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

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ABSTRACT

Oxidative modifications of low-density lipoproteins (LDLs) have a determinant role in atherogenesis and the study of agents that can modulate LDL oxidation is of pharmacological and therapeutic significance. Therefore, the aim of this study was to evaluate the antioxidant effect of the disubstituted diaryl diselenides, *p*-methoxyl-diphenyl diselenide (*p*-CH_3O–$C_6H_4Se)_2$ (DM) and *p*-chloro-diphenyl diselenide (*p*-Cl–$C_6H_4Se)_2$ (DC), on Cu^{2+}-induced LDL oxidation. Both compounds caused a dose-dependent inhibition of human serum and isolated LDL oxidation evidenced by the increasing of the lag phase of lipid peroxidation and decreased the lipid oxidation rate (V_{max}). The protein moieties from isolated LDL were also protected from Cu^{2+}-induced oxidation. Moreover, the disubstituted diaryl diselenides efficiently decreased the oxidized LDL (ox-LDL) induced foam cell formation in J774A.1 macrophage cells. Mechanistically, we have demonstrated that the antioxidant and antiatherogenic effects of DM and DC are related to formation of their selenol intermediates (RSeH) either by a direct reaction with endogenous thiols (GPx-like activity) or via their reduction by TrxR (using NADPH as electron donor). Considering the powerful effect of DM and DC against LDL-induced toxicity, they could be considered for developing of new therapeutic approaches to preventing and treating atherosclerosis and cardiovascular diseases.

1. Introduction

The relationship between inflammation and oxidative stress in the pathogenesis of cardiovascular disease has garnered much interest. In fact, oxidative stress is strongly implicated in all stages of atherosclerosis, from the initiation of fatty streaks to the rupture of vulnerable plaques (Steinberg and Witztum, 2010). 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 (Hansson and Libby, 2006).

LDL contains both protein and lipid components that can potentially be targeted by oxidative damage both during initiation and progression of atherosclerosis (Witzum, 1994). In this regard, it has been evidenced that the antioxidant capability of LDL can be increased by dietary antioxidant supplementation, i.e. LDL can incorporate endogenous and exogenous antioxidants in its supramolecular structure, decreasing its susceptibility to be oxidized. In fact, many endogenous and exogenous compounds have been reported to display beneficial effects against LDL oxidation (Noguchi et al., 2000).

Blood vessel cells are known to express antioxidant enzymes, which are involved in counteracting oxidative stress (Kobayashi et al., 2002). For instance, glutathione peroxidase (GPx) isoforms are key antioxidant enzymes expressed in different cell types, including the endothelial cells. GPx isoforms consume reduced glutathione to convert hydrogen peroxide to water and lipid peroxides to their respective alcohols (Flohe, 1988; Wilson et al., 1989). Of particular therapeutic significance, clinical points of evidence have indicated a protective role for GPx in atherogenesis. Accordingly, a reduction in red blood cell GPx1 activity has been associated with an increased risk of cardiovascular events in a prospective cohort study (Blankenberg et al., 2003), and atherosclerotic plaques of patients with carotid artery disease have reduced GPx1 activity (Lapenna et al., 1998). In animal studies, the lack of functional GPx1 has been shown to accelerate diabetes-associated atherosclerosis via the upregulation of proinflammatory and profibrotic pathways in ApoE−/−mice (Lewis et al., 2007). Furthermore, reduced GPx1 expression has been associated with an increase in cell-mediated oxidation of LDL (Guo et al., 2001) and with a decrease in the bioavailability of nitric oxide, which can contribute to endothelial dysfunction (Forgione et al., 2002).

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The discovery that selenium plays a pivotal role in GPx isoforms and other antioxidant enzymes has stimulated the development of new synthetic antioxidant organoselenium compounds. The first example of such compounds was ebselen (Muller et al., 1984), which has been extensively studied as antioxidant and anti-inflammatory agent (Nogueira et al., 2004). The antioxidant properties of ebselen and other diorganyl dichalcogenides have been linked to their glutathione peroxidase mimetic activity (Muller et al., 1984) and to the ability of ebselen to scavenge peroxynitrite ($ONOO^-$) (Sies and Masumoto, 1997). Moreover, ebselen is also a substrate for mammalian thioredoxin reductase (TrxR) and can be reduced by electrons derived from NADPH, forming its selenol intermediate that can efficiently decompose hydrogen peroxide (Zhao and Holmgren, 2002; Zhao et al., 2002).

Some points of evidence have suggested an antiatherogenic effect of organoselenium compounds. Lass et al. (1996) demonstrated the protective effect of ebselen against copper and peroxyl radical-induced LDL lipid oxidation. More recently, an interesting study showed that this organochalcogenide reduces atherosclerotic lesions in diabetic apoE−/−mice by modulating transcription factors such as NF-κB and AP-1 (Chew et al., 2009). In line with this, recent studies from our laboratory have demonstrated important *in vivo* and *in vitro* potential antiatherogenic properties of a simple diorganoselenium compound, diphenyl diselenide $(PhSe)_2$. This compound displayed beneficial effects against oxidation induced by copper ions or hydroxyl radical generator in human serum, isolated LDL, and rat aortic slices (de Bem et al., 2008), as well as reduced the oxidative stress in hypercholesterolemic rabbits (de Bem et al., 2009). Most importantly, the treatment with $(PhSe)_2$ reduced the atherosclerotic lesion in hypercholesterolemic LDLr−/−mice by modulating interconnected redox and inflammatory pathways (Hort et al., 2011).

Based on *in vitro* and *in vivo* antioxidant and anti-inflammatory properties found for ebselen and $(PhSe)_2$ in models of atherosclerosis, we hypothesize that new organoselenium compounds could also have potential pharmacological properties. Indeed, the disubstituted diaryl diselenides, *p*-methoxyl-diphenyl diselenide (p-CH_3O–$C_6H_4Se)_2$ (DM) and *p*-chloro-diphenyl diselenide (p-Cl–$C_6H_4Se)_2$ (DC), were derived from $(PhSe)_2$, by introducing functional groups (methoxy or chloro) into the aromatic ring (Paulmier, 1986). These chemical modifications could confer higher pharmacological efficiency and less toxicological effects, facts that justified the *in vitro* study proposed here. Recent studies have demonstrated that analogs of diphenyl diselenide exhibited antioxidant properties and caused antinociception in mice (Nogueira and Rocha, 2011). Given previous observations, the present study was carried out to evaluate the potential beneficial effects of DM and DC in protecting *in vitro* human serum and isolated LDL from oxidation as well from foam cells formation, which are the main elements involved in the early steps of atherogenesis. Moreover, the GPx-like activity of DM and DC and their reduction by TrxR was also evaluated in an attempt to delve into molecular mechanisms related to the aforementioned protective effects.

2. Materials and methods

2.1. Materials

The *p*-methoxyl-diphenyl diselenide (p-CH_3O–$C_6H_4Se)_2$ (DM) and *p*-chloro-diphenyl diselenide (p-Cl–$C_6H_4Se)_2$ (DC) was synthesized according to published methods (Paulmier, 1986). Analysis of the H NMR and 13C NMR spectra (data not shown) demonstrate that the compounds obtained presented analytical and spectroscopic data in full agreement with their assigned structure (Fig. 1). These drugs were dissolved in dimethylsulfoxide (DMSO) or ethanol and a 10 mM stock solution was stored at 4 °C for less than 2 weeks. Immediately before use, the compounds were diluted at the required concentrations for the different assays. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. LDL isolation and oxidation

This study was approved by our Ethic Committee at Federal University of Santa Catarina (no. 943/11). LDL was isolated from fresh human plasma by discontinuous density–gradient ultracentrifugation as described (de Bem et al., 2008). Briefly, plasma of nonfasted healthy normolipidemic voluntary donors collected with EDTA (1 mg/mL) was pooled and sucrose (final concentration, 0.5%) was added to prevent LDL aggregation. Five milliliters of EDTA-plasma adjusted to a density of 1.22 g/mL with solid KBr (0.326 g/mL) was layered on the bottom of a centrifuge tube. Then 5 mL EDTA-containing sodium chloride solution (density 1.006 g/mL) was overlaid on top of the plasma. Ultracentrifugation was run at 191.000*g* for 2 h at 4 °C, in a Himac CP80WX Hitachi ultracentrifuge, rotor 40ST. LDL particles were collected by aspiration of the yellow/orange band, which is located in the middle of sodium chloride solution just above the plasma main fractions. Then, LDL particles were dialyzed exhaustively overnight at 4 °C against 5 L of 148 mM phosphate buffer (Na_2HPO_4 8 mM, KH_2PO_4 1.4 mM, KCl 2.6 mM, NaCl 136 mM, pH 7.4) to remove the excess salt and the majority of the EDTA. Protein concentration in LDL solution was determined by Lowry et al. (1951).

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

2.3. Serum oxidation assay

Venous blood was drawn from nonfasted healthy normolipidemic voluntary donors into tubes containing no anticoagulant and centrifuged at 1.500*g* for 15 min. Serum samples were diluted 1:50 in 10 mM phosphate buffer, pH 7.4 and incubated at 37 °C with different DM or DC (0–20 μM) concentration. The oxidation was initiated by the addition of $CuSO_4$ (50 μM) and conjugated dienes (CDs) formation was monitored at 245 nm as previously described (Schnitzer et al., 1998).

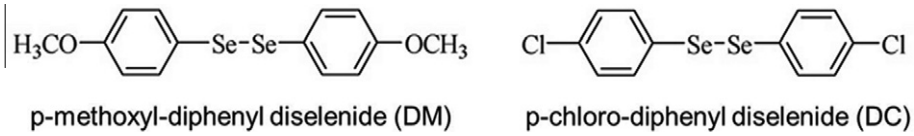

Fig. 1. Organoselenium compounds aromatic derivatives.