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Research paper

Diphenyl diselenide modulates oxLDL-induced cytotoxicity in macrophage by improving the redox signaling

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ABSTRACT

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

1. Introduction

Accumulation of modified low-density lipoprotein (LDL), such as oxidized LDL (oxLDL), in the arterial wall, and the recruitment of monocytes to the subendothelial space are known to be the main early events in the development of atherosclerosis [1].

Blood-circulating monocytes adhere to the endothelium, migrate to the intima, and differentiate to macrophages. Macrophages express receptors (CD36 and SR-A) that bind and internalize oxidized forms of LDL, and these processes give rise to the arterial foam cell, a hallmark of arterial lesion [2–6]. Macrophage activation and foam cell formation are involved in both the initiation and the progression and ultimate instability of advanced lesions. Macrophages taking up oxLDL could modify the production of inflammatory mediators, such as cytokines, proteases, reactive oxygen and nitrogen species (ROS and RNS), metalloproteinases (MMPs) [7–9] and other factors through oxidative sensitive signaling pathways [10].

In oxLDL-stimulated macrophages, the intracellular ROS/RNS are generated by several pathways, including the NADPH oxidase (NOX) system, the lipoxygenase/cyclooxygenase system, inducible $^{\bullet}NO$ synthase (iNOS), and mitochondrial respiratory chain [11]. Moreover, intracellular ROS generated by oxLDL stimulation can activate a wide variety of proinflammatory and proapoptotic pathways that are regulated by transcription factors, such as nuclear factor-κB (NF-κB) [12,13]. These events will culminate in the death of macrophages by activation of apoptosis.

Recent clinical studies have suggested an important antiatherogenic role for the antioxidant enzyme glutathione peroxidase (GPx) [14], which uses glutathione to reduce hydrogen peroxide to water and lipid peroxides to their respective alcohols [15], and it also acts as a peroxynitrite reductase [16]. Therefore, the investigation of antiatherogenic properties of synthetic organoselenium compounds with GPx-mimetic activity has attracted considerable attention. In this scenario, we have been studying the pharmacological properties of diphenyl diselenide $(PhSe)_2$, a

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simple diaryl diselenide with high GPx-mimetic activity [17]. In addition to its GPx-like activity, we recently demonstrated that $(PhSe)_2$ is able to promote the nuclear translocation of the Nuclear (erythroid-derived 2)-related factor (Nrf2) increasing the expression of enzymes related to antioxidant defenses, as gamma-glutamylcysteine synthetase (GGCS) and GPx [18].

This compound was very efficient in reducing the human LDL oxidation and Cu^{2+}-induced lipid peroxidation in rat aortic slices [19] and it decreased hypercholesterolemia and oxidative stress in cholesterol-fed rabbits [20]. Furthermore, the treatment with $(PhSe)_2$ reduced the atherosclerotic lesion in hypercholesterolemic LDLr −/− mice by modulating pathways related to antioxidant and anti-inflammatory responses [21]. In the present study, we therefore aimed at determining whether pretreatment with $(PhSe)_2$ attenuates oxLDL-induced effects in murine J774A.1 macrophage cells and, if so, whether the mechanisms underlying the process involve the redox signaling pathway.

2. Materials and methods

2.1. Materials

Diphenyl diselenide $(PhSe)_2$ was synthesized according to published methods [22]. Analysis of the ^1H NMR and ^{13}C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. LDL isolation and oxidation

This study was approved by our Ethic Committee at Federal University of Santa Catarina (n° 943/11). LDL was isolated from fresh human plasma by discontinuous density-gradient ultracentrifugation as previously described [19] and the protein concentration in LDL solution was determined by Lowry [23]. LDL isolated samples (1 mg of protein/mL) were oxidized at 37 °C in the presence of 10 μM $CuSO_4$ for 16 h, to produce oxLDL. Then EDTA 1.5 mM was added and the samples were dialyzed against 148 mM phosphate buffer for 24 h at 4 °C. LDL oxidation was monitored by following the thiobarbituric acid-reactive substances (TBARS) production.

2.3. Cell culture

Murine J774A.1 macrophage cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were maintained with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM glutamine, 10 mM HEPES, 100 units/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS) in a 5% CO_2 humidified atmosphere at 37 °C. The exposition of cells with $(PhSe)_2$ and/or oxLDL was carried out in DMEM without FBS.

2.4. Measurement of reactive oxygen species (ROS) production

Intracellular ROS production was detected using the non-fluorescent cell permeating compound, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). In order to evaluated the protective effect of $(PhSe)_2$ on ROS production induced by oxLDL, macrophage (2.0×10^5 cells/well) seeded in 24 well plates were firstly stimulated to an increasing concentration curve of oxLDL (25, 50, 100 and 200 μg/mL) for 1 h. After that, cells were incubated with DCFH-DA (10 μM) for 30 min at 37 °C, washed twice with PBS, harvested and collected for immediate determination of ROS generation by flow cytometry (FACS Canto II, BD Bioscience, United States). The protective effect of $(PhSe)_2$ was also evaluated by flow cytometry by pretreating cells for 24 h with $(PhSe)_2$ (1 μM) or vehicle before oxLDL (100 μg/mL) stimulation for 1 h. The results were expressed as percentage of control (non-stimulated cells; 100%).

2.5. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

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

2.6. Cell viability assay

The cell viability in the presence or absence of $(PhSe)_2$ was measured by MTT assay as described by Siqueira and cols [25]. Macrophage (1×10^4 cells/well) seeded in 96 well plates were pretreated with $(PhSe)_2$ (1 μM) or vehicle for 24 h, followed by stimulation with oxLDL (100 or 200 μg/mL) for additional 24 h. After this, 200 μL of MTT solution (0.5 mg/mL) was added and incubated for 2 h. Then, the MTT was removed and 200 μL of DMSO/well was added to dissolve the intracellular crystalline formazan product. The absorbance at 550 nm was read spectrophotometrically using a microplate reader. The results were expressed as a percentage of the absorbance of non-treated cells.

2.7. Foam cell formation assay

Foam cell formation assay was performed with the Oil Red O staining method [26]. Macrophages (2.5×10^5 cells/well) plated in coverslip in 12 wells plate were pretreated with $(PhSe)_2$ (1 μM) or vehicle for 24 h, and then stimulated with oxLDL (100 μg/mL) for additional 3 h. Following oxLDL incubation, cells were fixed with 4% paraformaldehyde and stained by 0.3% Oil Red O for 10 min. Hematoxylin was used as counterstaining. Images of cells were acquired using confocal microscopy (Leica DMI6000 B Microscope) using a 546 nm filter set. Ten images were captured from each group and the total pixels intensity was determined using NIH Image J 1.36b imaging software (National Institutes of Health, Bethesda, MD, USA), and lipid content was expressed as optical density (OD).

2.8. MMP activity measurement

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases, capable of degrading all the molecular components of extracellular matrix. To evaluate the activity of MMP-9, we used two different techniques, gelatin zymography and