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EFEITO DA HIPERCOLESTEROLEMIA SOBRE A FUNÇÃO COGNITIVA E A RELAÇÃO COM A FUNÇÃO MITOCONDRIAL E ESTRESSE OXIDATIVO EM CÓRTEX CEREBRAL DE CAMUNDONGOS DEFICIENTES PARA O RECEPTOR DE LDL

Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal de Santa Catarina como requisito parcial para obtenção do título de Mestre em Bioquímica.
Orientadora: Profª. Dra. Andreza Fabro de Bem
Co-orientador: Prof°. Dr. Marcelo Farina

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"Efeito da hipercolesterolemia sobre a função cognitiva e a relação com a função mitocondrial e estresse oxidativo em córtex cerebral de camundongos deficientes para o receptor de LDL"

por

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RESUMO

Nos últimos anos, evidências epidemiológicas, clínicas e experimentais indicam a associação entre a hipercolesterolemia e prejuízos cognitivos, como a demência associada à Doença de Alzheimer. Por outro lado, sabe-se que há uma importante participação da disfunção mitocondrial e estresse oxidativo na patogênese das doenças neurodegenerativas e da hipercolesterolemia. Neste contexto, nosso objetivo foi primeiramente avaliar a função cognitiva de camundongos deficientes para o receptor da lipoproteína de baixa densidade ($LDLr^{-/-}$), um modelo de hipercolesterolemia; e sua relação com a função mitocondrial e antioxidante. Para este fim, camundongos controle C57Bl/6 e $LDLr^{-/-}$ foram expostos à uma dieta padrão ou hipercolesterolêmica durante 30 dias e então submetidos ao teste de localização de objeto. Os camundongos $LDLr^{-/-}$ apresentaram prejuízo de aprendizado e memória espacial independentemente da dieta adotada. Além disso, os camundongos $LDLr^{-/-}$ expostos à dieta hipercolesterolêmica apresentaram uma significativa diminuição na atividade dos complexos mitocondriais I e II no córtex cerebral, a qual foi negativamente correlacionada com os respectivos níveis de colesterol plasmático. Este evento foi acompanhado pela diminuição nos níveis de glutationa (GSH), aumento na lipoperoxidação e desequilíbrio na atividade das enzimas integrantes do sistema antioxidante dependente da GSH, glutationa peroxidase (GPx) e glutationa redutase (GR) no córtex cerebral. Estes resultados indicam uma significativa relação entre a hipercolesterolemia, prejuízo cognitivo, e disfunção mitocondrial/estresse oxidativo no córtex cerebral. Considerando que o composto $(PhSe)_2$ vem demonstrando importante papel protetor para doenças cardiovasculares associadas a hipercolesterolemia, analisamos o seu possível efeito neuroprotetor frente ao estresse oxidativo induzido pela hipercolesterolemia no córtex cerebral de camundongos $LDLr^{-/-}$ expostos à dieta hipercolesterolêmica. Nossos resultados demonstraram que o tratamento oral com $(PhSe)_2$ (1 mg/kg) durante 30 dias aumentou significativamente os níveis de GSH e diminuiu a lipoperoxidação no córtex cerebral dos animais hipercolesterolêmicos. Este efeito antioxidante possivelmente está relacionado à sua atividade mimética da GPx. Tomados em conjunto, os resultados apontam este modelo animal de hipercolesterolemia como uma abordagem útil para compreender os eventos moleculares envolvidos na patogênese de doenças neurodegenerativas, bem como o papel neuroprotetor do $(PhSe)_2$.

Palavras-chave: demência, camundongos $LDLr^{-/-}$, hipercolesterolemia, prejuízo cognitivo, disfunção mitocondrial, estresse oxidativo, $(PhSe)_2$.

ABSTRACT

In recent years, epidemiological, clinical and experimental evidence have indicated an association between hypercholesterolemia and cognitive impairment, as well as development of dementia such as Alzheimer´s disease. On the other hand, it is known that there is an important involvement of mitochondrial dysfunction and oxidative stress in the pathogenesis of neurodegenerative diseases and hypercholesterolemia. In this context, our objective was to evaluate the cognitive performance in the low density lipoprotein receptor (LDLr)-deficient mice, a model of hypercholesterolemia; and its relationship with mitochondrial and antioxidant functions. Initially, wild type C57Bl/6 or $LDLr^{-/-}$ mice were fed with either standard or cholesterol-enriched diet for a 4-week period and tested for spatial learning and memory in the object location task. $LDLr^{-/-}$ mice displayed spatial learning and memory impairments regardless of diet. Moreover, $LDLr^{-/-}$ mice fed cholesterol-enriched diet presented a significant decrease in the mitochondrial complexes I and II activities in the cerebral cortex, which were negatively correlated with respective blood cholesterol levels. This event was accompanied by decreased in the glutathione (GSH) levels, lipoperoxidation and an imbalance between the peroxide-removing–related enzymes glutathione peroxidase (GPx)/glutathione reductase (GR) activities in the cerebral cortex. These findings indicate a significant relationship between hypercholesterolemia, cognitive impairment, and cortico-cerebral mitochondrial dysfunctional/ oxidative stress. Moreover, considering that the organoselenium compound $(PhSe)_2$ has shown important protective role in cardiovascular disease associated with hypercholesterolemia, we evaluated the potential neuroprotective effect against hypercholesterolemia-induces oxidative stress in the cerebral cortex of $LDLr^{-/-}$ mice fed with cholesterol-enriched diet. Our results demonstrated that the oral treatment with $(PhSe)_2$ 1 mg/kg during 30 days significantly increased the GSH levels and decreased the lipoperoxidation in the cerebral cortex of hypercholesterolemic mice. Taken together, the results suggest this mouse model of hypercholesterolemia a useful approach to comprehend the molecular events mediating neurodegenerative diseases pathogenesis. In addition, our results suggested the neuroprotective role of $(PhSe)_2$.

Key words: dementia, $LDLr^{-/-}$ mice, hypercholesterolemia, cognitive impairment, mitochondrial dysfunction, oxidative stress, $(PhSe)_2$.

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LISTA DE ABREVIATURAS

$^{\bullet}NO$	Óxido nítrico
$^{\bullet}OH$	Radical hidroxil
$(PhSe)_2$	Disseleneto de difenila
ApoE	Apolipoproteína E
$ApoE^{-/-}$	Camundongo deficiente para apolipoproteína E
ATP	Adenosina trifosfato
DA	Doença de Alzheimer
BHE	Barreira hematoencefálica
CR	Cadeia respiratória
eNOS	Óxido nítrico sintase endotelial
ERO	Espécies reativas de oxigênio
$FADH_2$	Dinucleotídeo de flavina adenina reduzida
GPx	Glutationa Peroxidase
GR	Glutationa Redutase
GSH	Glutationa reduzida
H_2O_2	Peróxido de hidrogênio
IDL	Lipoproteína de densidade intermediária
LDL	Lipoproteína de baixa densidade
$LDLr^{-/-}$	Camundongo deficiente para o receptor de LDL
MDA	Malondialdeído
NADH	Dinucleotídeo de nicotinamida adenina reduzida
NADPH	Dinucleotídeo fosfato de nicotinamida adenina reduzida
Nrf-2	Fator nuclear eritróide-2
$O_2^{\bullet-}$	Ânion superóxido
$ONOO^-$	Peroxinitrito

Se-OH	Selenol
SNC	Sistema nervoso central
SOD	Superóxido dismutase
TBARS	Substâncias reativas ao acido tiobarbitúrico
Vad	Demência Vascular
VLDL	Lipoproteína de densidade muito baixa

SUMÁRIO

1 INTRODUÇÃO

1.1 HIPERCOLESTEROLEMIA

O colesterol é um constituinte lipídico importante de todas as membranas celulares e bainhas de mielina, desempenhando papel crucial para integridade sináptica e função neuronal (Simons e Ikonen, 2000; Pfrieger, 2003). O colesterol também funciona como o substrato para síntese de ácidos biliares no fígado, e como o precursor de hormônios esteróides em tecidos endócrinos (Liscum e Underwood, 1995). Entretanto, apesar do seu papel em processos bioquímicos essenciais e no suporte estrutural de membranas, fortes evidências clínicas e experimentais suportam a estreita ligação entre níveis plasmáticos elevados de colesterol e o desenvolvimento e progressão de lesões ateroscleróticas (Brown e Goldstein, 1986; Stokes et al., 1987).

A hipercolesterolemia, particularmente os níveis elevados de colesterol presente na lipoproteína de baixa densidade (LDL), é um fator de risco bem estabelecido para incidência de aterosclerose e suas complicações patológicas (Ross e Harker, 1976; Libby, 2002). A associação entre a hipercolesterolemia e a aterosclerose foi estabelecida, em parte, com base em estudos sobre a hipercolesterolemia familiar, uma doença autossômica dominante, que têm como característica principal mutações no gene do receptor de LDL. 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 à deficiência no gene deste receptor a causa da hipercolesterolemia familiar. Heterozigotos para esta doença manifestam uma elevação de duas a cinco vezes nos níveis plasmáticos de LDL-colesterol, em função de um prejuízo funcional do receptor de LDL, resultando em uma deficiência na depuração sanguínea desta lipoproteína. Indivíduos homozigotos apresentam um aumento de quatro a seis vezes no colesterol plasmático e desenvolvem aterosclerose precocemente (Gotto e Grundy, 1999; Steinberg, 2002) (Figura 1).

Diferentes espécies animais têm sido utilizadas como modelos de hipercolesterolemia e aterosclerose, entre elas destacam-se coelhos e camundongos. A primeira evidência de aterosclerose experimental foi descrita em 1908 por Ignatowski, através da observação de lesões ateroscleróticas em coelhos alimentados com uma dieta rica em proteína animal. Apesar de não desenvolverem aterosclerose espontaneamente, os coelhos são ainda bastante utilizados como modelos de

hipercolesterolemia, por serem altamente responsivos à ingestão de colesterol e desenvolverem lesões em curto prazo. Porém, nesta espécie, os níveis plasmáticos de colesterol são muito elevados e as lesões formadas apresentam conteúdo lipídico e inflamatório muito superior às lesões observadas em humanos (Jawien et al., 2004).

Por sua vez, os camundongos tornaram-se uma espécie extensamente empregada nas investigações biomédicas, e sua utilização para o estudo do processo aterosclerótico apresenta inúmeras vantagens em relação aos demais modelos animais (Daugherty, 2002). No entanto, não há conhecimento de que camundongos do tipo selvagem desenvolvam aterosclerose de maneira espontânea. Além disso, muitas linhagens destes animais respondem fracamente à dieta rica em colesterol e desenvolvem apenas estrias gordurosas no arco aórtico (Paigen et al., 1990). Neste contexto, destaca-se o surgimento de camundongos geneticamente modificados, os quais solucionaram muitos problemas relacionados ao estudo experimental da aterosclerose (Ohashi et al., 2004). Os camundongos com deleção gênica de apolipoproteína E (apoE$^{-/-}$) ou do receptor de LDL (LDLr$^{-/-}$) são amplamente empregados na atualidade, e ambos fornecem uma ferramenta prática para o estudo da hipercolesterolemia e suas consequências.

Os camundongos LDLr$^{-/-}$, desenvolvidos em 1993 por Ishibashi e colaboradores, são um modelo de hipercolesterolemia familiar humana. Estes animais apresentam hipercolesterolemia, caracterizada por níveis moderados de LDL-colesterol, mesmo quando submetidos a uma dieta padrão, podendo desenvolver lesões ateroscleróticas a longo prazo. Ademais, são muito susceptíveis a modificações dietéticas quando alimentados com dieta rica em colesterol, tornando-se severamente hipercolesterolêmicos, com o desenvolvimento de intensa aterosclerose aórtica e xantomas subcutâneos (Ishibashi et al., 1993; Kowala et al., 2000; Daugherty, 2002). Algumas características deste modelo animal podem trazer vantagens para sua utilização, tais como: (1) a semelhança à condição humana de hipercolesterolemia familiar, causada por mutações no gene para o receptor de LDL; (2) o perfil de lipoproteínas plasmáticas, que se assemelha ao de humanos, estando a maior parte do colesterol confinado na fração LDL; e (3) o grau de dislipidemia intermediário, desenvolvendo lesões menos avançadas do que os camundongos apoE$^{-/-}$ (Zadelaar et al., 2007).

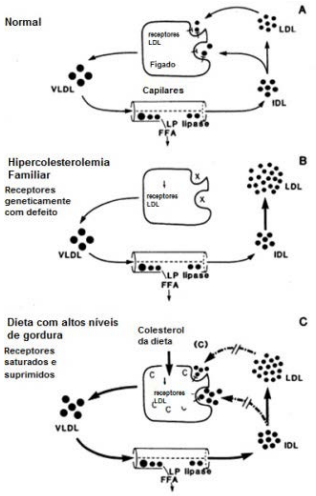

Figura 1. Modelo esquemático do mecanismo pelo qual os receptores de lipoproteína de baixa densidade (LDL) no fígado controlam a produção e o catabolismo de LDL plasmática. (A) em indivíduos normais, (B) em indivíduos com hipercolesterolemia familiar, e (C) em indivíduos consumindo uma dieta rica em gorduras saturadas e colesterol. VLDL, lipoproteína de muito baixa densidade; IDL, lipoproteína de densidade intermediária; LP lipase, lipase lipoprotéica; FFA; ácidos graxos livres (Adaptado de Brown e Goldstein, 1986)

1.1.1 Hipercolesterolemia e Doenças Neurodegenerativas

Evidências epidemiológicas e neuroquímicas suportam a associação entre alterações no metabolismo do colesterol e o aparecimento de prejuízos cognitivos, bem como de demência (Panza et al., 2006). Todavia, os mecanismos moleculares pelos quais os níveis de colesterol contribuem para patofisiologia de doenças neurodegenerativas ainda não estão totalmente elucidados (Wolozin, 2004; Panza et al., 2006; Duron e Hanon, 2008).

A demência é um dos distúrbios neurológicos com maior relevância em idosos e um dos principais problemas de saúde pública. Nas últimas décadas a expectativa de vida está aumentando. Em decorrência deste envelhecimento populacional estima-se que a prevalência global de demência quadruplique de 24,3 milhões em 2001 para 81,1 milhões de indivíduos afetados em 2040 (Ferri et al., 2005). Nos países ocidentais, as formas mais comuns de demência são a

Doença de Alzheimer (DA) e a Demência Vascular (Vad), com respectivas frequências de 70% e 15% entre todas as demências (Whitehouse et al., 1997).

Estudos epidemiológicos longitudinais com pacientes idosos indicam que indivíduos hipercolesterolêmicos durante a idade adulta são mais susceptíveis a desenvolver DA e Vad em idades avançadas (Kivipelto et al., 2001, 2002, 2005), e que quando recebem tratamento para doenças cardiovasculares como terapias que reduzem os níveis de colesterol (por exemplo as estatinas), apresentam uma menor prevalência de demência e diminuição da deterioração cognitiva (Sparks et al., 2005). Todavia, a relação entre hipercolesterolemia e funções cognitivas é mais complexa que uma simples relação linear.

Muitos dos fatores de risco vasculares clássicos, incluindo hipertensão, diabetes mellitus, e em particular a hipercolesterolemia, também são considerados fatores de risco para doenças neurodegenerativas, principalmente a DA (Casserly eTopol, 2004; Shobab et al., 2005; Beach et al., 2007). O grau de comprometimento destes fatores para o desenvolvimento das doenças neurodegenerativas ainda pode ser influenciado por fatores genéticos, como a presença do alelo ε4 da ApoE, que tem um papel bem estabelecido na doença arterial coronariana e no desenvolvimento de aterosclerose, mas também é fortemente associado com a DA (Davignon et al., 1988; Eichner et al., 2002).

A descoberta da disfunção neurovascular como parte integrante da DA, levou a um maior interesse ao que se tornou conhecido como “hipótese vascular” das doenças neurodegenerativas (Kolovou et al., 2002; Luthra et al., 2002). A existência de um componente vascular que reduz a perfusão cerebral tem sido proposta como um possível mecanismo envolvido na patofisiologia da DA (Humpel, 2011). Estudos epidemiológicos evidenciaram que há um aumento no risco de desenvolvimento de DA em indivíduos com aterosclerose grave (Hofman et al., 1997). Além disso, estudos demonstraram a relação entre doença arterial coronariana e a severidade de neuropatologias (Beeri et al., 2006). Esse envolvimento da aterosclerose poderia ser explicado pela ocorrência de doenças cerebrovasculares, como o acidente vascular cerebral, doença de pequenos vasos cerebrais, ou ainda ser resultante da hipoperfusão cerebral, situações estas relacionadas com o comprometimento da barreira hematoencefálica (BHE) (de la Torre, 2002, 2004). Sabe-se que a disfunção de células endoteliais que compõem a BHE também tem sido correlacionada com a severidade da DA (Dede et al., 2007).

Corroborando com estas evidências clínicas e epidemiológicas, estudos experimentais em animais utilizando primeiramente coelhos e em seguida camundongos transgênicos modelos de DA alimentados com uma dieta rica em colesterol, demonstraram que a hipercolesterolemia intensifica a patogênese da DA (Sparks et al., 1994; Refolo et al., 2000). De fato, Ullrich e colaboradores (2010) demonstraram que ratos hipercolesterolêmicos apresentam prejuízo de memória e desenvolvem uma patologia com características semelhantes às encontradas na DA. Recentemente, Ramirez e colaboradores (2011) utilizando camundongos ApoB100/LDLr$^{-/-}$ sugeriram a utilização da hipercolesterolemia como um biomarcador chave para o monitoramento de prejuízo cognitivo leve, e propuseram o uso destes camundongos transgênicos como um modelo de declínio cognitivo.

1.2 MITOCÔNDRIA

A mitocôndria é a mais complexa e dinâmica organela celular, indispensável para muitos processos biossintéticos e responsável pela maior produção líquida de energia (DiMauro e Schon, 2003; Spees et al., 2006). Esta organela tem uma estrutura basicamente membranosa, sendo seu envoltório formado por duas membranas, a membrana externa e a membrana interna. A membrana externa é mais permeável que a membrana interna, e entre ambas é determinado um espaço denominado intermembranoso onde ocorrem reações essenciais ao metabolismo celular. A membrana interna é formada por pregas que se expandem no espaço intramitocondrial (matriz mitocondrial) denominadas cristas mitocondriais (Lehninger et al., 2004; Rousset et al., 2004).

A produção energética mitocondrial é resultante de dois processos metabólicos estreitamente coordenados, o ciclo de Krebs e a cadeia transportadora de elétrons ou cadeia respiratória (CR). O ciclo de Krebs tem como função principal produzir as coenzimas NADH e $FADH_2$ para que sejam inseridas na CR; todas as enzimas envolvidas neste ciclo oxidativo se encontram localizadas na matriz mitocondrial (Di Donato, 2000). A mitocôndria produz mais de 90% da energia celular via fosforilação oxidativa (Chance et al., 1979); e essa produção bioenergética assume importância máxima no sistema nervoso central (SNC), devido à limitada capacidade glicolítica das células neuronais e sua alta dependência da fosforilação oxidativa (Moreira et al., 2010).

A fosforilação oxidativa é um processo que requer a ação orquestrada de cinco complexos enzimáticos distribuídos de forma

especial na membrana mitocondrial interna, os quais constituem a CR (Figura 2) (Alberts et al., 2002). Cada um destes complexos é constituído de várias subunidades protéicas que se encontram associados com uma variedade de grupamentos prostéticos com potencial de oxi-redução sucessivamente maiores (Lehninger et al., 2004). Durante este processo, NADH e/ou $FADH_2$ são oxidados provocando a transferência de seus elétrons para o complexo I (NADH desidrogenase) ou complexo II (Succinato desidrogenase), e então para o complexo III (Citocromo c redutase) via coenzima Q (CoQ). O complexo III transfere os elétrons da CoQ para o carreador móvel de elétrons, o citocromo c. O complexo IV (Citocromo c oxidase) é o complexo terminal da cadeia transportadora de elétrons, transferindo os elétrons a partir do ferrocitocromo c para o oxigênio molecular, o aceptor final de elétrons que através da adição de quatro elétrons é reduzido a H_2O (Barrientos et al., 2002; de Moura et al., 2010).

Concomitante com a transferência de elétrons entre os complexos I, III e IV ocorre à translocação de prótons através da membrana mitocondrial interna e a síntese endergônica de ATP, empregando como força motriz a energia armazenada como gradiente eletroquímico de prótons (Babcock e Wikstrom, 1992). Este gradiente determina uma polarização da membrana mitocondrial interna (potencial de membrana mitocondrial); que pode ser revertida pelo fluxo desses prótons através do complexo V (F_1-F_0 ATP sintase) (Figura 2). O fluxo de prótons leva à condensação do ADP e de fosfato inorgânico em ATP, que; por sua vez, é a moeda molecular de transferência de energia em uma célula (Saraste, 1990; Wallace, 1999).

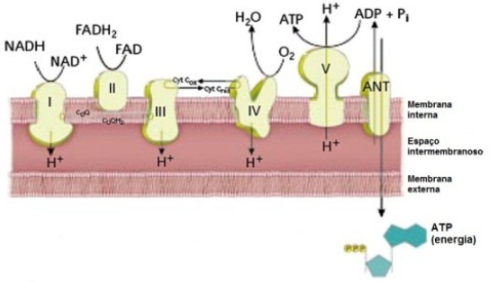

Figura 2. Cadeia respiratória (CR) mitocondrial. . Esquema da CR incorporada na membrana interna da mitocôndria. É composto por cinco complexos: complexo I, NADH desidrogenase; complexo II, Succinato desidrogenase; complexo III, Citocromo c redutase, complexo IV, Citocromo c

oxidase; e complexo V, F_1-F_0 ATP sintase. ANT é o transportador de nucleotídeos adenina (Adaptado de Pieczenik e Neustadt, 2007)

Na maioria dos tipos celulares, as mitocôndrias representam as principais fontes de espécies reativas de oxigênio (ERO), mesmo na presença de mecanismos de defesa antioxidantes; a Figura 3 ilustra as principais fontes celulares de ERO. Os ERO são continuamente gerados pela CR, isto porque uma consequência da fosforilação oxidativa é a geração de elétrons desemparelhados, principalmente no complexo I e em menor grau no complexo III. A interação destes elétrons com oxigênio molecular resulta na formação do ânion superóxido ($O_2^{-\bullet}$), ERO altamente reativa que é rapidamente interconvertida em outras espécies radicais e mediador de reações oxidativas em cadeia. Dismutação de $O_2^{-\bullet}$ produz peróxido de hidrogênio (H_2O_2), o qual pode ser completamente reduzido a H_2O ou parcialmente reduzido ao radical hidroxil ($OH^{\bullet}$), um dos mais fortes oxidantes da natureza (Turrens, 2003; Duchen, 2004).

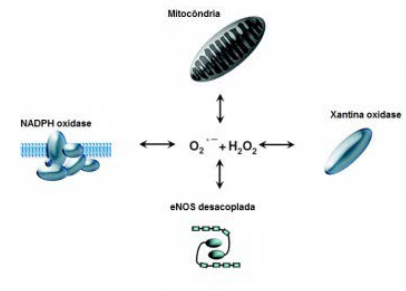

Figura 3. Principais fontes celulares de geração de espécies reativas de oxigênio (ERO). eNOS, Óxido nítrico sintase endotelial; $O_2^{-\bullet}$, ânion superóxido; H_2O_2, peróxido de hidrogênio (Adaptado de Ray e Shah, 2005)

1.2.1. Mitocôndria e Doenças Neurodegenerativas

A cognição humana depende, entre outras coisas, da habilidade do SNC em sustentar altas taxas de produção energética continuamente ao longo da vida mantendo um ambiente interno eletroquímico saudável (Dikalov, 2011). Por outro lado, a disfunção mitocondrial e altas concentrações de ERO têm papel importante na patogênese das mais

comuns doenças neurodegenerativas, incluindo a DA e as doenças cerebrovasculares (de Moura et al., 2010). Durante o desenvolvimento e progressão destas doenças neurodegenerativas, devido à hipoperfusão vascular cerebral, as mitocôndrias são danificadas tornando-se incapazes de manter a demanda energética da célula (Hirai et al., 2001), o que resulta em aumento na produção de ERO, interrupção da fosforilação oxidativa; e por fim diminuição dos níveis de ATP, necessários para homeostase energética normal (Schulz et al., 1997). Estes processos estão intimamente associados com morte e degeneração neuronal; danos, os quais podem levar a graves distúrbios neurológicos tais como prejuízo cognitivo e desenvolvimento de demência (Aliev et al., 2004).

A correlação positiva entre as doenças neurodegenerativas e as doenças cardiovasculares identifica a hipoperfusão vascular cerebral como fator desencadeador das doenças neurodegenerativas (de la Torre, 2008). Estudos experimentais em animais e culturas de células demonstram que as lesões vasculares induzidas pelo baixo fluxo sanguíneo cerebral causam maior geração de ERO mitocondrial, resultando em dano oxidativo e morte às células neuronais. Além disso, há crescentes evidências que a disfunção mitocondrial e a redução da atividade de algumas enzimas chaves mitocondriais, como os complexos respiratórios estão correlacionados com perda neuronal em indivíduos com doenças neurodegenerativas (Aliyev et al., 2005; Aliev 2009).

1.3 ESTRESSE OXIDATIVO E DEFESAS ANTIOXIDANTES

ERO são geradas tanto em resposta a condições fisiológicas como patológicas (Morgan et al., 2007). O estado redox das células é uma consequência de um crítico balanço entre a produção de ERO e as defesas antioxidantes (Emerit et al., 2004; Halliwell, 2006). A elevação na formação de ERO e/ou o prejuízo dos sistemas de defesas antioxidantes, resulta em estresse oxidativo potencialmente citotóxico (Sies, 1997; Droge, 2002; Turrens, 2003). Sob esta condição pró-oxidante, radicais altamente reativos danificam indiscriminadamente proteínas (Stadtman e Levine, 2000), lipídios (Rubbo et al., 1994), polissacarídeos (Kaur e Halliwell, 1994) e DNA (Richter et al., 1988; LeDoux et al., 1999), levando a morte celular.

A reação da ERO com os ácidos graxos poliinsaturados, presentes nas membranas celulares e nas lipoproteínas, inicia um processo em cadeia conhecido como peroxidação lipídica ou lipoperoxidação. As alterações nas membranas celulares devido à lipoperoxidação levam a

transtornos da permeabilidade, alterando o fluxo iônico e o fluxo de outras substâncias, o que resulta na perda da seletividade para entrada e/ou saída de nutrientes e substâncias tóxicas à célula, alterações do DNA, oxidação da LDL e comprometimento dos componentes da matriz extracelular (Barber e Harris, 1994). Os produtos gerados durante as fases do processo de lipoperoxidação também chamados de substâncias reativas ao ácido tiobarbitúrico (TBARS) podem ser avaliados e utilizados como um indicador do estresse oxidativo celular (Niki, 2009).

A produção excessiva de ERO superando os mecanismos de defesas antioxidantes tem sido implicada no desenvolvimento de aterosclerose e doenças cardiovasculares (Harrison et al., 2003). Um excesso de ERO em muitos sistemas celulares, incluindo as células das paredes vasculares e células da circulação sanguínea, é descrito em indivíduos com aterosclerose avançada (Forstermann, 2010). Neste sentido, o aumento do estresse oxidativo está associado com a presença dos fatores de risco cardiovasculares que estão envolvidos na formação das placas ateroscleróticas (Mansego et al., 2011). Portanto, o estresse oxidativo é um elo entre os fatores de risco cardiovasculares e as doenças vasculares. Nesse sentido, um grande número de evidências clínicas e experimentais propõe a associação entre o principal fator de risco cardiovascular: a hipercolesterolemia, e o estresse oxidativo (Csont et al., 2007; Hulsmans e Holvoet, 2010; Drummond et al., 2011).

O SNC é especialmente susceptível ao estresse oxidativo, esta susceptibilidade é devido ao grande conteúdo lipídico altamente peroxidável das bainhas de mielina e à alta taxa de metabolismo oxidativo cerebral (Halliwell, 1992, 2001, 2006). Neste contexto, o estresse oxidativo tem sido implicado em muitos mecanismos de neurotoxicidade, desempenhando papel importante em diferentes patologias neurodegenerativas. Elevados níveis de dano oxidativo ao DNA são encontrados em tecidos de pacientes com prejuízos cognitivos (Mao e Reddy, 2011), bem como tecidos post-mortem apresentam níveis aumentados de estresse oxidativo celular e perda sináptica em determinadas regiões cerebrais relacionadas com doenças neurodegenerativas (Hensley et al., 1995; Halliwell, 2006).

Em condições fisiológicas os níveis basais de ERO gerados são rapidamente eliminados por sistemas antioxidantes: superóxido dismutase (SODs), catalase, glutationa peroxidases (GPxs), glutationa redutase (GR), glutationa (GSH), vitamina E e C, tiol peroxidases entre outros (Dirnagl et al., 2003; Rhee et al., 2005). A GSH desempenha papel fundamental na proteção celular contra o estresse oxidativo, principalmente no SNC, exercendo suas funções através de mecanismos

diversos (Figura 4 apresenta as principais funções da GSH). Esta molécula efetivamente sequestra ERO, antes que estes iniciem as reações. Além disso, o sistema antioxidante dependente da GSH é o principal sistema antioxidante endógeno, desempenhando assim, papel central na proteção celular contra oxidantes. Neste ciclo (Figura 5), a GSH serve como um cofator essencial, trabalhando como doador de equivalentes redutores para a detoxificação de H_2O_2 ou outros peróxidos e produtos de peroxidação catalisada pela GPx. Neste processo, H_2O_2 é reduzido a H_2O pela reação da GPx com a GSH, a qual é oxidada a GSH dissulfeto (GSSG). GSSG é então reduzida novamente a GSH, pela ação da flavoenzima GR à custa da oxidação do NADPH (Dringen et al., 1999; Maher, 2005; Aoyama et al., 2008).

A mitocôndria possui 10% da massa celular, entretanto seu conteúdo de GSH é comparável ao restante do conteúdo presente na célula. O transporte do GSH citosólico para a matriz mitocondrial é o único determinante do conteúdo de GSH nesta organela (Lash, 2006). A depleção e/ou oxidação de GSH mitocondrial está associada a diversos estados patológicos, tais como, doenças cardiovasculares e distúrbios neurodegenerativos; sendo o estado redox da GSH crítico para que haja uma adequada função mitocondrial, uma vez que a GSH preserva a integridade de proteínas e lipídeos mitocondriais e controla a propagação de ERO mitocondrial (Circu e Aw, 2008).

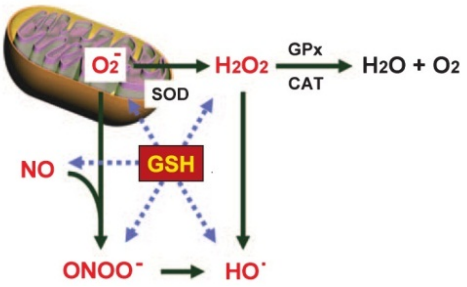

Figura 4. Principais mecanismos de defesa da glutationa (GSH) contra o estresse oxidativo. GSH é o principal antioxidante no SNC, o qual não enzimaticamente reage com ânion superóxido ($O_2^{\bullet-}$), óxido nítrico ($^{\bullet}NO$), radical hidroxil ($OH^{\bullet}$), e peroxinitrito ($ONOO^-$). GSH também reage com peróxido de hidrogênio (H_2O_2) ou outros peróxidos através da reação catalisada pela glutationa peroxidase (GPx)/ catalase (Adaptado de Aoyama et al., 2008)

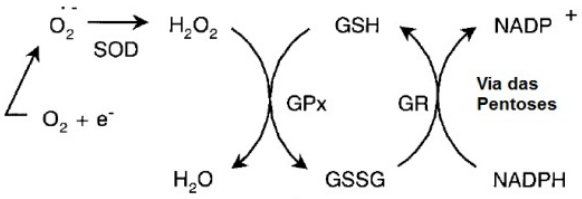

Figura 5. Sistema antioxidante dependente da glutationa (GSH). O ânion superóxido ($O_2^{\cdot -}$) sob a ação da superóxido dismutase (SOD) é convertido em peróxido de hidrogênio (H_2O_2), o qual é reduzido à água pela ação do sistema antioxidante dependente da GSH. GPx, Glutationa peroxidase; GR, Glutationa redutase; GSSG, Glutationa oxidada (Adaptado de O'Donovan e Fernandes, 2000)

As GPxs compreendem uma família de enzimas bem conhecidas por serem importantes componentes do sistema de defesa antioxidante humano. Nos seres humanos, foram descritas até o presente momento cinco tipos de GPxs: citosólica (GPx-1), gastrointestinal (GPx-2), plasmática (GPx-3), fosfolipídica (GPx-4), e a GPx do epitélio olfativo e tecido embriônico específica (GPx-6) (Kryukov et al., 2003). As GPx1-3 catalisam a redução de H_2O_2 e hidroperóxidos orgânicos, enquanto que a GPx-4 pode reduzir diretamente os hidroperóxidos de fosfolipídios e colesterol (Ursini et al., 1999).

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 está associada ao desenvolvimento de lesões mais severas (Lapenna et al., 1998). Em camundongos, a deficiência de 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 apoE$^{-/-}$ (Torzewski et al., 2007). De particular interesse, um recente estudo demonstrou que a atividade da GPx, que é crucial para a detoxificação do H_2O_2 no SNC, está diminuída em pacientes com DA quando comparados com indivíduos normais (Dringen et al., 2005; Kharrazi et al., 2008).

1.4 DISFUNÇÃO MITOCONDRIAL E HIPERCOLESTEROLEMIA

Alterações na função mitocondrial estão intimamente ligadas com doenças metabólicas. Recentes estudos propõem a relação entre hipercolesterolemia e alterações nas funções mitocondriais, tais como metabolismo energético, transporte de íons e estado redox (Vercesi et al., 2007). Logo, um dos possíveis mecanismos pelo qual a hipercolesterolemia induz estresse oxidativo pode estar relacionado à perturbação da função mitocondrial (McCommis et al., 2011).

Oliveira e colaboradores (2005) demonstraram que as mitocôndrias de tecidos isolados (cérebro, fígado e rim) de camundongos LDLr$^{-/-}$ hipercolesterolêmicos produzem taxas mais elevadas de ERO que mitocôndrias de controles. Em contraste as mitocôndrias dos controles, as mitocôndrias dos camundongos hipercolesterolêmicos não foram capazes de sustentar NADPH no estado reduzido. Este baixo conteúdo de nucleotídeos reduzidos nos hepatócitos dos camundongos LDLr$^{-/-}$ pode ser resultado da alta taxa de lipogênese, uma vez que estas células são deficientes em captar o colesterol exógeno. Os processos de lipogênese/ esteroidogênese consomem grande quantidade de equivalentes redutores do NADPH, uma vez que a biossíntese de um mol de colesterol oxida 24 mols de NADPH. De fato, a taxa de secreção hepática de triglicerídeos e colesterol *in vivo* foram duas vezes maior nos camundongos LDLr$^{-/-}$ que em camundongos controles. Além disso, a síntese *de novo* de colesterol e de outros lipídeos foi maior no fígado de camundongos LDLr$^{-/-}$ (Oliveira et al., 2005).

Uma das principais fontes de NADPH mitocondrial em animais é a isocitrato desidrogenase. Interessantemente, níveis endógenos mitocondriais de isocitrato e outros intermediários do ciclo de Krebs estão diminuídos no fígado de camundongos LDLr$^{-/-}$ e a suplementação das mitocôndrias destes camundongos com isocitrato efetivamente reverteu a falta de NADPH e o aumento de liberação de ERO observados; bem como aumentou o consumo de oxigênio nestas mitocôndrias. Ademais, a suplementação dos camundongos LDLr$^{-/-}$ *in vivo* com citrato, mostrou uma melhora na capacidade destes animais sustentarem o estado reduzido do NADPH, parcialmente revertendo a disfunção mitocondrial do fígado destes camundongos. Portanto, o estresse oxidativo em camundongos LDLr$^{-/-}$ hipercolesterolêmicos é, em parte, resultado da depleção dos substratos relacionados ao NADPH e de quantidades insuficientes de equivalentes redutores para

reconstituir os sistemas enzimáticos antioxidantes mitocondriais (Paim et al., 2008).

Este desequilibro redox mediado pela mitocôndria pode ser uma importante etapa na patogênese de muitas doenças que cursam com aumento de lipogênese e hipercolesterolemia, como a aterosclerose. O defeito no receptor de LDL leva a dois efeitos pró-aterogênicos: níveis aumentados de substratos oxidáveis extracelulares (LDL) e um desequilíbrio nos processos celulares redox. O ultimo fenômeno é responsável por estresse oxidativo local que desencadeia a oxidação de lipoproteínas, o que por sua vez induz dano mitocondrial. O ciclo vicioso resultante leva a morte celular e progresso da aterogênese (Figura 6) (Vindis et al., 2005; Zmijewski et al., 2005; Vercesi et al., 2007).

Neste sentido, acreditamos que a disfunção mitocondrial e o consequente estresse oxidativo desencadeados pela hipercolesterolemia possam desempenhar importante papel nos prejuízos cognitivos e processos neurodegenerativos.

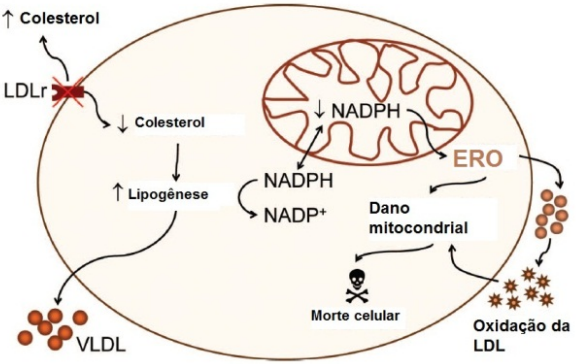

Figura 6: Liberação de espécies reativas de oxigênio (ERO) mitocondrial é aumentada em camundongos deficientes para o receptor de lipoproteína de baixa densidade (LDLr$^{-/-}$). A deficiência dos receptores de LDL resulta em deficiência de transporte de colesterol para dentro da célula, estimulando a lipogênese intracelular e secreção de lipoproteína de muito baixa densidade (VLDL), aumentando os níveis plasmáticos de lipídeos. NADPH é utilizado para a lipogênese, resultando em diminuição da razão de NADPH/NADP$^+$ citoplasmática e mitocondrial. Como muitos dos sistemas de remoção de ERO dependem de NADPH como uma fonte redox, ERO mitocondrial ficam

acumuladas e são liberados em níveis elevados. A produção aumentada de ERO nestas condições pode contribuir para dano oxidativo tecidual, oxidação da LDL e aterosclerose (Adaptado de Vercesi et al., 2007)

1.4 COMPOSTOS ORGÂNICOS DE SELÊNIO

O selênio é um elemento traço essencial, componente estrutural de enzimas com atividades antioxidantes, particularmente das isoformas da GPx (Flohe et al., 1973). Estas enzimas têm importantes papéis na defesa celular, protegendo contra processos oxidativos pela detoxificação de hidroperóxidos de hidrogênio ou lipídicos (Sies e Arteel, 2000; Klotz e Sies, 2003). Diferentes classes de compostos orgânicos de selênio exibem atividade mimética da GPx e decompõem H_2O_2 e hidroperóxidos orgânicos utilizando GSH ou outros tióis como doadores de hidrogênio (Wilson et al., 1989; Nogueira et al., 2004). A partir dos anos 80, o interesse em bioquímica, farmacologia e toxicologia de organoselênios aumentou de maneira significativa devido à variedade de atividades biológicas desempenhadas por esta classe de compostos (Nogueira e Rocha, 2011).

O primeiro exemplo desta classe de compostos foi o ebselen (Parnham e Kindt, 1984). O ebselen (Figura 7A) (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 GPx 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.

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). De particular importância, ebselen têm demonstrado ser neuroprotetor em estudos pré-clínicos e clínicos e em modelos animais de uma variedade de condições neuropatológicas (Saito et al., 1998; Davalos, 1999; Porciuncula et al., 2001; Centuriao et al., 2005; Yamagata et al., 2008; Yin et al., 2011).

1.4.1 Disseleneto de Difenila

O disseleneto de difenila $(PhSe)_2$ (Figura 7B), 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., 2003a).

O mecanismo catalítico para a detoxificação de peróxidos pelo $(PhSe)_2$ foi proposto. A atividade tiol-peroxidase do $(PhSe)_2$ parece ser cineticamente semelhante a reação catalisada pela enzima. 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 (Figura 7C) (Nogueira e Rocha, 2010).

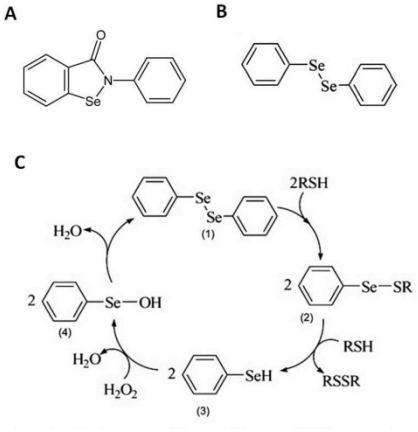

Figura 7. Estrutura química e mecanismo catalítico de organoselênios. Estrutura química do (A) ebselen e do (B) disseleneto de difenila ($(PhSe)_2$). (C) Mecanismo catalítico do $(PhSe)_2$ para a detoxificação de peróxido de hidrogênio (H_2O_2). O $(PhSe)_2$ reage com um grupamento tiol (ex. glutationa (GSH)) originando um selenopersulfato, o qual reage com um segundo equivalente de GSH formando um selenol (Se-H). Finalmente, este selenol reduz o H_2O_2 ou peróxidos lipídicos liberando uma molécula de H_2O, originando um ácido selenínico (Se-OH). Este Se-OH, por sua vez, libera uma molécula de H_2O, fechando assim o ciclo catalítico (Adaptado de Nogueira et al., 2004)

Uma das primeiras evidências das propriedades farmacológicas do $(PhSe)_2$ ocorreu em 2003, quando Nogueira e colaboradores demonstraram as atividades anti-inflamatória e antinociceptiva deste composto em roedores (Nogueira et al., 2003b). Desde então, estudos têm relatado o importante papel protetor deste composto em uma variedade de modelos experimentais associados à produção exacerbada de ERO e estresse oxidativo tais como, inflamação, diabetes, neurotoxicidade e hepatotoxicidade (Ghisleni et al., 2003; Burger et al., 2004; Meotti et al., 2004; Borges et al., 2005), além de diversas outras propriedades farmacológicas.

Nosso grupo de pesquisa tem demonstrado que o $(PhSe)_2$ possui importantes propriedades farmacológicas, que o tornam uma molécula interessante no manejo de doenças cardiovasculares. de Bem e colaboradores (2009) demonstraram que o tratamento por via oral com $(PhSe)_2$ reduz a hipercolesterolemia e o estresse oxidativo em coelhos alimentados com uma dieta rica em colesterol. 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 $LDLr^{-/-}$ e diminuir a formação de células espumosas, produção de mediadores inflamatórios e ERO em macrófagos expostos a LDL oxidada (Hort et al., 2011).

A atividade deste composto no SNC tem sido estudada, uma vez que este composto tem característica lipofílica e pode atravessar a BHE, levando ao aumento dos níveis de selênio no cérebro após tratamentos agudos ou crônicos (Jacques-Silva et al., 2001; Maciel et al., 2003). De fato, o $(PhSe)_2$ demonstrou ter atividade neuroprotetora em inúmeros estudos, tais como o de Posser e colaboradores (2008) que demostraram efeitos protetores do $(PhSe)_2$ *in vitro* contra dano oxidativo induzido por H_2O_2 em fatias hipocampais, e o de da Silva e colaboradores (2011), onde o $(PhSe)_2$ reverteu dano oxidativo e disfunção mitocondrial em cérebros de camundongos expostos ao acetaminofeno. Além disso, administração sistêmica de $(PhSe)_2$ em camundongos melhorou propriedades cognitivas, facilitando a memória de longa duração no teste de reconhecimento de objeto (Rosa et al., 2003).

Diante das importantes propriedades farmacológicas descritas para o $(PhSe)_2$, bem como da participação do estresse oxidativo nos processos neurodegenerativos, hipotetizamos que este composto possa ser uma molécula neuroprotetora efetiva contra danos oxidativos cerebrais induzidos pela hipercolesterolemia em camundongos $LDLr^{-/-}$.

2 OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo geral deste trabalho foi investigar os efeitos da hipercolesterolemia sobre parâmetros comportamentais e bioquímicos em córtex cerebral de camundongos $LDLr^{-/-}$. Ademais, avaliamos o potencial efeito neuroprotetor de um composto mimético da GPx, $(PhSe)_2$, neste modelo experimental.

2.2 OBJETIVOS ESPECÍFICOS

- Investigar os efeitos da hipercolesterolemia sobre a função cognitiva avaliada através do teste comportamental localização do objeto em camundongos $LDLr^{-/-}$ alimentados com dieta padrão ou hipercolesterolêmica;
- Investigar os efeitos da hipercolesterolemia sobre a função mitocondrial em córtex cerebral de camundongos $LDLr^{-/-}$ alimentados com dieta padrão ou hipercolesterolêmica;
- Investigar os efeitos da hipercolesterolemia sobre parâmetros de estresse oxidativo em córtex cerebral de camundongos $LDLr^{-/-}$ alimentados com dieta padrão ou hipercolesterolêmica;
- Investigar os efeitos neuroprotetores do composto orgânico de selênio $(PhSe)_2$, contra estresse oxidativo induzido pela hipercolesterolemia em córtex cerebral de camundongos $LDLr^{-/-}$ alimentados com dieta hipercolesterolêmica.

3 JUSTIFICATIVA

Nos próximos anos, devido ao aumento da expectativa de vida e consequente “envelhecimento populacional”, enfrentaremos um grande incremento na prevalência de prejuízos cognitivos bem como de demência associadas à idade tal como a DA. Estudos epidemiológicos demonstram um aumento exponencial na prevalência de demência em indivíduos acima de 70 anos, chegando a 45% em indivíduos com 95 anos ou mais. Desse modo uma melhor compreensão acerca dos mecanismos envolvidos nos processos de neurodegeneração e busca por novos alvos terapêuticos torna-se relevante.

Um elevado número de estudos epidemiológicos e bioquímicos suporta a associação entre os fatores de risco cardiovasculares, particularmente a hipercolesterolemia com a predisposição a estes distúrbios neurológicos. Sabe-se que a hipercolesterolemia está relacionada com aumento do estresse oxidativo, e o principal mecanismo proposto para esse aumento de produção de ERO é a disfunção mitocondrial. Ademais, a disfunção mitocondrial e altas concentrações de ERO têm papel importante na patogênese das mais comuns doenças neurodegenerativas, incluindo a DA e as doenças cerebrovasculares. A relação entre tais eventos e como os mesmos contribuem na patogênese das doenças neurodegenerativas ainda são desconhecidos.

O estudo do eventual potencial neuroprotetor do composto orgânico de selênio $(PhSe)_2$ é de grande importância, tendo em vista que várias propriedades farmacológicas já foram descritas para este composto. Nossos trabalhos anteriores comprovam sua propriedade anti-inflamatória e antioxidante, ação hipocolesterolêmica e sua capacidade de inibir a oxidação de LDL humana isolada in vitro e o estresse oxidativo em coelhos hipercolesterolêmicos, bem como sua capacidade de reduzir as lesões ateroscleróticas em camundongos $LDLr^{-/-}$. Além disso, outros estudos apontarem esta molécula como um efetivo agente neuroprotetor. Baseado nestas evidências, acreditamos na possibilidade futura deste composto orgânico de selênio como eficiente agente neuroprotetor em patologias relacionadas ao estresse oxidativo, em especial as decorrentes da hipercolesterolemia.

4 RESULTADOS

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo e manuscrito 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 manuscrito.

4.1 ARTIGO 1

Positive correlation between elevated plasma cholesterol levels and cognitive impairments in LDL receptor knockout mice: relevance of cortico-cerebral mitochondrial dysfunction and oxidative stress

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Neuroscience 197 (2011) 99–106

POSITIVE CORRELATION BETWEEN ELEVATED PLASMA CHOLESTEROL LEVELS AND COGNITIVE IMPAIRMENTS IN LDL RECEPTOR KNOCKOUT MICE: RELEVANCE OF CORTICO-CEREBRAL MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE STRESS

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Abstract—Convergent epidemiological, clinical, and experimental findings indicate that hypercholesterolemia contributes to the onset of Alzheimer's disease (AD)-like dementia, but the exact underlying mechanisms remains unknown. In this study, we evaluated the cognitive performance of mice submitted to a model of hypercholesterolemia, as well as its relationship with mitochondrial dysfunction and oxidative stress, two key events involved in AD pathogenesis. Wild-type C57bl/6 or low density lipoprotein receptor (LDLr)-deficient mice were fed with either standard or cholesterol-enriched diet for a 4-week period and tested for spatial learning and memory in the object location task. LDLr$^{-/-}$ mice displayed spatial learning and memory impairments regardless of diet. Moreover, LDLr$^{-/-}$ mice fed cholesterol-enriched diet presented a significant decrease in the mitochondrial complexes I and II activities in the cerebral cortex, which were negatively correlated with respective blood cholesterol levels. Additionally, hypercholesterolemic LDLr$^{-/-}$ mice presented a significant decrease in glutathione levels, about 40% increase in the thiobarbituric acid-reactive substances levels, as well as an imbalance between the peroxide-removing–related enzymes glutathione peroxidase/glutathione reductase activities in the cerebral cortex. These findings indicate a significant relationship between hypercholesterolemia, cognitive impairment, and cortico-cerebral mitochondrial dysfunctional/oxidative stress. Because of the involvement of such alterations in AD patients, our data render this mouse model of hypercholesterolemia a useful approach to comprehend the molecular events mediating AD pathogenesis. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: Alzheimer's disease, low density lipoprotein receptor (LDLr)-deficient mice, hypercholesterolemia, cognitive impairment, mitochondrial dysfunctional, oxidative stress.

There is increasing evidence linking abnormal cholesterol metabolism with cognitive impairments, especially in Alzheimer's disease (AD) (Herrmann and Knapp, 2002; Rojo et al., 2006). Cholesterol is essential for building and maintaining cell membranes; nevertheless, hypercholesterolemia is associated with negative health outcomes, especially those related to vascular disease (Stary, 1989; Sharrett et al., 2006).

Clinical studies have indicated that individuals with hypercholesterolemia are more prone to develop AD (Evans et al., 2000; Yaffe et al., 2002), and a previous case-control study showed a protective effect of lipid-lowering agents on the incidence of dementia (Cramer et al., 2008). Furthermore, experimental studies using New Zealand white rabbits (Sparks et al., 2000) and transgenic mouse models of AD (Refolo et al., 2000; Levin-Allerhand et al., 2002) have demonstrated that diet-induced hypercholesterolemia could enhance brain amyloid-beta (Aβ) protein accumulation. However, the exact mechanisms underlying the accumulation of cholesterol increasing the susceptibility to dementia are still not fully understood.

The mitochondria represent a biologically important source and target for reactive oxygen species (ROS), mainly in the CNS (Kowaltowski and Vercesi, 1999; Brookes et al., 2004). The rate of ROS production is modulated by mitochondrial energetic state and is favored by high membrane potential values (Cadenas and Davies, 2000). ROS production is also largely increased in cases of respiratory chain inhibition, as observed in mitochondrial disease, or in experimental and animal models of oxidative phosphorylation (OXPHOS) deficiencies (Leonard and Schapira, 2000; Fontanesi et al., 2009). The CNS is especially susceptible to ROS-induced damage because of its greater oxygen availability and consumption, high levels of membrane polyunsaturated fatty acids susceptible to oxidative damage, its relatively low levels of antioxidant defenses, and high content of redox metals (Valko et al., 2007). In this way, the concept that mitochondrial damage and dysfunction are relevant in chronic, age-related diseases is not new (Ballinger, 2005). Mitochondrial dysfunction is one of the earliest and most prominent features in hypercholesterolemia (Madamanchi and Runge, 2007; Vercesi et al., 2007) and AD (Hauptmann et al., 2009). Moreover, recent findings in the field support an involve-

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Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; LDLr, low density lipoprotein receptor; NADH, beta-nicotinamide adenine dinucleotide reduced dipotassium salt; NAD/PH, beta-nicotinamide adenine dicnucleotide phosphate sodium salt; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substance; TC, total cholesterol.

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ment of mitochondrial-dependent mechanisms in the pathogenesis of both the hypercholesterolemia and AD. One useful tool for the study of the impact of high circulating cholesterol levels on metabolic and functional parameters in different organs is the use of the hypercholesterolemic LDL receptor knockout mice ($LDLr^{-/-}$), which represent a model of familial hypercholesterolemia (FH), a major autosomal dominant disorder associated with increased risk of premature coronary heart disease (Zadelaar et al., 2007). Recent studies provided evidence that mitochondria from various tissues from the hypercholesterolemic $LDLr^{-/-}$ mice generate higher amounts of ROS when compared to those from wild-type mates, thus suggesting that mitochondrial ROS may be involved in the early steps of atherogenesis in this model (Oliveira et al., 2005; Paim et al., 2008).

In the present study, we aimed to investigate the effects of hypercholesterolemia on cognitive function as well as its relationship with mitochondrial function and oxidative stress condition in cerebral cortex of $LDLr^{-/-}$ mice fed with a standard or cholesterol-enriched diet.

EXPERIMENTAL PROCEDURES

Animals

Wild-type C57bl/6 and low density lipoprotein receptor knockout ($LDLr^{-/-}$) mice were obtained from Universidade Estadual de Campinas (UNICAMP, São Paulo, Brazil), by homologous recombination, as previously described by Ishibashi et al. (1993). The progenitors were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The animals were kept at 21±2 °C under a 12-h light/12-h dark cycle with free access to food and water. Efforts were made to minimize the number of animals used and their suffering. All procedures used in the present study compiled with the guidelines on animal care of the local Ethics Committee on the Use of Animals (CEUA/UFSC), which follows the NIH publication "Principles of Laboratory Animal Care." Process number: 23080.040932/2010-28.

Experimental protocol

Male wild-type C57bl/6 mice (3-month old; 24–26 g) fed with a standard commercial diet (Nuvilab CR-1, Nuvital Nutrientes S/A, Paraná, Brazil) were used as control. Male $LDLr^{-/-}$ mice (3-month old; 24–26 g) were randomly divided into two experimental groups (n=6–8 animals per group for the behavioral task and n=5 animals per group for the biochemical analyses), which were fed either a standard or a cholesterol-enriched chow (20% fat, 1.25% cholesterol, 0.5% cholic acid) as described by Hort et al. (2011). After 30 days, mice were tested on a cognitive paradigm (object location task), and after an overnight food deprivation, the blood was collected from the ocular plexus for posterior determination of total cholesterol (TC) levels. The animals were then euthanized by decapitation, their brains were immediately removed from the skull, and the cerebral cortices were dissected for the posterior biochemical analyses.

Object location task. The experimental apparatus used in this study was an open-field box (50 cm wide×50 cm deep×40 cm high) made of transparent polyvinyl chloride (PVC), placed in a dimly lit (7 lx) and sound-isolated room. Identical plastic rectangles (4 cm high×4.5 cm wide) were used as objects.

The protocol was based on the previous studies described by Assini et al. (2009). The mice were placed in the center of the apparatus with two identical objects for 5 min. The objects were placed 7 cm away from the walls of the open field. Exploration of the objects was timed by a stopwatch when mice sniffed, whisked, or looked at the objects from no more than 1 cm away. After the training phase, the mice were removed from the apparatus for 180 min. After the delay, one object was moved to a new location. The time spent exploring the objects in new (novel) and old (familiar) locations was recorded during 5 min. Sessions were recorded and later analyzed. All locations for the objects were counterbalanced among the groups. After each trial, the experimental apparatus was cleaned only with dry paper, thus ensuring that it was saturated with the smell of the animals. In order to analyze the cognitive performance, a location index was calculated as previously described by Mural et al. (2007): (Tnovel×100)/(Tnovel+Tfamiliar), where Tnovel is the time spent exploring the displaced object and Tfamiliar is the time spent exploring the non-displaced object (Mural et al., 2007).

Determination of TC levels. TC was measured in plasma using enzymatic kit according to the manufacture's instructions (Gold Analisa Diagnóstica Ltda., Minas Gerais, Brazil).

Cerebral cortex supernatant preparation. For the analyses of oxidative stress parameters, cerebral cortex was homogenized in 20 mM phosphate buffer (pH 7.4). Homogenates were centrifuged at 1000×g for 10 min at 4 °C to discard nuclei and cell debris. The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and immediately used for the analyses.

For the measuring of respiratory chain complex activities, mitochondrial suspensions from cerebral cortex were prepared. Briefly cerebral cortex samples were homogenized in 10 volumes (1:10, w/v) of phosphate buffer (pH 7.4), containing 0.3 M sucrose, 5 mM MOPS, 1 mM EGTA, and 0.1% bovine serum albumin. The homogenates were centrifuged at 1500×g for 10 min at 4 °C, and the pellet was discarded. The supernatant was centrifuged at 15000×g in order to concentrate mitochondria in the pellet, which was finally dissolved in the same buffer (Latini et al., 2005).

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

Glutathione determination. Glutathione (GSH) levels were determined as described by Ellman (1959) with slight modifications. GSH were measured in tissue homogenates after protein precipitation with 1 volume of 10% trichloroacetic acid (1000×g for 10 min). An aliquot of the protein-free supernatant was added to 800 mmol l^{-1} phosphate buffer, pH 7.4, and 500 mmol l^{-1} DTNB (5,5′-dithio-bis-2-nitrobenzoic acid). Color development resulting from the reaction between DTNB and thiols was read at 412 nm after 10 min. A standard curve of reduced GSH was used in order to calculate the GSH levels in the samples, and the results were expressed as nmol GSH.mg $protein^{-1}$.

Determination of thiobarbituric acid-reactive substances. Thiobarbituric acid-reactive substances (TBARS) were determined in tissue homogenates as described by 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% TBA. 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 nmol MDA .mg $protein^{-1}$.

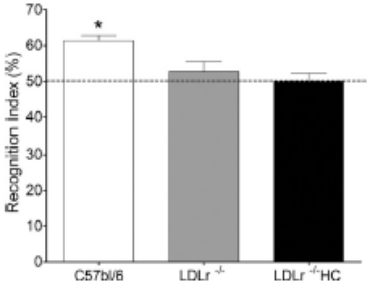

Fig 1. Effects of hypercholesterolemia on object location memory in wild-type C57bl/6 and LDLr$^{-/-}$ mice treated with standard or cholesterol-enriched diet. Each value represents the mean±SEM of six to eight animals in each group. * $P<0.05$ versus chance level (50% of displaced object investigation in test trial).

Glutathione reductase assay. Glutathione reductase (GR) activity was determined by the method described by Carlberg and Mannervik (1975), using oxidized glutathione (GSSG) as substrate. 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 GSSG, and the rate of GSSG reduction was indirectly determined through monitoring the NADPH disappearance at 340 nm. GR activity was expressed as nmol NADPH oxidized. min^{-1}.mg protein^{-1}, 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 the protocols developed by Wendel (1981) using tert-butyl hydroperoxide 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 GSH, 0.2 U ml^{-1} GR, 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 nmol NADPH oxidized. min^{-1}.mg protein^{-1}, using an extinction coefficient of 6.22×10^3 M^{-1}.cm^{-1} for NADPH.

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

Statistical analysis

Data are expressed as means±SEM. Statistical analyses were carried out using one-way analysis of variance (ANOVA). Following significant ANOVAs, multiple post hoc comparisons were performed using the Duncan's test. Pearson's correlations (r, P) were calculated for associations between cholesterol levels and respiratory chain enzyme activities (the plot includes values from all the animals groups). The object location task was analyzed by one-sample t-tests to determine whether the location index was different from chance performance (50%). The accepted level of significance for the tests was $P\leq0.05$. All tests were performed using the Statistica® software package (StatSoft Inc., Tulsa, OK, USA).

RESULTS

Hypercholesterolemia disrupts cognitive performance of mice in the object location task

Hypercholesterolemic LDLr$^{-/-}$ mice displayed spatial learning and memory impairments regardless of diet. As depicted in Fig. 1, the wild-type group showed a location index significantly higher than chance performance ($t=8.124$, $P=0.0001$), while LDLr$^{-/-}$ mice treated with standard diet ($t=0.9498$, $P=0.3739$) or cholesterol-enriched diet ($t=0.0312$, $P=0.9761$) were not able to identify the spatial alteration in the open field. The cholesterol-enriched diet did not trigger cognitive deficits in wild-type mice (data not show).

TC levels

Plasma cholesterol levels of wild-type and LDLr$^{-/-}$ mice are shown in Fig. 2. As expected, the cholesterol level of LDLr$^{-/-}$ mice treated with a standard diet were significantly higher when compared to wild-type group ($P\leq0.05$). Furthermore, when the LDLr$^{-/-}$ mice were fed with a cholesterol-enriched diet, the cholesterol levels were around three-fold higher than LDLr$^{-/-}$ mice fed with standard diet ($P\leq0.05$). By the other hand, the cholesterol levels were not modified in wild-type mice treated with a cholesterol-enriched diet (data not show).

Hypercholesterolemia inhibits the respiratory chain complexes I and II activities in cerebral cortex

The effect of hypercholesterolemia on cerebral cortex respiratory chain complexes is shown in Fig. 3. LDLr$^{-/-}$ mice treated with a cholesterol-enriched chow presented a significant inhibition of the activities of complexes I and II of the respiratory chain in cerebral cortex when compared to wild-type group ($P\leq0.05$) and to LDLr$^{-/-}$ mice treated with standard chow ($P\leq0.05$) (Fig. 3A). Additionally, the activities of complexes I and II in cerebral cortex were negatively

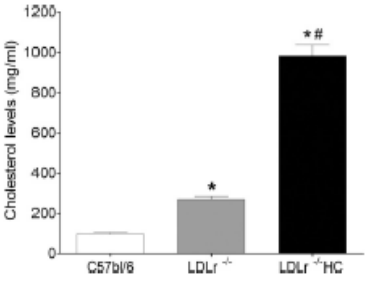

Fig 2. Plasma cholesterol levels of wild-type C57bl/6 and LDLr$^{-/-}$ mice treated with standard or cholesterol-enriched diet. Each value represents the mean±SEM of five to six animals in each group. * $P\leq0.05$ compared to wild-type C57bl/6 mice; # $P\leq0.05$ compared to LDLr$^{-/-}$ mice treated with standard diet (One-way ANOVA followed by the Duncan multiple range test).

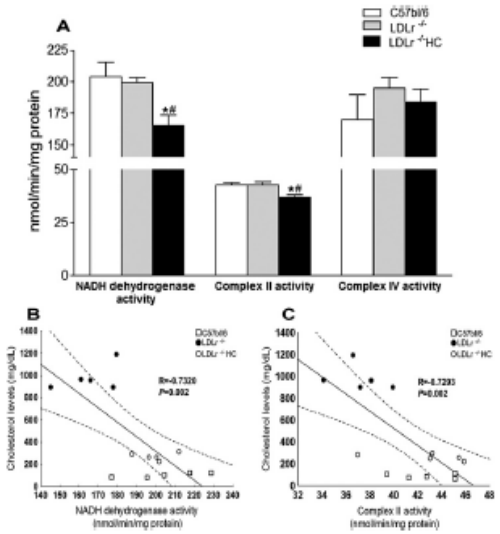

Fig 3. Effect of hypercholesterolemia on (A) respiratory chain complexes activities in cerebral cortex homogenates from wild-type C57bl/6 and LDLr$^{-/-}$ mice treated with standard or cholesterol-enriched diet. Each value represents the mean ± SEM of five to six animals in each group. * $P \leq 0.05$ compared to wild-type C57bl/6 mice; # $P \leq 0.05$ compared to LDLr$^{-/-}$ mice treated with standard diet (one-way ANOVA followed by the Duncan multiple range test). Significant correlation between (B) cholesterol levels and cerebral NADH dehydrogenase activity and (C) cholesterol levels and cerebral complex II activity.

correlated with blood cholesterol levels ($R = -0.7320$, $P \leq 0.002$ and $R = -0.7293$, $P \leq 0.002$, respectively; Fig. 3B and C, respectively), suggesting a potential link between hypercholesterolemia and impaired respiratory chain function in cerebral cortex of LDLr$^{-/-}$ mice.

Hypercholesterolemia modified oxidative stress-related parameters in cerebral cortex

The effect of hypercholesterolemia on cerebral oxidative stress parameters is shown in Fig. 4. Hypercholesterolemic mice presented a significantly impairment of the GSH-dependent antioxidant system in the cerebral cortex. GSH levels were significantly decreased when LDLr$^{-/-}$ mice were treated with a cholesterol-enriched chow when compared to wild-type group ($P \leq 0.05$) and to LDLr$^{-/-}$ mice treated with standard chow ($P \leq 0.05$) (Fig. 4A). The activities of the peroxide-removing–related enzymes, GPx and GR, are depicted in Fig. 4C, 4D, respectively. GPx activity was significantly reduced in hypercholesterolemic mice when compared to wild-type group ($P \leq 0.05$) and to LDLr$^{-/-}$ mice treated with standard chow ($P \leq 0.05$), while GR activity was significantly increased in both LDLr$^{-/-}$ mice treated with a standard or a cholesterol-enriched diet when compared to wild-type group ($P \leq 0.05$). Moreover, regardless of chow, LDLr$^{-/-}$ mice presented an increase in approximately 40% in cerebral lipid peroxidation ($P = 0.26$), assessed through the TBARS measurement, when compared to wild-type group (Fig. 4B); nevertheless, this difference did not reach statistical significance.

DISCUSSION

Confirming previous studies (Mulder et al., 2004), the present findings demonstrate spatial learning and memory deficits of hypercholesterolemic LDLr$^{-/-}$ mice when compared to their C57bl/6 wild-type controls. More importantly, our results demonstrate for the first time that hypercholesterolemia impaired mitochondrial electron transfer chain activity in the cerebral cortex, which was negatively correlated with blood cholesterol levels. We also present evidence that cholesterol-enriched diet induces impairment in the GSH-dependent antioxidant defenses in the cerebral cortex.

Early literature data has demonstrated that a cholesterol-enriched diet impairs spatial learning and the ability to store long-term memory in rodents (Ullrich et al., 2010; Granholm et al., 2008). In the present study, LDLr$^{-/-}$ mice treated with either standard or cholesterol-enriched diet were not able to identify the spatial alteration in the open

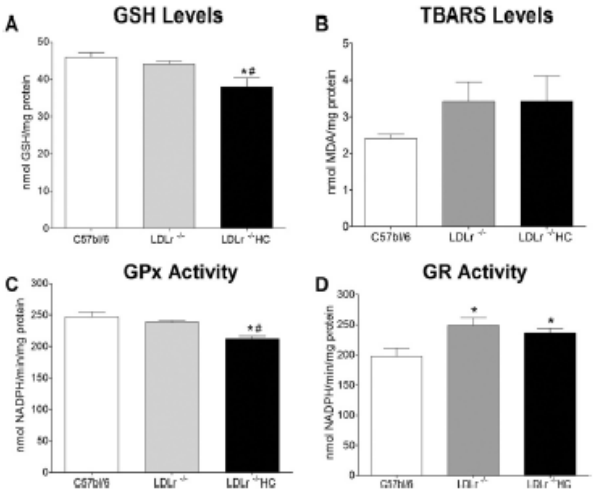

Fig 4. Effect of hypercholesterolemia on oxidative stress parameters in cerebral cortex homogenates from wild-type C57bl/6 and LDLr$^{-/-}$ mice treated with standard or cholesterol-enriched diet. Each value represents the mean ± SEM of five to six animals in each group. * $P \leq 0.05$ compared to wild-type C57bl/6 mice; # $P \leq 0.05$ compared to LDLr$^{-/-}$ mice treated with standard diet (one-way ANOVA followed by the Duncan multiple range test). (A) GSH levels, (B) TBARS levels, (C) GPx activity, and (D) GR activity.

field. However, unlike LDLr$^{-/-}$ mice, a 1-month treatment with cholesterol-enriched diet to wild-type mice is not able to modify cholesterol levels and to trigger cognitive deficits (data not show). In accordance with these observations, previous studies have demonstrated the absence of plasma cholesterol modification in wild-type mice receiving a high cholesterol diet (Ishibashi et al., 1994; Elder et al., 2008).

The object location/placement memory is proposed as a one-trial spatial memory model where the animals could learn without reinforcers (such as food) shaping the training process (Murai et al., 2007). The task has been widely used to evaluate the cognitive function of a variety of transgenic mice (Faivre et al., 2011). This task, which took advantage of the preference normal mice show for an object that has moved from its previous position, revealed an impairment following radiofrequency lesions of the fornix and cytotoxic lesions of the cingulate cortex (Ennaceur et al., 1997). Also, it has long been known that the medial prefrontal cortex is important for spatial working memory (Brozoski et al., 1979), and, in this line of evidence, Nelson et al. (2011) demonstrated that lesions to the prelimbic cortices impaired performance in the object-location task. Nevertheless, this memory test, as others cognitive tasks, provides just a qualitative index (i.e. the animals either learned or did not learn), and, thus, probably for this reason we could not determine a further decrease in cognitive function when LDLr$^{-/-}$ mice were exposed to a high cholesterol diet.

The exact mechanisms responsible for the cognitive deficit induced by hypercholesterolemia in mice are still unknown. One possible explanation was elicited by Evola et al. (2010) using apoE (apolipoprotein E) knockout mice (a widely used model of hypercholesterolemia) and by Thirumangalakudi et al. (2008) using LDLr$^{-/-}$ mice fed with high-fat/cholesterol diet. They argued that a noxious oxidative-inflammatory cycle in the vasculature could have deleterious consequences for brain function and cognition. In this line of evidence, Hafezi-Moghadam et al. (2007) showed that the genetic deletion of apoE in aged hypercholesterolemic mice induces a destabilizing effect on the cerebral microcirculation leading to blood–brain barrier leakage. Failure of the blood–brain barrier, with leakage of serum components into and through the walls of small cerebral vessels, can lead to neuronal and glial damage with persistent activation of microglia and astrocytes, which may be responsible for cognitive impairment (Rapp et al., 2008).

Neuroinflammatory processes also lead to increased production of ROS, which are detrimental to neurons (Witte et al., 2010). The mitochondria are victims of extensive oxidative damage and inflammation is likely to further contribute to mitochondrial dysfunction (Witte et al., 2010). Neurons are vulnerable to mitochondrial defects because

they require high levels of energy for the survival and specialized function (Chen and Chan, 2006). Of major interest, there is ample literature supporting a crucial role of mitochondrial dysfunction in AD, with altered energy metabolism and ROS production being the major correlates (Moreira et al., 2010; Lin and Beal, 2006).

On the other hand, changes in brain cholesterol levels seem not to be responsible for the observed cognitive impairments of LDLr$^{-/-}$ mice. There is evidence that the homeostasis of brain cholesterol is independent from the circulating cholesterol (Di Paolo and Kim, 2011). Moreover, previous studies demonstrated that even when submitted to high cholesterol, high-fat or high-fat/high-cholesterol diets, the LDLr$^{-/-}$ mice do not present any significant rise on brain cholesterol levels (Elder et al., 2007).

An interesting observation from our study was that a three-fold increase in serum cholesterol levels was enough to elicit a decrease in the behavioral performance without any significant impairment in mitochondrial function. However, a 10-fold increase (observed in LDLr$^{-/-}$ plus high-cholesterol diet) decreased mitochondrial complex function and induced GSH depletion, but did not further decrease performance in the recognition test. Based on these findings, one could suppose the occurrence of a "threshold phenomenon" for memory performance and mitochondrial function. However, based on the well-known relationship of cognitive impairment with either mitochondrial dysfunction or cholesterol dyshomeostasis, and considering the consistent literature, we realize that the occurrence of a threshold phenomenon for the memory performance and mitochondrial function can be ruled out. For example, in the study by Mulder et al. (2004), LDLr$^{-/-}$ mice displayed impaired hippocampal-dependent memory functions (demonstrated in several behavioral tests) when compared to LDLr$^{+/+}$ littermates. Mice lacking the LDL receptor displayed a decrease in the number of synaptophysin-immunoreactivity presynaptic buttons in the hippocampus CA1 region. These authors provided evidence for a role of the LDL receptor in the maintenance of normal synaptic plasticity in the hippocampus during processes of learning and memory. Since cholesterol is required for cell homeostasis, the reduced cellular uptake of cholesterol observed in LDLr$^{-/-}$ mice might underlie deleterious consequences in neuronal membrane function. Therefore, the lack of brain LDL receptor rather than the effect of the elevated serum cholesterol levels (two to three times higher than in the wild-type animals) on brain mitochondrial functions could be responsible for the cognitive deficits in LDLr$^{-/-}$ mice fed with normal diet. A similar event was already observed when evaluating the influence of hypercholesterolemia on the cognitive performance of LDLr$^{-/-}$ mice and its relationship with neuroinflammatory changes (Thirumangalakudi et al., 2008). The authors observed that memory deficits occurred regardless of diet, but pro-inflammatory markers were exacerbated in LDLr$^{-/-}$ treated with a hypercholesterolemic diet.

Furthermore, the relatively small period of exposure to this two-fold increase in cholesterol levels by LDLr$^{-/-}$ mice treated with standard chow (only 3 months) has to be considered. This short-term exposure may explain the absence of brain mitochondrial dysfunction and oxidative stress. Thus, raising the serum cholesterol with the aid of a cholesterol-enriched diet would be expected to further accelerate the emergence of a noxious oxidative-inflammatory cycle in the vasculature. In our study, a cholesterol-enriched diet increased the total plasma cholesterol levels (around 10 times) in LDLr$^{-/-}$ mice, leading to a significant decrease in the mitochondrial complexes I and II activities in the cerebral cortex. Of high interest, we presented evidence that this inhibition of complexes I and II activities was negatively correlated with respective blood cholesterol levels. A previous study conducted in liver tissue of LDLr$^{-/-}$ mice by Oliveira et al. (2005) demonstrated that mitochondrial dysfunction and oxidative stress triggered by hypercholesterolemia are due to low content of pyridine nucleotides in the reduced state, which were presumably consumed by augmented lipogenesis. It is noteworthy that cortico-cerebral GPx activity was decreased in LDLr$^{-/-}$ mice fed a high cholesterol diet in our methodological approach, which uses saturated amounts of the pyridine nucleotide substrate (NADPH), thus suggesting that not only the levels of this coenzyme but also the enzyme catalytic activity was decreased as a result of hypercholesterolemia.

It is well documented that mitochondrial electron transport chain is the major source of cellular ROS and oxidative stress, with ROS generation occurring mainly at complex I (Sanz et al., 2006; Pamplona and Barja, 2007). Cellular oxidative stress and its downstream consequences are also a well-documented feature of AD pathophysiology (Sultana and Butterfield, 2011), and the CNS is especially prone to ROS-induced damage (Gemma et al., 2007). Here we demonstrated an imbalance in GPx/GR activity that might be related to hypercholesterolemia-induced oxidative stress, mainly by causing lipid peroxidation and GSH-dependent antioxidant system impairment. Several reports have shown a link between high dietary exposures to fat and/or cholesterol and oxidative stress in the brain of mice and rats (Crisby et al., 2004; Montilla et al., 2006) and our present data may support a role of mitochondrial dysfunction in the consequent oxidative imbalance induced by hypercholesterolemia.

GSH is an abundant intracellular antioxidant and scavenger of ROS (Dringen and Hirrlinger, 2003). Previous studies have shown that the GSH system may be activated as a response to oxidative stress in the brains of AD patients (Lovell et al., 1995; Aksenov et al., 2001). Additionally, hypercholesterolemia has been demonstrated to trigger oxidative stress by increasing the generation of ROS leading to a depletion of GSH levels, besides impairing the mitochondrial transport of GSH, resulting in mitochondrial GSH depletion and mitochondrial oxidative stress (Colell et al., 1997; Marí et al., 2008; Fernández et al., 2009). Corroborating these findings, Esposito et al. (2000) demonstrated that isolated liver mitochondria from GPx1-deficient mice have increased rates of H_2O_2 production, reduced mitochondrial respiratory control ratio, and decreased mitochondrial power output index.

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CONCLUSIONS

To our knowledge, this is the first study to report a strong correlation between high levels of blood cholesterol and mitochondrial dysfunction in the cerebral cortex. This event was related with cognitive impairments, as well as lipid peroxidation, GSH depletion, and changes on peroxide-removing–related enzymes GPx and GR in the cerebral cortex of hypercholesterolemic LDLr$^{-/-}$ mice. In conclusion, our findings provide new evidence about the positive correlation between elevated plasma cholesterol levels and cognitive impairments in hypercholesterolemic LDLr$^{-/-}$ mice, which are mediated, at least in part, by mitochondrial dysfunctional and oxidative stress in cerebral cortex. In addition, they render this mouse model of hypercholesterolemia a useful approach to comprehend the molecular events mediating AD pathogenesis.

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4.2 MANUSCRITO 1

Diphenyl diselenide, a glutathione peroxidase mimetic, prevents cortico-cerebral oxidative stress induced by hypercholesterolemia in the LDL receptor knockout mice

Manuscrito em fase final de preparação.

Diphenyl diselenide, a glutathione peroxidase mimetic, prevents cortico-cerebral oxidative stress induced by hypercholesterolemia in the LDL receptor knockout mice.

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Abstract

Considerable evidence supports an association between hypercholesterolemia and brain oxidative stress. The interaction between antioxidant function and oxidative stress has emerged as a common causative factor in the pathology of several brain disorders. In the low density lipoprotein receptor knockout ($LDLr^{-/-}$) mice, cholesterol-enriched diet leads to memory impairment, as well, brain mitochondrial dysfunction and oxidative stress. In the present study, we evaluated the potential neuroprotective effect of diphenyl diselenide $(PhSe)_2$, a glutathione peroxidase (GPx) mimetic, against hypercholesterolemia-induced cortico-cerebral oxidative stress in the $LDLr^{-/-}$ mice submitted to a model of hypercholesterolemia. Our results showed that oral treatment with $(PhSe)_2$ prevented the cortico-cerebral oxidative stress in the 3 months-old $LDLr^{-/-}$ mice fed with cholesterol-enriched diet, significantly increased the GSH content, and decreased the lipid peroxidation. These antioxidant actions seem to be in part related to its thiol peroxidase-like activity. Based on our findings, in addition to its protective effects in cardiovascular diseases associated with hypercholesterolemia, $(PhSe)_2$ can be pointed as a promising neuroprotective molecule against hypercholesterolemia-induced effects deleterious on brain.

Keywords:
Hypercholesterolemia, cerebral oxidative stress, diphenyl diselenide, low density lipoprotein receptor knockout mice.

Introduction

Cholesterol is an important lipid constituent of all cellular membranes and myelin (Simons and Ikonen, 2000). Cholesterol also functions as the substrate for the synthesis of bile acids in the liver, and as the precursor of steroid hormones in endocrine tissue (Jackson et al., 1997). Meanwhile, despite its role in essential biochemical processes and support of membrane structure, elevated plasma cholesterol levels are associated with negative health outcomes especially its association with vascular disease (Lusis, 2000, Grundy et al., 2004, Lewington et al., 2007). Furthermore, alterations in cholesterol metabolism have been implicated with deleterious consequences of brain function (Sparks et al., 2000, Wolozin, 2004b, a, Ullrich et al., 2010). For instance, epidemiological and neurochemical investigations are providing increasing evidences that altered cholesterol metabolism contributes to the development of Alzheimer's disease (Pappolla et al., 2002, Di Paolo and Kim, 2011).

Indeed, there is compelling findings indicating a causal link between high dietary cholesterol intake and brain oxidative stress (Crisby et al., 2004, Montilla et al., 2006, Amin et al., 2011). Previous reports demonstrated that hypercholesterolemia triggers a neuroinflammatory response and brain oxidative stress, ultimately resulting in cognitive impairments and neurodegeneration (Sparks and Schreurs, 2003, Thirumangalakudi et al., 2008, Lu et al., 2009, Lu et al., 2010) For instance, Lu et al. (2010) observed that hypercholesterolemia increases reactive oxygen species (ROS) and reduces superoxide dismutase (SOD) activity in the brain of aged C57Bl/6 mice, and these alterations were attenuated by antioxidant compounds. In this context, we recently observed an imbalance in the glutathione (GSH)-dependent antioxidant defenses and an enhanced lipid peroxidation in the cerebral cortex of hypercholesterolemic low density lipoprotein receptor knockout ($LDLr^{-/-}$) mice (de Oliveira et al., 2011).

However, even though several epidemiological studies demonstrated a connection between cholesterol-lowering agents (e.g. statins) and a significant reduction in the prevalence of neurologic disorders in general population (Jick et al., 2000, Hajjar et al., 2002, Rodriguez et al., 2002, Dufouil et al., 2005, Wolozin et al., 2007), preliminary clinical studies reported modest success (Simons et al., 2002, Sparks et al., 2005, Sparks et al., 2006). In this scenario, there is compelling evidences that organoselenium compounds are promising pharmacological agents (Nogueira et al., 2004, Rosa et al., 2007). Of

particular significance, organoselenium compounds can mimicry endogenous antioxidant enzymes, such as glutathione peroxidase (GPx).

Diphenyl diselenide $(PhSe)_2$ is a simple diorganoilselenium compound that displays GPx-like activity, and presented neuroprotective and antioxidant activities *in vitro* (Rossato et al., 2002, Meotti et al., 2004, Puntel et al., 2007, Posser et al., 2008) and *in vivo* (Barbosa et al., 2006, Barbosa et al., 2008). Of particular relevance, our research group reported that oral treatment with lower doses of $(PhSe)_2$ potently reduced the formation of atherosclerotic lesion in the $LDLr^{-/-}$ mice (Hort et al., 2011), as well as, led to beneficial effects on serum total cholesterol and on several parameters related to oxidative stress in hypercholesterolemic rabbits (de Bem et al., 2009). Moreover, we demonstrated that $(PhSe)_2$ inhibited human LDL oxidation and that this phenomenon was related to its GPx-like activity (de Bem et al., 2008). Based on the above mentioned effects of $(PhSe)_2$, we hypothesized that this compound may present neuroprotective effects against hypercholesterolemia- induces cortico-cerebral oxidative stress. To test this hypothesis, in the present study we examined the effect of $(PhSe)_2$ on cortico-cerebral oxidative stress in the 3-months-old $LDLr^{-/-}$ mice fed daily with cholesterol-enriched diet during 30 days.

Material and methods

Chemicals

Diphenyl diselenide $(PhSe)_2$ was synthesized according to the literature 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.

Animals

C57Bl/6 low density lipoprotein receptor knockout ($LDLr^{-/-}$) mice were obtained by homologous recombination, as previously described by Ishibashi et al. (1993). The progenitors were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). Animals were maintained at controlled room temperature (21 ± 2°C) under a 12 h light/12 h dark cycle with free access to food and water. All experiments were

undertaken in accordance with the guidelines on animal care of the local Ethics Committee on the Use of Animals (CEUA/UFSC), which follows the NIH publication “Principles of Laboratory Animal Care”.

Experimental protocols

In order to evaluate the neuroprotective effects of $(PhSe)_2$ against hypercholesterolemia-induces cortico-cerebral oxidative stress, male 3-months-old $LDLr^{-/-}$ mice weighing 24–26 g were randomly divided into two experimental groups, that were fed with a standard or a cholesterol-enriched diet (20% fat, 1.25% cholesterol, 0.5% cholic acid) (de Oliveira et al., 2011). Half of the animals from each group were concomitantly treated daily with $(PhSe)_2$ (1 mg/kg; o.g.), and the other half with vehicle (canola oil), totalizing four experimental groups. Following 30 days of treatments, the animals were food-deprived overnight and the blood was collected by cardiac puncture in heparinized tubes for posterior determination of plasma cholesterol levels. After that, the mice were sacrificed by decapitation, and the cerebral cortex was removed for the biochemical analyses.

Plasma cholesterol levels

Whole blood was centrifuged at 3,000 x *g*, at room temperature for 10 min and the obtained plasma was used to measure total cholesterol and non-HDLcholesterol levels. Total and HDL plasma cholesterol were measured in plasma by an enzymatic method (Labtest Diagnostica®, Lagoa Santa-MG, Brazil). The concentration of non-HDL-cholesterol was calculated using the equation: (LDL + VLDL + IDL) = TC – HDL.

Cerebral cortex homogenates preparation

Cerebral cortex was removed and homogenized (1:10 w/v) in HEPES buffer (20 mM, pH 7.0). The tissue homogenates were centrifuged at 16,000 x *g*, at 4°C for 20 min and the supernatants obtained were used for the determination of glutathione peroxidase (GPx) and glutathione reductase (GR) activities and for the quantification of the levels of reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS).

Reduced glutathione levels

Glutathione (GSH) levels were determined as described by Ellman (1959) with slight modifications. GSH was measured in cerebral cortex homogenates after precipitation with 1 vol. of 10% trichloroacetic acid and centrifuged at 1,000 x *g* at 4 ºC for 10 min. An aliquot of the protein-free supernatant was added to 800 mmolM phosphate buffer, pH 7.4, and 500 mmolM1 DTNB (5,5'-dithio-bis-2-nitrobenzoic acid). Color development resulting from the reaction between DTNB and thiols was read at 412 nm after 10 min. A standard curve of reduced GSH was used in order to calculate the GSH levels in the samples, and the results were expressed as nmol GSH.mg protein^{-1}.

Thiobarbituric acid-reactive substances levels

Thiobarbituric acid-reactive substances (TBARS) were determined in tissue homogenates as described by 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% TBA. 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 nmol MDA .mg protein^{-1}.

Glutathione reductase activity

Glutathione reductase (GR) activity was determined according to previously described by Carlberg and Mannervik (1985). The enzyme activity was assessed in a solution containing 50mM 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 nmol NADPH oxidized.min^{-1}.mg protein^{-1}, using an extinction coefficient 6.22 x 103 M-1.cm^{-1} for NADPH.

Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was measured according to the protocols developed by 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 1mM 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 nmol NADPH oxidized.min^{-1}.mg $protein^{-1}$, using an extinction coefficient of 6.22 x 103 M^{-1} cm^{-1} for NADPH.

Protein determination

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

Statistical analysis

All data are expressed as mean ± S.E.M. The statistical evaluation was performed using two-way analysis of variance (ANOVA). Following significant ANOVAs, multiple post-hoc comparisons were performed using the Duncan's test. The accepted level of significance for the tests was $P \leq 0.05$. All tests were performed using the Statistica software package (StatSoft Inc., Tulsa, OK, USA).

Results

Plasma cholesterol concentration

In previous studies we demonstrated that when the 3 months-old $LDLr^{-/-}$ mice are submitted to a standard diet they presents a two- to four-fold increase in plasma cholesterol concentration when compared to the wild type mice. Moreover, $LDLr^{-/-}$ mice submitted to a cholesterol-enriched diet during 30 days presents around three-fold increase in total cholesterol levels compared to $LDLr^{-/-}$ mice fed with a standard diet (de Oliveira et al., 2011; Hort et al., 2011). Therefore, we investigated the effects of $(PhSe)_2$ treatment in the plasma cholesterol concentration of the hypercholesterolemic $LDLr^{-/-}$ mice.

Two-way ANOVA indicated only a significant effect for the diet factor on total plasma cholesterol levels [F (1, 16) = 212.68, $P \leq 0.05$],

and on non-HDL-cholesterol levels [F (1, 16) = 140.32, $P \leq 0.05$]. Subsequent post-hoc comparisons indicated that $LDLr^{-/-}$ mice submitted to the cholesterol-enriched diet presented a significantly increase in the total cholesterol and non-HDL cholesterol levels when compared to the $LDLr^{-/-}$ mice submitted to the standard diet. Meanwhile, as observed in Table 1, $(PhSe)_2$ treatment did not alter total plasma cholesterol levels and non-HDL cholesterol levels in the $LDLr^{-/-}$ mice regardless of diet .

Effects of $(PhSe)_2$ against hypercholesterolemia-induced cortico-cerebral oxidative stress

In a previous report, we demonstrated that 3 months-old $LDLr^{-/-}$ mice submitted to a cholesterol-enriched diet during 30 days presented a significant inhibition of the mitochondrial complexes I and II activities, as well as, an increased lipid peroxidation and an impairment in the GSH-dependent antioxidant system in the cerebral cortex, characterized by a diminishing in the reduced glutathione levels and a decrease in the GPx activity (de Oliveira et al., 2011). Herein, we demonstrated that the treatment with a GPx mimic compound, $(PhSe)_2$, protects against hypercholesterolemia- induced cortico-cerebral oxidative stress.

Two-way ANOVA indicated a significant effect for the treatment factor [F (1, 27) = 73.31, $P \leq 0.05$], and for the interaction between diet and treatment factors [F (1, 27) = 34.77, $P \leq 0.05$] on GSH levels. Subsequent post-hoc comparisons revealed that the cholesterol-enriched diet significantly reduced the GSH levels in the cerebral cortex of $LDLr^{-/-}$ mice ($P \leq 0.05$, Figure 1A), and $(PhSe)_2$ treatment prevent this reduction in the $LDLr^{-/-}$ mice fed with cholesterol enriched-diet ($P \leq 0.05$, Figure 1A). Furthermore, indicated a significant effect for the treatment factor [F (1, 15) = 122.68, $P \leq 0.05$], and for the interaction between diet and treatment factors [F (1, 15) = 4.84, $P \leq 0.05$] on GPx activity. Subsequent post-hoc comparisons revealed that the cholesterol-enriched diet significantly reduced the GPx activity in the cerebral cortex of $LDLr^{-/-}$ mice ($P \leq 0.05$,Figure 1C), while the $(PhSe)_2$ treatment increased the GPx activity in the $LDLr^{-/-}$ mice fed with a standard diet but not in the $LDLr^{-/-}$ mice fed cholesterol-enriched diet. Finally, Two-way ANOVA indicated a significant effect for the diet factor [F (1, 16) = 10.50 , $P \leq 0.05$] and for the interaction between diet and treatment factors [F (1, 16) = 7.42 , $P \leq 0.05$] on GR activity in cerebral cortex of $LDLr^{-/-}$ mice. Subsequent post-hoc comparisons revealed that treatment with $(PhSe)_2$ decreased the GR activity in the $LDLr^{-/-}$ mice fed cholesterol-enriched diet ($P \leq 0.05$, Figure 1C). In addition, two-way ANOVA indicated a significant effect for the

treatment factor [F (1, 25) = 12.496, $P \leq 0.05$] on lipid peroxidation in cerebral cortex of $LDLr^{-/-}$ mice. Subsequent post-hoc comparisons revealed a decreased lipid peroxidation in the cerebral cortex of $LDLr^{-/-}$ mice fed cholesterol-enriched diet following treatment with $(PhSe)_2$ ($P \leq 0.05$, Figure 1B).

Discussion

There are considerable evidences that hypercholesterolemia is associated with brain oxidative stress in humans and in rodents models (Stokes et al., 2002, Lu et al., 2009, Lu et al., 2010, Ramirez et al., 2011). Furthermore, an imbalance between antioxidant functions and oxidative stress is implicated in diverse brain pathologies (Harish et al., 2011). In this context, several studies have reported the beneficial effects of organoselenium compounds against pathological conditions associated with oxidative stress (e.g., inflammation, diabetes, neurotoxicity and hepatotoxicity) (Wilson et al., 1989, Nogueira et al., 2004, Nogueira and Rocha, 2011). Our present results demonstrated that $(PhSe)_2$ effectively prevented the cortico-cerebral oxidative stress induced by cholesterol-enriched diet in the 3-months-old $LDLr^{-/-}$ mice. Here, the antioxidant action of $(PhSe)_2$ was linked to a reduction in lipid peroxidation and increased in GSH content in cortico-cerebral homogenates of $LDLr^{-/-}$ mice submitted to a hipercholesterolemic diet.

Hypercholesterolemia has been demonstrated to trigger oxidative stress by increasing reactive oxygen species (ROS) generation and decreasing oxidative defenses (Colell et al., 1997, Mari et al., 2008, Fernandez et al., 2009). One potential mechanism by which hypercholesterolemia may induce oxidative stress is related to a disruption of mitochondrial function (McCommis et al., 2011). Previous studies showed that mitochondria obtained from various tissues of the hypercholesterolemic $LDLr^{-/-}$ mice generate higher amounts of ROS when compared to those of wild-type mates (Oliveira et al., 2005, Paim et al., 2008, de Oliveira et al., 2011). The authors suggested that higher rates of lipogenesis in the $LDLr^{-/-}$ mice deplete the mitochondrial reducing equivalents from NADPH leading to a state of oxidative stress.

However, since the homeostasis of brain cholesterol is independent from the circulating cholesterol (Di Paolo and Kim, 2011), the exact mechanisms responsible for the cortico-cerebral oxidative stress induced by hypercholesterolemia in the $LDLr^{-/-}$ mice remains unclear. One possible explanation is that the increased metabolic flux to the brain during hypercholesterolemia can orchestrate the blood-brain

barrier (BBB) disruption, recruitment of inflammatory immune cells from peripheral blood and microglial cells activation leading to neuroinflammation (Kalayci et al., 2009). In fact, inflammation of brain capillary endothelial cells may play a potent role in the neurodegenerative diseases, and it is well known that endothelial cells strongly respond to inflammatory stimuli (Moser et al., 2004) especially involving production of ROS (Iadecola, 2004). In this line of evidence, (Thirumangalakudi et al., 2008) using $LDLr^{-/-}$ mice fed with high-fat/cholesterol diet demonstrated that a noxious oxidative-inflammatory cycle in the vasculature could have deleterious consequences for brain function. Indeed, recently we proposed an oxidative stress condition in the cortex cerebral of hypercholesterolemic $LDLr^{-/-}$ mice after 30 days feeding with high cholesterol diet, which was characterized by impairment in the GSH metabolism and lipid peroxidation (de Oliveira et al., 2011).

In mammalian cells, glutathione (GSH) and the GPxs constitute one of the most important antioxidant defense systems (Raes et al., 1987). GSH is an abundant intracellular antioxidant and scavenger of ROS, providing the neuronal cell with important protection against oxidative damage (Brigelius-Flohe, 1999, Lima et al., 2006). Herein, we showed that $(PhSe)_2$ significantly increased the GSH content in the cerebral cortex of 3-months-old $LDLr^{-/-}$ mice fed with cholesterol-enriched diet, which possibly contributed to the enhanced detoxification of lipid peroxidation products as demonstrated by decreased in the MDA levels. In this way, there are two major pathways involved in regulating of cellular GSH contents: one is GR, which catalyzes the reduction of GSSG to GSH via consumption of NADPH (Carlberg and Mannervik, 1985), and the other is γ-glutamyl cysteine synthase (γGCS), which is the rate-limiting enzyme in GSH synthesis (Griffith and Mulcahy, 1999). In this study, the treatment with $(PhSe)_2$ paradoxically decreased the GR activity, which may occurred due to increase in the GSH levels by increased biosynthesis, not requiring regeneration. Furthermore, the $(PhSe)_2$ treatment increased the GPx activity in the $LDLr^{-/-}$ mice fed standard diet. In this scenario, we can speculate that $(PhSe)_2$ might modulate intracellular signaling pathways related to antioxidant response. In line with this, recent studies demonstrated that the organoselenium compound ebselen activates the Nrf-2, a transcription factor orchestrating antioxidant and cytoprotective responses on oxidative stress that ultimately increases the expression of genes encoding enzymes of the glutathione redox system, such as γGCS and GPx (Tamasi et al., 2004, Kim et al., 2009).

GPx is an antioxidant defense enzyme that can directly detoxify H_2O_2 and lipid hydroperoxides at the expense of GSH (Brigelius-Flohe, 1999), and accumulating evidence suggested that GPx functions as the primary protection against acute oxidative stress, particularly in neuropathological situations (De Haan et al., 2003, Ran et al., 2006). In line of this, another possible mechanism associated with the observed $(PhSe)_2$ neuroprotection in the hypercholesterolemic $LDLr^{-/-}$ mice, is the its capacity of mimic endogenous antioxidant enzymes, such as GPx (Nogueira et al., 2004, Borges et al., 2006, de Freitas and Rocha, 2011). In this regard, Posser et al. (2008) demonstrated that $(PhSe)_2$ exerts protective effects against H_2O_2-induced oxidative damage in hippocampal slices, and this neuroprotective effect seems to be related to its thiol-peroxidase-like activity. Indeed, we previously demonstrated that $(PhSe)_2$ inhibited human LDL oxidation and that this phenomenon was related to its GPx-like activity (de Bem et al., 2008). The GPx-like activity of $(PhSe)_2$ seems to be kinetically identical to that of the enzyme reaction (Nogueira et al., 2004, Barbosa et al., 2006, Nogueira and Rocha, 2011). In brief, $(PhSe)_2$ reacts with 2 thiol equivalents, such as GSH, to generate a product characterized as selenol. The selenol reacts with H_2O_2 or organic peroxides to form water and a seleninic acid, which spontaneously produces another molecule of water and turns to $(PhSe)_2$ (Nogueira et al., 2004, Posser et al., 2008, Nogueira and Rocha, 2011). Then, $(PhSe)_2$ might be conferring its protective effect by decomposing lipid hydroperoxides.

Of particular pharmacological importance, previous studies suggest that $(PhSe)_2$, a very lipophilic compound that can cross the BBB, leads to increasing selenium levels into the brain after acute and chronic treatments (Jacques-Silva et al., 2001, Maciel et al., 2003). Furthermore, toxicological studies have been conducted to establish the safety of this organoselenium compound. The level of toxicity induced by $(PhSe)_2$ depends on the route of administration and the species (rat, mice, or rabbits) (Nogueira et al., 2003, de Bem et al., 2007, Straliotto et al., 2010). In fact, the antioxidant effect of $(PhSe)_2$ showed in this study was obtained within a very safe dose range. Recently, Savegnago et al. (2007) characterized that the LD_{50} for $(PhSe)_2$ to mice by the oral route was >312 mg/kg and that this oral treatment did not provide evidence for renal or hepatic toxicity. The quantification of plasma levels of $(PhSe)_2$ after oral administration of 500 mg/kg in mice, showed a $(PhSe)_2$ peak at 30 minutes post dosing of approximately 10 µg/mL (Prigol et al., 2009).

Conclusions

In conclusion, the present findings constitute an in vivo evidence for neuroprotective role of low dose of $(PhSe)_2$ in cortico-cerebral oxidative stress induced by hypercholesterolemic diet in the 3-months-old $LDLr^{-/-}$ mice. The antioxidant effect of $(PhSe)_2$ is, at least in part, associated to the involvement of glutathione system. Further studies are warranted to elucidate the precise mechanism of $(PhSe)_2$ antioxidant effect in cerebral cortex of $LDLr^{-/-}$ mice. In addition, to its protective effects in cardiovascular diseases associated with hypercholesterolemia showed in ours previous studies, based on our findings $(PhSe)_2$ can be pointed as a promising neuroprotective molecule against hypercholesterolemia-induced effects deleterious on brain.

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Figures and Legends

Groups	Total Cholesterol (mg/ dL)	non-HDL cholesterol (mg/dL)
control + vehicle	211.6 ± 22.25	183.0 ± 22.70
control + $(PhSe)_2$	197.8 ± 13.45	183.9 ± 12.35
cholesterol + vehicle	833.9 ± 56.69*	764.7 ± 51.72*
cholesterol + $(PhSe)_2$	864.4 ± 42.51	834.5 ± 58.55

Table 1: Effect of $(PhSe)_2$ on plasma total cholesterol levels and non-HDL-cholesterol levels in the $LDLr^{-/-}$ mice fed standard or cholesterol-enriched diet . Each value represents the mean ± S.E.M. of four to five animals in each group. *$P \leq 0.05$ compared to 3-month-old $LDLr^{-/-}$ mice fed standard diet. (Two-way ANOVA followed by Duncan´s post-hoc test).

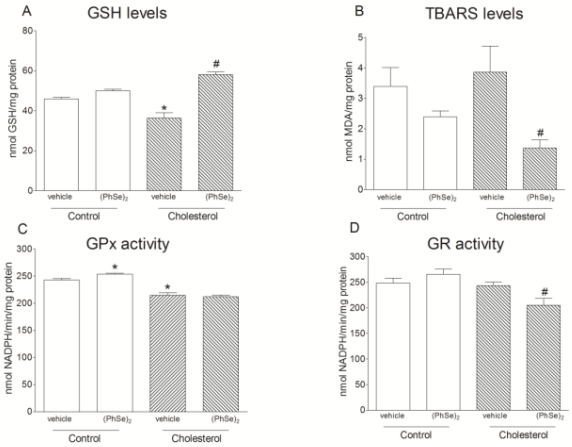

Figure 1: Effects of $(PhSe)_2$ on cerebral cortex oxidative stress parameters in the $LDLr^{-/-}$ mice fed standard or cholesterol-enriched diet: A) GSH levels, B) TBARS levels, C) GPx activity and D) GR activity. Each value represents the mean ± S.E.M. of four to six animals in each group. *$P \leq 0.05$ compared to 3-month-old $LDLr^{-/-}$ mice fed standard diet, #$P \leq 0.05$ compared to 3-month-old $LDLr^{-/-}$ mice fed cholesterol-enriched diet (Two-way ANOVA followed by Duncan´s post-hoc test).

5 DISCUSSÃO

A condição de hipercolesterolemia (monogênica e multifatorial) afeta 1 em cada 20 indivíduos na população em geral, por outro lado, a frequência da hipercolesterolemia familiar (HF) é de 1 em 500 para heterozigotos. Homozigotos são raros, com uma frequência de 1 em um milhão. A HF é uma patologia autossômica codominante caracterizada pela elevação isolada do colesterol presente na LDL plasmática e fortemente associada com alto risco de doença cardiovascular prematura (Brown e Goldstein, 1986; Civeira, 2004; Setia et al., 2012). Nos últimos anos, crescentes evidências clínicas e patológicas indicam que elevados níveis de LDL-colesterol podem contribuir para o desenvolvimento de prejuízos cognitivos e demência (Pappolla et al., 2002; Mielke et al., 2005, Whitmer et al., 2005, Zambon et al., 2010). Nesse contexto, uma ferramenta útil para o estudo do impacto dos altos níveis plasmáticos de colesterol sobre parâmetros metabólicos e funcionais em diferentes órgãos é o uso de camundongos LDLr$^{-/-}$, os quais representam muito bem um modelo de HF (Ishibashi et al., 1993; Zadelaar et al., 2007).

De fato, Mulder e colaboradores (2004) demonstraram que camundongos LDLr$^{-/-}$ apresentam prejuízo de aprendizado e memória quando comparados com camundongos controles em diversos testes comportamentais que avaliam memória espacial e de trabalho. Neste sentido, os resultados apresentados na primeira parte desta dissertação, referem-se à avaliação da função cognitiva, bem como da função mitocondrial e antioxidante em córtex cerebral de camundongos LDLr$^{-/-}$ expostos à dieta padrão ou hipercolesterolêmica durante 30 dias. O conjunto de resultados apresentados no Artigo 1 demonstram que independentemente da dieta adotada os camundongos LDLr$^{-/-}$ apresentam prejuízo de aprendizado e memória espacial no teste de localização do objeto. Além disso, a dieta hipercolesterolêmica causou inibição na atividade dos complexos I e II da CR no córtex cerebral de camundongos LDLr$^{-/-}$, evento que foi associado com lipoperoxidação, diminuição dos níveis de GSH e desequilíbrio na atividade das enzimas integrantes do sistema antioxidante dependente da GSH. Estes últimos resultados caracterizaram uma situação de estresse oxidativo no córtex cerebral destes camundongos hipercolesterolêmicos. De particular importância, a disfunção mitocondrial observada no córtex cerebral dos camundongos foi negativamente correlacionada com os níveis plasmáticos de colesterol.

Dados da literatura demonstram que a ingestão de uma dieta rica em colesterol prejudica o aprendizado espacial e a habilidade de retenção da memória de longo prazo em roedores (Granholm et al., 2008; Ullrich et al., 2010; Freeman et al., 2011). Nossos dados demonstram que camundongos LDLr$^{-/-}$ alimentados com dieta padrão ou hipercolesterolêmica não são capazes de identificar uma alteração espacial no campo aberto. A memória de localização/relocalização de objeto é proposta como um modelo de memória espacial com apenas um treinamento (Murai et al., 2007). O teste de localização do objeto é amplamente usado para avaliar a função cognitiva em camundongos transgênicos, levando em consideração a preferência de camundongos normais pelo objeto que foi movido de sua posição anterior (Faivre et al., 2011). Vários estudos demonstraram que lesões corticais levam ao prejuízo do desempenho neste teste em diferentes protocolos experimentais, uma vez que o córtex pré-frontal medial é importante para a memória espacial de trabalho (Brozoski et al., 1979; Ennaceur et al., 1997; Nelson et al., 2011). No entanto, uma desvantagem deste e de outros conhecidos testes cognitivos é fornecer apenas um índice qualitativo (aprender ou não aprender), então, provavelmente por esta razão não podemos determinar uma maior redução na função cognitiva dos camundongos LDLr$^{-/-}$ que foram expostos à dieta hipercolesterolêmica.

Como a homeostase do colesterol cerebral é independente do colesterol plasmático, o exato mecanismo responsável pelo prejuízo cognitivo induzido pela hipercolesterolemia ainda permanece desconhecido (Elder et al., 2007; Di Paolo e Kim, 2011). Uma possível explicação foi proposta por Evola e colaboradores (2010) usando camundongos ApoE$^{-/-}$ e por Thirumangalakudi e colaboradores (2008) usando camundongos LDLr$^{-/-}$ expostos á uma dieta com alto teor de gordura e/ou colesterol. Estes autores propuseram que um nocivo ciclo oxidativo e inflamatório na vasculatura poderia ter consequências deletérias para função cerebral e cognição. Na mesma linha de evidência, Hafezi-Moghadam e colaboradores (2007) demonstraram que a deleção genética da ApoE em camundongos idosos induz um efeito desestabilizador sobre a microvasculatura cerebral levando a disfunção da BHE. Portanto, alterações na BHE decorrentes da hipercolesterolemia resultam em extravasamento de componentes séricos para dentro e através da parede dos pequenos vasos cerebrais e recrutamento de células imune inflamatórias da circulação sanguínea, causando dano neuronal e glial com persistente ativação de micróglia e

astrócitos. Estes eventos podem ser direta ou indiretamente responsáveis pelo prejuízo cognitivo (Rapp et al., 2008).

O processo neuroinflamatório também pode levar ao aumento da produção de ERO, as quais são prejudiciais para os neurônios. As mitocôndrias são vítimas de extensivo dano oxidativo, enquanto a inflamação provavelmente também contribui intensificando a disfunção mitocondrial (Witte et al., 2010). Os neurônios são vulneráveis a defeitos na função mitocondrial porque requerem altos níveis de energia para sua sobrevivência e função especializada (Chen e Chan, 2006). Um grande número de evidências suporta o papel crucial da disfunção mitocondrial no desenvolvimento de prejuízos cognitivos e progressão para demência; com a diminuição no metabolismo energético e produção de ERO como principais eventos desencadeadores (Lin e Beal, 2006; Moreira et al., 2010). A dieta hipercolesterolêmica promoveu um aumento de 10 vezes no colesterol dos camundongos LDLr$^{-/-}$, o que provocou uma redução na atividade dos complexos I e II no córtex cerebral destes camundongos. De particular importância, nossos resultados apresentam evidência que esta inibição na atividade dos complexos I e II foi negativamente correlacionada com os níveis plasmáticos de colesterol. No entanto, os camundongos LDLr$^{-/-}$ alimentados com dieta padrão apesar de apresentarem prejuízo cognitivo; uma elevação de duas vezes nos níveis de colesterol plasmático não foi capaz de causar disfunção mitocondrial, bem como estresse oxidativo. Portanto, o aumento do colesterol sérico com o auxílio de uma dieta rica em colesterol acelera as consequências de um nocivo ciclo oxidativo e inflamatório na vasculatura.

Recentes estudos conduzidos em mitocôndrias isoladas de vários tecidos (cérebro, fígado e rim) de camundongos LDLr$^{-/-}$ demonstraram que a disfunção mitocondrial e estresse oxidativo causados pela hipercolesterolemia são devido ao baixo conteúdo de nucleotídeos piridinas no estado reduzido (NADPH), os quais são depletados pela lipogênese aumentada (Oliveira et al., 2005; Paim et al., 2008). As mitocôndrias são ao mesmo tempo alvos e fontes biologicamente importantes de ERO principalmente no SNC (Kowaltowski e Vercesi, 1999; Kowaltowski et al., 2001) e por outro lado, o aumento dos níveis de ERO causa disfunção mitocondrial (Tan et al., 1998). Entretanto, a produção de ERO aumenta quando os complexos respiratórios estão inibidos (Leonard e Schapira, 2000; Fontanesi et al., 2009).

Estresse oxidativo celular e suas consequências são também características bem estabelecidas da patofisiologia de diversas patologias neurodegenerativas, e o SNC é especialmente susceptível ao

dano induzido por ERO (Harish et al., 2011; Sultana e Butterfield, 2011). Nossos resultados indicaram um desequilíbrio na atividade das enzimas GPx e GR, caracterizado por uma diminuição na atividade da GPx e um aumento da atividade da GR no córtex cerebral dos camundongos expostos à dieta hipercolesterolêmica, o que pode estar relacionado com o estresse oxidativo induzido pela hipercolesterolemia, principalmente por causar lipoperoxidação e prejudicar o sistema antioxidante dependente da GSH. Muitos estudos tem mostrado uma relação entre a exposição a dietas com alto teor de gordura e/ou colesterol e estresse oxidativo em cérebros de camundongos e ratos (Crisby et al., 2004; Montilla et al., 2006) e os nossos resultados podem estabelecer o papel da disfunção mitocondrial no consequente desequilíbrio oxidativo induzido pela hipercolesterolemia.

GSH e as GPxs constituem um dos mais importantes sistemas de defesa antioxidante (Raes et al., 1987; Brigelius-Flohe, 1999). A GSH é um importante antioxidante e sequestrador de ERO intracelular, proporcionando a célula neuronal uma importante proteção contra os danos oxidativos (Dringen e Hirrlinger, 2003). Estudos anteriores demonstraram que o sistema GSH pode ser ativado como uma resposta ao estresse oxidativo em cérebros de indivíduos com doenças neurodegenerativas (Lovell et al., 1995; Aksenov et al., 2001). Adicionalmente, a hipercolesterolemia desencadeia estresse oxidativo por aumentar a geração de ERO levando à depleção dos níveis de GSH, além de prejudicar o transporte mitocondrial de GSH, resultando em depleção de GSH mitocondrial e estresse oxidativo mitocondrial (Colell et al., 1997; Mari et al., 2008; Fernandez et al., 2009). Corroborando com estas informações, Esposito e colaboradores (2000) demonstraram que mitocôndrias isoladas de fígados de camundongos deficientes de GPx-1 têm altas taxas de produção de H_2O_2 e reduzida taxa de respiração mitocondrial. A GPx é um enzima de defesa antioxidante que pode diretamente detoxificar H_2O_2 ou hidroperóxidos à custa de GSH. Várias evidências propõem que a GPx funciona como uma proteção primária contra estresse oxidativo agudo, principalmente em situações neuropatológicas (Brigelius-Flohe, 1999; De Haan et al., 2003; Ran et al., 2006). De acordo com nossos dados podemos indicar uma nova evidência sobre a associação entre níveis plasmáticos elevados de colesterol e prejuízos cognitivos em camundongos $LDLr^{-/-}$ hipercolesterolêmicos, o que foi mediado em parte pela disfunção mitocondrial e estresse oxidativo também encontrados no córtex cerebral destes camundongos expostos à uma dieta com alto teor de colesterol.

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, 2011). Dentre estes compostos, o $(PhSe)_2$, primeiramente utilizado como intermediário em reações de química orgânica, demonstrou ações farmacológicas interessantes em diversos modelos experimentais relacionados à produção exacerbada de espécies reativas (Wilson et al., 1989; Nogueira et al., 2004; Nogueira e Rocha, 2010), especialmente em modelos de hipercolesterolemia, apresentando um potencial efeito protetor em doenças cardiovasculares (de Bem et al., 2008; de Bem et al., 2009; Hort et al., 2011). O conjunto de dados apresentados no manuscrito 1 indicam um significativo efeito neuroprotetor do tratamento com $(PhSe)_2$ sobre o estresse oxidativo induzido pela hipercolesterolemia no córtex cerebral dos camundongos $LDLr^{-/-}$ expostos à dieta hipercolesterolêmica. Este efeito antioxidante foi relacionado ao aumento dos níveis de GSH e diminuição da lipoperoxidação.

O $(PhSe)_2$, atua como mimético da GPx, reagindo com tióis para gerar selenol, o intermediário responsável pelo efeito antioxidante (Mugesh e Singh, 2000). De fato, Posser e colaboradores (2008) demonstraram efeito neuroprotetor do $(PhSe)_2$ em fatias hipocampais expostas ao dano oxidativo induzido pelo H_2O_2, e este efeito foi relacionado a sua atividade mimética da GPx. Nesse sentido, o $(PhSe)_2$ pode exercer sua proteção por decompor lipoperóxidos. O tratamento oral de 30 dias com $(PhSe)_2$ na dose de 1 mg/kg, não foi capaz de modificar a atividade da GPx no córtex cerebral dos camundongos $LDLr^{-/-}$ expostos à dieta hipercolesterolêmica, no entanto aumentou os níveis de GSH, seu substrato, o que possivelmente contribui para o aumento da detoxificação dos produtos de lipoperoxidação, diminuindo os níveis de MDA. Além disso, a diminuição na atividade da GR, enzima responsável por regenerar a forma reduzida da GSH, também foi observada no córtex cerebral dos camundongos $LDLr^{-/-}$ expostos à dieta hipercolesterolêmica tratados com $(PhSe)_2$. Esta redução pode ser resultado do aumento na síntese de GSH nestes tecidos, podendo estar relacionada ao aumento da atividade da enzima gama-glutamilcisteína sintetase, passo limitante na síntese de GSH. Desta forma, acreditamos que os efeitos protetores do $(PhSe)_2$ frente ao estresse oxidativo induzido pela hipercolesterolemia no córtex cerebral dos camundongos $LDLr^{-/-}$ expostos à dieta hipercolesterolêmica vão além de sua atividade mimética da GPx.

Como já demonstrado recentemente para o ebselen, outro importante composto orgânico de selênio (Tamasi et al., 2004; Kim et al., 2009); acreditamos que o $(PhSe)_2$ possa ser capaz de promover a translocação do fator Nrf-2, sinalizando diversas respostas celulares vinculadas à ativação dos elementos de resposta antioxidante (ARE), entre elas o aumento nos níveis de GSH e na atividade de enzimas antioxidantes. A hipótese para este mecanismo é que o $(PhSe)_2$, da mesma forma que o ebselen, reage com grupamentos sulfidrila da proteína inibitória Keap-1, liberando o Nrf-2 e ativando vias de sinalização vinculadas ao sistema redox (Zhao e Holmgren, 2002; Tamasi et al., 2004). Confirmando nossos dados, estudos anteriores verificaram um aumento na concentração de GSH em diferentes tecidos, em animais tratados com $(PhSe)_2$ e expostos a danos oxidativo (Borges et al., 2006, de Bem et al., 2007; Straliotto et al., 2010). Nossos dados, constituem uma evidência do papel neuroprotetor in vivo do $(PhSe)_2$ sobre o estresse oxidativo induzido pela hipercolesterolemia no córtex cerebral dos camundongos $LDLr^{-/-}$ expostos à dieta hipercolesterolêmica. E este efeito antioxidante do $(PhSe)_2$ em parte está associado com o envolvimento do sistema GSH. Portanto, em adição aos seus efeitos protetores demonstrados anteriormente em modelos de doenças cardiovasculares desencadeadas pela hipercolesterolemia, baseados em nossos dados podemos apontar o $(PhSe)_2$ como uma molécula neuroprotetora contra efeitos deletérios centrais induzidos pela hipercolesterolemia.

Finalmente, os resultados obtidos neste trabalho de mestrado apontam a utilização dos camundongos $LDLr^{-/-}$ hipercolesterolêmicos como um modelo para o entendimento dos mecanismos envolvidos na patogênese de demências como a DA, bem como o composto orgânico de selênio, $(PhSe)_2$ como um efetivo agente neuroprotetor.

6 CONCLUSÕES

Os resultados obtidos neste trabalho indicam que:

- Os camundongos $LDLr^{-/-}$ hipercolesterolêmicos independentemente da dieta adotada apresentam prejuízo cognitivo em teste de memória espacial;
- A exposição de camundongos $LDLr^{-/-}$ à uma dieta hipercolesterolêmica durante 30 dias leva à diminuição da atividade dos complexos I e II da cadeia respiratória mitocondrial no córtex cerebral destes camundongos;
- A função mitocondrial nos camundongos $LDLr^{-/-}$ foi negativamente correlacionada com os respectivos níveis de colesterol destes camundongos;
- A disfunção cognitiva e mitocondrial nos camundongos $LDLr^{-/-}$ expostos à uma dieta hipercolesterolêmica foram associadas com um quadro de estresse oxidativo no córtex cerebral destes camundongos caracterizado por um desequilíbrio no sistema antioxidante vinculado a glutationa bem como peroxidação lipídica;
- O tratamento com composto orgânico de selênio, $(PhSe)_2$ (1 mg/kg) durante 30 dias preveniu o estresse oxidativo no córtex cerebral de camundongos $LDLr^{-/-}$ expostos à uma dieta hipercolesterolêmica.

7 PERSPECTIVAS

Alguns aspectos relacionados a este trabalho ainda precisam ser melhor elucidados, desta forma, nosso grupo propõe algumas perspectivas:

- Primeiramente, avaliar o efeito do $(PhSe)_2$ contra o prejuízo cognitivo e a disfunção mitocondrial induzida pela dieta hipercolesterolêmica em camundongos $LDLr^{-/-}$;
- Analisar a influência da hipercolesterolemia na toxicidade induzida pelo peptídeo β-amilóide em camundongos $LDLr^{-/-}$, através da injeção intracerebroventricular do peptídeo β-amilóide, a qual é uma ferramenta amplamente utilizada como modelo de Doença de Alzheimer em roedores;
- Desenvolver cultivo primário de neurônios hipocampais de camundongos $LDLr^{-/-}$, para analisar a resposta dos mesmos a toxicidade do peptídeo beta-amilóide.

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