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Research paper

Diphenyl diselenide protects endothelial cells against oxidized low density lipoprotein-induced injury: Involvement of mitochondrial function

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ABSTRACT

Elevated levels of oxidized low density lipoprotein (oxLDL) are considered to be one of the major risk factors for atherosclerosis and cardiovascular morbidity. The early stages of atherosclerosis are initiated by the accumulation of oxLDL and the induction of toxic effects on endothelial cells, resulting in endothelial dysfunction. The aim of this study was to investigate how diphenyl diselenide (DD), an organoselenium compound, protect vascular endothelial cells against the toxic effects of oxLDL *in vitro*. Our data showed that the treatment of bovine endothelial aortic cells (BAEC) with DD (0.1–1 μM) for 24 h protected from oxLDL-induced reactive species (RS) production and reduced glutathione (GSH) depletion. Moreover, DD (1 μM) *per se* improved the maximal mitochondrial respiratory capacity and prevented oxLDL-induced mitochondrial damage. In addition, DD could prevent apoptosis induced by oxLDL in BAEC. Results from this study may provide insight into a possible molecular mechanism underlying DD suppression of oxLDL-mediated vascular endothelial dysfunction.

1. Introduction

Damage to the structure or function of the endothelium is considered to be among the root causes of atherosclerosis. The subsequent combination of inflammation, cell death, cell proliferation, fibrosis and eventual calcification contributes to the formation of an atheromatous plaque, which can cause clinical sequel, leading to stenosis and rupture, and causing a thrombus [1,2].

A key factor in the initial endothelial damage is the infiltration of oxidized low density lipoprotein (oxLDL) into the arterial endothelium. OxLDL exerts its toxic effects via many mechanisms and has damaging effects on various aspects of endothelial function [3]. Multiple lines of evidence have demonstrated that oxidative stress plays a central role in oxLDL-induced endothelial toxicity and apoptosis. In oxLDL-stimulated endothelial cells, intracellular reactive oxygen and nitrogen species (ROS/RNS) are generated by several pathways, including the NADPH oxidase system, peroxisomes, the lipoxygenase system and the mitochondrial respiratory chain [4]. ROS and RNS can affect the mitochondria, which themselves also generate these species. Increased production of ROS and impaired mitochondrial function are associated with oxLDL-induced endothelial dysfunction and loss of bioenergetic control [5,6].

Vascular endothelial cells obtain a large portion of their energy from the anaerobic glycolytic metabolism of glucose and oxidative phosphorylation is not the main source of ATP generation [7]. In physiological conditions, mitochondria in endothelial cells are highly coupled and normally function at a sub-maximal capacity, indicating that they possess a considerable bioenergetic reserve. This reserve capacity is important in offering protection against oxidative and nitrosative stress that typically occurs in the endothelium during a pathological process associated with vascular dysfunction [6,8,9]. Previous reports have demonstrated that oxLDL

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induces mitochondrial dysfunction in endothelial cells. Modified forms of LDL (extensively oxidized or glycated LDL) reduce the activity of multiple enzymes in mitochondrial respiratory chain complexes in porcine aortic endothelial cells, which result in impaired oxygen consumption, disrupt in mitochondrial membrane potential and ROS generation [10–12].

Cellular antioxidant enzymes play a central role in the control of oxidative stress. In endothelial cells, glutathione (GSH) and the glutathione peroxidases (GPx) constitute an important antioxidant defense system. Accumulating evidence suggests that disturbances in this system may accelerate atherosclerosis [13,14]. In mice, GPx-1 deficiency causes endothelial dysfunction accompanied by increased oxidative stress, as well as abnormal vascular and cardiac function and structure [15]. On the other hand, overexpression of GPx-4 inhibits the development of atherosclerosis by decreasing lipid peroxidation and inhibiting the sensitivity of vascular cells to oxidized lipids [16].

Considering that GPx is a key enzyme in protecting vessels against oxidative stress and atherogenesis, modulation of GPx activity can be an alternative to prevent atherosclerosis development. Different classes of organoselenium compounds exhibit GPx-like activity and can decompose hydrogen peroxide (H_2O_2) and organic hydroperoxides using reduced GSH or other thiol compounds as hydrogen donors [17]. The first example of such compound was ebselen [18], which has been studied in view of its pharmacological and cardioprotective properties [19]. This compound reduces atherosclerotic lesions in aorta of diabetic apoE knockout mice [20] and also limits the development of diabetes-associated atherosclerosis and diabetic nephropathy in apoE/GPx-1 deficient mice [21].

Another compound with GPx-like activity, diphenyl diselenide (DD) that has been studied by our research group, has demonstrated potent cardioprotective properties. DD reduced hypercholesterolemia and oxidative stress in cholesterol-fed rabbits [22] and was able to inhibit human LDL oxidation *in vitro* by its GPx-like activity [23]. Recently, Rocha and collaborators propose that the hypolipidemic effect of DD could be related to its ability in regulate the phosphorylation/inactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and LDL receptor expression in HepG2 cells [24]. In line with this, we showed that DD efficiently prevents atherosclerosis and endothelial dysfunction in LDL receptor knockout mice [25] and protects macrophages against atherogenic signaling triggered by oxLDL [26]. These *in vivo* and *in vitro* protective effects of DD could be in part attributed to its antioxidant and anti-inflammatory properties. Moreover, recently we showed that DD could augment cellular antioxidant defense by activating the nuclear translocation of Nrf-2 and then protects endothelial cells against peroxynitrite-mediated toxicity [27].

Considering the promising actions of DD in different atherogenic experimental models, in this study we focus on investigate the possible mechanisms involved in the protective effect of DD against endothelial damage mediated by oxLDL, a key factor to endothelial dysfunction and atherosclerosis. Our data indicated that DD prevents oxidative stress, mitochondrial dysfunction and consequently apoptosis in oxLDL-stimulated bovine endothelial aortic cells (BAEC).

2. Materials and methods

2.1. Chemicals and reagents

$CuSO_4$, EDTA, HCl, KH_2PO_4 and perchloric acid were purchased from Nuclear (São Paulo, Brazil). 2,7-dichlorofluorescein acetate, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), dimetil sulfoxide (DMSO), oligomycin, *ortho*-phthaldehyde, propidium iodide and tetra-ethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from Sigma (St. Louis, MO, USA). Annexin-V-FITC, Dulbecco's Modified Eagle medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), L-glutamine, HEPES, penicillin and streptomycin were obtained from GIBCO (Carlsbad, CA, USA).

2.2. Diphenyl diselenide

Diphenyl diselenide (DD) was synthesized according to published methods [28]. Analysis of the H 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 DD (99.9%) was determined by GC/HPLC.

2.3. Oxidized LDL (oxLDL) preparation

LDL was isolated from fresh human plasma by discontinuous density-gradient ultracentrifugation, as described by de Bem et al. [23], and protein concentration in LDL solution was determined using the method of Lowry et al. [29]. Isolated LDL 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, 1.5 mM EDTA was added and the samples dialyzed against 148 mM phosphate buffer for 24 h at 4 °C. The experimental protocol was approved by the institutional ethics committee of the Universidade Federal de Santa Catarina (n° 943/10).

2.4. Cell culture

Bovine aortic endothelial cells (BAEC) were obtained from Genlantis (Torreyana, San Diego, CA, USA). All endothelial cells were characterized positive for uptake of acetylated LDL. Cells were cultivated in DMEM supplemented with 2 mM glutamine, 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) at 37 °C, in a humidified atmosphere of 5% CO_2. Cells were subcultured at confluences and used between the fourth and seventh passage. The exposition of cells with DD and/or oxLDL was carried out in DMEM with 0.5% of FBS.

2.5. Cell viability assay (MTT)

Cell viability in the presence or absence of DD was measured by MTT assays, as described by Mosmman [30]. BAEC were plated onto a 96-well plate at equal density (1.0×10^4 cells) in DMEM medium. After 24 h, the cells were treated with increasing concentrations of DD (0.1–50 μM) for 24 h in a 5% CO_2 humidified atmosphere at 37 °C. After this, 200 μl of MTT solution (0.5 mg/ml) in fresh medium was added and incubated for 3 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 control cells and all experiments were performed in triplicate.

2.6. Measurement of reactive species (RS) production

Intracellular RS production was detected using the non-fluorescent cell permeating compound, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is cell membrane permeable. Once inside the cells, DCFH-DA is hydrolyzed by cellular esterases to form DCF, which is trapped intracellularly due to its membrane impermeability. DCF then reacts with intracellular RS to form the fluorescent product. To examine the effects of DD, cells (3.0×10^5 cells/well) were pretreated with DD (0.1, 0.5 or 1 μM) or

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