

Chronic hyperbaric oxygen treatment elicits an anti-oxidant response and attenuates atherosclerosis in apoE knockout mice

Bhalchandra J. Kudchodkar, Anson Pierce¹, Ladislav Dory*

Department of Molecular Biology & Immunology, The University of North Texas Health Science Center, Fort Worth, TX 76104, USA

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Abstract

We previously demonstrated that hyperbaric oxygen (HBO) treatment inhibits diet-induced atherosclerosis in New Zealand White rabbits. In the present study we investigate the mechanisms that might be involved in the athero-protective effect of HBO treatment in a well-accepted model of atherosclerosis, the apoE knockout (KO) mouse. We examine the effects of daily HBO treatment (for 5 and 10 weeks) on the components of the anti-oxidant defense mechanism and the redox state in blood, liver and aortic tissues and compare them to those of untreated apoE KO mice.

HBO treatment results in a significant reduction of aortic cholesterol content and decreased fatty streak formation. These changes are accompanied by a significant reduction of autoantibodies against oxidatively modified LDL and profound changes in the redox state of the liver and aortic tissues. A 10-week treatment significantly reduces hepatic levels of TBARS and oxidized glutathione, while significantly increases the levels of reduced glutathione, glutathione reductase (GR), transferase, Se-dependent glutathione peroxidase and catalase (CAT). The effects of HBO treatment are similar in the aortic tissues. These observations provide evidence that HBO treatment has a powerful effect on the redox state of relevant tissues and produces an environment that inhibits oxidation. The anti-oxidant response may be the key to the anti-atherogenic effect of HBO treatment.

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1. Introduction

There is mounting evidence that atherosclerosis is a chronic, immune-mediated inflammatory response in the arterial intima to tissue damage [1]. Tissue damage may result through increased formation of reactive oxygen species (ROS) in response to altered shear stress, ischemia/reperfusion or exposure to modified (oxidized) LDL [2].

Superoxide ($O_2^{\bullet-}$) production is the first step in the generation of ROS. Potential sources of ROS in the vasculature include NAD(P)H oxido-reductases, xanthine oxidase, nitric oxide synthases, cyclooxygenases, lipoxygenases, mitochondria

oxidation and autooxidation of tissue metabolites [3]. NAD(P)H oxidases present in various arterial cell types may also be a critical source of oxygen radicals [3]. Although $O_2^{\bullet-}$ can spontaneously form hydrogen peroxide, this reaction is greatly accelerated by the action of superoxide dismutases (SODs). Hydrogen and lipid peroxides are then reduced by catalase (CAT) or glutathione peroxidases (GPx), respectively. GPx *iso*-enzymes are expressed ubiquitously and play an important role in reducing H_2O_2 , and lipid hydroperoxides. Glutathione-S-transferase (GST) facilitates the excretion of oxidized lipids and other biomolecules out of the cell, thus preventing cytotoxicity.

Hyperbaric oxygen (HBO) therapy is approved by the Undersea and Hyperbaric Medical Society for a limited number of diseases/conditions, including air or gas embolism, carbon monoxide poisoning, clostridial myonecrosis, exceptional anemia resulting from blood loss, necrotizing soft

* Corresponding author. Tel.: +1 817 735 0180; fax: +1 817 735 2118.

E-mail address: ldory@hsc.unt.edu (L. Dory).

¹ Present address: Department of Cellular & Structural Biology, UT HSC at San Antonio, TX, USA.

tissue infections, osteoradio-necrosis and thermal burns. It is associated with a number of positive effects: it improves transcutaneous oxygen pressure, diabetic foot ulcers and other characteristics seen in peripheral atherosclerosis. It is also protective in several animal models of organ ischemia and endotoxemia [4,5]. HBO treatment may also reverse the subendothelial hypoxia known to be associated with ischemia and atherosclerosis [6]. On the other hand, the increased availability of oxygen in tissues as a result of HBO treatment leads to increased formation of ROS. Generally, HBO therapy with moderate pressures (2–2.5 ATA) of short duration (60–90 min) is safe, without serious complication. The bulk of the studies that investigate the effect of HBO treatment on the anti-oxidant enzymes in experimental animals involve a single dose of high-pressure HBO until convulsions or death are observed. Repeated, short exposures to HBO at pressures of <3 atm are safe and reduce oxidative stress, possibly by a combined effect on the pro- and anti-oxidant enzyme activities [7].

We previously demonstrated that HBO treatment prevents the progression and accelerates the regression of diet-induced atherosclerosis in New Zealand White rabbits [8]. This effect appears to be mediated by, at least in part, reducing the extent of lipid oxidation in the plasma and tissue compartments, rather than by changes in plasma lipid and lipoprotein profiles. Based on these results we hypothesized that chronic and intermittent treatment with HBO induces components of the anti-oxidant defense response, leading to decreased formation of oxidized substrates (including oxLDL) and an attenuated immune response. In the present study we test this hypothesis and demonstrate that HBO reduces atherosclerosis without affecting the elevated plasma cholesterol levels in female apoE knockout (KO) mice. The decrease in fatty streak lesions is accompanied by a decrease in the titer of autoantibodies against oxidatively modified LDL, an increase in the activities of a number of anti-oxidant enzymes and a concomitant increase in tissue glutathione levels. Overall, and perhaps paradoxically, HBO treatment shifts the tissue environment to a more reduced redox state, resembling that of the WT, normal mice and in direct contrast to the oxidative environment seen in untreated apoE KO mice. Our results suggest that the induction of anti-oxidant enzyme activities (or their restoration) by HBO treatment is strongly atheroprotective.

2. Materials and methods

2.1. Animals

Four weeks old female apolipoprotein E KO mice, backcrossed for 10 generations to a C57BL/6 background and C57BL/6 mice (wild-type, WT) were purchased from Jackson laboratories (Bar Harbor, Maine). Mice were maintained in a pathogen-free environment on a 12 h light, 12 h dark cycle. They were provided with standard rodent chow and

water *ad libitum*. After 5 weeks of age, the WT mice were randomly assigned to two groups ($n = 12$ each) and the apoE KO mice were assigned to four groups. One group of apoE KO mice received HBO treatment for a period of 5 weeks ($n = 10$), while the other for a period of 10 weeks ($n = 14$). The other two groups of apoE KO mice and the two groups of WT mice remained untreated and were compared to the HBO-treated mice. While the untreated apoE KO mice were used as a control group for the comparison with the HBO-treated mice, we used WT mice to determine the baseline, age-matched values for tissue anti-oxidant components we were examining. The effects of the apoE gene deletion on the redox state of the various tissues have not been reported.

2.2. HBO treatment

Mice were treated in their cages, placed into a specialized HBO chamber for animals, with 100% oxygen for 90 min at 2.4 atm, 5 days/week for 5 or 10 weeks. The desired pressure in the chamber was reached over 15 min; after the 90 min treatment period the pressure was released over 15 min. Untreated apoE KO mice and WT mice were handled exactly the same way, except they were not placed into the hyperbaric chamber.

2.3. Tissue and sample preparation

At the end of the treatment periods, mice from each group (untreated and HBO-treated apoE KO as well as WT mice) were anesthetized by subcutaneous injection of ketamine (80 mg kg⁻¹) and xylazine (10 mg kg⁻¹). Blood was collected from the retro-orbital plexus into heparinized tubes. The abdomen and chest were opened, and the organs were perfused with ice-cold PBS. Tissues were excised, frozen in liquid nitrogen and kept at -80°C until analysis. All animal procedures were approved by the Institutional Animal Care and Use Committee.

2.4. Assessment of atherosclerosis

Atherosclerosis or fatty streak formation was evaluated by: (a) measuring total aortic cholesterol content; and (b) *en face* analysis of Oil Red O-stained sections of the aortic arc. For cholesterol content, aliquots of aortic homogenate, prepared as described below, were extracted with chloroform/methanol (2:1, v/v) and cholesterol content determined by the enzymatic method using a microtiter plate [9]. *En face* analyses of lesions were carried out as described [10]. The extent of lesioned surface area was determined by analysis with Image Pro Plus (Media Cybernetics).

2.5. Biochemical analyses of plasma, liver and aorta

2.5.1. Plasma and plasma lipoproteins

Plasma lipoprotein distribution and cholesterol content were determined by FPLC chromatography of plasma fol-

lowed by determination of cholesterol content in each fraction.

2.5.2. Detection of anti-oxLDL antibodies by ELISA

The levels of immunoglobulin (Ig) autoantibodies binding to native LDL (nLDL), copper-oxidized LDL (oxLDL) and malondialdehyde modified LDL (MDA-LDL) were determined [11,12] in pooled plasma samples using 1:80 and 1:480 dilution for oxLDL and MDA-LDL, respectively. The amount of IgM bound to nLDL, oxLDL and MDA-LDL antigen was detected with biotin-conjugated goat anti-mouse IgM. Data are expressed as relative absorbance units at 630 nm and are corrected for the non-specific binding to native LDL.

2.5.3. Tissue homogenates

Frozen liver and aortic tissues were ground with mortar and pestle in liquid nitrogen. The powdered samples were transferred into a Dounce homogenizer and homogenized in 10 volumes of extraction buffer (ice-cold 50 mM potassium phosphate buffer pH 7.4, containing a protease inhibitor cocktail and 1 mM EDTA) at 3–5 °C. The homogenates were centrifuged at $10,000 \times g$ at 4 °C for 10 min and the supernatant was divided into aliquots and stored at –80 °C prior to use.

2.5.4. Measurement of lipid oxidation

Lipid oxidation in liver tissue homogenates was measured by determining thiobarbituric acid-reactive substances (TBARS), as described [13].

2.5.5. Assay of reduced glutathione (GSH) content

The GSH content of the liver homogenate was measured spectrophotometrically and that of the aortic homogenate was determined fluorometrically [14]. Liver and aortic tissue GSH content was calculated using concurrently run standard curves and expressed as nmoles of GSH/mg of sample protein.

2.5.6. Assay of oxidized glutathione (GSSG) content

N-ethylmaleimide (NEM) was added (50 mM final) to the deproteinized supernatant of the liver homogenate. After incubation for 1 h at RT excess NEM was removed by successive extractions (10×) with ethyl ether (1:1, v/v). The ether-free residue was dissolved in Tris-EDTA buffer (pH 7.6) containing 1 mM NADPH and 6 U of glutathione reductase (GR). Reduced glutathione levels were determined, as described above. Tissue GSSG content was calculated using concurrently run standard curves and expressed as nmoles of GSSG/mg of sample protein.

2.5.7. Glutathione redox state

The redox state of the liver was determined as the ratio of oxidized glutathione/reduced glutathione $\times 2$ (GSSG/2GSH). The glutathione redox couple is two to four orders of magnitude more abundant than any other redox couple in the cell [15].

2.6. Enzyme assays

2.6.1. Plasma paraoxonase 1 (PON-1) and arylesterase activities

Paraoxonase (PON1) activity towards paraoxon is specific for PON-1 and was assayed by measuring *p*-nitrophenyl release from the substrate paraoxon [8]. Plasma arylesterase activity, shared by PON-1 and PON-3 was measured using phenylacetate as substrate [16]. Blanks were included to correct for the nonenzymatic hydrolysis of the substrate. The enzyme activity was calculated from the molar extinction coefficients: $17,100 \text{ M}^{-1} \text{ cm}^{-1}$ for PON-1 and $1310 \text{ M}^{-1} \text{ cm}^{-1}$ for arylesterase. One unit of the enzyme activity equals 1 μmol of substrate hydrolyzed (L min^{-1}).

2.6.2. Catalase activity

Catalase activity was measured by the method of Aebi [17]. The decomposition of H_2O_2 was monitored at 240 nm, at 25 °C for 1 min. One unit of catalase activity equals 1 μmol of H_2O_2 consumed (min mg^{-1}) of sample protein.

2.6.3. Oxidized glutathione reductase (GR) activity

GR activity was measured, as described, using blanks that did not contain GSSG [18]. The oxidation of NADPH was followed at 25 °C at 340 nm for 3 min. GR activity was calculated using the extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$, and expressed as nanomoles of NADPH consumed (min mg^{-1}) of sample protein.

2.6.4. Glutathione *S*-transferase (GST) activity

Tissue GST activity was measured, as described by Habig and Jakoby [19]. The increase in the absorbance due to the formation of the conjugate between 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) was monitored at 340 nm at 25 °C. GST activity was calculated using the extinction coefficient of $9600 \text{ M}^{-1} \text{ cm}^{-1}$, and expressed as nanomoles of GSH-CDNB conjugate formed (min mg^{-1}) of sample protein.

2.6.5. Glutathione peroxidase (GPx) activity

GPx activity was determined using a glutathione reductase-coupled assay [20]. The total GPx activity was determined using cumyl hydroperoxide as the substrate and the activity of Se-dependent GPx was measured with H_2O_2 as substrate. The activity of non-Se-dependent GPx was obtained by calculation. NADPH consumption was monitored at 340 nm for 4 min at 25 °C. Blanks were run using homogenization buffer in place of the tissue homogenate. GPx activity was calculated using the extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as nanomoles of NADPH consumed (min mg^{-1}) of sample protein.

2.6.6. Protein

Tissue homogenate protein content was determined by the Lowry method [21], using bovine serum albumin as standard.

2.6.7. Statistical analysis

Values reported in the text and in the table represent means \pm SEM. Data obtained from apoE KO mice (untreated or HBO-treated) are expressed as percent of those measured in age-matched WT mice. One-way ANOVA (Bonferroni/Dunn test) and Student's *t*-test were performed to see whether any significant differences occurred within and between groups. A probability $p \leq 0.05$ was accepted as statistically significant. All analyses were performed with the use of Stat View 4.5 software (Abacus Concepts, Berkeley, CA). Although apoE KO mice were treated for 5- and 10-week periods, data are shown only for the 10-week period. Unless otherwise stated, the 5-week data are similar to the 10-week data.

3. Results

3.1. Body and spleen weights, plasma cholesterol levels and paraoxonase activities

As shown in Table 1, HBO treatment has no effect on body weight or plasma cholesterol concentrations of the apoE KO mice. On the other hand, HBO treatment significantly reduces spleen weight (expressed as percent of body weight) and increases plasma PON-1 and arylesterase activities. These findings are in agreement with our previous findings in cholesterol-fed rabbits [8]. It may be of interest that apoE KO mice, when compared to the WT mice, have a significantly reduced body weight, increased spleen weight and significantly suppressed plasma PON-1 and arylesterase activities. It is also of interest that HBO treatment of the apoE KO mice "corrected" these values to be similar to and in some cases indistinguishable from those of the WT mice. Plasma cholesterol levels were markedly higher in apoE KO mice when compared to WT mice ($p < 0.0001$), and HBO treatment had no effect on them or on the distribution of cholesterol among the lipoprotein fractions, as analyzed by FPLC chromatography (data not shown).

The hypercholesterolemia of the apoE KO mice is associated with a profound decrease in plasma PON-1 activity (a 28% decrease), an observation similar to the one we made in cholesterol-fed rabbits [8]. HBO treatment restores plasma PON-1 activities of the apoE KO mice to normal, WT lev-

els, but it is less effective in restoring plasma arylesterase activity.

3.1.1. Autoantibodies to oxidized LDL

The levels of autoantibodies to oxidized LDL have been shown to reflect the levels of oxLDL in circulation and correlate positively with the progression and the regression of experimental atherosclerosis in mouse models [11,12]. In accord with these reports the circulating levels of antibodies to both, anti-oxLDL (Fig. 1A) and anti-MDA-LDL (Fig. 1B) were significantly higher ($p < 0.0001$) in apoE KO mice when compared to WT mice. After 10 weeks of HBO treatment the levels of circulating autoantibodies against both oxLDL and MDA-LDL declined by 45% ($p < 0.001$) and 50% ($p < 0.001$), respectively and were not significantly different from those of WT mice.

3.2. Oxidation products and anti-oxidant enzymes of the liver

There is no single measure of the intracellular net oxidant load under conditions of oxidative stress. Lipids as well as other biomolecules, such as glutathione, are oxidized and accumulate in plasma and tissues under oxidative stress. The data shown in Figs. 2 and 3 are expressed as percent of the age-matched control (WT) values. This allows for a direct comparison of the effects of HBO treatment on the variables of the apoE KO mice, as well as an immediate comparison of the apoE KO mice to WT animals. Although only the results after the 10 week treatment period are shown, changes in the same direction, albeit of lower magnitude were observed after 5 weeks of treatment.

3.2.1. Lipid oxidation and redox environment

As expected and shown in Fig. 2, hepatic TBARS levels are significantly higher in the apoE KO mice, when compared to WT mice (34%, $p < 0.0001$). HBO treatment results in a marked reduction in the TBARS formation in the livers of apoE KO mice to levels not different from those of WT mice.

Cellular levels of oxidized glutathione (GSSG) are known to increase under oxidative stress. Like TBARS, the GSSG content of the liver is markedly higher in apoE KO mice (39%, $p < 0.0001$), but HBO treatment completely reverses this accumulation to levels indistinguishable from those of

Table 1

Body and spleen weights, plasma cholesterol concentrations and paraoxonase activities in wild type (WT) and apoE KO mice

	Wild-type	ApoE KO	ApoE KO + HBO
Body weight (g)	21.2 \pm 0.3	19.8 \pm 0.4*	20.1 \pm 0.4*
Spleen weight (%BW)	0.40 \pm 0.01	0.47 \pm 0.01*	0.43 \pm 0.01*.#
Plasma chol. (mg dL ⁻¹)	80.7 \pm 0.8	411 \pm 11*	454 \pm 26*
PON-1 (mmol L ⁻¹ min ⁻¹)	147.7 \pm 7.3	105.7 \pm 4.4*	145.9 \pm 7.5#
Arylestrase (mmoles L ⁻¹ min ⁻¹)	72.6 \pm 4.2	46.6 \pm 0.7*	60.3 \pm 2.8*.#

Values represent averages \pm SEM of 12 WT, 9 apoE KO and 10 apoE KO + HBO-treated mice for body and spleen weight values. The remaining values are averages \pm SEM of 5 animals in each group.

* Significantly different ($p < 0.05$) from WT values.

Significantly different ($p < 0.05$) from untreated apoE KO values.

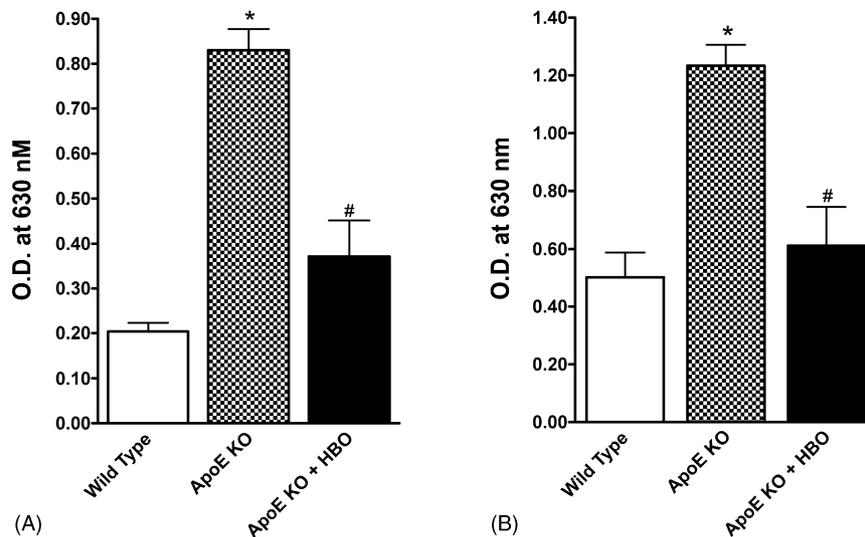


Fig. 1. Titers of autoantibodies against Cu-oxLDL and MDA-LDL. Mouse anti-oxLDL or MDA-LDL IgM was quantified by an ELISA using goat anti-mouse IgM conjugated with biotin, followed by incubation with avidin-conjugated horseradish peroxidase. Optical density at 630 nm was corrected for native LDL. Open bars represent the WT animals, while the stippled and full bars represent apoE KO mice in the absence or presence of HBO treatment, respectively (average \pm SEM of 5 animals in each group). (*) Significantly different from WT mice. (#) Significantly different from untreated apoE KO mice.

WT mice. Conversely, hepatic GSH levels in the apoE KO mice are significantly lower (16%, $p < 0.005$) when compared to the WT mice. Treatment with HBO restores GSH to normal, WT levels. These results indicate that the liver glutathione redox state in the apoE KO mice is shifted profoundly to an oxidative environment: a 67% increase in the GSSG/2GSH ($p < 0.0001$), when compared to age-matched WT mice. HBO treatment completely reverses this trend.

3.2.2. Enzymes

HBO treatment completely restores (raises) the hepatic glutathione reductase and catalase activities in apoE KO mice to normal (WT) values. HBO treatment also increases hepatic

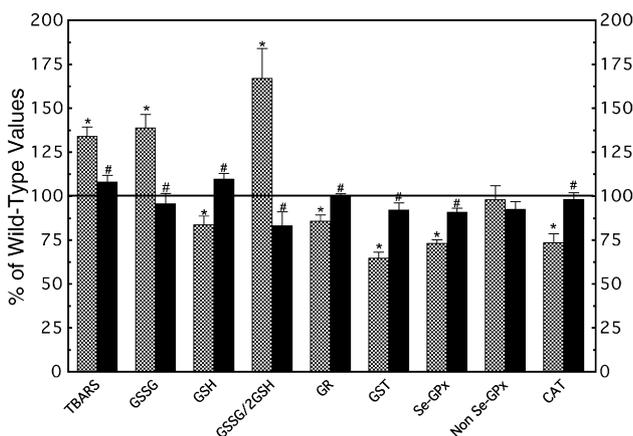


Fig. 2. Oxidation products and anti-oxidant enzymes of the liver. Enzymes, metabolites or metabolite ratios are expressed as a percent of those values found in age-matched WT mice ($n = 12$). Stippled bars represent untreated apoE KO mice ($n = 9$), while the full bars the HBO-treated mice ($n = 10$). Each bar is an average \pm SEM. (*) Significantly different from WT mice (represented by the line through 100%). (#) Significantly different from untreated apoE KO mice.

glutathione *S*-transferase activity by 40%, to levels indistinguishable from the values seen in WT mice. Similarly, selenium-dependent GPx-1 (Se-GPx) activity is increased by HBO treatment to nearly WT values. In contrast, the activity of the selenium-independent GPx (nonSe-GPx) is not affected by HBO treatment or the apoE gene ablation.

3.3. Levels of reduced glutathione and the activities of anti-oxidant enzymes in the aorta

3.3.1. Redox environment

As shown in Fig. 3, a 10-week HBO treatment increases aortic GSH levels by 44% ($p < 0.001$) to levels significantly higher than even those of the WT mice (by 20%, $p < 0.04$).

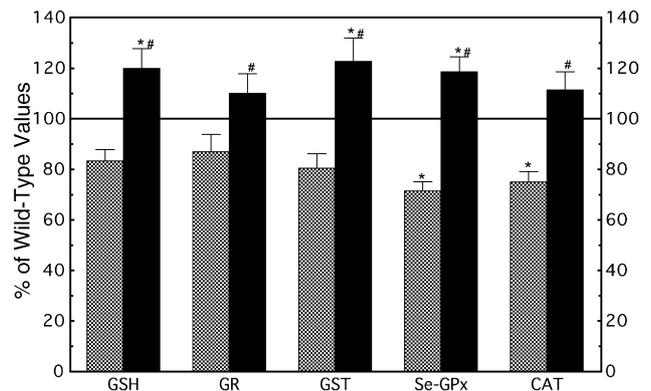


Fig. 3. Levels of reduced glutathione and the activities of anti-oxidant enzymes in the aorta. Reduced glutathione and the enzymes are expressed as a percent of those values found in age-matched WT mice ($n = 12$). Stippled bars represent untreated apoE KO mice ($n = 9$), while the full bars the HBO-treated mice ($n = 10$). Each bar is an average \pm SEM. (*) Significantly different from WT mice (represented by the line through 100%). (#) Significantly different from untreated apoE KO mice.

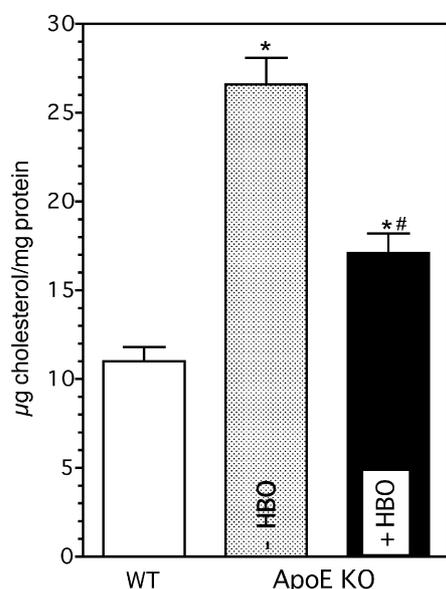


Fig. 4. Total aortic cholesterol content. Total aortic cholesterol content was determined in extracts of aortic homogenate, as described in the Materials and Methods. The effects of a 10-week HBO treatment of apoE KO mice (full bar, $n=10$) is compared to the untreated apoE KO mice (stippled bar, $n=9$) and WT mice (open bar, $n=7$). (*) Significantly different from WT mice. (#) Significantly different from untreated apoE KO mice.

We were unable to measure the levels of oxidized (GSSG) glutathione levels due to the scarcity of the aortic tissue.

3.3.2. Enzymes

HBO treatment has a profound effect on the aortic anti-oxidant enzymes: a 26, 52, 66 and 49% increase for GR, GST, Se-GPx and CAT, respectively. The levels of all of these enzymes in the aortic tissue of the HBO-treated apoE KO mice are significantly higher than even those of the WT mice.

The decreased levels of these enzymes in the untreated apoE KO mice are consistent with the increased oxidative stress observed in the apoE KO genotype.

3.4. Aortic fatty streaks and cholesterol

Fatty streak lesions in HBO-treated or untreated apoE KO mice were assessed by two independent means: (a) aortic cholesterol content; and (b) by *en face* analyses of the aortic arch. As shown in Fig. 4, the levels of cholesterol in the aortic tissue of the 15-week-old apoE KO mice are 2.4-fold higher ($p<0.0001$) than those of age-matched WT mice, reflecting significant fatty streak formation and atherosclerosis. HBO treatment leads to a marked decrease in aortic cholesterol content (37%, $p<0.0001$), but it is still substantially higher than the cholesterol content of the WT aorta.

En face analyses of the aortic arches obtained from four control (untreated) or treated apoE KO mice are shown in Fig. 5. The affected surface area in the untreated animals at 15 weeks of age was $6.9 \pm 1.4\%$, compared to $3.2 \pm 0.7\%$ in HBO-treated mice, a significant ($p<0.03$) decline of 53%. Gross examination of the fatty streak lesions with a stereomicroscope also reveals that lesions in untreated mice were more raised, when compared to lesions found in HBO-treated mice (not shown). Thus both measurements of atherosclerosis are in agreement and demonstrate the effectiveness of HBO treatment in reducing the development of atherosclerosis.

4. Discussion

In the present study we extend our previous observations [8] to a well-accepted murine model of atherosclerosis: female apoE KO mice fed a regular mouse chow. By including age-matched WT mice we are able to examine not only

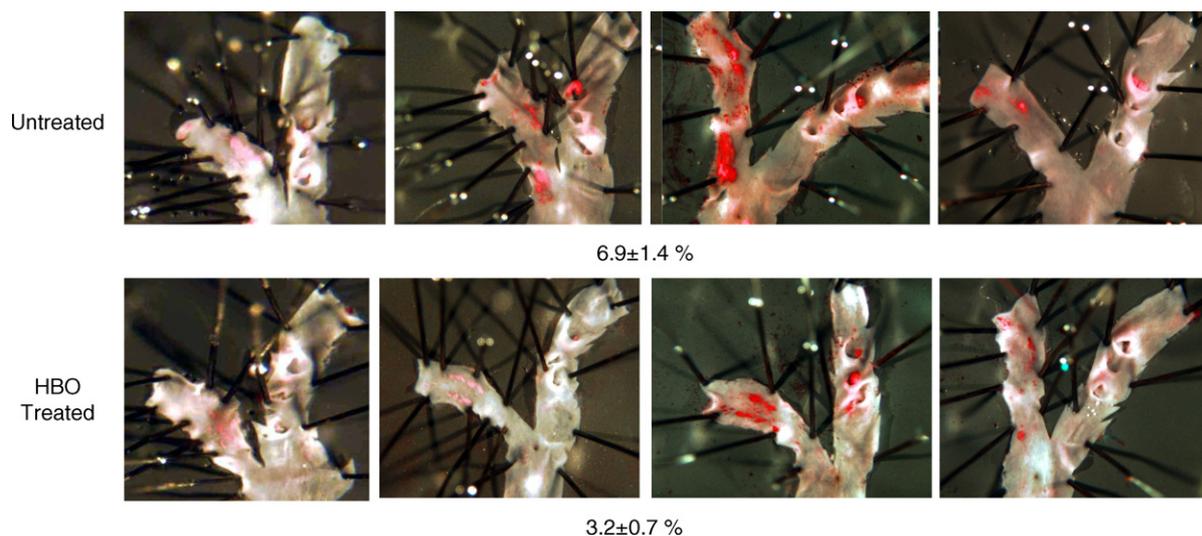


Fig. 5. Oil-Red O-stained aortic arches of 4 control (untreated apoE KO mice) and 4 HBO-treated mice. The average \pm SEM of the area of aorta involved in fatty streak formation is shown below each set of pictures. The total area of aorta examined in each group was similar and statistically not different. HBO treatment dramatically reduces the Oil-Red-O-stained lesions by 53% ($p<0.03$), as determined by analysis with Image Pro Plus.

the effects of HBO treatment on anti-oxidant enzyme activities in apoE KO mice, but also examine the effects of the apoE gene ablation on these activities.

Relative to the WT mice, the ablation of the apoE gene is accompanied by a substantial and statistically significant decrease in hepatic anti-oxidant enzymes, including GST, Se-GPx and CAT and a corresponding increase in TBARS, GSSG and the GSSG/2GSH ratio. These changes confirm a significant shift, in the liver, to a more oxidizing environment. Although the data on the aortic tissue are incomplete, a similar trend is observed. Another good indicator of the more oxidizing environment is the over 4- and 2-fold increase in the titers of the anti-oxLDL and MDA LDL antibodies, respectively. In agreement with these observations, others have demonstrated that the ablation of apoE expression leads to an increase in the oxidative stress, both in the vasculature as well as in the neuronal tissues in this animal model [22–24].

HBO treatment of the apoE KO mice was initiated at 5 weeks of age and continued for a period of up to 10 weeks. At 5 weeks of age they are essentially disease-free, but in the absence of treatment measurable fatty streak formation occurs over the next 10-week period, as shown by our results and reported by others [25]. HBO treatment has no effect on plasma total or individual lipoprotein cholesterol levels in apoE KO mice.

Serum paraoxonase circulates in blood in association with HDL and is synthesized in the liver. Both activities of PON1 (paraoxonase and arylesterase) possess anti-oxidant properties, as both reduce lipid peroxides in oxidized lipoproteins and tissues. Oxidative stress inactivates both activities, but can be reversed by anti-oxidants [26]. The restoration of plasma PON1 activities in apoE KO mice by HBO is in agreement with these reports.

HBO treatment significantly increases the hepatic levels of all measured anti-oxidant enzymes and reduced glutathione. Conversely and as expected, HBO treatment dramatically reduces the levels of hepatic TBARS and oxidized glutathione. As a result of these changes, the hepatic redox state is dramatically shifted towards a reducing environment. Changes of similar nature are observed in the aortic tissues. Although we were unable to measure all of the variables that were measured in the liver due to insufficient amounts of tissue, significant increases in aortic reduced glutathione as well as four anti-oxidant enzymes (GR, GST, Se-GPx and CAT) also indicate a shift to a reducing environment. The net and overall result of such changes is a reduction in lipid oxidation and inflammatory events. The dramatic reduction of the anti-oxLDL and MDA-LDL antibodies by the 10-week HBO treatment to levels not different from WT mice provides additional support for the notion of reduced oxidative stress. These changes are associated with reduced aortic cholesterol content by 37% and an over 50% decrease in aortic fatty streaks. Other anti-oxidants, including vitamin E, DPPD (*N,N'*-diphenyl 1,4-phenylenediamine and polyphenols) have been shown to reduce atherosclerosis in this animal model [22,27,28].

The importance of tissue GSH levels and redox state in modulating atherogenesis is evident from other studies. While a reduction in tissue GSH increases the lesion size, an increase in tissue GSH and GPx decreases the lesion size [29]. Similarly, caloric restriction which retards the pro-oxidizing shift in the redox state of glutathione [30] has been shown to reduce the production of superoxide and peroxides in the arterial wall and attenuate atherosclerosis in apoE KO mice without altering the elevated plasma cholesterol levels [31]. High levels of anti-oxidant activities have been linked, both in human and mouse studies, to a resistance towards atherosclerotic lesion development [32,33].

The present study does not investigate the molecular mechanisms underlying the HBO-mediated elevation of the endogenous anti-oxidants. The coordinated induction of anti-oxidants might be mediated by ROS and lipid peroxides, as has been demonstrated for glutathione, GPx1, catalase, GST etc [34].

Atherosclerosis is a complex, polygenic disease in which the involvement of oxidative stress and inflammation is well established but the specific mechanism is not fully understood. The apoE KO mice provide a particularly useful model for the study of this disease. It is now well established that the ablation of the apoE gene in these mice leads not only to prolonged circulation of cholesterol-rich lipoproteins (due to a lack of a receptor ligand) but also, and independent of its role in lipoprotein metabolism, increase in the oxidative stress in various tissues. It is not surprising that every variable related to oxidation we measured confirms that apoE KO mice are under increased oxidative stress relative to the WT mice. This is true for plasma components as well liver and aortic enzymes, markers of oxidation and glutathione ratios. In the present report we also demonstrate that HBO treatment dramatically reduces fatty streak formation along with a dramatic reduction of oxidative stress, as measured by a variety of parameters. Although the precise mechanism of HBO mediated protection is not known, our studies suggest a significant role for the induction of anti-oxidant enzymes. We are presently in the process of addressing these issues.

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