O chumbo é absorvido primariamente pelo trato gastrointestinal e respiratório. É transportado através da corrente sanguínea e depositado na matriz óssea, deslocando o cálcio, o que pode causar efeitos tóxicos continuados mesmo depois de cessada a exposição. Este elemento tem uma meia-vida de 35 dias no sangue, 50 dias nos tecidos moles, e de 20-30 anos nos ossos (Landrigan, 1994). Como este metal afeta vários órgãos e sistemas no organismo, os mecanismos de toxicidade propostos envolvem processos bioquímicos, que incluem a habilidade do chumbo de ligar-se a proteínas alvo que são, geralmente, proteínas que ligam cálcio e zinco (Godwin, 2001).

Os principais efeitos do chumbo são principalmente manifestados no sistema nervoso central. Estudos demonstraram que a exposição a esse metal causa déficit cognitivo e distúrbios comportamentais (Tong et al., 2000; Mendola et al., 2002).

O chumbo pode induzir morte celular no hipocampo, devido a apoptose, sendo que um componente glutamatérgico é indubitavelmente envolvido na neurotoxicidade induzida por este elemento (Sharifi et al., 2002). De fato, Cory-Sletcha e colaboradores (1995) demonstraram que concentrações sanguíneas de chumbo menores que 10 μg/dL resultam em defeitos neurológicos. Este metal causa uma variedade de efeitos, como modulação da regulação gênica, de sistemas de segundos mensageiros e outras vias de transdução e alterações nos sistemas catecolaminérgicos (Shellenberger et al., 1984), opióides, GABAérgicos e glutamatérgicos.

2.3.2. Mercúrio

O mercúrio (Hg) é responsável pelas exposições ocupacionais que ocorrem na produção de cloro, soda cáustica, equipamentos elétricos e eletrônicos (baterias, retificadores, relés, interruptores), aparelhos de controle (termômetros, barômetros, esfingonanômetros), tinta látex, amálgamas dentários, fungicidas, herbicidas, lâmpadas de mercúrio, processos de mineração, entre outros (Salgado, 1996; Broussard et al., 2002).

As três principais formas químicas de mercúrio encontradas em nosso ambiente são o vapor de mercúrio (mercúrio elementar), mercúrio inorgânico e compostos orgânicos do metal (Klaassen, 1996).

A população em geral está primariamente exposta ao mercúrio inorgânico, através das amálgamas dentários, sendo os dentistas e seus auxiliares o grupo de maior risco de exposição (Brune e Evje, 1985). Dependendo do nível de contaminação, o ar e a água também podem se tornar importantes fontes de exposição ao metal. Além disso, a alimentação também contribui para a contaminação pelo mercúrio, através da ingestão de peixes que acumulam o metil-mercúrio (MeHg), uma forma orgânica do metal, que causa efeitos neurotóxicos (Who, 1990).

A principal via de absorção do mercúrio no organismo humano é a pulmonar, devido à exposição a vapores ou partículas de mercúrio. Em menor proporção, a absorção ocorre pela via dérmica (Salgado et al., 1996). Os principais órgãos de deposição de mercúrio no organismo são os rins e o cérebro para o mercúrio elementar, os rins para o mercúrio inorgânico e o cérebro para os organomercuriais (Salgado et al., 1996). Entretanto, níveis menores de mercúrio se acumulam no fígado, pulmão, coração, baço e intestino (Larini et al., 1997).

O mercúrio pode causar vários danos ao organismo, como danos ao sistema reprodutivo (Underwood, 1977; Anderson et al., 1992), ao sistema hepático (Huang et al., 1996), ao sistema nervoso (Lorscheider et al., 1995) e ao sistema renal (Perottoni et al., 2004), sendo este último o alvo primário do metal (Emanuelli et al., 1996; Clarkson, 1997).

O mercúrio inorgânico possui grande afinidade por grupos –SH de biomoléculas endógenas (Clarkson, 1997). Deste modo, ele pode se complexar com estruturas que contêm estes grupos sulfidrilas, como a cisteína e a glutationa (Zalups, 2000) e também com as metalotioneínas (MT) (Yoshida et al., 1999), que são proteínas de baixo peso molecular que podem se complexar tanto com metais essenciais (zinco e cobre) como com metais pesados (mercúrio, arsênico e cádmio) (Foulkes, 1982).

Os sintomas de intoxicação por este metal envolvem distúrbios sensoriais, ataxia, tremor e distúrbios mentais. De fato, o MeHg e Hg^{2+} causam severas alterações no sistema nervoso central, os quais podem ser devido à sua habilidade em alterar a transmissão sináptica (Atchison and Hare, 1994; Aschner et al., 2000;

Gassó et al., 2000; Porciúncula et al., 2003a; Moretto et al., 2005; Fitsanakis et al., 2005; Kaur et al., 2006), produzindo morte neuronal que é parcialmente mediada pelo glutamato (Yamashita et al., 1997).

Esses dados corroboram com os trabalhos de Miyamoto e colaboradores (2001) que demonstraram que a administração de MK-801, um antagonista não-competitivo de receptores NMDA, foi capaz de melhorar o dano induzido por mercúrio em ratos.

Além disso, o mercúrio é capaz de inibir a captação de glutamato em fatias de córtex de ratos, *ex vivo* (Farina et al., 2003), bem como inibir a ligação de glutamato em membranas sinápticas (Soares et al., 2003), em experimentos *in vitro.* Estes estudos estimulam ainda mais pesquisas sobre o envolvimento do sistema glutamatérgico na neurotoxicidade induzida pelo mercúrio.

2.3.3. Cádmio

A exposição das populações humanas a uma variedade de metais tóxicos é um problema de saúde pública (Goyer et al., 1996). De todos os metais tóxicos encontrados no ambiente e utilizados industrialmente, o cádmio (Cd) é um dos que apresenta maior interesse clínico, uma vez que as intoxicações por este metal são geralmente intratáveis (Jones & Cherian, 1990).

A contaminação ambiental com cádmio é devido ao seu amplo uso industrial em processos como produção de plásticos, pigmentos, baterias que contém cádmio e em processos de mineração (Adriano et al., 2001). Esse elemento é absorvido no organismo em pequenas quantidades, entretanto ele pode se acumular nos tecidos devido à sua longa meia-vida biológica (Perry et al., 1962).

O cádmio pode causar toxicidade em vários órgãos como fígado, rim, pulmão, ovários, testículos e SNC.

A intoxicação aguda por cádmio produz primariamente injúria hepática e testicular, enquanto a exposição crônica produz dano renal e osteotoxicidade (Rikans et al., 2000). Dessa forma, sob condições de exposição mais prolongada ao cádmio, este metal se deposita primariamente no fígado, onde ele induz e se liga às metalotioneínas (MT), podendo também causar efeitos hepatotóxicos. Com o tempo, o complexo CdMT hepático é lentamente liberado na circulação (Toyama and Shaikh, 1981) e posteriormente, após filtração glomerular, este complexo é degradado e os

íons cádmio liberados se ligam a metalotioneínas renais pré-existentes ou àquelas recentemente sintetizadas (Cherian, 1978).

Apesar do fígado, rim e testículos serem os alvos primários dos efeitos tóxicos deste metal, estudos demonstraram que o cérebro também poderia ser afetado pela exposição ao cádmio. Alguns relatos sobre seus efeitos no SNC são observados em estudos clínicos com crianças e trabalhadores expostos ao metal e também na doença de Itaí-Itaí (Shukla et al., 1984). Em estudos com animais foi demonstrado que o cádmio causa extensos danos histopatológicos no córtex cerebral e cerebelar (Gabbiani et al., 1967). O cádmio afeta o equilíbrio de excitação-inibição na neurotransmissão sináptica, entretanto ainda são escassos os estudos que relatam os efeitos do cádmio sobre o sistema nervoso central. O mecanismo pelo qual este metal causa alterações cerebrais é pouco entendido, entretanto muitas evidências indicam que a geração de espécies reativas de oxigênio está envolvida na indução do dano tecidual causado por cádmio (Koizumi & Li, 1992). Baseado no exposto acima, mais estudos são necessários para avaliar os efeitos neurotóxicos do cádmio, bem como os mecanismos envolvidos.

2.4. Agentes quelantes

A terapia com agentes quelantes é a forma mais efetiva de tratamento para intoxicações com metais (Flora e Kumar, 1993). Os compostos sulfidrílicos como o dimercaprol (British Anti-Lewisite, BAL), a D-penicilamina (Klaassen, 1996) e derivados do dimercaprol como o ácido meso 2,3-dimercaptosuccínico (DMSA) (Endo e Sakata, 1995; Flora, 1999; Frumkin et al., 2001) e o ácido 2,3-dimercapto-1-propanosulfônico (DMPS) (Pingree et al., 2001) são os agentes quelantes mais utilizados na terapêutica.

O uso terapêutico de agentes quelantes em casos de intoxicação por metais tóxicos vem sendo praticado há aproximadamente 40 anos. Estes compostos são utilizados clinicamente como antídotos contra intoxicações agudas e crônicas por metais. Os agentes quelantes, além de aumentarem a excreção do metal tóxico, também reduzem a toxicidade dos metais por impedir a ligação destes a moléculas celulares alvo (Aposhian et al., 1995). Entretanto, o tratamento prolongado com agentes quelantes pode causar distúrbios hematopoiéticos (Flora e Kumar, 1993), desequilíbrio do metabolismo celular, na síntese de DNA, RNA e proteínas (Fischer et

al., 1975), ou ainda alteração da homeostase dos elementos traços (Cantilena e Klaassen, 1982). Considerando o descrito acima, questões referentes ao uso destes agentes permanece em discussão, incentivando a pesquisa nesta área.

2.4.1. 2,3- Dimercaptopropanol (BAL)

Os primeiros relatos do uso de agentes quelantes em casos de intoxicações datam da época da Segunda Guerra Mundial, na Inglaterra. Em 1946, Stocken e Thompson descreveram o uso do 2,3-dimercaptopropanol (BAL) (Figura 4) como um antídoto para intoxicações pelo dicloro-vinil arsênio. Este composto é um potente agente tóxico presente em gases de guerra, conhecido como Lewisite, o qual é capaz de atuar nos pulmões, nos rins e em outros órgãos internos. Segundo relatos, o BAL proporcionava 100 % de sobrevivência em animais expostos topicamente ao Lewisite quando comparado a outros quelantes menos efetivos, como o monotiol 2-mercaptoetanol (Stocken e Thompson, 1946).

As primeiras aplicações clínicas do BAL foram descritas para o tratamento de dermatites, decorrentes do uso terapêutico de compostos orgânicos contendo arsênio, usual na época para o tratamento de pacientes com sífilis (Longcope et al., 1946). O MELARSOPROL® é uma preparação farmacêutica contendo BAL, utilizada no tratamento da doença do sono causada pelo *Trypanossoma brucei gambiense*, demonstrando eficácia em eliminar os tripanossomas circulantes e inclusive do sistema nervoso central (Jennings et al., 1996; Pepin et al., 1995).

Entretanto, devido à sua lipossolubilidade, o BAL pode atravessar a membrana celular e atingir os espaços intracelulares (Andersen, 1989), causando redistribuição de metais, como o arsênio, o mercúrio (Aaseth et al., 1995; Hoover e Aposhian, 1983) e o chumbo (Cory-Slechta et al., 1987) dos órgãos periféricos para o cérebro.

Além disso, modelos experimentais mostraram que a administração de doses elevadas deste composto produz convulsões e morte sugerindo, que a modulação de receptores GABAérgicos e glutamatérgicos esteja envolvida no mecanismo de neurotoxicidade induzida pelo BAL (Nogueira et al., 2000). De fato, este agente quelante inibe a captação e aumenta a liberação de glutamato em sinaptossomas de ratos (Nogueira et al., 2001a).

Uma vez que a utilização do BAL apresenta diversas limitações devido à sua toxicidade, outros agentes quelantes, potencialmente menos tóxicos, têm sido

investigados (Keith et al., 1997). Derivados estruturais do BAL, o DMPS e o DMSA, são mais hidrossolúveis e possivelmente menos tóxicos do que o BAL (Andersen, 1989).

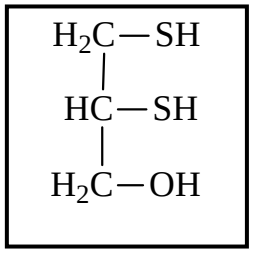


Figura 4- Estrutura química do 2,3- dimercaptopropanol (BAL).

2.4.2. Ácido 2,3-dimercapto-1-propanosulfônico (DMPS)

O DMPS (Figura 5) tem sido usado na antiga URSS desde 1958 e encontra-se disponível, comercialmente na Europa, como DIMAVAL®. Além disso, este composto tem sido utilizado na Alemanha para o tratamento de intoxicações por mercúrio (Campbell et al., 1986). Este quelante apresenta-se como sólido cristalino e estável (Aposhian et al., 1992) e é facilmente administrado por via oral (Aposhian et al., 1996). A DL_{50} em camundongos é 5,22 $mmol.kg^{-1}$ para o DMPS (Aposhian et al., 1981), portanto esse quelante é mais seguro que o BAL (0,73 $mmol.kg^{-1}$). Devido a estas características, o DMPS é considerado menos tóxico que o BAL (Aposhian et al., 1992).

O DMPS apresenta dois grupos -SH vicinais e caracteriza-se pela maior solubilidade em água (Nadig et al., 1985) e limitada solubilidade lipídica (Aposhian et al., 1983). A presença de dois grupos -SH vicinais é reconhecida como a estrutura essencial para a eficácia do agente quelante (Muckter et al., 1997). Em intoxicações agudas por mercúrio, por exemplo, uma das terapêuticas fundamentais é a utilização de compostos que apresentem na sua estrutura grupos -SH (Schwartz et al., 1992; Klaassen, 1996). Estes compostos ditiólicos possuem a capacidade de complexar este metal pesado e aumentar a velocidade de excreção renal e biliar (Jugo, 1980; Kojima et al., 1989; Shimada et al., 1993).

O DMPS foi descrito como uma droga efetiva no tratamento de intoxicações por mercúrio (Kostygou, 1958). Segundo diversos relatos, é uma alternativa segura e eficaz para substituir o BAL (Toet et al., 1994; Campbell et al., 1986; Cherian et al., 1988), apresentando menor toxicidade local e sistêmica (Hruby e Donner, 1987) e não causando redistribuição de mercúrio para o cérebro de ratos (Buchet e Lauwerys, 1989; Aposhian et al., 1996). Em um estudo anterior, entretanto, foi demonstrado que a associação entre o DMPS e o mercúrio pode ser perigosa, podendo afetar o sistema renal, devido à formação de um complexo Hg-DMPS (Brandão et al., 2006), que poderia ser mais facilmente transportado pelos túbulos renais. De fato, Nogueira e colaboradores (2003b) reportaram a formação de um complexo entre cádmio ou mercúrio e DMPS. Este complexo pode apresentar atividade pró-oxidante maior do que os componentes isolados (Miller e Woods, 1993; Putzer et al., 1995).

Considerando o descrito acima, permanece em aberto a questão de qual seria a droga de escolha, em casos de intoxicação com diferentes metais pesados (Muckter et al., 1997).

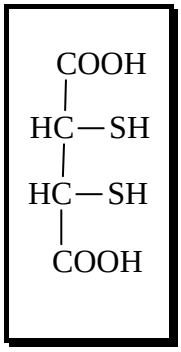


Figura 5- Estrutura química do ácido 2,3-dimercapto-1-propanosulfônico (DMPS).

2.5. Organocalcogênios

A partir da década de 30, os organocalcogênios têm sido alvo de interesse para os químicos orgânicos em virtude da descoberta de aplicações sintéticas (Petragnani et al., 1976; Comasseto, 1983) e de propriedades biológicas desses compostos (Parnham & Graf, 1991; Kanda et al., 1999), os quais são importantes intermediários e reagentes muito utilizados em síntese orgânica (Paulmier, 1986; Braga et al., 1996; 1997).

Conseqüentemente, o risco de contaminação ocupacional por organocalcogênios motiva estudos toxicológicos. Outro aspecto relevante é a tentativa crescente de desenvolvimento de compostos organocalcogênios que possuam atividade biológica e aplicações farmacológicas (Nogueira et al., 2003c).

2.5.1. Selênio

O elemento selênio foi descoberto em 1817, pelo químico sueco J. J. Berzelius. Esse elemento químico é um calcogênio do grupo 16 da tabela periódica, podendo apresentar-se sob quatro estados de oxidação: selenato (Se^{+6}), selenito (Se^{+4}), selênio elementar (Se^{0}) e seleneto (Se^{-2}).

O selênio é um elemento traço essencial, cuja essencialidade nutricional foi demonstrada em 1957, em ratos (Schwartz e Foltz, 1957). Anteriormente, o único interesse biológico prático para o selênio era que altos níveis deste elemento causavam toxicidade (Levander & Burk, 1994). Nos últimos anos, têm sido descrito que baixos níveis de selênio podem levar à predisposição para o desenvolvimento de algumas doenças, tais como câncer, esclerose, doença cardiovascular, cirrose e diabetes (Navarro-Alarcón e López-Martinez, 2000). Neste contexto, a suplementação de dietas com selênio tem sido aceita pela comunidade científica, sendo que a Junta de Alimentação e Nutrição da Academia de Ciências dos Estados Unidos propõe uma ingestão diária de 50- 200 μg (Food and Nutrition Board,1989).

Este calcogênio apresenta um grande número de funções biológicas, sendo a mais importante como antioxidante. As pesquisas recentes têm procurado estabelecer a função e a biologia molecular de selenoproteínas. Já é conhecido que o selênio está presente como resíduo de selenocisteína no sítio ativo das enzimas

glutationa peroxidase (Wingler e Brigelius-Flohé, 1999), tioredoxina redutase (Holmgren, 1985), 5'-deiodinase (Behne e Kyriakopoulos, 1990).

2.5.1.1. Ebselen e Disseleneto de Difenila

O conceito de que moléculas contendo selênio podem ser melhores nucleófilos (e, portanto antioxidantes) do que os antioxidantes clássicos têm levado ao desenvolvimento de organocalcogênios sintéticos (Arteel & Sies, 2001).

O ebselen (2-fenil-1,2-benzilsoselenazol-3(2H)-ona) (Figura 6) é um composto orgânico de selênio que exibe atividade catalítica e propriedades antioxidantes similares à glutationa peroxidase (Parnhan, 1990). Esse composto possui baixa toxicidade, pois ele não libera selênio de sua molécula (Parnhan & Graf, 1987). De fato, Wendel e colaboradores demonstraram que em animais deficientes de selênio, a atividade da enzima glutationa peroxidase não aumentava pela suplementação com ebselen.

Este composto reage com grupos tióis, como a glutationa (Ullrich et al., 1996), inibe a peroxidação lipídica (Parnhan & Graf, 1987), inibe a lipoxigenase (Parnhan & Graf, 1987), bloqueia a produção de ânion superóxido e desempenha um papel protetor contra o peroxinitrito (Masumoto & Sies, 1996).

O ebselen tem sido usado como antioxidante, como neuroprotetor em culturas de neurônios (Tan et al., 1997), no tratamento clínico de pacientes com isquemia aguda (Yamaguchi et al., 1998; Kondoh et al., 1999) e como antiinflamatório (Parnham & Graf, 1991). Devido aos diversos indicativos clínicos de redução de danos cerebrais após aneurisma, este composto aponta como um promissor agente neuroprotetor (Saito et al., 1998; Porciúncula et al., 2001; 2003b).

Engman e colaboradores (1992) demonstraram que disselenetos e diteluretos de diarila apresentam maior atividade do tipo tiol peroxidase, quando comparados ao ebselen, motivando o uso terapêutico destes compostos.

O disseleneto de difenila (Figura 7), um composto orgânico de selênio, demonstrou ser mais ativo como mimético da glutationa peroxidase (Meotti et al., 2004) e menos tóxico em roedores que o ebselen (Nogueira et al., 2003a; Meotti et al., 2003). Além disso, nosso grupo de pesquisa tem demonstrado que o disseleneto de difenila possui outras propriedades farmacológicas, tais como efeitos anti-úlcera

(Savegnago et al., 2005), antiinflamatório e antinociceptivo (Nogueira et al., 2003c; Zasso et al., 2005), hepatoprotetor (Borges et al., 2005; 2006), entre outras.

A toxicologia destes compostos é bastante similar. O mecanismo proposto para explicar a toxicidade de compostos de selênio, envolve a oxidação de grupos –SH de moléculas biologicamente ativas (Blais et al., 1972; Young et al., 1981). De fato, diversos trabalhos demonstraram que compostos orgânicos de selênio, inibem um grande número de enzimas sulfidrílicas, incluindo a 5-lipoxigenase (Björnstedt et al., 1996), δ-aminolevulinato desidratase (Nogueira et al., 2003d), esqualeno monooxigenase (Gupta and Porter, 2001) e Na^+, K^+- ATPase (Borges et al., 2005).

Da mesma forma, os compostos orgânicos de selênio e telúrio afetam um grande número de processos neurais, entre eles a modulação do sistema glutamatérgico (Nogueira et al., 2001c; 2002).

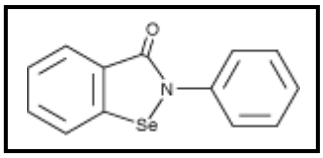


Figura 6- Estrutura química do 2-fenil-1,2-benzilsoselenazol-3(2H)-ona ou Ebselen

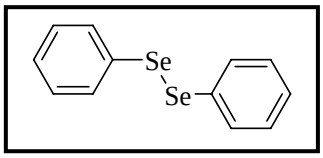


Figura 7- Estrutura química do Disseleneto de Difenila

2.5.2. Telúrio

O telúrio foi descoberto em 1782; entretanto a inclusão deste átomo em moléculas orgânicas ocorreu no início do século XIX. Esse elemento químico é um calcogênio do grupo 16 da tabela periódica, podendo apresentar-se sob quatro estados de oxidação: telurato (Te^{+6}), telurito (Te^{+4}), telúrio elementar (Te^{0}) e telureto (Te^{-2}). Ao contrário do selênio, este elemento não possui função biológica descrita até o momento (Taylor et al., 1996).

Industrialmente, o telúrio é utilizado no manufaturamento de semicondutores e outros componentes eletrônicos (Clayton & Clayton, 1981). Além disto, são empregados na síntese de fármacos e explosivos, na vulcanização de borracha, em lubrificantes, entre outros (Paz, 1989; Taylor et al., 1996).

Os compostos organotelúrio apresentam propriedades antiinflamatórias e antivirais (Sredni et al., 1987; Sun et al., 1996). Estudos têm demonstrado que, tal como o ebselen, os diorgano diteluretos, podem apresentar atividade tiol peroxidase (Engman et al., 1992; Kanda et al., 1999). Consequentemente, o emprego farmacológico destes agentes poderá crescer nos próximos anos, desde que não apresentem características tóxicas.

Casos de intoxicação ocupacional por telúrio são raros, entretanto, quando ocorrem, os sintomas são dores de cabeça, náuseas, alteração de freqüência cardíaca e odor característico de alho (Muller et al., 1989; Taylor et al., 1996).

O mecanismo proposto para explicar a toxicidade de compostos orgânicos de telúrio é similar aos organoselênios, e envolve a oxirredução de grupamentos –SH de moléculas biologicamente ativas (Blais et al., 1972; Young et al., 1981). De fato, os compostos de telúrio, inibem enzimas sulfidrílicas, incluindo δ-aminolevulinato desidratase (Barbosa et al., 1998; Nogueira et al., 2003d), esqualeno monooxigenase (Laden and Porter, 2001) e Na^{+}, K^{+}- ATPase (Borges et al., 2005).

Além disso, nosso grupo demonstrou que o ditelureto de difenila apresenta efeitos neurotóxicos (Nogueira et al., 2001c; 2002; Moretto et al., 2003) e demonstrou ser teratogênico em ratos (Stangherlin et al., 2005).

3- OBJETIVOS

Considerando que: - Os mecanismos pelos quais os metais pesados e os compostos organocalcogênios desencadeiam ações tóxicas ainda não estão bem descritos;

- Alguns trabalhos sugerem que a oxidação de grupos –SH de moléculas biologicamente ativas possa ser o provável mecanismo de toxicidade destes compostos;

- Os transportadores e receptores glutamatérgicos apresentam modulação redox;

Este estudo visa investigar os efeitos causados pelos metais pesados (Hg^{2+}, Cd^{2+}e Pb^{2+}) e pelos organocalcogênios (disseleneto de difenila, ditelureto de difenila e ebselen) sobre o sistema glutamatérgico em plaquetas, com o intuito de esclarecer se estes compostos podem modular os receptores e transportadores glutamatérgicos através de seus sítios redox, bem como investigar se as plaquetas podem servir como biomarcadores precoces frente à exposição a agentes tóxicos.

4- ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos, os quais encontram-se aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos. Os artigos estão dispostos da mesma forma que foram publicados na edição das revistas científicas (**Artigos 1, 3 e 5**), ou na forma que foi submetido, que é o caso do **Artigo 2 (ainda não publicado)**. O **artigo 4** encontra-se em fase de redação.

4.1 – Efeito causado pelos metais pesados sobre o sistema glutamatérgico em plaquetas, *in vitro*

4.1.1 - Artigo 1

HEAVY METALS MODULATE GLUTAMATERGIC SYSTEM IN HUMAN PLATELETS

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Neurochemical Research, *In press*

HEAVY METALS MODULATE GLUTAMATERGIC SYSTEM IN HUMAN PLATELETS

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Running title: Heavy metals affect glutamatergic system in platelets

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Abstract

Research strategies have been developed to characterize parameters in peripheral tissues that might easily be measured in humans as surrogate markers of damage, dysfunction or interactions involving neural targets of toxicants. The similarities between platelet and neuron may even be clinically important, as a number of biochemical markers show parallel changes in the central nervous system (CNS) and platelets. The purpose of our research was to investigate the effect of Hg^{2+}, Pb^{2+} and Cd^{2+} on the [^{3}H]-glutamate binding and [^{3}H]-glutamate uptake in human platelets. The involvement of oxidative stress in the modulation of glutamatergic system induced by heavy metals was also investigated. The present study clearly demonstrates that Hg^{2+}, Cd^{2+} and Pb^{2+} inhibited [^{3}H]-glutamate uptake in human platelets. Hg^{2+} inhibited [^{3}H]-glutamate binding, while Cd^{2+} and Pb^{2+} stimulated [^{3}H]-glutamate binding in human platelets. Hg^{2+}, Cd^{2+} and Pb^{2+} increased lipid peroxidation levels and reactive oxygen species (ROS) measurement in platelets. The present limited results could suggest that glutamatergic system may be used as a potential biomarker for neurotoxic action of heavy metals in humans.

Keywords: Platelets, heavy metals, glutamate binding, glutamate uptake, SNC.

1. INTRODUCTION

Glutamate is established as the major neurotransmitter in the mammalian brain that mediates fast synaptic transmission and induces neuronal plasticity (1). Given their high concentrations and importance in neuronal signaling, this amino acid is regulated at many levels: their storage in protective intracellular vesicles and their almost immediate removal from the extracellular space, following release into the synapse (2). Glutamate transporter proteins are of critical importance for the removal of extracellular glutamate concentrations in the synaptic cleft, are essential to facilite fast glutamatergic transmission and to prevent excitotoxicity (3). Alterations in glutamate uptake, due to malfunctioning or decreased expression of glutamate transporters, have been implicated in the pathogenesis of various neurological diseases (4- 6).

Consequently, research strategies have been developed to characterize parameters in peripheral tissues that might easily be measured in humans as surrogate markers of damage, dysfunction or interactions involving neural targets of toxicants (7). Blood platelets have been repeatedly suggested as an excellent model for various aspects of the synaptic apparatus (8). Platelets contain a high affinity, Na+-dependent glutamate system (9), and express excitatory amino acid transporters (EAAT1, EAAT2 and EAAT3) (10, 11). The glutamate uptake has been consistently shown to be dramatically affected in platelets peripheral model in different neurological conditions (12-15).

Heavy metals, such as mercury, lead and cadmium are environmental contaminants that increase significantly with the development of modern industries. Cadmium is one of the most abundant non-essential elements due to its immense use

in various industrial applications (16). When compared to lead and arsenic, cadmium is reported as an environmental pollutant of toxicological importance (17). This metal affects the degree and balance of excitation-inhibition in synaptic neurotransmission (18). Of the hazards associated with exposure to cadmium, central nervous system (CNS) disorders have been reported in the case of Itai-itai disease (19). In addition, there is scant evidence on the effects of cadmium accumulation on brain amino acid metabolism (20).

Mercurial compounds are global environmental contaminants deriving from natural processes, which possess neurotoxic potential (21). In fact, MeHg and Hg^{2+} cause severe alterations in the CNS that may be due to their ability to disrupt synaptic transmissions (22), producing neuronal death that is partially mediated by glutamate (23). In addition, *in vivo* studies showed that MeHg increased glutamate extracellular levels in frontal cortex of rats (24), and inhibited glutamate uptake by astrocytes (3,21).

In this context, another metal, lead, can alter synaptic transmission and cause severe neurological damage (25). Lead is a ubiquitous environmental toxicant. Severe and acute lead poisoning can cause encephalopathy, convulsion, coma, and even death. Despite several efforts to reduce lead levels in the environment, lead exposure continues to be a major public health problem, particularly in urban areas in the US as well as in third world countries. Lead adversely affects the central nervous system, and low level exposure is associated with behavioral abnormalities, decreased hearing, and cognitive impairment (26).

The similarities between platelet and neuron may even be clinically important, as a number of biochemical markers show parallel changes in the CNS and platelets (27). Taking into account that glutamatergic system is disrupted by heavy metals (28), the

purpose of our research was to investigate the effect of Hg^{2+}, Pb^{2+} and Cd^{2+} on the glutamate binding and uptake in human platelets. The major mechanism involved in heavy metals induced neurotoxicity currently studied is oxidative stress (29, 30). Thus, the involvement of oxidative stress in heavy metals-modulate glutamatergic system was investigated.

2. MATERIALS AND METHODS

2.1. Chemicals

[^{3}H]Glutamate (49 Ci/mM) was purchased from Amersham International (Amersham, Bucks, UK). All other chemicals ($HgCl_2$, $CdCl_2$, Pb acetate) were of analytical grade and obtained from standard commercial suppliers. Heavy metals were dissolved in 0.9% NaCl.

2.2. Blood samples

The human blood was obtained from 30 healthy volunteer donors of the Banco de Sangue, Hospital da Universidade Federal de Santa Maria, Santa Maria, RS, Brazil. The samples used in experiments for [^{3}H]-glutamate binding assay were transferred immediately after be obtained into a tube containing citrate/phosphate/dextran-adenine (CPD-A1).

2.3. [^{3}H] Glutamate uptake by platelets

All uptake assays were performed using platelet suspension prepared as described by Mangano & Schwarcz (9) and immediately assayed. To determine [^{3}H]-glutamate uptake by platelets, 50 μl of the platelet suspension were preincubated with Tris-citrate buffer (112.8 mM NaCl, 4.5 mM KCl, 1.1 mM KH_2PO_4, 25 mM Tris/HCl, 11

mM citrate and 10.2 mM glucose; pH 6.5) for 15 min at 37 °C in the presence or the absence of Hg^{2+}, Pb^{2+} or Cd^{2+} (1-200 μM). The uptake assay was initiated by the addition of 20 μl [^{3}H]-glutamate (0.54 nmol [^{3}H]-glutamate with 4.46 μM unlabeled glutamate) and incubation continued for 10 min. Uptake was stopped by rapid cooling of the tubes and the cold suspension was centrifuged at 49,500 x g for 10 min. The platelet pellet was washed with cold incubation medium. Sodium dodecyl sulfate (SDS) (1%) and scintillation liquid were added to the pellet and radioactivity was measured. Specific [^{3}H]-glutamate uptake was calculated as the difference between the uptake obtained in the incubation medium described above and the uptake obtained with a similar incubation medium in which NaCl was replaced by choline chloride. The IC_{50} values for metals inhibition were calculated.

2.4. [^{3}H] Glutamate binding by platelets

All binding assays were performed using platelet membranes prepared as previously described (28) and stored at – 70°C for up to 2 months. On the day of the binding assay, membranes were rapidly thawed in a water bath (37°C), homogenized with 3 volumes of 10 mM Tris/Acetate, pH 7.4, and centrifuged 3 times 27.000 x g for 15 min. The final pellet was resuspended in the same buffer and was used for the binding assay. Platelets were incubated in 0.5 mL reaction mixture containing 50 mM Tris/acetate, pH 7.4, and 40 nM [^{3}H]-glutamate in the presence or the absence of Hg^{2+}, Pb^{2+} or Cd^{2+} (10-200 μM). Incubation was carried out at 30°C for 30 min, and the reaction was stopped by rapid cooling of the tubes and the cold suspension was centrifuged at 27.000 x g for 15 min. The platelet pellet was washed with ice-cooled milli-Q water. Sodium dodecyl sulfate (SDS) (0.1%) and scintillation liquid were added to the pellet and radioactivity was measured. Non-specific binding (obtained by

incubating membranes with nonradioactive glutamate in concentration 1000 times greater than radioactive glutamate) typically amounted to 10-20% of total binding. The IC_{50} and ED_{50} values for metals were calculated.

2.5. Lipid Peroxidation

The lipid peroxidation assay was performed using the platelet suspension prepared as described by Mangano & Schwarcz (9). Platelets were incubated at 37 °C for 1 hour in the presence or the absence of Hg^{2+}, Pb^{2+} or Cd^{2+} (200 μM). Thiobarbituric acid-reactive species (TBARS) were determined as described by Ohkawa et al., (31).

2.6. ROS Measurement

To estimate the oxidation of reactive oxygen species, platelets were incubated with 10 μl of dichlorofluorescein (1 mM) in the presence or the absence of Hg^{2+}, Pb^{2+} or Cd^{2+} (50-200 μM). The reactive oxygen species (ROS) levels were determined by a spectrofluorimetric method, using 2',7'- dichlorofluorescein diacetate (DCHF-DA) assay. The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) was measured for the detection of intracellular ROS. The DCF fluorescence intensity emission was recorded at 520nm (with 480 nm excitation).

2.7. Protein Quantification

Protein concentration was measured by the method of Lowry et al. (32), using serum albumin as the standard.

2.8. Statistical Analysis

Statistical analysis was performed using one-way ANOVA followed by the Duncan's test. Values of $P < 0.05$ were considered statistically significant. The IC_{50} value for Hg^{2+} was reported as geometric mean accompanied by its 95% confidence limits. The IC_{50} and ED_{50} values were determined by linear regression from individual experiments using "GraphPad Software" (GraphPad software, San Diego, CA, USA).

3. Results

The results showed in Figure 1 indicate that Hg^{2+} at 1 μM significantly inhibited about of 38% [^{3}H]-glutamate uptake. The IC_{50} value (95% confidence) calculated for Hg^{2+} was 16.66 (15.62-17.77) μM and the maximal inhibitory effect was about of 98% at 100 μM.

At 10 μM, Cd^{2+} caused a significant inhibition in [^{3}H]-glutamate uptake by human platelets (Figure 1). The maximal inhibitory effect was around of 20% at 10 μM (IC_{50} > 200 μM).

In contrast, Pb^{2+} at 100 μM inhibited [^{3}H]-glutamate uptake (Figure 1). The maximal inhibitory effect was about of 20% at 100 μM (IC_{50} > 200 μM).

Hg^{2+} was the most effective inhibitor of [^{3}H]-glutamate uptake in human platelets when the potential inhibitory of metals was compared.

Figure 2 demonstrates that Hg^{2+} at 50 μM totally inhibited [^{3}H]-glutamate binding in human platelets. The IC_{50} value (95% confidence) calculated for Hg^{2+} was 12.66 (11.63 -13.77) μM and the maximal inhibitory effect was about of 98% at 20 μM.

In contrast, Cd^{2+} and Pb^{2+} significantly stimulated [^{3}H]-glutamate binding in human platelets. Cd^{2+}, at 200 μM, caused a significant stimulation of [^{3}H]-glutamate binding (Figure 2). The maximal effect was about of 18% at 100 μM (IC_{50} > 200 μM).

Pb^{2+} at 100 μM stimulated about of 27% [^{3}H]-glutamate binding. The ED_{50} value (95% confidence) calculated for Pb^{2+} was 105.97 (88 -127.6) μM. The maximal stimulatory effect was about of 70% at 200 μM (Figure 2).

Cd^{2+}, Pb^{2+} and Hg^{2+} increased reactive oxygen species levels in human platelets. Pb^{2+} and Hg^{2+}, at 200 μM, caused a significant increase (about of 50 and 40%, respectively) in TBARS levels (Table 1).

4. Discussion

Platelets have been extensively studied as models of synaptic apparatus, since they release and reuptake neurotransmitters such as serotonin and dopamine (33, 34), express serotonin (35) and glutamate receptors (36, 37) and have been used as clinical markers for neuropsychiatric disorders (12-15).

In fact, blood cells such as platelets are ideally suited for monitoring a chemical's neurotoxic effects because of their easy accessibility and because they share a number of functions similar to those of CNS neurons (38). The present study clearly demonstrates that Hg^{2+}, Cd^{2+} and Pb^{2+} inhibited [^{3}H]-glutamate uptake in human platelets. The inhibitory potency for [^{3}H]-glutamate uptake inhibition could be suggested as follow $Hg^{2+} > Cd^{2+} = Pb^{2+}$, as verified by the calculated IC_{50} values [16.66 μM (Hg^{2+}), > 200 μM (Cd^{2+} and Pb^{2+})]. In addition, we found that Hg^{2+} inhibited [^{3}H]-glutamate binding, while Cd^{2+} and Pb^{2+} stimulated [^{3}H]-glutamate binding in human platelets. To the best of our knowledge this is the first report documenting the effect of heavy metals on glutamate neurotransmission in human platelets. Although several groups have studied the effects of heavy metals on glutamatergic system in rat brain (3, 21, 28).

One important finding of this study is that Hg^{2+} was the most inhibitory metal of [^{3}H]-glutamate uptake in human platelets. Accordingly, several authors have reported that mercury compounds inhibited the glutamate uptake in different structures (39-41). Moretto et al. (41) have also reported that 50 μM of Hg^{2+} inhibited glutamate uptake in rat cortex slices. However, based on the calculated IC_{50} value we infer that Hg^{2+} was more inhibitory in platelets than was in brain, suggesting that platelets are more susceptible to Hg^{2+}. Thus, the platelets could be biomarkers of Hg^{2+} exposure that could provide early warning signs of Hg^{2+} neurotoxicity before considerable damage to nervous system has occurred.

The literature data have shown that mercury compounds induce the inhibition of GLT, GLAST and EAAC (30). Consequently, the mercury inhibitory effect can be due to a direct interaction with glutamate transporter proteins or by promoting oxidative stress that oxidizes the glutamate transporter proteins. In agreement, we showed that Hg^{2+} increased lipid peroxidation levels and ROS measurement in platelets, suggesting that the oxidative stress could be involved in metal effect on platelet glutamatergic system. We also found that Hg^{2+} inhibited [^{3}H]-glutamate binding in human platelets. These results agree with Soares and collaborators (28), which demonstrated that 10 μM of Hg^{2+} inhibited [^{3}H]-glutamate binding in brain synaptic membranes.

In this study, we reported that Cd^{2+} inhibited the [^{3}H]-glutamate uptake by platelets. It is difficult to compare the results obtained with Cd^{2+} in platelets, since there are only a few reports that demonstrate the effect of cadmium in the glutamatergic system. It is known that cadmium inhibits the voltage-dependent calcium channels in vitro (42) and this metal might affect the release of neurotransmitters from neuron terminals.

We also found that Pb^{2+} changed the glutamate uptake only at high concentrations. Lead adversely affects the CNS and low level exposure is associated with behavioral abnormalities, decreased hearing, and cognitive impairment (26). The glutamatergic component is undoubtedly involved in Pb-induced neurotoxicity (43). In fact, Struzynska et al. (44) demonstrated that the expression of neuronal and glial glutamate transporters is changed in lead-exposed rats.

Cd^{2+} and Pb^{2+} stimulated [^{3}H]-glutamate binding in human platelets, which may indicate a neurotoxic effect. As a matter of fact, the stimulation of glutamatergic neurotransmission, an exacerbation of the physiologic effects, could lead an increase of Ca^{2+} influx and consequently neurotoxic effects. On the contrary, Cd^{2+} and Pb^{2+} inhibited the [^{3}H]-glutamate binding in brain synaptic membranes (28). Comparing the results reported by Soares (28) and those found in this study, we can suggest that the effect of Cd^{2+} and Pb^{2+} on platelet glutamatergic system appears does not reflect what happens in the CNS.

In addition, a comparison between blood levels of individuals exposed to Hg^{2+} and Pb^{2+} (45, 46), but not to Cd^{2+}, and the IC_{50} levels obtained experimentally in this study indicates the suitability of this method as a biomarker of neurotoxic effects.

Taking into account our results, the oxidative stress could be one of the mechanisms involved in heavy metals-induced toxic effects in [^{3}H]-glutamate uptake. However, the potential discrepancies on the results found in uptake and binding must be interpreted with caution. We believe that the mechanisms involved in heavy metals action in uptake are probably different from those in binging. In fact, an increase in ROS inhibited glutamate uptake but stimulated glutamate binding suggesting that uptake and binding are differently affected by heavy metals in concentrations that stimulated oxidative stress. Therefore, the oxidative stress seems to be only related to

the inhibitory effect of Hg^{2+} in platelet [^{3}H]-glutamate binding. On the other hand, the mechanism implicated in Pb^{2+} and Cd^{2+} stimulation in [^{3}H]-glutamate binding appears not be related to the oxidative stress. Therefore, one can speculate that the modulation of glutamatergic receptors in human platelets involves complex mechanisms which could tentatively explain the effects induced by cadmium and lead in glutamate binding.

In conclusion, we suggest that the impairment of glutamatergic system caused by heavy metals lead ultimately to an increase of glutamate concentration which can evoke excytotoxic actions. The present limited results could suggest that glutamatergic system may be used as a potential biomarker for neurotoxic action of heavy metals in humans. However, based on the discrepancies found in some effects in platelets when compared with those obtained in brain these results must be interpreted with caution.

Acknowledgements

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Legends

Figure 1. Effect of Hg^{2+}, Cd^{2+} and Pb^{2+} on [^{3}H]-glutamate uptake in human platelets. Data are the mean ± SD of three separate determinations performed in triplicate using platelets from different individuals. The control value was 150 ± 16 pmol/mg protein/30 min. (*) Significantly different from control (without metals), $P < 0.05$.

Figure 2. Effect of Hg^{2+}, Cd^{2+} and Pb^{2+} on [^{3}H]-glutamate binding in human platelets. An aliquot of membrane was incubated with 40 nM [^{3}H]glutamate at 30 °C for 30 min in the presence of Hg^{2+}, Cd^{2+} and Pb^{2+}. Data are the mean ± SD of three separate determinations performed in triplicate using platelets from different individuals. The control value was 2.1 ± 0.5 pmol/mg protein/30 min. (*) Significantly different from control (without metals), $P < 0.05$.

Tables

Table 1- Effect of Hg^{2+}, Cd^{2+}, Pb^{2+} on TBARS levels and ROS measurement in human platelets

Metals	TBARS	ROS
Hg^{2+}	140.6 ± 19.8*	138.3 ± 21.0*
Cd^{2+}	99.3 ± 17.0	133.6 ± 9.4*
Pb^{2+}	153.6 ± 5.0*	153.1 ± 33.0*

Platelets were incubated at 37 °C for 1 hour in the presence or the absence of Hg^{2+}, Pb^{2+} or Cd^{2+} (200 μM). Data are the mean±SD of three separate determinations performed in triplicate using platelets from different individuals. TBARS and RS measurement are expressed as percentage of control values (100%). The control value for TBARS assay was 3.45 ± 0.76 nmol MDA/mg protein. (*) Significantly different from control (without metals), $P < 0.05$.

Figures

Figure 1

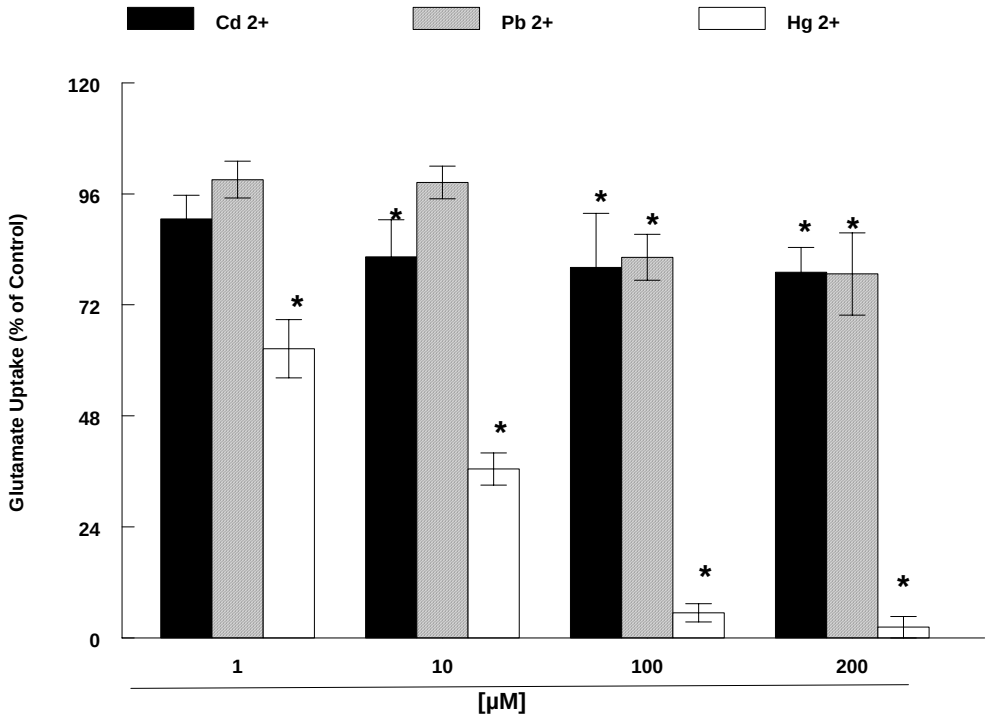
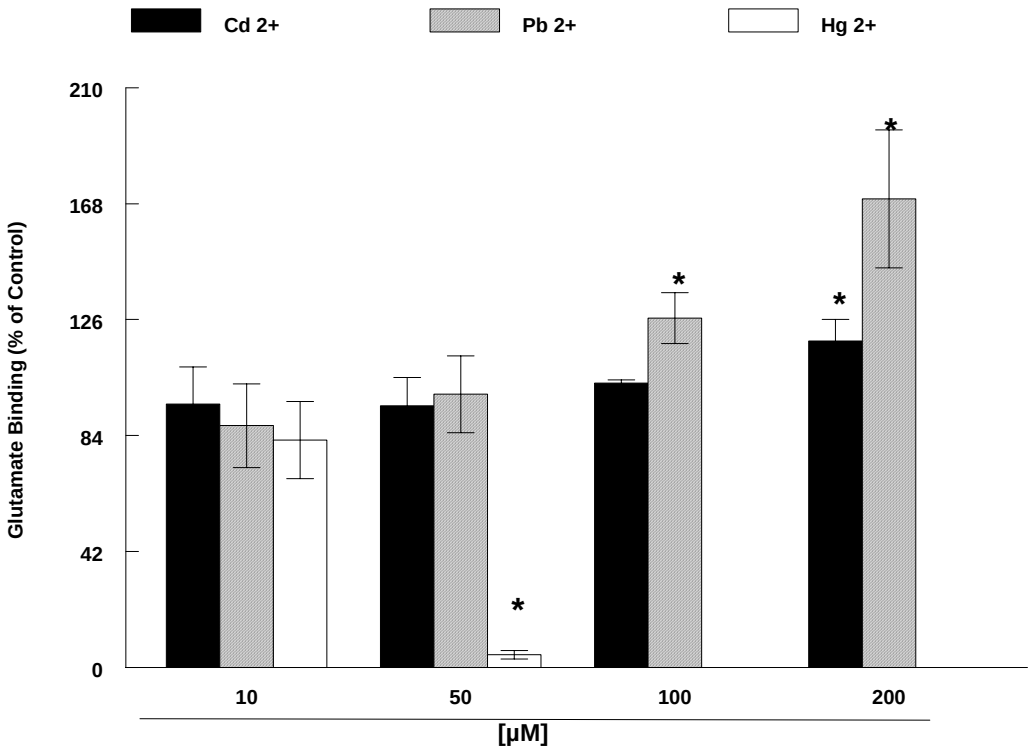


Figure 2


Cd 2+
Pb 2+
Hg 2+
Glutamate Binding (% of Control)
210
168
126
84
42
0
*
*
*
*
10
50
100
200
[µM]

4.1.2 – Artigo 2

THE ROLE OF THIOL REDUCING AGENTS ON MODULATION OF GLUTAMATE BINDING INDUCED BY HEAVY METALS IN PLATELETS

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Submetido à Biometals

The Role of Thiol Reducing Agents on Modulation of Glutamate Binding

Induced by Heavy Metals in Platelets

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Abstract

Taking into account that Hg^{2+}, Pb^{2+} and Cd^{2+} modulated glutamatergic system in human platelets, we investigated if thiol-reducing agents (BAL, DMPS, DTT and GSH) are capable of altering Hg^{2+}, Pb^{2+} and Cd^{2+} effects on platelet glutamatergic system. BAL, a dithiol chelating agent therapeutically used for the treatment of heavy metals poisoning, was capable of protecting the [^{3}H]-glutamate binding against the effects caused by Pb^{2+} and Hg^{2+}. DMPS, another dithiol reducing chelating agent therapeutically used for the treatment of heavy metals poisoning, was capable of protecting the effect caused by Cd^{2+}, Pb^{2+} and Hg^{2+}. The similar effect was observed with addition of DTT on [^{3}H]-glutamate binding in human platelets. These dithiol reducing agents (BAL, DMPS and DTT) alone did not alter [^{3}H]-glutamate binding. In contrast, GSH, a monothiol reducing agent, caused a significant inhibition on [^{3}H]-glutamate binding at all concentrations tested. GSH did not modify heavy metals effect on [^{3}H]-glutamate binding in platelets. The findings of the present investigation indicate that dithiol-reducing agents (BAL, DMPS and DTT) are capable of altering Hg^{2+}, Pb^{2+} and Cd^{2+} effects on platelet glutamatergic system, supporting the capacity of these compounds for chelating metal ions.

Keywords: Platelets, heavy metals, glutamate binding, chelating agents

1. INTRODUCTION

Research strategies have been developed to characterize parameters in peripheral tissues that might easily be measured in humans as surrogate markers of damage, dysfunction or interactions involving neural targets of toxicants (Manzo et al., 1995). Human platelets have been shown to accumulate glutamate in a manner similar to that in as synaptosomal preparations (Mangano and Schwarcz, 1981). In addition, it has been speculated that defects in central synaptic function may be demonstrable in platelet preparations. Platelets express glutamate receptors that have an anti-aggregating role and glutamate transporters that are analogous to those found in the brain (Ferrarese et al., 1999; 2000; 2001). Glutamate is established as the major neurotransmitter in the mammalian brain and plays a fundamental role in a variety of neurophysiological processes, including learning and memory formation (Lipton and Rosenberg, 1994; Danbolt et al., 2001).

The increasing number of chemicals present in the habitational and occupational environment elevates the risk of toxic human exposure. Therefore heavy metals have become one of many contaminants found in our environment. Cadmium is an important inorganic environmental pollutant and the potential for human exposure has generally increased with the increasing use of this metal (Waalkes et al., 1991). The exposure to cadmium induced damage to numerous tissues such as liver, kidney, lung, ovaries and testes. In central nervous system (CNS), this metal affects the degree and balance of excitation-inhibition in synaptic neurotransmission (Minami et al., 2001). However, there are a few reports that demonstrate the effect of cadmium in the glutamatergic system.

In this context, another metal, lead, is a ubiquitous environmental toxicant and can alter synaptic transmission and cause severe neurological damage (Gong et al.,

1997). This metal is also known to have a wide-range of effects, from modulation of gene regulation, second messenger systems and other signal transduction pathways, to alterations in the catecholaminergic, opioid, GABAergic and glutamatergic systems (Cory-Slechta, 1997; Aschner, et al., 2005; Struzynska et al., 2005).

Mercurial compounds are global environmental contaminants deriving from natural processes, which possess neurotoxic potential (Aschner et al., 2000). In fact, MeHg and Hg^{2+} cause severe alterations in the CNS that may be due to their ability to disrupt synaptic transmissions (Atchison et al., 1994), producing neuronal death that is partially mediated by glutamate (Yamashita et al., 1997). In addition, *in vitro* studies showed that Hg^{2+} inhibited glutamate binding on brain synaptic membranes (Soares et al., 2003).

These metals may react with free protein cysteine thiol groups, which are involved in the function of many enzymes, structural proteins and receptors (Aposhian et al., 1990; Erskine et al., 2000). Of particular importance, the glutamatergic receptors could be modulated by the action of reducing or oxidizing agents (Aizenman et al., 1989). In this way, NMDA function, appears to be highly sensitive to the oxidizing potential of the extracellular environment (Dingledine et al., 1999) and these receptors were described in platelets (Franconi et al., 1996; Skerry et al., 2001) and in megakaryocytes (Genever et al., 1999; Skerry et al., 2001). In fact, Borges and collaborators (2007) demonstrated that Hg^{2+}, Pb^{2+} and Cd^{2+} modulated glutamatergic system in human platelets.

Taking into account that heavy metals disrupted glutamatergic system in platelets, we investigated if thiol-reducing agents (BAL, DMPS, DTT and GSH) are capable of altering Hg^{2+}, Pb^{2+} and Cd^{2+} effects on platelet glutamatergic system.

2. MATERIALS AND METHODS

2.1. Chemicals

[^{3}H]Glutamate (49 Ci/mM) was purchased from Amersham International (Amersham, Bucks, UK). 2,3-Dimercaptopropanol (BAL), 2,3-dimercapto-propane-1-sulfonic acid (DMPS), dithiothreitol (DTT) and reduced glutathione (GSH) were purchased from Sigma (St. Louis, MO, USA). All other chemicals ($HgCl_2$, $CdCl_2$, Pb acetate) were of analytical grade and obtained from standard commercial suppliers. Heavy metals were dissolved on 0.9% NaCl.

2.2. Blood samples

The human blood was obtained from the 30 healthy volunteer donors of the Banco de Sangue, Hospital da Universidade Federal de Santa Maria, Santa Maria, RS, Brazil. The samples used in experiments for [^{3}H]-glutamate binding assay were transferred immediately after be obtained into a tube containing citrate/phosphate/dextran-adenine (CPD-A1).

2.3. [^{3}H]-Glutamate binding by platelets

All binding assays were performed using platelet membranes prepared as described by Jones and Matus (1974) and stored at – 70 °C for up to 2 months. On the day of the binding assay, the membranes were rapidly thawed in a water bath (37 °C), homogenized with 3 volumes of 10 mM Tris/Acetate, pH 7.4, and centrifuged 3 times 27.000 x g for 15 min. The final pellet was resuspended in the same buffer and was used for the binding assay. Platelets were incubated in 0.5 mL reaction mixture containing 50 mM Tris/acetate, pH 7.4, and 40 nM [^{3}H]-glutamate in the presence or the absence of Hg^{2+} (50 μM), Pb^{2+} or Cd^{2+} (200 μM). The dose of heavy metals was

chosen based on previous study of our group (Borges et al., 2007). Incubation was carried out at 30 °C for 30 min, and the reaction was stopped by rapid cooling of the tubes and the cold suspension was centrifuged at 27.000 x g for 15 min. The platelet pellet was washed with ice-cooled milli-Q water. Sodium dodecyl sulfate (SDS) (0.1%) and scintillation liquid were added to the pellet and radioactivity was measured. Non-specific binding (obtained by incubating membranes with nonradioactive glutamate in concentration 1000 times greater than radioactive glutamate) typically amounted to 10-20% of total binding.

2.4.1 Effect of thiol reducing agents

To investigate a possible involvement of cysteinyl groups in metal effect on glutamate binding, platelets were incubated in the presence of Hg^{2+} (50 μM), Pb^{2+} or Cd^{2+} (200 μM) (Borges et al., 2007) and with reducing agents (BAL, DMPS, DTT and GSH- 1 mM). Binding was then immediately started by adding platelets membranes.

Incubation was carried out at 30 °C for 30 min, and the reaction was stopped by rapid cooling of the tubes and the cold suspension was centrifuged at 27,000 x g for 15 min. The platelet pellet was washed with ice-cooled milli-Q water. Sodium dodecyl sulfate (SDS) (0.1%) and scintillation liquid were added to the pellet and radioactivity was measured.

The effect caused by reducing agents (DMPS, BAL, DTT and GSH) alone was tested and these agents were used in different concentrations (10- 1000 μM).

Aiming at studying the effect of thiol reducing agents (BAL, DMPS, DTT and GSH- 1 mM) on reversing heavy metals-induced effects on [^{3}H]-glutamate binding, Hg^{2+} (50 μM), Pb^{2+} or Cd^{2+} (200 μM) (Borges et al., 2007) were pre-incubated with platelet membranes for 10 min at 30 °C. After that, the reducing agents were added to

the reaction tubes. Incubation was carried out at 30 °C for 30 min, and the reaction was stopped by rapid cooling of the tubes and the cold suspension was centrifuged at 27.000 x g for 15 min. The platelet pellet was washed with ice-cooled milli-Q water. Sodium dodecyl sulfate (SDS) (0.1%) and scintillation liquid were added to the pellet and radioactivity was measured.

2.5. Protein Quantification

Protein concentration was measured by the method of Lowry et al. (1979), using serum albumin as the standard.

2.7. Statistical Analysis

Statistical analysis was performed using a one-way or two-way analysis of variance (ANOVA), followed by Duncan's multiple range test when appropriate. Values of $p<0.05$ were considered statistically significant.

3. Results

At 200 μM, Cd^{2+} (Table 1) and Pb^{2+} (Figure 1) significantly stimulated [^{3}H]-glutamate binding in human platelets, about 17% and 60%, respectively. In contrast, Hg^{2+} at 50 μM totally inhibited [^{3}H]-glutamate binding in human platelets (Figure 2).

BAL, at all concentrations tested, did not alter [^{3}H]-glutamate binding (data not shown). Two-way ANOVA of [^{3}H]-glutamate binding did not reveal interaction between BAL and Cd^{2+} (Table 1).

In contrast, two-way ANOVA of [^{3}H]-glutamate binding revealed a significant interaction between BAL and Pb^{2+} ($F(1,11)= 6.52$, $p< 0.026$), BAL and Hg^{2+} ($F(1,9)=$

213.64, p< 0.001). In fact, BAL was capable of protecting the [^{3}H]-glutamate binding against the effects caused by Pb^{2+} and Hg^{2+} (Figures 1 and 2).

DMPS did not alter [^{3}H]-glutamate binding in human platelets (data not shown). Two-way ANOVA of [^{3}H]-glutamate binding showed a significant interaction between DMPS and Cd^{2+} (F (1,8)= 19.53, p< 0.002) (Table 1), DMPS and Pb^{2+} (F (1,9)= 5.12, p< 0.049) (Figure 1) as well as DMPS and Hg^{2+} (F (1,9)= 400.3, p< 0.001) (Figure 2).

Two-way ANOVA of [^{3}H]-glutamate binding revealed a significant interaction between BAL and Cd^{2+} (F (1,9)= 12.46, p< 0.006), BAL and Pb^{2+} (F (1,8)= 28.56, p< 0.001) as well as BAL and Hg^{2+} (F (1,9)= 272.68, p< 0.001). BAL was capable of restoring the effect caused by heavy metals (Figure 3). Similarly, two-way ANOVA of [^{3}H]-glutamate binding revealed a significant interaction between DMPS and Cd^{2+} (F (1,9)= 8.64, p< 0.016), DMPS and Pb^{2+} (F (1,8)= 14.04, p< 0.005), DMPS and Hg^{2+} (F (1,9)= 25.31, p< 0.001) (Figure 3).

DTT, a sulfhydryl agent commonly used as a reducing of thiol groups, was tested for its ability to prevent metals-induced effects on [^{3}H]-glutamate binding. Two-way ANOVA of [^{3}H]-glutamate binding revealed a significant interaction between DTT and Cd^{2+} (F (1,8)= 16.78, p< 0.003) (Table 1), DTT and Pb^{2+} (F (1,9)= 6.26, p< 0.033) (Figure 1) as well as DTT and Hg^{2+} (F (1,9)= 502.25, p< 0.001) (Figure 2). In fact, DTT was capable of preventing the effect caused by Cd^{2+}, Pb^{2+} and Hg^{2+} on [^{3}H]-glutamate binding in human platelets.

Regarding the experiments aiming to restore heavy metal effects on [^{3}H]-glutamate binding, similar results to those obtained on protecting experiments were observed (data not shown).

GSH, a monothiol reducing agent, caused a significant inhibition on [^{3}H]-glutamate binding at all concentrations tested (about of 80%) (data not shown). GSH

did not modify heavy metals effect on [^{3}H]-glutamate binding (Figures 1 and 2, Table 1).

4. Discussion

This study clearly demonstrated that dithiol reducing agents, BAL, DMPS and DTT, were effective in ameliorate the effect caused by Hg^{2+}, Cd^{2+} and Pb^{2+} on [^{3}H]-glutamate binding in platelets. Conversely, the monothiol reducing agent, GSH, was unable to protect against the effect induced by heavy metals.

These heavy metals may react with free protein cysteine thiol groups, which are involved in the function of many enzymes, structural proteins and receptors (Aposhian et al., 1990; Erskine et al., 2000). In fact, the formation of stable mercaptides with the thiol-disulfide site of glutamate receptors could be a reasonable explanation for the binding inhibition induced by Hg^{2+}. Of particular importance, the glutamatergic receptor, NMDA, appears to be highly sensitive to the oxidizing potential of the extracellular environment (Dingledine et al., 1999). NMDA receptors have been reported in platelets (Franconi et al., 1996; Skerry et al., 2001) and megakaryocytes (Genever et al., 1999; Skerry et al., 2001), supporting their importance in regulating platelet activation and aggregation (Zoia et al., 2004).

One important finding of this study is that DTT was effective in protecting the effect caused by Hg^{2+} on [^{3}H]-glutamate binding in platelets, suggesting that this heavy metal interacts with cysteinyl residues that are important for redox modulation of NMDA-receptor responses. On the contrary, DTT did not prevent the effect caused by Hg^{2+} on [^{3}H]-glutamate binding in brain synaptic membranes (Soares et al., 2003). These results reinforce the assumption that glutamatergic receptors in platelets are

modulated by thiol-disulfide status but this system seems to be more sensitive to DTT in platelets than in synaptic membranes.

The monothiol reducing agent, GSH, did not protect the inhibition induced by Hg^{2+} in glutamate binding, suggesting that this metal mainly react with sulfhydryl groups localized in the deeper regions of the receptor. For instance, DTT (-0.55 mV), the thiol with the highest electronegative redox potential, was more efficient in protecting heavy metals induced toxicity than was GSH (-0.23 mV). These data suggest that thiol electronegative redox potential can partially explain the differences on the capacity of thiols in restoring effect caused by heavy metals. In fact, mercurial compounds inhibited glutamatergic system in rat astrocytes and this effect was reversed by the addition of the cell permeant DTT, while addition of the non-permeant GSH had no effect (Albrecht et al., 1993).

Another interesting finding of this study is that GSH inhibited [^{3}H]-glutamate binding. Two hypothesis can be postulated to, at least in part, explain the effect caused by GSH in platelets; the first hypothesis is that GSH displaces glutamate from specific sites by means of their γ-glutamyl moiety. Accordingly, Janáky and collaborators (2000) have reported glutamate displacement by GSH in cerebral cortical synaptic membranes. Oja and collaborators (1988) have also demonstrated that both GSH and GSSG inhibit the Na^+- and temperature-independent binding of [^{3}H]-glutamate to synaptic membranes. The second is that GSH increases the generation of ROS in platelets. This evidence is partly sustained by reports that demonstrated that BSO (L-buthionine sulfoximine), which blocks GSH synthesis, reduced the amount of ROS in platelets (Wachowicz et al., 2002).

The use of BAL, a dithiol chelating agent therapeutically used for the treatment of heavy metals poisoning, was effective in protecting the effects caused by Hg^{2+} on [^{3}H]-

glutamate binding. Conversely, Soares et al. (2003) have reported that BAL was inefficient in protecting the inhibition caused by heavy metals on glutamate binding from brain synaptic membranes.

In addition, our results demonstrated that BAL did not present effect on [^{3}H]-glutamate binding in human platelets. However, BAL can produce neurotoxic effects in a variety of situations (Aposhian et al., 1995; Nogueira et al., 2000). In fact, BAL inhibited [^{3}H]-glutamate binding in synaptic membranes (Nogueira et al., 2001), suggesting that brain is more susceptible to BAL than platelets.

DMPS, another dithiol reducing chelating agent therapeutically used for the treatment of heavy metals poisoning, was capable of protecting the effect caused by Hg^{2+} on [^{3}H]-glutamate binding in human platelets. However, this chelating agent did not prevent the inhibition caused by Hg^{2+} in brain (Soares et al., 2003).

The stimulatory effect caused by Cd^{2+} and Pb^{2+} on [^{3}H]-glutamate binding in platelets was protected by the addition of BAL and DMPS, supporting the capacity of these compounds for chelating metal ions. DTT protected the stimulation on [^{3}H]-glutamate binding caused by heavy metals. However, GSH did not modify the stimulatory effect caused by Cd^{2+} and Pb^{2+} on [^{3}H]-glutamate binding, demonstrating that are necessary two thiol groups for chelating these metal ions.

In conclusion, the findings of the present investigation indicate that dithiol-reducing agents (BAL, DMPS and DTT) are capable of altering Hg^{2+}, Pb^{2+} and Cd^{2+} effects on platelet glutamatergic system, by chelating metal ions and/or reducing thiol groups important for the activity of glutamatergic receptors.

Acknowledgements

The financial support by UFSM, FAPERGS, CAPES and CNPq is gratefully acknowledged. J.B.T.R, C.W.N. are the recipients of CNPq fellowships.

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Legends

Figure 1. Effect of reducing agents on [^{3}H]-glutamate binding stimulated by Pb^{2+} in human platelets. An aliquot of membrane was incubated with 40 nm [^{3}H]glutamate at 30 °C for 30 min in the presence or the absence of Pb^{2+} (200 μM) simultaneously with reducing agents (BAL, DMPS, DTT and GSH- 1 mM). Data are the mean ± SD of three separate determinations performed in triplicate using platelets from different individuals. (*) Significantly different from control, $P < 0.05$. (#) Significantly different from Pb^{2+}.

Figure 2. Effect of reducing agents on [^{3}H]-glutamate binding inhibited by Hg^{2+} in human platelets. An aliquot of membrane was incubated with 40 nm [^{3}H]glutamate at 30 °C for 30 min in the presence or the absence of Hg^{2+} (50 μM) simultaneously with reducing agents (BAL, DMPS, DTT and GSH- 1 mM). Data are the mean ± SD of three separate determinations performed in triplicate using platelets from different individuals. (*) Significantly different from control, $P < 0.05$. (#) Significantly different from Hg^{2+}.

Figure 3. Restoring effect of reducing agents on effect caused by heavy metals on [^{3}H]-glutamate binding in human platelets. Hg^{2+} (50 μM), Pb^{2+} or Cd^{2+} (200 μM) were pre-incubated with platelets membranes for 10 min at 30 °C. After this time, reducing agents were added to reactions tubes. Data are the mean ± SD of three separate determinations performed in triplicate using platelets from different individuals. (*) Significantly different from control, $P < 0.05$. (a) Significantly different from Cd^{2+}. (b) Significantly different from Pb^{2+}. (c) Significantly different from Hg^{2+}.

Tables

Table 1. Protective effects of reducing agents on [^{3}H]-glutamate binding stimulated by Cd^{2+}

	% of Control
DMPS	100.3 ± 4.2#
BAL	104.6 ± 5.5
DTT	109.3 ± 3.0
GSH	22.3 ± 5.5*#
Cd^{2+} 200 μM	117.8 ± 4.8*
Cd^{2+} + DMPS	96.0 ± 3.6#
Cd^{2+} + BAL	117.3 ± 20.0*
Cd^{2+} + DTT	111.0 ± 4.3#
Cd^{2+} + GSH	24.1 ± 6.5*#

Platelets were incubated in the presence or the absence of Cd^{2+} (200 μM) simultaneously with reducing agents (BAL, DMPS, DTT and GSH- 1 mM) at 30 °C for 30 min. Data are the mean ± SD of three separate determinations performed in triplicate using platelets from different individuals. (*) Significantly different from control, $P < 0.05$. (#) Significantly different from Cd^{2+}.

Figures

Figure 1

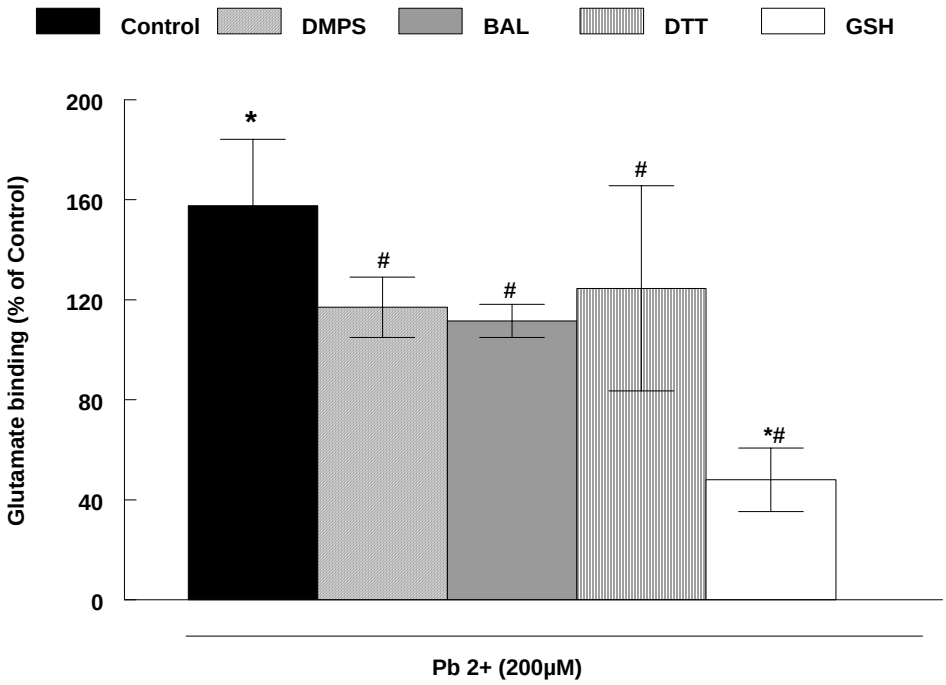


Figure 2

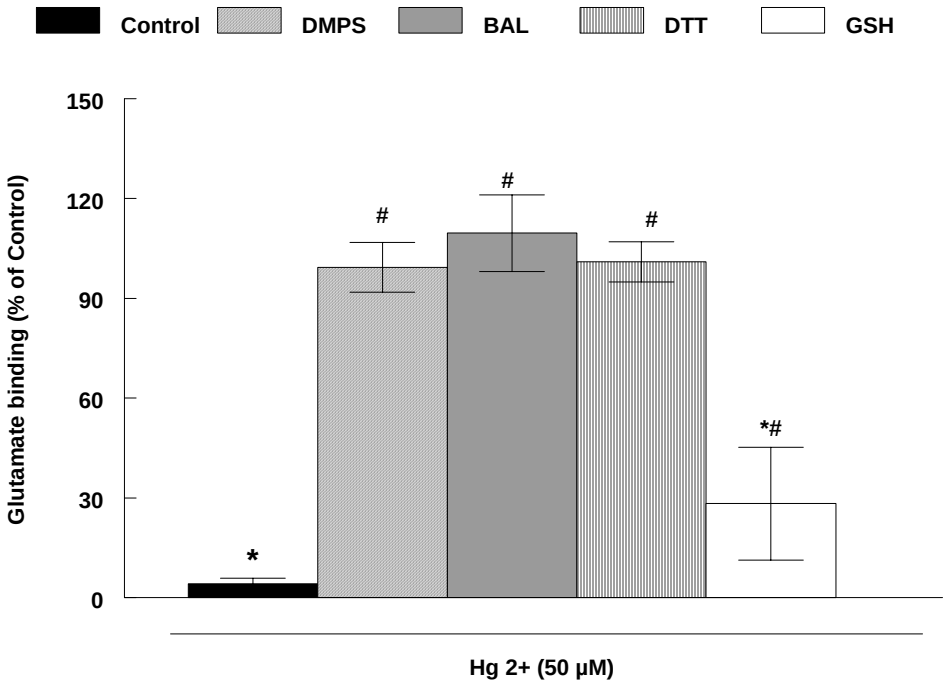
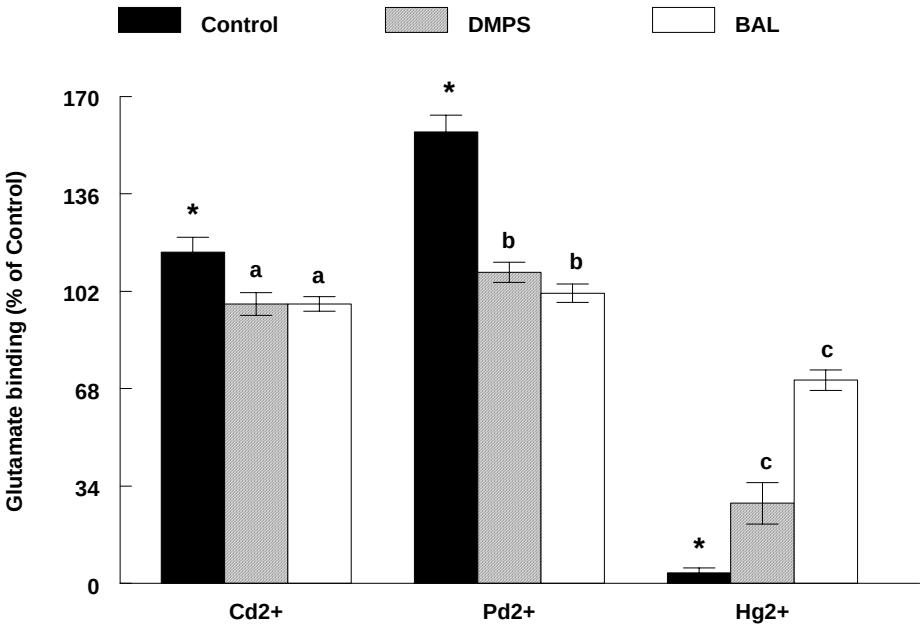

Control
DMPS
BAL
DTT
GSH
150
120
90
60
30
0
Glutamate binding (% of Control)
*
#
#
#
*#
Hg 2+ (50 µM)

Figure 3


Control
DMPS
BAL
Glutamate binding (% of Control)
170
136
102
68
34
0
*
a
a
*
b
b
*
c
c
Cd2+
Pd2+
Hg2+

ANEXO

Figure 1

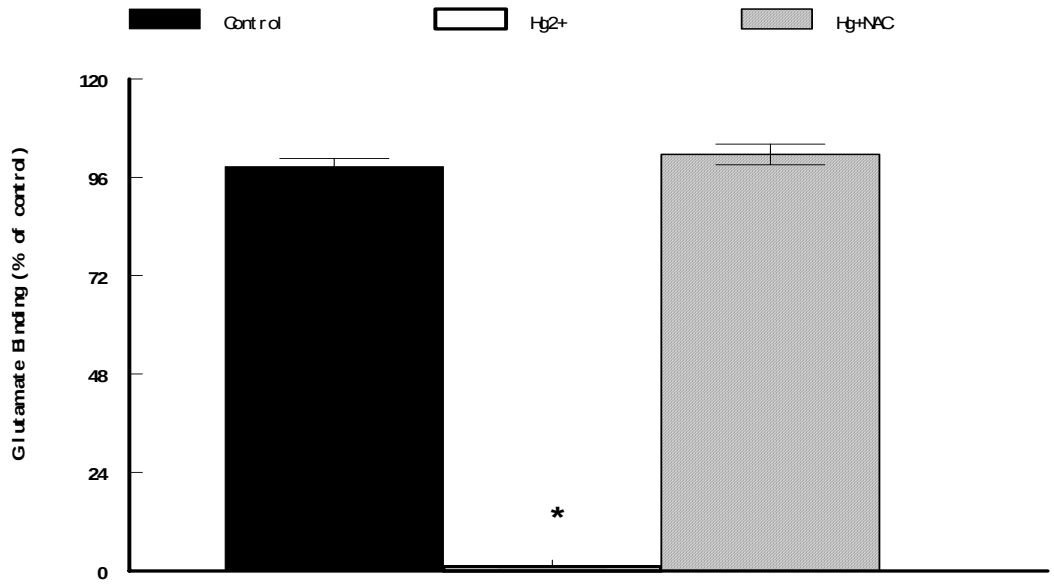


Figure 1. Effect of NAC on [^{3}H]-glutamate binding inhibited by Hg^{2+} in human platelets. An aliquot of membrane was incubated with 40 nm [^{3}H]glutamate at 30 $^{\circ}$C for 30 min in the presence or the absence of Hg^{2+} (50 μM) simultaneously with NAC (1 mM). Data are the mean $\pm$ SD of three separate determinations performed in triplicate using platelets from different individuals. (*) Significantly different from control, $P < 0.05$.

4.2- Efeito causado pelos organocalcogênios sobre o sistema glutamatérgico em plaquetas, *in vitro*

4.2.1 – Artigo 3

ORGANOCHALCOGENS AFFECT THE GLUTAMATERGIC NEUROTRANSMISSION IN HUMAN PLATELETS

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Neurochemical Research

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Organochalcogens Affect the Glutamatergic Neurotransmission in Human Platelets

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(Accepted January 27, 2004)

Blood platelets have repeatedly been suggested as an excellent model for various aspects of the synaptic apparatus. Considering that organochalcogens affect some parameters of glutamatergic neurotransmission in rats, in the current study we evaluated the effect of diphenyl diselenide $(PhSe)_2$, diphenyl ditelluride $(PhTe)_2$, and Ebselen on glutamatergic neurotransmission in human platelets. $(PhTe)_2$ and $(PhSe)_2$ caused a significant inhibition, but Ebselen did not interfere in Na-independent glutamate binding. Dithiothreitol (DTT) did not completely prevent the [^{3}H]glutamate binding inhibition caused by 100 μM $(PhTe)_2$. $(PhSe)_2$, $(PhTe)_2$, and Ebselen (100 μM) significantly inhibited [^{3}H]glutamate uptake, whereas organochalcogens at 1 and 10 μM had no significant effect on the [^{3}H]glutamate uptake in human platelets. In this study, platelets were demonstrated to be a suitable model for neurotoxicological research, and, to the best of our knowledge, this is the first report documenting the toxic effects of organochalcogens in human platelets.

KEY WORDS: Glutamate; neurotoxicity; organoselenium; organotellurium; platelets.

INTRODUCTION

The metalloid elements have interested humans in many ways. In particular, those of the periodic table group 16 (Se and Te) have long histories and many uses. These metalloids are extensively produced and used by industry and agriculture (1).

Selenium was discovered as an element showing great toxicity, and for many years it was treated solely as a toxic element. High doses of selenium produce a toxic syndrome of dermatitis, loose hair, diseased nails, and a peripheral neuropathy (2,3). Despite the fact that selenium is considered to be highly toxic, there is a well-established nutritional requirement for it in animals and humans at low concentrations (4,5). The nutritional requirement for selenium derives from the fact that several enzymes are selenium-dependent and involves the selenium-containing amino acid analog of cysteine, selenocysteine (6,7). Studies have focused on not only the relevant biological role selenium plays in metabolism, but also its potential role in low concentrations as an antioxidant (8) and in high concentrations as an anticancer agent (9). The use of selenium in these ways has been popularized by its availability either as a nutritional supplement or in combination with antioxidant vitamins.

The biological functions of tellurium, if any, are unknown, despite the relatively large amounts found in the human body. One suggestion is that tellurium may act as a metabolic antagonist to selenium (10). Tellurium has been described as a toxic element. In fact, long-term treatment of adult rats with elemental tellurium has been reported to induce cerebral lipofuscinosis (11). Inorganic tellurium compounds are highly toxic to the CNS of rodents (12). As well, they disrupt cholesterol synthesis in Schwann cells through the inhibition of squalene monooxygenase (13).

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Besides, seleno- and telluro- organic compounds affect a number of neuronal processes. The exposure to high doses of diphenyl ditelluride and diphenyl diselenide causes CNS effects in mice (14–16) and modifies the functionality of the glutamatergic system *in vitro* and *in vivo* (17,18).

Diphenyl diselenide and diphenyl ditelluride are simple synthetic intermediaries useful in organic synthesis, as opposite to Ebselen, a complex organoselenium compound. They exhibit thiol peroxidase-like activity (19,20) and due to this and other properties, a variety of selenide and telluride analogs have been considered for potential use as pharmaceutical drugs (21,22).

Although organochalcogen compounds have been proposed as potential neuroprotective drugs (23), they can also lead to neurotoxic effects (24–26). Therefore, it is important to evaluate the potential toxic effects of these compounds using platelets as a model of the synaptic apparatus.

Blood platelets have repeatedly been suggested as an excellent model for various aspects of the synaptic apparatus (27). Platelets express a variety of cell surface receptors, many of which are similar in both structure and effector coupling to their neuronal counterparts (28). The similarities between platelet and neuron may even be clinically important, as a number of biochemical markers show parallel changes in the CNS and platelets during disease states (29,30).

Considering that organochalcogens affect some parameters of glutamatergic neurotransmission in rats, in the current study we evaluated the effect of diphenyl diselenide, diphenyl ditelluride, and Ebselen on glutamatergic neurotransmission on human platelets. Even though there have been significant researches on Se and Te toxicity in animals, the mode of the actions of Se and Te at cellular and molecular levels is not yet fully understood, especially in humans.

EXPERIMENTAL PROCEDURE

Chemicals. [^{3}H]glutamate (49 Ci/mmol) was purchased from Amersham International (Amersham, Bucks, UK). Diphenyl diselenide and diphenyl ditelluride were synthesized as described by Paulmier (31). Ebselen was synthesized according to Engman (32). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that all the compounds obtained presented analytical and spectroscopic data in full agreement with their assigned structures. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Blood Samples. The human blood was obtained from the healthy volunteer donors of the Banco de Sangue, Hospital da Universidade Federal de Santa Maria, Santa Maria, RS, Brazil.

Blood was collected from a forearm vein into a tube containing citrate/phosphate/dextran-adenine (CPD-A1). The samples were prepared as described by Mangano and Schwarcz (27) with slight modifications. Platelet-rich plasma was obtained by centrifugation of the tube at 7000 $\times$ *g* for 10 min at 4°C, and the pellets were suspended in ice-cold 0.32 M sucrose.

[^{3}H]Glutamate Binding Assay. Platelets were incubated in 0.5 ml reaction mixture containing 50 mmol/l Tris/acetate, pH 7.4, and 40 nmol/L [^{3}H]glutamate in the presence of organochalcogens (10 and 100 μmol/L). Incubation was carried out at 30°C for 30 min, and the reaction was stopped by centrifugation at 27,000 $\times$ *g* for 15 min. The pellet and the wall of the tubes were quickly and carefully washed with ice-cooled milli-Q water. Sodium dodecyl sulfate (SDS; 0.1%) was added to the dry pellet, and radioactivity incorporated was measured using a liquid scintillation counter. Organochalcogens (diphenyl diselenide, diphenyl ditelluride, and Ebselen) at two concentrations (10 and 100 μmol/L) were added to reaction tubes. Glutamate binding assay was immediately started by adding platelet membranes. A set of experiments using dithiothreitol (DTT) (1 mM) was evaluated by adding DTT before platelets membrane are added to reaction tubes. Nonspecific binding (obtained by incubating membranes with nonradioactive glutamate in concentration 1000 times greater than radioactive glutamate) typically amounted to 10%–15% of total binding. We measured [^{3}H]glutamate binding in the absence of Na^+ in order to determine the binding specifically to glutamatergic receptors without the involvement of binding to glutamate transporters (which is Na^+ dependent).

[^{3}H]Glutamate Uptake. The uptake assay was performed using platelet suspension prepared as described by Mangano & Schwarcz (27). An aliquot of the platelet suspension (50 μl) was preincubated in Tris/citrate (buffered salt solution, composition in mM: NaCl, 112.8; KCl, 4.5; $MgSO_4$, 1.1; KH_2PO_4, 1.1; Tris/Cl, 25; citrate, 11; and glucose, 10.2; pH 6.5) in the presence of organochalcogens (1–100 μmol/L) at 37°C for 15 min. Uptake assay was initiated by addition of 20 μl [^{3}H]glutamate, and incubation continued for 10 min. Uptake was stopped by rapid cooling of the tubes, and the cold suspension was centrifuged at 49,500 $\times$ *g* for 10 min. The platelet pellet was washed with cold incubation medium. SDS (1%) and scintillation liquid were added to the pellet, and radioactivity incorporated was measured. Specific [^{3}H]glutamate uptake was calculated as the difference between the uptake obtained in the incubation medium described above and the uptake obtained with a similar incubation medium in which NaCl was replaced by choline chloride.

Statistical Analysis. Data are expressed as means ± SEM. Statistical analysis was performed using ANOVA followed by the Duncan's test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Figure 1 shows a significant reduction in Na-independent glutamate binding caused by $(PhSe)_2$ (100 μM) and $(PhTe)_2$ (10 and 100 μM). Ebselen did not interfere on [^{3}H]glutamate binding; also, 10 μM $(PhSe)_2$ had no significant effect on [^{3}H]glutamate binding in human platelets (Fig. 1).

Dithiothreitol, a sulfhydryl reagent commonly used as a thiol group protector, was tested for its ability to prevent diphenyl ditelluride–induced inhibition on [^{3}H]glutamate binding in platelets. Although 1 mM DTT did not completely prevent the binding inhibition to

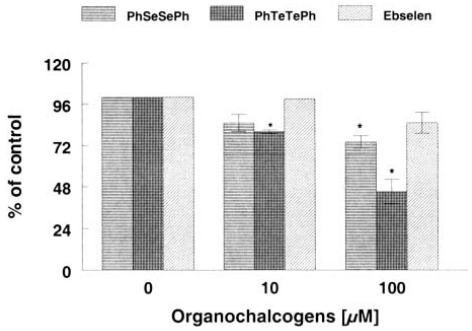

Fig. 1. Effects of $(PhSe)_2$, $(PhTe)_2$, and Ebselen on [^{3}H]glutamate binding. An aliquot of platelet-rich plasma was incubated with 40 nM [^{3}H]glutamate at 30°C/30 min in the presence of $(PhSe)_2$, $(PhTe)_2$, and Ebselen (10 and 100 μM). The control value was 1.3 ± 0.3 pmol/mg protein per 30 min. Data are mean ± SEM (bars) for four separate determinations. (*) Significantly different from control, $P < 0.05$.

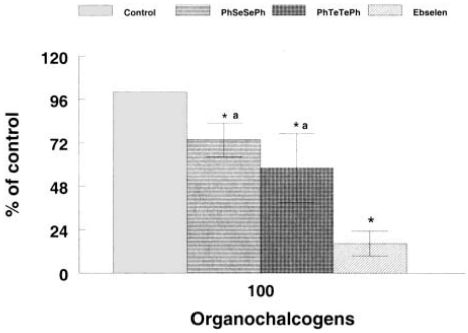

Fig. 3. Effects of $(PhSe)_2$, $(PhTe)_2$, and Ebselen on [^{3}H]glutamate uptake by human platelets. Glutamate uptake is expressed as percentage of control. The control value was 98 ± 6.0 pmol/mg protein per 30 min. Data are mean ± SEM from four independent experiments. (*) Significantly different from control, $P < 0.05$, (*) Significantly different from Ebselen, $P < 0.05$.

control levels, the degree of inhibition in the presence of 1 mM DTT is significantly less than in its absence with 100 μM $(PhTe)_2$ (Fig. 2).

$(PhSe)_2$, $(PhTe)_2$, and Ebselen (100 μM) significantly inhibited [^{3}H]glutamate uptake (Fig. 3), whereas organochalcogens at 1 and 10 μM had no significant effect on the [^{3}H]glutamate uptake in human platelets (data not shown). The inhibitory potency of organochalcogens on [^{3}H]glutamate uptake could be suggested as Ebselen > $(PhTe)_2$ = $(PhSe)_2$.

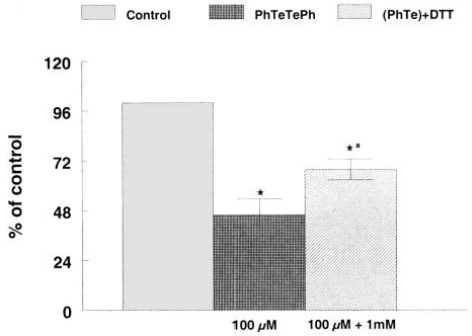

Fig. 2. Effect of dithiotreitol (DTT) on [^{3}H]glutamate binding inhibited by $(PhTe)_2$ on human platelets. An aliquot of platelet-rich plasma was incubated with 40 nM [^{3}H]glutamate at 30°C for 30 min in the presence of 100 μM $(PhTe)_2$ and 1 mM DTT, simultaneously added in the medium assay. The control value was 1.3 ± 0.3 pmol/mg protein per 30 min. Data are the mean ± SEM of five experiments performed in triplicate. (*) Significantly different from control, $P < 0.05$, (*) Significantly different from $(PhTe)_2$, $P < 0.05$.

DISCUSSION

In this study, the neurotoxic effect of organochalcogens in human platelets was tested, and it was found to occur at middle to high concentrations. Human blood platelets have frequently been used as a peripheral model of central neurons in the field of biological psychiatry (29,30). In this study, platelets were demonstrated to be a suitable model for neurotoxicological research, and, to the best of our knowledge, this is the first report documenting toxic effects of organochalcogens in human platelets.

Glutamate is the major excitatory amino acid transmitter in the human central nervous system; it is believed to play important roles in several physiological and pathological processes (33). For its physiological actions, a series of coordinating events at different levels have to function in synchrony, with the objectives of maintaining the glutamate levels at nonpathological concentrations and/or at adequate time in the synaptic cleft. In this way, increasing concentrations of the glutamate in the synaptic cleft may produce neurotoxic effects associated with an overstimulation of the glutamatergic system and has been implicated in neurodegenerative diseases (34).

Diphenyl diselenide and diphenyl ditelluride inhibited Na-independent glutamate binding in human platelets.

In brain synaptosomes, we have previously reported the effects of these compounds on [^{3}H]glutamate binding at similar concentrations as those seen in the current study (17); these results suggest that platelets are as sensitive as brain synaptosomes.

Taking into account that the toxicity of selenium could be related to the oxidation of thiols of biological importance (35,36) and that neuronal effects of diphenyl diselenide and diphenyl ditelluride may occur through a change in the thiol-disulfide balance of glutamatergic receptors, the effect of DTT on [^{3}H]glutamate binding was examined. The lack of a total protective effect of DTT against the inhibitory effect of $(PhTe)_2$ on [^{3}H]glutamate binding in platelets allows us to suggest that the toxicological properties of this compound is not exclusively related to oxidation of –SH groups on glutamate receptors. Alternatively, $(PhTe)_2$ could oxidize some cysteinyl residues located in a hydrophobic site of receptor not accessible to the more hydrophilic DTT. This result is in accordance with our previous results obtained in rat brain synaptic membranes (17).

The removal of glutamate from the synaptic cleft, which occurs primarily by high affinity of sodium-dependent membrane transporters, is the major mechanism for modulating of glutamate actions and to maintain extracellular glutamate concentration below neurotoxic levels (37). Organochalcogens tested inhibited [^{3}H]glutamate uptake in human platelets. This may indicate that organochalcogens have the ability to increase the extracellular glutamate and that glutamatergic system must be involved in the neurotoxicity induced by these compounds. We have demonstrated that diphenyl diselenide induces seizures in mice (16) and that organochalcogens are neurotoxic agents in rats (18,20,38).

Ebselen did not affect [^{3}H]glutamate binding. However, Ebselen inhibited [^{3}H]glutamate uptake in human platelets, which would be undesirable in neurotoxic situations where the compound has recently been used (23). In fact, Ebselen demonstrated the highest inhibitory potency of organochalcogens tested on [^{3}H]glutamate uptake.

In conclusion, our study on the platelet model suggests that diphenyl diselenide, diphenyl ditelluride, and Ebselen could be neurotoxic compounds in humans. Consequently, detailed toxicological studies must be considered before suggesting the pharmacological use of these organochalcogens.

ACKNOWLEDGMENTS

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4.2.2 – Artigo 4

CHANGES ON GLUTAMATERGIC SYSTEM IN HUMAN PLATELETS BY ORGANOCHALCOGENS: EFFECT OF REDUCING AGENTS

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Em fase de redação

Changes on glutamatergic system in human platelets by organochalcogens: Effect of reducing agents

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1. Introduction

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) and is involved in the pathogenesis of acute and chronic neurological disorders, when its extracellular concentration rises to toxic levels (Choi et al., 1992). Excitotoxicity results from overstimulation of glutamate receptors due to high extracellular glutamate concentration resulting from excessive release and/ or inhibition of uptake (Rainesalo et al., 2003). Glutamate uptake is the process responsible for the maintenance of extracellular glutamate concentration below neurotoxic levels. Defects in glutamate uptake were described in autoptic brain samples in neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS) (Bristol et al., 1996; Rothstein et al., 1992) and Alzheimer's disease (Masliah et al., 1996).

Human platelets have been shown to accumulate glutamate in a manner similar to that in as synaptossomal preparations (Mangano and Schwarcz, 1981) and were extensively studied as models of synaptic apparatus. A high affinity glutamate uptake, with kinetic characteristics similar to brain synaptossomes, was also shown in platelets, which appear the major site of clearance of glutamate from blood.

Platelets express both mRNA and protein for the three major glutamate transporters, namely $EAAT_1$, $EAAT_2$, $EAAT_3$ (Zoia et al., 2004) and express serotonin and glutamate receptors (Franconi et al., 1996; Skerry et al., 2001). NMDA receptors were described in platelets (Franconi et al., 2005; Skerry et al., 2001) and in megakaryocytes (Genever et al., 1999; Skerry et al., 2001), demonstrated their importance in regulating platelet activation and aggregation (Zoia et al., 2004).

Organoselenium compounds have been reported to possess chemical an biological antioxidant properties. Several reports heve been published on glutathione

peroxidase (GPx)-mimetic activity of chalcogen compounds, which, like the native enzyme, rely on the redox cycling of selenium or tellurium moiety of the compounds (Parnham and Sies, 2000; Nogueira et al., 2004). Besides, seleno and telluro organic compounds affect a number of neuronal processes. In fact, diphenyl diselenide and diphenyl ditelluride modifies the functionality of the glutamatergic system *in vitro* and *in vivo* (Nogueira et al., 2001; 2003; 2004). In fact, Borges and collaborators (2004) demonstrated that diphenyl diselenide, diphenyl ditelluride and ebselen modulated glutamatergic system in human platelets.

Taking into account that organochalcogens changed glutamatergic system in platelets, we investigated the mechanism involved in toxicity induced by organochalcogens.

2. Materials and Methods

2.1. Chemicals

Diphenyl diselenide, diphenyl ditelluride and Ebselen were synthesized according to literature methods (Paulmier, 1986; Engman, 1989; Petragnani, 1994). These drugs were dissolved in dimethylsulfoxide (DMSO). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that all the compounds obtained presented analytical and spectroscopic data in full agreement with their assigned structures. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Platelet isolation

The blood samples used in glutamate binding assay was obtained from the healthy volunteer donors of the Banco de sangue, Hospital da Universidade Federal de Santa Maria, Santa Maria, RS, Brazil. Blood was collected from a forearm vein into a tube containing citrate/phosphate/dextran-adenine (CPD-A1).

The human blood used in uptake assay was collected by venipuncture in tubes containing K_3 -EDTA solution. The samples were transferred immediately after obtained to polypropylene tubes and blood platelets were isolated as described by Mangano and Schwarcz (1981).

2.3. [^{3}H]-Glutamate uptake by platelets

All uptake assays were performed using platelet suspension prepared as described by Mangano & Schwarcz (1981) and immediately assayed. To determine [^{3}H]-Glutamate uptake by platelets, a 50 μl of the platelet suspension were preincubated with Tris-citrate buffer (NaCl 112.8 mM, KCl 4.5 mM, KH_2PO_4 1.1 mM, Tris/HCl 25 mM, citrate 11 mM and glucose 10.2 mM; pH 6.5) in the presence or absence of diphenyl ditelluride, Ebselen (100 μM) and diphenyl diselenide (200 μM) for 15 min at 37 ^{0}C. Uptake assay was initiated by addition of 20 μl [^{3}H]-glutamate (0.54 nmol [^{3}H]-glutamate with 4.46 μM unlabeled glutamate) and incubation continued for 10 min. Uptake was stopped by rapid cooling of the tubes and the cold suspension was centrifuged at 49.500 x g for 10 min. The platelet pellet was washed with cold incubation medium. Sodium dodecyl sulfate (SDS 1%) and scintillation liquid were added to the pellet and radioactivity was measured. Specific [^{3}H] glutamate

uptake was calculated as the difference between the uptake obtained in the incubation medium described above and the uptake obtained with a similar incubation medium in which NaCl was replaced by choline chloride. To investigate a possible involvement of thiol groups in the inhibitory action of organochalcogens, a set of experiments using dithiotreitol (DTT) (3 mM) and glutathione reduced (GSH) (3 mM) was evaluated by adding these compounds before or after addition of [^{3}H]-glutamate .

2.4. [^{3}H] Glutamate binding assay

All binding assays were performed using platelet membranes prepared as described by Jones and Matus (1974) and stored at – 70 °C for up to 2 months. On the day of the binding assay, the membranes were rapidly thawed in a water bath (37 °C), homogenized with 3 volumes of 10 mM Tris/Acetate, pH 7.4, and centrifuged 3 times 27.000 x g for 15 min. The final pellet was resuspended in the same buffer and was used for the binding assay. Platelets were incubated in 0.5 mL reaction mixture containing 50 mM Tris/acetate, pH 7.4, and 40 nM [^{3}H]-glutamate in the presence or the absence of of diphenyl diselenide and Ebselen (200 μM). Incubation was carried out at 30 °C for 30 min, and the reaction was stopped by rapid cooling of the tubes and the cold suspension was centrifuged at 27.000 x g for 15 min. The platelet pellet was washed with ice-cooled milli-Q water. Sodium dodecyl sulfate (SDS) (0.1%) and scintillation liquid were added to the pellet and radioactivity was measured. Non-specific binding (obtained by incubating membranes with nonradioactive glutamate in concentration 1000 times greater than radioactive glutamate) typically amounted to 10-20% of total binding.

To investigate a possible involvement of thiol groups in the inhibitory action of diphenyl diselenide and Ebselen, a set of experiments using dithiotreitol (DTT- 1 mM) and glutathione reduced (GSH- 1 mM) was evaluated by adding these compounds before or after addition of [^{3}H]glutamate.

2.5. Protein Quantification

Protein concentration was measured by the method of Lowry et al. (32), using serum albumin as the standard.

2.6. Statistical Analysis

Statistical Analysis was performed using ANOVA followed by the Duncan's test. Values of $P < 0.05$ were considered statistically significant.

3. Results

Diphenyl ditelluride, Ebselen (100 μM) and diphenyl diselenide (200 μM) significantly inhibited [^{3}H]-glutamate uptake in human platelets, about 45%, 72% and 51% ,respectively (Figures 1, 2 and 3)..

Dithiotreitol, a sulfhydryl reagent commonly used as a thiol group protector, did not prevent the inhibition caused by diphenyl ditelluride (Figure 1). However, the inhibition caused by Ebselen and diphenyl diselenide was completely prevented by addition of DTT (Figure 2 and 3).

A physiological thiol-containing peptide, GSH, did not protect [^{3}H]-glutamate uptake against the inhibitory effect of diphenyl ditelluride, diphenyl diselenide and Ebselen (data not shown).

The effect of DTT and GSH in restoring [^{3}H] glutamate uptake inhibition caused by organochalcogens was evaluated. DTT and GSH did not recover organochalcogen-induced uptake inhibition (data not shown).

At 200 μM, ebselen and diphenyl diselenide significantly inhibited [^{3}H]-glutamate binding in human platelets, about 95% and 60%, respectively. DTT did not modify organochalcogens effect on [^{3}H]-glutamate binding (Figure 4).

Regarding the experiments aiming to restore organochalcogens effects on [^{3}H]-glutamate binding, similar results to those obtained on protecting experiments were observed (data not shown).

GSH did not modify organochalcogens effect on [^{3}H]-glutamate binding (data not shown). However, this reducing agent caused a significant inhibition on [^{3}H]-glutamate binding (about of 75 %) (data not shown).

4. Discussion

Acknowledgements

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Legends

Figure 1. Effect of dithiotreitol on [^{3}H] glutamate uptake inhibited by diphenyl ditelluride in human platelets. Data are the mean ± SD of three separate determinations performed in triplicate using platelets from different individuals. (*) Significantly different from control, $P < 0.05$.

Figure 2. Effect of dithiotreitol on [^{3}H] glutamate uptake inhibited by diphenyl diselenide in human platelets. Data are the mean ± SD of three separate determinations performed in triplicate using platelets from different individuals. (*) Significantly different from control, $P < 0.05$.

Figure 3. Effect of dithiotreitol on [^{3}H] glutamate uptake inhibited by Ebselen in human platelets. Data are the mean ± SD of three separate determinations performed in triplicate using platelets from different individuals. (*) Significantly different from control, $P < 0.05$.

Figure 4. Effect of dithiotreitol on [^{3}H]-glutamate binding inhibited by Ebselen and diphenyl diselenide in human platelets. An aliquot of membrane was incubated with 40 nm [^{3}H]glutamate at 30°C for 30 min in the presence or the absence of organochalcogens (200 μM) simultaneously with reducing agent (DTT- 1 mM). Data are the mean ± SD of three separate determinations performed in triplicate using platelets from different individuals. (*) Significantly different from control, $P < 0.05$.

Figures

Figure 1

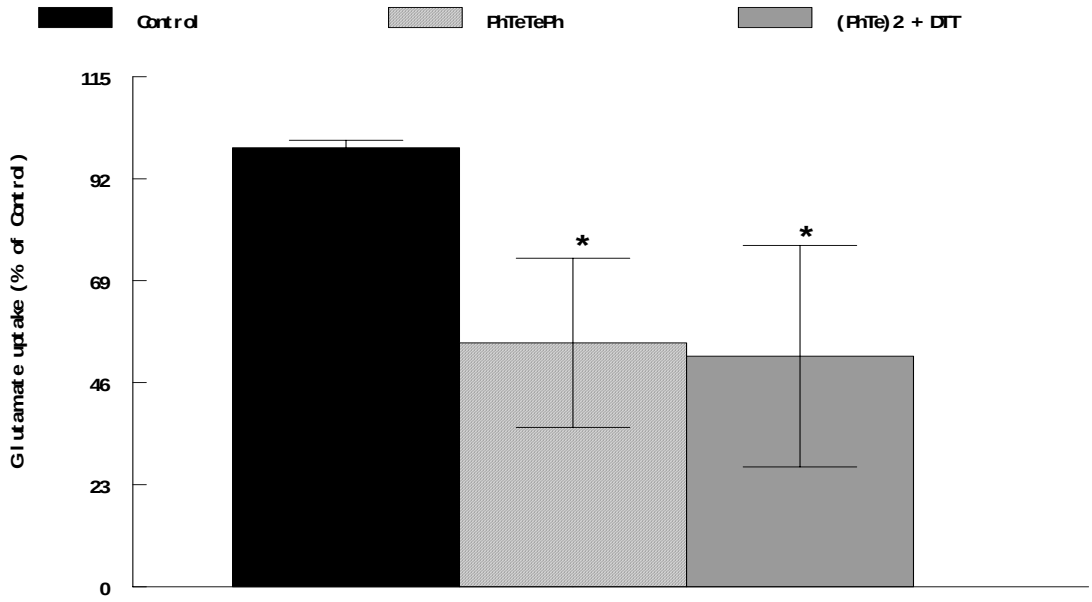


Figure 2

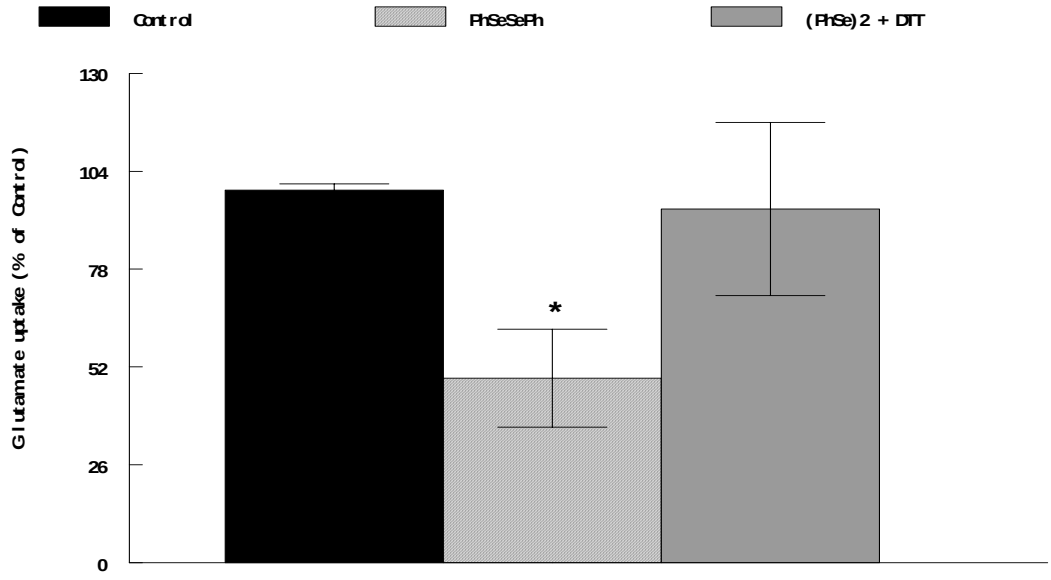

Control
PhSeSePh
(PhSe)2 + DTT
Glutamate uptake (% of Control)
130
104
78
52
26
0
*

Figure 3

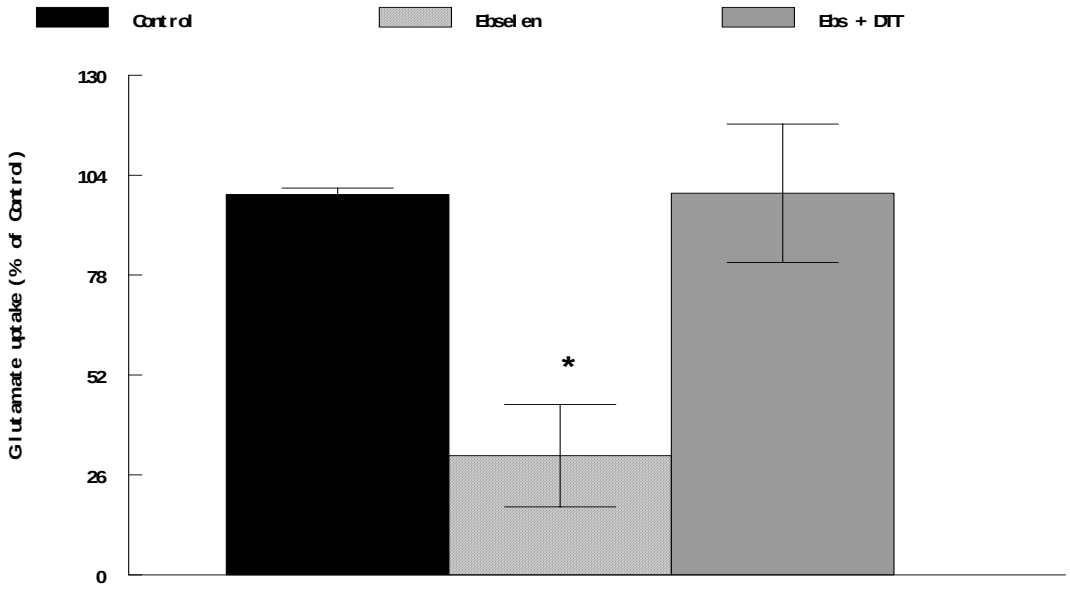
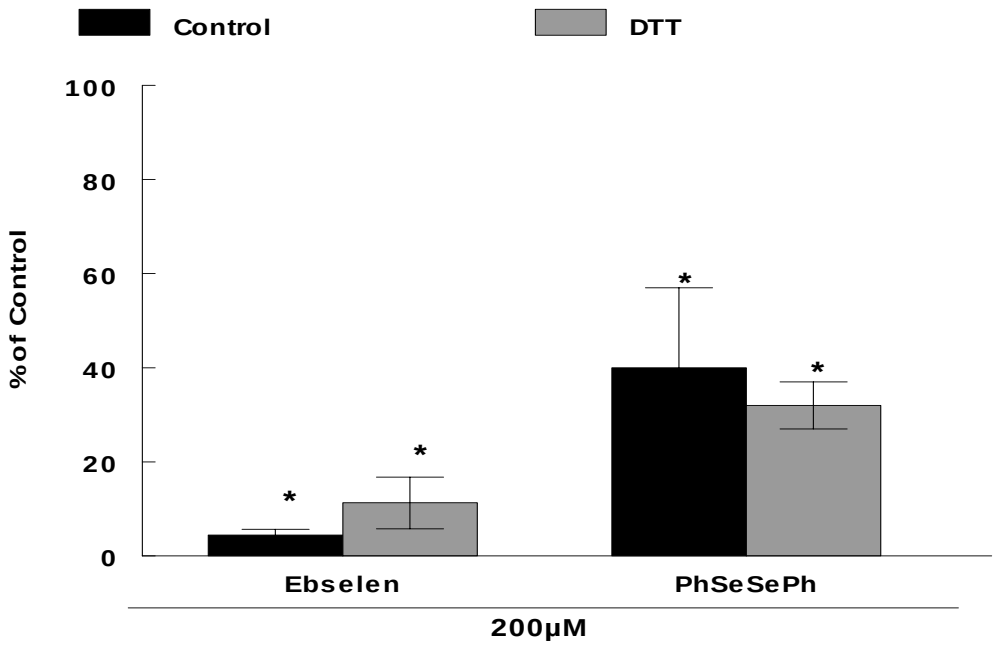

Control
Ebselen
Ebs + DTT
Glutamate uptake (% of Contrd)
130
104
78
52
26
0
*

Figure 4


Control
DTT
100
80
60
40
20
0
%of Control
*
*
*
*
Ebselen
PhSeSePh
200μM

5- DISCUSSÃO

O uso de biomarcadores periféricos para investigação de alterações que ocorrem na função sináptica central está sendo bastante utilizado, principalmente em doenças neurodegenerativas (Ferrarese et al., 1999, 2000, 2001; Nascimento et al., 2006). De fato, foi demonstrado que as plaquetas são um excelente modelo do aparato sináptico (de Gaetano & Garattini, 1978), e ainda apresentam vantagens por serem facilmente obtidas de forma pouca invasiva. Entretanto, existem poucos relatos referentes à utilização desses marcadores em casos de exposição a agentes tóxicos. Devido a isso, torna-se importante o estudo com biomarcadores periféricos que possam estar alterados precocemente frente à exposição a agentes tóxicos.

Baseando-se nestas perspectivas, um dos nossos objetivos foi avaliar o efeito dos metais pesados sobre o sistema glutamatérgico em plaquetas. Os resultados obtidos no ARTIGO 1 indicam que os metais pesados (Hg^{2+}, Cd^{2+} e Pb^{2+}) inibem a captação de glutamato em plaquetas e que o Hg^{2+} demonstrou ser mais inibitório que os outros metais testados. De fato, vários trabalhos demonstram que este metal inibe a captação de glutamato em cérebro de ratos (Moretto et al., 2001, 2005; Aschner et al., 1990). É importante salientar que as concentrações de Hg^{2+} que inibiram a captação de glutamato em plaquetas foram menores que as utilizadas em experimentos realizados com fatias de córtex de ratos (Moretto et al., 2005), demonstrando que as plaquetas poderiam servir como um marcador precoce dos efeitos neurotóxicos causados pelo mercúrio. Outros autores demonstraram que formas orgânicas de mercúrio inibem a atividade da enzima monoamino oxidase em diferentes regiões do cérebro de ratos e que, resultados similares foram encontrados utilizando as plaquetas desses animais (Chakrabarti et al., 1998), confirmando a validade deste modelo experimental.

O Hg^{2+} também inibiu a união específica de glutamato em plaquetas. Resultados similares foram obtidos em trabalhos utilizando membrana sináptica de ratos (Soares et al., 2003). Entretanto, o Cd^{2+} e o Pb^{2+} estimularam a união específica de glutamato. Esses resultados contrastam com os dados obtidos em experimentos utilizando membranas sinápticas de ratos (Soares et al., 2003). De fato, esses resultados foram surpreendentes, uma vez que esses metais são cátions divalentes e apresentam algumas propriedades similares às apresentadas pelo Hg^{2+}. Portanto,

seria esperado que esses metais inibissem a união específica de glutamato em plaquetas.

Os mecanismos pelos quais os metais pesados desencadeiam ações neurotóxicas não estão totalmente bem descritos. Entretanto, sabe-se que esses metais podem induzir efeitos tóxicos por causarem um aumento no estresse oxidativo e conseqüente, dano celular. Devido a isso, investigou-se o envolvimento do estresse oxidativo na modulação do sistema glutamatérgico induzido pelos metais pesados. O Hg^{2+}, o Cd^{2+} e o Pb^{2+} causaram um aumento nos níveis de espécies reativas de oxigênio, sugerindo que o estresse oxidativo, pelo menos em parte, pode estar envolvido no efeito induzido por estes metais sobre o sistema glutamatérgico em plaquetas. Esses resultados corroboram com os dados obtidos no Anexo do ARTIGO 2, no qual foi demonstrado que a adição de N-acetilcisteína (NAC), um antioxidante bastante utilizado em condições de estresse oxidativo (Aruoma et al., 1989), foi capaz de proteger do efeito causado pelo Hg^{2+} sobre a união específica de glutamato em plaquetas.

Tendo em vista que os metais reagem com grupos –SH, os quais são importantes para manter a função de muitas enzimas, proteínas e receptores (Aposhian et al., 1990; Erskine et al., 2000), o segundo objetivo desta tese foi avaliar se o efeito dos metais pesados sobre a união específica de glutamato estaria relacionado com a oxidação de grupos –SH presentes nos receptores glutamatérgicos. De particular importância, os receptores glutamatérgicos do tipo NMDA, uma vez que estes receptores foram descritos em plaquetas (Franconi et al., 1996; Skerry et al., 2001), e são altamente sensíveis a agentes redutores ou oxidantes (Dingledine et al., 1999).

Segundo os resultados obtidos neste estudo (ARTIGO 2), o efeito causado pelos metais pesados sobre a união específica de glutamato em plaquetas foi protegida ou revertida pela adição dos agentes redutores ditiólicos (BAL, DMPS e DTT), sugerindo que esse agentes podem estar atuando de duas formas; como quelantes, já que todos eles apresentam 2 grupos –SH vicinais em sua estrutura, o que parece ser essencial para a eficácia do agente quelante (Muckter et al., 1997) ou como agente redutor de grupos –SH presentes na estrutura dos receptores glutamatérgicos. O uso de um agente redutor monotiólico, o GSH, não foi capaz de proteger dos efeitos causados pelos metais pesados sobre a união específica de glutamato. De fato, Albrecht e colaboradores (1993) demonstraram que a inibição causada por

compostos de mercúrio sobre o sistema glutamatérgico, em astrócitos de ratos, também não foi protegida pela adição de GSH.

Os agentes redutores ditiólicos (BAL, DMPS e DTT) não alteraram a união específica de glutamato em plaquetas. Em contraste, dados da literatura demonstraram que o BAL, um agente quelante utilizado em casos de intoxicações por metais pesados, inibe a união específica de glutamato em membranas sinápticas, inibe a captação de glutamato e aumenta a liberação de glutamato em sinaptossomas de ratos (Nogueira et al., 2001 a, b). Estes resultados sugerem que o cérebro é mais suscetível aos efeitos tóxicos causados pelo BAL do que as plaquetas.

Os resultados obtidos com o DMPS em plaquetas foram de acordo com o que foi demonstrado em SNC, já que alguns estudos demonstraram que esse composto não afeta a neurotransmissão glutamatérgica em cérebro de ratos (Nogueira et al., 2001 a, b; Soares et al., 2003).

Outro resultado interessante deste estudo, é que a GSH inibiu a união específica de glutamato em plaquetas. Duas hipóteses podem ser postuladas para explicar esses resultados. A primeira hipótese é que a GSH poderia deslocar o glutamato de sítios específicos dos receptores glutamatérgicos, através do resíduo γ-glutamil. De acordo com isso, Janáky e colaboradores (2000) demonstraram que o glutamato é deslocado por GSH em membranas sinápticas de córtex de porcos. Além disso, outros autores reportaram que ambas formas de GSH (oxidada e reduzida) inibem a união específica de glutamato em membranas sinápticas cerebrais de ratos (Oja et al., 1988). A segunda hipótese para explicar essa inibição, poderia ser por um aumento na geração de espécies reativas de oxigênio (EROS) nas plaquetas. Esta evidência é parcialmente sustentada por estudos que demonstraram que o BSO (sulfoxamina de L-butionina), um bloqueador da síntese de GSH, reduz a quantidade de EROS nas plaquetas durante a sua ativação (Wachowicz et al., 2002).

Paralelo aos estudos com os metais pesados investigou-se o efeito dos compostos orgânicos de selênio (disseleneto de difenila e ebselen) e de telúrio sobre o sistema glutamatérgico em plaquetas. Estes compostos são reagentes muito utilizados em laboratórios de química como intermediários em reações de síntese orgânica (Paulmier, 1986; Braga et al., 1996; 1997) e em virtude da descoberta de suas propriedades biológicas (Parnham & Graf, 1991; Kanda et al., 1999; Nogueira et al., 2004), têm sido alvo de estudos em laboratórios de farmacologia.

Os mecanismos pelos quais os organocalcogênios desencadeiam ações tóxicas não estão bem descritos. Entretanto, existem várias evidências que demonstram que estes compostos podem oxidar grupos –SH de moléculas biologicamente ativas (Painter, 1941; Tsen & Tappel,1958; Ganther, 1971). Portanto, poderiam afetar o sistema glutamatérgico por atuar em receptores e transportadores neuronais desencadeando efeitos neurotóxicos.

Os resultados obtidos no ARTIGO 3, indicam que o disseleneto de difenila e o ditelureto de difenila inibem a união específica de glutamato em plaquetas. O efeito causado pelo ditelureto de difenila foi maior que o causado pelo disseleneto de difenila. De fato, os compostos orgânicos de telúrio são mais reativos que os compostos organoselênio, devido a sua maior eletronegatividade em relação ao átomo de carbono, associado com um maior volume atômico (Comasseto et al., 1997). Resultados similares aos encontrados em plaquetas foram encontrados em experimentos utilizando membranas sinápticas de cérebro de ratos (Nogueira et al., 2001c).

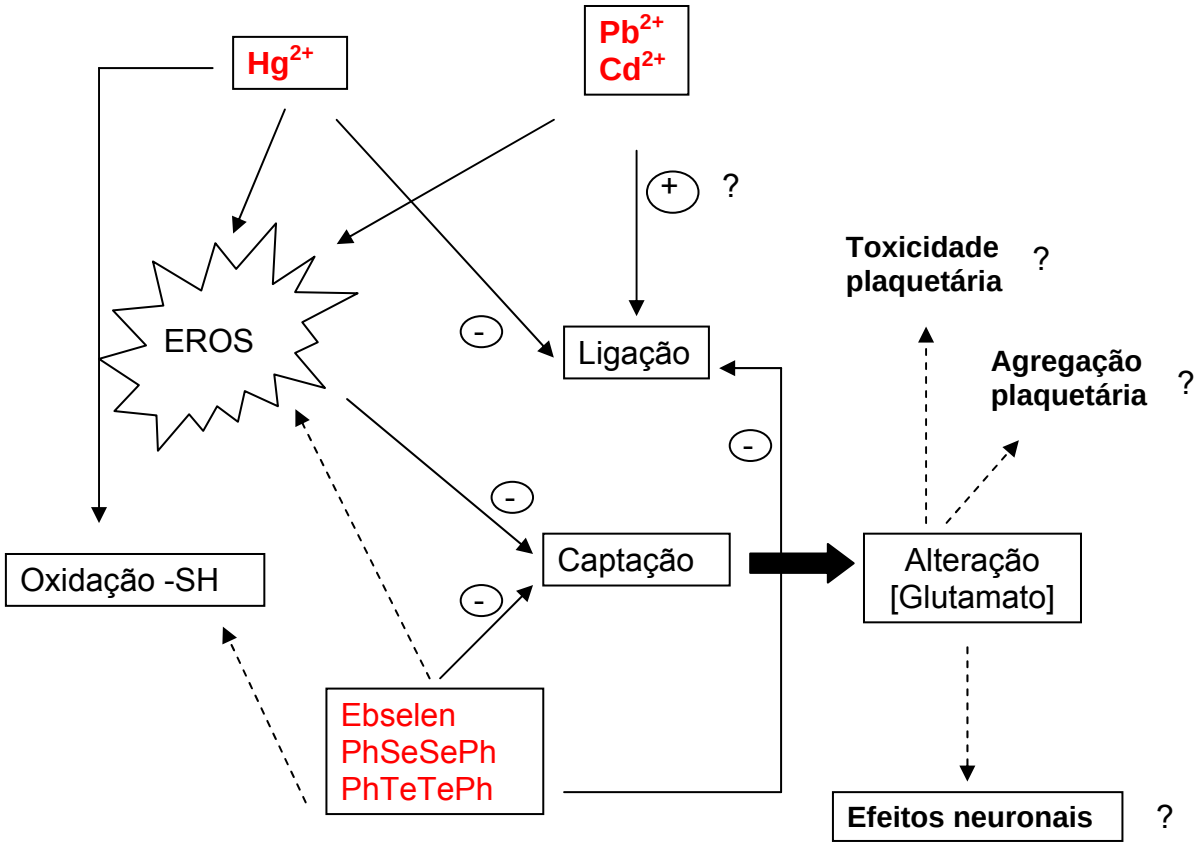
O efeito inibitório causado pelos organocalcogênios na união específica de glutamato não foi revertida pela adição de DTT ou GSH (ARTIGO 4). Da mesma forma, o DTT não foi capaz de proteger da inibição causada por estes compostos em preparações utilizando cérebro de ratos (Nogueira et al., 2001c). A ausência de efeito protetor dos agentes redutores, sugere que as propriedades toxicológicas dos organocalcogênios sobre o sistema glutamatérgico não está exclusivamente relacionada à oxidaçào de grupos –SH presentes nos receptores glutamatérgicos. Uma explicação para este fato, é que os organocalcogênios podem oxidar resíduos cisteinil localizados em sítios hidrofóbicos do receptor, não acessíveis para os agentes redutores utilizados.

A captação de glutamato pelos transportadores (EAAT) é o mais importante mecanismo para controlar os níveis de glutamato, uma vez que o acúmulo deste neurotransmissor no espaço extracelular pode ser excitotóxico aos neurônios. Alterações destes transportadores induzem uma cascata de eventos, os quais podem levar a uma disfunção e conseqüente, morte celular. O disseleneto de difenila, o ditelureto de difenila e o ebselen inibiram a captação de glutamato em plaquetas (ARTIGO 3). O ebselen foi mais inibitório que os outros organocalcogênios testados sob este parâmetro de avaliação do sistema glutamatérgico. Estes resultados também foram surpreendentes, uma vez que os compostos orgânicos de telúrio são

mais reativos que os de selênio (Comasseto et al., 1997). Entretanto, é importante salientar que o ebselen também demonstrou ser mais inibitório que o ditelureto de difenila em sinaptossomas de ratos (Nogueira et al., 2002), sugerindo que o sistema glutamatérgico de plaquetas pode responder de forma similar ao do SNC. Além de atuar na captação, outros eventos podem ser modulados pelos organocalcogênios. De fato, Nogueira e colaboradores (2002) também demonstraram que os organocalcogênios inibem a captação vesicular de glutamato em cérebro de ratos.

Considerando os resultados obtidos neste trabalho, nós podemos sugerir que as plaquetas podem servir como marcadores periféricos em casos de exposição por agentes tóxicos e, ainda representam um bom modelo para investigar os mecanismos envolvidos nas alterações causadas por estes compostos.

Esquema 1- Visão geral dos efeitos causados pelos metais pesados e organocalcogênios, bem como suas interações estudadas neste trabalho.



As linhas cheias (–) indicam os efeitos demonstrados neste trabalho.

As linhas pontilhadas (---) indicam possíveis efeitos baseados em estudos da literatura.

6- CONCLUSÕES

De acordo com os resultados apresentados nesta tese podemos inferir o que segue:

- Os metais pesados Hg^{2+}, Cd^{2+} e Pb^{2+} inibiram a captação de [^{3}H]-glutamato em plaquetas *in vitro*. Investigando o possível mecanismo de inibição causada por estes compostos, verificou-se que o estresse oxidativo poderia estar envolvido, em maior ou em menor grau na inibição, dependendo do composto em questão.
- Entre os metais testados, o Hg^{2+} apresentou maiores efeitos tóxicos sobre o sistema glutamatérgico, considerando que em baixas concentrações este metal inibe a captação e a união específica de [^{3}H]-glutamato.
- Os agentes redutores sulfidrílicos (BAL, DMPS e DTT) foram efetivos em proteger do efeito causado pelos metais pesados sobre a união específica de [^{3}H]-glutamato em plaquetas.
- O agente redutor monotiol, GSH, é capaz de modular a união específica de [^{3}H]-glutamato em plaquetas.
- O disseleneto de difenila, ditelureto de difenila e ebselen modulam o sistema glutamatérgico em plaquetas.
- O mecanismo de ação tóxica dos organocalcogênios sobre o sistema glutamatérgico, não está exclusivamente relacionado à oxidação de grupos –SH dos transportadores e receptores glutamatérgicos.
- As plaquetas podem servir como marcadores periféricos em casos de exposição por agentes tóxicos e ainda, representam um bom modelo para investigar os mecanismos envolvidos nas alterações causadas por estes compostos.

7- PERSPECTIVAS

Tendo em vista os bons resultados obtidos com a utilização de um modelo periférico para avaliar a neurotrasmissão glutamatérgica, poderíamos aprofundar ainda mais estes estudos em relação ao mecanismo de ação dos compostos testados. Ainda, avaliar outros sistemas que possam ser afetados por esses compostos utilizando como modelo as plaquetas. Dessa forma, poderíamos realizar este estudo a partir da concretização dos seguintes objetivos:

- Identificar os possíveis mecanismos envolvidos no efeito destes compostos sobre o sistema glutamatérgico em plaquetas.
- Avaliar o efeito de metais pesados e organocalcogênios sobre a captação de serotonina em plaquetas de humanos.
- Investigar o efeito de metais pesados e organocalcogênios sobre a captação de serotonina em plaquetas de ratos expostos a estes compostos.
- Estudar o efeito destes compostos sobre a atividade da enzima monoamina oxidase em plaquetas.

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9- ANEXO

9.1 – Artigo 5

Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na^+, K^+-ATPase activity in rats

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Effect of diphenyl diselenide, diphenyl ditelluride and ebselen on cerebral Na^+, K^+-ATPase activity in rats

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Abstract

In the present study, we investigated the in vitro effect of diphenyl ditelluride, diphenyl diselenide and ebselen on Na^+, K^+-ATPase activity of rat brain. The results demonstrated that all compounds significantly inhibited (in the μM range) Na^+, K^+-ATPase activity. Diphenyl ditelluride, at low concentrations, provoked an increase in Na^+, K^+-ATPase activity. Dithiothreitol (DTT), at 3 mM, protected the inhibition caused by diphenyl ditelluride, diphenyl diselenide and ebselen in Na^+, K^+-ATPase activity. Post-incubation of diphenyl diselenide-treated homogenate with DTT completely recovered enzyme activity. DTT was able to recover the enzyme inhibition induced by 20 μM of diphenyl ditelluride, but was partially able to recover inhibition induced by high concentrations of organotellurium compound. Conversely, DTT did not recover ebselen-induced Na^+, K^+-ATPase inhibition. The mechanism of inhibition by diphenyl diselenide, diphenyl ditelluride and ebselen in Na^+, K^+-ATPase activity revealed: decreased maximal velocity and K_m. Cerebral Na^+, K^+-ATPase is a potential molecular target for the toxic effect of organochalcogens and the inhibition may occur through a change in the crucial thiol groups of this enzyme.

Keywords: Na^+, K^+-ATPase; Organoselenium; Organotellurium; Ebselen; Toxicity

1. Introduction

Selenium, as a trace element, is essential for normal growth and development of the organism. The nutritional need for trace amounts of selenium has been recognized for many years (Schwartz and Foltz, 1957); however, its antioxidant function was unknown until Rotruck et al., 1973 discovered that selenium is an integral component of the active site of the mammalian enzyme glutathione peroxidase (GSH-Px).

Elemental tellurium is a rare trace element, which is used as an industrial component of many alloys, in the rubber compounding, in microchip industry and eletronics and in photovoltaic energy systems. It is also used in glass and steel production and as a gasoline antiknock aditive (Fairhill, 1969).

Organochalcogen compounds have been described to possess very interesting biological activities. Several reports have been published on glutathione peroxidase (GSH-px)-mimetic activity of chalcogen compounds, which, like the native enzyme, rely on the redox cycling of selenium or tellurium moiety of the compounds (Parnham and Sies, 2000; Klotz and Sies, 2003; Nogueira et al., 2004).

In fact, ebselen (a mimetic of GPx) has been demonstrated to exert a protective role against brain ischemia and stroke (Daiber et al., 2000; Muller et al., 1984;

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Yamaguchi et al., 1998) and, in experimental models, against glutamate excitotoxicity (Porciúncula et al., 2001; Rossato et al., 2002a,b). Accordingly, we have recently demonstrated that diphenyl diselenide, a simple organochalcogenide, possesses neuroprotective and anti-inflammatory activities (Ghisleni et al., 2003; Nogueira et al., 2003b, 2004). Contrasting with these selenium containing compounds, diphenyl ditelluride, an analogous molecule of diphenyl diselenide, was extremely toxic to rodents and caused marked neurotoxic effects in mice after acute or prolonged exposure (Maciel et al., 2000; Nogueira et al., 2001, 2003a; Meotti et al., 2003).

Although the specific molecular targets that mediate organochalcogens toxicity are not known (Nogueira et al., 2004), organoselenium and organotellurium compounds can interact directly with low molecular thiols, oxidizing them to disulfides (Goeger and Ganther, 1994). In fact, reduced cysteinyl residues from proteins can also react with these compounds, which may cause, in the case of the enzymes, the loss of their catalytic activity (Björnstedt et al., 1996; Park et al., 2000; Gupta and Porter, 2001; Nogueira et al., 2003c).

Therefore, Na^+, K^+-ATPase (EC 3.6.1.37), a sulfhydryl-containing enzyme, could be sensitive to oxidizing agents (Carfagna et al., 1996; Folmer et al., 2004). Na^+, K^+-ATPase is an enzyme embedded in the cell membrane and responsible for the active transport of sodium and potassium ions in the nervous system. This process regulates the cellular Na^+/K^+ concentrations and hence their gradients across the plasma membrane, which are required for vital functions such as membrane co-transports, cell volume regulation and membrane excitability (Doucet, 1988; Jorgensen, 1986). This dimeric enzyme exists in several isoforms in brain and consumes the greater part of available ATP (Bertorello and Kats, 1995). The inactivation of Na^+, K^+-ATPase leads to partial membrane depolarization allowing excessive Ca^{2+} entry inside neurons with resultant toxic events like excitotoxicity (Beal et al., 1993).

Several experiments have shown the effects of metals such as Ba^{2+}, Hg^{2+}, Cd^{2+} and Al^{3+} on the Na^+, K^+-ATPase activity since the enzyme discovery (Lai et al., 1980; Somló and Hassón-Voloch, 1987; Anner and Moosmayer, 1992; Pedrenho et al., 1996).

Based on the considerations above, the present study investigated the effects of diphenyl ditelluride, diphenyl diselenide and ebselen on Na^+, K^+-ATPase activity in rat brain. We also study the kinectics of the inhibition caused by organochalcogens in this enzyme activity. The data reported here demonstrated that all compounds tested significantly inhibited Na^+, K^+-ATPase activity. Thus, this study corroborates our continuous efforts in investigating the mechanisms involved in toxicity induced by organochalcogens.

2. Materials and methods

2.1. Chemicals

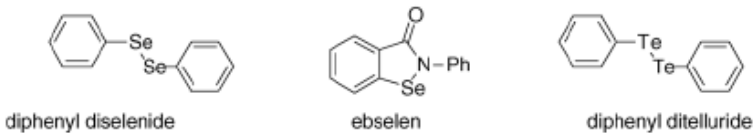
Diphenyl diselenide, diphenyl ditelluride and ebselen (Fig. 1) were synthesized according to literature methods (Paulmier, 1986; Engman, 1989; Petragnani, 1994). These drugs were dissolved in dimethylsulfoxide (DMSO). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that all the compounds obtained presented analytical and spectroscopic data in full agreement with their assigned structures. Ouabain and adenosine tri phosphate (ATP) were obtained from Sigma. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Male adult Wistar rats (200–250 g) from our own breeding colony were used. Animals were kept in separate animal rooms, on a 12-h light:12-h dark cycle, at a room temperature of 22 °C, and with free access to food and water. The animals were used according to the guidelines of the Comite on Care and Use of Experimental Animal Resources, School of Medicine, Veterinary, and Animal Science of the University of Sao Paulo, Brazil.

2.3. Na^+, K^+-ATPase activity

Immediately after the sacrifice, the brain was removed and the homogenate was prepared in 0.05 M Tris–HCl buffer (pH 7.4). The homogenate was centrifuged at 4000 × g at 4 °C for 10 min and supernatant was used for assay of protein Na^+, K^+-ATPase. The



diphenyl diselenide ebselen diphenyl ditelluride

Fig. 1. Structure of diphenyl diselenide, ebselen and diphenyl ditelluride.

reaction mixture for Na^+, K^+-ATPase activity assay contained 3 mM MgCl, 125 mM NaCl, 20 mM KCl and 50 mM Tris–HCl, pH 7.4, in a final volume of 500 μl. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 0.1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow (1925). All the experiments were conducted at least four times and similar results were obtained.

2.4. Effect of dithiotreitol on Na^+, K^+-ATPase inhibition induced by organochalcogens

To investigate a possible involvement of cysteinyl groups in the inhibitory action of diphenyl diselenide, diphenyl ditelluride and ebselen the protective effect of DTT (3 mM) was examined. The homogenate was pre-incubated with DTT and diphenyl diselenide, ebselen (1–100 μM) or diphenyl ditelluride (1–40 μM) at 37 °C for 10 min. After that, the reaction was initiated by addition of ATP (3 mM).

To check whether post-incubation of inhibitor-treated homogenates with DTT will recover Na^+, K^+-ATPase activity, organochalcogens and enzyme (homogenate) were incubated at 37 °C for 10 min, after that ATP and DTT (3 mM) were added to the medium and incubated for 30 min.

2.5. Kinetic determinations

The kinetics of the interaction of organochalcogens and Na^+, K^+-ATPase was determined using the Lineweaver–Burk double reciprocal plot, plotting $1/v$ against $1/s$, analyzed over a range of ATP (0.5–3 mM) in the absence and in the presence of organochalcogens (the concentrations used were: one concentration below the IC_{50} values, approximately the IC_{50} and one concentration above the IC_{50}). K_m and V_{max} values were obtained using $1/v$ versus $1/s$.

2.6. Statistical analysis

The results were expressed as mean ± S.D. for four independent experiments performed in duplicate and were analyzed by ANOVA, followed by Duncan's multiple-range test when appropriate. Differences between groups were considered significant when $p < 0.05$. Pearson's linear regression coupled to ANOVA was used to verify concentration-dependent effects. Values of the correlations coefficients and their corresponding probability value were obtained by the linear regression analysis.

3. Results

Ebselen in concentrations higher than 20 μM significantly inhibited cerebral Na^+, K^+-ATPase activity, the maximal inhibitory effect (98%) was observed at 100 μM (Fig. 2). Regression analysis indicated that ebselen inhibited the enzyme in a concentration-dependent manner ($\beta = -0.69$; $p < 0.05$).

Diphenyl ditelluride at low concentrations (1–4 μM) significantly increased Na^+, K^+-ATPase activity. In contrast, this compound caused inhibition of the enzyme activity at concentrations up to 20 μM (Fig. 3). Regression analysis indicated that diphenyl ditelluride inhib-

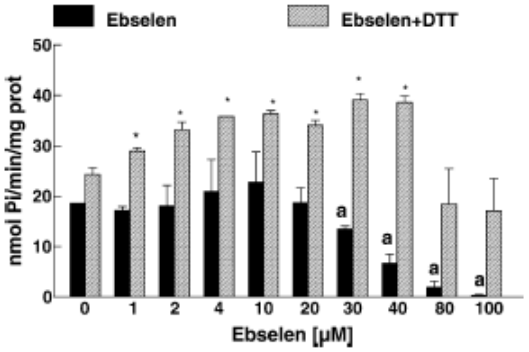


Fig. 2. Effect of ebselen (1–100 μM) on Na^+, K^+-ATPase activity in rat brain. Results are expressed as mean ± S.D. for four independent experiments performed in duplicate; $^a p < 0.05$ compared to the control (ANOVA/Duncan). $^* p < 0.05$ compared to tubes without DTT (dithiotreitol) (ANOVA/Duncan).

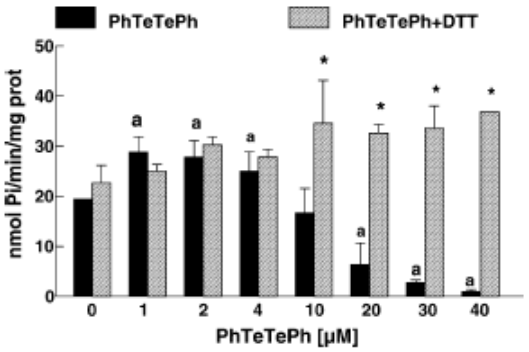


Fig. 3. Effect of diphenyl ditelluride (PhTeTePh) (1–40 μM) on Na^+, K^+-ATPase activity in rat brain. Results are expressed as mean ± S.D. for four independent experiments performed in duplicate; $^a p < 0.05$ compared to the control (ANOVA/Duncan). $^* p < 0.05$ compared to tubes without DTT (dithiotreitol) (ANOVA/Duncan).

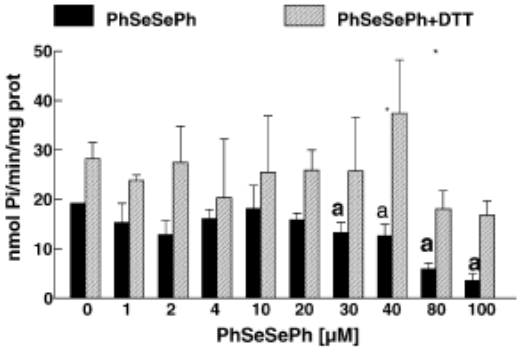


Fig. 4. Effect of diphenyl diselenide (PhSeSePh) (1–100 μM) on Na^+, K^+-ATPase activity in rat brain. Results are expressed as mean ± S.D. for four independent experiments performed in duplicate; $^a p < 0.05$ compared to the control (ANOVA/Duncan). $^* p < 0.05$ compared to tubes without DTT (dithiotreitol) (ANOVA/Duncan).

ited the enzyme in a concentration-dependent manner ($\beta = -0.90$; $p < 0.05$).

Diphenyl diselenide (100 μM) significantly inhibited, about 80%, cerebral Na^+, K^+-ATPase activity. At low concentrations, diphenyl diselenide did not alter enzyme activity (Fig. 4).

Dithiotreitol (3 mM), a sulfhydryl reagent commonly used as a thiol group protector, was able to prevent the inhibition caused by ebselen, diphenyl ditelluride and diphenyl diselenide in Na^+, K^+-ATPase activity (Figs. 2–4). Post-incubation of diphenyl diselenide-treated homogenate with DTT completely recovered Na^+, K^+-ATPase activity (Fig. 5). DTT was able to recover enzyme inhibition induced by 20 μM of diphenyl ditelluride, but was partially able to recover inhibition

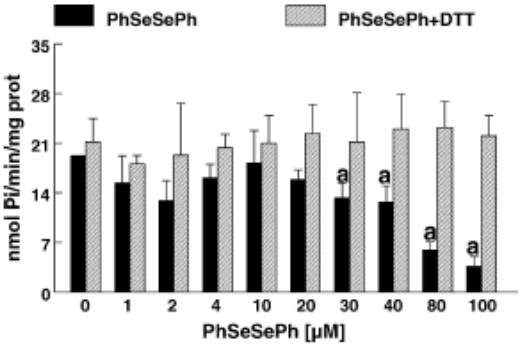


Fig. 5. Effect of DTT in restoring cerebral Na^+, K^+-ATPase inhibition caused by diphenyl diselenide (PhSeSePh) (1–100 μM). The organochalcogens and enzyme (homogenate) were incubated at 37 °C for 10 min, after that ATP and DTT (3 mM) were added to the medium and incubated for 30 min. Results are expressed as mean ± S.D. for four independent experiments performed in duplicate; $^a p < 0.05$ compared to the control (ANOVA/Duncan).

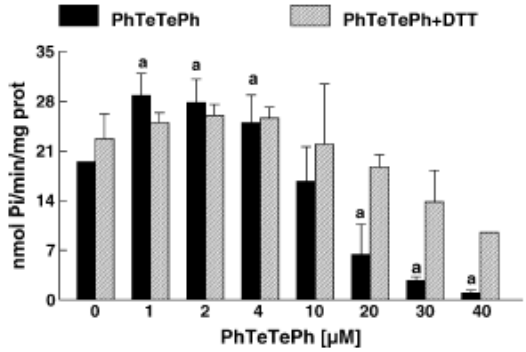


Fig. 6. Effects of DTT in restoring cerebral Na^+, K^+-ATPase inhibition caused by diphenyl ditelluride (PhTeTePh) (1–40 μM). The organochalcogens and enzyme (homogenate) were incubated at 37 °C for 10 min, after that ATP and DTT (3 mM) were added to the medium and incubated for 30 min. Results are expressed as mean ± S.D. for four independent experiments performed in duplicate; $^a p < 0.05$ compared to the control (ANOVA/Duncan).

induced by high concentrations of organotellurium compound (Fig. 6). Conversely, DTT did not recover ebselen-induced Na^+, K^+-ATPase inhibition (Fig. 7).

The Lineweaver–Burk double-reciprocal plot was analyzed over the range of 0.5–3 mM ATP in the absence and presence of organochalcogens. The K_m values and maximal velocity in the absence of organochalcogens was 0.62 mM and 20 nmol Pi released per min per mg protein, respectively. Data indicated that ebselen (25–45 μM), diphenyl ditelluride (5–25 μM) and diphenyl diselenide (60–80 μM) decreased maximal velocity and K_m. K_m value in the presence of Ebselen, diphenyl ditelluride or diphenyl diselenide

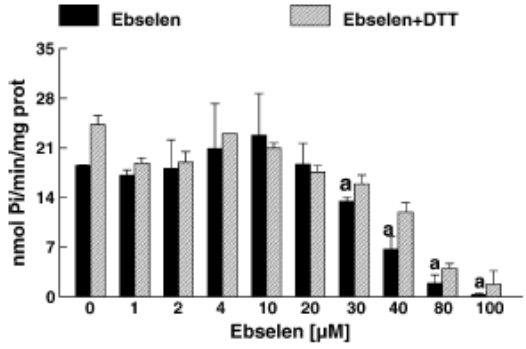


Fig. 7. Effects of DTT in restoring cerebral Na^+, K^+-ATPase inhibition caused by ebselen (1–100 μM). The organochalcogens and enzyme (homogenate) were incubated at 37 °C for 10 min, after that ATP and DTT (3 mM) were added to the medium and incubated for 30 min. Results are expressed as mean ± S.D. for four independent experiments performed in duplicate; $^a p < 0.05$ compared to the control (ANOVA/Duncan).

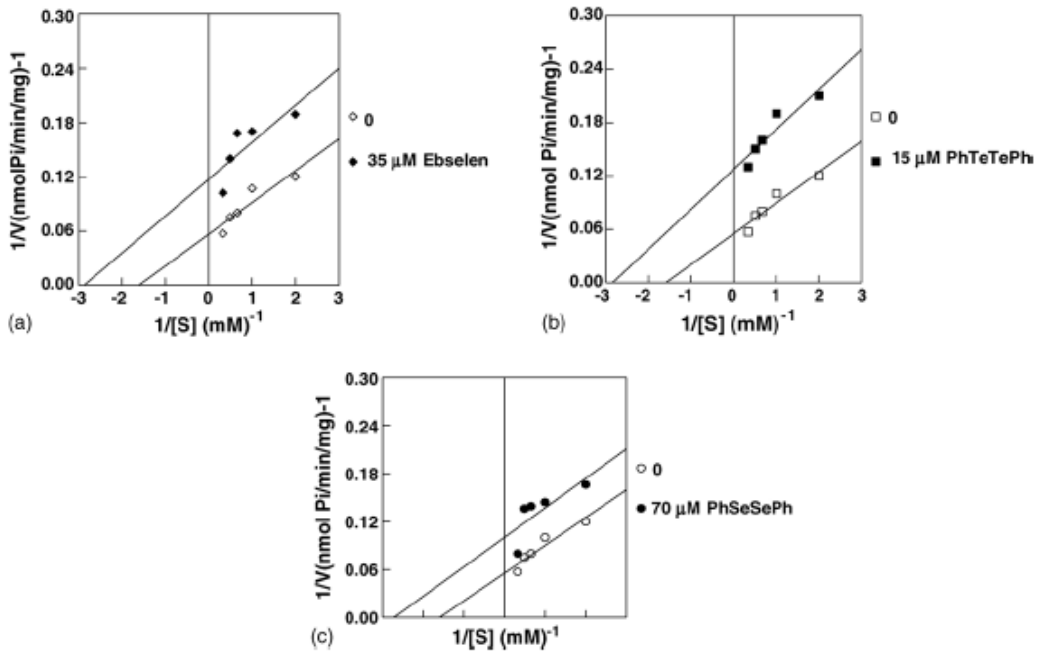


Fig. 8. Kinetic analysis of Na^+, K^+-ATPase activity inhibition caused by organochalcogens in rat brain. The graph represent double-reciprocal plot of Na^+, K^+-ATPase activity for ATP concentrations (0.5–3 mM): in the absence and presence of 35 µM ebselen (a), 15 µM diphenyl ditelluride (PhTeTePh), (b) and 70 µM diphenyl diselenide (PhSeSePh) (c). All the experiments were conducted at least four times and similar results were obtained.

was 0.35 mM. The V_{max} values were 8.3, 7.7 and 9.1 nmol Pi released per min per mg protein for ebselen, diphenyl ditelluride and diphenyl diselenide, respectively (Fig. 8 a–c). The correlation coefficients for ebselen, diphenyl diselenide and diphenyl ditelluride were $r = 0.8014$, ($F(1,8) = 14.363$, $p < 0.00531$); $r = 0.8001$ ($F(1,8) = 14.239$, $p < 0.00544$); $r = 0.9337$ ($F(1,8) = 54.432$, $p < 0.00008$), respectively.

4. Discussion

These results clearly indicate that diphenyl diselenide, diphenyl ditelluride and ebselen inhibit Na^+, K^+-ATPase by interacting with cysteinyl residues that are important for enzyme activity. The importance of thiol groups for Na^+, K^+-ATPase catalysis has been reported. In fact, –SH groups of this enzyme is highly susceptible to oxidative stress (Yufu et al., 1993) and oxidizing agents (Carfagna et al., 1996).

Therefore, organochalcogens can interact directly with low molecular thiols oxidizing them to disulfides (Goeger and Ganther, 1994). Reduced cysteinyl residues from proteins can also react with simple diselenides and ditellurides, wich can cause, in the case of enzymes, the loss of catalytic activity. Besides, organoselenium and organotellurium compounds have been reported to inhibit a number of sulfhydryl dependent enzymes, including 5-lipoxygenase (Björnstedt et al., 1996), protein kinase JNK1 (Park et al., 2000), δ-aminolevulinate dehydratase (Barbosa et al., 1998; Nogueira et al., 2003c) and squalene monooxygenase (Gupta and Porter, 2001; Laden and Porter, 2001).

In this study, dithiotreitol, a sulfhydryl reagent commonly used as a thiol group protector, prevented Na^+, K^+-ATPase activity inhibition induced by organochalcogens. Thus, a co-incubation of organochalcogens with an excess of DTT is most likely to prevent inhibition of ATPase because of direct interaction of DTT with the inhibitor. Similarly, we have reported that δ-aminolevulinate dehydratase of rats and human erythrocytic cells inhibited by organochalcogens were completely protected by DTT (Barbosa et al., 1998; Nogueira et al., 2003c).

Furthermore, DTT indeed recovered Na^+, K^+-ATPase activity when the inhibitor was diphenyl diselenide, partially recovered the activity when the inhibitor was diphenyl ditelluride and did not recover enzyme activity when ebselen was the inhibitor. This different behavior

could be explained by the fact that sulfur-tellurium bound exhibits an easier heterolytic cleavage towards nucleophylic reagents than the sulfur-selenium bonds due to the large volume and the greater ionic character of the tellurium atom and the easy polarization of the bounds (Moro et al., 2005). In addition, it is possible that the DTT merely binds extracellularly to these compounds and that there intracellular concentrations are reduced. It should be also considered that the binding of DTT to ebselen, diphenyl diselenide and diphenyl ditelluride can reduce the effective concentrations of these compounds within the intracellular compartment.

Besides low concentrations of diphenyl ditelluride enhanced Na^+, K^+-ATPase activity, one interpretation of this interesting finding is that diphenyl ditelluride could be an antioxidant agent. In fact, the antioxidant property of organochalcogens depends on the concentration tested (Meotti et al., 2003, 2004). Thus, it is possible that the handling of the enzyme oxidizes crucial sulphydryl groups and low concentrations of diphenyl ditelluride reduce them, causing the increment in enzyme activity.

Several studies have been carried out in the effect of different xenobiotics on the Na^+, K^+-ATPase activity (Lai et al., 1980; Somló and Hassón-Voloch, 1987; Anner and Moosmayer, 1992; Pedrenho et al., 1996). Accordingly, all tested organochalcogens act as Na^+, K^+-ATPase inhibitors, present a similar inhibitory potency (in the μM range) and the inhibition follows a similar mechanism. In fact, diphenyl diselenide, diphenyl ditelluride and ebselen decreased both maximal velocity and K_m.

In addition, organoselenium and organotellurium compounds affect a number of neuronal processes (Kice and Lee, 1978) and the exposure to high doses of these compounds causes central nervous system effects in mice (Nogueira et al., 2003c) and modifies the functionality of the glutamatergic system in vitro and in vivo (Nogueira et al., 2001, 2002). Similarly, the modification of the glutamatergic system by organochalcogens results, at least in part, on changes in the thiol-disulfide balance. Taking into account that maintenance of Na^+, K^+-ATPase activity is critical for normal brain function and the reduction of its activity is related to selective neuronal damage (Lees, 1993) the effect of organochalcogens on this enzyme may contribute to understand the neurotoxicity induced by organochalcogens.

In conclusion, cerebral Na^+, K^+-ATPase is a potential molecular target for the toxic effect of organochalcogens and the inhibition may occur through a change in the thiol groups of this enzyme.

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