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Atherosclerosis xxx (2008) xxx–xxx

ATHEROSCLEROSIS

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Diphenyl diselenide, a simple glutathione peroxidase mimetic, inhibits human LDL oxidation *in vitro*

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Received 29 August 2007; received in revised form 27 February 2008; accepted 29 February 2008

Abstract

Oxidative modification of low-density lipoprotein (LDL) represents an important factor in atherogenesis. In the present study, we have investigated the antioxidant capability of diphenyl diselenide $(PhSe)_2$, a simple organoseleno compound, against copper (Cu^{2+}) and peroxyl radical-induced human LDL oxidation *in vitro*. In initial studies using human serum, $(PhSe)_2$ caused a dose-dependent inhibition of Cu^{2+}-induced lipid peroxidation, which was correlated to thiol consumption. $(PhSe)_2$ increased lipid peroxidation *lag phase* and decreased lipid peroxidation *rate* in isolated human LDL, evaluated by measuring both conjugated diene (CD) and thiobarbituric acid reactive substances (TBARS) levels. Consistent with these observations, $(PhSe)_2$ showed a marked inhibitory effect on 2,2-azobis(2-amidinopropane dihydrochloride) (AAPH)-induced oxidation of LDL or parinaric acid (PnA) incorporated into LDL. $(PhSe)_2$ also displayed a dose-dependent protective effect against Cu^{2+}-induced lipid peroxidation in rat aortic slices. Interestingly, besides the antioxidant effects of $(PhSe)_2$ toward the lipid moieties of LDL, which was related to its thiol-peroxidase activity, protein moieties from human isolated LDL were also protected against Cu^{2+}-induced oxidation. The results presented herein are the first to show that (i) $(PhSe)_2$ inhibits lipid peroxidation in human isolated LDL *in vitro*, (ii) this phenomenon is related to its thiol-peroxidase activity, and (iii) this chalcogen also prevents the oxidation of protein moieties of human LDL. Taken together, such data render $(PhSe)_2$ a promising molecule for pharmacological studies with respect to the atherogenic process.

Keywords: Diphenyl diselenide; Selenium; Ebselen; Glutathione peroxidase; LDL oxidation; Atherosclerosis

1. Introduction

Atherosclerosis remains the most common cause of death in industrialized countries. This affects the vascular wall and leads to coronary artery diseases and cerebrovascular accidents (stroke) [1]. There is increasing evidence that oxidative modifications of low-density lipoprotein (LDL) play a pivotal role in the development of atherosclerosis. Moreover, elevated levels of oxidized LDL have been positively correlated to the severity of acute coronary events and have been considered a biochemical marker for coronary heart disease [2].

Although the molecular bases related to the triggering process of LDL oxidation remain unclear, the major mechanisms currently explored are metal ions dyshomeostasis, changes in lipoxygenase- and myeloperoxidase-related pathways, reactive oxygen and nitrogen species generation, and alterations in the thiol status [3].

Since LDL oxidation plays a key role in the pathogenesis of atherosclerosis, antioxidants that can inhibit this oxidative process might be useful in preventing atherosclerosis-related pathological conditions, such as coronary artery diseases and

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doi:10.1016/j.atherosclerosis.2008.02.030

stroke [4]. In this regard, it has been evidenced that antioxidant capability of LDL can be easily increased by dietary antioxidant supplementation. In fact, many endogenous and exogenous compounds have been reported to display beneficial effects against LDL oxidation [5].

Selenium (Se) is an essential nutrient associated with the function of major metabolic pathways in the cell, where it is incorporated as selenocysteine at the active site of a wide range of proteins [6]. Selenium intake is inversely correlated to the incidence of atherosclerosis and coronary heart disease [7]. In endothelial cells, selenium can protect from oxidative damage by altering the expression of selenoproteins which have antioxidant function, including cytoplasmic glutathione peroxidase (cyGPX), phospholipid hydroperoxide glutathione peroxidase (PHGPx) and thioredoxin reductase (TR) [8].

The interest in organoselenium chemistry and biochemistry has increased in the last three decades mainly due to the fact that several organoselenium compounds possess antioxidant activity [9], which is interplayed with its glutathione-peroxidase-like activity [10,11]. The first example of such a compound was ebselen [12,13], and this compound has been extensively studied in reason of its antioxidants and anti-inflammatory properties [14,15]. Considering the pharmacological properties of ebselen, we have been studying the pharmacological properties of another organoselenium compound, diphenyl diselenide $(PhSe)_2$, which also exhibits thiol-peroxidase-like activity which is about two times that of ebselen [10,16].

This organoselenium caused minimal toxicity when administrated acutely to mice and rats [17] and after a long term exposure to rabbits [18,19] and has been reported to present lower toxicity than ebselen [11]. Recently, studies from our laboratory have demonstrated that $(PhSe)_2$ caused a significant reduction in blood glucose and glycated protein levels in diabetic rats [20]. Moreover, the *in vitro* and *in vivo* antioxidant potential of $(PhSe)_2$ was already demonstrated in several tissues and models, including brain, liver and platelets [11,21].

Given the earlier observations, the present study was carried out to evaluate the potential beneficial effects of $(PhSe)_2$ in protecting *in vitro* oxidation using different methodological approaches: human serum, human isolated LDL, and rat aortic slices (rich in endothelial cells), which are the main elements involved in the early atherosclerosis development *in vivo*. The thiol-peroxidase capability of $(PhSe)_2$ was also evaluated in an attempt to delve into molecular mechanisms related to the aforementioned antioxidant effects.

2. Materials and methods

2.1. Materials

Diphenyl diselenide $(PhSe)_2$ was synthesized according to a method previously described [22]. Analyses of the ^1H NMR and ^{13}C NMR spectra showed that the obtained compound had analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/HPLC. $(PhSe)_2$ was solubilized in ethanol PA and a 10-mM stock solution was stored at 4 °C for less than 2 weeks. Immediately before use, $(PhSe)_2$ was diluted in ethanol PA at the required concentrations for the different assays. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Serum oxidation

The study was approved by our Ethic Committee at Universidade Federal de Santa Maria. Blood samples were collected from healthy and normolipidemic volunteers after a 12-h overnight fasting period. Samples were left to clot in the dark at room temperature for 30 min and then centrifuged at 1500 × *g* for 15 min. The serum was removed and immediately used in the oxidation assays and oxidations were performed in serum rather than in plasma to avoid potential interferences of substances such as EDTA, heparin, or citrate. Briefly, serum samples were diluted 1:4 in 10 mM potassium phosphate buffer, pH 7.4 and incubated at 37 °C with $CuSO_4$ (100 μM) and/or $(PhSe)_2$ (0–200 μM). The total volume was 9 mL. At different time points, aliquots (200 μL) were removed for evaluating thiobarbituric acid reactive substances (TBARS) levels and consumption of the total thiol groups (–SH). Serum TBARS levels and thiol groups were determined based on Ohkawa et al. [23] and Ellman [24], respectively.

2.3. Thiol-peroxidase activity of $(PhSe)_2$ and spectroscopy studies

The thiol-peroxidase activity of $(PhSe)_2$ was measured according to a method previously described by Wilson et al. [10]. $(PhSe)_2$ (50 μM) was incubated at 37 °C in a medium containing 50 mM potassium phosphate buffer, pH 7.0, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM reduced glutathione (GSH), 1 U of GR and 0.25 mM NADPH (final volume of reaction = 1 mL). The reaction was initiated by addition of 0.5 μmol of hydrogen peroxide. The activity was followed by the decrease of NADPH absorption at 340 nm. Appropriate controls were carried out without $(PhSe)_2$ and were subtracted.

In order to evaluate the potential chemical interaction between $(PhSe)_2$, GSH and H_2O_2, $(PhSe)_2$ (20 μM) was incubated with GSH (200 μM) in 10 mM potassium phosphate buffer (pH 7.4). The reaction was performed at 37 °C in a quartz cuvette and monitored spectrophotometrically (250–400 nm) using a PerkinElmer Lambda 6 spectrophotometer. In additional experiments, H_2O_2 (200 μM) was added 1 min after the reaction of $(PhSe)_2$ with GSH. The reaction mixture had 2 mL. Temperature was maintained using a circulating water bath. Readings were done against a reference cuvette containing phosphate buffer.

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2.4. LDL isolation

LDL was isolated from fresh human plasma by discontinuous density-gradient ultracentrifugation as described by Silva et al. [25], with slight modifications. Plasma of non-fasted healthy normolipidemic donors collected with EDTA (1 mg/mL) was pooled and sucrose (final concentration, 0.5%) was added to prevent LDL aggregation. Five milliliters of EDTA–plasma adjusted to a density of 1.22 g/mL with solid KBr (0.326 g/mL) was layered on the bottom of a centrifuge tube. Then 5 mL EDTA-containing sodium chloride solution (density 1.006 g/mL) was overlaid on top of the plasma. Ultracentrifugation was run at 65,000 rpm for 2 h at 4 °C, in a Hitachi ultracentrifuge. LDL particles were collected by aspiration of the yellow band, which is located in the middle of sodium chloride solution just above the plasma main fractions. Then, 4 mL of LDL particles was dialyzed exhaustively overnight at 4 °C against 6 L of 10 mM phosphate buffer to remove the excess salt and the majority of the EDTA. Protein concentration in LDL solution was determined by Lowry et al. [26]. The purity of LDL preparation was verified by agarose gel electrophoresis. Isolated LDL was stored at −20 °C not longer than 2 weeks.

2.5. LDL oxidation

Incubation of LDL with copper ions initiates lipid peroxidation and causes extensive oxidation of the LDL lipids. Cu^{2+}-mediated oxidation is frequently used to assess the susceptibility of LDL to oxidation which is regarded as a possible risk factor for atherosclerosis [27].

LDL oxidation was monitored by following the CD formation and the TBARS production. LDL samples (50 μg protein/mL) were pre-incubated at 37 °C in a medium containing 10 mM potassium phosphate buffer, pH 7.4 and different concentrations of $(PhSe)_2$ (0–40 μM). After 10 min, $CuSO_4$ (1.6, 5 or 10 μM) was added to the reaction medium and aliquots were removed at different time points for evaluating CD and TBARS production. In another set of experiments, $(PhSe)_2$ (40 μM) was added at different time points (0, 45 or 90 min) in an attempt to evaluate whether $(PhSe)_2$ could inhibit Cu^{2+}-induced LDL oxidation once the process was started.

Potential antioxidant effects of $(PhSe)_2$ against LDL oxidation were also evaluated after the addition of an azo initiator, 2,2-azobis(2-amidinopropane dihydrochloride) (AAPH) (1 mM), whose slow decomposition generates peroxyl radical at a constant rate in aqueous medium and induces the chain oxidation of human LDL by a free radical mediated mechanism.

In the studies of CD formation, the temporal change in absorbance at 234 nm (which refers to the CD concentration) is divided into three phases: a lag phase, a propagation phase, and a terminal phase. The lag phase was defined as the intercept of the tangent of the slope of the absorbance curve in propagation phase with the time axis, and was expressed in min. The rate of propagation was obtained from the slope of the absorbance curve during the propagation phase, and the terminal phase corresponds to the extensive oxidation of lipids of LDL. Absorbance at 234 nm was normalized with respect to the absorbance at the beginning of the oxidation process.

2.6. Measurement of parinaric acid (PnA) fluorescence

PnA is a polyunsaturated fatty acid that has been used successfully as a fluorescent probe to monitor the initial stages of lipid peroxidation by peroxyl radicals from AAPH [28]. *Cis*-PnA (9,11,13,15-octadecatetraenoic acid) fluorescence was monitored in a PerkinElmer LS 50 spectrofluorometer provided with a thermostated cuvette containing a magnetic stirring device. The excitation and emission wavelengths were 324 and 413 nm, respectively (slit widths: 3.5 nm).

Preliminary studies were performed in order to determine the concentration of LDL and PnA that insure totally incorporation of the probe into LDL and a linear fluorescent response with PnA concentration. The assays were performed at 37 °C in 2 mL of potassium phosphate buffer (110 mM NaCl, 20 mM phosphate, pH 7.4) containing 45 μg of LDL protein and an aliquot of ethanolic solution of PnA (1.5 μM final concentration). The incorporation of the probe was carried out by gentle stirring for 1 min. Increasing concentrations of $(PhSe)_2$ were added to the mixture and the oxidation reaction was initiated by addition of AAPH (10 mM final concentration). The capacity of $(PhSe)_2$ to protect LDL from oxidation was determined by the inhibition of probe fluorescence decay [28].

2.7. Measurement of LDL-Trp fluorescence

The time course of tryptophan (Trp) fluorescence emission intensity is used to monitor Cu^{2+}-induced apolipoprotein LDL oxidation. The fluorescence spectra of native LDL display a single band centered at approximately 332 nm, which is assigned to the Trp residues in apo B. Loss of Trp fluorescence is a marker for oxidations at the protein core of LDL [29]. Cu^{2+}-induced LDL oxidation was performed in a similarly way to item described in Section 2.5, excepting that 3.3 μM $CuSO_4$ and different $(PhSe)_2$ concentrations (0–30 μM) were used. Trp fluorescence was measured at different time points (0–360 min) using a Shimatzo spectrofluorometer (excitation at 282 nm and emission at 331 nm).

2.8. Aortic slices oxidation

Adult Wistar rats were decapitated, thoracic aorta were quickly dissected and then removed, rinsed and submerged in a petri dish filled with ice-cold isosmotic phosphate buffer (NaCl 124 mM, Na_2HPO_4 10 mM, NaH_2PO_4 5 mM, KH_2PO_4 5 mM, glucose 10 mM, pH 7.4) and cleaned of adherent fat and connective tissue in an ice-bath. Transverse propsections (400 μm) were prepared using a McIlwain

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tissue chopper. Aortic slices (10 slices) were incubated in isosmotic phosphate buffer with 20 or 40 μM of $(PhSe)_2$ and/or 10 μM $CuSO_4$ for 2 h. After incubations, aorta slices were washed with saline and homogenized in 400 μL of acetic acid buffer (pH 3.5). Lipid oxidation in aorta homogenates was measured by determining TBARS, as described by Ohkawa et al. [23]. Experimental procedures involving animals were approved by the local Animal Care Committee.

2.9. Statistical analysis

Values are expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) was used for multiple comparisons, followed by the Duncan's multiple range test when the *F*-value was significant. Only significant *F*-values are given in the text. Linear regression analysis was also used to test dose-dependent effects. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1. Effects of $(PhSe)_2$ on serum oxidation

The protective effects of $(PhSe)_2$ against Cu^{2+}-induced TBARS generation and thiol consumption in blood serum are depicted in Fig. 1A. Cu^{2+} (100 μM) caused a time-dependent increase of serum TBARS levels (Fig. 1A, right, *Y* axis) and a time-dependent decrease of total thiol groups (Fig. 1A, left, *Y* axis). Interestingly, even though $(PhSe)_2$ displayed a concentration-dependent inhibitory effect toward Cu^{2+}-induced TBARS generation ($F_{(4,10)} = 21.18$; $p < 0.001$; $\beta = -0.877$), there was a stimulation of thiol groups consumption by $(PhSe)_2$.

3.2. Spectroscopy studies

In order to delve into molecular mechanisms involved with the beneficial role of $(PhSe)_2$ against LDL lipid peroxidation, *in vitro* experiments of light/UV spectroscopy concerning the chemical interaction between $(PhSe)_2$, GSH and H_2O_2 were investigated spectrophotometrically. The characteristic spectra of 20 μM $(PhSe)_2$ (line A, Fig. 1B) and of 200 μM GSH (line B, Fig. 1B) was changed after their reaction (line C, Fig. 1B), probably due to the formation of phenyl selenol intermediate ($PhSe^-$). Interestingly, after the addition of H_2O_2 (200 μM), the strong and broad absorption peak at 270 nm of such intermediate was abolished, indicating its chemical interaction with H_2O_2 (Fig. 1B, spectrogram D). Consistent with this observation, $(PhSe)_2$ displayed a concentration-dependent glutathione peroxidase-like activity (Fig. 1B, inset), which was indirectly measured by NADPH consumption in the presence of GSH, purified

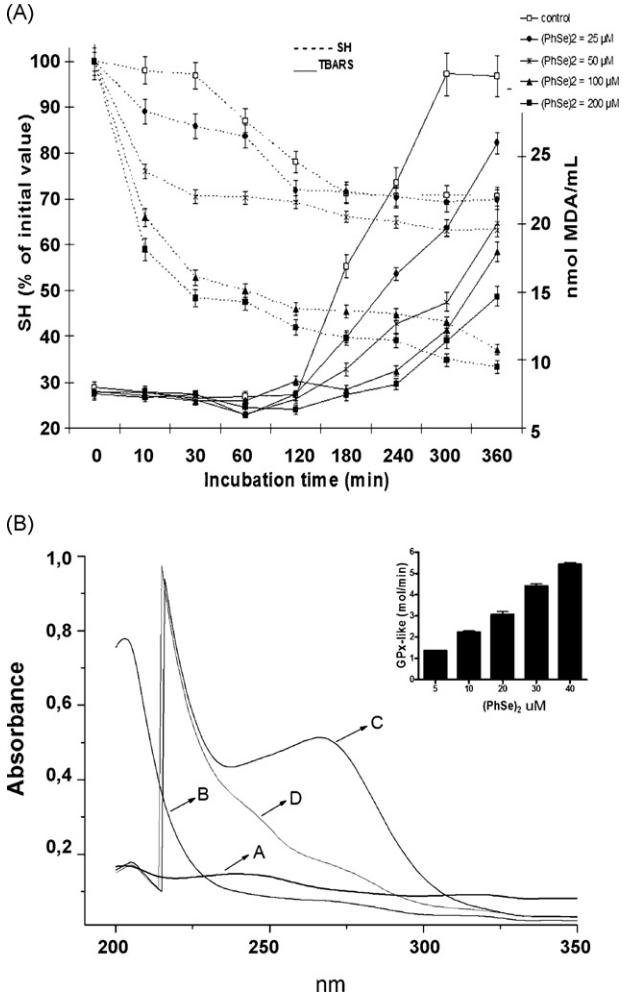

Fig. 1. (A) Effects of $(PhSe)_2$ on Cu^{2+}-induced lipid peroxidation and thiol consumption in human serum. At different time points (indicated in the abscissa axis) TBARS (solids line) and SH (broken lines) content were analyzed (see Section 2). TBARS levels are expressed as nmol of malondialdehyde (MDA). Total thiol (–SH) content is expressed as percent of control, whose basal concentration was 437 ± 25 nmol/mL. Results are represented as mean ± standard error of mean (S.E.M.) from at least three independent experiments. (B) The comparative spectra of $(PhSe)_2$ and the products of its interaction with GSH and/or H_2O_2. (A) $(PhSe)_2$ (20 μM); (B) GSH (200 μM); (C) $(PhSe)_2$ (20 μM) + GSH (200 μM) + 1 min at 37 °C; (D) $(PhSe)_2$ (20 μM) + GSH (200 μM) + 1 min + H_2O_2 (200 μM) + 1 min at 37 °C. Spectra of 20 μM $(PhSe)_2$ or 200 μM GSH were not modified by addition of 200 μM H_2O_2. For details, see Section 2. The inset graph shows glutathione peroxidase activity of $(PhSe)_2$.

GSH reductase and hydrogen peroxide. The peroxidase-like activity of $(PhSe)_2$ was approximately twice when compared to that of ebselen (data not show), these results are in agreement with the previous study of Wilson et al. [10], where they showed that $(PhSe)_2$ is about 1.6 times more effective as GPx-mimetic than ebselen. Moreover, $(PhSe)_2$ catalytically increased GSH oxidation in the presence of H_2O_2 in a concentration-dependent manner (data not show). Taken together, these data indicate that the chemical interaction between $(PhSe)_2$ and GSH produces an intermediate that is capable of interacting with peroxides. This process allows for

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the detoxification of peroxides at the expenses of sulfhydryl groups from GSH.

3.3. Effects of $(PhSe)_2$ on the LDL oxidation

3.3.1. $(PhSe)_2$ effects on AAPH-mediated lipid LDL oxidation

The effects of $(PhSe)_2$ on AAPH-mediated lipid LDL oxidation is depicted in Fig. 2A. AAPH caused a significant increase of CD formation within time (Fig. 2A). $(PhSe)_2$ (3–50 μM) showed a marked inhibitory effect on AAPH-induced lipid LDL oxidation in a concentration-dependent manner. In fact, a significant negative correlation ($F_{(7,16)} = 83.21$; $p < 0.001$; $\beta = -0.944$) was observed for $(PhSe)_2$ concentrations and CD levels (inset Fig. 2A).

3.3.2. $(PhSe)_2$ effects on PnA fluorescence

Fig. 2B shows that low concentrations of $(PhSe)_2$ (2–4 μM) caused a concentration-dependent inhibition ($F_{(3,8)} = 684.27$; $p < 0.001$; $\beta = 0.215$) of the fluorescence intensity decay of PnA in the presence of AAPH when compared to the control condition (absence of $(PhSe)_2$). The inset of Fig. 2B indicates a typical control assay where the decrease of the fluorescence of PnA following the addition of AAPH reflects its oxidative degradation.

3.3.3. $(PhSe)_2$ effects on Cu^{2+}-induced LDL oxidation

The effects of $(PhSe)_2$ on Cu^{2+}-induced lipid oxidation in isolated LDL are depicted in Fig. 3. $(PhSe)_2$ inhibited Cu^{2+}-induced generation of both CDs (Fig. 3, left) and TBARS (Fig. 3, right) in a concentration-dependent manner. Cu^{2+} displayed concentration-dependent oxidative effects toward lipids, evaluated by measuring CDs (Fig. 3). LDL oxidation showed an expected oxidation pattern of an initial *lag phase* followed by a *propagation phase* and *a decomposition phase* (Fig. 3, left), as originally described by Esterbauer et al. [4]. Interestingly, $(PhSe)_2$ caused concentration-dependent increases in *lag phase* and decreases in the *oxidation rate*, evidenced by changes in the propagation phase slope (Cu^{2+} 1.6 μM: $F_{(3,8)} = 104.05$; $p < 0.001$; $\beta = -0.910$; Cu^{2+} 5 μM: $F_{(3,8)} = 650.72$; $p < 0.001$; $\beta = -0.956$; Cu^{2+} 10 μM: $F_{(3,8)} = 41.46$; $p < 0.001$; $\beta = -0.932$).

In another set of experiments, $(PhSe)_2$ (40 μM) was added at different time points (0, 45 or 90 min) in an attempt to evaluate whether $(PhSe)_2$ could inhibit Cu^{2+}-induced lipid LDL oxidation once the process was started. Fig. 4A shows a significant inhibitory effect of $(PhSe)_2$ toward Cu^{2+}-induced lipid LDL oxidation when present at the beginning of the oxidation process. Interestingly, $(PhSe)_2$ inhibited Cu^{2+}-induced lipid LDL oxidation when added to the reaction medium at 45 and 90 min after the oxidation process was started.

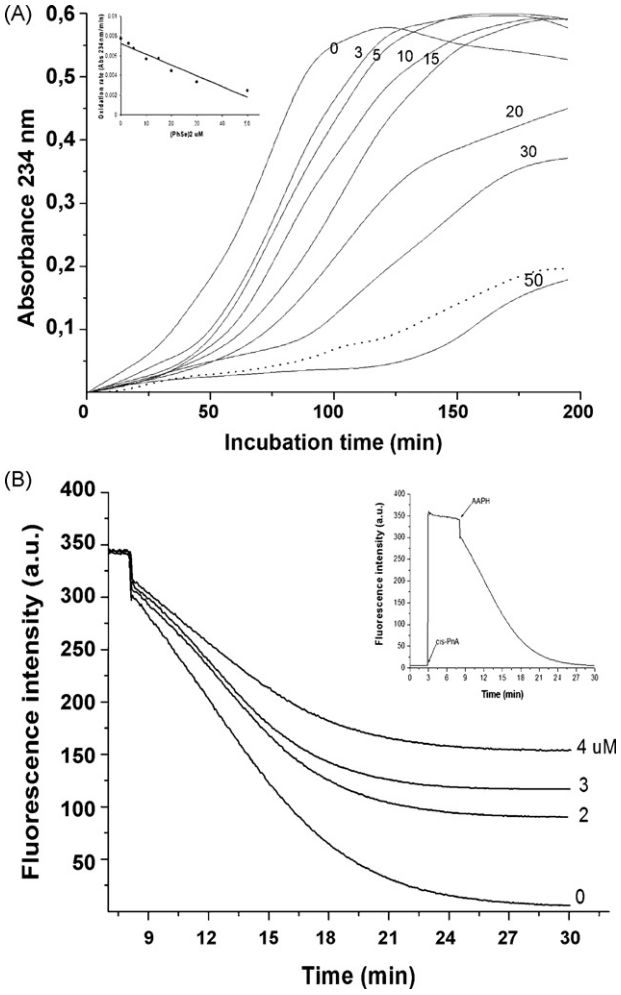

Fig. 2. (A) Effects of $(PhSe)_2$ on AAPH-induced conjugated dienes formation in human LDL. At different time points, conjugated dienes were analyzed. LDL samples (50 μg protein/mL) were incubated at 37 °C with 1.0 mM AAPH in the absence (control) or presence (3–50 μM) of $(PhSe)_2$ (broken lines represent control conditions without Cu^{2+} addition). Conjugated dienes are expressed as absorbance at 234 nm. Inset shows the significant correlation between oxidation rate and $(PhSe)_2$ concentrations ($F_{(7,16)} = 83.21$; $p < 0.001$; $\beta = -0.944$). (B) Effect of $(PhSe)_2$ on AAPH-induced oxidation of PnA incorporated into LDL. LDL samples (45 μg protein) were incubated at 37 °C with 1.5 μM PnA for 1 min under gentle stirring. AAPH (1.0 mM) and $(PhSe)_2$ (0, 2, 3, or 4 μM; 3 min after AAPH addition) were added to the reaction medium. Inset shows a typical control assay of PnA oxidation, incorporated into LDL, initiated by AAPH. The fluorescence intensity was recorded up to 30 min and shows the initial light scattering and the fluorescent signal decay following AAPH addition. Results are derived from a single representative experiment. Experiments were repeated at least three times, showing similar results.

3.4. Effects of $(PhSe)_2$ on the LDL-Trp fluorescence emission

Fig. 4B shows that protein moieties of LDL are oxidized within time in the presence of $CuSO_4$ (3.3 μM). This phenomenon was prevented by $(PhSe)_2$ in a concentration-dependent manner ($F_{(3,8)} = 104.05$; $p < 0.001$; $\beta = 0.954$). It is noteworthy that Cu^{2+}-induced apolipoprotein LDL oxida-

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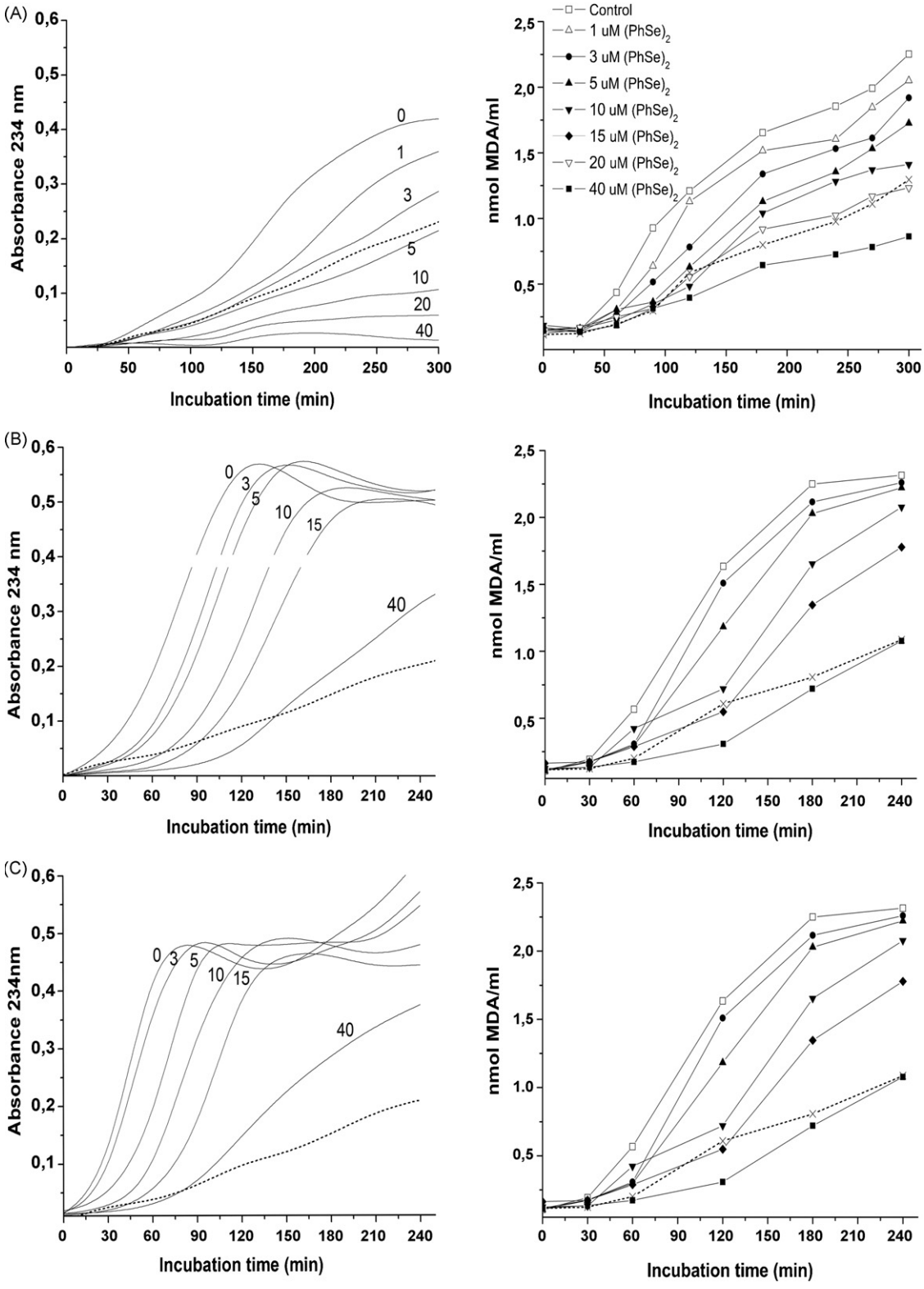

Fig. 3. Effects of $(PhSe)_2$ on Cu^{2+}-induced lipid peroxidation in human LDL. At different time points (indicated in the abscissa axis), conjugated dienes (left) and TBARS (right) were analyzed. LDL samples (50 μg protein/mL) were incubated in the presence of 1.6 μM (A), 5.0 μM (B) and 10 μM (C) $CuSO_4$ and in the absence (control) or presence (1–40 μM) of $(PhSe)_2$ (broken lines represent control conditions without Cu^{2+} addition). TBARS levels are expressed as nmol of malondialdehyde (MDA). Conjugated dienes are expressed as absorbance at 234 nm. Results are represented as mean ± standard error of mean (S.E.M.) from at least three independent experiments.

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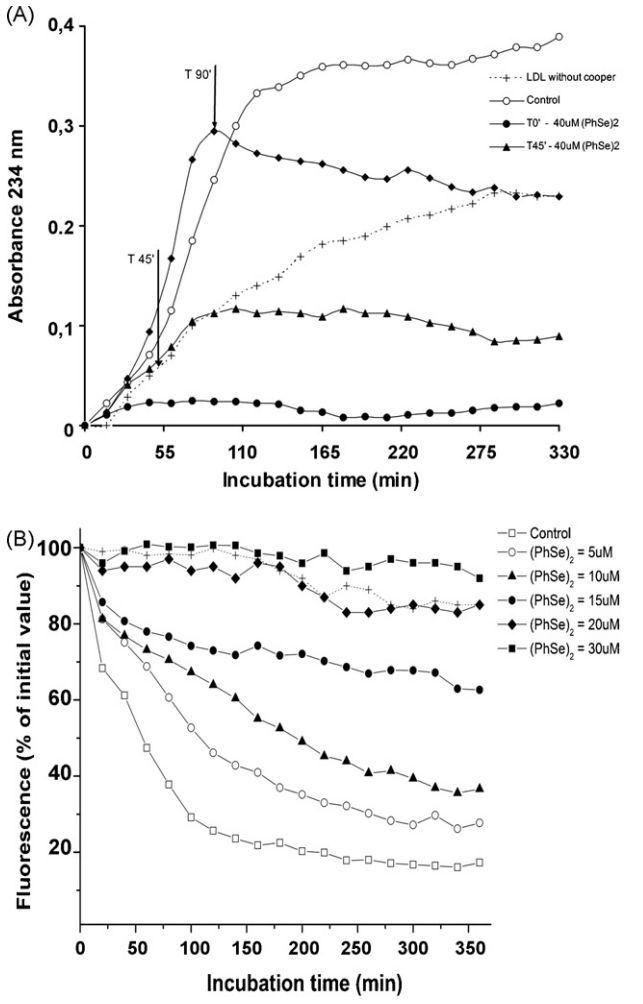

Fig. 4. (A) Effects of $(PhSe)_2$ on Cu^{2+}-induced conjugated dienes formation in previously oxidized human LDL. LDL samples (50 μg protein/mL) were incubated at 37 °C in the presence of 1.6 μM $CuSO_4$ and in the absence (control) or presence (40 μM) of $(PhSe)_2$, which was added at 0, 45 or 90 min after $CuSO_4$ addition. At different time points (indicated in the abscissa axis), conjugated dienes were analyzed. Results are expressed as absorbance at 234 nm. Results are derived from a single representative experiment. Experiments were repeated at least three times, showing similar results. (B) Effects of $(PhSe)_2$ on Cu^{2+}-induced loss of tryptophan fluorescence in human LDL. LDL samples (50 μg protein/mL) were incubated at 37 °C in the presence of 3.3 μM $CuSO_4$ and different $(PhSe)_2$ concentrations (0–30 μM). Tryptophan fluorescence (excitation at 282 nm and emission at 331 nm) was measured at different time points (0–360 min) (broken lines represent control conditions without Cu^{2+} addition). Data are expressed as percentage of the emission intensity measured before Cu^{2+} addition. Results are represented as mean ± standard error of mean (S.E.M.) from at least three independent experiments.

tion was almost completely abolished in the presence of 20 and 30 μM of $(PhSe)_2$.

3.5. Aortic slices oxidation

The effects of $(PhSe)_2$ on Cu^{2+}-induced lipid peroxidation in rat aortic slices are depicted in Fig. 5. One-way ANOVA showed that 10 μM Cu^{2+} increased ($p < 0.01$) lipid peroxidation in rat aortic slices. Co-incubation of aortic rats slices with $(PhSe)_2$ for 2 h was associated with a reduction in lipid peroxidation induced by copper ions ($F_{(2,13)} = 8{,}67$; $p < 0.01$). $(PhSe)_2$ was effective in decreasing Cu^{2+}-induced TBARS formation in a concentration-dependent manner (Fig. 5; $\beta = -0.740$, $p < 0.01$).

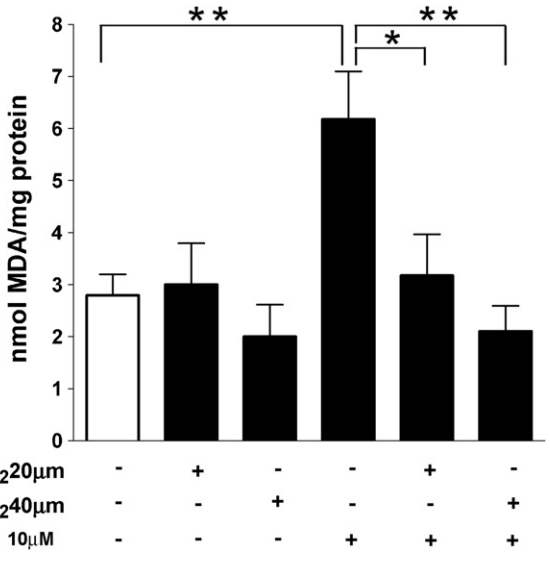

Fig. 5. Effect of $(PhSe)_2$ on Cu^{2+}-induced lipoperoxidation in rat aortic slices. Data are expressed as nmol of malondialdehyde (MDA) per mg of protein and presented as mean ± standard error of mean (S.E.M.) of 3–4 independent experiments. Controls are represented by an open bar. *$p < 0.05$; **$p < 0.01$ (One-way ANOVA followed by the Duncan's multiple range test).

4. Discussion

Oxidative damage to lipoproteins, in particular LDL, is known to play a role in a number of diseases associated with ageing and is in agreement with the oxidative stress theory of ageing [30]. Oxidized LDL is involved in the development of atherosclerosis through the formation of foam cells [31] and autoantibodies against oxidized LDL [32]. Since LDL oxidative damage is involved in the development of atherosclerosis, several recent studies have sought for the potential beneficial effects of antioxidant molecules against LDL oxidation [5,33]. Of particular importance, epidemiological studies have pointed to red wine polyphenols as promising molecules that could prevent the development of several coronary syndromes by inhibiting the atherogenic process [34].

There is considerable evidence suggesting that an elevated plasma copper concentration is associated with existing atherosclerosis and represents a factor of risk for disease in the future [35]. Catalytically active copper ion has been found in gruel taken from human advanced atherosclerotic lesions, indicating that the interior of human advanced atherosclerotic lesions is a highly pro-oxidant environment. Consequently, the use of copper ions to promote peroxidation of LDL under

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in vitro conditions can be considered a valid model for studying events occurring in atherosclerotic arterial wall [36]. In addition, AAPH-generated hydroxyl radical has been also reported as a good alternative for mimicking oxidative insults that occur toward lipid and protein moieties of LDL during the atherogenic process [37].

Even though several studies have reported the beneficial effects of organoseleno compounds against pathological conditions associated to oxidative stress (inflammation, gastric mucosal damage, neurotoxicity, and hepatotoxicity [11]), there are no reports regarding beneficial effects of $(PhSe)_2$ against human LDL oxidative damage. In fact, it appears that ebselen is the only organoseleno compound that has received considerable interest during the last decades [14,15]. In the present study, the choice for studding $(PhSe)_2$ as a potential beneficial molecule against human LDL oxidation was based on previous studies from our group, which has shown that $(PhSe)_2$ displays a higher capability of ebselen in detoxifying peroxides at expenses of sulfhydryl molecules [21].

Here, we observed that $(PhSe)_2$, an organoseleno compound with glutathione peroxidase-like activity, displayed beneficial effects against oxidation induced by copper ions or AAPH, a hydroxyl radical generator, on human serum and isolated LDL and rat aortic slices. $(PhSe)_2$ increased the oxidation lag phase and decreased the oxidation rate in isolated human LDL and these phenomena were evidenced by using different methodological approaches, including the TBARS measurement, CDs generation and incorporation of PnA into the lipoproteins.

On the other hand, $(PhSe)_2$ showed an antioxidant effect on Cu^{2+}-induced oxidation of human serum and this effect was in a concentration-dependent manner. Moreover, this effect showed a temporal correlation with the sulfhydryl groups consumption: the increase of TBARS levels started just when stopped the decrease of SH levels (120 min). This phenomenon suggests the need for available reduced sulfhydryl groups for the maintenance of the beneficial effect of $(PhSe)_2$ against Cu^{2+}-induced lipid peroxidation, reinforcing the idea of the involvement of thiol-peroxidase-like activity on the antioxidant effects of $(PhSe)_2$.

This idea is reinforced by the observed beneficial effects of $(PhSe)_2$ against the oxidation of endogenous and exogenous (incorporated PnA) LDL lipids. Inline with this, spectroscopic studies showed the direct chemical interaction between $(PhSe)_2$ and GSH, resulting in the formation of a chemical intermediary whose stability is affected by the presence of hydrogen peroxide. Furthermore, we demonstrated a concentration-dependent antioxidant effect of $(PhSe)_2$ on Cu^{2+}-oxidized rat aortic slices, indicating its antiatherogenic potential. Therefore, $(PhSe)_2$ behaves as a potent antioxidant in different models intimately related to the atherogenic process.

An interesting effect of this study was that $(PhSe)_2$ displayed significant beneficial effects in the different phases of Cu^{2+}-induced LDL oxidation. In fact, when added to the reaction medium at time zero (Fig. 3A), $(PhSe)_2$ significantly prevented LDL oxidation. Moreover, when added to the reaction medium at 45 or 90 min (partially oxidized LDL), $(PhSe)_2$ was capable of stopping CDs formation. Mechanistically, these results are of interest because they indicate that the thiol-peroxidase activity of $(PhSe)_2$ is enough important to prevent the generation of secondary products of lipid peroxidation, such as CDs.

Another significant (and maybe the most important) result from our study was the capability of $(PhSe)_2$ to prevent Cu^{2+}-induced loss of Trp fluorescence in human LDL. In this regard, it has been reported that the fluorescence spectrum of native LDL displays a single band centered at approximately 332 nm, which is assigned to the Trp residues in apo B and loss of Trp fluorescence is a marker for oxidations at the protein core of LDL [29]. The protective effect of $(PhSe)_2$ against Cu^{2+}-induced loss of Trp fluorescence indicates that, besides it's beneficial effects against oxidation of lipid moieties of LDL, this chalcogen also prevents the oxidation of protein moieties of human LDL, pointing to an additional mechanism that could contribute to the inhibition of the atherogenic process.

The results of the present study are the first to show that (i) $(PhSe)_2$ inhibits lipid peroxidation in human isolated LDL *in vitro*, (ii) this phenomenon is related to its thiol-peroxidase activity, and (iii) this chalcogen also prevents the oxidation of protein moieties of human LDL. Taken together, such data render $(PhSe)_2$ a promising molecule for pharmacological studies with respect to the atherogenic process.

Acknowledgements

The authors are grateful to Dr. Gilson Zeni to provide diphenyl disselenide for assays. The financial support by UFSM (FIPE), FAPERGS, CAPES and CNPq are gratefully acknowledged. M.F., J.B.T.R. and C.W.N. are the recipients of CNPq fellowships.

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