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Protective effect of diphenyl diselenide against peroxynitrite-mediated endothelial cell death: A comparison with ebselen

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ABSTRACT

Excess production of superoxide ($O_2^{\cdot-}$) and nitric oxide (·NO) in blood vessel walls may occur early in atherogenesis leading to the formation of peroxynitrite, a strong oxidant and nitrating agent. This study was designed to determine the effect of diphenyl diselenide $(PhSe)_2$, a synthetic organoselenium compound, in comparison with ebselen, on peroxynitrite-mediated endothelial damage. Experimental results showed that pre-incubation of BAEC (24 h) with low concentrations of $(PhSe)_2$ (0.5 and 1 μM) protected the cells from peroxynitrite-dependent apoptosis and protein tyrosine nitration. The intracellular levels of GSH were almost completely depleted by peroxynitrite and, although the compounds did not restore its normal levels, $(PhSe)_2$ *per se* significantly increased GSH in a concentration-dependent manner. Moreover, $(PhSe)_2$, which was about two times more active as a GPx mimic than ebselen, induced a significantly higher increase in both cellular GPx expression and activity. Taking into account the kinetics of the reaction between peroxynitrite and $(PhSe)_2$, our data indicate that $(PhSe)_2$ protects BAEC against peroxynitrite-mediated cell damage not by a direct reaction, but rather by increasing cellular GPx expression as a consequence of enhanced nuclear translocation of Nrf-2, which together with the increase in intracellular GSH, may work catalytically to reduce peroxynitrite to nitrite.

Introduction

Atherosclerosis and subsequent vascular diseases are the main cause of morbidity and mortality in the developed world. Vascular oxidative injury and LDL oxidation in the intima of arteries have been recognized as important steps in atherogenesis, leading to endothelial cell dysfunction [1,2]. Actually, it has been reported that an overproduction of reactive oxygen and nitrogen species in the vascular endothelium occurs very early in the atherogenic process, suggesting a link between reactive oxidant species, LDL oxidation, and endothelial cells dysfunction and apoptosis [3,4].

Peroxynitrite,[1] a biologically relevant oxidizing and nitrating agent, is formed from the diffusion-controlled reaction between nitric oxide (·NO) and superoxide ($O_2^{\cdot-}$). Of particular importance, peroxynitrite is a powerful reactive and short-lived species that can promote oxidative damage to most classes of biomolecules [5–7]. In addition to the generation of pro-oxidant species, the formation of peroxynitrite results in decreased bioavailability of ·NO, therefore diminishing both its salutary physiological functions [8,9] and its strong antioxidant actions over free radical and metal-mediated processes [10,11]. The ability of peroxynitrite to oxidize and covalently modify a wide range of biomolecules, including thiols [12], DNA, proteins and lipids, can affect important cellular functions in such

Abbreviations: $(PhSe)_2$, diphenyl diselenide; BAEC, bovine aortic endothelial cells; GSH, reduced glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; GGCS, gamma-glutamylcysteine synthetase.

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[1] The term peroxynitrite is used to refer to the sum of peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH), whose IUPAC recommended names are oxoperoxynitrate (−1) and hydrogen oxoperoxynitrate, respectively.

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a way that cell homeostasis may be compromised and subsequently apoptosis or necrosis may occur [13–15].

The involvement of peroxynitrite in atherosclerosis has been suggested either by its ability to oxidize LDL or by the extensive nitration of protein tyrosine, detected in human atherosclerotic lesions [16–18]. Therefore, it is important to counteract peroxynitrite-damaging effects. One of the possible strategies is to intercept this reactive species in order to prevent the damage to potentially sensitive biological targets [15,19]. Organoselenium compounds may fulfill such requirements, as it has been reported for ebselen, which efficiently reacts with peroxynitrite. In fact, the second-order rate constant for this reaction ($2.0 \times 10^6\ M^{-1}\ s^{-1}$, pH 7.4) is one of the highest for a low-molecular-weight compound with peroxynitrite, as known so far [20,21]. Some organoselenium compounds have been shown to prevent protein oxidation and nitration [22,23] and to protect DNA against peroxynitrite-mediated damage [24]. Additionally, it has been shown that ebselen prevents the recruitment of leukocytes into inflamed tissues by attenuating peroxynitrite-mediated IL-8 gene expression [25]. Moreover, clinical studies have demonstrated that ebselen improves neurological outcome in patients with acute ischemia, stroke or subarachnoid hemorrhage [26–28], pointing to the potential therapeutic significance of organoselenides.

Recently, it was demonstrated that ebselen protection is accomplished through the nuclear translocation of the transcription factor Nrf-2 triggering Nrf-2-dependent signaling [29–31]. Nrf-2 binds to the antioxidant responsive elements (ARE) and accelerates the transcription of the cognate genes, which can be classified into three separate classes, those that are involved in glutathione synthesis, detoxification enzymes, and those directly involved with the amelioration of oxidative stress [32]. Under basal conditions, Keap1, a protein associated with the actin cytoskeleton, binds very tightly to Nrf-2, anchors this transcription factor in the cytoplasm, and targets it for ubiquitination and proteasome degradation, thereby repressing the ability of Nrf-2 to induce phase-2 detoxification enzyme genes [33].

Diphenyl diselenide [$(PhSe)_2$], a simple organoselenium compound, has shown important antioxidant and pharmacological properties. In fact, in the presence of a reducing thiol, this compound reacts very efficiently with hydroperoxides and organic peroxides, mimicking the glutathione peroxidase (GPx) enzyme [34]. This GPx-like activity of $(PhSe)_2$ has been reported to be even higher than that of ebselen, displaying also a lower toxicity to mammalian cells [35,36]. Moreover, recent data from our group have demonstrated that $(PhSe)_2$, as ebselen, is a good substrate of hepatic and cerebral TrxR enzymes, a fact that contributed to its powerful antioxidant action [30,37,38]. Of note, we have recently shown that $(PhSe)_2$ protects human platelets against lipoperoxidation induced by sodium nitroprusside (SNP), by increasing GPx-like activity in these cells [39]. Additionally, we showed that oral treatment with low doses of $(PhSe)_2$ potently reduced the formation of atherosclerotic lesion in hypercholesterolemic, low-density lipoprotein (LDL) receptor knockout ($LDLr^{-/-}$) mice [40] and inhibited human LDL oxidation [41], facts that were related to its thiol-peroxidase activity. Of particular importance, $(PhSe)_2$ protected hippocampal slices from glucose–oxygen deprivation and its neuroprotective effect seems to be related to a normalization of iNOS overexpression induced by ischemia [42].

The aim of this study was to explore whether $(PhSe)_2$ displays a protective effect against peroxynitrite-mediated toxicity, in primary cultures of bovine aortic endothelial cells (BAEC). In addition, experiments to unveil the mechanics behind the protective effects observed with $(PhSe)_2$ were performed. Our data indicates that $(PhSe)_2$ is able to partially prevent programmed cell death in BAEC triggered by peroxynitrite in a more efficient way than ebselen, most probably through an Nrf-2-dependent increase in cellular GSH levels and GPx activity rather than a direct reaction with peroxynitrite.

Materials and methods

Materials

General laboratory chemicals and some specific ones, namely, collagenase, gelatin, streptomycin/penicillin, MTT, Hoechst 33,258, ethylene diamine tetra acetic acid (EDTA), ortho-phthaldeyde, GSH and glutathione reductase were obtained from Sigma Chemicals (St. Louis, MO). For cell culture, Dulbecco's modified Eagle's medium (DMEM), trypsin 0.25%, fungizone, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) pH 7.4, were purchased from Gibco-Invitrogen. $(PhSe)_2$ was synthesized as previously described [43]. 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.

Primary cultures of bovine aortic endothelial cells

Bovine aortic endothelial cells (BAEC) were obtained from thoracic aorta by treatment with collagenase (2 mg/ml). Cells were cultivated on gelatin-coated tissue culture plastic in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml fungizone at 37 °C, in a humidified atmosphere of 5% CO_2. Endothelial cells were identified by their cobblestone morphology and uptake of fluorescently labelled acetylated LDL. Cells were subcultured at confluences and used between the fourth and the seventh passage. Prior to the experiments, cells at 80% of confluence were starved in serum-free medium for 24 h.

Peroxynitrite synthesis

Peroxynitrite was synthesized by using a quenched flow reactor, as previously described [44] and was stored at −80 °C under N_2 atmosphere. Before use, $ONOO^-$ was spectrophotometrically quantified at 302 nm in 1 N NaOH ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$).

Peroxynitrite exposure

The exposure of BAEC to peroxynitrite was performed as previously described by Brito et al. [45]. In brief, cells were washed and equilibrated with potassium phosphate buffer (PBS) containing 0.9 mM calcium chloride and 0.5 mM magnesium chloride, pH 7.4 for 5 min. Afterwards, cells were exposed to peroxynitrite (final concentration 500 μM), for 10 min and then they were washed and replaced in culture medium for 6 h. The same volumes of either 10 mM NaOH (vehicle control) or pre-decomposed $ONOO^-$ ($ONOO^-$ was decomposed in PBS overnight) were used as controls. In a parallel set of experiments, BAEC were exposed to three bolus of 50 μM peroxynitrite in order to verify peroxynitrite-mediated nitration by determination of 3-nitrotyrosine residues in proteins. When required, cells were pre-incubated with $(PhSe)_2$ or ebselen in a medium free of fetal bovine serum (FBS) for 24 h. At the end of the indicated times, the incubation medium was gently removed and the cells were incubated with peroxynitrite as described above. In these conditions, the compounds were not present in the incubation medium throughout the experiment with peroxynitrite.