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ESTUDO DAS PROPRIEDADES TOXICOLÓGICAS E ANTIATEROGÊNICAS DE COMPOSTOS ORGÂNICOS DE SELÊNIO

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"Todo efeito tem uma causa. Todo efeito inteligente tem uma causa inteligente. O poder da causa inteligente está na razão da grandeza do efeito".

Allan Kardec

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RESUMO

A aterosclerose é uma doença inflamatória crônica caracterizada pelo acúmulo de lipídios e elementos celulares nas artérias de calibre médio e grande porte, sendo que a modificação oxidativa da lipoproteína de baixa densidade (LDL) desempenha um papel importante na patogênese dessa condição. Os compostos orgânicos de selênio estão recebendo grande atenção da comunidade científica devido as suas propriedades benéficas destes compostos em patologias associadas ao estresse oxidativo. Neste contexto, nosso objetivo foi avaliar as propriedades toxicológicas e antiaterogênicas de compostos orgânicos de selênio em modelos experimentais *in vivo* e *in vitro*. Inicialmente, avaliamos os efeitos da administração aguda do disseleneto de difenila $(PhSe)_2$ sobre parâmetros toxicológicos em coelhos. O tratamento de coelhos com 5 μmol/kg/dia não induziu toxicidade, no entanto, quando os animais foram tratados com 50 μmol/kg, sinais leves de toxicidade foram observados, e o tratamento com maior dose utilizada (500 μmol/kg) causou mortalidade em 85% dos animais, indicando intensa toxicidade. Assim estes resultados indicam que os efeitos toxicológicos da exposição aguda ao $(PhSe)_2$ em coelhos é dependente da dose. Considerando o envolvimento da LDL oxidada (LDLox) na patogênese da aterosclerose, investigamos o efeito protetor do $(PhSe)_2$ sobre danos citotóxicos induzidos pela exposição de macrófagos murinos J774 à LDLox. O pré-tratamento com $(PhSe)_2$ reduziu os efeitos citotóxicos desencadeados pela LDLox incluindo a geração de ROS, distúrbio da homeostase do $^{\bullet}NO$, ativação de metaloproteinases de matriz, formação de células espumosas e disfunção mitocondrial em macrófagos *in vitro*. Além disso, os efeitos da sinalização redox do $(PhSe)_2$ apresentados neste estudo, foram acompanhados por uma regulação da atividade de ligação do NF-κB. Considerando o efeito promissor de compostos orgânicos de selênio como moléculas antiaterogênicas, na última parte deste estudo, avaliamos o efeito de diaril disselenetos substituídos, *p*-metoxi-difenil disseleneto (DM) e *p*-cloro-difenil disseleneto (DC), na oxidação da LDL induzida por Cu^{2+}. Ambos os compostos causaram inibição dose-dependente na oxidação de soro humano e de LDL isolada. A parte proteica da LDL humana isolada também foi protegida contra a oxidação induzida por Cu^{2+}. Além disso, o DM e DC eficientemente diminuíram a formação de células espumosas induzidas por LDLox em macrófagos J774. O potencial efeito antiaterogênico destes compostos

foi relacionado à sua atividade mimética a GPx e sua propriedade de atuar como substrato para TrxR mamífera, duas importantes vias envolvidas na decomposição de peróxidos. Tomados em conjunto, os resultados deste trabalho sugerem um novo papel para os compostos orgânicos de selênio como potenciais agentes antiateroscleróticos.

Palavras-chave: Aterosclerose, Lipoproteína de baixa densidade, Macrófagos.

ABSTRACT

Atherosclerosis is a chronic inflammatory disease characterized by accumulation of lipids and cellular elements in the arteries of medium and large caliber. The oxidative modification of low density lipoprotein (LDL) plays an important role in the pathogenesis of this condition. Because of their possible beneficial role in the prevention of atherosclerosis and other oxidative stress-associated diseases, organoselenium compounds are receiving increased attention. In this context, our objective was to evaluate the toxicological and antiatherogenic properties of organoselenium compounds in experimental models *in vivo* and in *vitro*. Initially, we evaluated the effects of acute administration of diphenyl diselenide $(PhSe)_2$ on toxicological parameters in rabbits. The treatment of rabbits with 5 μmol/kg/day did not induce toxicity, however, when the animals were treated with 50 μmol/kg mild signs of toxicity were observed, and the concentration of 500 μmol/kg caused high mortality (85%) and toxicity in these animals. The results indicated that the toxicological effects of acute exposition of $(PhSe)_2$ in rabbit is dose-dependent. It has been reported that oxidized LDL (oxLDL) are involved in the pathogenesis of atherosclerosis, in the context, we investigated the mechanisms underlying the protective effect exerted by $(PhSe)_2$, on oxLDL-mediated cytotoxic effects in murine J774 macrophage cells. $(PhSe)_2$ pretreatment reduced atherogenic cytotoxic effects triggered by oxLDL including ROS generation, disturbance of NO homeostasis, activation of matrix metalloproteinase, foam cell formation and mitochondrial dysfunction in macrophages *in vitro*. Moreover, the redox signaling effects of $(PhSe)_2$ presented in this study, were accompanied by a down-regulation of NF-κB-binding activity. Considering the promising effect of organoselenium compounds as antiatherogenic molecule, in the last part of this study we evaluated the effect of the disubstituted diaryl diselenides, *p*-dimethoxy-diphenyl diselenide (DM) and *p*-dichloro-diphenyl diselenide (DC), on Cu^{2+}-induced LDL oxidation. Both compounds caused a dose-dependent inhibition on human serum and isolated LDL oxidation. The protein moieties from human isolated LDL were also protected against Cu^{2+}-induced oxidation. Moreover, DM and DC efficiently decreased the LDL induced foam cell formation in J774 macrophage cells. The potential antiatherogenic effect of these compounds is related to its GPx-like activity and its property to acts as a good substrate for mammalian TrxR, two important pathways involved

in peroxide decomposition. Taken together, the results suggest a new role for organoselenium compounds as potential anti-atherogenic agent.

Keywords: Atherosclerosis, Low density lipoprotein, macrophage.

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LISTA DE ABREVIATURAS E SIGLAS

$\Delta\Psi_m$	Potencial de membrana mitocondrial
$^{\bullet}NO$	Óxido nítrico
$^{\bullet}OH$	Radical hidroxil
$(PhSe)_2$	Disseleneto de difenila
ALT	Alanina aminotranferase
Apo B-100	Apolipoproteína B-100
Apo E -/-	Camundongo deficiente para apolipoproteína E
AST	Aspartato aminotransferase
CD36	Receptor *scavenger* CD36
DC	*p*-chloro-diphenyl diselenide
DCFH-DA	2',7'- Diclorodiidrofluoresceína diacetato
DL_{50}	Dose letal 50%
DM	*p*-methoxyl-diphenyl diselenide
DMEM	*Dulbecco's Modified Eagle Medium*
DMSO	Dimetilsulfóxido
eNOS	Óxido nítrico sintase endotelial
FITC	Isotiocianato de fluoresceína
GPx	Glutationa Peroxidase
GR	Glutationa Redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
GST	Glutationa S-transferase
H_2O_2	Peróxido de hidrogênio
HDL	Lipoproteína de alta densidade
HMG CoA	Hidroximetilglutaril coenzima A
HOCl	Ácido hipocloroso
iNOS	Óxido nítrico sintase induzível
JC-1	5,5',6,6'-tetracloro- 1,1',3,3'-tetraetilbenzimidazolilcarbocianina iodado
LDH	Lactato dehidrogenase
LDL	Lipoproteína de baixa densidade
LDLox	Lipoproteína de baixa densidade oxidada
LDLr -/-	Camundongo deficiente para o receptor de LDL
LOX-1	Receptor de LDL oxidada
LPS	Lipopolissacarídeo
MCP-1	Proteína quimiotática para monócitos - 1
M-CSF	Fator estimulador de colônia de macrófagos
MDA	Malondialdeído

MMP-9	Metaloproteinase de matriz-9
MTT	3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina
NADPH	Dinucleotídeo fosfato de nicotinamida adenina reduzida
NF-κB	Fator nuclear κB
NOS	Óxido nítrico sintase
Nrf-2	Fator nuclear eritróide-2
$O_2^{\bullet-}$	Radical ânion superóxido
$ONOO^-$	Peroxinitrito
PBS	Salina tamponada com fosfato
PUFAS	Ácidos graxos poliinsaturados
RNS	Espécies reativas de nitrogênio
ROS	Espécies reativas de oxigênio
Se-OH	Selenol
SFB	Soro fetal bovino
SOD	Superóxido dismutase
SRA	Receptor *scavenger* A
TBARS	Substâncias reativas ao acido tiobarbitúrico
TrxR	Tiorredoxina Redutase
VCAM-1	Molécula de adesão vascular - 1
VLDL	Lipoproteína de densidade muito baixa
δ-ALA-D	Deltaminolevulonato dehidratase
δ-GT	Gama glutamiltransferase

SUMÁRIO

1. INTRODUÇÃO

1.1 DOENÇAS CARDIOVASCULARES

As doenças cardiovasculares representam a principal causa de mortes nos países ocidentais. Apesar de ser um problema antigo dos países desenvolvidos, elevações relativamente rápidas e substanciais têm ocorrido em países em desenvolvimento, dentre os quais o Brasil. De acordo com as projeções da Organização Mundial de Saúde (WHO, 2004), esta tendência deve persistir agravando o quadro de morbidade e mortalidade elevadas nestes países (Sposito et al., 2007)

Estima-se que 17,1 milhões de pessoas morrem em todo mundo anualmente de doenças cardiovasculares, representando 29% do total de mortes. Deste número, aproximadamente 7,2 milhões são devido à doença arterial coronariana e 5,7 milhões ao infarto do miocárdio (WHO, 2004). Segundo a Associação Americana de Cardiologia, no ano de 2006, uma a cada 2,9 mortes nos Estados Unidos foi decorrente de doenças cardiovasculares (Lloyd-Jones et al., 2010).

De acordo com o Ministério da Saúde, as doenças cardiovasculares representam a principal causa de morte no Brasil, registrando cerca de 300 mil óbitos em 2006, quase 30% do total de mortes registradas no país. Estes dados fazem parte do Programa Saúde Brasil de 2008, publicação anual da Secretaria de Vigilância em Saúde (SVS).

A aterosclerose é a condição patológica que antecede a maior parte dos episódios cardiovasculares, nomeadamente infartos do miocárdio e acidentes vasculares cerebrais (AVC), na qual as artérias desenvolvem uma constrição do seu espaço interno e perdem elasticidade (Itabe, 2003, Hansson e Libby, 2006).

1.2 ATEROSCLEROSE

1.2.1 Aspectos gerais

A aterosclerose é uma doença progressiva caracterizada pelo acúmulo de material lipídico e elementos celulares, especialmente macrófagos e células musculares lisas, na túnica íntima das artérias de médio e grande calibre, levando à formação de placas ateroscleróticas. Os vasos sanguíneos mais comumente afetados e clinicamente mais relevantes incluem a artéria aorta e as artérias carótidas, coronárias e cerebrais. O caráter crônico do processo conduz à formação de lesões

que podem obstruir completamente o lúmen vascular, diretamente ou mediante complicação trombótica (Lusis, 2000, Stocker e Keaney, 2004).

A relação entre lipídeos e a aterosclerose dominou os estudos acerca desta patologia por muitos anos, onde fortes evidências experimentais e clínicas relatavam a ligação entre a hipercolesterolemia e o ateroma (Libby, 2002). Estudos realizados na última década, no entanto, chamam a atenção ao processo inflamatório envolvido na aterosclerose e em suas complicações. A aterosclerose foi reconhecida como uma doença inflamatória crônica que pode ter componentes infecciosos e autoimunes contribuindo para a evolução do processo (Ross, 1999, Libby, 2002). Estudos realizados em humanos e em modelos animais mostraram que o processo inflamatório está presente em todas as fases do processo aterosclerótico, incluindo a fase inicial, de progressão e as complicações trombóticas da lesão (Libby et al., 2010)

1.2.2 Características morfológicas da aterosclerose

A parede arterial é composta de três definidas camadas concêntricas que circundam o lúmen arterial, cada qual com uma composição distinta de células, e pela matriz extracelular. A camada imediatamente adjacente à luz é chamada de íntima, a camada intermediária é conhecida como a média e a camada mais externa compreende a adventícia arterial, separadas pela lâmina elástica interna (Stocker e Keaney, 2004).

As lesões iniciais ocorrem especialmente nas bifurcações e curvaturas das artérias, locais onde o fluxo sanguíneo ocorre de maneira turbilhonada e desorientada (Ross, 1999, Lusis, 2000). O processo aterosclerótico se inicia com uma disfunção endotelial, favorecendo o surgimento de uma resposta inflamatória que resulta em aumento da permeabilidade aos leucócitos, lipídeos e plaquetas circulantes (Kadar e Glasz, 2001).

A fase inicial da aterosclerose é caracterizada pelo surgimento de estrias gordurosas, as quais darão origem ao ateroma (Stary, 1990). Este processo inicia-se com a passagem da lipoproteína de baixa densidade (LDL) da circulação para a íntima dos vasos, através da camada endotelial (Ross, 1999). A LDL sofre modificações oxidativas através de ação enzimática ou não enzimática, levando à liberação de fosfolipídeos bioativos que podem ativar as células endoteliais. Por sua vez, as células endoteliais ativadas expressam vários tipos de moléculas de adesão para leucócitos (principalmente VCAM-1), que promovem o

rolamento e adesão destas células na superfície vascular. Após a adesão à camada endotelial, os leucócitos migram para a íntima em resposta a estímulos quimioatraentes, incluindo quimiocinas, dentre elas a MCP-1 (proteína quimiotática para monócitos-1), que recruta monócitos circulantes para o local da lesão (Hansson e Libby, 2006).

Na camada íntima, os monócitos adquirem características dos macrófagos teciduais e passam a expressar receptores sequestradores que ligam e internalizam partículas de lipoproteínas modificadas. O conjunto destes processos origina as células espumosas, que são o resultado do acúmulo de gotículas lipídicas dentro do citoplasma destes macrófagos (Brown e Goldstein, 1983, Ross, 1999, Libby, 2002). Dentro da íntima arterial, os macrófagos participam de muitas funções relacionadas à aterosclerose e suas complicações. Notavelmente, as células espumosas secretam citocinas pró-inflamatórias, que amplificam a resposta inflamatória no local da lesão, bem como intensificam a produção de espécies reativas de oxigênio (ROS). Além disto, os macrófagos e as células espumosas têm um papel fundamental nas complicações trombóticas da aterosclerose através da produção e secreção de metaloproteinases de matriz (MMP). Estas enzimas proteolíticas podem degradar a matriz extracelular resultando em ruptura da placa aterosclerótica, e assim o sangue circulante entra em contato com o fator tecidual pró-coagulante, levando à formação de um trombo sobrejacente (Libby, 2002) (Figura 1).

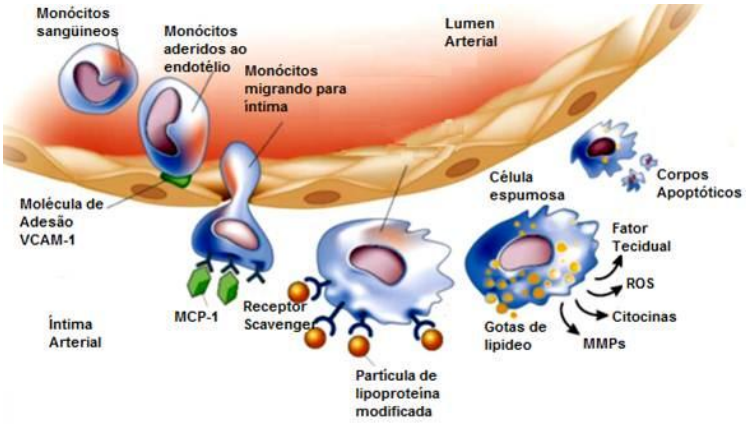

Figura 1: Recrutamento e ativação de macrófagos na lesão aterosclerótica. Monócitos da circulação se aderem às células endoteliais através de moléculas de adesão vascular (VCAM-1), atravessam a camada endotelial e na camada

íntima adquirem características de macrófagos teciduais. Os macrófagos expressam receptores que incorporam partículas de LDL modificadas e formam as células espumosas, componentes essenciais para o estabelecimento da lesão aterosclerótica. Estes macrófagos ativados, secretam componentes inflamatórios (citocinas, MMPs, ROS, etc) que intensificam e perpetuam o processo (Adaptado de Libby, 2002).

Com a ruptura da placa aterosclerótica, o material contido no interior da lesão entra em contato com o sangue circulante, o qual associado com as plaquetas ativadas pela trombina gerada a partir da cascata de coagulação, favorece a formação de trombos. Se o trombo obstruir o vaso persistentemente, o resultado pode acarretar eventos tais como: infarto agudo do miocárdio e acidente vascular cerebral. Por outro lado, a resposta de cicatrização desencadeada pela trombina gerada durante a coagulação sanguinea, pode estimular a proliferação de células da musculatura lisa e o estabelecimento de placas estáveis. O aumento da migração, proliferação e síntese de matriz extracelular por células musculares lisas aumenta a espessura da cápsula fibrosa e provoca uma maior expansão da íntima, levando a constrição do lúmen, consequentemente restringindo o fluxo, especialmente em situações de maior demanda cardíaca, levando a isquemia e sintomas como a angina de peito (Libby et al., 1996, Libby, 2002, Stocker e Keaney, 2004).

Quanto à classificação, as placas ateroscleróticas podem ser divididas em estáveis ou vulneráveis à ruptura. As placas estáveis possuem um pequeno núcleo lipídico, uma densa camada fibrosa e poucas células inflamatórias; enquanto as placas vulneráveis apresentam uma fina capa fibrosa e número elevado de células inflamatórias (Lusis, 2000, Stocker e Keaney, 2004) (Figura 2).

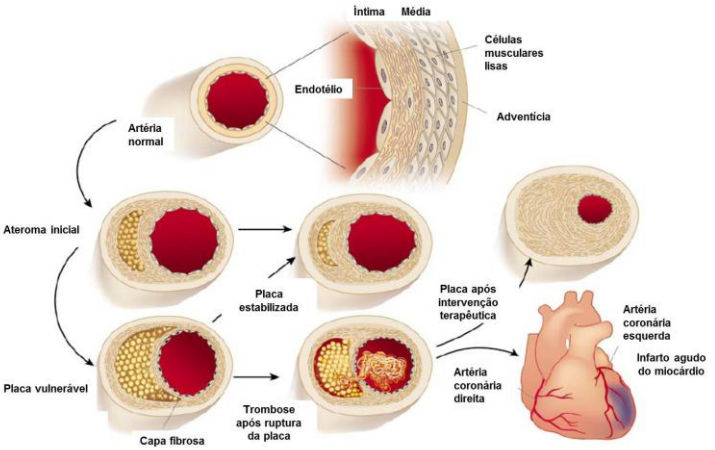

Figura 2. Progressão do ateroma. No ateroma inicial, o recrutamento de células inflamatórias e o acúmulo de lipídeos promovem uma expansão da parede arterial em direção ao lúmen do vaso. Se as condições inflamatórias e os fatores de risco, tais como a dislipidemia, persistirem, o núcleo lipídico aumenta e a capa fibrosa torna-se menos espessa, formando uma lesão vulnerável e susceptível à ruptura. Quando esta ruptura acontece, o sangue entra em contato com o conteúdo da lesão promovendo a formação de um trombo, que pode por sua vez pode obstruir o vaso de maneira persistente e resultar em infarto agudo do miocárdio (Adaptado de Libby, 2002).

1.3 LIPOPROTEÍNA DE BAIXA DENSIDADE (LDL) E A ATEROSCLEROSE

As LDLs são as principais lipoproteínas plasmáticas que transportam o colesterol e estão entre os agentes causadores da aterosclerose (Lusis, 2000). Estas lipoproteínas são constituídas por uma porção lipídica, composta principalmente por colesterol e uma única proteína - a apolipoproteína B-100 (apo B-100), e são responsáveis pelo fornecimento de colesterol para os tecidos periféricos. As células captam as LDLs através de receptores B/E que reconhecem a parte proteica da lipoproteína (Champe e Harvey, 1997). A ação destes receptores no fígado é responsável pela manutenção dos níveis de colesterol no sangue e depende da atividade da enzima Hidroximetilglutaril Coenzima A (HMG-CoA) redutase, que é a enzima chave para a síntese do colesterol hepático (Champe e Harvey, 1997, Sposito et al., 2007).

A associação entre o colesterol LDL e a aterosclerose foi estabelecida, em parte, com base em estudos sobre a hipercolesterolemia

familiar, uma doença autossômica dominante que afeta uma em cada 500 pessoas da população geral. No ano de 1985, os pesquisadores americanos Michael Brown e Joseph Goldstein conquistaram o prêmio Nobel de Medicina por caracterizarem o receptor de LDL e atribuírem a deficiência genética deste receptor à causa da hipercolesterolemia familiar. Heterozigotos para esta doença manifestam uma elevação de duas a cinco vezes nos níveis plasmáticos de LDL, em função de um prejuízo funcional do receptor de LDL, resultando em uma deficiência na depuração desta lipoproteína. Os indivíduos homozigotos apresentam um aumento de quatro a seis vezes no colesterol plasmático e desenvolvem aterosclerose precocemente (Gotto e Grundy, 1999, Steinberg, 2002)

1.3.1 Hipótese da modificação oxidativa da LDL

De acordo com a hipótese da modificação oxidativa da LDL, proposta por Steinberg e colaboradores (1989), as LDL oxidadas (LDLox) desempenham um papel fundamental na aterogênese.

As LDLs são partículas submicroscópicas com massa molecular relativa média de 3000 kDa, com diâmetro entre 22 e 28 nm e densidade entre 1.019 e 1.063 g/ml. A estrutura geral é de uma microemulsão esferoidal formada por uma camada externa de fosfolipídeos polares, colesterol livre ou não esterificado e proteína (Apo B-100), com um núcleo central de lipídeos neutros, predominantemente ésteres de colesterol e triacilgliceróis (Figura 3).

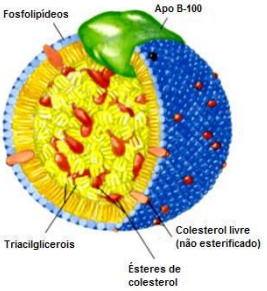

Figura 3: Representação esquemática de uma lipoproteína de baixa densidade (LDL). A partícula de LDL é formada de uma monocamada de fosfolipídeos e colesterol livre na camada externa onde também se encontra a maior proteína, a Apo B-100. Triacilgliceróis e ésteres de colesterol encontram-se na sua parte interna (Adaptado de Lehninger et al., 2006).

Os mecanismos das modificações das lipoproteínas *in vivo* ainda não estão completamente esclarecidos, todavia, um dos eventos iniciais na oxidação da LDL é a peroxidação dos lipídeos, particularmente, fosfolipídeos e ésteres de colesterol, os quais contêm ácidos graxos poliinsaturados (PUFAs). Um dos produtos finais da oxidação dos ácidos graxos é o dieno conjugado, o qual reage muito rapidamente com o oxigênio molecular iniciando uma reação auto-catalítica que leva a formação de hidroperóxidos. Os hidroperóxidos lipídicos, por clivagem da ligação carbono-carbono, podem ainda dar origem a fragmentos de cadeia curta, com 3 a 9 carbonos, incluindo aldeídos (malondialdeído e 4-hidroxinonenal) e cetonas, que podem conjugar-se com outros lipídeos ou proteínas (Porter, 1984, Yamaguchi et al., 2002). A presença de peróxidos lipídicos em extratos preparados de aortas ateroscleróticas foi descrita há mais de 50 anos e, desde então, foi sugerido que a peroxidação lipídica desempenha um papel ativo na patogênese da aterosclerose (Glavind et al., 1952).

Adicionalmente à oxidação lipídica, a apo B-100 fragmenta-se por cisão oxidativa. Os produtos formados na lipoperoxidação reagem com resíduos de lisina da apo B-100, formando bases de Schiff (Steinbrecher, 1987, Yamaguchi et al., 2002). As alterações conformacionais causadas pela perda da estrutura secundária ou alterações de carga elétrica, resultam em uma proteína com carga negativa aumentada. Por sua vez, este aumento na carga global negativa da apo B-100 resulta no aumento de reconhecimento pelo receptor *scavenger* dos macrófagos, levando ao acúmulo descontrolado de LDL por estas células e a consequente formação de células espumosas, resultando na lesão fisiopatológica primária da aterosclerose (Figura 4).

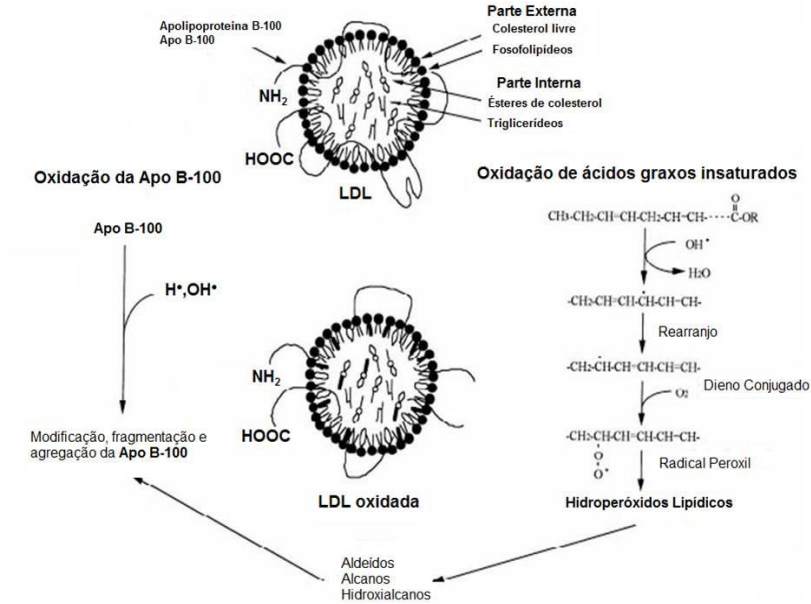

Figura 4: Modificação oxidativa da LDL. O dano oxidativo a LDL afeta ambos componentes lipídicos e proteicos da molécula. A oxidação da LDL leva à modificação, fragmentação e agregação da apo B-100. Por outro lado, a oxidação da LDL inicia-se através da peroxidação lipídica, principalmente de fosfolipídeos e ésteres de colesterol, resultando na formação de dienos conjugados e hidroperóxidos lipídicos (Adaptado de Yamaguchi et al., 2002).

Adicionalmente, as LDLox e seus produtos derivados, podem atrair monócitos e induzir a adesão destes ao endotélio, ação que pode desencadear o processo inflamatório implicado na aterosclerose. Está descrita ainda a capacidade das LDLox induzirem a migração e proliferação das células musculares lisas, por promoção da expressão de fatores de crescimento, impedirem a migração endotelial necessária para reparação do dano, e ainda promoverem a injúria celular, por via necrótica ou apoptótica. Ao promover a disfunção do endotélio, as LDLox interferem com o relaxamento vascular dependente do endotélio, por indução de perda da bioatividade do óxido nítrico ($^{\bullet}$NO) e, também, pela promoção da atividade pró-coagulante das células vasculares (Chisolm e Steinberg, 2000).

1.4 ESTRESSE OXIDATIVO E ATEROSCLEROSE

O estresse oxidativo pode ser definido como um distúrbio entre o balanço pró-oxidante/antioxidante em favor da situação pró-oxidante, levando a um dano oxidativo que compromete a resposta redox celular. Esta situação pode ser resultado de uma diminuição dos sistemas antioxidantes ou aumento da produção de espécies reativas de oxigênio (ROS) e de nitrogênio (RNS) (Halliwell, 1999).

As ROS/RNS mais relevantes e deletérias originadas nos sistemas biológicos incluem: o radical ânion superóxido ($O_2^{\bullet -}$), o radical hidroxil ($^{\bullet}OH$), óxido nítrico ($^{\bullet}NO$), peróxido de hidrogênio (H_2O_2), peroxinitrito ($ONOO^-$) e ácido hipocloroso (HOCl) (Halliwell, 1999). A utilização alterada do oxigênio e/ou a formação aumentada de ROS e/ou de RNS, contribuem para a progressão da aterogênese e das doenças cardiovasculares. Vários sistemas enzimáticos são importantes na geração destas espécies reativas, tais como a xantina oxidase, NADPH oxidases, citocromo P450, fontes mitocondriais (cadeia transportadora de elétrons) e as óxido nítrico sintases (NOS) (Harrison et al., 2003). Em nível mitocondrial, os complexos I e III da cadeia respiratória são as principais fontes produtoras de $O_2^{\bullet -}$ (Turrens, 2003).

A produção aumentada de ROS em situações de estresse oxidativo, pode induzir eventos celulares tóxicos, como a inativação do $^{\bullet}NO$, ativação de monócitos, modificações oxidativas do DNA e proteínas, oxidação de lipídeos, aumento da mitogenicidade e apoptose de células vasculares, e aumento da expressão e ativação de genes redox-sensíveis, tais como receptores para LDLox, moléculas de adesão, fatores quimiotáticos, citocinas pró-inflamatórias, reguladores do ciclo celular e metaloproteinases de matriz (Wassmann et al., 2004, Kaliora et al., 2006).

No ambiente vascular, as ROS são responsáveis pela indução das modificações oxidativas da LDL, convertendo esta lipoproteína em partículas mais aterogênicas (Rizzo et al., 2009). A oxidação da LDL, mediada por ROS envolve a peroxidação de ácidos graxos e modificação covalente da apo B-100 por produtos da peroxidação lipídica. Assim, a LDLox é rapidamente captada pelos macrófagos através dos receptores *scavenger* que, ao contrário do receptor que reconhece a LDL nativa, não apresenta hiporregulação em resposta ao excesso de colesterol celular, dando origem às células espumosas (Steinberg e Witztum, 2010).

A LDLox também pode induzir estresse oxidativo, levando a geração de $O_2^{\bullet -}$ pelas células endoteliais e musculares lisas,

intensificando o processo de disfunção endotelial e vascular (Williams e Fisher, 2005, Galle et al., 2006). Nas células endoteliais, o $^{\bullet}NO$ protege a vasculatura do desenvolvimento da aterosclerose através de suas propriedades vasodilatadoras, da inibição da expressão de moléculas de adesão, agregação plaquetária, proliferação de células musculares lisas e oxidação da LDL (Gaut e Heinecke, 2001). A produção intensa de $O_2^{\bullet-}$ pode favorecer a reação deste radical com o $^{\bullet}NO$ formando o $ONOO^-$, o qual atua como potente agente oxidante e nitrante, promovendo danos celulares e intensificando o processo pró-aterogênico (Yokoyama, 2004).

Como mecanismo de proteção ao estresse oxidativo e aos danos promovidos por este processo na vasculatura, as células possuem sistemas de defesa antioxidantes, tais como as enzimas superóxido dismutase (SOD), glutationa peroxidase (GPx), glutationa transferase (GST), catalase e as enzimas que sintetizam glutationa reduzida (GSH), além de outras (Kondo et al., 2009). Distúrbios nestes sistemas de defesa podem levar ao acúmulo de ROS e acelerar o desenvolvimento da aterosclerose (Hill e Singal, 1996). Por outro lado, a expressão aumentada de enzimas, como a GPx, SOD e a catalase, pode retardar o aparecimento de lesões em modelos animais de aterosclerose (Yang et al., 2004, 2009).

Modelos animais utilizando coelhos hipercolesterolêmicos demonstraram a primeira evidência indireta do papel da LDL na indução do estresse oxidativo. As aortas destes animais produziram significativamente mais $O_2^{\bullet-}$ do que as aortas controles (Ohara et al., 1993, Mugge et al., 1994). Adicionalmente, outros estudos demonstraram que quando células endoteliais, artérias isoladas e outras células sanguíneas foram incubadas com LDLox a formação de $O_2^{\bullet-}$ foi aumentada (Galle et al., 1995, Maeba et al., 1995). Cabe ressaltar ainda que, a LDL é quimicamente vulnerável às ROS, pois possuem grande quantidade de ácidos graxos poliinsaturados susceptíveis à oxidação (Kaliora et al., 2006).

A geração intracelular de ROS pode representar um evento chave nas ações citotóxicas da LDLox. Desta forma, estratégias terapêuticas para o tratamento de doenças relacionadas ao processo inflamatório e oxidativo, como a aterosclerose, deve incluir não só antioxidantes extracelulares que impeçam a oxidação da LDL, mas também antioxidantes permeáveis à membrana que podem inibir a geração de ROS no meio intracelular. Parte do fracasso dos antioxidantes nos ensaios clínicos relatados até o momento, pode estar associado à sua

incapacidade de inibir a geração intracelular de ROS em resposta a LDLox (Levitan et al., 2010).

1.5 ANTIOXIDANTES E ATEROSCLEROSE

Considerando a "teoria oxidativa da aterosclerose", antioxidantes presentes na dieta têm atraído considerável atenção como agentes preventivos e terapêuticos. Inúmeras evidências obtidas por estudos *in vitro*, *in vivo* e intervenções controladas demonstram que o consumo de antioxidantes previne a progressão da aterosclerose (Otero et al., 2002, Bleys et al., 2006, Frederiksen et al., 2007).

As enzimas antioxidantes, incluindo a superóxido dismutase (SOD), catalase e glutationa peroxidase (GPx) constituem o sistema central de defesa contra o estresse oxidativo (Forsberg et al., 2001). Distúrbios nestes sistemas de defesa podem levar ao acúmulo de ROS e acelerar o desenvolvimento da aterosclerose (Hill e Singal, 1996).

1.5.1 Glutationa Peroxidase e aterosclerose

A glutationa peroxidase (GPx) é uma selenoenzima presente em muitos tipos celulares e utiliza glutationa reduzida para converter H_2O_2 em H_2O, e peróxidos lipídicos em seus respectivos álcoois (Wilson et al., 1989, Stocker e Keaney, 2004). Além disso, a GPx pode atuar também como peroxinitrito redutase (Sies, 1999). Em mamíferos, existem quatro principais classes de GPx, que diferem entre si na distribuição tecidual e localização celular. Esta enzima contém selenocisteína em seu sítio ativo, essencial para sua atividade catalítica (Arthur, 2000, Margis et al., 2008).

Durante o ciclo catalítico da GPx, o grupo selenol/selenolato ($GPx\text{-}Se^-$) inicialmente interage com moléculas pro-oxidantes, como H_2O_2 ou hidroperóxidos orgânicos, formando um ácido selenínico (GPx-Se-OH) o qual reage com a primeira molécula de glutationa reduzida (GSH) formando selenopersulfato (GPx-Se-SG) que por sua vez sofre ataque da segunda molécula de GSH, regenerando o selenol/selenolato no sítio ativo da GPx e liberando a glutationa na sua forma oxidada (GSSG) (Nogueira e Rocha, 2010) (Figura 5).

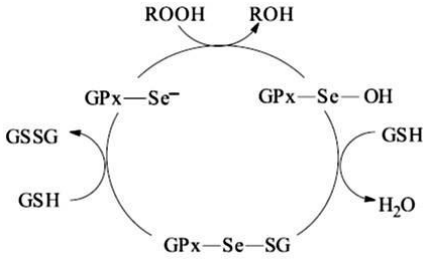

Figura 5. Ciclo catalítico da glutationa peroxidase (GPx). Fonte: Nogueira e Rocha 2010.

Estudos clínicos recentes têm demonstrado que a diminuição da atividade da enzima GPx-1 eritrocitária está associada ao risco aumentado de eventos cardiovasculares (Blankenberg et al., 2003, Espinola-Klein et al., 2007). Além disso, a atividade da GPx-1 está diminuída ou ausente em placas ateroscleróticas humanas e esta diminuição na atividade desta enzima está associada ao desenvolvimento de lesões mais severas (Lapenna et al., 1998).

Em camundongos, a deficiência genética da GPx-1 causa disfunção endotelial, acompanhada de aumento do estresse oxidativo, anormalidades funcionais e estruturais do sistema cardiovascular (Forgione et al., 2002), além de acelerar a progressão da lesão aterosclerótica em camundongos deficientes da apolipoproteína E (apoE -/-) (Torzewski et al., 2007). Por outro lado, a superexpressão da GPx-4, diminui a lesão aterosclerótica em camundongos apoE -/- (Guo et al., 2008).

Considerando que a GPx é uma enzima chave na proteção de vasos sanguíneos contra o estresse oxidativo e aterogênese, surge a hipótese de que compostos com atividade mimética desta enzima possam atuar beneficamente na prevenção do desenvolvimento da aterosclerose. Além disso, numerosos estudos têm verificado que a utilização de substâncias antioxidantes está relacionada à prevenção das doenças cardiovasculares (Glass e Witztum, 2001, Brigelius-Flohe et al., 2005, Sposito et al., 2007).

1.6 COMPOSTOS ORGÂNICOS DE SELÊNIO

O selênio é um elemento traço essencial, componente de enzimas com atividades antioxidantes, particularmente das isoformas da glutationa peroxidase (GPx) (Flohe et al., 1973) e do sistema tireoxina redutase (TrxR) (Engman et al., 1997). Estes sistemas enzimáticos têm importantes papéis na defesa celular, protegendo contra processos oxidativos pela detoxificação de hidroperóxidos de hidrogênio ou lipídicos e ainda apresentam específico envolvimento na defesa contra o peroxinitrito (Sies e Arteel, 2000, Klotz e Sies, 2003).

Diferentes classes de compostos orgânicos de selênio exibem atividade mimética da GPx e decompõem peróxido de hidrogênio e hidroperóxidos orgânicos utilizando GSH ou outros tióis como doadores de hidrogênio (Wilson et al., 1989, Nogueira et al., 2004). O conceito de que moléculas contendo selênio podem ser melhores nucleófilos (e, portanto antioxidantes) do que os antioxidantes clássicos têm levado ao desenvolvimento de organocalcogênios sintéticos (Sies e Arteel, 2000).

Nas últimas décadas, o interesse em bioquímica, farmacologia e toxicologia de compostos orgânicos de selênio aumentou de maneira significativa devido à variedade de propriedades biológicas desempenhadas por estes compostos (Parnham e Graf, 1991, Kanda et al., 1999, Nogueira et al., 2004).

1.6.1 Ebselen

O primeiro exemplo desta classe de compostos foi o ebselen (Parnham e Kindt, 1984). O ebselen (2-fenil-1,2-benzilsoselenazol-3(2H)-ona) é um composto orgânico de selênio cujas propriedades antioxidantes e anti-inflamatórias têm merecido destaque no campo da farmacologia. Este composto foi descrito e caracterizado como um mimético da enzima glutationa peroxidase na década de 80 (Muller et al., 1984), entretanto, apenas a partir da década de 90, cresceu enormemente o número de trabalhos demonstrando seus efeitos protetores em diferentes tipos celulares e para os mais diversos tipos de injúria.

O ebselen demonstrou ações anti-inflamatória e antioxidante em diversos modelos experimentais *in vivo* e *in vitro* (Schewe, 1995, Porciuncula et al., 2003, Brodsky et al., 2004). Além disso, foi empregado com sucesso em estudos clínicos em pacientes com aneurisma hemorrágico e isquemia aguda (Parnham e Sies, 2000).

Recentemente, um estudo utilizando camundongos apoE -/- mostrou o efeito antiaterogênico do ebselen na aterosclerose associada à hiperglicemia (Chew et al., 2009). Outros trabalhos verificaram que o ebselen restaura a função endotelial em ratos diabéticos (Brodsky et al., 2004) e diminui as lesões ateroscleróticas em camundongos transgênicos com expressão aumentada da NADPH oxidase (Khatri et al., 2004).

As atividades farmacológicas do ebselen podem ser explicadas em parte por sua capacidade de neutralizar eficientemente hidroperóxidos orgânicos e $ONOO^-$, mimetizando a enzima GPx (Muller et al., 1984, Masumoto e Sies, 1996).

1.6.2 Disseleneto de difenila $(PhSe)_2$

O disseleneto de difenila $(PhSe)_2$, assim como o ebselen, é um composto orgânico de selênio que reage eficientemente com hidroperóxidos e peróxidos orgânicos, através de reação similar a catalisada pela GPx. Todavia, o $(PhSe)_2$ demonstrou ser mais ativo como mimético da GPx (Wilson et al., 1989) e menos tóxico em roedores que o ebselen (Meotti et al., 2003, Nogueira et al., 2003).

O mecanismo catalítico para a detoxificação de peróxidos pelo $(PhSe)_2$ foi proposto. Inicialmente o $(PhSe)_2$ reage com um grupamento tiol (RSH) (por exemplo GSH) originando um selenilsulfeto, o qual reage com um segundo equivalente de GSH formando um selenol (Se-H), este selenol reduz o H_2O_2 ou peróxidos lipídicos em H_2O, fechando assim o ciclo catalítico (Nogueira e Rocha, 2010) (Figura 6).

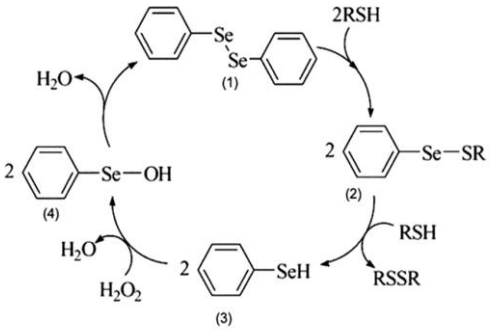

Figura 6. Ciclo catalítico proposto para o Disseleneto de difenila $(PhSe)_2$. Fonte: Nogueira e Rocha, 2010.

A tabela 1 demonstra importantes propriedades farmacológicas desempenhadas pelo $(PhSe)_2$. Este composto apresenta papel protetor em uma variedade de modelos experimentais associados à produção exacerbada de radicais livres (Rossato et al., 2002, Ghisleni et al., 2003, Burger et al., 2004, Meotti et al., 2004, Borges et al., 2005). Em modelo experimental de diabetes, o tratamento com $(PhSe)_2$ reduziu significativamente a hiperglicemia e a indução de estresse oxidativo em diferentes tecidos de roedores (Barbosa et al., 2006, Barbosa et al., 2008). Adicionalmente, este composto foi capaz atuar beneficamente em modelos experimentais de inflamação e dor (Nogueira et al., 2003, Savegnago et al., 2006, Savegnago et al., 2007, Savegnago et al., 2008), e na indução de hepatotoxicidade em roedores (Borges et al., 2005, Borges et al., 2008). O $(PhSe)_2$ também demonstrou proteger as plaquetas humanas contra peroxidação lipídica induzida pelo nitroprussiato de sódio, um doador de óxido nítrico, e reativou a atividade da GPx nestas células (Posser et al., 2006).

Recentemente, nosso grupo de pesquisa demonstrou que o tratamento por via oral com $(PhSe)_2$ reduz a hipercolesterolêmia e o estresse oxidativo em coelhos alimentados com uma dieta rica em colesterol (de Bem et al., 2009). O $(PhSe)_2$ também foi capaz de inibir a oxidação da LDL humana isolada *in vitro* e este efeito foi relacionado com a sua atividade tiol-peroxidase (de Bem et al., 2008). Além disso, verificamos que este composto foi capaz de reduzir a formação de lesões ateroscleróticas em camundongos hipercolesterolêmicos deficientes para receptor LDL (LDLr -/-) e diminuir a formação de células espumosas, produção de mediadores inflamatórios e ROS em macrófagos expostos a LDLox (Hort, 2011).

Tabela 1: Propriedades farmacológicas do Disseleneto de Difenila em diferentes modelos experimentais.

Modelo experimental	Tratamento	Espécie animal	Referência
Hepatotoxicidade	agudo	rato	(Borges et al., 2005);(2006)
Dano gástrico	agudo	rato	(Ineu et al., 2008)
Dano pulmonar	agudo	camundongo/rato	(Luchese et al., 2007); (2009)
Hiperglicemia	agudo	rato	(Barbosa et al., 2006)
Carcinogênese mamária	crônico	rato	(Barbosa et al., 2008)
Falência renal	agudo	rato	(Brandao et al., 2009)
Ansiedade	agudo	camundongo	(Savegnago et al., 2008)
Depressão	agudo	camundongo/rato	(Ghisleni et al., 2008); (Savegnago et al., 2008); (Acker et al., 2009)
Performance cognitiva	agudo	camundongo	(Rosa et al., 2003)
Memória espacial	sub-crônico	rato	(Stangherlin et al., 2008)
Discinesia orofacial	sub-crônico	rato	(Burger et al., 2006)
Hipercolesterolêmia	sub-crônico	coelho	(de Bem et al., 2009)
Hiperlipidemia	agudo	camundongo	(da Rocha et al., 2009)

Fonte: (Nogueira e Rocha, 2010).

Diversas evidências obtidas por estudos em modelos animais e intervenções controladas em humanos comprovam o envolvimento do estresse oxidativo na aterogênese e sugerem que o consumo de antioxidantes tenha um importante efeito no tratamento e prevenção das doenças cardiovasculares (Thomson et al., 2007). Neste contexto e diante das importantes propriedades farmacológicas descritas para o $(PhSe)_2$, bem como da participação da GPx no controle do processo aterogênico, hipotetizamos que este composto pode ser efetivo na prevenção e/ou tratamento da aterosclerose.

1.6.3 Organocalcogênios substituídos

Compostos orgânicos de selênio, como ebselen e o $(PhSe)_2$, têm demonstrado várias propriedades biológicas. Desta forma, a busca por novos compostos que possam mimetizar seus efeitos tem sido intensamente investigado. Desta forma, a introdução de um grupamento funcional no anel aromático do $(PhSe)_2$ pode prover novas alternativas de agentes terapêuticos.

Os compostos organocalcogênios substituídos ou diaril disseleneto substituídos, *p*-cloro-difenil disseleneto (*p*-Cl–$C_6H_4Se)_2$ - (DC) e *p*-metoxi-difenil disseleneto (*p*-CH_3O–$C_6H_4Se)_2$ - (DM) foram sintetizados a partir do $(PhSe)_2$, através da introdução de grupamentos funcionais metoxi e cloro ao anel aromático (Paulmier, 1986).

O primeiro estudo reportando propriedades farmacológicas de diaril disseleneto substituídos foi publicado em 2003. Neste estudo, a administração intraperitoneal de DM e *m*-tricloro-difenil disseleneto (*m*-CF_3–$C_6H_4Se)_2$ inibiram o edema de pata induzido por carragenina em ratos (Nogueira et al., 2003). A partir de então, outros estudos estão sendo conduzidos no intuito de verificar possíveis propriedades farmacológicas para estes compostos. Os compostos DM e DC, inibiram a atividade da enzima δ-aminolevulinato desidratase (δ-ALA-D) em homogenatos de cérebro de ratos, por interagirem diretamente com grupamentos tióis oxidando-os a dissulfetos (Bruning et al., 2009).

Estudo recente demonstrou que o DM possui efeito hepatoprotetor ao dano agudo induzido por lipopolissarídeo (LPS) (Wilhelm et al., 2009) e foi capaz de produzir efeito antinociceptivo em camundongos (Jesse et al., 2009). O tratamento com DM foi capaz de proteger da perda de memória e reforçar o aprendizado em modelo de demência esporádica do tipo doença de Alzheimer em camundongos induzido pela administração intracerebroventricular de estreptozotocina (Pinton et al., 2010).

Neste sentido, acreditamos que estes compostos (DM e DC) análogos estruturais do $(PhSe)_2$, possam apresentar importantes propriedades farmacológicas em modelos experimentais relacionados à aterosclerose. Por outro lado, estudos adicionais necessitam ser conduzidos para determinar os mecanismos pelos quais estes compostos atuam em sistemas biológicos.

1.7 TOXICIDADE DO $(PhSe)_2$

Recentemente, os pesquisadores Nogueira e Rocha, os quais estudam a mais de uma década as propriedades farmacológicas e toxicológicas de compostos de selênio, publicaram um artigo de revisão intitulado “Diphenyl Diselenide a Janus-Faced Molecule” (Nogueira e Rocha, 2010). Esta revisão aborda uma reflexão sobre o potencial terapêutico e tóxico destes compostos com particular ênfase ao $(PhSe)_2$. Estas moléculas apresentam dupla característica, sendo que seu comportamento é dependente da dose utilizada. Em baixas doses,

possuem efeitos benéficos e em altas doses são potencialmente tóxicos (Nogueira e Rocha, 2010).

Em relação ao mecanismo de toxicidade molecular do $(PhSe)_2$, este pode estar associado com a oxidação catalítica de importantes grupos tióis da GSH ou de diferentes proteínas ou enzimas endógenas sulfidrílicas, incluindo a 5-lipoxigenase (Bjornstedt et al., 1996), δ-ALA-D (Nogueira et al., 2003), esqualeno monooxigenase (Gupta e Porter, 2001) e Na^+,K^+-ATPase (Borges et al., 2005). A inibição da atividade destas enzimas pela oxidação de grupos tióis pelos compostos de disseleneto podem contribuir para sua toxicidade celular (Kade et al., 2009, Nogueira e Rocha, 2010).

A exposição em camundongos a altas concentrações de $(PhSe)_2$, causou hepatotoxicidade, depleção de grupamentos tióis em vários tecidos e inibição da enzima δ-ALA-D, sensível a oxidação a grupos sulfidrílicos (Maciel et al., 2000, Jacques-Silva et al., 2001). Em outro estudo, o $(PhSe)_2$, demonstrou ser mais tóxico em camundongo que em ratos quando administrado via intraperitoneal e não apresentou efeitos tóxicos quando administrado via subcutânea ou via oral nestas espécies (Meotti et al., 2003, Nogueira et al., 2003, Wilhelm et al., 2009).

A toxicidade do $(PhSe)_2$ é dependente da via de administração, da dose e da espécie de animal (rato, camundongo ou coelho) (Nogueira et al., 2003, de Bem et al., 2006, de Bem et al., 2007, Meotti et al., 2008). Trabalhos anteriores de nosso grupo demonstraram que o tratamento crônico com $(PhSe)_2$, por via oral induziu sinais sutis de toxicidade em coelhos expostos por longo período (8 meses). Neste estudo avaliamos diferentes marcadores de estresse oxidativo e de função renal e hepática em diversos tecidos dos animais expostos a 3 diferentes doses diárias de $(PhSe)_2$. Somente na maior dose utilizada (correspondente a 5 μmol/kg por dia) observamos pequenas alterações em marcadores do dano oxidativo, evidenciado pela diminuição dos níveis de ácido ascórbico a nível hepático e cerebral. Por outro lado, o tratamento crônico com o composto melhorou a resposta redox dos animais por aumentar os níveis de tióis não proteicos no fígado e nos eritrócitos (de Bem et al., 2007). O peso dos animais, bem como os marcadores periféricos de lesão hepática (AST, ALT) e renal (uréia e creatinina) não foram modificados pela exposição crônica dos animais ao $(PhSe)_2$ (de Bem et al., 2006). Em contraste com estudos realizados em camundongos e ratos, demonstrando a inibição da enzima sulfidrílica δ-ALA-D (Maciel et al., 2000, Jacques-Silva et al., 2001), o tratamento crônico com $(PhSe)_2$ em coelhos foi capaz de induzir aumento na atividade desta enzima em

eritrócitos, no fígado e no córtex cerebral destes animais (de Bem et al., 2006).

Neste sentido, a complementação dos estudos relacionados à avaliação das propriedades toxicológicas deste composto em modelos de não roedores (coelhos, por exemplo), torna-se imperativa.

2 OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo geral deste trabalho foi investigar os potenciais efeitos antiaterogênicos e toxicológicos de compostos orgânicos de selênio em modelos experimentais *in vivo* e *in vitro*.

2.2 OBJETIVOS ESPECÍFICOS

- Investigar os efeitos toxicológicos sobre parâmetros bioquímicos e marcadores do estresse oxidativo no sangue e em diferentes tecidos de coelhos expostos agudamente pela via intraperitoneal ao disseleneto de difenila $(PhSe)_2$;
- Investigar o mecanismo do possível efeito protetor do disseleneto de difenila $(PhSe)_2$ sobre a toxicidade induzida pela exposição à LDL oxidada (LDLox) em cultura de macrófagos murinos J774;
- Avaliar o possível efeito protetor *in vitro* dos diaril disselenetos substituídos *p*-metoxi-difenil disseleneto $(p\text{-}CH_3O\text{–}C_6H_4Se)_2$ e *p*-cloro-difenil disseleneto $(p\text{-}Cl\text{–}C_6H_4Se)_2$ (DM e DC, respectivamente) na proteção contra a oxidação da LDL humana isolada, relacionando o possível mecanismo de ação.

3 JUSTIFICATIVA

As doenças cardiovasculares, que tem como causa primária a aterosclerose, representam a principal causa de morbidade e mortalidade nos países ocidentais. Considerando a "hipótese oxidativa da aterosclerose", antioxidantes dietéticos têm atraído considerável atenção da comunidade acadêmica e da indústria farmacêutica como agentes preventivos e terapêuticos das doenças cardiovasculares.

Neste sentido, o interesse por estudos químicos e bioquímicos dos compostos orgânicos de selênio tem aumentado consideravelmente nas últimas três décadas, principalmente devido ao fato de que vários destes compostos apresentam importantes atividades antioxidantes, as quais possivelmente estão associadas à capacidade destes em mimetizar a atividade da enzima glutationa peroxidase.

O $(PhSe)_2$ é um composto sintético orgânico de selênio e várias propriedades farmacológicas já foram descritas para este composto, destacando-se as atividades antioxidante, antinflamatória, antinociceptiva, hepatoprotetora entre outras. Além disso, o $(PhSe)_2$ foi capaz de inibir a oxidação de LDL humana isolada *in vitro* e o estresse oxidativo em coelhos hipercolesterolêmicos, bem como reduziu as lesões ateroscleróticas em camundongos deficientes do receptor de LDL (LDLr -/-). Assim, o estudo farmacológico de novas moléculas semelhante quimicamente ao $(PhSe)_2$ é um grande incentivo a possibilidade da utilização destes compostos no tratamento das doenças cardiovasculares.

Por outro lado, o estudo das potenciais ações toxicológicas do $(PhSe)_2$ é de fundamental relevância. O tratamento com $(PhSe)_2$ não foi tóxico quando administrado agudamente em ratos e camundongos em doses que demonstraram efeitos anti-inflamatório e antinociceptivo. Cabe ainda ressaltar, que a administração crônica de $(PhSe)_2$ por via oral em coelhos não produziu sinais grosseiros de toxicidade. Por outro lado, a administração subcrônica de altas doses deste composto induziu sinais de toxicidade em ratos. Desta forma, torna-se indispensável a avaliação toxicológica do $(PhSe)_2$ por diferentes vias de administração e em diferentes espécies.

Baseado nestas evidências, acreditamos na possibilidade futura do emprego de compostos orgânicos de selênio como eficientes agentes preventivos e terapêuticos em patologias relacionadas ao estresse oxidativo, em especial a aterosclerose. Acreditamos que a concretização deste estudo contribua para novas possibilidades de tratamento das doenças cardiovasculares.

4. RESULTADOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo e manuscritos em fase de redação final, os quais encontram-se aqui organizados.

Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigo e manuscritos.

4.1 AVALIAÇÃO DOS EFEITOS TOXICOLÓGICOS DA EXPOSIÇÃO AGUDA PELA VIA INTRAPERITONEAL AO DISSELENETO DE DIFENILA $(PhSe)_2$ EM COELHOS;

Artigo:

Acute exposure of rabbits to diphenyl diselenide: a toxicological evaluation.

Artigo publicado no periódico: *Journal Applied Toxicology*, 2010.

4.2 AVALIAÇÃO DO EFEITO PROTETOR DO DISSELENETO DE DIFENILA $(PHSE)_2$ SOBRE A TOXICIDADE INDUZIDA PELA EXPOSIÇÃO À LDL OXIDADA (LDLox) EM CULTURA DE MACRÓFAGOS MURINOS J774;

Manuscrito 1, em fase final de preparação:

Diphenyl diselenide, a simple GPx-mimetic compound, potently protects macrophages from oxLDL-induced cytotoxicity

4.3 AVALIAÇÃO DO EFEITO PROTETOR *IN VITRO* DOS DIARIL DISSELENETOS SUBSTITUÍDOS *P*-METOXI-DIFENIL DISSELENETO (P-CH_3O-$C_6H_4Se)_2$ E *P*-CLORO-DIFENIL DISSELENETO (P-Cl-$C_6H_4Se)_2$ E (DM E DC, RESPECTIVAMENTE) NA PROTEÇÃO CONTRA A OXIDAÇÃO DA LDL HUMANA ISOLADA.

Manuscrito 2, em fase final de preparação :

Disubstituted diaryl diselenides as potential atheroprotective compounds: involvement of TrxR and GPx-like systems.

4.1 ARTIGO:

Acute exposure of rabbits to diphenyl diselenide: a toxicologgical evaluation.

Marcos Raniel Straliotto, Gianni Mancini, Jade de Oliveira, Evelise Maria Nazari, Yara Maria Rauh Müller, Alcir Dafre, Susana Ortiz, Edson Luiz Silva, Marcelo Farina, Alexandra Latini, João Batista Teixeira Rocha, Andreza Fabro de Bem. ***J Appl Toxicol.*** **(8):761-8, 2010.**

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Acute exposure of rabbits to diphenyl diselenide: a toxicological evaluation

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ABSTRACT: The simple organoselenium compound diphenyl diselenide $(PhSe)_2$ is a promising new pharmacological agent. However, few toxicological evaluations of this molecule have been reported. We evaluated the effects of acute administration of $(PhSe)_2$ on toxicological parameters in rabbits. Adult New Zealand rabbits were exposed to $(PhSe)_2$ (5–500 μmol kg^{-1}, intraperitoneally) once a day for 5 days. Exposure to 500 μmol kg^{-1} caused 85% mortality. Exposure to 50 μmol kg^{-1} of $(PhSe)_2$ increased the glutathione levels in the hippocampus, kidney, heart, muscle and blood, whereas lipoperoxidation (TBARS) decreased in the cerebellum and kidney after exposure to 5 μmol kg^{-1}. The activity of glutathione peroxidase increased in the heart and muscle of rabbits treated with 50 μmol kg^{-1} of $(PhSe)_2$ and glutathione reductase activity was reduced in the cerebellum, cerebral cortex and kidney. Treatment with $(PhSe)_2$ reduced the activity of δ-aminolevulinate dehydratase in the hippocampus and increased this activity in the heart, but did not alter the activity of complexes I and II of the respiratory chain in the liver and brain. Hepatic and renal biochemical and histological parameters were not modified by $(PhSe)_2$ and apoptosis was not detected in these tissues; however, the hepatic cells tended to accumulate fat vacuoles. These results indicated that acute toxicology to $(PhSe)_2$ in rabbit is dependent on the dose, which should motivate further experiments on the therapeutic properties of this compound. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: toxicity; diphenyl diselenide; antioxidant; oxidative stress; rabbits

INTRODUCTION

Oxidative stress is defined as an imbalance between oxidants and antioxidants in favor of oxidants, potentially leading to damage, and may play important roles in the etiology of degenerative diseases such as atherosclerosis, cancer and neurodegenerative diseases, and in the aging process (Sies, 1991). Therefore, the study of the antioxidant properties of natural or synthetic compounds is an important tool in the search for new pharmacological agents (Kinsella *et al.*, 1993).

Interest in organoselenide chemistry and biochemistry has increased in the past three decades, mainly due to their chemical and biological activities (Nogueira *et al.*, 2004; Parnham and Graf, 1987). The pharmacological properties of organoselenium compounds have been extensively reported using [2-phenyl-1,2-benzisoselenazole-3 (2H)-one], commonly known as ebselen (Nogueira *et al.*, 2003, 2004; Schewe, 1995; Yamaguchi *et al.*, 1998). Ebselen has glutathione peroxidase-like activity and considerable antioxidant properties (Muller *et al.*, 1984; Nogueira *et al.*, 2004). In addition to ebselen, other forms of organoselenium compounds have been studied in the past two decades (Nogueira *et al.*, 2004; Parnham and Graf, 1987).

Diphenyl diselenide is the simplest of the synthetic diaryl diselenides, and several studies by our group have proposed the pharmacological use of this compound (Nogueira *et al.*, 2004). We demonstrated that $(PhSe)_2$ inhibits human LDL oxidation *in vitro* (Bem *et al.*, 2008) and reduces oxidative stress in hypercholesterolemic rabbits (Bem *et al.*, 2009). In addition, Barbosa *et al.* (2006, 2008) showed that $(PhSe)_2$ causes a significant reduction in the levels of blood glucose and glycated proteins in diabetic rats. The anti-nociceptive, neuroprotective, hepatoprotective and anti-inflammatory properties of $(PhSe)_2$ have been demonstrated in different *in vitro* and *in vivo* experimental models (Borges *et al.*, 2005; Ghisleni *et al.*, 2003; Savegnago *et al.*, 2007; Zasso *et al.*, 2005). Furthermore, the antioxidant ability of $(PhSe)_2$ has been shown in several *in vitro* systems containing different types of

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tissue, including brain, liver and platelets (Borges *et al.*, 2005; Posser *et al.*, 2006).

The antioxidant potential of $(PhSe)_2$ can be explained in part by its glutathione peroxidase-like activity. The reaction catalyzed by the organoselenium compound is similar to that catalyzed by glutathione peroxidase (Nogueira *et al.*, 2004). This activity is of particular significance in living cells because it decomposes hydrogen peroxide, phospholipid hydroperoxide and other organic hydroxyperoxides, preventing the formation of the reactive and toxic hydroxyl and lipoperoxyl radicals (Draper and Hadley, 1990).

Of particular importance is the low toxicity of $(PhSe)_2$ in rabbits after long-term oral exposure (Bem *et al.*, 2006, 2007). Similarly, acute or subchronic exposure of $(PhSe)_2$ induces minor toxicological effects in rodents in supra-pharmacological doses (Nogueira *et al.*, 2003). In contrast, it causes toxic effects in rats after exposure to high doses (Meotti *et al.*, 2008), but is less toxic to rodents than ebselen (Meotti *et al.*, 2003; Nogueira *et al.*, 2003). Considering that few toxicological evaluations of this molecule have been published and that this simple diorganochalcogenide has been reported as a promising pharmacological agent, we evaluated the potential toxicological effects of acute exposure in rabbits.

MATERIALS AND METHODS

Materials

Diphenyl diselenide $(PhSe)_2$ was synthesized according to the literature methods (Paulmier, 1986). Analysis of the ^1H NMR and ^{13}C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Animals

Male and female adult New Zealand rabbits weighing 1.8–2.2 kg were used. The animals were kept in a separate room, housed in individual cages on a 12 h light–dark cycle, at a temperature of 22 ± 2°C and with free access to water. The animals were used according to guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Catarina.

Experimental Protocol

After 1 week of adaptation, the rabbits were randomly divided into four experimental groups ($n = 6$ for each; three male and three female) and treated intraperitoneally (i.p.) with $(PhSe)_2$, dissolved in DMSO (1 ml kg^{-1} weight), at doses of 0, 5, 50 and 500 µmol kg^{-1} body weight, once a day for 5 days. The animals from the control group received only vehicle by the same route. Animals were weighed daily during the experiment. The baseline of body weight was obtained just before beginning treatment. Food consumption was controlled and each animal received 100 g of regular chow per day.

Twenty-four hours after the last exposure of $(PhSe)_2$ (sixth day), the rabbits were anesthetized for blood collection by heart puncture. An aliquot of 2 ml was separated into vacuum tubes. Whole blood with heparin was used for determination of δ-aminolevulinic dehydratase (δ-ALA-D) activity, glutathione (GSH) levels and blood with EDTA was used for hematological parameters. Plasma was obtained by centrifugation at 2000*g* for 10 min (hemolyzed plasma was discarded) and used for biochemical assays. The samples of liver, kidney, heart, gastrocnemius muscle and brain structures (cerebral cortex, cerebellum and hippocampus) were homogenized in 20 mM phosphate buffer, pH 7.4 and centrifuged at 2000*g* for 10 min at 4 °C. The low-speed supernatants (S1) were stored at −80 °C for no more than 3 weeks and used for determination of biochemical and toxicological parameters.

For measuring the respiratory chain complex activities, liver and brain samples were homogenized in 1:20 (w/vol.) of phosphate buffer pH 7.4, containing 0.3 M sucrose, 5 mM MOPS, 1 mM EGTA and 0.1% bovine serum albumin. The homogenates were centrifuged at 1000*g* for 10 min at 4 °C, the pellet was discarded and the supernatants were kept at −80 °C until enzyme activity determination.

To determine lethal potential of $(PhSe)_2$, rabbits were observed for up to 5 days after administration of the compound. The LD_{50} was calculated by the method of Litchfield and Wilcoxon (1949).

Biochemical and Hematological Parameters

Plasma enzymes AST (aspartate aminotransferase), ALT (alanine aminotransferase), LDH (lactate dehydrogenase) and δ-GT (δ-glutamyl transferase) were used as biochemical markers for the early acute hepatic damage and determined by enzymatic methods. Renal function was analyzed by determining plasma urea and creatinine levels. The assays were performed in a LABMAX 240 biochemical analyzer using a commercial kit (Labtest, Diagnostica S.A., Minas Gerais, Brazil). Hematological parameters, hematocrit and hemoglobin levels were determined with an automated counter (Coulter T-890).

Histological Parameters

The rabbits were subject to a thorough necropsy evaluation. Liver and kidney weights were recorded and these tissues were fixed in 4% formalin. For light microscopy examination, the samples were embedded in paraffin, sectioned at 7 µm and stained with hematoxylin and eosin.

TUNEL Staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed using the TdT-FragEL in situ apoptosis detection kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions. In brief, paraffin sections were dewaxed, digested with proteinase K in 10 mM Tris pH 8.0 (1 : 400) for 4 min at room temperature and reacted with TdT enzyme for 90 min at 37 °C. Therefore, the sections were reacted with diaminobenzidine solution (DAB), counterstain with methyl green and mounted with Entelan. A positive control for apoptosis was run for TUNEL staining.

δ-Aminolevulinic Dehydratase Activity

δ-ALA-D activity was assayed according to the method of Sassa (1982). The enzyme activity was determined by measuring the

amount of porphobilinogen formed at 37 °C. The reaction was started by addition of the substrate (ALA) into blood or tissue homogenate samples and incubated for 90 min (blood) or 60 min (tissue homogenates). The reaction product (porphobilinogen) was determined using modified Ehrlich's reagents and the absorbance was measured at 555 nm.

Glutathione Determination

Glutathione levels were determined as described by Ellman (1959) with slight modifications. GSH were measured in blood and tissue homogenates after protein precipitation with 1 vol. of 10% trichloroacetic acid. An aliquot of the samples was added to 800 mmol l^{-1} phosphate buffer, pH 7.4, and 500 μmol l^{-1} DTNB (5,5′-dithio-bis-2-nitrobenzoic acid). Color development resulting from the reaction between DTNB and thiols reached a maximum within 5 min and was stable for more than 30 min. Absorbance was read at 412 nm after 10 min. A standard curve of reduced glutathione was used in order to calculate the GSH levels in the samples and the results were expressed as nmoles GSH per milligram of protein.

Determination of Thiobarbituric Acid Reactive Substances Levels

Thiobarbituric acid reactive substances (TBARS) were determined in the tissue homogenates by the method of Ohkawa *et al.* (1979), in which malondialdehyde (MDA), an end-product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. In brief, samples were incubated at 100 °C for 60 min in acid medium containing 0.45% sodium dodecyl sulfate and 0.6% thiobarbituric acid. After centrifugation, the reaction product was determined at 532 nm using 1,1,3,3-tetramethoxypropane as the standard and the results were expressed as nanomoles MDA per milligram of protein.

Glutathione Reductase Assay

Glutathione reductase (GR) activity was determined according to (Carlberg and Mannervik, 1985). The enzyme activity was assessed in a solution containing 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.2 mM NADPH and the supernatant containing 0.2–0.3 mg protein ml^{-1}. The reaction was initiated by the addition of 1 mM oxidized glutathione and a change in absorbance was measured at 340 nm. GR activity was expressed as nmoles of NADPH oxidized per minute per milligram of protein, using an extinction coefficient 6.22×10^3 M^{-1} cm^{-1} for NADPH.

Glutathione Peroxidase Assay

Glutathione peroxidase (GPx) activity was measured according to Wendel (1981) using *tert*-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM glutathione, 0.2 U ml^{-1} glutathione reductase, 1 mM azide, 0.2 mM tert-butyl-hydroperoxide, 0.2 mM NADPH and the supernatant containing 0.2–0.3 mg protein ml^{-1}. GPx activity was expressed as nmoles of NADPH oxidized per minute per milligram of protein, using an extinction coefficient 6.22×10^3 M^{-1} cm^{-1} for NADPH.

Determination of the Respiratory Chain Enzyme Activities

Complex I activity was measured in mitochondria preparation by the rate of NADH-dependent ferricyanide reduction at 420 nm (1 mM^{-1} cm^{-1}) as described in Cassina and Radi (1996). The activities of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) were determined according to the method of Fischer *et al.* (1985). The methods described to determine these activities were slightly modified, as detailed in a previous report (Latini *et al.*, 2005). The activities of the respiratory chain complexes were calculated as nmol per minute per milligram protein.

Protein Determination

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

Statistical Analysis

Results are presented as means ± SD. Data were analyzed using one-way analysis of variance (ANOVA) followed by the *post hoc* Duncan multiple range test when F was significant. For analysis of dose-dependent effects, linear regression was used. Differences between the groups were rated significant at $P \leq 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences 16.0 (SPSS) software.

RESULTS

The treatment with 5 and 50 μmol kg^{-1} of $(PhSe)_2$ (i.p.) for five consecutively days did not induce death or clinical signal of toxicity and did not alter the weight gain or food consumption in the rabbits (data not shown). Conversely, administration of 500 μmol kg^{-1} $(PhSe)_2$ caused 85% (5/6) death: two animals died in the third day, two in the fourth and one in the fifth day after $(PhSe)_2$ exposure. The animals of this group showed evident signals of toxicity. The food and water consumption was reduced by around 50% and the weight decreased approximately 15% with the treatment. The LD_{50} calculated at the 95% confidence interval was 311 μmol kg^{-1} (106–1083 μmol kg^{-1}). Considering the high mortality of the rabbits exposed to 500 μmol kg^{-1}, the results presented below refer to the doses of 5 and 50 μmol kg^{-1} of $(PhSe)_2$ and the control group.

Biochemical, Hematological and Histological Parameters

Acute exposure to $(PhSe)_2$ (5 and 50 μmol kg^{-1}) did not alter the activity of ALT, AST and δ-GT. However, the highest dose of $(PhSe)_2$ (50 μmol kg^{-1}) increased plasma LDH activity ($P < 0.05$, Table 1). The renal plasma-function markers urea and creatinine were not altered by $(PhSe)_2$ acute exposure (Table 1).

Blood cells (leucocytes, erythrocytes, and platelets), hematocrit and hemoglobin concentration were not altered by exposure to 5 and 50 μmol kg^{-1} (data not shown). Treatment with $(PhSe)_2$ did not cause significant alterations in the hepatic and

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Table 1. AST, ALT, LDH and γ-GT activity, urea and creatinine levels in serum of the rabbits treated with $(PhSe)_2$

Group	AST (UI^{-1})	ALT (UI^{-1})	LDH (UI^{-1})	γ-GT (UI^{-1})	Urea (mg dl^{-1})	Creatinine (mg dl^{-1})
Control	34.0 ± 16.1	60.6 ± 16.7	126.5 ± 23.7	24.3 ± 2.1	35.0 ± 10.1	0.8 ± 0,1
$(PhSe)_2$ μmol kg^{-1}						
5	23.0 ± 7.6	41.8 ± 10.3	116.3 ± 22.9	23.5 ± 2.0	31.3 ± 6.25	0.8 ± 0.1
50	32.8 ± 9.5	41.3 ± 18.7	168.0 ± 34.6*	22.5 ± 2.6	39.0 ± 20.6	0.7 ± 0.1

Data are reported as means ± SD of six animals.
*$P < 0.05$, as compared with control group (one-way ANOVA, Duncan's test).

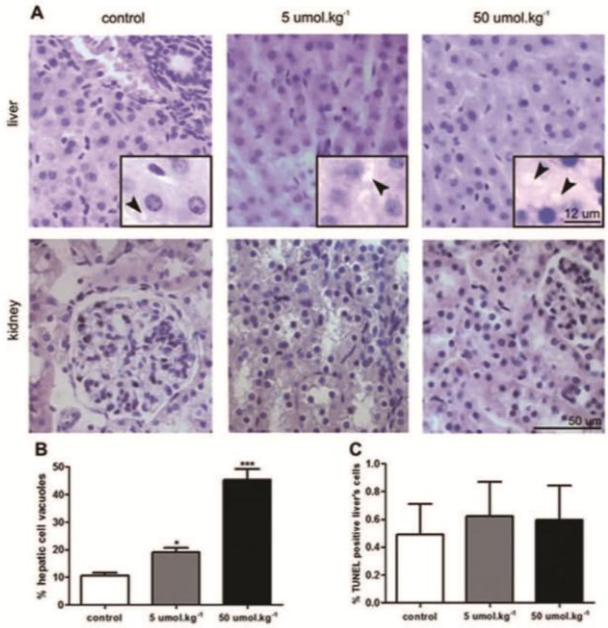

Figure 1. Histological sections stained with hematoxylin and eosin of rabbit's liver and kidney exposed to $(PhSe)_2$ (A). Hepatic cells showed cytoplasmic vacuoles (arrowhead in squares). The accompanying graph (B) presents the effect of $(PhSe)_2$ on percentage of hepatic cell vacuoles. The apoptosis was determined by TUNEL staining in the liver (C) of the rabbits exposed to $(PhSe)_2$. Data are expressed as means ± SD of six animals per group. *$P < 0.05$; ***$P < 0.001$, as compared with control group (one-way ANOVA, Duncan's test).

renal morphology and inflammatory cell infiltration were not detected by routine histology analyze. However, hepatic cells tended to accumulate slight or moderate levels of fat vacuoles after exposure to 5 ($P < 0.05$) and 50 ($P < 0.001$) μmol kg^{-1} $(PhSe)_2$, (Fig. 1A, B). The fat vacuole content of the grid was considered to be any nonstaining area of cytoplasm with a sharply defined border.

Figure 1(C) also shows the liver TUNEL staining. Acute exposure to $(PhSe)_2$ did not induce apoptosis in the liver and kidney (data not shown) of rabbits.

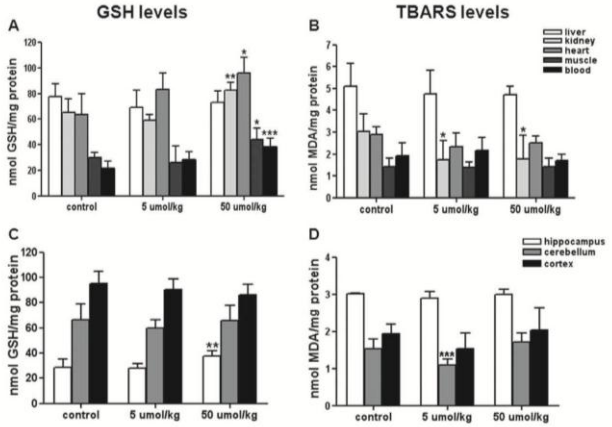

Figure 2. Effect of acute exposure of $(PhSe)_2$ on (A and C) GSH and (B and D) TBARS levels in peripheral and central tissue of rabbits. Data are expressed as means ± SD of six animals per group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ as compared with control group (one-way ANOVA, Duncan's test).

Oxidative Stress Parameters

Glutathione and TBARS levels

Figure 2 shows the GSH levels and lipid peroxidation, assessed through the TBARS measurement in peripheral and central tissue, of the rabbits treated with $(PhSe)_2$. Exposure to 50 μmol kg^{-1} increased GSH levels in the kidney ($P < 0.01$; $\beta = 0.588$), heart ($P < 0.05$; $\beta = 0.653$), muscle ($P < 0.05$; $\beta = 0.506$), blood ($P < 0.001$; $\beta = 0.777$) (Fig. 2A), and hippocampus ($P < 0.01$; $\beta = 0.655$) (Fig. 2C). Interestingly, a linear regression analysis demonstrated that $(PhSe)_2$ showed a dose-dependent effect on GSH elevation in these types of tissue.

Exposure to $(PhSe)_2$ did not induce lipid peroxidation in all tissues analyzed, and inhibited spontaneous lipid oxidation in the kidney ($P < 0.05$; Fig. 2B) and cerebellum ($P < 0.001$; Fig. 2D) of the rabbits.

GR and GPx activities

Figure 3 shows the activities of the two GPx and GR in peripheral and central tissue of the rabbits treated with $(PhSe)_2$. GPx activity was significantly increased in the heart ($P < 0.05$; $\beta = 0.671$) and muscle ($P < 0.01$; $\beta = 0.718$) of $(PhSe)_2$-treated rabbits (Fig. 3A), and this increase was dose-dependent.

Conversely, the activity of GR was significantly reduced in the kidney ($P < 0.05$; $\beta = -0.642$) (Fig. 3B), cortex ($P < 0.01$; $\beta = -0.748$), and cerebellum ($P < 0.01$; $\beta = -0.663$) (Fig. 3D) of the rabbits exposed to $(PhSe)_2$, and this decrease was dose-dependent.

δ-ALA-D activity

Acute treatment with 5 μmol kg^{-1} of $(PhSe)_2$ caused a significant increase in δ-ALA-D activity in the heart, whereas exposure to 50 μmol kg^{-1} significantly decreased the activity of this enzyme in the hippocampus ($P < 0.05$, Table 2).

Electron-transfer chain activity

Table 3 shows the effect of acute exposure to $(PhSe)_2$ in the respiratory chain function in rabbit cerebral cortex and hepatic mitochondrial preparations. The activities of complexes I and II were not altered by the treatment with $(PhSe)_2$.

DISCUSSION

The search for new drugs capable of affecting the pathologies related to oxidative stress has gained momentum over the years, resulting in numerous reports on significant activities of natural or synthetic antioxidant agents (Kinsella *et al.*, 1993). $(PhSe)_2$ is considered a potential antioxidant compound and has been successfully employed in models of hypercholesterolemia (Bem *et al.*, 2009), diabetes (Barbosa *et al.*, 2006, 2008), LDL oxidation (Bem *et al.*, 2008), pain (nociception) (Savegnago *et al.*, 2007), inflammation (Savegnago *et al.*, 2006), hepatotoxicity (Borges *et al.*, 2005, 2007), toxicity induced by metals (Freitas *et al.*, 2009), and others.

The results of this study clearly indicated that acute i.p. exposure of rabbits to $(PhSe)_2$ has different toxicological implications depending on the doses utilized. Rabbits treated with a dose of

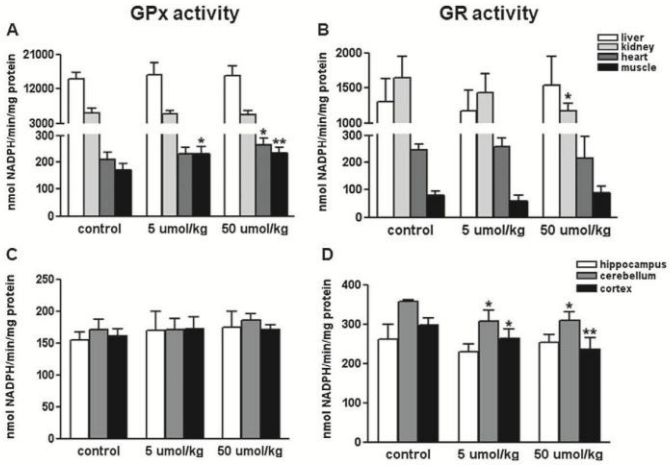

Figure 3. Effect of acute exposure of $(PhSe)_2$ on (A and C) GPx and (B and D) GR activities in peripheral and central tissue of rabbits. Data are expressed as means ± SD of six animals per group. *$P < 0.05$; **$P < 0.01$ as compared with control group (one-way ANOVA, Duncan's test).

Table 2. δ-ALA-D activity in cortex, cerebellum, hippocampus, liver, kidney, heart and muscle of the rabbits treated with $(PhSe)_2$

		δ-ALA-D (nmol PBG h^{-1} mg^{-1} protein)	
Group	Control	5 μ mol kg^{-1}	50 μ mol kg^{-1}
Cortex	1.00 ± 0.09	0.95 ± 0.14	0.79 ± 0.26
Cerebellum	0.76 ± 0.03	0.74 ± 0.05	0.80 ± 0.04
Hippocampus	0.95 ± 1.14	0.84 ± 0.36	0.45 ± 0.08*
Liver	3.55 ± 0.91	4.27 ± 0.89	3.85 ± 0.91
Kidney	4.45 ± 1.30	4.13 ± 0.55	3.48 ± 1.26
Heart	0.85 ± 0.08	1.00 ± 0.10*	0.90 ± 0.08
Muscle	0.15 ± 0.07	0.14 ± 0.04	0.21 ± 0.19

Data are reported as means ± SD of six animals.
*$P < 0.05$, as compared with control group (one-way ANOVA, Duncan's test).

5 μmol kg^{-1} showed no toxicological effects, whereas those treated with 50 μmol kg^{-1} showed slight toxicological effects. In contrast, the highest does (500 μmol kg^{-1}) was associated with elevated mortality (85%) and toxicological effects.

Previous studies by our group have shown that the toxicity of $(PhSe)_2$ is dependent on the route of administration, the dose, and the species of animal (rat, mouse, or rabbit) (Bem *et al.*, 2006, 2007; Meotti *et al.*, 2008; Nogueira *et al.*, 2003). Previous results from our laboratory have demonstrated that rabbits treated orally with $(PhSe)_2$ (0.05 to 5 μmol kg^{-1}) for a long period displayed minor signs of toxicity, characterized by a decrease in the ascorbic acid content in the liver and brain, only at the highest dose tested; however, no animals died (Bem *et al.*, 2006, 2007). In agreement with this, $(PhSe)_2$ administered by the i.p. route did not lead to death or modified weight gain in rabbits exposed to 5 and 50 μmol kg^{-1}.

The hepatic and renal functions were evaluated by plasma biochemical markers and by histological analysis. Hepatic and renal morphology and function were not modified, and infiltrating inflammatory cells were not present in the liver and kidney of rabbits treated with $(PhSe)_2$. Exposure to 5 and 50 μmol kg^{-1} $(PhSe)_2$ caused fat vacuole accumulation in the liver of the animals. An amount of fat vacuoles in less than 33% of hepatocytes is considered light, steatosis up to 33% is considered moderate, and up to 66% severe (Brunt *et al.*, 1999). In this study, we found 45% fat vacuoles in the liver of animals exposed to 50 μmol kg^{-1} of $(PhSe)_2$; however, this condition was not followed by loss of function of this organ, as demonstrated by the main-

Table 3. Complex I and complex II activity in cortex and liver of the rabbits treated with $(PhSe)_2$

Group	Complex I (nmol min^{-1} mg^{-1} protein) Cortex	Liver	Complex II (nmol min^{-1} mg^{-1} protein) Cortex	Liver
Control	85.2 ± 9.4	289.1 ± 62.5	2.3 ± 0.5	3.2 ± 0.6
$(PhSe)_2$ µmol kg^{-1}				
5	81.1 ± 7.5	238.7 ± 21.4	2.0 ± 0.4	3.2 ± 0.4
50	78.1 ± 5.5	241.9 ± 23.0	2.1 ± 0.5	3.5 ± 0.5

Data are reported as means ± SD of six animals.

tenance of the plasma enzymes, morphological integrity and absence of inflammatory cells. Similarly, apoptosis was not altered (as assessed by TUNEL staining) in the liver and was not detected in the kidney.

The organoselenium compound $(PhSe)_2$ acts as a mimic of glutathione peroxidase (GPx), a selenoenzyme which, at the expense of GSH, catalyzes the reduction of a variety of hydroperoxides and protects the cell membranes from oxidative damage. In fact, $(PhSe)_2$ efficiently reacts with thiols to generate selenol, the intermediate responsible for the antioxidant effect of $(PhSe)_2$ (Mugesh and Singh, 2000). Our study showed a beneficial effect of $(PhSe)_2$ on the antioxidant system.

The *in vivo* acute treatment with $(PhSe)_2$ was able, in a dose-dependent manner, to increase the activity of GPx in the muscle and heart and to increase the levels of the substrate of this enzyme (GSH) in several tissues analyzed, i.e. kidney, heart, muscle, blood and hippocampus. The decrease in the GR activity, enzyme responsible to regenerate the reduced form of glutathione (GSH), observed in the kidney and in brain structures, is associated with the increase in GSH synthesis and can be explained by a regulatory mechanism. This increase in the GSH levels in different organs after acute $(PhSe)_2$ exposure could be a result of the increase of δ-glutamylcysteine synthetase activity, a rate-limiting step in glutathione synthesis. According previous reports, ebselen, an analog of $(PhSe)_2$, caused an increase in glutathione levels in hepatic cell line and this effect was attributed to its ability to activate nrf-2-dependent responses (Tamasi *et al.*, 2004). Additionally, $(PhSe)_2$ was able to inhibit spontaneous lipid oxidation in the kidney and cerebellum, as demonstrated by the decrease in TBARS levels. This improvement in the antioxidant system of the rabbits may be associated with the pharmacological effects demonstrated by $(PhSe)_2$.

Convincing evidence has indicated that δ-ALA-D is extremely sensitive to the presence of pro-oxidant agents (Fachinetto *et al.*, 2006) which oxidized the SH groups located at the active center of the enzyme, which are essential for the enzyme activity (Barnard *et al.*, 1977). Because this enzyme is very sensitive to xenobiotics, δ-ALA-D activity was used as a marker of toxicity. In this study, i.p. administration of a high dose (50 µmol kg^{-1}) of $(PhSe)_2$ induced inhibition of δ-ALA-D activity only in the hippocampus, indicating a possible sign of toxicity. By contrast, we observed an increase in the activity of this enzyme in the heart, which may be associated with the increase in GSH levels and GPx activity in this tissue. In fact, it has been shown that δ-ALA-D activity might be modulated by the thiol status (Barnard *et al.*, 1977).

Evidence indicates that several toxicants disrupt the activity of the mitochondrial respiratory chain and subsequently induce the generation of reactive oxygen species and oxidative stress (Latini *et al.*, 2007). These observations led us to investigate in more detail the participation of cerebral and hepatic energy metabolism in acute $(PhSe)_2$ exposure, by measuring the activity of complexes I and II. The activity of these enzymatic complexes was not modified by $(PhSe)_2$, consistent with the low toxicity of this organoselenium compound at the doses evaluated.

Overall, our results indicated that the toxicity of acute exposure to $(PhSe)_2$ in rabbit is dependent on the dose. Considering that pharmacological effects of $(PhSe)_2$ including antioxidant, anti-nociceptive, anti-inflammatory, neuroprotective and hepatoprotective properties have been reported at doses of up to 10 µmol kg^{-1} (Bem *et al.*, 2008, 2009; Borges *et al.*, 2005; Ghisleni *et al.*, 2003; Savegnago *et al.*, 2006, 2007; Zasso *et al.*, 2005), this toxicological study encourages further experiments on the therapeutic properties of this compound.

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4.2 MANUSCRITO 1:

Diphenyl diselenide inhibits oxLDL-induced cytotoxic effects in macrophage cells by improving the redox signaling

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Diphenyl diselenide inhibits oxLDL-induced cytotoxic effects in macrophage cells by improving the redox signaling

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Running title: Diphenyl diselenide inhibits oxLDL-induced cytotoxic effects in macrophage

Key words: diphenyl diselenide, selenium, atherosclerosis, macrophage, glutathione peroxidase, LDL oxidation, inflammation, oxidative stress.

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ABSTRACT

It has been reported that oxidized LDL (oxLDL) are involved in the pathogenesis of atherosclerosis, and that macrophages as well as other cells of the arterial wall can oxidize LDL *in vitro*, depending on the balance between intracellular prooxidant generation and antioxidant defense efficiency. Because of their possible beneficial role in the prevention of atherosclerosis and other oxidative stress-associated diseases, organoselenium compounds as diphenyl diselenide $(PhSe)_2$, are receiving increased attention. In the present work, we investigated the mechanisms underlying the protective effect exerted by $(PhSe)_2$, on oxLDL-mediated cytotoxic effects in murine J774 macrophage-like cells. $(PhSe)_2$ pretreatment reduced atherogenic signaling triggered by oxLDL in macrophages *in vitro*, namely: ROS generation, disturbance of $^{\bullet}NO$ homeostasis, activation of matrix metalloproteinase, foam cell formation and mitochondrial dysfunction. Moreover, the redox signaling effects of $(PhSe)_2$ presented in this study, were accompanied by a down-regulation of NF-κB-binding activity. The relative strong performance of $(PhSe)_2$ makes it an ideal candidate for further, expanded trials as a new generation of antioxidant to prevent the atherosclerotic process.

1. INTRODUCTION

Atherosclerosis is a progressive disease characterized by the accumulation of lipids, inflammatory cells and fibrous elements in the large arteries (Libby, 2002). It is the underlying cause of most cardiovascular diseases, including coronary artery disease (CAD), ischemic gangrene, abdominal aortic aneurysms, and many cases of heart failure and stroke (Hansson e Libby, 2006).

Oxidative stress-mediated low density lipoprotein (LDL) modification play a pivotal role in initiation of the atherosclerotic process (Levitan et al., 2010). The blood circulating monocytes adhere to the endothelium and migrate to the intima and are differentiated to macrophages. Macrophages express high level of scavenger receptors (CD36, SR-A and LOX-1) that bind oxidized LDL (oxLDL), and these processes give rise to the arterial foam cell, a hallmark of the arterial lesion (Yamada et al., 1998; de Villiers e Smart, 1999; Ross, 1999; Lusis, 2000; Libby, 2002). The activation of macrophage and foam cell formation are involved in both the initiation and the progression and ultimate instability of advanced lesions. Macrophages taking up oxLDL could modify the production of inflammatory mediators, such as cytokines, proteases, reactive oxygen and nitrogen species (ROS and RNS) (Hansson et al., 2002; 2005) and other factors through oxidative sensitive signaling pathways (Geng et al., 2010). In macrophages oxLDL-stimulated, the intracellular ROS/RNS are generated by several pathways, including the NADPH oxidase (NOX) system, the lipoxygenase/cyclooxygenase system, inducible $^{\bullet}$NO sintase (iNOS) and mitochondrial dysfunction (Levitan et al., 2010). The activation of a wide variety of pro-inflammatory and pro-apoptotic pathways in atherosclerotic lesions may be regulated by transcription factors, such as NF-κB, which is activated by intracellular ROS generated by oxLDL stimulation (Karin, 1999; Robbesyn et al., 2003). Furthermore, the raise in the matrix metalloproteinase-9 (MMP-9) expression induced by oxLDL in macrophages, which may influence vascular remodeling and plaque disruption, was associated with increased nuclear binding of transcription factor NF-κB (Xu et al., 1999).

Recent clinical studies have suggested an important antiatherogenic role for the antioxidant enzyme glutathione peroxidase (GPx) (Blankenberg et al., 2003), which uses glutathione to reduce hydrogen peroxide to water and lipid peroxides to their respective alcohols (Flohe, 1988), and it also acts as a peroxynitrite reductase (Sies, 1999). Then, the investigation of antiaherogenic properties of synthetic

organoselenium compounds, with GPx-mimetic activity have been attracted considerable attention. In this scenario, we have been studying the pharmacological properties of diphenyl diselenide $(PhSe)_2$, a simple diaryl diselenide with high GPx-mimetic activity (Nogueira e Rocha, 2010).

This compound was very efficient in reducing the human LDL oxidation and Cu^{2+}-induced TBARS production in rat aortic slices (de Bem et al., 2008) and decreased the hypercholesterolemia and oxidative stress in cholesterol-fed rabbits (de Bem et al., 2009). Furthermore, the treatment with $(PhSe)_2$ reduced the atherosclerotic lesion in hypercholesterolemic LDLr-/- mice by modulating pathways related to antioxidant and anti-inflammatory responses (Hort, 2011). In the present study, we therefore aimed to determine whether pretreatment with $(PhSe)_2$ attenuates oxLDL-induced citotoxicity in J774 macrophage cells and, if so, whether the mechanisms underlying the process involve the redox signaling pathway.

2. MATERIALS AND METHOD

2.1 Materials

Diphenyl diselenide $(PhSe)_2$ was synthesized according to published methods (Paulmier, 1986). Analysis of the 1H NMR and 13C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2 Oxidized LDL (oxLDL) preparation

This study was approved by our Ethic Committee at Federal University of Santa Catarina (nº 943/11). LDL was isolated from fresh human plasma by discontinuous density-gradient ultracentrifugation as previously described (de Bem et al., 2008) and the protein concentration in LDL solution was determined by Lowry (Lowry et al., 1951). LDL isolated samples (1 mg of protein/mL) were oxidized at 37°C in the presence of 10 μM $CuSO_4$ for 16 hours, to produce oxLDL. Then EDTA 1.5 mM was added and the samples were dialyzed against 148 mM phosphate buffer for 24 hours at 4°C.

2.3 Cell culture

Murine J774 macrophage-like cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were maintained with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM glutamine, 10 mM HEPES, 100 units/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum (FBS) in a 5% CO_2 humidified atmosphere at 37°C. The exposition of cells with $(PhSe)_2$ and/or oxLDL was carried out in DMEM without FBS.

2.4 Measurement of Reactive Oxygen Species (ROS) production

Intracellular ROS production was detected using the non-fluorescent cell permeating compound, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is cell membrane permeable. Once inside the cells, DCFH-DA is hydrolyzed by cellular esterases to form DCF, which is trapped intracellularly due to its membrane impermeability. DCF then reacts with intracellular ROS to form the fluorescent product, 2',7'-dichlorohydrofluroscein. In order to evaluated the protective effect of $(PhSe)_2$ on ROS production induced by oxLDL, J774 macrophage cells (2.0×10^5 cells/well) seeded in 24 well plates were firstly stimulated to a increasing concentration curve of oxLDL (25, 50, 100 and 200 µg/mL) for 1 hour. After that, cells were incubated with DCFH-DA (10 µM) for 30 min at 37°C, washed twice with PBS, harvested and collected for immediate determination of ROS generation by flow citometry (FACS Canto II, BD Bioscience, United States).

The protective effect of $(PhSe)_2$ were also evaluated by flow citometry by pretreating cells for 24 hours with $(PhSe)_2$ (1 µM) or vehicle before oxLDL (100 µg/mL) stimulation for 1 hour. The results were expressed as percentage of control (non-stimulated cells; 100%). The time course of ROS production induced by oxLDL were evaluated fluorimetrically for 60 min in a plate reader (Tecan, Grödig/Salzburg, Austria). The fluorescence intensity was measured at 488 nm excitation and 525 nm emission and the results were expressed intensity of fluorescence (arbitrary units).

2.5 Cell viability assay

J774 macrophage cells ($1x10^4$ cells/well) seeded in 96 well plates were incubated in a medium without FBS in absence or presence of increasing $(PhSe)_2$ concentrations (0.1 - 100 µM) for 24 hours. Cell viability was then assessed by the MTT test according to (Denizot e Lang, 1986). Briefly, after the incubation period, the medium was

removed, the cells were washed with PBS and further, 200 µL of MTT solution (0.5 mg/mL) was added to each well and incubated for 2 hours at 37ºC. Formazan crystals were dissolved in DMSO and the absorbance was read at 550 nm using a microplate reader (Reader Labsystems Multiskan EX). Results were expressed as a percentage of control cells.

2.6 Apoptosis assay

The apoptotic cell death induced by oxLDL in J774 macrophage cells was performed by flow cytometry using Annexin-V-FITC according to manufacturer's instructions. Cells (2.0 x 10^5 cells/well) seeded in 24 well plates were incubated in a medium without FBS in absence or presence of increasing concentrations of oxLDL (25, 50, 100 and 200 µg/ml) for 24 hours. Then, the cells harvested by a scraper were, resuspended in binding buffer (0.1 M Hepes/NaOH, 1.4 M NaCl, 25 mM $CaCl_2$, pH 7.4) with Annexin V-FITC staining for 15 min at room temperature and analyzed by flow cytometry (FACS Canto II, BD Biosciences, United States). For each experimental condition, 10.000 events were collected. Data were analyzed using WinMID 2.9 software (USA, 2000). Results were expressed as percentage of apoptotic J774 macrophage cells of control.

2.7 Measurement of mitochondrial membrane potencial ($\Delta\Psi_m$)

The mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using the lipophilic cationic probe fluorochrome JC-1 (Reers et al., 1991). In presence of physiological mitochondrial membrane potentials, JC-1 forms aggregates that fluoresce with an emission peak at 588 nm. Loss of membrane potential favors the monomeric form of JC-1, which has an emission peak at 530 nm. To examine the effect of $(PhSe)_2$ on modifications in $\Delta\Psi_m$ induced by oxLDL, J774 macrophage cells were plated into 24 well plates (2.0 x 10^5 cells/well) and pretreated with $(PhSe)_2$ (1 µM) or vehicle for 24 hours, followed by stimulation with oxLDL (100 µg/mL) or FCCP 1 µM (a positive control) for additional 3 hours. After, cells were incubated with JC-1 (5 µM) for 20 min at 37°C and JC-1 fluorescence intensity was measured using a fluorimetric microplate reader (Tecan, Grödig/Salzburg, Austria) with excitation at 488 nm and an emission at 525 nm and 590 nm. Mitochondrial membrane potential was inferred from the ratio of fluorescence intensity red/green. The images were acquired from three randomly chosen fields using an inverted epifluorescence microscope (Olympus IX70).

2.8 Foam cell formation assay

Foam cell formation assay was performed with the Oil-Red O staining method (Koopman et al., 2001). J774 macrophage cells (2.5 x 10^5 cells/well) plated in coverslip in 12 wells plate were pretreated with $(PhSe)_2$ (1 µM) or vehicle for 24 hours, hand then stimulated to oxLDL (100 µg/mL) for additional 3 hours. Following oxLDL incubation, cells were fixed with 4% paraformaldehyde and stained by 0.3% Oil-Red O for 10 min. Hematoxylin was used as counterstaining. Images of cells were acquired using confocal microscopy (Leica DMI6000 B Microscope) using a 546 nm filter set. Ten images were captured from each group and the total pixels intensity was determined using NIH ImageJ 1.36b imaging software (National Institutes of Health, Bethesda, MD, USA), and lipid content was expressed as optical density (OD).

2.9 MMP activity measurement

Matrix metalloproteinase (MMP)-9 activity in J774 macrophages cells was measured using two different methods.

2.9.1 Gelatin Zimography

The gelatin zymography were performed in conditioned media of J774 macrophage cells as previously described (Kleiner e Stetler-Stevenson, 1994). Cells (2.5 x 10^5 cells/well) were seeded in 12 wells plate and then preincubated with $(PhSe)_2$ (1 µM) for 24 hours, followed by stimulation with oxLDL (100 µg/ml) for additional 24 hours. Supernatants (50 µg/mL of total protein) were subjected to SDS-PAGE in 7.5% (w/v) polyacrylamide gels copolymerized with 1.5% gelatin as a substrate. After electrophoresis, the gels were stained with Coomassie Brilliant Blue. Proteolytic activity in a particular gel location yielded a clear band against the blue background of the stained gelatin. The MMP-9 were identified by molecular size using high-molecular mass (200 kDa) standards (Bio-Rad, Hercules, CA, USA). Gels were scanned and the intensity of the bands were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA). The results were expressed as intensity of MMP-9 relative to control.

2.9.2 Collagenase IV assay

The activity of MMP-9 (gelatinase or collagenase IV activity) in conditioned supernadants was also determinate using an Enzimatic Gelatinase/Collagenase fluorimetric assay kit (Molecular Probes, USA). Briefly, conditioned supernadants (50 µg/mL of protein), prepared as described for zymographic analyses, were incubated with MMP specific

peptide substrate, based on the instructions included with the kit. The cleavage of substrates by MMP result in increased fluorescence signal measured using a fluorescence microplate reader (Tecan, Grödig/Salzburg, Austria) with excitation at 495 nm and emission at 515 nm. Data were reported as the collagenase IV activity relative to the control.

2.10 Measurement of $^{\bullet}$NO production

Nitrite accumulation, an indicator of $^{\bullet}$NO production, was measured in the conditioned culture medium by the Griess reaction (Schulz et al., 1999). J774 macrophage cells (2.0 x 10^5 cells/well), cultured in 24 well plates were preincubated with $(PhSe)_2$ (1 μM) for 24 hours and then stimulated with oxLDL (100 or 200 μg/ml) for additional 24 hours. The supernadants were collected to quantification of nitrite. Briefly, 100 μL of supernadants were mixed with 100 μL of Griess reagent (equal volumes of 1% (w/v) sulphanilic acid in 30% (v/v) acetic acid and 0.1% (w/v) N-(1-naphthyl) ethylenedianime in 60% (v/v) acetic acid), incubated at room temperature for 30 min in the dark, and the absorbance at 540 nm was measured in an microplate reader (Tecan, Grödig/Salzburg, Austria). In short, diazonium ions are produce when acidified nitrite reacts with sulphanilic acid. These diazonium ions form chromophore agents on reacting N-(1-naphthyl) ethylenedianime. The amount of nitrite was calculated from a standard curve using freshly prepared sodium nitrite in culture medium and the results were expressed as percentage of control.

2.11 Immunofluorescence

The effect of $(PhSe)_2$ on oxLDL-induced NF-κB nuclear translocation was evaluated by immunofluorescence. J774 macrophage cells (2.5 x 10^5 cells/well) seeded in coverslips in 12 wells plate were preincubated with $(PhSe)_2$ (1 μM) or vehicle for 24 hours, followed by stimulation with oxLDL (100 μg/ml) for additional 1 hour. After which cells were fixed with 4% paraformaldehyde and blocked with 4% of albumin in 0,05% (v/v) Tween 20-PBS (PBS-T20) for 15 min. The nuclear translocation of NF-κB was evaluated by immunofluorescence using an anti-NF-κB rabbit monoclonal p-65 antibody (1:100) in PBS-T20 for 24 hours at 4ºC, followed by FITC conjugated donkey antirabbit IgG secondary antibody (1:100) for 1 hour. Cell nuclei were stained with Hoechst 33258. The images were acquired from eight to ten randomly chosen fields using a fluorescent microscope (Olympus BX41).

2.12 Statistical analysis

All data are expressed as mean ± SEM from at least three independent experiments. Differences between mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by the *post-hoc* Duncan multiple range test when P was significant. Statistical significance was considered at $P < 0.05$.

3. RESULTS

3.1 Effect $(PhSe)_2$ on oxLDL-stimulated intracellular ROS production in J774 macrophage cells

As expected, oxLDL (25 - 200 µg/mL) caused a concentration-dependent increase in intracellular ROS production in J774 macrophage cells (Fig. 1A). Then we evaluated the effect of $(PhSe)_2$ pretreatment on oxLDL-mediated intracellular ROS accumulation. Firstly, we followed fluorimetrically the time course of ROS production induced by oxLDL in J774 cells. The Figure 1B shows that oxLDL (100 µg/mL) induces an linear ROS generation under the first 30 min getting a constant production until 60 min, and the pretreatment with $(PhSe)_2$ efficiently reduces this ROS production. In the another series of experiments, using flow citometry, we demonstrated that the pretreatment with $(PhSe)_2$ (1 and 5 µM) revealed a significant decrease in the intensity of DCF fluorescence after 1 hour of oxLDL exposition (Fig. 1C-D).

3.2 Effect of $(PhSe)_2$ on cell viability

In order to evaluate the potential beneficial effects of $(PhSe)_2$ on oxLDL mediated toxicity in J774 macrophage cells, we started our experiments by assessing the appropriate non-toxic concentration of $(PhSe)_2$ to be used in the study. Thus, a 24 hours concentration-response study was conducted by exposing J774 macrophage cells to concentrations of $(PhSe)_2$ ranging from 0.5 to 100 µM. Cell viability was evaluated by the MTT assay. As shown in Figure 2A, there was no significant decrease in viability over a 24 hours exposure to $(PhSe)_2$ concentrations below 10 µM. However, for higher concentrations, a concentration-dependent decrease in cell viability was observed.

3.3 OxLDL induces apoptosis in J774 macrophage cells

Using flow cytometric analysis, effects of oxLDL on J774 macrophage apoptosis and necrosis were determined. As shown in the

histogram (Fig. 2B), exposure of J774 cells to different oxLDL concentration by 24 hours elicited a significant increase in apoptosis. The necrosis induction was minimal after oxLDL exposition (data not show). The Figure 2C shows a concentration-dependent effect on apoptosis induced by oxLDL. The percentage of apoptotic cells strongly increase after the exposure with 100 and 200 μg/mL of oxLDL (around 3 times). Then, we elect these oxLDL concentrations for the next experiments. The effect of $(PhSe)_2$ on oxLDL-induced apoptosis will be evaluated in the next stage of this study.

3.4 Effect $(PhSe)_2$ oxLDL-mediated mitochondrial dysfunction

Mitochondrial dysfunction and the ensuing bioenergetic crisis are likely to promote ion pump failure and loss of membrane integrity. Mitochondrial membrane potential ($\Delta\Psi_m$) in J774 cells was monitored. Seen from Figure 3A, oxLDL (100 μg/mL) significantly induced the decrease of mitochondrial $\Delta\Psi_m$ after 3 hours of exposition, by decreasing the JC1 red fluorescence, suggesting oxLDL-induced mitochondrial depolarization. The depolarizing effect of oxLDL was prevented by pretreatment of J774 macrophage cells with $(PhSe)_2$ (Fig. 3A-B, pictures and chart). Considering that FCCP (1 μM) is a known depolarizing agent, it was used as a positive control.

3.5 $(PhSe)_2$ inhibits the oxLDL-mediated foam cell formation

J774 macrophages cells incubated with oxLDL resulting in accumulation of lipid droplets in cytoplasm that could be detected by Oil Red O staining assay (Fig. 4). To test the inhibitory potential of $(PhSe)_2$ in the foam cell formation, cells were incubated with oxLDL (100 μg/mL) for 3 hours, with or without $(PhSe)_2$ pretreatment. As expected, oxLDL uptake by macrophages and following foam cell formation was induced in macrophages exposed to oxLDL (Fig. 4A-B, pictures and chart). Importantly, the pretreatment with $(PhSe)_2$ significantly reduces the foam cell formation.

3.6 $(PhSe)_2$ modulates oxLDL triggered MMP-9 activity

Next we focus on the effect of $(PhSe)_2$ on MMP-9 activity in J774 macrophage cells, since oxLDL has been reported to increase MMP-9 activity (Yamamoto et al., 2007). In gelatin zymography, densitometric analysis showed that the pretreatment with $(PhSe)_2$ significantly reduced the MMP-9 activity (Fig. 5A). In addition, in order to obtain quantitative inhibitory data related to this, a fluorescent assay

was used. In the same way, $(PhSe)_2$ abolished the oxLDL-induced elevation of MMP-9 activity verified by the fluorescent enzymatic assay in conditioned supernadants (Fig. 5B).

3.7 Effect of $(PhSe)_2$ on oxLDL-induced $^{\bullet}NO$ production

The cumulative $^{\bullet}NO$ production induced by oxLDL was evaluated in J774 macrophage cells by measuring nitrite levels in the conditioned media. Figure 6 shows that only the higher oxLDL concentration utilized in this study (200 µg/mL) caused a significant increase in $^{\bullet}NO$ production and the pretreatment with $(PhSe)_2$ was able to prevent it. However, the exposition of macrophages to oxLDL 100 µg/ml for 24 hours, did not show any significant increase in $^{\bullet}NO$ production.

3.8 $(PhSe)_2$ modulates oxLDL-induced NF-κB nuclear translocation

In order to evaluate whether $(PhSe)_2$ was blocking the oxLDL-induced NF-κB activation in J774 macrophage cells, we investigated the NF-κB translocation to the nucleus by immunofluorescence assay. As already demonstrated (Huang et al., 2010), the exposition of macrophages to oxLDL by 1 hour induced the NF-κB nuclear translocation. Figure 7 also shows that the pretreatment with $(PhSe)_2$ (1 µM) blocked the NF-κB nuclear translocation, with respect to cell controls treated with oxLDL alone. As shown in this figure, NF-κB was retained in the cytoplasm of oxLDL-stimulated cells pretreated with $(PhSe)_2$.

4. DISCUSSION

Atherosclerosis is considered to be a chronic inflammatory disease (Ross, 1999), and growing evidence indicates that chronic and acute overproduction of reactive oxidative species (ROS) under pathophysiological conditions is related to the development of cardiovascular diseases (Madamanchi et al., 2005). It has been reported that ROS generated by oxLDL triggers macrophages activation, process that can accelerate the atherosclerosis progression (Napoli, 2003).

Taking into account the inverse association of GPx activity and the risk for cardiovascular events in humans (Blankenberg et al., 2003) and animal models (Torzewski et al., 2007), the investigation of antiatherogenic properties of organoselenium compounds, with GPx like activity, is very relevant. In this way, we have been studying the

antiatherogenic properties of $(PhSe)_2$. This compound was able to inhibited, in a very effective way, the human LDL oxidation *in vitro* (de Bem et al., 2008) and reduced the oxidative stress in hypercholesterolemic rabbits (de Bem et al., 2009). Most important, the atherosclerotic lesions in hypercholesterolemic LDLr-/- mice were reduced by $(PhSe)_2$ oral treatment (Hort, 2011).

In the present study we have shown that, several critical ROS-sensitive signaling events are involved in oxLDL-induced macrophage citotoxicity, including disturbance of $^{\bullet}NO$ homeostasis, activation of MMP, stimulation of cholesterol accumulation, mitochondrial dysfunction, activation of nuclear NF-κB and consequent apoptosis. Moreover, in this work we show that $(PhSe)_2$, a simple organoselenium compound, elicits cytoprotective effects against oxLDL-stimulated J774 macrophage cells by inhibiting ROS generation and modulating the consequent cytotoxic signaling pathways.

To determine the imposition of oxidative stress following oxLDL-stimulation macrophage, we analyzed the ROS generation by flow citometry. Our results revealed a significant increase in ROS levels (Fig. 1A) and consequent apoptotic cell death (Fig. 2B-C) after oxLDL exposure. This ROS overproduction can be responsible to trigged different pathways related to inflammatory process and cell death, considering that ROS generation is the earliest apoptotic signal (Ou et al., 2010). By the other hand, when macrophages were pretreated with $(PhSe)_2$ the ROS generation induced by oxLDL was significantly reduced (Fig. 1B-C-D), indicating that the cytoprotective effects of this compound could be related to its known antioxidant activity (Nogueira e Rocha, 2010).

Mitochondria are easily affected by reactive nitrogen/oxygen species (RNS/ROS), whereas it also generate ROS by themselves, so the protection of mitochondria against oxidative damage becomes increasingly essential, playing an important role in apoptotic mechanism (Perfettini et al., 2005). In the present study we found that oxLDL induced damage to mitochondrial function by decreasing $\Delta\Psi_m$ in J774 cells and, $(PhSe)_2$ was able to prevent this damage (Fig. 3), probably by decreasing the ROS generation. It has been well documented that increased ROS levels cause mitochondrial dysfunction and down regulation of anti-apoptotic proteins (Tan et al., 1998).

OxLDL could promote atherogenesis by its cytotoxicity, its inhibitory effects on macrophage motility, and its uptake by the macrophage scavenger receptor, resulting in stimulation of cholesterol accumulation and hence foam cell formation, which is the hallmark of

early atherosclerosis lesions (Quinn et al., 1985). In this way, here we showed that the pretreatment of J774 macrophages with $(PhSe)_2$ significantly decrease the oxLDL uptake and consequent foam cell formation (Fig. 4).

The activity of MMP-9, a zinc dependent endopeptidase, is markedly increased after myocardial infarction (Kai et al., 1998) and rupture of abdominal aneurysms (Wilson et al., 2006), therefore MMP secretion from macrophage could contribute to plaque rupture. It has been documented that oxLDL regulates MMP-9 expression in macrophages cell lines. In agreement with this, we showed by two different methodological approach that oxLDL induced a markedly increase in MMP-9 activity which was attenuated by the treatment with $(PhSe)_2$ (Fig. 5).

Endothelial NO synthase (eNOS), a key regulator of vascular wall homeostasis, produces $^{\bullet}NO$ at concentrations in the nanomolar range under normal physiological conditions. At these low level, $^{\bullet}NO$ has been shown to have anti-inflammatory and protective effects by inhibiting the activation of NF-κB (Peng et al., 1995). In contrast, inducible NO synthase (iNOS) produces $^{\bullet}NO$ at concentrations in the micromolar range. High levels of $^{\bullet}NO$ have been shown to have pro-inflammatory effects. In addition, high levels of $^{\bullet}NO$ have the opportunity to react with superoxide leading to peroxynitrite formation and cell toxicity (Chandel et al., 2000). A recent study shows that oxLDL enhance the expression of iNOS and NO production in macrophages (Persson et al., 2009). In our experimental condition, the exposition of macrophages to oxLDL (200 μg/ml) for 24 hours, caused a significant increase in $^{\bullet}NO$ levels while the pretreatment with $(PhSe)_2$ prevented it (Fig. 6). However, we did not evaluate the iNOS expression in this study. Consistent with these observations, Shin et al. 2009 have recently reported that another diselenide, the bis-(3-hydroxyphenyl) diselenide (DSE-B) potently inhibits $^{\bullet}NO$ production and reduced the protein levels of iNOS in macrophages stimulated with LPS (Shin et al., 2009).

It has been shown that iNOS derived overproduction of $^{\bullet}NO$ can lead to activation of NF-κB (Li et al., 2001). Furthermore, the NF-κB activation can be a subsequent process of the increased abundance of ROS stimulated by oxLDL, which in turn leads to the up-regulation of several major pro-inflammatory mediators such as COX-2, iNOS, TNF-α and MCP-1 (Terkeltaub et al., 1994; Ou et al., 2007). According, in the present study, we have shown that oxLDL could lead to the activation of NF-κB (Fig. 7), which could be a consequence of oxLDL

ROS production. By the other hand, the redox signaling effects of $(PhSe)_2$ presented in this study, were accompanied by a down-regulation of NF-κB-binding activity (Fig. 7). Consistent with this, we recently demonstrated that the oxLDL-mediated increase of TNF-α and MCP-1, inflammatory mediators trigged by the NF-κB activation, were prevented by $(PhSe)_2$ in macrophage cells (Hort, 2011). Moreover, as presented before, DSE-B, another diaryl diselenide, reduces the expression of pro-inflammatory cytokines, including TNF-α, through down-regulation of NF-κB-binding activity in LPS-stimulated macrophages (Shin et al., 2009).

Taken together, we propose a possible cytoprotective effect of $(PhSe)_2$ in J774 macrophage cells. This organoselenium compound protects against atherogenic signaling triggered by oxLDL through inhibiting oxLDL-induced ROS generation and subsequent NF-κB activation, which in turn activated downstream cytotoxic signaling events. Our findings suggest that $(PhSe)_2$ might be a candidate agent for further development in the prevention of cardiovascular diseases.

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FIGURES AND LEGENDS

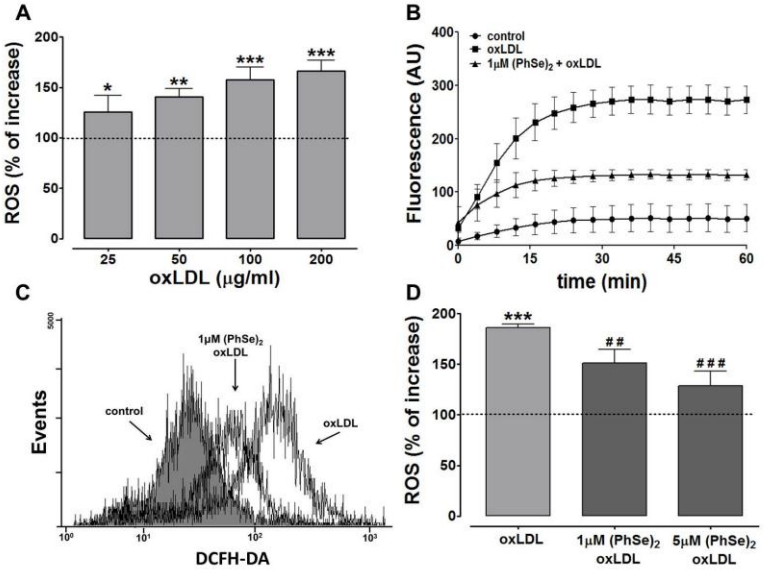

Figure 1. Effect of $(PhSe)_2$ on oxLDL-induced reactive oxygen species (ROS) production in macrophage J774 cells. (A) J774 macrophage cells were exposure with different concentrations of oxLDL (25 - 200 µg/mL) for 1 hour, after DCFH-DA (10 µM) was added to the medium for 30 min and amount of ROS was determinate by flow cytometry. The ROS amount was expressed as percentage of increase of control. (B) Time course of ROS production induced by oxLDL 100 µg/mL in J774 macrophage cells. Cells were pretreated with 1 µM of $(PhSe)_2$ or vehicle for 24 hours and then exposure to oxLDL (100 µg/mL). At the same time, DCFH-DA (10 µM) were added at the medium and the fluorescence was followed fluorimetrically by 60 min. (C) Representative flow citometry histogram of ROS generation induced by oxLDL in J774 macrophage cells. The cells were pretreated with 1 µM and 5 µM of $(PhSe)_2$ or vehicle for 24 hours and then exposure to oxLDL (100 µg/mL) for additional 1 hour. (D) Percentage of ROS production induced by oxLDL measured flow cytometrically. Each bar represents the mean ± SEM of at least three independent experiments. *p<0.05; **p<0.01; ***p<0.001 indicates the difference when compared with control (non-treated cells); $^{\#\#}$p<0.01; $^{\#\#\#}$p<0.001 indicates the difference when compared with oxLDL exposure cells (one-way ANOVA).

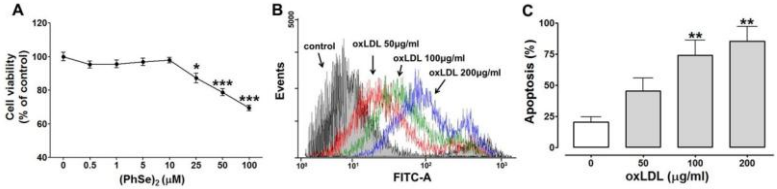

Figure 2. Effect of $(PhSe)_2$ or oxLDL on J774 macrophage cell viability. (A) J774 macrophage were treated with different concentrations of $(PhSe)_2$ (0.5 – 100 μM) for 24 hours and the cell viability was determined by MTT assay. (B) Representative flow cytometric histogram of apoptosis induced by oxLDL in J774 macrophage cells. The cells were treated with different concentrations of oxLDL (50, 100 or 200 μg/mL) for 24 hours and apoptosis was assessed by Anexinn V-FITC staining. (C) Percentage of apoptotic J774 macrophage cells. Each bar represents the mean ± SEM of at least three independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$ indicates the difference when compared with control (non-treated cells) (one-way ANOVA).

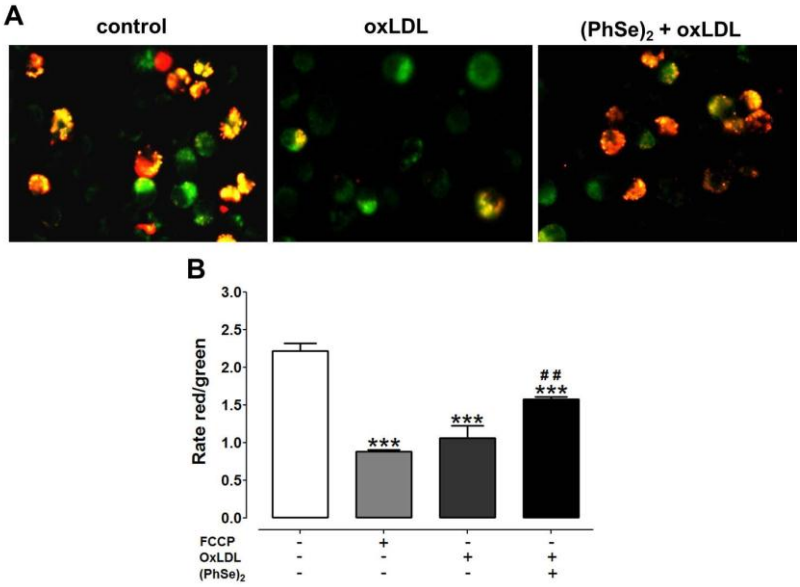

Figure 3. Effect of $(PhSe)_2$ on oxLDL-induced mitochondrial membrane potential ($\Delta\Psi_m$) disruption. (A) J774 macrophages cells were pretreated with $(PhSe)_2$ (1 μM) or vehicle for 24 hours and then exposure to oxLDL (100 μg/mL) for additional 3 hour. FCCP (1 μM) was utilized as a positive control. (A) the change in $\Delta\Psi_m$ was assessed based on the signal intensity from monomeric and J-aggregate JC-1 fluorescence. *Left*, no treatment; *middle*, oxLDL; *right*, $(PhSe)_2$ + oxLDL - magnification 400x. (B) JC-1 fluorescence was quantified fluorimetricaly at 488 nm excitation and 590 nm and 525 nm emission. Each bar represents the mean ± SEM of the ratio (red/green fluorescence) of at least three independent experiments. ***$p<0.001$ indicates the difference when compared with control; $^{\#\#}p<0.01$ indicates the difference when compared with cells treated with oxLDL 100 μg/mL (one-way ANOVA).

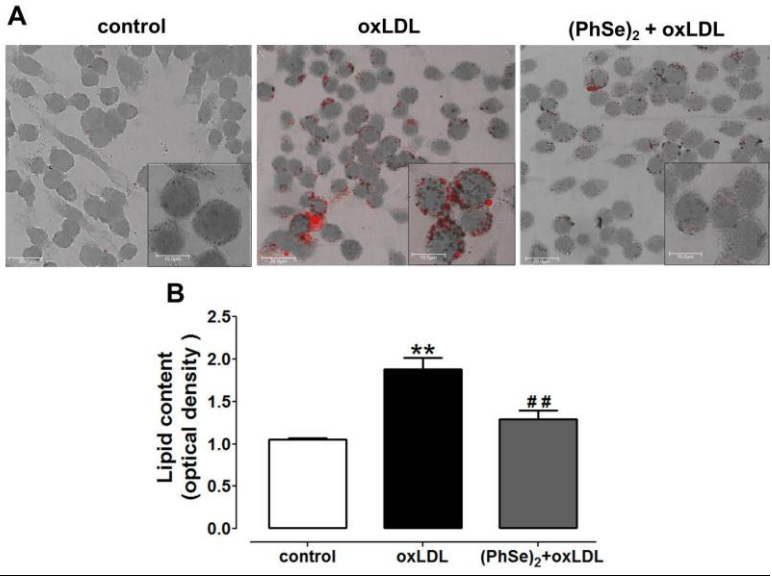

Figure 4. Effect of $(PhSe)_2$ on oxLDL-induced foam cell formation. (A) After pretreatment for 24 hours with $(PhSe)_2$ (1 µM) or vehicle, oxLDL (100 µg/mL) was added to the medium and incubated for 3 hours, followed by exposure with Oil Red O. Representative confocal micrographs of Oil Red O-derived fluorescence in J774 macrophage cells. *Left*, no treatment; *middle*, oxLDL; *right*, $(PhSe)_2$ + oxLDL - Magnification x63, oil immersion objective using a 546 nm filter set (Leica DMI6000 B Microscope). (B) Quantification of foam cells lipid content (optical density). Each bar represents the mean ± SEM of at least three independent experiments. $^{**}p<0.001$ compared with control and $^{\#\#}p<0.001$ indicates the difference when compared with oxLDL-treated cells (one-way ANOVA).

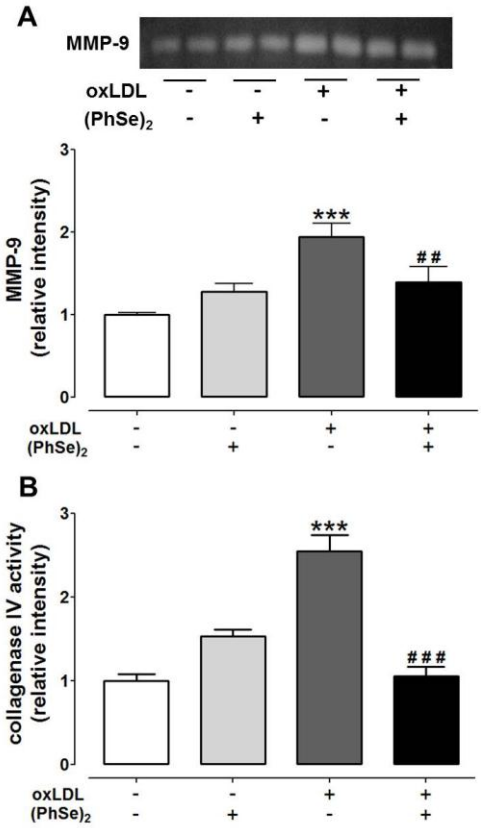

Figure 5. Effect of $(PhSe)_2$ on oxLDL-induced MMP-9 activity. (A) MMP-9 activity was measured in the supernatant by zymography. After pretreatment for 24 hours with $(PhSe)_2$ (1μM) or vehicle, oxLDL (100 μg/mL) was added to the medium for additional 24 hours. The bottom panels show the densities of bands of MMP-9 (85 KD). (B) The collagenase IV activity was measured in the conditioned culture medium using an EnzChek Gelatinase/Collagenase Assay kit. Each bar represents mean ± SEM of at least three independent experiments. ***$p<0.001$ compared with control and $^{##}p<0.01$; $^{###}p<0.001$ indicates the difference when compared with oxLDL-treated group (one-way ANOVA).

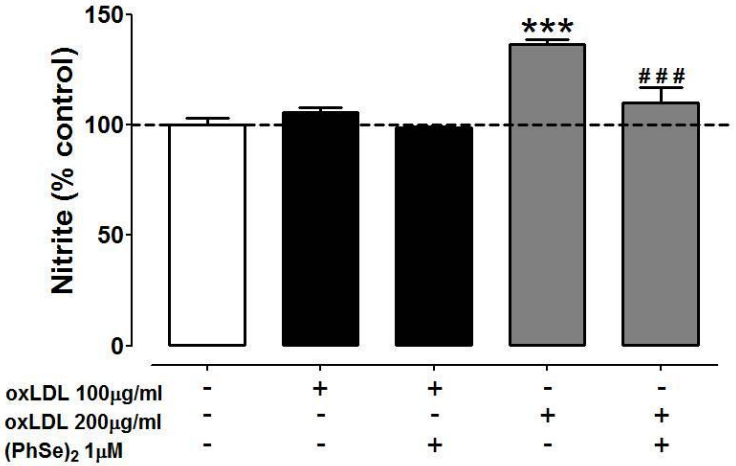

Figure 6. Effect of $(PhSe)_2$ on oxLDL-induced $^{\bullet}NO$ production. J774 macrophages cells were pretreated with $(PhSe)_2$ (1 μM) or vehicle for 24 hours and then exposure to oxLDL (100 or 200 μg/mL) for additional 24 hours. Nitrite accumulation, an indicator of $^{\bullet}NO$ production, was measured in the conditioned culture medium and was expressed as percentage of control. Each bar represents the mean ± SEM of at least three independent experiments. ***$p<0.001$ compared with control and $^{###}p<0.001$ indicates the difference when compared with oxLDL-treated group (one-way ANOVA).

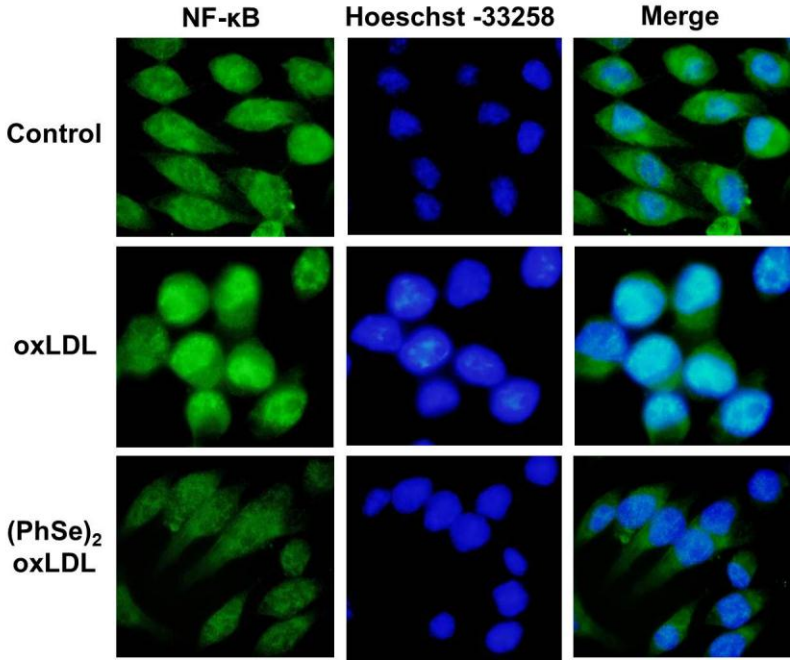

Figure 7. Effect of $(PhSe)_2$ on oxLDL-induced NF-κB nuclear translocation. J774 macrophages cells were pretreated with $(PhSe)_2$ (1 μM) or vehicle for 24 hours and then exposure to oxLDL (100 μg/mL) for 1 hour. Nuclear translocation of NF-κB was evaluated by immunofluorescence (green). Nuclear counterstaining was made with Hoechst-33258 (blue). A representative merge of the two fluorescence images (green and blue) is shown. Magnification 1000x. The representative images were from three independent experiments.

4.3 Manuscrito 2:

Disubstituted diaryl diselenides as potential atheroprotective compounds: involvement of TrxR and GPx-like systems

Marcos Raniel Straliotto, Gianni Mancini, Jade de Oliveira, Sergio Melin Sgrott, Afonso Bayne, Alexandra Latini, João Batista Teixeira Rocha, Andreza Fabro de Bem.

Disubstituted diaryl diselenides as potential atheroprotective compounds: involvement of TrxR and GPx-like systems

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Running title: Disubstituted diaryl diselenides as potential atheroprotective compounds

Key words: disubstituted diaryl diselenides, selenium, glutathione peroxidase, thioredoxin reductase, LDL oxidation, atherosclerosis.

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ABSTRACT

Since increasing evidence indicates that oxidative modification of low-density lipoproteins (LDL) is an important determinant in atherogenesis, the aim of this study was to evaluate the effect of the disubstituted diaryl diselenides, DM and DC, on Cu^{2+}-induced LDL oxidation. Both compounds caused a dose-dependent inhibition on human serum and isolated LDL oxidation evidenced of the lag phase of lipid peroxidation increase and the oxidation rate (Vmax) decrease. The protein fraction from human isolated LDL were also protected against Cu^{2+}-induced oxidation. Moreover, the disubstituted diaryl diselenides efficiently decreased the LDL induced foam cell formation in J774 macrophage cells. In order to establish the mechanistic approach, we demonstrated that the potential antiatherogenic effect of these compounds is related to its GPx-like activity and its property to act as a good substrate for mammalian TrxR, two important pathways involved in peroxide decomposition.

Key words: disubstituted diaryl diselenides, selenium, glutathione peroxidase, thioredoxin reductase, LDL oxidation, atherosclerosis.

1. INTRODUCTION

The relation between inflammation and oxidative stress in the pathogenesis of cardiovascular disease has aroused much interest. Oxidative stress is strongly implicated in all stages of atherosclerosis, from the initiation of fatty streaks to the rupture of vulnerable plaques [1]. Oxidative modifications of the low-density lipoprotein (LDL) play a pivotal role in the development of atherosclerosis by inducing expression of adhesion proteins and proinflammatory cytokines and by causing the formation of lipid-loaded foam cells from macrophages [2]. LDL contains both a protein and a lipid component, either of which may be a target for oxidative damage during atherosclerosis [3]. In this instance, it has been evidenced that the antioxidant capability of LDL can be easily increased by dietary antioxidant supplementation. In fact, many endogenous and exogenous compounds have been reported to produce beneficial effects against LDL oxidation [4].

Blood vessels cells are known to express antioxidant enzymes to counteract oxidant stress [5]. Glutathione peroxidase (GPx), the key antioxidant enzyme within many cells, including the endothelium, consumes reduced glutathione to convert hydrogen peroxide to water and lipid peroxides to their respective alcohols [6]. Clinical evidences suggest a protective role for GPx in atherogenesis. In a prospective cohort study, reductions in red blood cell GPx1 activity were associated with an increased risk of cardiovascular events[7] while atherosclerotic plaques of patients with carotid artery disease have reduced GPx1 activity [8]. In animal studies, the lack of functional GPx1 accelerates diabetes-associated atherosclerosis via upregulation of proinflammatory and profibrotic pathways in ApoE-/- mice. It also showed that reduce GPx1 expression increased cell-mediated oxidation of LDL [9] as well as decreased the bioavailability of nitric oxide, leading to endothelial dysfunction [10].

Since the discovery that selenium plays a crucial role in GPx enzymes, design and development of new synthetic organochalcogenides catalytic antioxidant have attracted considerable attention. The first example of such compound was ebselen [11], which has been extensively studied in view of its pharmacological properties [12]. The antioxidant property of ebselen and others organochalcogenides has been linked to its glutathione peroxidase mimetic activity [11] and to its ability to act as a scavenger of peroxynitrite ($ONOO^-$) [13]. Moreover, ebselen is also a substrate for mammalian thioredoxin reductase (TrxR) and can be reduced by electrons derived from NADPH, forming its

selenol intermediate that can efficiently decompose hydrogen peroxide [14, 15].

Some points of evidence have suggested the antiatherogenic effect of organochalcogenides. Lass and col. (1996) demonstrated the protective effect of ebselen against copper and peroxyl radical-induced LDL lipid oxidation [16]. More recently, an interesting study showed that this organochalcogenide reduces atherosclerotic lesions in diabetic apoE -/- mice by modulating transcription factors such as NF-kB and AP-1 [17]. In line with this, previous studies from our laboratory have demonstrated important antiatherogenic properties of the simple diorganoilselenium compound, diphenyl diselenide $(PhSe)_2$. This compound presented beneficial effects against oxidation induced by copper ions or hydroxyl radical generator, on human serum and isolated LDL and rat aortic slices [18]. It also reduced the oxidative stress in hypercholesterolemic rabbits [19]. Most importantly, the treatment with $(PhSe)_2$ reduced the atherosclerotic lesion in hypercholesterolemic LDLr -/- mice by modulating pathways related to antioxidant and anti-inflammatory responses [20].

Encouraged by the successful results found for ebselen and $(PhSe)_2$ in *in vitro* and *in vivo* models of atherosclerosis, we decided to study the protective effect of new organochalcogenide in preventing the LDL oxidation. The disubstituted diaryl diselenides, *p*-methoxyl-diphenyl diselenide $(p\text{-}CH_3O\text{-}C_6H_4Se)_2$ (DM) and *p*-chloro-diphenyl diselenide $(p\text{-}Cl\text{–}C_6H_4Se)_2$ (DC) were synthesized since $(PhSe)_2$, by the introduction of functional groups (methoxyl or chloro) into the aromatic ring [21]. Recent studies demonstrated that DM improved the hepatic antioxidant defense system, decreased liver damage induced by LPS/D-GalN [22] and produced antinociception in mice [23]. Futhermore, DM and DC inhibited d-ALA-D activity by interacting directly with thiols oxidizing them to disulfides [24]. Given the earlier observations, the present study was carried out to evaluate the potential beneficial effects of DM and DC in protecting *in vitro* human serum and isolated LDL oxidation as well as foam cells formation, which are the main elements involved in the early steps of atherogenesis. The GPx-like activity and the capability to be a substrate for TrxR was also evaluated in an attempt to delve into molecular mechanisms related to the aforementioned protective effects.

2. MATERIALS AND METHOD

2.1 Materials

The *p*-methoxyl-diphenyl diselenide (*p*-CH_3O–$C_6H_4Se)_2$ (DM) and *p*-chloro-diphenyl diselenide (*p*-Cl–$C_6H_4Se)_2$ (DC) was synthesized according to published methods [21]. Analysis of the H NMR and 13C NMR spectra showed that the compounds obtained presented analytical and spectroscopic data in full agreement with their assigned structure (Fig. 1). These drugs were dissolved in dimethylsulfoxide (DMSO) or ethanol and a 10mM stock solution was stored at 4°C for less than 2 weeks. Immediately before use, the compounds were diluted at the required concentrations for the different assays. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2 LDL isolation and oxidation

This study was approved by our Ethic Committee at Federal University of Santa Catarina (nº 943/11). LDL was isolated from fresh human plasma by discontinuous density-gradient ultracentrifugation as described[18]. Briefly, plasma of nonfasted healthy normolipidemic voluntary donors collected with EDTA (1 mg/mL) was pooled and sucrose (final concentration, 0.5%) was added to prevent LDL aggregation. Five milliliters of EDTA-plasma adjusted to a density of 1.22 g/mL with solid KBr (0.326 g/mL) was layered on the bottom of a centrifuge tube. Then 5 mL EDTA-containing sodium chloride solution (density 1.006 g/mL) was overlaid on top of the plasma. Ultracentrifugation was run at 191.000xg for 2 h at 4ºC, in a Himac CP80WX Hitachi ultracentrifuge, rotor 40ST. LDL particles were collected by aspiration of the yellow/orange band, which is located in the middle of sodium chloride solution just above the plasma main fractions. Then, LDL particles was dialyzed exhaustively overnight at 4°C against 5 L of 148 mM phosphate buffer (Na_2HPO_4 8 mM, KH_2PO_4 1.4 mM, KCl 2.6 mM, NaCl 136 mM, pH 7.4) to remove the excess salt and the majority of the EDTA. Protein concentration in LDL solution was determined by Lowry et al [26].

LDL isolated samples (1 mg of protein/mL) were oxidized at 37°C in the presence of 10 µM $CuSO_4$ for 16 hours. Then EDTA 1.5 mM was added and the samples were dialyzed against 148 mM phosphate buffer for 24 hours at 4°C, result in oxidized LDL (oxLDL). Isolated LDL and oxLDL were stored at -20°C not longer than 2 weeks.

2.3 Serum oxidation

Venous blood was drawn from nonfasted healthy normolipidemic voluntary donors into tubes containing no anticoagulant and centrifuged at 1.500xg for 15 min. Serum samples were diluted 1:50 in 10 mM phosphate buffer, pH 7.4 and incubated at 37°C with different DM or DC (0 - 20 μM) concentration. The oxidation was initiated by the addition of $CuSO_4$ (50 μM) and conjugated dienes (CD) formation was monitored at 245 nm as previously described [27].

2.4 LDL oxidation

LDL oxidation was monitored by following the CD formation. LDL samples (50 μg protein/mL) were preincubated at 37°C in a medium containing 10 mM potassium phosphate buffer, pH 7.4 and different concentrations of DC (0 - 50 μM) or DM (0 - 30 μM). After 10 min, $CuSO_4$ (5 or 10 μM) was added to the reaction medium and the reaction was monitored for 6 hours for evaluating CD production. The oxidation was monitored by measuring the increase in absorbance at 234 nm due to CD formation as previously described [28].

2.5 Determination of lag phase and oxidation rate (Vmax)

In the studies of CD formation, there are several parameters which can be obtained from CD vs. time profiles. The value of the lag phase was defined as the intercept of the tangent of the slope of the absorbance curve in propagation phase with the time axis, and was expressed in min. The oxidation rate (Vmax) was obtained from the slope of the absorbance curve during the propagation phase [29].

2.6 Measurement of LDL-Trp fluorescence

The time course of tryptophan (Trp) fluorescence emission intensity is used to monitor Cu^{2+}-induced apolipoprotein LDL oxidation. The fluorescence spectra of native LDL display a single band centered at approximately 332 nm, which is assigned to the Trp residues in ApoB-100 [30]. Loss of Trp fluorescence is a marker for oxidations at the protein core of LDL [30, 31]. The kinetics of LDL oxidation was followed by measuring the decrease of Trp-fluorescence, corresponding to the decomposition of this amino acid, after the addition of 3.3 μM $CuSO_4$, in absence or presence of different DM (0 - 15 uM) or DC (0 - 20 uM) concentrations. Trp fluorescence was measured at different time points (0 - 360 min) using a spectrofluorometer (Tecan, Grödig/Salzburg, Austria) (excitation at 282 nm and emission at 331 nm). The parameter "half-time" ($t_{max/2}$) was used to characterize the fluorescence changes in

quantitative terms for practical purposes. It is defined as the time needed to observe a reduction in fluorescence of 50% of the difference between initial and residual fluorescence intensity [32].

2.7 Cell culture and Foam cell formation assay

J774 murine macrophage-like cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were maintained with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM glutamine, 10 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum (FBS).

For the foam cells formation, J774 cells were plated in 12-well plate at equal density (2 x 10^5 cells per well) in DMEM medium supplemented with 10% fetal bovine serum (FBS) to 24 hours. After that, the medium were replaced by DMEM medium without FBS and the cells were incubated with DM or DC (1 µM) or vehicle for 24 hours in a 5% CO_2 humidified atmosphere at 37°C. Oxidized LDL (oxLDL) (100 µg/mL) was then added to the medium for additional 3 hours.

Foam cell formation assay was performed with the Oil-Red O staining method[33]. Following oxLDL incubation, cells were fixed with 4% paraformaldehyde and stained by 0.3% Oil-Red O for 10 min. Hematoxylin was used as counterstaining. Images of cells were acquired using a light microscopy with oil immersion objective using Olympus microscope (1000x). Ten images were captured from each group and the total pixels intensity was determined using NIH Image J 1.36b imaging software (National Institutes of Health, Bethesda, MD, USA), and lipid content was expressed as optical density (OD).

2.8 GSH consumption

GHS comsumpition was determined according to the Ellman's method [34]. DM or DC (100 µM) were incubated (37ºC) in a reaction medium containing 1 mM of reduced glutathione (GSH) in 10 mM potassium phosphate buffer, pH 7.4. An aliquot (10 µL) from each sample was removed at different times points and added to 800 mM phosphate buffer, pH 7.4, and 500 µM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB). Samples were read at 412 nm after 10 min.

2.9 Spectroscopy studies

In order to evaluate the potential chemical interaction between the disubstituted diaryl diselenides (DM and DC), GSH and H_2O_2, DM and DC (10 µM) were incubated with GSH (200 µM) in 10 mM

potassium phosphate buffer (pH 7.4). The reaction was performed at 37°C and monitored spectrophotometrically (190 - 360 nm) using a spectrophotometer UV/vis (Hitachi, U-2001). In additional experiments, H_2O_2 (200 μM) was added 1 min after the reaction of DM and DC with GSH.

2.10 Glutathione-peroxidase (GPx) like activity

The GPx-like activity of DM and DC were measured according to a method previously described by Wilson et al [35]. DM and DC (1 - 30 μM) were incubated at 37°C in a medium containing 50 mM potassium phosphate buffer, pH 7.0, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM reduced glutathione (GSH), 1 mM azide, 0.2 U of GR and 0.25 mM NADPH. The reaction was initiated by addition of 0.2 mM of hydrogen peroxide. The activity was followed by the decrease of NADPH absorption at 340 nm. Appropriate controls were carried out without the disubstituted diaryl diselenides and were subtracted.

2.11 Thioredoxin reductase assay

TrxR activity was determined according Zhao et al [14] . Activity was performed in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, 100 μL of TrxR (10 μg protein/mL of reaction medium of rat liver homogenate) and 100 μM of NADPH. Enzyme reaction was started with the addition of different amount of disubstituted diaryl diselenides DM and DC (0 - 100 μM) as a substrate for enzyme. The activity was followed by the decrease of NADPH absorption at 340 nm. Appropriate controls were carried out without the disubstituted diaryl diselenides and were subtracted.

2.12 Statistical Analysis

Results are presented as means ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) followed by the *post-hoc* Duncan multiple range test when P was significant. Linear regression analysis was also used to test concentration-dependent effects. A value of $p < 0.05$ was considered to be significant. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences 16.0 (SPSS) software.

3. RESULTS

3.1 Effect of disubstituted diaryl diselenides on Cu^{2+}-induced LDL and human serum oxidation

Incubating native human LDL with Cu^{2+} resulted in the oxidation of LDL polyunsaturated fatty acids, as indicated by the formation of CD. The kinetic profile of the oxidation was characterized by an initial lag phase followed by a propagation phase, where the rate of CD formation was maximal, and then by a decomposition phase. The effects of disubstituted diaryl diselenides on Cu^{2+}-induced (10 μM) lipid oxidation in isolated LDL are depicted in Figure 2. DM (Fig. 2A) and DC (Fig. 2B) inhibited Cu^{2+}-induced generation of CD in a concentration-dependent manner. Both compounds caused a concentration-dependent increases in *lag phase* (DM: β=0.970, $p<0.001$; DC: β=0.979, $p<0.001$), and decreases the oxidation rate - Vmax (DM: β=-0.993 , $p<0.001$; DC: β=-0.919, $p<0.001$), evidenced by changes in the propagation phase slope. It is noteworthy that Cu^{2+}-induced lipid LDL oxidation was almost completely abolished in the presence of 20 μM of DM. Similar protocol were conducted using a lower Cu^{2+} concentration (5 μM), and both compounds showed the same inhibitory pattern on LDL induced oxidation (data not show).

Serum oxidation was determined by CD formation at 245 nm. No oxidation occurred in serum when the medium did not contain Cu^{2+} ions. The effects of disubstituted diaryl diselenides on Cu^{2+}-induced human serum oxidation are depicted in Figure 3. Interestingly, progressively higher concentrations of DM and DC (0 - 20 μM) inhibited the human serum oxidation and reduced its susceptibility to lipid peroxidation, as observed by a concentration-dependent increase in the *lag phase* (DM: β=0.946, $p<0.001$; DC: β=0.845, $p<0.001$) and a reduction in the oxidation rate - Vmax (DM: β=-0.986, $p<0.001$; DC: β=-0.985, $p<0.001$), respectively (Fig. 3A and 3B, upper and lower panels). In this oxidation system, DM was more effective in inhibited serum oxidation than DC.

3.2 Effect of disubstituted diaryl diselenides on LDL-tryptophan fluorescence kinetics

Figure 4 shows that protein moieties of LDL are oxidized within time in the presence of $CuSO_4$ (3.3 μM), decreasing the kinetic of tryptophan fluorescence. This phenomenon was prevented by DM or DC in a concentration-dependent manner (DM: β=0.984, $p<0.001$; DC:

β=0.975, $p<0.001$), evaluated by 50% inhibition of fluorescence tryptophan ($t_{max/2}$) (Fig. 4A and B, inset).

3.3 Disubstituted diaryl diselenides inhibits the oxLDL mediated foam cell formation

As expected, oxLDL uptake by macrophages and following foam cell formation was induced in macrophages exposed to oxLDL. Figure 5 shows that the pretreatment for 24 hours with 1 μM of DM or DC significantly reduced ($p<0.001$) the uptake of oxLDL by J774 macrophages cells, indicating the effect of these compounds in this important step involved in the atherogenic process.

3.4 Spectroscopy studies

In order to delve into molecular mechanisms involved with the beneficial role of DM and DC against LDL lipid peroxidation, *in vitro* experiments of light/UV spectroscopy concerning the chemical interaction between DM or DC, GSH and H_2O_2 were conducted. Initially, we evaluated the consumption of GSH (1 mM) by incubation with 100 μM of both disubstituted diaryl diselenides (DM or DC). In fact, both diaryl diselenides diphenyl diselenide caused a significant GSH consumption after 20 min of reaction (Fig. 6A).

Once determined that the compounds are capable of reacting with GSH, causing its consumption, we evaluate this interaction spectrophotometrically. The characteristic spectra of DM 10 μM (Fig. 6B, line A) or DC 10 μM (Fig. 6C, line A), and of 200 μM GSH (line B) was changed after their reaction (line C), probably due to the formation of phenyl selenol intermediate ($PhSe^-$). Interestingly, after the addition of H_2O_2 (200 μM), the strong and broad absorption peak at 280 nm of the intermediate produced by DC and GSH interaction was abolished, indicating its chemical interaction with H_2O_2 (Fig 6A and B, line D).

Consistent with this observation, only DC displayed a concentration-dependent GPx-like activity (Fig. 6C, inset), which was indirectly measured by NADPH consumption in the presence of GSH, purified GSH reductase and hydrogen peroxide. Taken together, these data indicate that the chemical interaction between DC and GSH produces an intermediate that is capable of interacting with peroxides. This process allows for the detoxification of peroxides at the expenses of sulfhydryl groups from GSH. By the other hand, besides the ability of DM reach with GSH, it not displayed GPx-like activity, suggesting that the protective effect of this compound against LDL toxicity is related to other mechanism.

Then, we also studied the kinetics of disubstituted diaryl diselenides free radical scavenging activity by allowing DM and DC to react with DPPH, a stable free radical. Both disubstituted diaryl diselenides, in its original form, not exhibit radical DPPH scavenger capacity (data not show), showing that antioxidant activity of these compounds is not related to their chemical structured (ability to give electrons).

3.5 Disubstituted diaryl diselenides are substrates for hepatic mammalian Thioredoxin Reductase (TrxR)

Figure 7 show the effect of disubstituted diaryl diselenides on the oxidation of NADPH in the presence of partially purified hepatic mammalian TrxR. DM and DC (at concentrations of 0 - 100 μM) stimulated NADPH oxidation in a concentration-dependent manner (DM: β=-0,671, $p<0.001$; DC: β=-0.647, $p<0.001$) in the presence of TrxR, indicating that they are substrates for hepatic mammalian TrxR. Oxidation of NADPH observed in the presence of these disubstituted diaryl diselenides were almost completely blocked by 1 uM $AuCl_3$ (more than 90 %).

4. DISCUSSION

Since LDL oxidation and foam cell formation play a key role in the pathogenesis of atherosclerosis, antioxidants that can inhibit these oxidative processes might be useful in preventing atherosclerosis-related pathological conditions, such as coronary artery diseases and stroke [35]. Several studies have reported the beneficial effects of organoseleno compounds against pathological conditions associated to oxidative stress (inflammation, diabetes, neurotoxicity, and hepatotoxicity)[36]. In fact, during the last decades, ebselen was the unique organoseleno compound that raised interest in studies involving atherosclerosis models. Recently, Chew et al. (2009) demonstrated the effect of this organochalcogenide in reducing atherosclerotic lesions in diabetic apoE -/- mice [17]. In this regard, we have been studying the potential antiatherogenic properties of other organoseleno compounds in different *in vivo* and *in vitro* models related to atherosclerosis. A recent study from our group demonstrated that a simple diaryl diselenide, $(PhSe)_2$, was more effective than ebselen in reducing the atherosclerotic lesion in hypercholesterolemic LDLr -/- mice, considering that we used a dose of $(PhSe)_2$ around 100 times lower than that of the ebselen study [20].

In the present work, the decision to study the disubstituted diaryl diselenides, DM and DC, as potential beneficial molecules against human LDL oxidation was based on their chemical similarity to $(PhSe)_2$. Herein, both disubstituted diaryl diselenides showed beneficial effects against oxidation induced by copper ions on human serum and isolated LDL and prevented *in vitro* foam cell formation. A large body of evidence indicates that oxLDL plays a key role in both the early and more advanced inflammatory stages of atherosclerosis lesions [37]. The progression of atherosclerosis is slowed when oxidation is inhibited, and there is a correlation between the ability of LDL to resist oxidation and the severity of coronary atherosclerosis [38, 39].

Our results showed that DM and DC prevented copper-induced isolated LDL peroxidation in a concentration-dependent manner by decreasing the formation of conjugated dienes, thus extending the lag phase and lowering the oxidation rate (Vmax). Besides, the treatment of human serum with DM or DC also inhibited copper-mediated oxidation in a concentration-dependent manner.

Another significant result from our study was the capability of both disubstituted diaryl diselenides to prevent copper-induced loss of Trp fluorescence in human LDL. It has been reported that the fluorescence spectrum of native LDL shows a single band centered at approximately 332 nm, which is due to the Trp residues in apo B-100 and loss of Trp fluorescence is a marker for oxidations at the protein core of LDL [30]. The protective effect of DM and DC against copper-induced loss of Trp fluorescence indicates that besides their beneficial effects against oxidation of the lipid fraction of LDL, these chalcogens also prevent the oxidation of the protein fraction of human LDL, pointing to an additional mechanism that could contribute to the inhibition of the atherogenic process.

OxLDL could promote atherogenesis due to its cytotoxicity and its uptake by the macrophage scavenger receptor, resulting in stimulation of cholesterol accumulation and hence foam cell formation, which is the trigger point for early atherosclerosis lesions [40]. In this way, here we showed that the pretreatment of J774 macrophages with DM and DC significantly decreases the oxLDL uptake and consequent foam cell formation. By, retarding LDL oxidation and consequently, avoiding the damages, i.e, macrophage cell death and foam cell formation, atherosclerosis could be prevented.

The generation of ROS by vascular cells takes place after the binding of LDL particles to scavenger receptors such as CD36 [41] and LOX-1 [42]. These findings are in accordance with our recent study which

demosntrated that the simple diaryl diselenides, $(PhSe)_2$ prevented oxLDL ROS production and cytokine generation in J774 macrophages [20].

To clarify the mechanism by which DM and DC reduces LDL oxidation, we investigated its ability to oxidize thiols and performed spectroscopy studies. In fact, we show in this study that both disubstituted diaryl diselenides caused GSH consumption *in vitro* system. In line with this, spectroscopic studies showed the direct chemical interaction between DM and DC with GSH, resulting in the formation of a chemical intermediary (probably the phenyl selenol) whose stability is affected by the presence of hydrogen peroxide. The selenol produced can decompose peroxides using either reduced glutathione or other thiols. Although the thiol oxidase activities of the diselenides can indicate a potential pro-oxidant property and, consequently, can give some indication about its potential toxicity [12], its interaction with thiols is critical to form the selenol/selenolate for degradation of peroxides. Consistent with this observation, DC showed a concentration-dependent GPx-like activity (Fig. 6C, inset), however this enzymatic-like effect was not present in DM, suggesting that an additional mechanism is required to explain its protective effect against LDL induced oxidation. Moreover, we demonstrated by DPPH assay, that these compounds do not show a direct free radical scavenging activity, reinforcing the need for a sulfhydryl interaction to produce their antioxidant effect.

Although the DC GPx-like activity is partially responsible for its antioxidant effects, the mechanism by which DM acts as a protective molecule needs another explanation. In the present study, we also observe that DM and DC can be reduced by TrxR, demonstrating that these compounds can be a substrate for mammalian TrxR. These findings are in agreement with previous studies of Holmgren group which demonstrated that ebselen and its diselenide were good substrates for human TrxR [14, 15]. Furthermore, they have also clearly demonstrated that the antioxidant activity of ebselen and its diselenide is related to their reductions by mammalian TrxR producing ebselen selenol and therefore that the TrxR pathway could more efficiently decompose hydrogen peroxide than the GPx-like pathway [15]. Consequently, the potential participation of disubstituted diaryl diselenides as substrates for TrxR could explain their antioxidant and pharmacological properties.

In conclusion, DM and DC demonstrated a strong potency to inhibit the lipid and protein oxidation induced by copper in human isolated LDL. Moreover, these compounds exert a direct effect on

macrophage cholesterol metabolism by reducing oxLDL cellular uptake and consequently the foam cell formation. Our findings on the possible mechanism involved in the antioxidant effect of the disubstituted diaryl diselenides DM and DC against LDL toxicity, will contribute towards the development of new therapeutic approaches to prevent and treat atherosclerosis and cardiovascular diseases.

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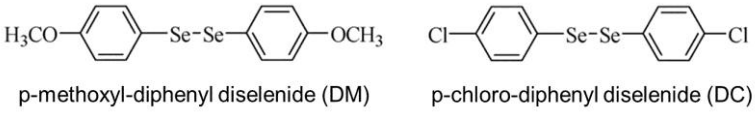
FIGURES AND LEGENDS



p-methoxyl-diphenyl diselenide (DM) p-chloro-diphenyl diselenide (DC)

Figure 1. Organoselenium compounds aromatic derivatives.

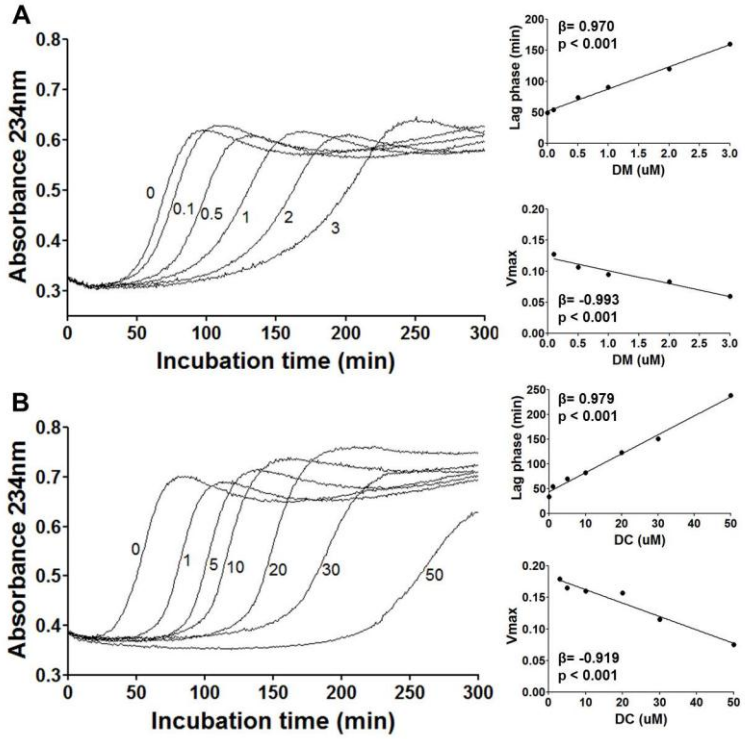


Figure 2. Effect of DM (A) and DC (B) on Cu^{+2}-induced lipid peroxidation in human LDL. At different time points, conjugated dienes were analyzed. LDL samples (50 µg protein/ml) were incubated in the presence of 10 µM $CuSO_4$ in the absence or presence (0.1-3 µM) of DM or (1-50 µM) of DC. CD are expressed as absorbance at 245 nm. The upper and the lower panels (A and B) show the effect of DM and DC on the lag phase and oxidation rate (Vmax) of CD formation, respectively. Experiments were repeated at least three times, showing similar results. Linear regression of lag phase and Vmax was used in order to verify the concentration-dependent effect.

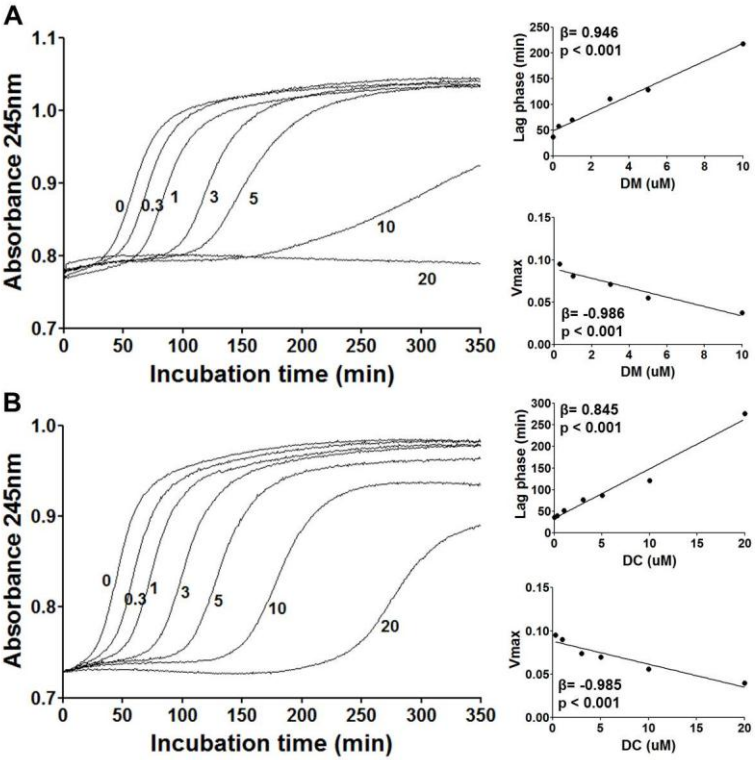


Figure 3. Effect of DM (A) and DC (B) on Cu^{+2}-induced lipid peroxidation in human serum. At different time points, conjugated dienes were analyzed. Serum diluted 50-fold in PBS 10 mM (pH 7.4), were incubated in the presence of 50 µM $CuSO_4$ and in the absence or presence (0.1-20 µM) of DM or DC. CD are expressed as absorbance at 245 nm. The upper and the lower panels (A and B) show the effect of DM and DC on the lag phase and oxidation rate of CD formation, respectively. Experiments were repeated at least three times, showing similar results. Linear regression of lag phase and Vmax was used in order to verify the concentration- dependent effect.

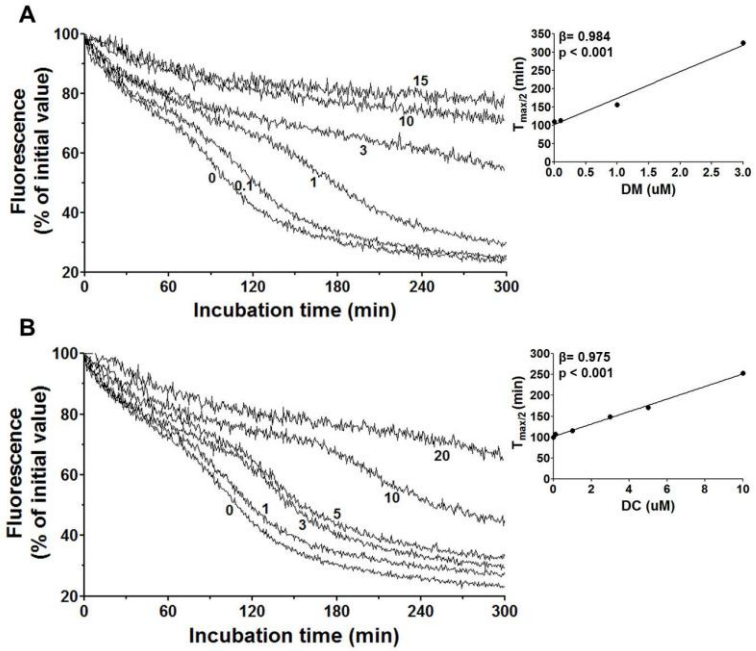


Figure 4. Effect of DM (A) and DC (B) on Cu^{+2}-induced loss of tryptophan fluorescence in human LDL. LDL samples (50 µg protein/ml) were incubated at 37°C in the presence of 3.3 µM $CuSO_4$ and (0-15 µM) of DM (A) and (0-20 µM) DC (B). Tryptophan fluorescence (excitation at 282 nm and emission at 331 nm) was measured at different time points (0-300 min). Inset shows the 50% inhibition ($T_{max/2}$) of loss of tryptophan fluorescence. Date are expressed as percentage of the initial value of emission intensity measured before Cu^{+2} addition. Experiments were repeated at least three times, showing similar results. Linear regression of loss of tryptophan fluorescence was used in order to verify the concentration-dependent effect.

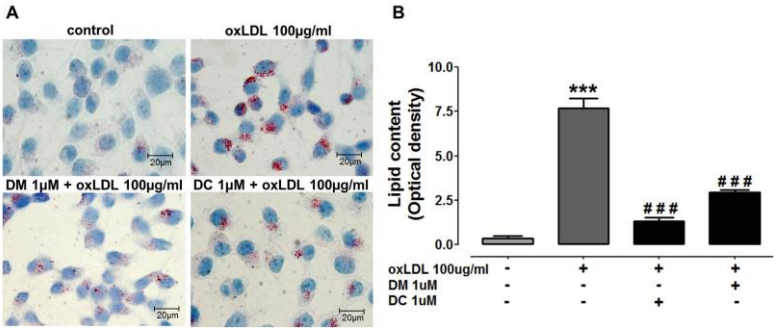


Figure 5. Effect of DM and DC on foam cell formation. (A) J774 macrophage cells were preincubated for 24 hours with 1 µM of the DM or DC or vehicle, then, oxLDL 100 µg/mL was added to the medium for 3 h. The cells were stained with Oil Red O and hematoxilin and observed under light microscope (magnification 1000x). Representative images were from three independent experiments. (B) Quantification of foam cells lipid content (optical density). The data presented were means ± SEM. ***$p<0.001$ compared with control and $^{###}p<0.001$ indicates the difference when compared with oxLDL-treated cells (one-way ANOVA).

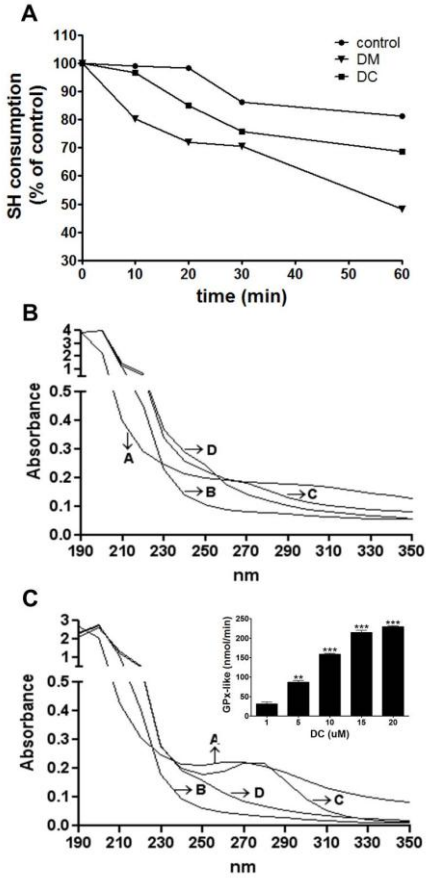


Figure 6. Effect of DM and DC on GSH consumption and spectroscopy study. (A) At different time points (0-150 min) SH content was analyzed after incubation of DM or DC (100 µM) with glutathione (1 mM). The results were expressed as percentage of control. Experiments were repeated at least three times, showing similar results. The comparative spectra of DM (B) and DC (C) and the products of its interaction with GSH and/or H_2O_2. (A) DM or DC (10 µM); (B) GSH (200 µM); (C) DM or DC (10 µM) + GSH (200 µM) + 1 min at 37 °C; (D) DM or DC (10 µM) + GSH (200 µM) + 1min + H_2O_2 (200 µM) + 1 min at 37°C. The inset graph (in panel C) shows GPx-like activity of DC. Results are represented as mean±SEM from at three independent experiments (One-way ANOVA).

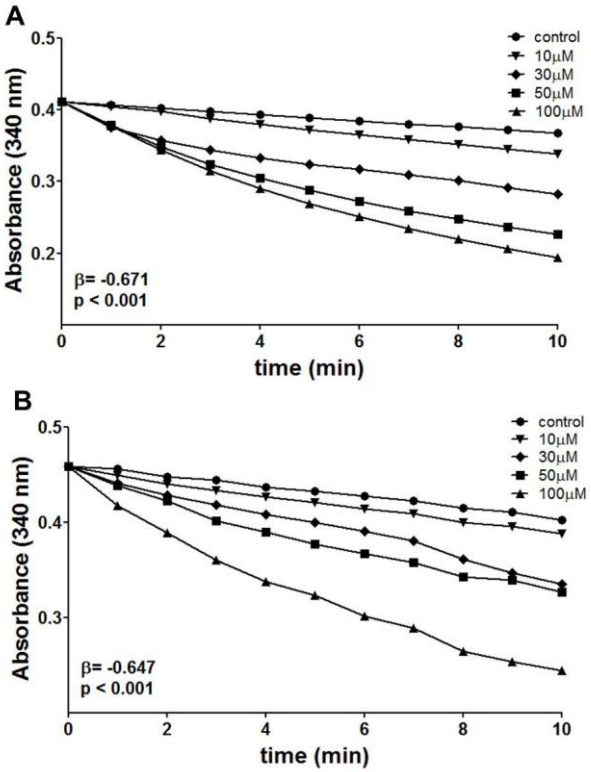


Figure 7. Reduction of DM (A) and DC (B) by NADPH catalyzed by hepatic mammalian TrxR. Enzyme (10μg/ml) was incubated (37°C) in a reaction medium containing 50 mM Tris/HCl buffer (pH 7.5), 1mM EDTA, and 100μM NADPH. Enzyme reaction was started with the addition of different amount of disubstituted diaryl diselenides (DM or DC at 0-100μM). The results are expressed as absorbance at 340nm. Linear regression of slopes were used in order to verify the concentration-dependent effect. The data were means ± SEM from three repeats from independent experiment.

5. DISCUSSÃO

A descoberta de novos compostos capazes de atuarem beneficamente em patologias relacionadas ao estresse oxidativo tem ganhado destaque nos últimos anos, resultando em numerosos estudos relacionados à atividade de agentes antioxidantes naturais ou sintéticos (Kinsella et al., 1993). Assim, o interesse por estudos químicos e bioquímicos de compostos orgânicos de selênio tem aumentado consideravelmente nas últimas três décadas, principalmente devido ao fato de que vários destes compostos apresentam importante atividade antioxidante (Andersson et al., 1994, Sies e Arteel, 2000), a qual possivelmente está relacionada à capacidade destes em mimetizar a atividade da enzima glutationa peroxidase (GPx) (Wendel, 1981, Muller et al., 1984).

O primeiro exemplo desta classe de compostos foi o ebselen, extensivamente estudado na última década. Este composto de baixa toxicidade (Muller et al., 1988), demonstrou propriedades antiinflamatória, antiaterosclerótica e citoprotetora em modelos experimentais *in vivo* e *in vitro* (Schewe, 1995, Takasago et al., 1997, Porciuncula et al., 2003, Brodsky et al., 2004). Neste sentido, nosso grupo de pesquisa vem investigando há mais de uma década as propriedades farmacológicas e toxicológicas destes compostos, com especial ênfase ao estudo do disseleneto de difenila $(PhSe)_2$. Este composto, primeiramente utilizado como intermediário em reações de química orgânica, demonstrou ações farmacológicas interessantes em diversos modelos experimentais relacionados à produção exacerbada de espécies reativas. As atividades farmacológicas do $(PhSe)_2$ são atribuídas em parte a sua ação mimética da enzima GPx (Spector et al., 1989), o que caracteriza este composto como um potente antioxidante. O efeito farmacológico do $(PhSe)_2$ foi demonstrado recentemente em modelos experimentais de hipercolesterolemia (de Bem et al., 2009), diabetes (Barbosa et al., 2006, Barbosa et al., 2008), oxidação de LDL (de Bem et al., 2008), dor (nonicepção) (Savegnago et al., 2007), inflamação (Savegnago et al., 2006), hepatotoxicidade (Borges et al., 2005, Borges et al., 2007), toxicidade induzida por metais (Freitas et al., 2009), entre outros.

Por outro lado, a utilização do $(PhSe)_2$ pode ocasionar toxicidade dependente da dose, da via de administração e da espécie animal utilizada, caracterizando um efeito dual da utilização deste compostos (Meotti et al., 2003, Nogueira et al., 2003, de Bem et al., 2006, de Bem et al., 2007). Em baixas doses, possuem efeitos benéficos

e em altas, são potencialmente tóxicos (Nogueira e Rocha, 2010). Em relação ao mecanismo de toxicidade molecular do $(PhSe)_2$, este pode estar associado com a oxidação catalítica de importantes grupos tióis da GSH ou de diferentes proteínas ou enzimas endógenas sulfidrílicas, incluindo a 5-lipoxigenase (Bjornstedt et al., 1996), δ-ALA-D (Nogueira et al., 2003), esqualeno monooxigenase (Gupta e Porter, 2001) e Na+,K+-ATPase (Borges et al., 2005).

Neste sentido, os resultados apresentados na primeira parte desta dissertação, referem-se à avaliação toxicológica do $(PhSe)_2$ em coelhos. Os animais receberam via intra-peritoneal (i.p.), diferentes concentrações de $(PhSe)_2$, de forma aguda durante 5 dias. O conjunto de resultados apresentados no Artigo 1 demonstram que a toxicidade do $(PhSe)_2$ neste modelo experimental foi dependente da dose. As doses utilizadas neste estudo foram 5, 50 e 500 μmol/kg por dia. O tratamento dos coelhos com 5 μmol/kg/dia não induziu toxicidade, no entanto, quando os animais foram tratados com 50 μmol/kg sinais leves de toxicidade foram observados. Por outro lado, a concentração de 500 μmol/kg causou uma elevada mortalidade (85%) e efeitos tóxicos nestes animais.

Estudos anteriores realizados pelo nosso grupo de pesquisa avaliaram a toxicidade do $(PhSe)_2$ em coelhos expostos cronicamente por via oral a baixas doses (de Bem et al., 2006, de Bem et al., 2007). Estes trabalhos demonstraram que a administração oral de $(PhSe)_2$ nas doses de 0,03, 3 e 30 ppm na ração (o equivalente a 0,05, 0,5 e 5 μmol/kg) por um longo período (8 meses), foi relativamente segura, mesmo na maior dose utilizada neste protocolo de estudo (30 ppm), a qual está muito acima dos valores nutricionais preconizados para a ingestão de Se em humanos. Sinais leves de toxicidade, caracterizados pela diminuição no conteúdo de ácido ascórbico no fígado e cérebro, foram observados somente na maior dose testada, não ocasionando morte nestes animais (Bem et al., 2006, Bem et al., 2007).

Nossos dados demonstram que a exposição aguda ao $(PhSe)_2$ em coelhos não induziu modificações nos marcadores periféricos de função renal (uréia e creatinina) e hepática (AST e ALT). Porém, quando avaliamos histologicamente o fígado dos animais, observamos que o tratamento com 50 μmol/kg induziu a formação vacúolos lipídicos no fígado, entretanto, esta condição não foi refletida pelo aumento da atividade das enzimas marcadores de lesão hepática no sangue periférico, indicando, de certa forma integridade morfológica e funcional. A presença de células inflamatórias e apoptóticas não foram observadas no tecido hepático.

O $(PhSe)_2$, atua como mimético da GPx, reagindo com tióis para gerar selenol, o intermediário responsável pelo efeito antioxidante (Mugesh e Singh, 2000). O tratamento agudo *in vivo* com $(PhSe)_2$ em coelhos, foi capaz de aumentar por um lado a atividade da GPx, de maneira dose-dependente, no músculo e coração, e por outro lado os níveis de glutationa (GSH), substrato desta enzima, nos rins, coração, músculo, sangue e hipocampo. A diminuição da atividade da GR, enzima responsável por regenerar a forma reduzida da GSH, foi observada nos rins e estruturas cerebrais. Esta redução pode ser resultado do aumento na síntese de GSH nestes tecidos, podendo estar relacionada ao aumento da atividade da enzima δ-glutamilcisteína sintetase, passo limitante na síntese de GSH. Estudos utilizando o ebselen, análogo do $(PhSe)_2$, observaram que o aumento dos níveis de glutationa em linhagem de células hepáticas foi devido a capacidade deste composto ativar resposta antioxidante dependente do fator de transcrição Nrf2 (Tamasi et al., 2004).

Um importante marcador de toxicidade é a enzima δ-ALA-D, que contém em seu sítio ativo grupos -SH extremamente sensíveis a presença xenobióticos ou pró-oxidantes, levando a inibição da sua atividade (Barnard et al., 1977, Fachinetto et al., 2006). A administração aguda de 50 μmol/kg de $(PhSe)_2$ foi capaz de inibir a atividade da enzima δ-ALA-D somente no hipocampo, indicando um possível sinal de toxicidade. De forma contrária, a atividade desta enzima aumentou no coração, a qual pode estar associada ao aumento dos níveis de GSH neste tecido, preservando a forma reduzida dos grupamentos tióis desta enzima.

Evidências indicam que vários toxicantes podem levar a distúrbios na atividade da cadeia transportadora de elétrons mitocondrial e consequentemente induzir a geração de ROS e estresse oxidativo (Latini et al., 2007). Para isso, investigamos a atividade dos complexos I e II da cadeia respiratória, no fígado e no cérebro destes animais. Observamos que o tratamento agudo (i.p) com $(PhSe)_2$ em coelhos não foi capaz de modificar a atividade destes complexos enzimáticos, confirmando a baixa toxicidade deste composto nas doses de 5 e 50 μmol/kg sobre parâmetros de função mitocondrial. Desta forma, juntamente com demais evidências já avaliadas anteriormente, podemos afirmar que a toxicidade da exposição aguda via i.p. ao $(PhSe)_2$ em coelhos é dependente da dose utilizada.

Vários estudos têm reportado efeitos benéficos dos compostos orgânicos de selênio contra condições patológicas associadas ao estresse oxidativo (inflamação, diabetes, neurotoxicidade e hepatoxicidade)

(Nogueira e Rocha, 2010). Nas últimas décadas, os compostos organoselênios Ebselen e $(PhSe)_2$ receberam destaque em estudos envolvendo modelos experimentais de aterosclerose. Recentemente, estudos revelaram que o ebselen foi eficiente em diminuir lesões ateroscleróticas em camundongos diabéticos apoE -/- (Chew et al., 2009). Nosso grupo também demonstrou que o $(PhSe)_2$, foi mais potente que o ebselen na redução de lesões ateroscleróticas em camundongos hipercolesterolêmicos LDLr -/-, considerando que a dose usada deste composto foi cerca de 100 vezes menor que a dose estudada do ebselen (Hort, 2011).

A oxidação da LDL e a formação de células espumosas possuem um papel importante na patogênese da aterosclerose. Antioxidantes que possam inibir este processo oxidativo poderão ser usados na prevenção de condições patológicas relacionado com aterosclerose, tais como doenças arterial coronariana e infartos (Esterbauer et al., 1992). Neste contexto, buscamos estudar as propriedades antiaterogênicas de compostos orgânicos de selênio em diferentes modelos *in vitro* relacionados com aterosclerose.

Várias evidências indicam que a LDLox participa tanto no estágio inicial quanto nos estágios mais avançados do processo aterogênico (De Lorgeril e Salen, 2000). A progressão da aterosclerose está diminuída quando a oxidação da LDL está inibida, e existe uma correlação entre a habilidade da LDL resistir a oxidação e a severidade da doença coronariana (Regnstrom et al., 1992, Heinecke, 1998). Estudos prévios do nosso grupo demonstraram que o $(PhSe)_2$ foi muito eficiente na inibição da oxidação da LDL humana isolada e de anéis de aorta de ratos (de Bem et al., 2008). Estes dados indicam a possível utilização do $(PhSe)_2$ como um agente farmacológico para o tratamento e/ou prevenção das doenças cardiovasculares relacionadas à aterosclerose. Para melhor compreender o mecanismo de ação do $(PhSe)_2$ na prevenção de eventos relacionados ao processo aterosclerótico, na segunda parte desta dissertação, avaliamos o efeito deste composto sobre a toxicidade da LDLox em cultivo de macrófagos murinos (J774).

Através dos resultados apresentados no manuscrito 1, podemos verificar primeiramente que a citotoxicidade induzida pela LDLox em macrófagos é, em parte, relacionada a super-produção de espécies reativas (ROS/RNS) geradas pela LDLox. Dentre estes eventos celulares podemos destacar distúrbios na homeostase do $^{\bullet}NO$, ativação de MMP, acúmulo de colesterol intracelular, disfunção mitocondrial, ativação do fator de transcrição NF-κB e consequente apoptose celular. Baseado nas

propriedades antioxidantes apresentadas anteriormente em relação ao $(PhSe)_2$, buscamos avaliar a resposta citoprotetora deste composto em macrófagos expostos à LDLox.

Nossos resultados demonstram que a exposição de macrófagos a LDLox, induziu um aumento significativo na geração de ROS e consequente morte celular por apoptose nestas células. Este aumento na produção de ROS pode ser responsável pela ativação de diferentes vias relacionadas ao processo inflamatório e morte celular (Ou et al., 2010). Por outro lado, quando os macrófagos foram pré-tratados com $(PhSe)_2$ a geração de ROS induzida pela LDLox foi significativamente reduzida, indicando que o efeito citoprotetor deste composto pode estar relacionado à sua atividade antioxidante (Nogueira e Rocha, 2010).

As mitocôndrias são ao mesmo tempo alvos e fontes biologicamente importantes de ROS/RNS (Kowaltowski et al., 2001, Zmijewski et al., 2005) e por outro lado, o aumento dos níveis de ERO causa disfunção mitocondrial e diminuição de proteínas anti-apoptóticas (Tan et al., 1998). A exposição à LDLox induziu o processo de disfunção mitocondrial, demonstrada pela diminuição do potencial de membrana mitocondrial ($\Delta\Psi_m$) nos macrófagos J774. Entretanto, o pré-tratamento com $(PhSe)_2$ foi capaz de prevenir esta disfunção, provavelmente pela diminuição da geração de ROS.

O processo aterogênico promovido pela LDLox, pode ser consequência de sua toxicidade e efeitos inibitórios na motilidade dos macrófagos e sua incorporação por receptores *scavenger*. O acúmulo de colesterol intracelular leva a formação das células espumosas, que são os primeiros marcadores morfológicos e inflamatórios das lesões ateroscleróticas precoces (Quinn et al., 1985). Como demonstrado nos resultados apresentados no manuscrito 1, o pré-tratamento de macrófagos J774 com $(PhSe)_2$, significativamente diminuiu a incorporação de LDLox e consequentemente a formação de células espumosas.

Outro evento importante que ocorre precocemente no processo aterogênico é o aumento da atividade de metaloproteinases de matriz (MMP), como observado após infarto agudo do miocárdio e ruptura de aneurisma abdominal (Kai et al., 1998, Wilson et al., 2006). Assim, a secreção de MMP por macrófagos pode contribuir para a ruptura da placa aterosclerótica. Neste trabalho observamos que o aumento da produção de MMP-9 em macrófagos expostos a LDLox foi significativamente diminuído pelo $(PhSe)_2$.

Um estudo recente demonstrou que a LDLox aumenta a expressão da iNOS e consequentemente a produção de $^{\bullet}NO$ em

macrófagos (Persson et al., 2009). Altos níveis de $^{\bullet}NO$ induzem situações pró-inflamatórias, além disso, este radical pode reagir com $O_2^{\bullet-}$ levando a formação de $ONOO^-$ e toxicidade celular (Chandel et al., 2000). Através da análise de nossos resultados, podemos observar que a exposição de macrófagos J774 a 200 μg/ml de LDLox causou um aumentou significativo na produção de $^{\bullet}NO$, enquanto o pré-tratamento com $(PhSe)_2$ impediu este aumento.

Trabalhos recentes demonstram ainda que o aumento na produção de $^{\bullet}NO$ pela iNOS pode levar a ativação de NF-κB (Li et al., 2001). Além disso, a ativação do NF-κB, indutor de vários mediadores pró-inflamatórios, pode ser um evento posterior da superprodução de ROS pela LDLox (Terkeltaub et al., 1994, Ou et al., 2007). Em nosso trabalho, observamos que a LDLox leva a ativação do NF-κB, o que poderia ser uma consequência da produção de ROS induzida por LDLox. Por outro lado, o $(PhSe)_2$ demonstrou capacidade de regular a ativação do fator NF-κB em macrófagos J774 expostos a LDLox, possivelmente devido às propriedades antioxidantes apresentadas pelo $(PhSe)_2$ como mimético da GPx.

O conjunto de dados apresentados no manuscrito 1 indicam um significativo efeito do $(PhSe)_2$ sobre a citotoxicidade induzida pela LDLox em macrófagos J774. Estes resultados são muito relevantes para o entendimento dos mecanismos moleculares da ação antiaterogênica do $(PhSe)_2$ demonstrada recentemente por nosso grupo em camundongos LDLr -/- (Hort, 2011).

A terceira e última parte desta dissertação, se refere ao estudo da possível ação antiaterogênica de novos compostos orgânicos de selênio. O interesse em estudar os compostos diaril disselenetos dissubstituídos, *p*-methoxyl-diphenyl diselenide (DM) e *p*-chloro-diphenyl diselenide (DC), no processo de oxidação da LDL, foi baseado na sua similaridade química com o $(PhSe)_2$. Ambos os compostos, DM e DC, foram capazes de reduzir a peroxidação lipídica da LDL humana isolada induzida por cobre, de maneira concentração-dependente, avaliada pela diminuição da formação de dienos conjugados, verificado através do aumento da *Phase lag* de oxidação e diminuição da taxa de oxidação (Vmax). Da mesma forma, ambos compostos inibiram, de maneira dependente da concentração, a oxidação de soro humano induzida por cobre. Estes resultados corroboram com nosso estudo anterior que demonstrou o efeito inibitório do $(PhSe)_2$ sobre a oxidação da LDL isolada e de soro humano, induzida por cobre (de Bem et al., 2008).

Outro importante resultado apresentado no manuscrito 2, foi a capacidade do DM e DC em prevenir a perda da fluorescência do triptofano na LDL humana oxidada por cobre. A LDL nativa apresenta um espectro de fluorescência com uma banda central em aproximadamente 332 nm, a qual é decorrente do resíduo de triptofano presente na apo B-100. A perda desta fluorescência é um marcador da oxidação da proteína na camada externa da LDL (Reyftmann et al., 1990). Os efeitos do DM e DC indicam que além de prevenir a oxidação das moléculas lipídicas da LDL, estes compostos também diminuem a oxidação da porção proteica da LDL humana, apontando para um mecanismo adicional que pode contribuir para a inibição do processo aterogênico. Este efeito é semelhante ao relatado anteriormente para o $(PhSe)_2$ (de Bem et al., 2008), confirmando a similaridade de efeitos dos diaril disselenetos substituídos em relação ao $(PhSe)_2$.

Para esclarecer os mecanismos pelos quais os compostos DM e DC reduzem a oxidação de LDL, investigamos sua capacidade em oxidar tióis através de estudo espectroscópico. Ambos os compostos consumiram GSH em sistema *in vitro*, e mostraram uma interação química direta com GSH, resultando na formação de um intermediário químico (provavelmente selenol), cuja estabilidade foi afetada pela presença de H_2O_2. Este selenol produzido pode decompor peróxidos usando GSH ou outros tióis. De acordo com estas observações, o DC apresentou uma atividade enzimática mimética à GPx dependente da concentração, o que pode explicar, em parte, seu efeito antioxidante, entretanto, este efeito não foi observado com o composto DM, sugerindo que um mecanismo adicional seja necessário para explicar o seu efeito protetor contra a oxidação induzida da LDL.

Podemos observar que o DM e DC podem ser reduzidos pela TrxR, demonstrando que estes compostos tem uma atividade como substrato para TrxR mamífera. Estes resultados estão de acordo com estudos prévios realizados por Zhao e Holmgren (2002) que demonstraram que o ebselen e seus disselenetos são bons substratos para a TrxR humana. Além disso, este mesmo grupo verificou que a atividade antioxidante destes compostos, está relacionada com sua redução pela TrxR e tioredoxina (Trx) produzindo ebselen-selenol e que a via da TrxR pode ser mais eficiente para decompor H_2O_2 que a via mimética a GPx (Zhao et al., 2002). Desta forma, a atuação dos diaril disselenetos substituídos como substrato para TrxR pode explicar também sua atividade antioxidante e propriedades farmacológicas.

Finalmente, os resultados obtidos neste trabalho de mestrado contribuem para a fundamentação das bases toxicológicas e

farmacológicas da aplicabilidade de compostos orgânicos de selênio como agentes antiaterogênicos.

6. CONCLUSÕES

Os resultados obtidos neste trabalho indicam que:

A exposição de coelhos adultos New Zealand ao tratamento agudo por 5 dias via intra-peritoneal com $(PhSe)_2$ (5 - 500 μmol/kg), demonstrou que a toxicidade é dependente da dose utilizada.

Os compostos orgânicos de selênios diaril disselenetos substituídos DM e DC, preveniram a oxidação de LDL isolada e soro humano de maneira dose-dependente, e esta atividade pode ser atribuída a suas propriedades antioxidantes relacionadas aos sistemas enzimáticos GPx e TrxR.

Em cultivo de macrófagos J774, os compostos DM, DC e $(PhSe)_2$ foram capazes de prevenir a formação de células espumosas induzidas por LDLox.

O pré-tratamento com $(PhSe)_2$ reduziu a formação intracelular de de ROS, o dano mitocondrial, a produção de MMP-9 e a ativação do fator de transcrição NF-κB em macrófagos J774 expostos a LDLox.

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