Hyperbaric Oxygen Reduces the Progression and Accelerates the Regression of Atherosclerosis in Rabbits

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Abstract—We studied the effect of hyperbaric oxygen (HBO) treatment on the extent of diet-induced accumulation of lipid oxidation products in rabbit plasma and tissues, on plasma paraoxonase activity, and on the extent of progression and regression of atherosclerotic lesions in the rabbit aorta. HBO treatment of cholesterol-fed rabbits dramatically reduces the development of arterial lesions despite having little or no effect on plasma or individual lipoprotein cholesterol concentrations. Compared with no treatment in cholesterol-fed animals, HBO treatment also substantially reduces the accumulation of lipid oxidation products (conjugated dienes, trienes, and thiobarbituric acid–reactive substances) in plasma, in the low density lipoprotein and high density lipoprotein fractions of plasma, in the liver, and in the aortic tissues. In addition, HBO treatment prevents the decrease in plasma paraoxonase activity observed in rabbits fed cholesterol-rich diets. Similarly, in regression studies, HBO treatment has no effect on the rate of plasma (or lipoprotein) cholesterol decline but significantly accelerates aortic lesion regression compared with no treatment. Direct measures of aortic cholesterol content support these morphological observations. On the basis of these results, we conclude that repeated, but relatively short, exposure to HBO induces an antioxidant defense mechanism(s) that is responsible for retarding the development or accelerating the regression of atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2000;20:1637-1643.)

Key Words: hyperbolic oxygen ■ atherosclerosis ■ paraoxonase ■ hypercholesterolemia ■ lipid oxidation

Atherosclerosis is the leading cause of death in Western society. Several lines of evidence suggest that oxidized lipoproteins play an important role in the etiology of this disease. Oxidized LDL is a powerful atherogenic agent that is able to bypass the normal feedback–regulated cellular cholesterol uptake by peripheral cells and lead to foam cell production.1–3 It has been isolated from atherosclerotic lesions.4–5 Oxidized LDLs potentiate monocyte–endothelial cell interaction,6 regulate cytokine expression,7,8 and stimulate chemotactic factor expression.9,10 They have also been shown to induce apoptosis in vascular cells.11–13 In contrast, the addition of antioxidants to the diet has been shown to decrease the progression of atherosclerosis in animal models.14–16 Oxidized HDLs appear to lose their ability to protect against atherosclerosis,17 including their ability to promote reverse cholesterol transport,18 one of the major putative mechanisms of protection against peripheral cholesterol accumulation.19 Myeloperoxidase, a heme protein secreted by phagocytes, appears to be a major catalyst for the in vivo formation of oxidized lipoproteins.20,21 Active enzyme has been found in atherosclerotic lesions.22

Hyperbaric oxygen (HBO) treatment is primarily used in the treatment of carbon monoxide poisoning and healing of problematic wounds in diabetic patients.23–25 Because diabetic patients are already at significantly increased risk of atherosclerosis,26,27 we wondered whether the additional periodic exposure to 100% oxygen at higher pressures would further increase this risk.

Repeated exposure to HBO or hyperoxia may be expected to lead to increased formation of hydrogen peroxide, a major reactant in the myeloperoxidase-catalyzed formation of hypochlorous acid,28,29 which, in turn, may modify plasma LDL and promote its rapid uptake by macrophages. Similar modification of HDL may reduce its ability to promote cholesterol efflux from peripheral cells.19

On the other hand, repeated exposure to HBO may induce the production of antioxidant enzymes/reagents by tissues,30,31 including the arterial wall, which, in turn, may reduce the extent of formation of oxidized lipoproteins and atherosclerosis.

In the present study, we tested the hypothesis that HBO accelerates the progression of atherosclerosis in rabbits fed an atherogenic diet. Surprisingly, we found that repeated exposure to HBO for short periods of time significantly reduces the progression of fatty streak formation compared with no exposure to HBO. Equally significant is our finding that HBO accelerates the regression of preestablished lesions. These observations extend our understanding of the etiology of atherosclerosis and have important implications in the treatment of this disease.

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Methods

Animals and Diets
Male New Zealand White rabbits (initial weight 1.5 to 2 kg) were used for these studies. All animals were individually caged in stainless-steel wire-bottomed cages in a temperature- and humidity-controlled room on a 12-hour light/dark cycle. Our animal care facility is accredited by the American Association for the Accreditation of Laboratory Animal Care, and all procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

Two separate experimental protocols were used to assess the effects of HBO treatment on the progression and regression of atherosclerosis. In the progression studies, atherosclerosis was induced by feeding the animals laboratory rabbit chow supplemented with 1% cholesterol and 10% hydrogenated coconut oil. A slightly less atherogenic diet was used for the regression studies (rabbit chow supplemented with 0.5% cholesterol). Dyets, Inc, supplied both diets. The change in the diet composition was to reduce, in the regression study, the extent of hepatic hyperlipidemia and liver damage noted in 2 animals during the progression study.

Experimental Protocol

Progression Studies
Two separate progression studies were carried out. After 2 weeks of acclimatization, the animals were randomly divided into 3 groups. The animals in the control group (n=3, study 1; n=4, study 2) were fed standard rabbit chow diet; the remaining animals were fed the atherogenic diet. Half of the rabbits fed the atherogenic diet also received daily HBO treatment (n=5 in both studies). The other half of the cholesterol-fed animals (n=5 and 6 in studies 1 and 2, respectively) received mock HBO treatment (no oxygen) to provide a similar amount of overall handling. This portion of the studies lasted 10 weeks. HBO treatment was initiated 1 week before the start of the diet. The data from studies 1 and 2 were pooled for analyses, but selected parameters were measured in only 1 of the studies.

Regression Study
All 18 animals used in the regression study were fed the atherogenic diet, as described above. After 10 weeks on the diet, 6 animals, selected at random, were euthanized, and the aortic fatty streak formation and cholesterol deposition were studied, as described below. This group provided baseline values for the atherosclerotic lesions developed before the start of regression with and without HBO treatment. The remaining 12 animals were randomly divided into 2 equal groups and switched to the regular chow diet to induce the regression of established fatty streaks in the presence or absence of daily HBO treatment. The regression period lasted 10 weeks.

HBO Treatment
The HBO treatment was administered in a specialized HBO chamber for animals. HBO treatment (5 days per week) consisted of a 15-minute compression period to 2.5 atm (absolute) or a pressure equivalent to 49.5 feet of sea water, followed by 90 minutes of 100% oxygen at 2.5 atm (absolute), followed by a 15-minute decompression period. The total treatment time was 120 minutes. A similar amount of overall handling. This portion of the studies was initiated 1 week before the start of the diet. The data from studies 1 and 2 were pooled for analyses, but selected parameters were measured in only 1 of the studies.

Atherosclerotic Lesion Evaluation
At the end of the experiment, the rabbits were anesthetized with ketamine and xylazine (35 and 10 mg/kg body wt, respectively), and blood was obtained by cardiac puncture. The abdomen and chest were opened, and the organs were perfused with ice-cold saline. The liver was removed, blotted dry, weighed, frozen in liquid nitrogen, and stored at −70°C until analysis. The aorta from the ascending arch to the iliac bifurcation was removed, washed with ice-cold PBS, and dissected free of adventitia and adipose tissue.

In the progression study, a small segment of arch was transferred to the fixative (4% formaldehyde and 1% glutaraldehyde in PBS, pH 7.4). The fixed segment of aortic arch was embedded in paraffin, cut into thin sections, and stained with hematoxylin and eosin. Six slides from each segment were evaluated by light microscopy by an observer blinded to the study protocol. The remaining aorta was cut into 3 segments (arch, thoracic, and abdominal), weighed, and stored at −70°C until analysis.

In the regression study, the aortas were opened longitudinally, pinned flat on a waxed bed immersed in ice-cold PBS, and photographed. The total aortic area and the intimal area covered with atherosclerotic lesion were quantified from enlarged scanned photographs by use of Diversity version 1.0 software (PDI) and a Scannmaster-SM3 scanner (Howtek, Inc).

After photography, the aortas were cut into 3 segments as described above, blotted dry, weighed, and stored at −70°C until analysis.

Biochemical Analyses of Plasma, Liver, and Aorta

Plasma and Plasma Lipoproteins

In both studies, blood was collected by cardiac puncture at the time of euthanasia and transferred into heparinized tubes and tubes containing EDTA (1.5 mg/mL). In addition, in the regression study, small samples of blood (3 mL) were collected in heparinized tubes from the ear artery before the start of the atherogenic diet and at intervals thereafter until the end of the study. Blood was collected after a 12- to 16-hour fast. Plasma was obtained by low-speed centrifugation at 4°C. Lipoproteins were isolated within 24 hours of obtaining the plasma. Aliquots of plasma were adjusted to a density of 1.3 g/mL with the use of KBr and 1 mmol/L CaCl₂. Plasma was then sequentially layered under NaCl-KBr salt solutions, and the lipoprotein fractions were separated by density gradient ultracentrifugation, essentially as previously described. 33 Cholesterol content of plasma and of the lipoprotein fractions was determined by an enzymatic procedure adapted to the microtiter plate assay. 34 Aliquots of plasma containing butylated hydroxytoluene (100 μg/mL) were kept at −20°C for thiobarbituric acid-reactive substance(s) (TBARS) assays (see below).

Aorta and Liver

The entire segment of aorta was finely minced with scissors, and an aliquot was taken to measure lipid oxidation products. The remaining tissue was homogenized in a phosphate buffer, and the lipid was extracted from the homogenates as described by Folch et al. 35 The lipid-containing fraction was dried under nitrogen and then redissolved in ethanol, and after appropriate dilutions, total and free cholesterol levels were determined by an enzymatic assay described above. Esterified cholesterol was calculated as the difference between total and free cholesterol. The same procedures were used for pieces of liver obtained from different lobes.

Assessment of Lipid Oxidation

Lipid oxidation in plasma, plasma lipoproteins, liver, and aortic tissue segments was measured by determining TBARS. TBARS were determined essentially as described by Buege and Aust, 36 by use of the standard curve of malondialdehyde equivalents generated by acidic hydrolysis of 1,1,3,3-tetraethoxypropane. The extent of lipid oxidation in the plasma and lipoprotein fractions was also assessed by the determination of conjugated diene and triene concentrations after absorption of the isopropanol extract at 233 nm (for conjugated dienes) and 277 nm (conjugated trienes). Calculations were made with the use of molar extinction coefficients:

\[
2.8 \times 10^4 \text{ mol \cdot L}^{-1} \cdot \text{cm}^{-1} \quad \text{and} \quad 2.3 \times 10^4 \text{ mol \cdot L}^{-1} \cdot \text{cm}^{-1}
\]

for conjugated dienes and trienes, respectively. 37

Determination of PON Activity

Paraoxonase (PON) activity in the plasma and lipoprotein fractions was determined by an adaptation of the spectrophotometric method of Furlong et al. 38 to the microtiter plate assay method. Aliquots (10 μL) of diluted plasma (1:10) or the lipoprotein fraction (0- to 5-fold) were placed in microtiter plate wells in triplicate, and the reaction was initiated by adding 190 μL of the substrate (1.2 mmol/L paraoxon in 0.26 mmol/L Tris-HCl, pH 8.5, and 25 mmol/L CaCl₂). After a mixing, the plate was read immediately at 405 nm to establish the total rate of hydrolysis. The enzyme activity was calculated from the linear portion of the plot (A₄₀₅/time) by use of the molar extinction coefficient for p-nitrophenol: 17 100 μmol \cdot L^{-1} \cdot cm^{-1}. One
unit of PON activity equals 1 μmol of p-nitrophenol released per liter per minute.

Statistical Methods
Values reported in the text and in the Table represent mean±SEM. One-way ANOVA followed by Bonferroni/Dunn tests was performed to see whether any significant differences occurred among groups. A value of $P \leq 0.05$ was accepted as statistically significant. All analyses were performed with the use of StatView 4.5 statistical software (Abacus Concepts).

Results

Progression Studies
Atherosclerosis was induced in a group of rabbits by a 10-week regimen of cholesterol-supplemented rabbit chow (1% cholesterol). Half of these animals were exposed daily (5 times per week) to HBO; a third group of animals on a normal rabbit chow served as a control. At the end of the progression study, the body weights of cholesterol-fed HBO-treated rabbits (3.3±0.07 kg) were similar to the body weights of rabbits (3.3±0.11 kg) and significantly ($P<0.05$) higher than the body weights of cholesterol-fed untreated rabbits (2.8±0.07 kg). The liver weights were markedly increased in cholesterol-fed rabbits; HBO treatment had no additional effect.

As expected, the cholesterol-rich diet induced severe hypercholesterolemia during the 10-week study; plasma cholesterol levels increased >20-fold in the cholesterol-fed rabbits (Figure 1A). The majority of the increase (>67%) was accounted for by the VLDL + IDL fraction, whereas >30% of the increase was in the LDL fraction. The levels of HDL cholesterol decreased in both cholesterol-fed groups, but the decrease was not statistically significant in the HBO-treated group (data not shown). Overall, HBO treatment had little or no effect on plasma cholesterol concentrations (Figure 1A) or distribution (not shown), with the exception of HDL cholesterol noted above. The total liver cholesterol content was >10-fold higher in cholesterol-fed animals, and HBO treatment resulted in an additional, although not significant, increase in hepatic cholesterol content (Figure 1B). In contrast, compared with cholesterol-fed animals not treated with HBO, HBO-treated animals exhibited a profound and significant ($P<0.05$) reduction in the accumulation of cholesterol in the 3 aortic segments examined (Figure 1C through 1E). The aortic arch cholesterol content increased >9-fold as a result of cholesterol feeding but only 3-fold when accompanied by daily HBO treatment. Overall, HBO treatment reduced the diet-induced accumulation of cholesterol in the examined segments of aorta by >50%.

We also examined the levels of TBARS, a measure of overall lipid oxidation, in plasma and various tissues. HBO treatment resulted in a marked reduction in TBARS formation in all compartments examined compared with no treatment. As shown in Figure 1F, plasma TBARS were elevated 11-fold in the untreated cholesterol-fed rabbits but only 3-fold in cholesterol-fed HBO-treated rabbits. Significantly, HBO treatment prevented the increase in liver TBARS seen in rabbits receiving the cholesterol-rich diet (Figure 1G). HBO treatment was also very effective in preventing the diet-related increases in aortic tissue TBARS (Figure 1H through 1J). Pooled data from the 2 progression studies on the plasma concentration of conjugated dienes, trienes, and PON activities are presented in the Table. Cholesterol feeding induced a 3-fold increase in plasma-conjugated diene and triene concentrations, and HBO treatment inhibited these increases by >60%. Similarly, plasma TBARS concentrations increased 11-fold in the cholesterol-fed rabbits, but HBO treatment inhibited this increase by >70%. Concomitant with increases in plasma TBARS and conjugated diene and triene concentrations, plasma PON activity in cholesterol-fed rabbits decreased by >40%. In contrast, PON activity in HBO-treated rabbits remained unchanged.

The concentrations of dienes, trienes, TBARS, and PON activities in specific lipoprotein fractions were assessed in study 2, and the results are shown in the Table. Although in the majority of cases a convincing trend is observed, the smaller number of animals in the second study precluded many of the reported differences to reach statistical significance at $P<0.05$. Treatment with HBO inhibited the diet-
induced increases in LDL- and HDL-conjugated dienes and trienes. It was also very effective in decreasing the extent of TBARS accumulation in these lipoprotein fractions. HBO treatment had little or no effect on the levels of lipid oxidation products in the VLDL+IDL fraction of plasma lipoproteins.

The cholesterol-rich diet reduced total plasma PON activities by >40%. Because the great majority of plasma PON is associated with the HDL fraction, it is not surprising that this reduction came at the expense of HDLs. The large decrease in the HDL-associated PON was accompanied by small increases in PON activities in the LDL and VLDL+IDL fractions. Overall, HBO treatment completely prevented the diet-induced reduction of HDL-associated PON activity.

In agreement with the significantly lower arterial tissue cholesterol, histomorphological examination of fixed aortic arches from HBO-treated rabbits revealed little if any fatty streak formation, the initial stage of atherogenesis. Figure 2 shows typical representative sections of aortic arches from the 3 groups of rabbits. In the absence of HBO, cholesterol feeding led to a massive accumulation of macrophage-foam cells in the subendothelial layer (Figure 2c), typical of extensive fatty streaks and suggestive of progressive atherogenesis. Only occasional and small groups of macrophage-foam cells were observed in the aortic arches of HBO-treated rabbits (Figure 2b).

Regression Studies
To examine the effect of HBO treatment on existing lesions, 18 rabbits were fed a cholesterol-rich diet for 10 weeks. Plasma cholesterol concentrations rose rapidly during the 10-week induction period, nearly to the levels seen in the cholesterol-fed rabbits in the progression study, despite a lower content of cholesterol (0.5% versus 1% cholesterol). At this point, 6 control animals were euthanized to provide reference values for the various variables examined. The remaining animals were switched to normal rabbit chow for an additional 10-week period; half of these animals were exposed daily to HBO. Plasma cholesterol concentrations fell rapidly and in a nearly identical manner in both groups of rabbits during this period (Figure 1K). Liver cholesterol content (Figure 1L) also fell rapidly in both groups of rabbits, but HBO treatment resulted in a significantly greater decrease than that produced by rabbit chow alone. Rabbit chow alone was ineffective in significantly lowering thoracic or abdominal aortic cholesterol content but was effective in significantly lowering aortic arch cholesterol content. In contrast, the combination of rabbit chow and HBO treatment effectively and significantly lowered the cholesterol content in all 3 sections of the aorta (Figure 1M through 1O).

To examine the extent of regression of the fatty streak lesions in all 3 sections of the aorta, whole fresh aortas were opened and pinned flat on a wax bed and photographed. The photographs were scanned, and the raised areas (lesions) were evaluated by computer-aided planimetry. The results are shown in Figure 3. Rabbit chow alone resulted in a 30% reduction in raised opaque areas. The combination of rabbit chow and HBO treatment resulted in 60% reduction in fatty streak lesions. These observations correlate well with the direct measures of aortic cholesterol content.

Discussion
It is now well established that a major force in the peripheral accumulation of cholesterol is the oxidation of cholesterol-rich lipoproteins, such as LDL. Thus, modified LDL is subject to nonregulated uptake by macrophages within the
vessel wall, leading to the formation of macrophage-foam cells, which are building blocks in fatty streak formation and the initiation of atherogenesis.

The present studies examine the effect of HBO on the development of aortic fatty streaks in cholesterol-fed rabbits as well as on the regression of preestablished lesions. We found that HBO treatment protects against the development of fatty streaks in cholesterol-fed rabbits. Accumulation of cholesterol, especially cholesteryl esters, in macrophage-foam cells is the hallmark of the atherosclerotic lesion. A cholesterol-rich diet markedly increased free and cholesteryl ester levels in all 3 segments of the aorta examined. This accumulation was dramatically reduced in the rabbits treated with HBO. The lipid data are supported by histomorphological examination of the sections taken from similar areas of the aortic arch segments. Of the 5 HBO-treated rabbits examined, 3 rabbits showed a complete absence of lesions, whereas the other 2 rabbits had far less pronounced lesion thickness and frequency compared with the cholesterol-fed untreated rabbits, all of whom showed marked lesion formation. The scarcity and mildness of lesions in the treated group suggest that HBO treatment arrests the development of fatty streaks in cholesterol-fed rabbits.

The differences in lesion formation seen in HBO-treated animals, compared with untreated animals, were not due to differences in plasma total or individual lipoprotein (VLDL, IDL, LDL, and HDL) cholesterol levels. These values were not significantly different in the 2 cholesterol-fed groups of animals. A major difference between the 2 cholesterol-fed groups of rabbits was the content of oxidized products in the LDL and HDL fractions. Compared with LDL and HDL from HBO-treated cholesterol-fed rabbits, LDL and HDL isolated from untreated cholesterol-fed rabbits were both enriched severalfold in conjugated dienes, trienes, and TBARS. The abundance of mildly oxidized LDL in untreated but not HBO-treated animals may explain the accelerated fatty streak formation in these animals. The increased content of oxidized products in the HDL fraction of the untreated rabbits may further decrease the protection normally afforded by HDL and the potentially antioxidant enzymes it may carry. These modifications are consistent with increased potential for the development of fatty streaks and atherosclerosis.

The reduction in the concentration of oxidation products in the tissue and plasma of HBO-treated rabbits is not surprising and is in agreement with previous reports. Furthermore, beneficial effects of hyperoxia (increased oxygen concentrations at normal pressure) on the progression and regression of atherosclerosis have been noted in the past. The previously reported changes have been modest relative to the results observed in control animals and compared with the results reported in the present study with the use of HBO.

The reduced oxidation of tissue and plasma lipids may well be a result of induced antioxidant activity. PON may play a role in this protection. Recent studies suggest that serum PON is an antioxidant enzyme involved in the detoxification of lipid peroxides. The profound decrease in HDL-associated (and total) PON activity in the untreated cholesterol-fed rabbits correlates well with the increased formation of oxidation products. Similar findings have been reported in atherosclerosis-susceptible (C57BL/6J) mice but not in atherosclerosis-resistant (C3H/HeJ) mice. It remains to be seen whether the observed decrease in plasma PON activities of the untreated cholesterol-fed rabbits is the result of decreased

![Figure 2. Hematoxylin- and eosin-stained paraffin-embedded thin sections of aortic arch from control (a), cholesterol-fed HBO-treated (b), and cholesterol-fed (c) rabbits. Note the complete absence of macrophage-foam cells in the subendothelial layer in section taken from a control animal (a) and occasional small accumulation visible in section taken from a cholesterol-fed HBO-treated rabbit (b). In contrast, aortic arch sections taken from cholesterol-fed rabbits not treated with HBO (c) had an extensive and continuous subendothelial layer of macrophage-foam cells.](http://atvb.ahajournals.org/)

![Figure 3. Extent of total aortic atherosclerosis in control and regressed rabbits, as seen at the end of the induction period (cholesterol-fed [Chol. fed] rabbits) or 10-week regression period (untreated or HBO-treated rabbits). Atherosclerosis was evaluated as described in Methods. a, P<0.05 vs Chol. fed non-regressed animals. b, P<0.05 vs regressed animals not treated with HBO.](http://atvb.ahajournals.org/)
enzyme mass (reduced expression) or inhibition by the high concentration of lipid peroxides.  

The maintenance of normal plasma PON activity in HBO-treated rabbits may be a result of a counteracting mechanism inducing the expression of additional PON or be secondary to the reduced levels of lipid peroxides. Our data do not exclude the possibility that other enzymes, induced by the intermittent exposure to HBO, may also play an important role in “detoxifying” tissue or circulating lipoprotein-associated lipids.

Indeed, brief exposure to HBO/hyperoxia has been shown to increase the levels of glutathione in blood and to induce the expression of a number of antioxidant enzymes in tissues. These include heme oxygenase, which recently has been shown to offer protection from the development of atherosclerosis and to reduce oxidation products in HDL. Alternatively, the protective effect of HBO treatment may be mediated by the suppression of specific enzymes responsible for lipid oxidation. Our future studies will specifically address the regulation of expression, by exposure to HBO, of a number of candidate enzymes.

Although the finding that HBO inhibits the formation of fatty streaks has important implications for the prevention of atherosclerosis, the use of this treatment to accelerate regression could have profound implications for therapy of established disease. Our results demonstrate that HBO treatment markedly accelerates the regression process by a mechanism independent of plasma cholesterol concentrations.

These data suggest that HBO suppresses the recruitment and proliferation of macrophages and the formation of foam cells in atherosclerotic lesions, thereby inhibiting the initial development of atherosclerosis. This is brought about by increased protection against the formation of lipid-derived oxidation products. These observations further support the importance of oxidative processes in promoting atherogenesis. Elucidation of the details of this mechanism will provide new insights into the pathophysiology of atherosclerosis.

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