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EFEITO DE COMPOSTOS ORGANOCALCOGÊNIOS E DERIVADOS DA GUANINA EM MODELOS DE DANO CEREBRAL EM RATOS

Tese de Doutorado

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*Dedico este trabalho a **Julien**,*
inseparável companheiro de jornada

“Aprendi com a primavera a me deixar cortar.

E a voltar inteira”.

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LISTA DE ABREVIATURAS

AMPA → Ácido α-amino-3-hidroxi-5metil-4-isoxazolepropionato

AMPc → 3',5'- Adenosina monofosfato cíclico

ATP →Adenosina trifosfato

Ca^{2+} →Íon cálcio

CCDV→canais de cálcio dependentes de voltagem

Cl^-→Íon cloro

DTT→dithiothreitol

EAAC1 →transportador de aminoácido excitatório

EAAT4 →transportador de aminoácido excitatório

EAAT5→transportador de aminoácido excitatório

EAAC(EAAT3)→transportador de aminoácido excitatório

DAG →Diacilglicerol

GLT1/EAAT2 →transportador de glutamato

GLAST/EAAT1 →transportador de glutamato-aspartato

GSH→ glutationa reduzida

GPx→glutationa peroxidase

GFAP →Proteína glial fibrilar ácida

GMPc →3', 5'-Guanosina monofosfato cíclico

GUO→guanosina

Hg^0→mercúrio metálico ou elementar

Hg^{1+}→ mercúrio mercuroso

Hg^{2+} →mercúrio mercúrico

HI→hipóxia-isquemia

IP3 → Inositol trifosfato

K^+ →Íon potássio

KCl→cloreto de potássio

KA→ácido caínico

Mg^{++} → íon magnésio

MTT→ 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide

Na^+ → Íon sódio

Na($^+$)/K($^+$)-ATPase →sódio-potássio-adenosinatrifosfase

NMDA →N-metil-D-aspartato

$(PhSe)_2$ →disseleneto de difenila

$(PhTe)_2$ →ditelureto de difenila

RE →retículo endoplasmático

SNC→Sistema nervoso central

SH→ grupamento sulfidrílico

RESUMO

Os mecanismos envolvidos nas atividades toxicológicas e/ou farmacológicas dos compostos orgânicos de selênio são pouco conhecidos. Os compostos orgânicos de selênio (disseleneto de difenila e ebselen) e organotelúrio (ditelureto de difenila) foram alvo dos trabalhos realizados*"in vitro",* neste estudo. Os compostos organocalcogênios apresentaram efeitos diversos sobre o influxo de $^{45}Ca^{2+}$ medido em sinaptossomas de cérebro de rato, dependendo das condições e agentes despolarizantes usados. Ebselen, $(PhSe)_2$ e $(PhTe)_2$ alteram a captação de $^{45}Ca^{2+}$ de maneira distinta quando expostos a aminopiridina ou KCl. Enquanto $(PhTe)_2$ inibe a captação de cálcio em todas as condições experimentadas, $(PhSe)_2$, apresenta este efeito apenas quando incubado em condições basais ou sob a ação de aminopiridina. Ebselen, por sua vez, aumenta a captação de cálcio em altas concentrações em condições basais e sob a ação de aminopiridina, porém, apresenta efeito inverso quando os sinaptossomas são despolarizados por KCl. Ebselen evitou a inibição da captação de $^{45}Ca^{2+}$ "*in vitro"* provocada por cloreto de mercúrio(HgCl) em sinaptossomas de cérebro de rato em condições basais do ensaio, no entanto, ebselen não afetou a inibição da captação de glutamato "*in vitro"* por HgCl, indicando que ebselen pode atuar dependendo das proteínas-alvo consideradas.Os compostos de mercúrio, MeHg e HgCl, inibiram a captação de glutamato em córtex cerebral de ratos de 17 dias e ebselen reverteu somente o efeito do MeHg porém, não, o do HgCl. Disseleneto de difenila não alterou os parâmetros avaliados na exposição de ambos os compostos de mercúrio.Os compostos de mercúrio estudados provocaram a morte celular das fatias de córtex, porém, ebselen protegeu as fatias dos efeitos lesivos provocados por MeHg e não pelo HgCl.

A hipoxia–isquemia neonatal reduziu a captação de glutamato de fatias de hipocampo de ratos, após 3 – 5 dias do insulto. Esta inibição foi recuperada aos 60 dias de idade. A guanosina evitou a redução da captação de glutamato em fatias de hipocampo de ratos submetidos à hipoxia–isquemia neonatal quando administrada em quatro doses consecutivas:imediatamente, 24 h, 48 h após o insulto HI. Estes resultados indicam que a HI neonatal influencia a captação de glutamato logo após o insulto e a guanosina previne esta ação.

ABSTRACT

The mechanisms underlying the toxicity and/or pharmacology of organic forms of selenium (diphenyl diselenide and Ebselen) is not very well known. The organic forms of selenium (diphenyl diselenide and Ebselen) and organic forms of tellurium were investigated " *in vitro*". These compounds display differents effects depending on the condition and the depolarizing agent used. Ebselen, $(PhSe)_2$ e $(PhTe)_2$ changes in Ca^{2+} influx into synaptosomes of different form when exposed the 4-AP or high K^+ . While $(PhTe)_2$ decrease Ca^{2+} influx in all conditions tested. $(PhSe)_2$, display this effect only when non-depolarizing conditions or in 4-AP estimulation. Contrary, Ebselen, increase the Ca^{2+} influx in high concentrations under non-depolarizing concentrations and with 4-AP , however, shown inverse effect when synaptosomes under high KCl. Ebselen protects Ca^{2+} influx blockage caused by Hg^{2+} .into brain synaptosomes under non-depolarizing conditions, nevertheless, ebselen does not protect glutamate uptake inhibition caused by Hg^{2+} suggesting that its protection is dependent on the target protein considered. The mercury compounds, MeHg e HgCl, inhibited the glutamate net uptake from the cerebral cortex of 17-day-old rats; and ebselen reverted the MeHg-induced inhibition of glutamate net uptake but did not protect the inhibition caused by Hg^{2+}. Diphenyl diselenide $(PhSe)_2$, was observed that this compound did not revert the action of MeHg or Hg^{2+}. Ebselen protected slices from the deleterious effects of MeHg, but not of Hg^{2+}on cell viability. Conversely, ebselen did not modify the reduction of MTT caused by MeHg and Hg^{2+}.The protective effect of ebselen on MeHg-induced inhibition of glutamate net uptake seems to be related to its ability in maintaining cell viability. The neonatal hypoxia-ischemia (HI) in 7-day-old pups on the high-affinity [^{3}H] glutamate uptake into hippocampal slices at different times

after insult was examined. Immediately following and 1 day after the insult there was no effect. But at 3 to 5 days after the HI insult, glutamate uptake into the hippocampus was markedly reduced; however, after 30 or 60 days the glutamate uptake into hippocampal slices returned to control levels. Also, this study demonstrated the effect of the nucleoside guanosine (Guo) on the [^{3}H] glutamate uptake in neonatal HI injury, maintaining the [^{3}H] glutamate uptake at control levels when injected before and after insult HI. We conclude that neonatal HI influences glutamate uptake a few days following insult, and that guanosine prevents this action.

APRESENTAÇÃO

Esta tese constitue-se de: item I - INTRODUÇÃO, ou seja, a fundamentação que originou o trabalho e no item II encontra-se OBJETIVOS GERAIS.

O ítem III - OBJETIVOS ESPECÍFICOS; o IV- MATERIAIS e MÉTODOS e o V- RESULTADOS estão sob a forma de artigos publicados conforme discriminados no SUMÁRIO.

O ítem VI - DISCUSSÃO apresenta comentários gerais e a integração dos resultados obtidos neste trabalho.

No ítem VII - CONCLUSÕES são apresentadas as conclusões dos manuscritos. E, no ítem VIII - REFERÊNCIAS BIBLIOGRÁFICAS refere-se somente às citações contidas na Introdução e Discussão desta tese.

I. INTRODUÇÃO

I.1.SISTEMA GLUTAMATÉRGICO

I.1.1. CAPTAÇÃO DE GLUTAMATO

O glutamato é o aminoácido encontrado em maior concentração no Sistema Nervoso Central(SNC) de mamíferos, onde participa de funções metabólicas idênticas às exercidas em outros tecidos, como biossíntese de proteínas, formação de redes neuronais durante o desenvolvimento, alterações plásticas associadas a aprendizagem e a memória (Meldrum et al., 1999, Ozawa et al.,1998). Principal neurotransmissor excitatório do sistema nervoso central (SNC) de mamíferos, sua síntese ocorre nos terminais pré-sinápticos, e é estocado nas vesículas sinápticas (Meldrum, 2000).

O papel excitatório do glutamato ocorre por sua atuação sobre os receptores glutamatérgicos localizados nas membranas pré e pós-sinápticas, e nas membranas gliais. A neurotransmissão glutamatérgica é mediada através da interação do glutamato com duas classes distintas de receptores: ionotrópicos e metabotrópicos, classificados de acordo com suas propriedades farmacológicas e funcionais (Conn e Pinn, 1997).Os receptores ionotrópicos são canais iônicos que permeiam cátions quando ativados e que promovem a despolarização da membrana sináptica, desencadeando uma resposta excitatória. São subdivididos em N-metil-d-aspartato (NMDA), -amino-3-hidroxi-5-metil-4-isozaol-ácido propiônico (AMPA), e ácido caínico (KA), com base na sua sensibilidade a agonistas específicos. Os receptores metabotrópicos estão acoplados a proteínas ligantes de nucleotídeos da guanina (proteínas G) e modulam a atividade de efetores intracelulares, tais como: adenilato ciclase e fosfolipase C, responsáveis pela produção de segundos-mensageiros (AMPc, diacilglicerol e IP3) (Ozawa,1998).

A concentração de glutamato na fenda sináptica é finamente regulada através de sistemas de captação localizados nas membranas astrocíticas e

terminais pré-sinápticas, pois não há enzimas extracelulares capazes de metabolizá-lo (Anderson and Swanson, 2000, Attwell, 2000, Chen and Swanson, 2003, Rothstein et al., 1994 Ullensvang et al.,1997). Através da ação distintamente regulada destes sistemas o glutamato é armazenado nas vesículas, diminuindo sua concentração na fenda sináptica. Quando as membranas pré-sinápticas são despolarizadas, o glutamato é liberado na fenda sináptica. Após a captação astrocitária, o glutamato é transformado pela glutamina sintetase em glutamina que é transportada para os neurônios onde é convertida através da glutaminase a glutamato que pode ser captado pelas vesículas sinápticas e liberado novamente, recomeçando o processo (Anderson e Swanson, 2000). Os astrócitos são responsáveis pela maior parte do glutamato captado (Schousboe, 1981).

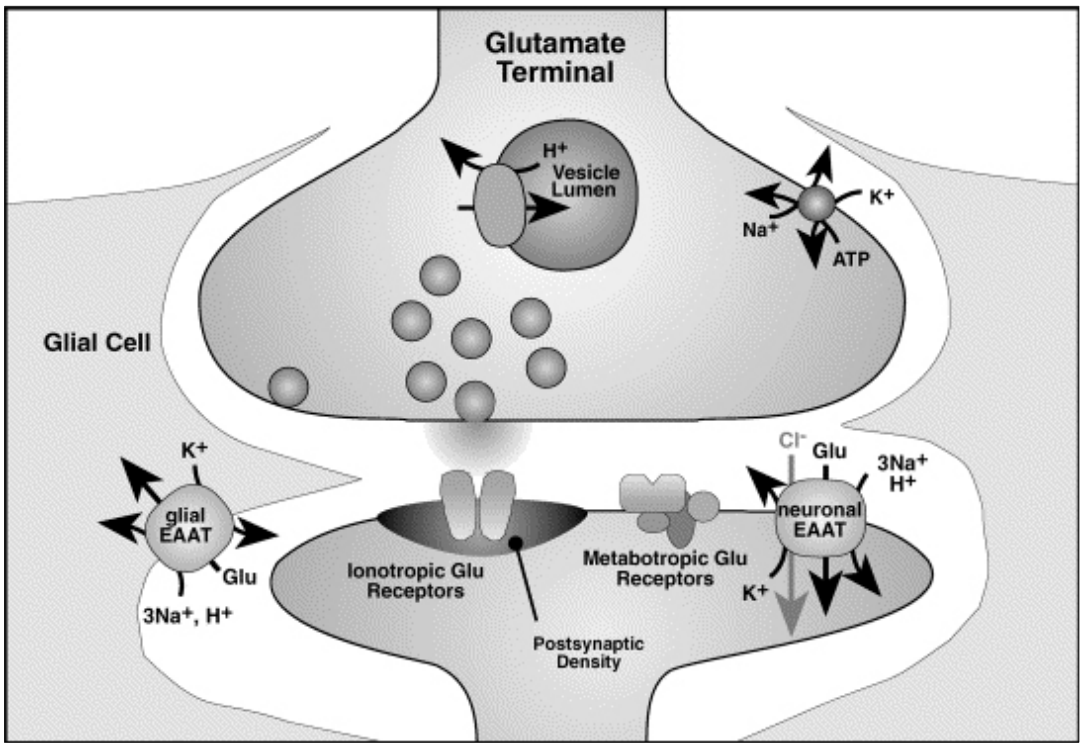

FIGURA 1 – **Sinapse glutamatérgica**. Mecanismo de transporte de glutamato e localização de seus transportadores nos astrócitos e neurônios. Os transportadores

neuronais estão localizados, na sua maioria, fora da região do neuropil (Fonte:Amara e Fontana, 2002)

Nitidamente, a captação de moléculas de glutamato representa uma função vital na manutenção de altos níveis de precursores de glutamato e baixas concentrações extracelulares deste neurotransmissor (Dichter & Wilcox,1997).

A neurotransmissão glutamatérgica é finalizada quando a concentração de glutamato na fenda sináptica é reduzida através da sua difusão e principalmente através de sua remoção por ação de dois sistemas de transporte: um carreador com alta afinidade e dependente de Na^+, localizado nas membranas sinápticas e gliais (Rothstein et al., 1994, Robinson and Dowd, 1997) e outro com baixa afinidade e independente de Na^+, localizado nas membranas das vesículas sinápticas.

I.1.1.2. Glutamato como neurotoxina

Lucas and Newhouse (1957) foram os primeiros a sugerir que o glutamato poderia ser uma neurotoxina ao demonstrar que injeções de L-glutamato podem destruir a camada interna da retina de camundongo. Estas observações foram replicadas e ampliadas por Olney (1969) ao verificar que uma hiperativação do sistema glutamatérgico pode ocasionar dano neuronal, processo conhecido como excitotoxicidade**.** Entre as causas deste processo estão a hipersensibilidade ao glutamato devido a deficiências no metabolismo energético ou nas defesas antioxidantes (Greene and Greenamyre, 1996), anormalidades nos receptores glutamatérgicos (aumento na densidade e modulação) e/ ou um aumento na concentração extracelular deste neurotransmissor (aumento na liberação ou redução na captação) (Rothstein et al., 1992, Battaglioli et al., 1993, Robinson and Dowd, 1997). Desde então, este processo tem sido relacionado a várias neuropatologias agudas e crônicas como epilepsia, isquemia cerebral, tumores do sistema nervoso central, esclerose amiotrófica lateral, doença de Alzheimer, de Huntington, de Parkinson, neurodegeneração associada a infecções por HIV (Greenamyre. and Young, 1989, Rothstein et al., 1992. Lipton and Rosemberg,

1994, Price, 1999, Maragakis and Rothstein, 2001, 2004, Mattson et al., 1999, 2002, Segovia et al., 2001, Brewer, 2000; Ingram et al., 2001, Meldrum e Garthwate, 1991, Wang et al., 2003).

I.1.1.3.Transportadores de glutamato

Atualmente, cinco subtipos de transportadores de glutamato foram caracterizados: GLAST/EAAT1, GLT1/EAAT2, expressos nos astrócitos. GLAST é o principal transportador presente durante o desenvolvimento do Sistema Nervoso Central (Furuta et al.,1997), enquanto que GLT1 é responsável por 90% de todo o transporte nos tecidos adultos (Tanaka et al., 1997). Apenas GLT1, pode ser também expresso em células bipolares de retina (Rauen e Kanner, 1994, Rauen et al., 1996). Estes dois transportadores são quantitativamente os principais transportadores de glutamato, sendo responsáveis pela maior parte de sua captação no SNC (Anderson e Swanson, 2000).

A distribuição e concentração do GLAST e GLT-1 no SNC variam de acordo com a região (Gegelashvili e Schousboe, 1998) e conforme o estágio de desenvolvimento cerebral (Furuta et al., 1997a, Ullensvang et al., 1997, Maragakis e Rothstein, 2001). Ambos os transportadores são expressos numa mesma célula, mas em proporções diferentes dependendo da região (Lehre et al., 1995). GLT1 é o maior transportador de glutamato em todas as regiões cerebrais (Danbolt et al.,1998).

Recentes estudos evidenciaram alterações na expressão dos transportadores de glutamato durante o desenvolvimento do sistema nervoso central. As expressões de GLAST e GLT-1 estão reduzidas em ratos jovens e aumentam com o desenvolvimento aos níveis dos adultos, e a expressão de EAAC1 é maior nos cérebros dos ratos neonatos do que nos cérebros adultos (Furuta et al., 1997). No cérebro imaturo ou em desenvolvimento, os níveis de GLAST predominam sobre os de GLT-1, o qual não é detectado no nascimento (Levy et al., 1995, Ullensvang et al., 1997). As concentrações de GLAST e GLT-1 aumentam drasticamente no período de maior atividade de sinaptogênese (do final

da segunda ao final da quarta semana pós-natal) atingindo níveis adultos após trinta e cinco dias de vida (Furuta et al., 1997a, Ullensvang et al., 1997). Camundongos Knock-out para GLT-1 apresentam convulsões letais nas primeiras horas de vida (Tanaka et al., 1997). Da mesma forma, animais com deficiência de GLAST demonstraram uma suscetibilidade aumentada à injúria cerebelar, assim como uma redução da coordenação motora (Watase et al., 1998).

Nos neurônios e glia encontramos EAAC1/EAAT3, enquanto que o EAAT4, é predominantemente encontrado nas células de Purkinje no cerebelo. E, finalmente, EAAT5, encontrado na retina (Danbolt, 2001). Portanto, a captação de glutamato em cérebro de rato é baixa no nascimento e aumenta aos níveis de adulto nas primeiras semanas de vida (Ullensvang et al., 1997).

I.2. MODELO DE DANO AO SISTEMA NERVOSO CENTRAL POR MERCÚRIO

I.2.1.MERCÚRIO

O mercúrio é uma toxina ambiental capaz de provocar amplos e variados efeitos adversos à saúde humana, especialmente ao sistema nervoso central. Apresenta-se em três formas diferentes: vapor de mercúrio (a temperatura ambiente) metálico ou elementar (Hg^0), compostos inorgânicos de mercúrio, ou sal de mercúrio (Hg^{2+}), ou (Hg^{1+}) mercuroso, e as formas orgânicas de mercúrio, Metil, Etil, Fenilmercúrio (MeHg, EhHg, PhHg), respectivamente. A solubilidade, reatividade, efeitos biológicos e toxicidade variam entre estas formas.

No meio ambiente as emissões vulcânicas, o desgaste da crosta terrestre, a evaporação de fontes de água, são responsáveis pela presença de mercúrio na natureza. Outras fontes como descargas industriais, a queima de carvão, petróleo, dos crematórios, a mineração, bem como a produção de cloroalcalis (NaOH, cloro puro), cimento, fotografias, tintas, fungicidas, baterias, lâmpadas fluorescentes, germicidas, instrumentos médicos podem servir como fonte de exposição a este tóxico (Aucott et al., 2003, Betsinger et al., 2000, Hoover and Goldwater, 1966).

Recentes relatos da literatura sugerem que o mercúrio das amálgamas dentárias pode provocar nefrotoxicidade, mudanças comportamentais, alterações na pele e mucosas entre outras complicações. O desenvolvimento de doença de Alzheimer ou esclerose múltipla tem sido ligada a exposições a baixas concentrações de mercúrio. A suscetibilidade genética influencia na determinação dos efeitos negativos das amálgamas dentárias (Yip et al., 2003, Counter e Buchanan, 2004, Mutter et al., 2004, 2005).

Geralmente, o MeHg no meio ambiente é formado por microorganismos a partir do Hg elementar depositado no ar ou na água de fontes naturais ou humanas. O consumo de peixe é a forma primária de exposição ao Hg orgânico (Clarkson, 1997, Langford e Ferner, 1999). Cabe salientar que no Brasil as áreas de mineração representam uma fonte de exposição considerável às populações nativas e índios que vivem na região Norte, como demonstra recente estudo de Gonçalves e Gonçalves, 2004.

I.2.1.1.Absorção, metabolismo, distribuição:

O mercúrio elemental vaporiza a temperatura ambiente e ao ser inalado passa através das membranas dos alvéolos pulmonares ao sangue onde se distribue nos eritrócitos, sistema nervoso central (SNC) e rins. Pouco absorvido pelo trato gastrointestinal e pela derme. Atravessa a placenta e pode chegar ao feto. No SNC, ao ser transformado na forma mercúrica (Hg^{++}) é pouco difusível no cérebro. A maioria dos mercuriais orgânicos são absorvidos pela ingestão, inalação e através da pele. São lipossolúveis e absorvidos pelo trato gastrointestinal. As formas orgânicas de mercúrio também atravessam a barreira hematoencefálica e a placenta.

I.2.1.2.Toxicidade

Em altas concentrações, o mercúrio elemental ao ser inalado causa pneumonia, insuficiência respiratória e morte. A absorção de sais de mercúrio

pode ser fatal, por ulceração gastrointestinal e hemorragia, podendo acumular-se nos rins levando a síndrome nefrótica (Goldman e Shannon, 2001). Os compostos orgânicos de mercúrio provocam incoordenação motora, parestesias, surdez, mudez, tremores, coma, morte. Bebês que nasceram de mães intoxicadas durante a gravidez apresentam retardo mental, cegueira, mudez e convulsões durante o desenvolvimento (Amin-Zaki et al., 1974).

I.2.1.3.Aspectos bioquímicos dos mercuriais:

Um aspecto comum do Hg^{2+} e do MeHg consiste na sua capacidade de romper a regulação neuronal dos íons divalentes, especialmente o Ca^{2+}. Ambos MeHg e Hg^{2+} alteram a regulação dos níveis de cálcio intracelular, a liberação de neurotransmissores e a função dos canais iônicos ligados à voltagem (Sirois e Atchison, 1996,. 2000). Apesar de pouco conhecidos os mecanismos pelos quais a sobrecarga do íon cálcio provocada pelo MeHg pode acarretar morte celular, a desorganização do citoesqueleto, a ativação de proteases, fosfolipases, e a disfunção mitocondrial estariam entre os fatores prováveis de rompimento da homeostase deste íon (Orrenius e Nicotera,1994, Oyama e cols.,1994). Bloqueadores de canais de Ca^{2+} dependentes de voltagem podem prevenir disfunções neurológicas em ratos submetidos a tratamento *in vivo* por MeHg (Sakamoto e cols., 1996).

Vários dados experimentais demonstram o envolvimento do mecanismo excitotóxico na neurotoxicidade por mercuriais. O mercúrio exerce um efeito inibitório sobre a habilidade dos astrócitos retirar ou depurar o glutamato liberado no espaço extracelular (Kim and Choi, 1996, Aschner, 1996). Vários estudos apontam que os astrócitos são um alvo importante para o Hg^{2+} e que a excitotoxicidade seria provocada pela disfunção astrocitária (Kim e Choi, 1995, Chen e Swanson, 2003). MeHg preferencialmente acumula-se nos astrócitos onde provoca o edemaciamento (“swelling”) celular e inibe a captação de aminoácidos excitatórios (Aschner et al., 2000). Igualmente, cloreto de mercúrio inibe a captação de glutamato em cultura de astrócitos (Brookes, 1988), e inibe em 60%

os transportadores GLT, GLAST e EAAC em lipossomos (Trotti et al., 1997). Além disso, antagonistas do receptor NMDA,ácido D-2-amino-5-fosfonovalerico (um antagonista competitivo NMDA), e ácido 7-cloroquinurenico (um antagonista do sitio de glicina associado com o receptor NMDA) podem inibir os efeitos tóxicos induzidos por MeHg em culturas neuronais cerebrais (Rajanna et al.,1997).

Outro exemplo foi evidenciado por Juarez et al., 2002 que ao utilizar MeHg através de microdiálise no córtex frontal de rato obteve aumento da liberação de glutamato no dialisado, por alterações nos mecanismos de captação/depuração dos aminoácidos excitatórios.

Além disso, a alta afinidade do MeHg por grupamentos sulfidrílicos (SH) (Clarkson, 1997, Ascher et al., 2000) torna proteínas contendo cisteína suscetíveis a modificações estruturais e funcionais,como por exemplo, os transportadores GLT1 e GLAST (Pines et al., 1992). Também, Ascher et al., 1996 sugerem que a maior inibição astrocitária seria causada pelo Hg^{2+} inorgânico do que pelo MeHg pois o MeHg seria demetilado a Hg^{2+} inorgânico dentro dos astrócitos (Aschner et al., 1996). Por fim, $HgCl_2$ diminue a atividade da glutamina sintetase, enzima astrocítica responsável pela conversão do glutamato a glutamina (Allen et al., 2001). Esta inibição poderia afetar a depuração do glutamato extracelular contribuindo para a desregulação do metabolismo glutamatérgico (Fitsanakis et al., 2005). Portanto, é notório que a neurotoxicidade induzida pelo MeHg pode ser atribuída, pelo menos em parte, a desregulação da homeostase dos aminoácidos excitatórios e que os mecanismos excitotóxicos estão envolvidos.

Sanfeliu e cols., 2001 demonstraram em culturas de células que os astrócitos são mais resistentes a neurotoxicidade provocada por MeHg enquanto que os neurônios foram mais responsivos a ação de antioxidantes. Outrossim, no cérebro em desenvolvimento o MeHg é tóxico para o cerebelo e córtex levando a necrose focal e apoptose (Castoldi et al., 2001, Costa et al.,2004).

I.2.3.CAPTAÇÃO DE CÁLCIO

Quase tudo que fazemos é controlado pelo Ca^{2+} - como nos movemos, como nossos corações batem e como nossos cérebros processam informações e guardam memórias. Para que tudo isso seja feito, o Ca^{2+} atua como um mensageiro intracelular, relacionando informações dentro das células para regular sua atividade. Por exemplo, o Ca^{2+} inicia a vida na fertilização e controla o desenvolvimento e diferenciação das células em tipos especializados. Ele media a atividade subseqüente dessas células e finalmente, está invariavelmente envolvido na morte celular. Para coordenar todas essas funções, os sinais de Ca^{2+} devem ser versáteis e ao mesmo tempo precisamente regulados. Essa extraordinária versatilidade se dá devido a possibilidade do íon atuar nos diferentes contextos de espaço, tempo e amplitude (Berridge et al., 1998). Os níveis intracelulares de cálcio em condições basais são mantidos baixos, porém, a estimulação fisiológica por hormônios, neurotransmissores, fatores de crescimento, resulta em níveis aumentados necessários para ativar rotas de transdução de sinal dependente de cálcio. (Berridge et al., 1998).

I.2.3.1.Canais de Cálcio

Os canais de Ca^{2+} caracterizam-se por sua seletividade, localização e abrem/fecham-se de acordo com sinais elétricos, mecânicos e químicos provocando mudanças conformacionais. São altamente plásticos funcionalmente e diferenciam-se conforme suas propriedades eletrofisiológicas e farmacológicas.

Alguns canais se abrem quando um neurotransmissor ou um hormônio ocasiona variações na diferença de potencial elétrico através da membrana e são denominados canais de cálcio dependentes de voltagem (CCDV). Outros se abrem quando o ligante interage com o receptor de membrana celular associado a canais de membrana e são chamados canais de cálcio ligados ao receptor. Neste caso, a abertura dos canais pode se dar pela ação de segundos mensageiros, pela fosforilação do canal, por ação da proteína G, pelo próprio Ca^{2+} intracelular ou pela ligação direta do hormônio ou neurotransmissor ao próprio canal (Kandel, 2000).

A grande diferença entre as concentrações do íon cálcio no meio extra e intracelular evidenciam a importância de mecanismos de regulação da entrada e saída deste íon. O influxo de cálcio do meio extracelular e a redistribuição dos estoques de cálcio citosólico podem aumentar o cálcio intracelular que ativam processos tóxicos que podem levar a morte celular. No sistema nervoso central a liberação de neurotransmissores é ativada pelo influxo de Ca^{2+} através dos canais de Ca^{2+} voltagem-dependentes que provocam um rápido influxo de Ca^{2+} para dentro das células quando a membrana é despolarizada. Quando um canal de cálcio se abre, uma nuvem altamente concentrada de Ca^{2+} se forma ao redor de seu poro, dissipando-se rapidamente por difusão depois do canal se fechar. Esses sinais localizados, que podem se originar de canais na membrana plasmática ou nos estoques internos, representam os eventos elementares – as unidades básicas da sinalização com Ca^{2+}. Além dos sinais de Ca^{2+} serem altamente regulados, sua distribuição nos diversos compartimentos celulares também o é, pois, como já foi mencionado o Ca^{2+} livre em altas concentrações leva a morte celular. A perda da habilidade regulatória destes mecanismos e o subseqüente aumento dos níveis de cálcio intracelular estariam envolvidos em eventos patológicos como trauma cerebral, acidente vascular cerebral, epilepsia, esclerose amiotrófica lateral. Além disso, os canais de cálcio desempenham papel fundamental nas funções neurológicas, sendo alvos para uma variedade de tóxicos orgânicos e inorgânicos, entre eles, metilmercúrio, chumbo, etanol. Mutações dos canais de cálcio estão associados a várias desordens neurológicas como enxaquecas e dificuldades de movimento(Audesirk et al., 2000)

I.2.3.1.Canais de cálcio dependentes de voltagem(CCDV):

Muitos processos neuronais são regulados pelo influxo de cálcio através dos canais de cálcio dependentes de voltagem (CCDV) como a fosforilação protéica, a expressão gênica, as variações na diferença de potencial elétrico e, especialmente, na liberação de neurotransmissores (Llinas,1988, Miller,1992, Chung et al.,2000, Sirois e Atchison, 2000, Lipscombe et al.,2004). Os canais de

cálcio dependentes de voltagem (CCDV) constituem-se de uma família de canais formados por proteínas de membrana cuja atividade é modulada pela variação do potencial de membrana. Quando a membrana é despolarizada eles promovem um rápido influxo de Ca^{2+} para dentro das células.

Atualmente são conhecidos cinco classes de CCDV, segundo suas propriedades eletrofisiológicas e farmacológicas que são designados por: L, N, T, P/Q, R (Fisher e Bourque, 2001).

Os canais do tipo *L* são ativados por alta voltagem e inativados lentamente. A abertura do tipo L é bloqueada por antagonistas dihidropiridinas e assegurado por agonistas dihidropiridinas. É sensível as fenilalquilaminas e benzodiazepinas

Os canais do tipo *T* são ativados por baixa voltagem e inativam-se rapidamente. Estão presentes em alta concentração nos neurônios. Função desconhecida.

Canal do tipo *N* caracteriza-se por ser ativado por alta voltagem como o canal tipo L, porém difere deste último por ser insensível às dihidropiridinas. Bloqueado por ω-conotoxina, toxina produzida pelo molusco marinho *Conus geographus.*

Os canais do tipo *P/Q* demonstram resistência às dihidropiridinas e às conotoxinas, presente nos neurônios, cujo bloqueador específico é a ω-Agatoxina GIVA. Participa da neurotransmissão. E, finalmente os canais do tipo *R* cujo antagonista é SNX-482. Participa da secreção neuroendocrina.

Atualmente existem muitas formas de se estudar as mudanças nas concentrações de cálcio intracelular, sua distribuição nos diferentes compartimentos celulares. A caracterização da distribuição e movimentos nas diferentes áreas pode se obtida com agentes inibitórios ou procedimentos relativamente específicos para o efluxo, influxo e ligação ao cálcio (Trump e Berezesky, 1992).

I.2.4. COMPOSTOS ORGANOCALCOGÊNIOS

Compostos calcogênios são importantes intermediários e reagentes muito utilizados em síntese orgânica (Paulmier, 1986; Braga et al.,1996, 1997).São formados pelos elementos oxigênio(O), enxofre(S), selênio(Se), telúrio(Te) e polônio (Po) localizados no grupo 16 da tabela periódica. Nestes últimos anos, este grupo de pesquisa dedicou-se a investigar as propriedades farmacológicas e/ou toxicológicas destes versáteis compostos. Nesta tese focalizamos dois destes elementos, selênio e telúrio nas suas formas orgânicas, uma vez que estes compostos tem sido alvo de expressivo interesse pelas propriedades específicas que apresentam e por serem intermediários em diversos processos de síntese orgânica.

I.2.4.1.Selênio

O selênio é um elemento traço, essencial na dieta, pois está ligado a selenoproteínas. Sua deficiência pode provocar a cardiopatia endêmica (Keshan) observada em áreas geográficas cujos solos são pobres neste elemento. Diversos estudos foram descritos evidenciando a relação entre o baixo nível de selênio e o desenvolvimento de algumas doenças como câncer, esclerose cardiovascular, alterações digestivas, cardiovasculares e reumáticas (Neve et al., 1987, Ortuño et al., 1996). No entanto, este micronutriente pode ocasionar toxicidade, como uma doença chamada “alkali disease” decorrente da ingestão de plantas seleníferas, que acumulam grandes quantidades de selênio (Spallhoz, 1993,)

O principal local de absorção do selênio parece ser o duodeno, seguido pelo jejuno e íleo. Além do trato gastrointestinal, o selênio pode ser absorvido por tecidos cutâneos ou por inalação dependendo da exposição e intoxicação ocupacional por compostos de selênio (Whanger et al., 1976) Em animais submetidos à intoxicação crônica por selênio observa-se sua deposição principalmente no fígado e rins (Wilber, 1980). É eliminado do organismo principalmente pela urina, fezes e ar expirado.

A similaridade nas propriedades físicas e químicas do selênio e enxofre; permite interações selênio-enxofre nos sistemas biológicos, entretanto, as diferenças em suas propriedades físico-químicas estabelecem suas funções

específicas (Stadtman, 1980). Os selenóis (R-SeH) correspondem as formas de tióis(R-SH), onde ocorre a substituição do átomo de enxofre pelo átomo de selênio (Klayman & Günther, 1973). As oxidações dos selenóis podem originar disselenetos.

O selênio participa do sítio ativo da enzima glutationa peroxidase (GSH-Px), na forma de selenocisteína (Flohé, 1989). Esta enzima é um dos principais sistemas antioxidantes do organismo. Daí deriva uma das principais funções biológicas do selênio: sua atividade antioxidante, eliminando radicais livres, e, conseqüentemente favorecendo a integridade das membranas, promovendo a redução do risco de câncer, o processo de envelhecimento e doenças degenerativas (Navarro-Alarcón & López-Martinez, 2000).

I.2.4.2. Disseleneto de difenila $(PhSe)_2$

Os compostos orgânicos de selênio podem substituir o S nos grupamentos tiólicos das cisteína, dithiothreitol e GSH originando selenocisteína, selenóis e dissulfetos (Walter et al., 1972). Vários relatos tem demonstrado atividade antioxidante de compostos orgânicos de selênio em diferentes modelos experimentais, tanto ao reagir com hidroperóxidos como com peroxinitritos (Andersson et al.,1994, Meotti et al., 2004, Roussyn et al, 1996). No entanto, apesar da atividade glutationa-peroxidase proteger as biomembranas da peroxidação lipídica, bem como, mediar os processos inflamatórios, estes compostos podem também oxidar proteínas sulfidrílicas favorecendo suas propriedades toxicológicas (Nogueira et al., 2004).

Cabe salientar ainda que o composto disseleneto de difenila pode interferir no sistema glutamatérgico tanto *in vitro* quanto *in vivo* inibindo a ligação de glutamato e MK-801 em membranas sinápticas de cérebro de rato (Nogueira et al., 2001). Evidências experimentais reforçam o interesse neste composto, pois, também demonstrou atividade antiinflamatória e antinociceptiva (Nogueira et al., 2003(a)). Além de apresentar maior atividade tiol peroxidase quando comparado ao Ebselen (Engman et al., 1992), compostos orgânicos de selênio apresentam

um processo de síntese rápido e econômico. Contrariamente, ao processo empregado na síntese de ebselen que necessita de várias etapas complexas. Além disso, Ghisleni et al, 2003 demonstraram efeito neuroprotetor em situações de isquemia *in vitro* em fatias hipocampais de rato.

Contudo, dados deste grupo demonstram que compostos orgânicos de selênio podem apresentar neurotoxicidade dependendo da forma de administração e da espécie animal estudada. Enquanto a administração intraperitoneal de disseleneto de difenila provoca convulsões em camundongos, a administração subcutânea não provoca efeitos. Já a administração intraperitoneal ou subcutânea em ratos não produz atividade convulsivante (Nogueira et al., 2003 (b))

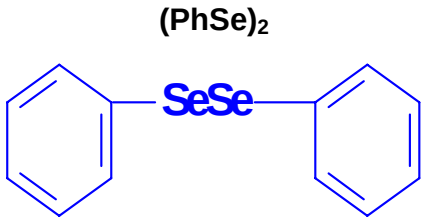

FIGURA 2. Estrutura de disseleneto de difenila.

I.2.4.3. Ebselen

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) é um composto orgânico de selênio cujas propriedades farmacológicas tem sido intensamente estudados em diferentes tipos celulares e para os mais diferentes tipos de injúria. A capacidade de ebselen mimetizar a selenoenzima glutationa peroxidase proporciona a base de algumas de suas propriedades biológicas (Muller et al., 1984, Wendel et al.,1984). Destaque especial merece suas propriedades antioxidantes e antiinflamatórias demonstradas em vários modelos experimentais *in vitro* e *in vivo* (Schewe,1995, Saito et al., 1998, Bosch-Morell et al., 1999, Rossato et al., 2002 a, b, Centurião et al., 2005).

O mecanismo exato pelo qual Ebselen é antioxidante não está completamente elucidado, porém, dados da literatura sugerem que parte deste

efeito ocorre através da redução de peróxido de hidrogênio e de hidroperóxidos orgânicos (Sies,1993, Muller et al., 1984), usando a GSH como fonte de elétrons para redução. Desta maneira o ebselen age de modo similar a GPx, utilizando GSH para decompor uma molécula potencialmente tóxica para a célula. Evidentemente que um excesso de ebselen pode ocasionar efeitos tóxicos para célula por depletar a GSH. Além de sua capacidade de mimetizar glutationa peroxidases, por sua alta reatividade com fosfolipídios e hidroperóxidos (Maiorino et al, 92, Noguchi et al., 1992) o ebselen inibe algumas das enzimas envolvidas no processo inflamatório. Ebselen provoca inibição da infiltração inflamatória de leucócitos, da liberação de citoquinas, da síntese de leucotrienos,da atividade das lipoxigenases (Kuhl et al. 1986) e da óxido nítricosintase (Wang et al., 1992).

Além disso, este composto de selênio demonstrou propriedades neuroprotetoras em cultura de neurônios (Tan et al., 1997, Porciúncula et al., 2001), inibiu a produção de substâncias reativas com o ácido tiobarbitúrico (TBARS) induzida pela administração de ácido quinolínico em cérebro de ratos (Rossato et al., 2002) e na exposição " *in vivo"* e "*in vitro"* a metilmercúrio (Farina et al., 2003a, b; Moretto et al. 2004, 2005)

Um dos aspectos mais relevantes a ser observado em relação ao ebselen são as investigações pré-clínicas já realizadas em humanos sujeitos à isquemia (Yamaguchi et al., 1998). Há controvérsia na literatura quanto aos resultados que demonstraram melhora significativa sem efeitos adversos expressivos. Vários modelos animais de isquemia apresentaram bons resultados no emprego deste composto orgânico de selênio (Dawson et al., 1995, Davalos, 1999, Saito et al., 1998). Contrariamente, Green e Ashwood (2005) ao administrarem ebselen antes do insulto provocado pela oclusão transitória da artéria cerebral obtiveram um pequeno efeito neuroprotetor ou um efeito nulo quando o insulto foi severo. Segundo os autores, isto explicaria o insucesso de alguns experimentos pré clínicos com o uso de ebselen.

EBSELEN

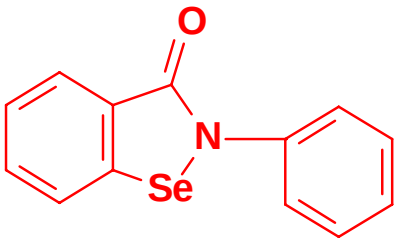

FIGURA 3 - Estrutura de ebselen.

Ebselen apresenta baixa toxicidade porque o átomo de selênio presente na sua molécula não é liberado durante a sua biotransformação e, deste modo, não é metabolizado pelo organismo como outros compostos, onde o selênio está mais disponível (Parnham & Graf, 1987). No organismo o transporte de ebselen se dá ligado à albumina (Wagner et al., 1994).

Apesar das auspiciosas propriedades antioxidantes de ebselen, Farina e col., 2004 demonstraram característica de hepatoxicidade somente para ratos jovens.

I.2.4.4.Interação selênio-mercúrio

Os compostos orgânicos de selênio tem sido apontados como redutor da toxicidade de metais pesados, entre eles mercúrio, chumbo e prata (Cuvin-Aralar e Furness, 1991). Conhece-se as propriedades protetoras do selênio contra a toxicidade de espécies inorgânicas de mercúrio observadas em diferentes tecidos, condições e espécies (Parizek e Ostadalová,1967, Magos, 1997, Gregus et al., 2001).

Em relação ao MeHg parece que o mesmo interage quimicamente com o selênio, apesar de não se conhecer exatamente este mecanismo (Watanabe et al., 1999, Bjornberg et al., 2003). Do mesmo modo, Moretto e cols (2004)

observaram que os compostos de selênio ebselen e disseleneto de difenila apresentaram efeitos protetores em relação a inibição da fosforilação dos neurofilamentos de córtex de ratos jovens provocados pela exposição ao MeHg.

I.2.4.5. Telúrio

O telúrio pertence ao grupo 16 da tabela periódica, assim como o enxofre e o selênio. Ao contrário do selênio, o telúrio não apresenta função fisiológica descrita até o momento (Taylor, 1996). No entanto, as configurações eletrônicas do selênio e do telúrio são semelhantes, e, por conseguinte, ambos apresentam algumas características similares, como a toxicidade (Van Vleet et al, 1982b).

O telúrio é empregado na manufatura de semicondutores, na indústria de componentes eletrônicos, síntese de fármacos e explosivos, na vulcanização de borracha, em lubrificantes sólidos e na petroquímica, entre outros (Clayton and Clayton, 1981,Taylor, 1996).

1.2.4.6. Ditelureto de difenila

Dados sobre a possível toxicidade de compostos orgânicos de telúrio são controversos. Tanto as formas orgânicas quanto inorgânicas são altamente tóxicas para o SNC de roedores(D'Gregorio e Miller, 1988, Jackson et al., 1989,Maciel et al., 2000) As formas orgânicas de telúrio são inibidores da esqualeno monooxigenase, alterando a biossíntese do colesterol e provocando desmielinização do sistema nervoso periférico (Laden e Porter, 2001).

Segundo, Maciel et al., 2000 o tratamento de camundongos com ditelureto de difenila revelou alterações histológicas acentuadas no cérebro. Em relação ao sistema glutamatérgico, ditelureto de difenila inibe a ligação de glutamato e MK-801 em membranas sinápticas de cérebro de rato in vitro e ex vivo após exposição aguda a baixas doses deste composto (Nogueira et al., 2001). Este estudo também sugere que estes diorganoteluretos quando administrados in vivo são mais reativos do que os compostos orgânicos de selênio devido a sua alta

eletronegatividade relacionada ao maior volume do átomo de telúrio. Já *in vitro* não foi observada esta correlação.

(PhTe) $_2$

FIGURA 4 - Estrutura de ditelureto de difenila

O efeito inibitório sobre a captação de glutamato em sinaptossomas e vesículas sinápticas também observada neste estudo sugere que este efeito possa estar relacionado a habilidade deste composto oxidar grupos tióis. O átomo de telúrio presente em compostos orgânicos é facilmente oxidado do estado divalente (+ii) até o estado tetravalente (+iv), o que torna estes compostos aptos a inibirem a peroxidação estimulada em hepatócitos de ratos e microssomas de rim de rato (Cotgreave et al., 1991, Andersson et al., 1994) e a peroxidação azo-induzida do ácido linolêico em metanol. Assim como seu análogo de selênio, ditelureto de difenila inibiu a peroxidação lipídica induzida por ácido quinolínico e nitroprussiato de sódio em cérebro de rato (Rossato et al., 2002b). Outro aspecto relevante deste composto relaciona-se ao efeito neuroproteror que este composto tem evidenciado em modelos neuronais de estresse oxidativo (Kanski et al., 2001).

I.3. MODELO DE DANO AO SISTEMA NERVOSO CENTRAL POR HIPÓXIA-ISQUEMIA

I.3.1. HIPÓXIA-ISQUEMIA NEONATAL:

O dano cerebral hipoxia-isquemia (HI) neonatal é a principal causa de mortalidade aguda e morbidade neurológica crônica em crianças. Segundo Vannucci and Hagberg (2004) 60% dos recém-nascidos prematuros com baixo peso apresentam encelopatia hipoxia-isquêmica. Estes bebês prematuros

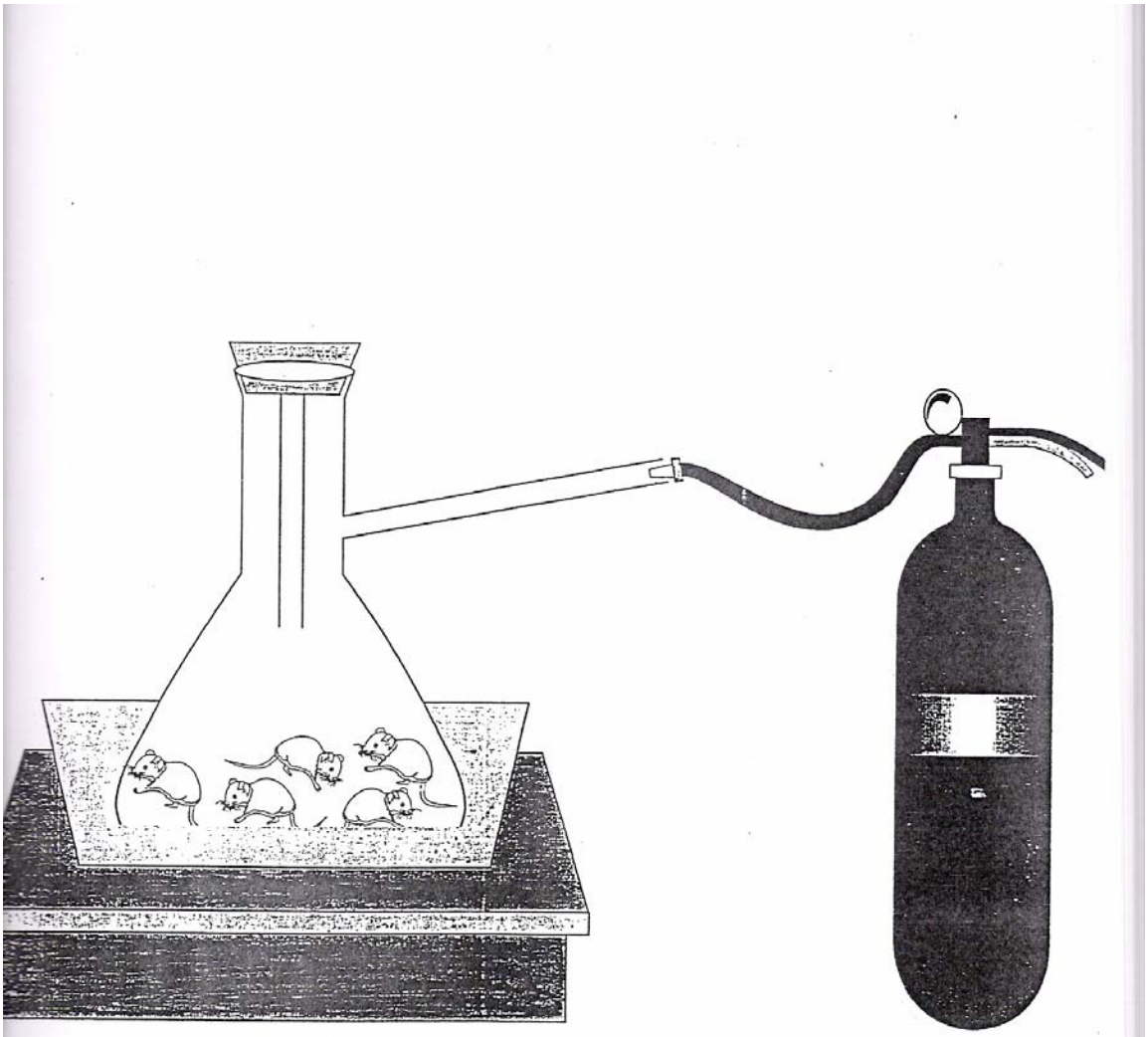

FIGURA 5 - Câmara de hipóxia. A mistura de 8% de oxigênio e 92% de nitrogênio passa através do fluxômetro, que libera (a uma razão de 5 litros por minuto) a mistura gasosa na câmara hipóxica onde estão os animais.

(Adaptado de Ruviaro,1996).

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apresentam alto risco de disfunções motoras e outros déficits neurológicos como retardo mental, déficits visuais corticais e convulsões epilépticas. É interessante salientar que os neonatos são um grupo heterogêneo de pacientes e o dano cerebral um heterogêneo grupo de desordens. Daí, a dificuldade de estratégias de intervenção apropriadas. Na maioria dos casos, os modelos de injúria cerebral em neonatos são influenciados pela natureza do insulto e a idade gestacional do bebê no decorrer na injúria (Amato e Donati, 2000).

I.3.1.2. Modelos animais de HI neonatal:

Os modelos animais promovem uma valiosa contribuição ao entendimento da patofisiologia do dano cerebral que advém na HI neonatal. O modelo animal mais usado para estudar a HI neonatal foi proposto por Rice et al., 1981 e emprega ratos ou camundongos de sete dias de idade. A associação da oclusão unilateral da artéria carótida com a exposição subseqüente a atmosfera hipóxica causa dano no estriado, córtex e hipocampo do hemisfério ipsilateral ao da oclusão da carótida. A duração da exposição ao ambiente hipóxico varia de trinta minutos a 2.5 horas dependendo da espécie animal. Geralmente, o hemisfério contralateral é preservado de dano neuropatológico e para a hipoxia ser efetiva é imprescindível a ligação da artéria carótida (Painter, 1995,Towfighi et al.,1995, Vannucci and Vannuccci, 1997).

I.3.1.3.Mecanismos bioquímicos do insulto HI neonatal:

Evidências sugerem que o insulto HI cerebral dispara uma cascata de eventos bioquímicos que podem ocasionar injúria e morte celular. A patofisiologia da injúria HI inicia-se quando o fornecimento cerebral de oxigênio e glicose são diminuídos. A despolarização provoca a liberação e acumulo de aa excitatórios e a produção aumentada de $[Ca^{2+}]_i$ e geração de radicais livres (Yager et al., 1997, Volpe, 2001, Vexler e Ferriero, 2001,Vannucci e Hagberg, 2004) A inibição da

fosforilação oxidativa leva a acidose e a diminuição da atividade da Na^+ K^+ ATPase , o aumento do Na^+ e como conseqüência há inchamento da célula.

As intensas alterações nos gradientes iônicos iniciam a ativação de processos danosos à célula, como p. ex. o aumento do influxo de cálcio ativa proteases, endonucleases e fosfolipases que hidrolisam fosfolipídios de membrana. Portanto, o influxo de cálcio dentro dos neurônios hipóxicos pode ser considerado um dos mais importantes mecanismos da injúria cerebral irreversível (Rothman and Olney, 1986, Cortey, 1995, Berger and Garnier, 2000).

Há um consenso na literatura de que a excitotoxicidade glutamatérgica propicia o desenvolvimento da morte neuronal na HI neonatal. A excessiva estimulação de receptores de glutamato, o acumulo de glutamato extracelular, abertura de canais NMDA estão intimamente ligados a excessiva excitação neuronal, bem como uma redução da atividade dos transportadores gliais e neuronais(Siesjo and Bengtsson,1989, Silverstein et al, 1991, Hagberg et al., 1994, Tan et al., 1996, Dirnagl et al., 1999, Murata et al., 2000,).

Evidências demonstram que o aumento dos níveis de glutamato causados pela HI envolvem a ruptura no sistema de captação de alta afinidade (Andine et al., 1994; Benveniste et al, 1984; Johnston, 2001; Massieu and Tapia, 1997; McDonald and Johnston, 1990), bem como o aumento da liberação (Nishizawa, 2001; Yager et al., 2002) e a reversão dos transportadores glutamatérgicos (Rossi, et al., 2000; Szatkowski et al., 1990).

Como o glutamato é um importante fator trófico para o cérebro imaturo e os receptores NMDA influenciam o desenvolvimento cerebral normal promovendo proliferação e migração de precursores neuronais e plasticidade sináptica, sua “over-stimulation” neste período ocasiona uma sensibilidade excitotóxica superior ao da de um cérebro adulto (Johnston, 1995, De Reuck, 1984).

I.3.1.4.Vulnerabilidade neuronal seletiva na HI

O cérebro do neonato é mais propenso à excitotoxicidade do que o cérebro adulto, provavelmente pela existência de muitos circuitos excitatórios que influem

na plasticidade neuronal durante o desenvolvimento, e/ou devido a liberação dos aa excitatórios (Ferriero, 2001, McDonald e Johnston,1990). A distribuição regional dos receptores glutamatérgicos no cérebro de ratos jovens corresponde ao modelo de vulnerabilidade seletiva durante a HI (Rice e Vannucci, 1981). Receptores NMDA expressos durante o desenvolvimento são compostos de sub unidades que permite que eles reajam mais facilmente a um estímulo do que em receptores de animais adultos (Monyer et al., 1993). Estas alterações nos receptores são parcialmente responsáveis pelo fato de os cérebros imaturos serem mais excitáveis e epileptogênicos do que os cérebros adultos (Holmes e Bem-Ari, 2001).

I.3.2. GUANOSINA

Os derivados da guanina estão associados ao sistema de transmissão de sinal transmembrana via proteínas G (Guderman et al.,1997). Quando ativadas por GTP, as proteínas G exercem dois efeitos simultâneos:modulam a atividade de efetores e diminuem a afinidade do agonista unido ao receptor. No SNC, esta forma de transdução do sinal celular está associada a subtipos de praticamente todos os receptores estudados: dopaminérgicos, glutamatérgicos, serotoninérgicos, purinérgicos, gabaérgicos, entre outros (Morris e Malbon, 1999).

Na presença de Guanosina foram observados efeitos tróficos sobre células neurais, importantes durante o desenvolvimento, na manutenção do sistema nervoso e em resposta a doença ou injúria (Neary et al.,1996, Rathbone et al.,1999). Os efeitos tróficos envolvem estimulação da proliferação astrocitária, síntese e liberação de fatores tróficos a partir de culturas de astrócitos e aumento da diferenciação de neurônios hipocampais" *in vitro"* (Ciccarelli et al., 2000).

Além da ação intracelular em proteínas G, várias evidências indicam que os derivados da guanina modulam o sistema glutamatérgico atuando do lado externo da membrana plasmática celular, sem o envolvimento de proteínas G. (Souza e Ramirez, 1991, Paz et al., 1994). Altas concentrações extracelulares de guanosina

são encontradas após isquemia e seus níveis permanecem elevados por vários dias após o insulto (Uemura et al.,1991). Também, Ciccarelli et al., 1999 observaram a elevação da concentração extracelular de guanosina em cultura de astrócitos em situações de hipóxia/hipoglicemia.

Recentes trabalhos tem indicado um efeito neuroprotetor da guanosina em situações de injúria. Estudos "*in vivo"* com Guanosina foram capazes de prevenir as convulsões provocadas por ácido quinolínico e α - dendrotoxina em camundongos (Schimidt et al., 2000, Lara et al., 2001). Ainda relacionados com o papel modulatório dos derivados da guanina sobre a hiperestimulação do sistema glutamatérgico usando cultura primária de astrócitos, Frizzo et al. (2001) demonstraram um aumento dose-dependente na captação basal de glutamato na presença de guanosina. Em fatias de córtex parietal de ratos de 10 dias, submetidos ou não à isquemia, a guanosina aumentou a captação de glutamato em ambas situações (Frizzo et al., 2002). Da mesma forma, o efeito da guanosina em relação à captação de glutamato pode ser idade dependente, visto que Gottfried et al. (2002) apontaram um aumento na captação de glutamato em cultura cortical de astrócitos com 10 dias de cultivo, mas não naquelas com 40 dias e Thomazi et al. (2004) demonstraram que o efeito da guanosina foi dependente da idade e estrutura estudada.

II.OBJETIVOS GERAIS

Considerando que:

- É fundamental para a célula a manutenção da homeostase do glutamato e do íon cálcio a fim de evitar a excitotoxicidade e morte celular que podem advir da exposição ao mercúrio e/ou à hipóxia-isquemia.

-Vários trabalhos sugerem que os compostos organocalcogênios e a guanosina podem atuar como neuroprotetores em situações de injúria.

Este estudo teve por objetivo investigar:

-os efeitos dos compostos organocalcogênios sobre o influxo de cálcio em sinaptossomas de cérebro de rato;

-os efeitos de ebselen sobre o influxo de cálcio e de glutamato em sinaptossomas de cérebro de rato expostos ao cloreto de mercúrio;

- os efeitos de ebselen sobre a captação de glutamato em fatias de córtex de ratos em desenvolvimento expostos ao metilmercúrio;

-os efeitos de ebselen sobre a viabilidade celular das fatias expostas aos compostos de mercúrio;

-o efeito da guanosina sobre a captação de glutamato em fatias de hipocampo de ratos submetidos à hipoxia–isquemia neonatal; com a finalidade de esclarecer se estes compostos podem atuar sobre os canais de cálcio dependentes de voltagem e os transportadores de glutamato, e visando possíveis intervenções terapêuticas que possam ser implementadas evitando o dano cerebral ou/e suas graves seqüelas, respectivamente.

III.OBJETIVOS ESPECÍFICOS:

Encontram-se inseridos nos artigos científicos.

IV. MATERIAIS E MÉTODOS:

Estão inseridos nos artigos científicos às páginas 29(artigo 1), 36(artigo 2), 44 e 45 (artigo 3), 54 e 55 (artigo 4)

V.RESULTADOS

V.1. Artigo 1

Moretto, M.B., Rossato, J.I., Nogueira, C.W., Zeni, G., Rocha, J.B.T. **2003**. Voltage-dependent ebselen and diorganochalcogenides inhibition of $^{45}Ca^{2+}$- influx into brain synaptosomes. ***J. Bioch. Mol. Tox***. 17, 154-160.

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Voltage-Dependent Ebselen and Diorganochalcogenides Inhibition of $^{45}Ca^{2+}$ Influx into Brain Synaptosomes

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ABSTRACT: By mediating the Ca^{2+} influx, Ca^{2+} channels play a central role in neurotransmission. Chemical agents that potentially interfere with Ca^{2+} homeostasis are potential toxic agents. In the present investigation, changes in Ca^{2+} influx into synaptosomes by organic forms of selenium and tellurium were examined under nondepolarizing and depolarizing conditions induced by high KCl concentration (135 mM) or by 4-aminopyridine (4-AP). Under nondepolarizing conditions, ebselen (400 μM) increased Ca^{2+} influx; diphenyl ditelluride (40–400 μM) decreased Ca^{2+} in all concentrations tested; and diphenyl diselenide decreased Ca^{2+} influx at 40 and 100 μM, but had no effect at 400 μM. In the presence of KCl as depolarizing agent, ebselen and diphenyl ditelluride decreased Ca^{2+} influx in a linear fashion. In contrast, diphenyl diselenide did not modify Ca^{2+} influx into isolated nerve terminals. In the presence of 4-AP (3 mM) as depolarizing agent, ebselen (400 μM) caused a significant increase, whereas diphenyl diselenide and diphenyl ditelluride inhibited Ca^{2+} influx into synaptosomes. The results can be explained by the fact that the mechanism through which 4-AP and high K^+ induced elevation of intracellular Ca^{2+} is not exactly coincident. The mechanism by which diphenyl ditelluride and ebselen interact with Ca^{2+} channel is unknown, but may be related to reactivity with critical sulfhydryl groups in the protein complex. The results of the present study indicate that the effects of organochalcogenides were rather complex depending on the condition and the depolarizing agent used.
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KEYWORDS: **Synaptosomes; Ca^{2+} Influx; Ca^{2+} Channel; Ebselen; Diphenyl Diselenide; Diphenyl Ditelluride; 4-Aminopyridine; Depolarization**

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INTRODUCTION

Ca^{2+} is one of the most important intracellular signaling agent and is used by virtually all eukaryotic cells to trigger a variety of cellular processes, including neurotransmitter release, hormone secretion, gene expression, and excitation–contraction coupling [1–6].

In neurons, particularly at the presynapse, Ca^{2+} movements across cell membranes contributes decisively to the Ca^{2+} signals that control cell functioning. In fact, adequate cytoplasmic Ca^{2+} concentration is maintained by the concerted action of different Ca^{2+} transport proteins, including pumps and translocators [2,7–10]. Likewise, transient increase in cytosolic Ca^{2+} can result from Ca^{2+} flux in the direction of its gradient concentration by entry from extracellular medium or by release of Ca^{2+} from intracellular Ca^{2+} stores, through specific Ca^{2+} channels [1,4,11–13].

By mediating the Ca^{2+} influx that triggers exocytotic fusion, Ca^{2+} channels play a central role in neurotransmission [14,15]. Ca^{2+} channels consist of a complex of protein subunits, including a α_1 subunit that constitutes the voltage-dependent ion-conducting pore of the channel, and a group of associated proteins, which modulate channel properties such as voltage dependence, inhibitor sensitivity, kinetics of inactivation, and channel targeting [16,17].

During the last decades, several points of evidence have been accumulated showing that persistent increases in intracellular Ca^{2+} can activate toxic processes that culminate in cell death [18–20]. Consequently, chemical agents that potentially interfere with Ca^{2+} homeostasis are potential toxic agents. In line with this, an infinite number of neurotoxicants can modify the activity of Ca^{2+}-handling proteins, including the Ca^{2+} pumps and channels [21–24].

In addition to their important role as synthetic intermediaries in organic synthesis [25–29], which increase the risk of occupational exposure to chalcogenides,

organic forms of selenium and tellurium have been pointed out by several investigators as potential pharmacological therapeutic agents. It has been suggested that utilization of the redox activity of the selenium and tellurium atoms of such compounds provides antioxidants of considerable potency, suitable as tools in free-radical biology and medicine [30–37]. In fact, a variety of organochalcogenides can protect the brain from oxidative stress in vitro and in vivo [36,38,39]. In vivo, ebselen, a more complex organochalcogenide, has been demonstrated to present antioxidant activity in animal models of ischemia [32,35,40]. Most importantly, in humans, ebselen has been used with success in preclinical trials for the treatment of cerebral ischemia and stroke [41,42].

In contrast to ebselen, the use of organotellurides has been questioned by our group, because tellurium is extremely neurotoxic [34,43]. Furthermore, organic forms of tellurium and selenium are potent inhibitors of squalene monooxygenase [44,45]. However, the neurotoxicological properties of simple organoselenides and tellurides have not yet been fully investigated. Recent data from our laboratory have shown that simple organochalcogenides have convulsant activity [46] and ebselen interferes with the glutamatergic system both in vitro and ex vivo [34,47]. The effects of diphenyl diselenide, diphenyl ditelluride, and ebselen are rather complex and depend on whether the exposure was done in vitro or in vivo and whether the release of glutamate from previously loaded synaptosomes was measured in the presence or absence of depolarizing K^+ concentration [34]. However, the mechanism(s) underlying these changes in glutamate release is still unknown.

The main objective of the present study was to determine whether the previous published effect of diphenyl ditelluride, diphenyl diselenide, and ebselen on glutamate transport in synaptosomal preparations could be at least in part related to changes in the Ca^{2+} influx at presynaptic level, under basal and depolarizing conditions. Of particular importance for our working hypothesis, a variety of agents that changes Ca^{2+} channel activity in isolated nerve terminals also changes neurotransmitter release, including glutamate [15,48–52]. The influence of organochalcogenides on Ca^{2+} influx stimulated by 4-aminopyridine (4-AP), a depolarizing drug that functions inhibiting K^+ channels [53–56], was also examined. Furthermore, in view of the fact that organochalcogenides can react with thiol groups of proteins [63,64] and that classical sulfhydryl blocking agents such as mercurials modify Ca^{2+} influx in isolated presynapses [48–51], we can suppose that organochalcogenides may change Ca^{2+} independently of changes in glutamate movements in synaptosomes.

MATERIALS AND METHODS

Drugs

The organochalcogenides diphenyl diselenide [$(PhSe)_2$], diphenyl ditelluride [$(PhTe)_2$], and ebselen [2-phenyl-1,2-benzisoselenazol-3(2*H*)-one] were synthesized in our laboratory. Ebselen was synthesized using the expedient method described by Engman [57]. Diphenyl ditelluride and diphenyl diselenide were synthesized using the method described by Petragnani [58] and Paulmier [59], respectively. Stock solutions were prepared in dimethylsulfoxide (DMSO) just before use. Final concentration of DMSO was 0.5% and did not modify Ca^{2+} influx when compared with influx measured in the absence of DMSO.

4-AP was obtained from Sigma (St. Louis, MO) and [^{45}Ca]$CaCl_2$ was purchased from New England Nuclear. All other reagents were of analytical grade.

Preparation of Crude Synaptosomes

Adult male Wistar rats (age 2–3 months and weighing 200–250 g) from our own breeding colony were used. Animals were housed at constant temperature (22°C) under a regular light–dark schedule (lights on 7.00 a.m. to 7.00 p.m.). Food and water were freely available. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine, and Animal Science of the University of São Paulo, Brazil.

Male Wistar rats were decapitated and their brains removed and forebrains processed, as described previously by Rocha [60] with some modifications. In brief, the tissue was homogenized in 10 volumes of Tris-HCl 5 mM, 0.32 M sucrose, buffered at pH 7.4. The homogenate was first centrifuged (20 min, 1000 *g*) and synaptosomes were then isolated from the supernatant by centrifugation at 12,000 *g* for 20 min. The synaptosomal pellet was then resuspended in the medium described above. All of the above procedures were performed at 0–4°C. Protein was determined using the Bradford method [61].

Calcium Uptake Assays

$^{45}Ca^{2+}$ uptake was carried out essentially as described by Eason and Aronstam [62] with little modifications. In short, two salt solutions were used in these studies: (1) Na^{2+} (unstimulated) buffer contained 130 mM NaCl, 5 mM KCl, 1.2 mM Na_2HPO_4, 0.1 mM $CaCl_2$, 10 mM glucose, and 20 mM Tris-HCl, pH 7.4; (2) K^+ (stimulated) buffer, where the NaCl was omitted and the KCl concentration raised to 135 mM.

To measure calcium influx, 25 μL of synaptosomes was added to prewarmed Na^+ or K^+ buffer mixtures containing 2–3 μCi $^{45}Ca^{2+}$ to give a final protein concentration of 0.4–0.6 mg/mL. After 30 s, the samples were filtered on Whatman GF/B glass-fiber filters. The filters were washed with 9 mL of cold low K^+ medium (nondepolarizing) or high K^+ medium (depolarizing) in accord to incubation medium. Ca^{2+} influx was also determined using 10-s incubation and the effects of organochalcogenides were essentially similar to those obtained using 30-s incubation ($n = 2$ for each chalcogenide). The only exception was the fact that 4-AP did not induce an increase in Ca^{2+} influx when 10-s incubations were used. The radioactivity content was determined by liquid scintillation counting. All experiments were performed in triplicate.

Statistical Analysis

All values were presented as mean ± SEM. ANOVA-followed Duncan's post hoc test was used to evaluate difference between groups. The obtained P values less than 0.05 were regarded as significant.

RESULTS

Ca^{2+} influx into synaptosomes increased proportionally as K^+ concentration increased in the reaction mixture (Figure 1) and was more than doubled at 135 mM of KCl. Trend analysis revealed a significant linear effect of KCl concentrations for Ca^{2+} influx into synaptosomes ($P < 0.01$). These results are essentially identical to those reported by Eason and Aronstam [62].

Ca^{2+} influx into synaptosomes increased proportionally with increasing concentrations of 4-AP (Figure 2). Trend analysis revealed a significant linear effect of the dose of 4-AP for Ca^{2+} influx ($P < 0.05$).

Under nondepolarizing conditions, ebselen (up to 100 μM) did not change Ca^{2+} influx, but at a high concentration (400 μM) produced an accentuated increase in Ca^{2+} influx into synaptosomes. Similar results were obtained when 4-AP was used as depolarizing agent. In contrast, ebselen caused a dose-dependent inhibition of Ca^{2+} influx stimulated by 135 mM KCl (Figure 3). One-way ANOVA yielded a significant effect of ebselen ($P < 0.01$, for all conditions; Figure 3). The dose–effect relationship was assessed by partitioning the total sum of squares into trend components and a significant linear effect was obtained for the inhibitory effect of ebselen on K^+-evoked Ca^{2+} influx into synaptosomes ($P < 0.05$).

Diphenyl ditelluride inhibited Ca^{2+} influx into synaptosomes both when nondepolarizing and depolarizing conditions using 135 mM KCl or 3 mM 4-AP were used. One-way ANOVA yielded a significant effect of telluride ($P < 0.01$, for all conditions; Figure 4). Dose–effect relationship was assessed by partitioning the total sum of squares into trend components and

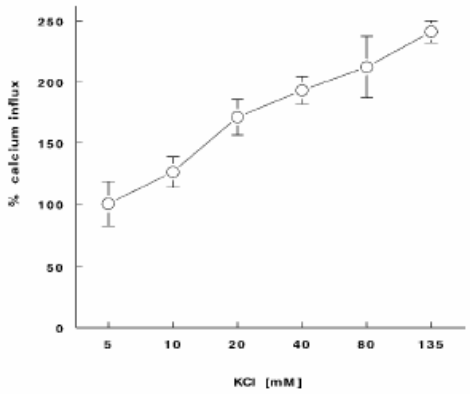

FIGURE 1. Calcium uptake (% of control) into synaptosomes as a function of K^+. Synaptosomes were prepared as described in Materials and Methods section and diluted in medium containing 2–3 μCi of ^{45}Ca (total [Ca] = 0.1 mM, containing 1.2 mM Na_2HPO_4, 0.1 mM $CaCl_2$, 10 mM glucose, and 20 mM Tris-HCl, pH 7.4; and indicated concentration of KCl). NaCl concentration was adjusted in such a way that the final KCl plus NaCl concentration was 135 mM. Value of 100% influx was 1.03 ± 0.12 nmol $^{45}Ca^{2+}$/mg protein/30 s. Data are expressed as mean ± SEM for four independent experiments carried out in triplicate. Duncan's multiple range test revealed a significant effect of KCl from 20 to 135 mM.

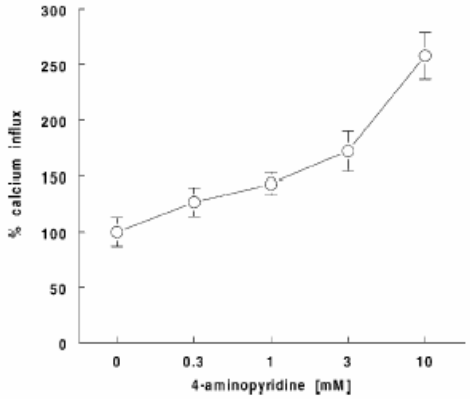

FIGURE 2. Calcium uptake (% of control) into synaptosomes as a function of 4-AP concentration. Value of 100% influx was 0.93 ± 0.2 nmol $^{45}Ca^{2+}$/mg protein/30 s. Data are expressed as mean ± SEM for three independent experiments carried out in triplicate. Duncan's multiple range test revealed a significant effect of 4-AP from 1 to 10 mM.

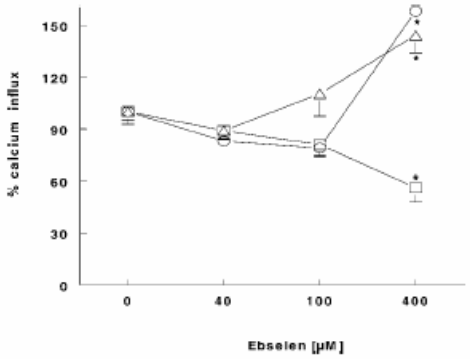

FIGURE 3. Influence of ebselen (0–400 μM) on Ca^{2+} influx induced by high K^+ (135 mM, squares), or 4-AP (3 mM, triangles) into synaptosomes. Values are expressed as % of calcium uptake. Values of 100% influx were 1.15 ± 0.14 nmol $^{45}Ca^{2+}$/mg protein/30 s (nondepolarizing condition, circles); 2.8 ± 0.16 nmol $^{45}Ca^{2+}$/mg protein/30 s (high K^+ medium, squares); and 1.57 ± 0.16 nmol $^{45}Ca^{2+}$/mg protein/30 s (4-AP, triangles). Data are expressed as mean ± SEM for three to six independent experiments carried out in triplicate. $^*P < 0.05$, difference from control (without ebselen) by Duncan's multiple range test.

the analysis revealed a significant linear effect of the dose for depolarizing condition with KCl ($P < 0.05$).

Diphenyl diselenide (40 and 100 μM) decreased Ca^{2+} influx into synaptosomes in a nondepolarizing (low K^+ medium) condition. The effect under depolarizing conditions varied depending on the depolarizing agent. In fact, at low concentrations of $(PhSe)_2$ a significant inhibition of Ca^{2+} influx was observed when 4-AP was used as the depolarizing agent. In contrast, $(PhSe)_2$ did not modify Ca^{2+} influx when KCl was used as the depolarizing agent (Figure 5). One-way ANOVA revealed a significant effect of $(PhSe)_2$ ($P < 0.01$).

DISCUSSION

The results of the present study indicate that the effects of organochalcogenides were rather complex depending on the condition and the depolarizing agent used. In fact, $(PhSe)_2$ reduced Ca^{2+} influx into isolated nerve endings when a nondepolarizing condition was used or when 4-AP was used as depolarizing agent. Ebselen, at high concentrations, increased Ca^{2+} influx into synaptosomes when a nondepolarizing condition or 4-AP was used as the depolarizing agent. In contrast, ebselen caused a concentration-dependent inhibition of Ca^{2+} influx when KCl was used as the depolarizing agent. Differing from the other two compounds, $(PhTe)_2$ inhibited Ca^{2+} influx into synaptosomes both under a nondepolarizing and a depolarizing condition, regardless of the depolarizing agent used.

Apparently ebselen and diphenyl ditelluride interact with Ca^{2+} channels that were gated by depolarizing

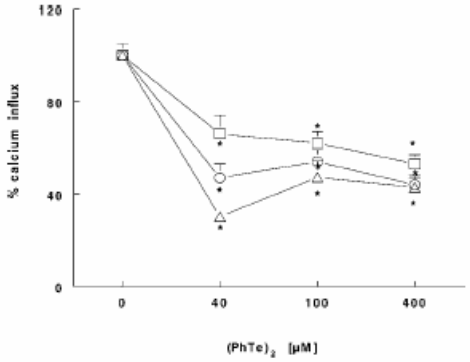

FIGURE 4. Influence of $(PhTe)_2$ (0–400 μM) on Ca^{2+} influx induced by high K^+ (135 mM, squares), or 4-AP (3 mM, triangles) into synaptosomes. Values are expressed as % of calcium uptake. Values of 100% influx were 1.11 ± 0.20 nmol $^{45}Ca^{2+}$/mg protein/30 s (nondepolarizing condition, circles); 2.83 ± 0.09 nmol $^{45}Ca^{2+}$/mg protein/30 s (high K^+ medium, squares); and 1.61 ± 0.14 nmol $^{45}Ca^{2+}$/mg protein/30 s (4-AP, triangles). Data are expressed as mean ± SEM for three to five independent experiments carried out in triplicate. $^*P < 0.05$, difference from control (without telluride) by Duncan's multiple range test.

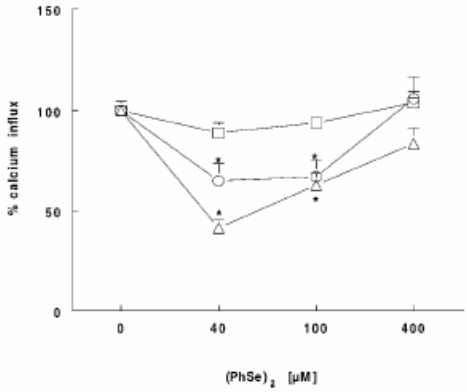

FIGURE 5. Influence of $(PhSe)_2$ (0–400 μM) on Ca^{2+} influx induced by high K^+ (135 mM, squares), or 4-AP (3 mM, triangles) into synaptosomes. Values are expressed as % of calcium uptake. Values of 100% influx were 1.13 ± 0.19 nmol $^{45}Ca^{2+}$/mg protein/30 s (nondepolarizing condition, circles); 2.85 ± 0.14 nmol $^{45}Ca^{2+}$/mg protein/30 s (high K^+ medium, squares); and 1.58 ± 0.12 nmol $^{45}Ca^{2+}$/mg protein/30 s (4-AP, triangles). Data are expressed as mean ± SEM for three to six independent experiments carried out in triplicate. $^*P < 0.05$, difference from control (without organoselenide) by Duncan's multiple range test.

concentration of K^+, whereas diphenyl diselenide did not inhibit Ca^{2+} influx under similar conditions. As pointed out above, changes in Ca^{2+} movements can interfere and disrupt a variety of neurophysiological processes [18–21,24]. Consequently, the previously reported, high neurotoxicity of $(PhTe)_2$ can be tentatively linked to its effect on Ca^{2+} fluxes at the presynapse determined experimentally in the present study using both nondepolarizing and depolarizing conditions induced either by KCl or 4-AP. Furthermore, these results indicated that $(PhTe)_2$, $(PhSe)_2$, and ebselen interact in a distinct way with Ca^{2+} channels. The differences are tentatively linked to the superior chemical reactivity of tellurides in relation to selenides analogous [31]. Alternatively, $(PhTe_2)$, because of its higher lipophilicity in relation to ebselen, may have access to the Ca^{2+} channels by more than one route, including diffusion through the membrane and entry through the channel pore, while ebselen gets access to its target on the protein channel only after channel activation by K^+.

The mechanism by which $(PhTe)_2$ and ebselen interact with Ca^{2+} channel is unknown, but may be related to reactivity with critical sulfhydryl groups in the protein complex. In fact, there is an expressive quantity of data showing that ebselen and $(PhTe)_2$ react with sulfhydryl groups of proteins [63–65]. Furthermore, *N*-ethylmaleimide, methyl mercury, and other mercurials, which have high affinity for sulfhydryl groups, are potent inhibitors of Ca^{2+} channels [24,62,66]. In most cases, the influence of organochalcogenides on Ca^{2+} influx measured in the presence of 4-AP was comparable to that measured in its absence. However, there was an exception: diphenyl diselenide did not change Ca^{2+} influx measured in the presence of high K^+ concentration, whereas diphenyl diselenide at low concentrations inhibited Ca^{2+} influx into synaptosomes in the presence of 4-AP. This result can be explained by the fact that the mechanism through which 4-AP and high K^+ induced elevation of intracellular Ca^{2+} is not exactly coincident [67–71]. Of toxicological and pharmacological significance, these observations might suggest potential targets for $(PhSe)_2$ activity. 4-AP, at least in part, depolarizes synaptosomes by a mechanism that involves voltage-gated Na^+ channels and, in line with this, TTX, an antagonist of Na^+ currents, abolishes or reduces the physiological, biochemical, and pathological effect of 4-AP [72–74]. Consequently, it is possible to speculate that $(PhSe)_2$ can directly block these sodium channels and inhibit Ca^{2+} influx into synaptosomes.

In conclusion, the results of the present investigation indicate that the effect of organochalcogenides on Ca^{2+} entry into synaptosomes is rather complex and varies considerably depending on the depolarizing condition of the medium. In fact, changes in Ca^{2+} flux caused by organochalcogenides might result from a direct interaction of these compounds with Ca^{2+} channel proteins or even indirectly by changes in the activity of proteins that modulates synaptosomal membrane depolarization, including Na^+ channels. Diphenyl ditelluride is extremely neurotoxic for rodents [34,64], whereas ebselen and diphenyl diselenide do not exhibit gross neurotoxic effect in rodents even when administered in doses higher than diphenyl ditelluride. Ebselen has been used with success in the treatment of ischemia stroke and subarachnoid hemorrhage [19,41]. Diphenyl ditelluride was the only compound tested that inhibited Ca^{2+} influx under nondepolarizing and depolarizing conditions. Whether the broad-spectrum action of diphenyl ditelluride is related to its potent neurotoxicity deserves further investigation. One important find of the present investigation is that the inhibitory and stimulatory effect of ebselen on Ca^{2+} influx under depolarizing and nondepolarizing conditions, respectively, is coincident with previous results from our laboratory showing that ebselen stimulates glutamate release under nondepolarizing conditions and inhibits its release under depolarizing conditions. Since a variety of agents that changes Ca^{2+} channel activity in isolated nerve terminals [15,48,50,51] also change neurotransmitter release [49] it is plausible to suppose that the effects of ebselen on glutamate release [47] and Ca^{2+} influx are at least in part related.

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V.2. Artigo 2

Moretto, M. B.; Franco, J.; Posser, T.; Nogueira, C.W.; Zeni, G.; and Rocha,J.B.T.**2004**. Ebselen Protects Ca^{2+} Influx Blockage But Does Not Protect Glutamate Uptake Inhibition Caused By Hg^{2+} ***Neurochem. Res***. 10,1-6.

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Ebselen Protects Ca^{2+} Influx Blockage But Does Not Protect Glutamate Uptake Inhibition Caused By Hg^{2+}

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The goal of this study was to investigate the isolated and combined effect of ebselen and Hg^{2+} on calcium influx and on glutamatergic system. We examined the *in vitro* effects of 2 phenyl-1,2-benzisoselenazol-3(2H)-ona), (Ebselen) on $^{45}Ca^{2+}$ influx in synaptosomes of rat at rest and during depolarization and glutamate uptake into synaptosomes. Entry of ^{45}Ca was measured during exposure to mercury in non-depolarizing and depolarizing solutions. Ebselen abolished the inhibition of $^{45}Ca^{2+}$ influx on non-depolarizing conditions; however, ebselen did no modify inhibition uptake of $^{45}Ca^{2+}$ caused by Hg^{2+} in high K^+ depolarizing medium. Ebselen did not modify glutamate uptake inhibition caused by Hg^{2+} in synaptosomes. These results indicate that ebselen has an *in vitro* protective effect against Hg^{2+} induced inhibition of Ca^{2+} influx into synaptosomes, depending on the depolarizing conditions of the assay. The effects of Hg^{2+} on glutamate uptake were not modified by ebselen, suggesting that its protection is dependent on the target protein considered.

KEY WORDS: Calcium uptake; ebselen; glutamate uptake; mercury; synaptosomes.

INTRODUCTION

Mercury (Hg) contamination of the environment has received considerable attention because of its inherent toxicity to living forms (1–5). Mercurial compounds have been shown to be particularly damaging to the developing brain, resulting in degenerative alterations and biochemical changes, both in humans and in experimental animals (6–10).

Functional alterations of signal transduction can also occur after exposure to chemical toxicants and environmental pollutants (11–13). Ca^{2+} channels and Ca^{2+} transport systems are affected by interaction with metals. Due to the crucial role that Ca^{2+} plays in neurotransmitter release and the fact its action is controlled primarily by voltage-dependent Ca^{2+} channels, much attention has been directed at examining effects of mercurials on voltage-dependent Ca^{2+} channels (14). Blockage of Ca^{2+} channels has been proposed to mediate the reduction in evoked transmitter release observed following treatment with mercurials and has been investigated in several model systems, using a variety of techniques (15–17).

Alterations of the neurotransmitter release and re-uptake systems in CNS have been reported in a variety of neuropathological processes associated with heavy metal toxicity (18–22). For instance, glutamate, an excitatory amino acid which plays a variety of neurophysiological roles, including the development of the central nervous system, synapse induction and elimination, and cell migration, differentiation and death (23) has its release/re-uptake altered by mercury. Inhibition of glutamate transport is considered a potential indirect cause of excitotoxic damage by glutamate in the CNS. The mercuric ion,

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the form in which metallic mercury vapor is believed to exert its neurotoxic action, is a known inhibitor of amino acid transport (24). Mercury lowers the threshold for glutamate neurotoxicity presumably by inhibiting glutamate uptake (25–28).

The seleno-organic compound Ebselen has anti-inflammatory and anti-oxidant properties, which have been demonstrated in a variety of *in vivo* and *in vitro* models (29–34). In addition, Ebselen has glutathione peroxidase-like activity (35) and reduces excitotoxicity provoked by glutamate in rat neurons (36–39). Ebselen reverses the Me-Hg-induced glutamate release and reduces the inhibition of glutamate uptake caused by methylmercury in slices obtained from animals exposed to methylmercury (40,41).

Recently, data from our laboratory showed that organoilchalcogenides induced changes in the Ca^{2+} influx into isolated pre-synaptic terminals in a voltage-dependent manner (42). These results indicate that ebselen can interact with Ca^{2+} channels and, consequently, can be considered as a potential modifying agent of inorganic mercury actions at these Ca^{2+} channels.

As pointed out above, inhibition of calcium uptake by Hg^{2+} can play an important role in cellular homeostasis, it follows that intracellular Ca^{2+} levels disruption by mercurials can lead to potentially deleterious consequences for the cell. The present study investigates the possible protection of ebselen against mercurial-induced blockage of Ca^{2+} influx and on the glutamate uptake into brain synaptosomes.

EXPERIMENTAL PROCEDURE

Animals. Male albino adult Wistar rats (200–250 g) from our own breeding colony were maintained at 25°C, on a 12 h light/12 h dark cycle, with free access to food and water.

Synthesis of Ebselen. Ebselen was synthesized using the expedient method described by Engman (43)

Preparation of synaptosomes. Male Wistar rats were decapitated and their brains removed and forebrains processed (44) with some modifications. In brief, the tissue was homogenized (12 strokes at 900 rpm) in 10 vol of 5 mM Tris–HCl, 0.32 M Sucrose, buffered at pH 7.4. The homogenate was first centrifuged (20 min, 1000 *g*) and synaptosomes were then isolated from the supernatant by centrifugation at 12000 g for 20 min. The synaptosomal pellet was then resuspended in the medium described above. All of the above procedures were performed at 0°–4°C. Protein was estimated using the method of Bradford (45).

Calcium uptake assays. Ca^{2+} uptake was carried out essentially as described by Eason and Aronstam (46) with minor modifications. In short, two salt solutions were used in these studies: **(1)** Na (non-depolarized) buffer contained 130 mM NaCl, 5 mM KCl, 1.2 mM Na_2HPO_4, 0.1 mM $CaCl_2$, 10 mM Glucose, and 20 mM Tris–HCl, pH 7.4; **(2)** K^+ (Depolarizing) buffer, where the NaCl was omitted and the KCl concentration raised to 135 mM. To measure calcium influx, 25 µl of synaptosomes were added to pre-warmed Na or K buffer mixtures containing 2–3 µCi $^{45}Ca^{2+}$ to give a final protein concentration of 0.4–0.6 mg/ml. After 30 s, the samples were filtered on Whatman GF/B glass fiber filters. The filters were washed three times with 3 ml of cold Na buffer containing 0.1 mM $La(NO_3)_2$ and their radioactivity content determined by liquid scintillation counting. All experiments were performed in triplicate.

[^{3}H]Glutamate uptake by synaptosomes. Preparation of rat brain synaptosomes was performed according two methods: (a) as described by Shank and Campbell(47), which was enriched in synaptic terminals and contained only small amounts of myelin and few, if any, free mitochondria. This fraction of cellular material is referred to as low-density synaptosomes (LDS); (b) P_2 pellet was obtained as described above (44).

Incubation procedure for uptake experiments. The final pellet for both preparations was resuspended in 0.3 M sucrose, 15 mM Tris/acetate buffer (pH 7.4), and incubated in HBSS (Hepes buffered salt solution, composition in mM: 27 HEPES, 133 NaCl, 2.4 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 12 Glucose, 1.0 $CaCl_2$), pH 7.4 (adjusted with HCl), in the presence of [^{3}H] glutamate (final concentration 100 nM) in the absence or in the presence of 100 µM $HgCl_2$, 100 µM Ebselen (PZ 51), for 1 min at 37°C. The reaction was stopped by filtration through GF/B filters. The filters were washed three times with 3 ml of ice-cold 15 mM Tris/acetate buffer (pH 7.4) in 155 mM ammonium acetate. The radioactivity retained on the filters was measured in a Packard scintillation counter. 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 choline chloride replace NaCl.

Statistical analysis. Data were analyzed statistically by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test, when appropriate. Differences between groups were considered to be significant when $P < 0{,}05$.

RESULTS

Hg^{2+} caused a significant reduction on K^+-induced Ca^{2+} influx into brain synaptosomes (Table 1). In the absence of depolarizing K^+ con-

Table I. Calcium uptake (% of control) into synaptosomes as a function of concentration of Hg^{2+}.

Concentration Hg^{2+} (µM)	Ca^{2+} uptake (% of mercurial-free control)
0,1	89 (± 12)
1,0	95 (± 11)
10	97 (± 9.5)
40	77 (±4.5)
75	43 (± 4)*
100	40 (± 4)*

Note: Synaptosomes were prepared as described in Materials and Methods. $^{45}Ca^{2+}$ uptake is expressed as percentage of untreated control. Values are the means of three experiments with each experimental value being the average of three replicates. Data are expressed as mean ±SEM and $P < 0.05$, difference from control by Duncan's multiple range test.

centrations, Hg^{2+} caused a similar inhibition of Ca^{2+} influx (data not shown). In a recent work (42), we showed that under non-depolarizing condition, ebselen (100 μM) did not modify Ca^{2+} influx into synaptosomes. Here we showed that ebselen, under non-depolarizing condition, abolished the inhibitory effect of Hg^{2+} on Ca^{2+} influx into synaptosomes (Fig. 1A). Under K^+-induced depolarizing condition, ebselen caused a small but significant inhibition (30%) of Ca^{2+} influx into synaptosomes. However, ebselen did not modify the inhibitory effect caused by Hg^{2+} on Ca^{2+} influx measured in the presence of high concentration of K^+ (Fig. 1B).

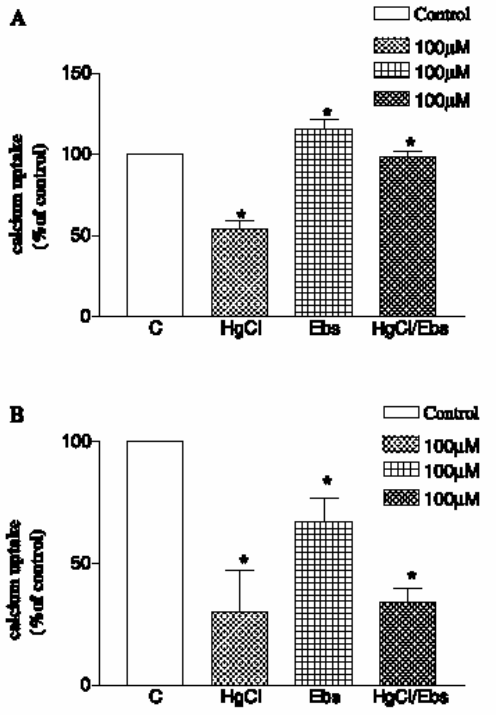

FIG. 1. (A) Effect of Ebselen on Hg^{2+}- induced block of basal calcium influx into brain synaptosomes (under non-depolarizing). $^{45}Ca^{2+}$ uptake is expressed as percentage of untreated control. Values are the means of three experiments with each experimental value being the average of three replicates. Data are expressed as mean ± SEM and $P < 0,05$, difference from control by Duncan's multiple range test. (B) Influence of Ebselen on Hg^{2+}-induced block calcium influx into brain synaptosomes (under depolarizing medium). $^{45}Ca^{2+}$ uptake is expressed as percentage of untreated control. Values are the means of three experiments with each experimental value being the average of three replicates. Data are expressed as mean ± SEM and $P < 0,05$, difference from control by Duncan's multiple range test.

The [^{3}H] glutamate uptake into crude brain synaptosomes was drastically inhibited by 50 μM $HgCl_2$ and Ebselen (50 μM) did no modify control or $HgCl_2$-inhibited glutamate uptake into synaptosomes. (Fig. 2A). We also performed experiments with a highly purified synaptosome fractions prepared from rat brain, and a similar result was obtained when compared to that obtained using crude synaptosomal fraction (Fig. 2B).

DISCUSSION

The highly reactive nature of mercurials, due to interaction with various functional groups present

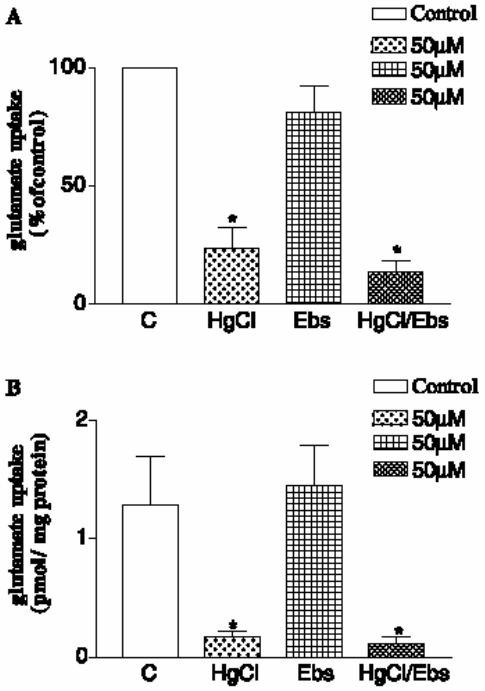

Fig. 2. (A) Effect of Ebselen in vitro on [^{3}H] glutamate uptake by brain crudes synaptosomes of rats. Glutamate uptake is expressed as percentage of total radioactivity content. Data are mean ± SEM from three independent experiments done in triplicate.(*) $P < 0,05$ compared to control, ANOVA/Duncan. (B) Effect of Ebselen *in vitro* on [^{3}H] glutamate uptake by Low-Density Synaptosomes (LDS) of brain of rats. Glutamate uptake is expressed as pmol/min/mg of protein. Data are mean ± SEM from three independent experiments done in triplicate.(*) $P < 0,05$ compared to control, ANOVA/Duncan.

in biological membranes and proteins, allows for the potential disruption of numerous functional processes within the nervous system, as well as other sites in the body (14). In view of their location on the plasma membrane and strict regulation by various intracellular mediators, Ca^{2+} channels are a likely target of mercurials (48). In this study, we have observed that mercury decreases calcium influx and glutamate uptake into synaptosomes *in vitro*. These results confirm data from literature (24,49,50) and we shown for the first time, in this study, that ebselen, *in vitro*, can counteract the inhibitory effect of Hg^{2+} on Ca^{2+} influx into synaptosomes. However, the protective effect of ebselen depended on the depolarizing condition of the medium. In fact, under non-depolarizing conditions ebselen cancelled the inhibition of Ca^{2+} influx into brain synaptosomes mediated by Hg^{2+}. However, under high-K^+ depolarizing condition, ebselen caused a small reduction and did no modify the inhibitory effect of Hg^{2+} on Ca^{2+} influx into synaptosomes. These results may indicate that Hg^{2+} interacts in a distinct way with Ca^{2+} channels, depending on the state of cell depolarization. In line with this, Atchison and co-workers have previously demonstrated that the nature of Ca^{2+} influx inhibition by mercurials is dependent upon the presence of depolarizing agents (51).

Hg^{2+} caused a marked inhibition of glutamate uptake, which is in agreement with literature data (23,52). In fact, glutamate transporter seems to be very sensitive to oxidants agents (53), including mercurials. In contrast to that observed with Ca^{2+} influx, ebselen did not cancel the marked inhibition of glutamate uptake caused by Hg^{2+}. Recent studies from our laboratory have shown that *in vivo* treatment with ebselen recuperates glutamate uptake inhibited by methylmercury experimental poisoning (40,41). Ebselen protection was tentatively attributed to its antioxidant properties. The mechanism underlying the neuroprotection afforded by ebselen is still not completely understood, but it is well established that ebselen inhibits both nonenzymatic and enzymatic lipid peroxidation in cells and has anti-inflammatory activity in various animal models (54,55). It also directly inhibits 5-lipoxygenase, nitric oxide synthases, NADPH oxidase, protein kinase C, and ATPase by chemically modifying an SH-group forming a selenosulfide complex (56–60). Ebselen is an excellent substrate for mammalian thioredoxin reductase and a highly efficient oxidant of reduced thioredoxin and catalyzes H_2O_2 reduction suggesting that the antioxidant and many actions of ebselen are to a large extent, due to reactions with the thioredoxin system (61,62).

The *in vitro* absence of ebselen protection against Hg^{2+} inhibition of glutamate uptake demonstrates indirectly that they are not interacting chemically in the reaction medium. In fact, if ebselen had reacted in a stable way with Hg^{2+}, the protective effect against Hg^{2+} would appear for both Ca^{2+} influx (regardless of the depolarizing condition of the medium) and glutamate uptake. With regard Hg^{2+} effect on Ca^{2+} influx, there is a methodological problem that must be considered. In particular, because we employed the crude mitochondrial fraction (which contains not only synaptosomes but also mitochondria and myelin particles), the effects observed might be attributable, at least in part, to the presence of mitochondria and/or myelin, both of which can take up calcium (63–65).

In conclusion, our experiments confirm the inhibitory effect of Hg^{2+} on Ca^{2+} influx and glutamate uptake into isolated pre-synaptic terminals. And, now we showed that under non-depolarizing conditions ebselen, a seleno-organic compound with neuroprotective actions, protects against mercurial-induced blockage of Ca^{2+} influx calcium into brain synaptosomes. However, ebselen did not protect glutamate uptake from the inhibitory effect of Hg^{2+}. Thus, *in vitro*, the protective effect of ebselen depends on the neural component considered. In fact, the inhibitory effect of Hg^{2+} on Ca^{2+} influx was abolished, whereas ebselen did not protect glutamate transport into synaptosomes against the inhibitory action of Hg^{2+}.

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V.3. Artigo 3

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Ebselen protects glutamate uptake inhibition caused by methyl mercury but does not by Hg^{2+}

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Abstract

Alterations of the neurotransmitter release systems in CNS have been reported in a variety of neuropathological processes associated with heavy metal toxicity. Neurotoxic effects of mercurials were investigated in vitro in cerebral cortex slices from young rats. The present study indicates that: (i) the environmental contaminants methylmercury (MeHg) and mercuric chloride (Hg^{2+}) (50 μM) inhibited the glutamate net uptake from the cerebral cortex of 17-day-old rats; (ii) ebselen (10 μM) reverted the MeHg-induced inhibition of glutamate net uptake but did not protect the inhibition caused by Hg^{2+}. At same time, we investigated another diorganochalcogenide, diphenyl diselenide $(PhSe)_2$ and it was observed that this compound did not revert the action of MeHg or Hg^{2+}; (iii) in addition, we observed that exposure of slices to 50 μM MeHg and Hg^{2+} for 30 min followed by Trypan blue exclusion assay resulted in 58.5 and 67.5% of staining cells, respectively, indicating a decrease in cell viability. Ebselen protected slices from the deleterious effects of MeHg, but not of Hg^{2+} on cell viability. Conversely, ebselen did not modify the reduction of MTT caused by MeHg and Hg^{2+}; (iv) the protective effect of ebselen on MeHg-induced inhibition of glutamate net uptake seems to be related to its ability in maintaining cell viability.

Keywords: Organochalcogens; Methylmercury; Glutamate; Ebselen; Cell death

1. Introduction

Mercurial compounds are global environmental contaminants deriving from natural processes and from anthropogenic activities, which possess neuro-

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toxic potential (Takeuchi et al., 1989; Harada, 1995; Aschner et al., 2000). Methylmercury (MeHg) is the principal environmental form of mercury because it can be bioaccumulated in the food chain and cause adverse effects on human health (El-Demerdash, 2001; Allen et al., 2001; Clarkson et al., 2003). MeHg can be demethylated to inorganic mercury (Hg^{2+}) in the brain after long-term MeHg exposure. Human exposure to mercury vapor can occur from the use of dental amalgam as well as in industries, which use mercury (Clarkson, 1997).

Methylmercury is a highly neurotoxic compound and the mechanism underlying its toxicity is not fully understood. The main mechanisms involved in MeHg neurotoxicity currently explored are related to oxidative stress, the disruption of ion intracellular calcium homeostasis and the inhibition of glutamate uptake by astrocytes (Brookes, 1988; Aschner et al., 1990; Sirois and Atchison, 2000; Gassó et al., 2001; Juárez et al., 2002). In fact, MeHg and Hg^{2+} cause severe alterations in the central nervous system that may be due to their ability to disrupt synaptic transmissions (Atchison and Hare, 1994; Gassó et al., 2000), producing neuronal death that is partially mediated by glutamate (Yamashita et al., 1997).

Although MeHg produces neuronal death (Vahter et al., 1995; Limke and Atchison, 2002), mercurials are located predominantly within astrocytes (Takeuchi et al., 1989; Leyshon-Shorlan et al., 1994). One of the most important aspects of MeHg neurotoxicity is its ability to damage the developing nervous system (Charleston et al., 1994; Vitarella et al., 1996; Philbert et al., 2000; Castoldi et al., 2001). Developing cortical neurons have been well documented to be extremely vulnerable to the toxic effect of methylmercury, leading to alterations of the structure and functionality of the nervous system (WHO, 1990; Castoldi et al., 2001; Sanfeliu et al., 2003). Indeed, in the developing brain, methylmercury is toxic to the cerebral and cerebellar cortex, causing focal necrosis of neurons the destruction of glial cells and the inhibition of axonal development (Goldman and Shannon, 2001; Castoldi et al., 2001; Heidemann et al., 2001). In addition, in vivo and in vitro MeHg exposure can cause apoptosis and necrosis with distinct morphological and molecular features (Nakada and Imura, 1983; Miura et al., 1999; Dare et al., 2000).

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS). It is involved in most brain functions (Ozawa et al., 1998; Anderson and Swanson, 2000; Meldrum, 2000; Danbolt, 2001) such as memory and learning (Izquierdo and Medina, 1997), development and aging (Segovia et al., 2001), and adaptation to the environment (Danbolt, 2001; Mattson et al., 2002; Warren, 2002). At high concentrations, glutamate may also act as an excitotoxin by providing the excessive stimulation of its receptors (Anderson and Swanson, 2000; Meldrum, 2000; Danbolt, 2001; Maragakis and Rothstein, 2001). Glutamate toxicity has been related to neuronal death in ischemia, hypoxia, hypoglycemia and trauma (Choi, 1988; Ikonomidou et al., 1989) and with many chronic neurodegenerative disorders of the CNS, including Huntington's and Alzheimer's, and *status epilepticus* (Brewer, 2000; Danbolt, 2001; Ingram et al., 2001; Maragakis and Rothstein, 2001; Segovia et al., 2001).

Glutamate uptake is the process responsible for the maintenance of extracellular glutamate concentrations below neurotoxic levels. The subtypes of glutamate transporters are expressed regionally in distinct patterns, with glial and neuronal transporter expression appearing in a coordinated manner during CNS development (Danbolt, 1994, 2001; Ullensvang et al., 1997; Furuta et al., 1997).

Organoselenium compounds have been reported to possess chemical and biological anti-oxidant properties (Muller et al., 1984; Sies, 1993; Bosch-Morell et al., 1999, 2002; Mugesh and Singh, 2000; Mugesh et al., 2001). Of particular importance, the organoselenium compound ebselen (2-phenyl-1,2-benzisoselenazole-3[2]-one), a lipid-soluble seleno-organic compound that is a potent anti-oxidant agent, has shown to be neuroprotective in pre-clinical studies and in a variety of in vitro and in vivo animal models of neuropathological conditions, including ischemia (Dawson et al., 1995; Takasago et al., 1997; Saito et al., 1998; Yamaguchi et al., 1998; Davalos, 1999; Parnham and Sies, 2000; Imai et al., 2003; Porciúncula et al., 2003), quinolinic acid- or glutamate-induced lipoperoxidation (Porciúncula et al., 2001; Rossato et al., 2002a,b), alterations on glutamatergic homeostasis at the pre-synaptic level (Nogueira et al., 2001), and exposure to methylmercury (Farina et al., 2003a,b). Similarly to ebselen, diphenyl diselenide, a simple organochalcogenide

has anti-oxidant properties (Nogueira et al., 2004) and, of particular importance, recently, we showed that ebselen and diphenyl diselenide abolished the inhibition caused by methylmercury on intermediate filament protein phosphorylation from brain slices of suckling rats (Moretto et al., 2005).

Taking into account the vast number of reports showing that the uptake and release of neurotransmitters are disrupted by mercury, the purpose of our research was to investigate whether the deleterious effect of methylmercury (MeHg) and mercuric chloride (Hg^{2+}) on the glutamate uptake could be modified by diphenyl diselenide $(PhSe)_2$ and ebselen in the cerebral cortex of developing rats. In addition, although it is well documented that sulfhydryl agents, including MeHg and Hg^{2+} can oxidize glutamate transporters (Albrecht et al., 1993; Sarafian and Verity, 1991; Aschner et al., 2000), there is little data regarding cell viability after short-term exposure to mercurials. Thus, we sought to determine whether the neurotoxicity of MeHg and Hg^{2+} could also be attributed to a secondary effect on slice viability.

2. Materials and methods

2.1. Chemicals

L-^{3}[H] glutamate was purchased from Amersham International. The organochalcogenides diphenyl diselenide [$(PhSe)_2$] and ebselen [2-phenyl-1,2-benzisoselenazol-3(2H)-one] were synthesized in our laboratory. Ebselen and diphenyl diselenide was synthesized using the method described by Engman (1989) and Paulmier (1986), respectively. Stock solutions were prepared in dimethylsulfoxide (DMSO) just before use. The final concentration of DMSO was 0.5% and this did not modify the glutamate uptake. Other chemicals were of analytical reagent grade and purchased from Sigma.

2.2. Animals

Seventeen-day-old Wistar rats were obtained from our own breeding colony. They were maintained at approximately 25 °C, on a 12-h light/12-h dark cycle, with free access to food and water. All experiments were conducted in accordance with the Guiding Principles of the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989.

2.3. Glutamate net uptake

Cortex slices (400 μm) were obtained using a McIlwain chopper. The slices were washed with HBSS solution containing (in mM) 137 NaCl, 0.63 $Na_2HPO_4{\cdot}7H_2O$, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2{\cdot}2H_2O$, 0.41 $MgSO_4{\cdot}7H_2O$; 0.49 $MgCl_2{\cdot}6H_2O$ and 5.55 glucose, adjusted to pH 7.2. The glutamate net uptake was performed according to Frizzo et al. (2002). Slices were preincubated at 35 °C for 23 min in the presence or absence of 10, 50 μM MeHg, HgCl or 10 μM ebselen, $Ph(Se)_2$. In some experiments, 50 μM MeHg plus 10 μM ebselen or $Ph(Se)_2$ and 50 μM HgCl plus 10 μM ebselen or $Ph(Se)_2$ were used to better evaluate the effect of these mercurials on glutamate net uptake into the cortical slices. Uptake was started by adding 0.33 μCi/ml L-[^{3}H] glutamate with 100 μM unlabeled glutamate in HBSS to the slice medium. The reaction was stopped after 7 min by two ice-cold washes with 1 ml HBSS, immediately followed by the addition of 0.5 M NaOH, which was kept overnight. Sodium independent uptake (non-specific uptake) was determined by using *N*-methyl-D-glucamine instead of sodium chloride. Sodium dependent net uptake was calculated as the difference between the net uptake measured in a medium containing sodium and the uptake measured in a similar medium in the absence of sodium. Radioactivity incorporated was determined with a Wallac scintillation spectrometer. Protein concentration was measured by the method of Peterson (1977). All experiments were performed in triplicate.

2.4. Trypan blue exclusion assay

At the final of incubation accordingly described above each slice was transferred to a tube containing 400 μl of Trypsin/EDTA (Gibco) and maintained at 37 °C. After 5 min, it was added fetal calf serum to inactivate trypsin. Thus, the slices were mechanically dissociated by sequential passage through a Pasteur pipette and the cell suspension allowed to settle during 10 min to remove residual intact tissue. An aliquot (50 μl) of the cell suspension was transferred to another

tube followed by addition of 2.5 μl of 1.3% trypan blue solution. After 2 min, cells were counted in a hemocytometer by phase-contrast in an inverted light microscope at 100× magnification. Each value indicates the percentage of viable cells obtained from a mean of the number of viable cells counted in four squares of the chamber in three separated experiments.

2.5. Tetrazolium salt method (MTT assay)

The viability assay was performed by the colorimetric 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Slices from 17-day-old rats were preincubated at 35 °C for 30 min in HBSS in the presence or absence of 50 μM MeHg or HgCl, 10 μM ebselen or $(PhSe)_2$ and 50 μM MeHg or Hg^{2+}. Immediately after preincubation, 0.5 mg/ml of MTT was added to the medium containing the slices, followed by an incubation at 37 °C for 45 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide (DMSO) and measured at 490 and 630 nm. Only viable slices are able to reduce MTT.

2.6. Data analysis

Data were analyzed using one-way ANOVA for multiple group comparison. Post hoc analysis was carried out by Tukey multiple range test. Values of $p < 0.05$ were considered statistically significant.

3. Results

MeHg and Hg^{2+} inhibited glutamate net uptake into cerebral cortex slices from young rats (Fig. 1). At lower concentrations (1 and 10 μM) mercurials did not change glutamate net uptake (data not shown). Ebselen, which did not affect glutamate net uptake per se, abolished the inhibitory effect of MeHg, but did not alter the effect caused by Hg^{2+} (Fig. 2A), whereas $(PhSe)_2$ did not alter the inhibition caused by 50 μM MeHg and 50 μM Hg^{2+} on glutamate net uptake into cerebral cortex slices from young rats (Fig. 2B).

In vitro exposure of cortical slices to 50 μM MeHg or Hg^{2+} caused a marked reduction of cell viability of about 60 and 70%, respectively. In a similar way to that observed for glutamate net uptake, ebselen abol-

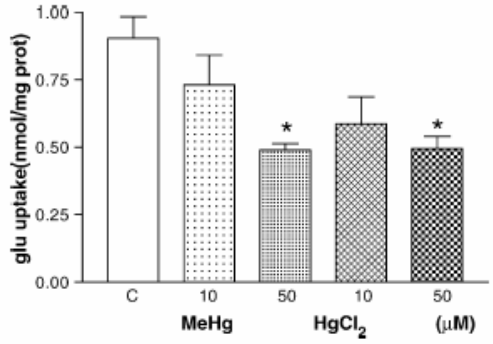

Fig. 1. Effect of methylmercury (MeHg) and mercury chloride (Hg^{2+}) on glutamate uptake into cortical slices from 17-day-old rats. Results are reported as mean ± S.E.M. of six animals in each group. Statistically significant differences as determined by one-way ANOVA followed by the Tukey test is indicated.

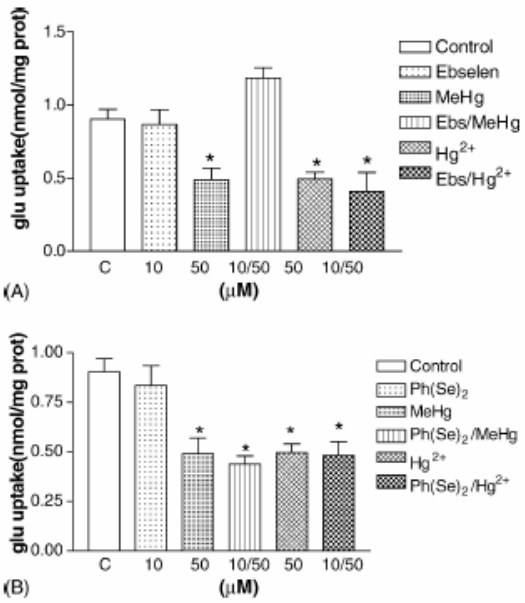

Fig. 2. Influence of ebselen (A) and $(PhSe)_2$ (B) against the inhibitory effects of MeHg and Hg^{2+} on glutamate uptake into brain cortical slices of 17-day-old rats. Results are reported as mean ± S.E.M. of six animals in each group. Statistically significant differences as determined by one-way ANOVA followed by the Tukey test is indicated.

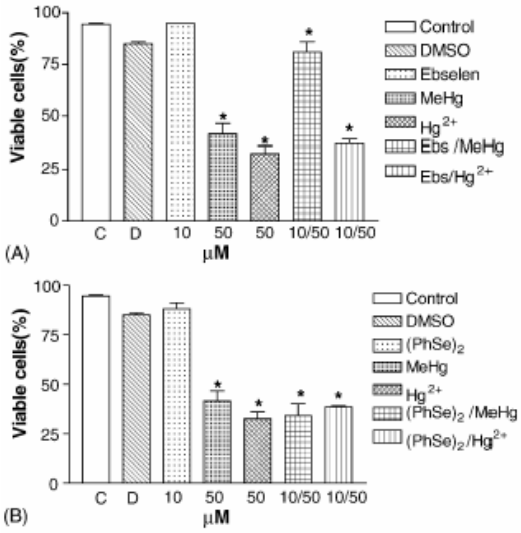

Fig. 3. (A and B) Influence of ebselen and $(PhSe)_2$ on the effects of mercurials on cell viability of cortex cerebral slices of young rats was made according to Section 2. Each value indicates the percentage of viable cells obtained from a mean of the number of viable cells counted in four squares of the chamber in three separated experiments. Data are expressed as mean ± S.E.M. for three independent experiments. (*) is different of control and C-vehicle, (#) is different of MeHg ($^*p < 0.05$) statistically significant differences as determined by one-way ANOVA followed by the Tukey test is indicated.

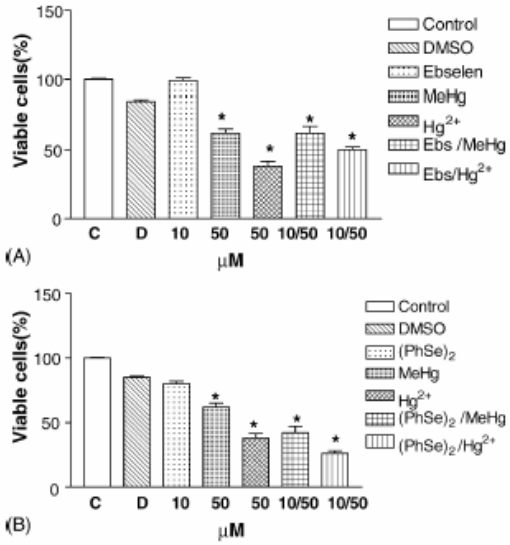

Fig. 4. (A and B) Effect of ebselen and mercurials on cell viability of cortical slices from 17-day-old rats as determined by the MTT method. Data are expressed as mean ± S.E.M. for six independent experiments. (*) is different of control and C-vehicle, $^*p < 0.05$, difference from control by Tukey multiple range test.

ished the loss of cell viability caused by MeHg, but had no protective effect against Hg^{2+} toxicity. Moreover, 10 μM $(PhSe)_2$ plus 50 μM $HgCl_2$ or 50 μM MeHg did not modify the effects of both mercury compounds (Fig. 3A and B). Conversely, using similar incubation conditions to that described above for the other experiments, 10 μM ebselen or $(PhSe)_2$ did not cause a protective effect against the toxicity of MeHg or Hg^{2+} in the MTT assay (Fig. 4A and B).

4. Discussion

The present study demonstrates that mercury compounds inhibited glutamate net uptake, which is in accordance with previous reports from the literature (Brookes, 1992; Aschner et al., 1990; Moretto et al., 2004) (Fig. 1). It is becoming increasingly clear that glutamate transporter dysfunction plays a major role in some neurological diseases and mercury poisoning (Aschner et al., 2000; Danbolt, 2001). Indeed, there are points of evidence indicating that astrocytic glutamate uptake is a mediator of mercury neurotoxicity (Albrecht and Matyja, 1996; Danbolt, 2001; Fitsanakis and Aschner, 2005).

The uptake of glutamate is mainly accomplished by Na^+-dependent high affinity systems mediated by a family of proteins known as amino acid transporters. It has been shown that mercury compounds induce the inhibition of GLT, GLAST and EAAC (Trotti et al., 1997). Here, we showed for the first time that the inhibitory effect of mercurials on glutamate uptake into brain cortical slices may be secondary to their deleterious effects on cell viability. These are surprising results because the period of exposure to mercurials was very short and indicates their direct effect on cell targets. Although glutamate has been implicated in mercury toxicity (Aschner et al., 1996; Kim and Choi, 1995; Allen et al., 2001), our results cannot be explained only in terms of increased excitotoxicity because the short period of exposure to the toxicants.

Thus, mercurials act at least in two distinct ways: one of these is by a direct interaction with glutamate transporter proteins; the other by promoting oxidative stress that oxidizes the glutamate transporter proteins. In fact, if one considers that the reactivity of Hg^{2+} is very high (Onyido et al., 2004) and that this form of mercury is expected to interact with all the available external sites, little Hg(II) should be available to be internalized. Although MeHg can be internalized more easily than Hg^{2+}, we cannot exclude that MeHg is also being internalized only to a limited extent. The blockage of glutamate transport in slices (particularly in astrocytes), which are the main site of glutamate uptake and also the main target of MeHg (Aschner et al., 1993; Aschner, 1996) via an external site of action is not unsound, because substances with low cellular permeability, such as DTNB, are also inhibitors of glutamate uptake (Trotti et al., 1997). Ebselen counteracted the deleterious effects of MeHg on cell viability and on glutamate uptake into brain slices, whereas $(PhSe)_2$ was devoid of effect (Fig. 2A and B). This indicates that although these organochalcogens share pharmacological and neurochemical properties (Wang et al., 1992; Gladilin et al., 2000; Parham and Sies, 2000; Bosch-Morell et al., 1999, 2002; Rossato et al., 2002a,b; Zhao and Holmgren, 2002a; Zhao et al., 2002b; Nogueira et al., 2003a,b; Nogueira et al., 2004), their mechanisms of action are not exactly equivalent (Moretto et al., 2003, 2004, 2005; Porciúncula et al., 2003; Ghisleni et al., 2003). Regarding a possible chemical interaction between ebselen and mercurials (which we could call of a one-dentate chelating interaction via selenol derived from ebselen), we can say that this is improbable for three reasons: (a) the concentration of ebselen used is lower than that of mercury (1:5). So, if all the ebselen was transformed into its selenol intermediate (via some "ebselen reductase" activities that has not yet been demonstrated in brain slices), the quantity of free mercurials will be high. Furthermore, we can suppose that during the short-term period of exposure to the two compounds the quantity of ebselen selenol formed (if any) is far from complete, consequently the actual concentration of mercury (which we consider only ebselen as the site of interaction) would be close to 50 uM. (b) Diphenyl diselenide, which shares several chemical properties with ebselen (including the possibility of being metabolized or reduced chemically to a selenol form) was ineffective against the neurotoxicity of the two mercurials considered.

Evidence that oxidative stress plays a part on MeHg neurotoxicity has been reported (Olivieri et al., 2000; Gassó et al., 2001; Shanker et al., 2004). In fact, MeHg has a high affinity for thiols, which results in the depletion of intracellular glutathione leading to the accumulation of reactive oxygen species (ROS) (Juárez et al., 2002). Oxidative stress can by itself inhibits the astrocytic glutamate uptake mechanisms through a direct action on the transporter proteins. Recently, it has been shown that the inhibition of excitatory amino acid transport induced by MeHg in vivo can be reversed by ebselen, and the protective effect of ebselen on MeHg-induced inhibition of glutamate uptake could be related to its ability to detoxify H_2O_2 (Farina et al., 2003b).

One the other hand, it is well established in vitro, that the chronic low-level inhibition of glutamate uptake results in neuronal death (Nishizawa, 2001; Rothstein et al., 1993), possibly through cellular processes subsequent to increase of intracellular calcium and reactive oxygen species. The Trypan blue exclusion and MTT assays used here showed a MeHg-induced decrease in cell viability. Paradoxically, ebselen showed a protective effect against MeHg toxicity only with Trypan blue exclusion assay (Figs. 3A, B and 4A, B). These results may indicate that energetic failure occurs before cell death and that probably ebselen did not modulate redox activity of the cells that are measured by the MTT assay. It is probable that ebselen maintains the cellular viability by inhibiting the oxidative stress induced by MeHg. We have demonstrated that the protective effect of ebselen against glutamate excitotoxicity in neurons in culture is mediated by an inhibition of lipoperoxidation (Porciúncula et al. 2001). So, we can suppose that ebselen is delaying cellular death by decreasing oxidative stress, but was unable to restore to normal levels the mitochondrial activity as determined by the MTT reduction.

Furthermore, the protective effect of ebselen can not be explained only in terms of glutathione-peroxydase-like activity, because $(PhSe)_2$ is an even better mimetic of GPx than ebselen (Wilson et al., 1989; Meotti et al., 2004; Nogueira et al., 2004). One possible explanation for the protection of ebselen may be related to its ability to negatively modulate the NMDA receptor activation (Herin et al., 2001) or by directly interacting with MeHg.

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V.4. Artigo 4

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Regular Article

Hypoxic-ischemic insult decreases glutamate uptake by hippocampal slices from neonatal rats: Prevention by guanosine

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Abstract

Brain injury secondary to hypoxic-ischemic disease is the predominant form of damage encountered in the perinatal period. The impact of neonatal hypoxia-ischemia (HI) in 7-day-old pups on the high-affinity [^{3}H] glutamate uptake into hippocampal slices at different times after insult was examined. Immediately following, and 1 day after the insult there was no effect. But at 3 to 5 days after the HI insult, glutamate uptake into the hippocampus was markedly reduced; however, after 30 or 60 days the glutamate uptake into hippocampal slices returned to control levels. Also, this study demonstrated the effect of the nucleoside guanosine (Guo) on the [^{3}H] glutamate uptake in neonatal HI injury, maintaining the [^{3}H] glutamate uptake at control levels when injected before and after insult HI. We conclude that neonatal HI influences glutamate uptake a few days following insult, and that guanosine prevents this action.

Keywords: Neonatal hypoxia-ischemia; Glutamate uptake; Guanosine; Hippocampal slices

Introduction

Perinatal brain hypoxic-ischemic (HI) injury is relevant to morbidity and mortality in humans, often leading to mental retardation, seizures, and motor impairment (cerebral palsy), neuro-developmental impairment and disability (Amato and Donati, 2000; Fukamachi et al., 2001; Vannucci, 1990; Vexler and Ferriero, 2001). In neonates and children, hypoxia is a major complicating factor associated with low birth weight and other medical problems, such as those encountered in sudden infant death syndrome (Kalaria et al., 1993; Ottaviano et al., 2001; Sizonenko et al., 2003). The brain of the fetus is extremely susceptible to disorders involving oxygen supply (Valkounova et al., 2001). The vulnerability of the developing brain to HI damage is different from that seen in adult brain and is thought to be due in part to the release of excitatory amino acids (Fukamachi et al., 2001; Grow and Barks, 2002; Johnston, 2001; McDonald and Johnston, 1990; Vexler and Ferriero, 2001).

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS). It is involved in most brain functions (Meldrum, 2000; Nedergaard et al., 2002; Ozawa et al., 1998) such as memory and learning (Izquierdo and Medina, 1997), development and aging (Segovia et al., 2001), and adaptation to the environment (Danbolt, 2001; Mattson et al., 2002; Warren, 2002). Glutamate exerts its signaling role by acting on glutamate receptors located on the neural cell surface in such a way that glutamate concentration in the surrounding extracellular space usually determines the extent of receptor stimulation. The amount of glutamate in the synaptic cleft depends on the balance between its release by presynaptic neurons and its uptake that occurs mainly

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through transporters located in the astrocyte cell membrane and also in the presynaptic neuronal terminal since there is no known extracellular enzyme capable of significantly metabolizing glutamate (Attwell, 2000; Chen and Swanson, 2003; Danbolt, 2001; Rothstein et al., 1994; Ullensvang et al., 1997). The uptake of glutamate is mainly accomplished by Na^+-dependent high affinity systems mediated by a family of transporters (Danbolt, 2001). When present in high concentrations in the synaptic cleft, glutamate may lead to excitotoxicity, a process corresponding to glutamate receptor over-stimulation that subsequently leads to neuronal damage (Danbolt, 2001; Furuta et al., 1997; Maragakis and Rothstein, 2001, 2004; Mattson et al., 2002; Segovia et al., 2001). Indeed, excitotoxicity has been related to various acute and chronic neurodegenerative disorders (Brewer, 2000; Danbolt, 2001; Ingram et al., 2001; Maragakis and Rothstein, 2004; Segovia et al., 2001). Recent evidence suggests that glutamate excitotoxicity is the major mechanism for neuronal death after neonatal HI. In this situation, there is an increase in the extracellular glutamate levels, and its uptake is impaired by oxygen free radicals produced during hypoxia (Painter, 1995; Palmer, 1995; Volpe, 2001). Experimentally, it has been shown that the rat hippocampus is most susceptible at around the second postnatal week (Danbolt, 2001).

Experiments performed by our group have provided evidence that the nucleoside guanosine (Guo) enhances glutamate uptake by rat cortical astrocyte cultures and brain cortical slices from P10 rats under physiological and excitotoxic conditions (Frizzo et al., 2001, 2002, 2003; Oliveira et al., 2004). In vivo studies show that systemic administration of Guo prevented seizures elicited by quinolinic acid and alpha-dendrotoxin, which over stimulate the glutamatergic system, strongly suggesting a neuro-modulatory role of Guo in this system (Lara et al., 2001; Schmidt et al., 2000; Soares et al., 2004; Vinadé et al., 2003). As pointed out above, one of the most important effects of hypoxic ischemia is the increase in extra cellular glutamate levels; thus, mechanisms that enable maintenance of glutamate homeostasis after ischemia might protect against neuronal damage. The aim of this study was to investigate putative alteration on glutamate uptake by hippocampal slices of developing rats subject to HI, and the potential protective effect of guanosine against the HI-induced disturbance in the uptake.

Materials and methods

Chemicals

L-^{3}[H] glutamate was purchased from Amersham International. Other chemicals were of analytical reagent grade and purchased from Sigma.

Animals

Seven-day-old Wistar rats, weighting 12-16 g from the Department of Biochemistry, ICBS, UFRGS Animal House were used. They were fed ad libitum and maintained on a 12 h light/12 h dark cycle, at room temperature. All animal procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA). As recently reviewed by Hurn et al. (2005), long-term damage produced by unilateral HI in seven-day-old rats (as we used) is not affected by gender, since no difference in outcome between male and female pups was observed. Thus in this study we used both male and female rats.

Hypoxic-ischemic (HI) injury

In this research, we utilized the most widely used experimental model of neonatal cerebral hypoxia-ischemia—the Rice-Vannucci Model (Rice et al., 1981; Vannucci and Vannucci, 1997). It consists of the association of unilateral occlusion of the common carotid artery with exposure to a hypoxic atmosphere in order to produce unilateral damage in the rat brain. Animals were anesthetized with halothane. The left common carotid artery was identified through a longitudinal neck incision, isolated from the vagus nerve and permanently occluded with surgical silk thread. After a 2-h recovery period, groups of four pups were placed into a 1500 mL chamber and exposed to an 8% oxygen-92% nitrogen atmosphere delivered at 5 l/min for 1.5 h, with the chamber partially immersed in a 37°C water bath to maintain a constant thermal environment. Rats surviving hypoxia (the mortality rate was less than 5%) were returned to their dams. The pups were killed by decapitation immediately or 1, 3, 5, 30, or 60 days after the hypoxic insult. The brain was removed and the left hippocampus was used in all experimental assays. In this neonatal model, cerebral hemispheres of rats receiving hypoxia are differentially affected: the left hemisphere will present neuronal death since it suffers ischemia (due to carotid occlusion) in association with hypoxia, while the right hemisphere (receiving only hypoxia) suffers no overt morphological damage (Bômont et al., 1992; Rice et al., 1981). The time interval from death to assay was about 10 min.

The rat model of neonatal ischemic-hypoxia as modified by Rice et al., 1981, reproducibly caused unilateral brain injury. This model is useful for the study of neurochemical events associated with neuronal death in the affected hemisphere, as compared to the contra lateral, undamaged hemisphere (Moretto et al., 1999). However, based upon data shown in Fig. 2, in this research the controls consisted only of pups that had not suffered any insult.

The animals in Group H (hypoxic) were exposed exclusively to 1.5 h of hypoxic environment, while those in group O (occluded) were submitted exclusively to unilateral common carotid artery occlusion. Seven (7) animals were used in each experimental group. The only exception being in the experiment represented in Fig. 1, where the pups were killed at variable times after the HI insult. As the observed HI effect was time dependent, the number of pups necessary for clarifying this effect in each specific time was variable. In all other experiments, where the time was fixed as 3 days after HI insult, the number of pups used was invariable (7).

Guanosine treatment

The potential effect of guanosine was investigated using two protocols of drug administration:

a) Seven-day-old pups were treated with one intraperitoneal injection of guanosine (7.5 mg/kg) immediately before the surgical procedure for HI insult and they were killed three days after.
b) Seven-day-old pups were given intraperitoneal injections of guanosine (7.5 mg/kg) in four consecutive doses: immediately before, immediately after, 24 hours and 48 hours after HI insult. They were killed three days after the HI injury.

Control animals for both protocols (a and b) received NaCl 0.9% instead of guanosine and control groups for guanosine alone received only guanosine without suffering insult. During the experimental treatments, pups were maintained with their mothers. A dose of 7.5 mg/kg was used, based upon various previous studies from our group using other animal models, where a dose response curve established that 7.5 mg/kg exerted the maximum neuroprotective effect (Lara et al., 2001; Schmidt et al., 2000).

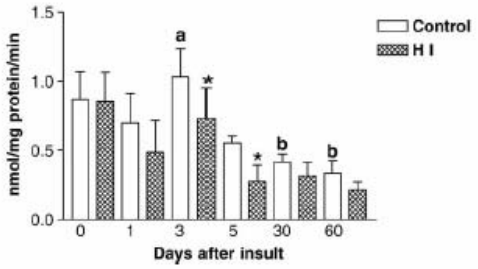

Fig. 1. Effect of neonatal HI insult on glutamate uptake by hippocampal slices. The uptake of glutamate by hippocampal slices from left brain hemisphere is shown at different times (immediately, 1, 3, 5, 30, or 60 days) after HI insult. Results are expressed as mean ± S.E.M. of 5 to 14 animals in each group: 7/8 immediately; 8/8 day 1; 14/14 day 3; 5/6 day 5; 5/6 day 30, 5/5 day 60, for control and HI, respectively. The statistically significant difference between control and the respective HI, as determined by two-way ANOVA followed by the Duncan test, is indicated by * ($P < 0.001$). The letters indicate comparisons between age groups: a, indicates difference from all other control groups; b, indicates difference from immediate to 3 days after HI.

Glutamate uptake

Slices (0.4 mm) were obtained by transversally cutting the middle of the hippocampus of the left hemisphere using a McIlwain chopper. For each animal, one slice/well was tested in triplicate.

The slices were washed with a HBSS solution containing (in mM) 137 NaCl, 0.63 Na2HPO4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH2PO4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, adjusted to pH 7.2. The glutamate uptake was performed according to Frizzo et al. (2002). Briefly, uptake was carried out at 35°C by adding 0.66 μCi/mL [^{3}H] glutamate with 100 μM unlabeled glutamate. The reaction was stopped after 5 minutes by two ice-cold washes with 1 mL HBSS, immediately followed by addition of 0.5 N NaOH, which was kept overnight. Sodium independent uptake was determined by using N-methyl-D-glucamine instead of sodium chloride, which was subtracted from the total uptake to obtain the sodium dependent uptake. Incorporated radioactivity was determined with a Wallac scintillation spectrometer. Protein concentration was measured following the method of Peterson (1977). All experiments were performed in triplicate.

Statistical analysis

Data were analyzed using two-way ANOVA for multiple group comparison. Post hoc analysis was carried out using the Duncan multiple range test. Values of $P < 0.05$ were considered statistically significant.

Results

The effect of neonatal HI on glutamate uptake by brain hippocampal slices

The uptake of glutamate by hippocampal slices from left brain hemisphere was investigated at different times (immediately, 1, 3, 5, 30, or 60 days) after HI insult (Fig. 1). Two-way ANOVA revealed a significant hypoxic-ischemic effect, depending on the time after the insult. Post-hoc comparisons using unpaired t test revealed that glutamate uptake was significantly decreased in the HI groups at 3 and 5 days after the insult ($P < 0.01$) compared to control groups. Thus, all further experiments were performed after 3 days following HI. Glutamate uptake change as a function of age (according Thomazi et al., 2004), reaching highest levels at 10 days old (3 days group) and decreasing from 37 days old (30 days group) (Fig. 1).

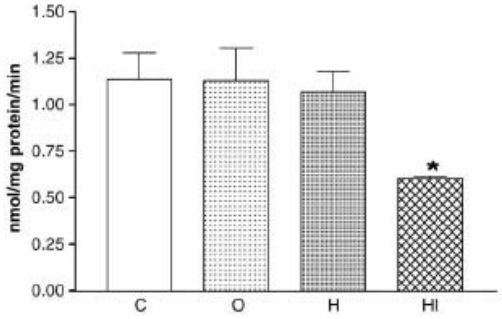

Fig. 2. Effect of hypoxia (H), carotid artery occlusion (O) or both (HI) on glutamate uptake by hippocampal slices measured 3 days after insult. Glutamate uptake was significantly lower in the HI group, as compared to all other groups ($P < 0.01$). Results are expressed as mean ± S.E.M. of 7 animals in each group. The statistically significant difference, as determined by one-way ANOVA followed by the Tukey test, is indicated by * ($P < 0.005$).

Effect of different brain insults on glutamate uptake

The effect of hypoxia (H), carotid artery occlusion (O) or both (HI) on glutamate uptake by hippocampal slices was investigated 3 days after injury (Fig. 2). Glutamate uptake was significantly lower than control only in the HI group ($P < 0.01$), while the O and C groups display the same results (Fig. 2). Glutamate uptake by hippocampal slices from right hemisphere was also investigated and no effect was observed (data not shown).

Guanosine (Guo) prevents the decrease of glutamate uptake

Pre-treatment of rats with a single Guo (i.p.) injection before HI did not affect glutamate uptake by hippocampal slices, measured 3 days after insult (Fig. 3). However, treatment of rats with four doses of Guo (i.p.) given, before

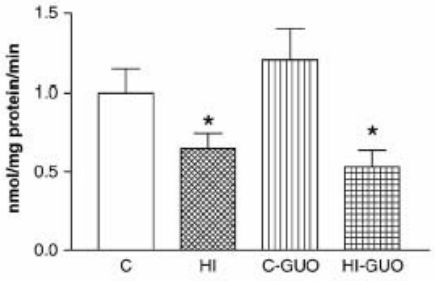

Fig. 3. Effect of a single guanosine administration (7.5 mg/ kg, i.p.) immediately before HI on glutamate uptake by hippocampal slices measured 3 days after insult. Data are expressed as mean ± S.E.M. for 7 animals in each group. The statistically significant difference compared to control, as determined by one-way ANOVA followed by the Tukey test, is indicated by * ($P < 0.005$).

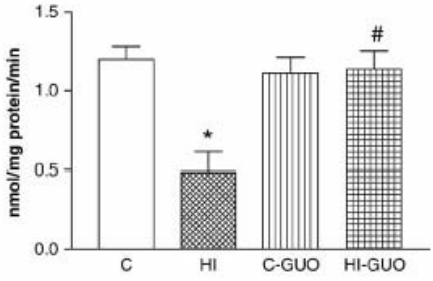

Fig. 4. Effect of guanosine (7.5 mg/kg, i.p.) administered in four doses: before, as well as immediately, 24 hours and 48 hours after HI on glutamate uptake by hippocampal slices determined 3 days after hypoxic insult. Data are expressed as mean ± S.E.M. of 7 animals in each group. The statistically significant difference, as determined by one-way ANOVA followed by the Tukey test, is indicated by * (from control) and # (from HI) ($P < 0.005$).

HI, immediately, 24 hours and 48 hours after HI prevented the decrease in the glutamate uptake caused by insult (Fig. 4), measured three days after the insult.

Discussion

There is evidence that the increase in the extracellular glutamate levels caused by hypoxia/ischemia (HI) involves impairment of glutamatergic high-affinity uptake system (Andine et al., 1994; Benveniste et al., 1984; Johnston, 2001; Massieu and Tapia, 1997; McDonald and Johnston, 1990), as well as an increase in Glu release (Nishizawa, 2001; Yager et al., 2002) and reversal of Glu transporters activity (Rossi et al., 2000; Shimada et al., 1999; Szatkowski et al., 1990). It follows that homeostasis of extra cellular Glu is critical in the post-ischemic period (Bruhn et al., 2000). It has been shown that stroke and ischemia increase the extra cellular glutamate levels by affecting the glutamate uptake, and that this effect seems to be involved in the neurotoxicity caused by ischemia (Maragakis and Rothstein, 2004).

During CNS development the expression of glutamate transporter subtypes is differentially regulated and regionally coordinated (Furuta et al., 1997). In the period of active synaptogenesis there is a strong increase in the levels of both GLT-1 and GLAST transporters (Danbolt et al., 2001). Astrocytes in vivo might adapt their ability to control synaptic levels of glutamate to environmental or injurious stimuli depending on their development and maturation stage (Stanimirovic et al., 1999). A previous age-related study of the developmental profile of basal glutamate uptake in rats showed higher activity in younger than in older animals (Thomazi et al., 2004). The results of the present study confirm these data and provide evidence that experimental brain hypoxia-ischemia in immature rats decreases glutamate uptake, depending on the time after the insult. HI decreased the

glutamate uptake between the 3rd and the 5th days after the insult (Fig. 1). Interestingly, it was also demonstrated that the glutamate uptake by hippocampal slices returned to control levels after 30-60d after HI. In the animals submitted only to hypoxic conditions or only to carotid artery occlusion, there was no effect on glutamate uptake (Fig. 2). As the energy involved in the process for clearing glutamate from the external space represents a large fraction of total synaptic ATP turnover, it is not surprising that complete ischemia leads to a decrease in glutamate uptake and a massive increase in extra cellular glutamate concentrations (Maragakis and Rothstein, 2004; Sibson et al., 1998). Moreover, when membrane gradients (ATP dependent) are collapsed, there is an efflux of glutamate via reverse uptake (Chen and Swanson, 2003).

Guanosine (Frizzo et al., 2001, 2002) has been shown to produce in vitro neuroprotective effects, and was effective at preventing seizures induced by over-stimulation of the glutamatergic system in adult animals (Lara et al., 2001; Schmidt et al., 2000; Soares et al., 2004; Vinadé et al., 2003). In our laboratory, it has also been found that Guo increases glutamate uptake by cortical astrocyte cultures, whether submitted to an injury process or not (Frizzo et al., 2001), and that this effect was higher at 10 days than 40 days (Gottfried et al., 2002). Results published in the literature show that Guo levels remain elevated for several days after toxic conditions (Dobolyi et al., 2000), such as brain ischemia (Uemura et al., 1991). In addition, astrocytes subjected to hypoxia/hypoglycemia release higher amounts of Guo than adenosine and for more extended periods, when compared to control cells (Ciccarelli et al., 1999). In this study, Guo was administered with the aim of correlating its demonstrated neuroprotective effects with its ability to avoid the decrease of brain glutamate uptake in our model of HI in young rats.

Our results showed that Guo prevents the decrease of glutamate uptake caused by HI. It can be hypothesized that endogenously released guanosine, by enhancing glutamate uptake (Frizzo et al., 2001, 2002, 2003), affects the glutamatergic system, preventing glutamatergic excitotoxicity. Furthermore, it is important to emphasize that, in the same model of ischemic injury, a decrease in the expression of hippocampal GLAST and GLT1 transporters has been observed (Fukamachi et al., 2001). All these studies reinforce the hypothesis that guanosine could increase glutamate uptake by affecting glutamatergic transporters on the cell surface of astrocytes.

This prevention was not observed when a single dose of Guo was administered immediately before the insult (Fig. 3), but uptake returned to control levels when Guo was applied in *four* consecutive doses: immediately before, immediately after, 24 hours and 48 hours after the insult (Fig. 4). In view of the importance of glutamate for the development of the nervous system, it is plausible that in this early stage, the mechanism of glutamate uptake is more susceptible to modulation. Whether this Guo effect was due to direct or indirect action on the transporters, and the mechanisms involved, is currently under investigation by our group.

The most important findings of the present research are that after HI insult, glutamate uptake by hippocampal slices from immature animals was significantly reduced and that this reduction was prevented by in vivo treatment with Guo. It is possible to suggest that this Guo effect could contribute to the maintenance of extra cellular glutamate in physiological concentrations, and so avoid excitotoxicity. Our findings, together with those from previous research (Rathbone's group, Souza's group), suggest the potential value of initiating clinical studies. However, enthusiasm should be tempered, given reports of amnesic effects with GUO in in vivo studies (Roesler et al., 2000, Vinadé et al., 2003).

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VI. DISCUSSÃO

Apesar de muitos relatos descreverem propriedades biológicas muito interessantes, os mecanismos pelos quais os compostos de selênio ou telúrio desencadeiam ações tóxicas não estão bem esclarecidos. Sabe-se que estes compostos podem oxidar grupamentos –SH de moléculas biologicamente ativas (Ganther, 1971). Assim, poderiam atuar sobre as proteínas constituintes dos canais de cálcio desencadeando efeitos neurotóxicos e/ou neuroprotetores. Os resultados obtidos no ARTIGO 1 demonstram que os compostos organocalcogêneos apresentam efeitos variáveis de acordo com as condições empregadas, pois, $(PhSe)_2$ diminuiu o influxo de cálcio em sinaptossomas *in vitro* em condições basais, enquanto que quando um agente despolarizante foi usado seu efeito foi variável. Assim, sob o estímulo de KCl, $(PhSe)_2$ não alterou o influxo de cálcio e sob ação de aminopiridina (um inibidor de canais de K) houve uma inibição em baixas concentrações (40 e 100μM) e em 400μM não houve alteração. É razoável pensar que este composto orgânico de selênio atua dependendo da concentração, sobre canais de cálcio afetando o movimento de cálcio que pode interferir em vários processos neurofisiológicos. Contrariamente, em condições basais e sob a ação de aminopiridina, ebselen em alta concentração (400μM) aumenta a captação de cálcio, diminuindo quando o agente despolarizante KCl é usado, também em 400μM. Já $(PhTe)_2$ inibiu a captação de cálcio em todas as condições estudadas. Não se sabe o mecanismo pelo qual estes compostos interagem com os canais de cálcio. A conseqüência fisiológica de uma inibição na captação de cálcio por sinaptossomas pode ser interpretada como um efeito lesivo à célula, pois, poderia interferir diretamente na exocitose ocasionando a ruptura na homeostase dos processos envolvidos na liberação de neurotransmissores na fenda sináptica. Analogamente, os mercuriais que são ávidos por grupamentos sulfidrílicos também bloqueiam os poros dos canais iônicos, ao mesmo tempo interagindo sobre diversas rotas de sinalização intracelular. É possível supor que $(PhTe)_2$, cujo efeito inibitório foi o mais acentuado dos três compostos analisados pode ser considerado neurotóxico para o sistema nervoso central. De fato, foi recentemente demonstrado em nosso grupo que ebselen e $(PhTe)_2$ reagem com

grupos sulfídrilas de proteínas (Barbosa et al., 1998, Maciel et al., 2000, Mugesh, et al., 2001). Além disso, os distintos modos pelos quais os compostos organocalcogênios interagem com os canais de cálcio podem estar relacionados a reatividade química destes compostos, pois $(PhTe)_2$ é muito lipofílico tendo possibilidade de acesso por mais de uma via, inclusive podendo difundir-se pela membrana enquanto ebselen acessaria o canal somente após um estímulo, neste caso a ativação por K^+

Outro dado interessante deste trabalho(ARTIGO 1) foi o efeito inibitório(sob ação de KCl) e estimulatório (sob ação de aminopiridina ou sob condições basais) apresentado por ebselen. Resultados recentes de nosso laboratório demonstraram que ebselen estimula a liberação de glutamato em condições basais e ao mesmo tempo, inibe esta liberação quando estimulado por KCl (Nogueira et al., 2002). Como a liberação de glutamato nos terminais nervosos é dependente de cálcio, pode-se deduzir que estes efeitos possam estar relacionados.]

Como já foi previamente citado os insultos provocados por neurotóxicos (mercúrio, chumbo) alteram a homeostase do cálcio podendo levar à morte neuronal. A maquinaria responsável pela regulação da homeostase do cálcio inclui organelas intracelulares, calcioATPases e os canais de cálcio dependentes de voltagem e aqueles que medeiam a passagem deste íon(NMDA). De acordo com o ARTIGO 2 os compostos orgânicos de selênio podem interagir com outro conhecido neurotóxico, o sal de mercúrio HgCl. Sob condições basais, ebselen extinguiu a inibição do influxo de cálcio provocado por cloreto de mercúrio ($HgCl_2$) em sinaptossomas, *in vitro*. Entretanto, sob condições de despolarização(por KCl) não apresentou este efeito. Devido a sua localização na membrana plasmática, os canais de cálcio são alvos para os mercuriais, e, conforme observamos a redução da captação de cálcio induzida pelo $HgCl_2$ foi revertida por ebselen, somente em condições basais.

Confirmando dados da literatura anteriormente citados nesta tese $HgCl_2$. produziu uma inibição acentuada na captação de glutamato em sinaptossomas, *in vitro* (ARTIGO 2) utilizando-se dois procedimentos diferentes no preparo da fração sinaptossomal. Os transportadores de glutamato são sensíveis a ação de agentes

oxidantes, inclusive os mercuriais. A ausência de proteção por parte de ebselen em relação a captação de cálcio em condições de despolarização, cabe uma ressalva: a fração sinaptossomal que utilizamos não estava isenta de contaminantes, como, mitocôndrias, que também podem captar cálcio, não nos permitindo supor um efeito apenas sobre os canais dependentes de voltagem.

No ARTIGO 3 utilizamos fatias de córtex, um sistema em que a interação neurônio-glia está presente, e, simultaneamente, a possibilidade de avaliar a captação de glutamato, que é mais acentuada nas células gliais. Investigamos o efeito de ebselen e $(PhSe)_2$ sobre a captação de glutamato em fatias de córtex de ratos em desenvolvimento expostas a MeHg e a HgCl. Ambos, os mercuriais inibiram a captação de glutamato, contudo, apenas ebselen reverteu o efeito inibitório sobre a captação de glutamato e a morte celular provocada por MeHg. Os efeitos sobre a captação podem ser secundários à viabilidade celular, e, esta, reveste-se de maior relevância pelo curto tempo de exposição dos mercuriais, indicando ser um efeito direto sobre as células alvo. Embora a excitotoxicidade glutamatérgica esteja expressivamente ligada a toxicidade de MeHg, nossos resultados não podem ser explicados somente em termos de aumento da excitotoxicidade devido ao curto período de exposição dos mercuriais.

Além de interagir com os transportadores de glutamato, os compostos de mercúrio promovem estresse oxidativo à célula. Ao mesmo tempo, que os astrócitos são os principais responsáveis pela captação de glutamato, também são os principais alvos para o MeHg. Podemos considerar uma possível interação entre ebselen e mercuriais pois, a concentração de ebselen é mais baixa do que a de mercúrio, e se houve a formação do intermediário selenol durante a reação, a quantidade de mercúrio livre seria muito alta. O tempo de exposição curto não indica tal efeito.

É mais provável que devido a alta afinidade por tióis apresentada por MeHg ocorra uma depleção de glutationa intracelular, e, conseqüentemente aumento nas espécies reativas de oxigênio. Ebselen poderia atuar por suas propriedades antioxidantes, já demonstradas em tratamentos “ex vivo” com MeHg, onde ebselen promoveria a detoxificação dos radicais livres formados pelo MeHg no córtex dos

animais tratados (Farina et al., 2003 b), inclusive por aumentar a atividade da GPx. Além disso, o ebselen protege da excitotoxicidade glutamatérgica em cultura de neurônios por inibir a lipoperoxidação produzida por MeHg, que foi atribuída a seu efeito como mimético da GPx (Porciúncula et al., 2001).

Ebselen mantém a viabilidade celular, provavelmente, inibindo o estresse oxidativo induzido por MeHg, observado pelo ensaio com o corante azul trypan. Porém, foi incapaz de restabelecer a atividade das desidrogenases como um todo nas células, medida pelo ensaio MTT. No entanto, o efeito protetor de ebselen não pode ser explicado somente em termos de sua atividade glutationa-peroxidase, pois $(PhSe)_2$ é um melhor mimético de GPx do que ebselen e não protegeu do dano provocado por ambos os mercuriais.

Outro aspecto interessante que se pode inferir de nossos resultados está relacionado a ausência de ação de ebselen sobre os efeitos provocados por HgCl. Isto poderia ser explicado pelo fato de Hg ser mais reativo e possuir uma maior afinidade por grupamentos –SH do que MeHg, reagindo com todos os sítios externos disponíveis, agindo diretamente sobre os transportadores ou oxidando as proteínas dos transportadores de glutamato. Daí, ser possível supor que MeHg e Hg atuem em parte, por distintos alvos moleculares, um modulado por ebselen e outro não.

Por fim, ao usarmos um modelo *in vivo* de dano cerebral, hipóxia-isquemia (HI) neonatal, observamos uma inibição significativa da captação de glutamato em fatias de hipocampo de animais jovens entre o terceiro e o quinto dia após sofrerem o insulto HI (ARTIGO 4). Múltiplas evidências relatam que a HI rompe a homeostase do sistema glutamatérgico. Isto pode ser mais relevante quando ocorre em animais em desenvolvimento, pois, a expressão dos transportadores de glutamato está aumentada durante o período de sinaptogênese. Nossos resultados também apontaram para uma recuperação na redução da captação de glutamato nos animais adultos.

Guanosina (GUO) tem evidenciado efeito neuroprotetor em diversos modelos animais. Interessantemente, no modelo HI neonatal, guanosina evitou a

redução da captação de glutamato nos animais que sofreram o insulto apenas quando administrada em quatro doses:antes, imediatamente, 24, 48 horas após a injúria HI neonatal, significando que o sistema glutamatérgico é sensível à GUO nos estágios precoces pós-insulto HI. É possível sugerir que a GUO mantenha as concentrações extracelulares de glutamato em níveis fisiológicos evitando a excitotoxicidade, apenas quando administrada em quatro doses.

VII.CONCLUSÕES

De acordo com os resultados apresentados neste trabalho, pode-se concluir que os compostos organocalcogênios atuam de forma complexa sobre o influxo de cálcio em sinaptossomas de cérebro de rato, dependendo das condições e agentes despolarizantes usados na preparação "*in vitro*". Ebselen, $(PhSe)_2$ e $(PhTe)_2$ alteram a captação de cálcio de maneira distinta quando expostos a aminopiridina ou KCl. Os mecanismos pelos quais aminopiridina ou KCl estimulam a entrada de cálcio são diferentes, levando, por conseguinte a originar diferentes propriedades farmacológicas e/ou toxicológicas para estes compostos.

Ebselen atenuou a inibição da captação de cálcio "*in vitro"* provocada por cloreto de mercúrio em sinaptossomas de cérebro de rato apenas em condições basais do ensaio. A inibição da captação de glutamato "*in vitro"* por HgCl não foi modificada por ebselen, sugerindo que esta proteção depende da proteína-alvo ou do sítio da membrana sinaptossomal considerada.

MeHg e HgCl inibiram a captação de glutamato em córtex cerebral de ratos de 17 dias e ebselen reverteu somente o efeito do MeHg mas, não o do HgCl. Já, disseleneto de difenila não alterou os parâmetros avaliados na exposição de ambos os compostos de mercúrio.

Os compostos de mercúrio estudados provocaram a morte celular das fatias de córtex, porém, ebselen protegeu as fatias dos efeitos lesivos provocados por MeHg e não pelo HgCl. Ebselen, semelhante ao observado em tratamento"*in vivo"* por MeHg, mantém a viabilidade celular protegendo as membranas celulares do estresse oxidativo provocado pela exposição das fatias apenas ao MeHg.

Confirmando dados da literatura, a hipoxia–isquemia neonatal reduziu a captação de glutamato de fatias de hipocampo de ratos, após 3 – 5 dias do insulto. Esta inibição foi recuperada aos 60 dias de idade.

A guanosina evitou a redução da captação de glutamato em fatias de hipocampo de ratos submetidos à hipoxia–isquemia neonatal quando administrada em quatro doses consecutivas: antes, imediatamente, 24 h, 48 h após o insulto HI. O cérebro é altamente vulnerável a insuficiência energética, portanto, medidas precoces que visem intervenções terapêuticas evitando o dano cerebral ou/e suas

graves seqüelas são imprescindíveis. GUO parece modular os níveis extracelulares de glutamato evitando a excitotoxicidade e suas conseqüências.

Ambos os modelos usados em nosso estudo (estudo "*in vitro*" com MeHg e HgCl e o modelo "*in vivo*" de HI neonatal) revelaram dano celular que faz jus a estudos adicionais sobre o efeito destes compostos na viabilidade celular.

VIII.PERSPECTIVAS

Analisando os resultados obtidos nesta tese com organocalcogênios sobre a captação de cálcio instiga-nos desvendar os possíveis mecanismos envolvidos nestes efeitos, utilizando bloqueadores de cálcio específicos para os diversos tipos de canais, assim como, seria interessante verificar a ação destes compostos “ex vivo”, em diferentes tipos de preparações e condições.

Da mesma forma, o tratamento “ex vivo” com cloreto de mercúrio na forma aguda, sub crônica ou crônica poderia revelar um outro perfil de ebselen sobre estas intoxicações no sistema glutamatérgico e no influxo de cálcio.

Quando observamos as nítidas diferenças entre os efeitos de ebselen em relação a morte celular e a captação de glutamato em relação aos compostos de mercúrio estudados, observa-se que há muitas possibilidades a investigar. Dentre elas, verificar se a morte celular observada nas fatias ocorre nos neurônios ou nos astrócitos, e, qual seria o alvo para ebselen em relação aos compostos de mercúrio; assim como, em qual estrutura cerebral isto poderia ter relevância .

O cerebelo também é alvo de deposição para intoxicações para MeHg, portanto, podemos verificar os efeitos “ex vivo” dos tratamentos de MeHg e HgCl, em diferentes idades do desenvolvimento cerebral e o possível efeito de organocalcogêneos , quando avaliado o sistema glutamatérgico , a morte celular, os mecanismos de controle de entrada de cálcio.

Uma importante linha de trabalho que ainda não foi explorada é o tratamento “ex vivo” com os compostos estudados nos parâmetros citados acima adicionados de estudos histológicos em diversas fases do desenvolvimento cerebral a fim de avaliar neuroproteção ou neurotoxicidade.

Em relação a guanosina, a relação entre a excitotoxicidade medida através da captação de glutamato em tempos pós-insulto superiores aos estudados, especialmente, em tempos curtos pós-insulto, pode assumir uma considerável importância futura, sob o ponto de vista clínico. Ações precoces em relação a dano HI minizam suas graves seqüelas, que podem ser irreversíveis, como retardo mental, epilepsia, etc. Além disso, o estudo de alterações histológicas provocada por este modelo de HI neonatal e o efeito provável de Guo pode garantir o seu provável papel neuroprotetor.

IX. REFERÊNCIAS BIBLIOGRÁFICAS

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