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Original article

Glutathione peroxidase-1 expression is up-regulated by ozone therapy in ApoE deficient mice

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ABSTRACT

The role of glutathione peroxidase-1 (GPx1) in limiting the oxidative risk for atherogenesis is increasingly recognized. Therapeutic strategies, designed to augment cellular endogenous defense systems have been identified as a promising approach to control oxidative stress-associated diseases, including atherosclerosis. Ozone therapy regulates oxidative metabolism and prevents oxidative stress-associated-chronic diseases. Thus, the present study was aimed to test the hypothesis that ozone-oxidative conditioning up-regulates GPx1 synthesis in apolipoprotein E deficient mice (apoE$^{-/-}$), protecting the vasculature against atherosclerosis. Male apoE$^{-/-}$ mice were treated with 1 mL of ozone/oxygen containing 40 μg/mL of ozone by rectal insufflation. As controls, mice were untreated or insufflated with oxygen only. Results showed a significant increase ($P < 0.05$) of aortic *GPx1* gene expression in ozone-treated mice, whereas only minor atherosclerotic lesions were observed. Furthermore, GPx1 activity and GSH levels were significant increased ($P < 0.05$) in ozone receiving group compared with controls. In addition, lipid peroxidation was attenuated by ozone treatment, whereas serum lipids were similar among experimental groups. These results altogether suggest that ozone therapy attenuated atherogenesis by a mechanism that involved, at least, the improvement of aortic GPx1 expression/activity. Further studies are needed in order to assess the possible link of cellular redox-sensitive pathways with the antiatherogenic effect of ozone therapy.

1. Introduction

The selenium-containing peroxidases are a broad group of enzymes that utilizes hydrogen peroxide (H_2O_2) as a substrate along with an endogenous source of reducing equivalence [1]. Glutathione peroxidases (GPx) are one of the best studied families of peroxidases. GPx are tetrameric proteins where each monomer contains one atom of selenium at the catalytic site [2]. These enzymes have been involved in the detoxification of H_2O_2 and other peroxides, including lipid peroxides [3].

Evidences from genetically modified mouse models have confirmed the critical role of GPx in vascular redox homeostasis. GPx1-deficient mice are susceptible to endothelial dysfunction and neointima formation, increased adventitial inflammation, and intimal collagen deposition [4,5]. When GPx1$^{-/-}$ mice were crossed with apoE$^{-/-}$ mice to examine the influence of GPx-1 deficiency in atherosclerosis, decreased GPx1 activity was associated with increased atherosclerotic lesion formation, increased vascular oxidative stress (OS), and decreased nitric oxide levels compared to apoE$^{-/-}$ mice with normal GPx1 activity [6].

Therapeutic strategies, designed to augment cellular endogenous defense systems have been identified as a promising approach to combat oxidative stress (OS)-associated pathologies, such as atherosclerosis. Evidences that antioxidant enzymes, nitric oxide pathways, and other subcellular activities could be modulated by low doses of ozone have been shown, supporting the effects of ozone therapy in many age-matched pathological conditions, including coronary artery disease [7–10]. The present study was aimed to test the hypothesis that ozone-oxidative conditioning (ozone-OC) up-regulates GPx1 synthesis in apoE$^{-/-}$ mice protecting the vasculature against atherosclerotic lesions development.

2. Materials and methods

2.1. Animals

ApoE$^{-/-}$ male mice, weighing 20–22 g and 4 weeks old, were obtained from the Center of Molecular Immunology (Havana, Cuba). The animals were adapted to laboratory conditions (60%

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humidity, 25 ± 1 °C) 1 week before the experimental procedures. The animals were housed in groups of 5, maintained on a 12 h light/darkness cycle, with free access to food and water. The study was approved by the Pharmacy and Food Sciences College Institutional Animal Ethical Committee. All procedures were performed in accordance with the guidelines stipulated by this Committee.

2.2. Ozone generation

Ozone was generated by OZOMED equipment manufactured by the Center of Ozone Research (Havana, Cuba). Ozone was obtained from medical grade oxygen, and was used immediately upon generation and represented only about 3% of the gas mixture (O_3/O_2). The ozone concentration was measured by using a built-in UV spectrophotometer set at 254 nm.

2.3. Experimental design

Mice were randomly distributed in three groups of 10 animals and supplemented with a hypercholesterolemic diet containing 1% of cholesterol (Sigma, St. Louis, USA). The first group was assumed as control without any treatment. On week 6, the second group was treated with 1 mL of ozone/oxygen containing 40 μg/mL of ozone by rectal insufflation once a day during 20 sessions in alternated days. The animals of the third group were treated with 1 mL of oxygen as vehicle control. Prior to ozone/oxygen or oxygen insufflation the rectum was stimulated to eliminate the excrements. At the end of the study, mice were anesthetized with ketamine hydrochloride (5 mg/kg intramuscular [i.m.]), and euthanized with an overdose of sodium pentobarbital (90 mg/kg, intravenous.) (Abbott Lab., Mexico SA CV, Mexico). Then, vascular system was perfused with ice cold NaCl 0.9% solution and aortas were used for redox biomarkers determination, histopathological study ($n = 5$) and quantitative polymerase chain reaction technique ($n = 5$).

2.4. Histopathological analysis

Aortic arches were rinsed in PBS 50 mM, pH 7.4, transversally cut, and fixed in 4% formaldehyde solution during 24 h. Samples were then embedded in paraffin. Five-micrometer tissue sections were cut, air-dried on glass slides, deparaffinized, and rehydrated. Finally, tissue sections were stained with hematoxylin and eosin (HE) under standard procedures. The sections were analyzed in an optic microscope Olympus BX51.

2.5. Quantitative real-time polymerase chain reaction (RT-qPCR)

Approximately 3 mg of frozen aortic tissue were homogenized at 20 Hz during 2 min in a Tissue Lyser II (Qiagen). Total RNA from homogenates was isolated with an RNeasy Plus Micro Kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was prepared from total RNA by Quantitect Reverse Transcription Kit (Qiagen). Genomic DNA was removed as described by the manufacturer. Nevertheless, to assess genomic DNA contamination, controls without reverse transcriptase were included. Oligonucleotide primers were designed based on the cDNA sequences reported in the GenBank database. Primers sets were the following: GPx1, for-5′-CCTCAAGTACGTCCGACCTG and rev-5′-CAATGTCGTTGCGGCACACC; β-actin, for-5′-CCCAAGGCC-AACCGCGAGAAGAT-3ánd rev-5′-GTCCCGGCCAGCCAGGTCCAG-3′.

The highly specific measurement of mRNA was carried out for genes GPx1 and β-actin using the RT-CyclerTM (Capital Bio, China). Each sample was run and analyzed by duplicate. Quantification was performed by REST software. GPx1 mRNA levels were adjusted as the values relative to β-actin, used as endogenous control.

2.6. Serum samples collection

Blood samples (1 mL) were obtained at the end of the study for biochemical analyses. Blood was obtained by cardiac punction. These samples were immediately centrifuged at 2500 g, at 4 °C for 10 min. The serum was collected and aliquots were stored at −80 °C until analysis.

2.7. Biochemical determinations

All biochemical parameters were determined by spectrophotometric methods using a Pharmacia 1000 Spectrophotometer (Pharmacia LKB, Uppsala, Sweden).

Total proteins levels were determined using the method described by Bradford [11] with bovine serum albumin as standard. GPx1 activity was determined as previously described [12]. After precipitation of thiol proteins, the reduced glutathione (GSH) levels were measured according to the method of Sedlak and Lindsay with Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid) [13]. Total peroxides (ROOH) were measured by Bioxytech H_2O_2-560 kit (Oxis International Inc., Portland, OR, USA) using xylenol orange to form a stable colored complex, which was measured at 560 nm. Meanwhile, total cholesterol (TC), low-density lipoprotein cholesterol (LDLc), and high-density lipoprotein cholesterol (HDLc) were determined by a commercial enzymatic kit (MBL International Corporation, MA, USA).

2.8. Statistical analysis

Statistical analysis was performed using the SPSS program for Windows (version 11.5, SPSS Inc.). Comparisons among multiple groups were achieved using one-way ANOVA followed by Kruskal-Wallis post-test. Data were expressed as the mean ± standard deviation (SD). A P-value of < 0.05 was considered statistically significant.

3. Results

At the end of the experiment macroscopic and microscopic examination of mice organs did not show any relevant disease or abnormalities. The body weight varied significantly in all experimental groups but differences between them were not observed (data not shown). The hematoxylin/eosin staining of aortic sections showed the presence of atherosclerotic lesions in non-treated and oxygen-receiving mice. The lesions were characterized by intimal thickening and vascular tissue damage (Fig. 1A and B). In contrast, only minor lesions were observed in 8/10 of ozonized mice (Fig. 1C), indicating a protective effect of ozone therapy against atherosclerosis development in apoE$^{-/-}$ mice.

At the end of the experiment the serum levels of TC, LDLc and HDLc did not differ among groups, showing no statistical differences (data not shown). Hence, the antiatherogenic effect of ozone treatment in this animal model was exerted by mechanisms other than serum lipid modulation.

The RT-qPCR experiments showed that GPx1 mRNA levels were increased in ozonized mice respect the non-treated and oxygen-treated animals (Fig. 2A). The insufflation with ozone/oxygen mixture increased GPx1 expression in aortas, demonstrating the capacity of low doses of ozone to induce the transcriptional expression of this antioxidant enzyme. Accordingly, aortic GPx activity and GSH concentration were higher in ozone-treated mice in comparison with controls (Fig. 2B and C respectively); whereas ozone therapy promoted a reduction of aortic ROOH levels (Fig. 2D).

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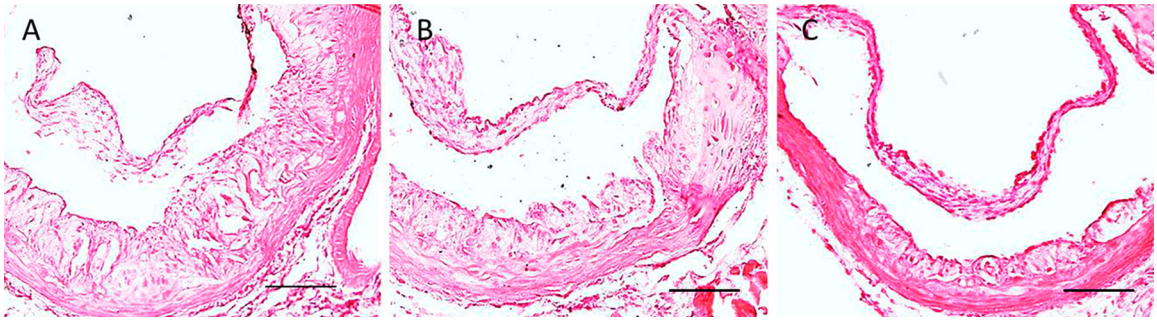

Fig. 1. Antiatherosclerotic effect of ozone-oxidative conditioning. Figures are representative cross sections of aortic arches from apoE$^{-/-}$ stained with hematoxylin/eosin. A. PBS-treated mice. B. oxygen-treated mice. C. ozone-treated mice. Magnification 20 ×, scale bar: 50 μm.

4. Discussion

Many factors including low-density lipoprotein oxidation, endothelial dysfunction, and inflammation are involved in atherogenesis. OS, a common denominator in these pathogenic processes, has been considered as a major contributor of atherosclerosis development [14]. The early stages of atherogenesis are characterized by an activation of antioxidant enzymes presumably in response to the increment of ROS and oxidized molecules. In contrast, in larger stages the redox imbalance persists and the expression/activity of antioxidant enzymes such as superoxide dismutase (SOD), GPx and catalase (CAT) are decreased [15]. Up to now, therapeutic approaches to atherosclerosis have been focused on risk factors, targeting mainly hyper-cholesterolemia and hypertension [16]. On the other hand, synthetic and natural antioxidants have been used to prevent or treat cardiovascular diseases associated with atherosclerotic development [17]. However, these therapeutic strategies have shown a low efficacy in different clinical trials and metanalyses [18].

During the last two decades the application of ozone therapy has demonstrated that the use of a low dose of ozone added to blood ex vivo [19,20] and also by rectal insufflation can be effective to properly quench the chronic OS in cardiovascular diseases [7–10,21]. Ozone acts in a hormetic mechanism and as a mild oxidant stressor, acting by a formation of second messengers, such as hydrogen peroxide and lipoperoxide compounds [22]. Low levels of lipid peroxidation (LPO) end-products induce cellular adaptive responses, inducing tolerance against subsequent OS by up-regulation of antioxidant mechanisms. LPO-end-products as well as reactive oxygen species (ROS) has been shown to play a key role as a regulator of gene expression [23]. In this scenario, ozone-derived reactive molecules are able to activate transcription nuclear factors, including nuclear related factor 2 (Nrf2) which interacts with the antioxidant response elements (ARE), leading to the synthesis of a great variety of antioxidant enzymes able to restore the redox homeostasis, including GPx [24].

In the present work we showed new evidences on the regulatory effects of ozone therapy on the antioxidant systems. Here we demonstrated the efficacy of ozone insufflation to prevent atherosclerotic development in apoE$^{-/-}$ mice, reinforcing the preclinical efficacy of ozone therapy against atherosclerosis development. Our results suggest that antiatherogenic effect of ozone-OC may be mediated, at least, by a mechanism related to the improvement of *GPx1* gene expression. Accordingly, aortic redox systems seem to be favored by ozone-OC since GPx1 activity and GSH levels were increased, whereas lipid damage was attenuated. Overexpression of GPx has been shown to be protective against OS in cultured cells and in vivo animal models [3]. Clinical studies have shown that low GPx1 activity is an independent predictor of fatal cardiovascular events in subjects without risk factors for atherosclerosis [25].

On the other hand, Biswas and coworkers [26] demonstrated that depressed GSH synthesis precedes OS and atherogenesis in ApoE$^{-/-}$ mice. GSH plays an important role in cardiovascular

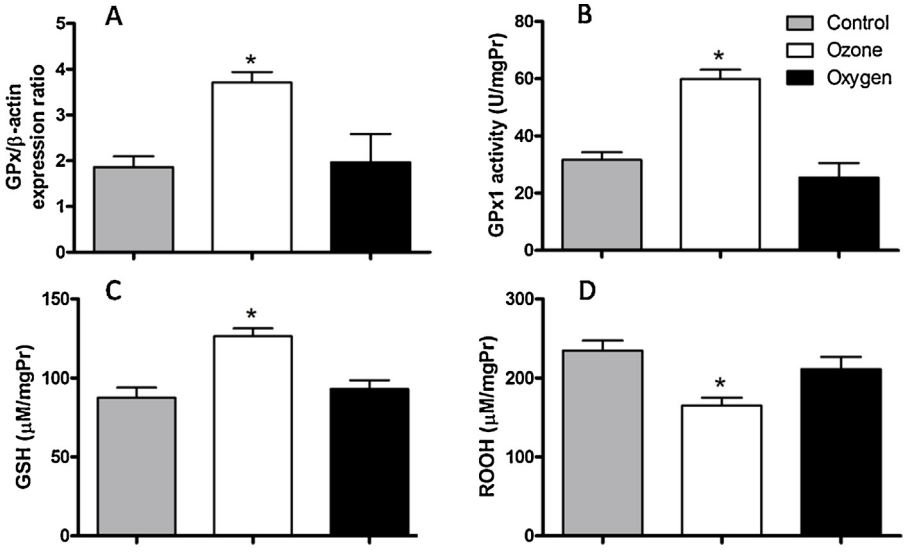

Fig. 2. Effect of ozone-oxidative conditioning on GPx1 expression/activity, GSH levels and total hydroperoxides. Ozone-oxidative conditioning stimulates *GPx1* gene expression, meanwhile GPx activity, GSH and ROOH levels were higher than in controls. Asterisks represent statistical differences ($P < 0.05$).

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system mediating several redox-based signaling reactions as well as gene expression within the cell, impeding atherogenic development [3]. Our results suggest that increased GSH synthesis in aortas from ozone-treated mice may result from the interaction of ozone-derived peroxides with ARE, which in turn contribute to antioxidants synthesis, contributing with GSH preservation and/or restoration. Thus, further investigations on the effects of ozone-OC on GSH/GSSG redox state and such enzymes involved in GSH metabolism are needed.

5. Conclusions

In summary, our results altogether showed that ozone therapy improved the antioxidant status of apoE$^{-/-}$ aortic tissue, preventing mice from atherosclerotic lesions development. Other studies are in progress to further clarify the mechanisms related with the antiatherosclerotic effects of ozone-OC.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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