Lipid Lowering Reduces Oxidative Stress and Endothelial Cell Activation in Rabbit Atheroma

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Background—Lipid lowering may reduce acute coronary events in patients in part by reducing vascular inflammation. Oxidative stress induces endothelial cell (EC) expression of vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemoattractant protein 1 (MCP-1) and reduces levels of atheroprotective NO, leading to monocyte recruitment and macrophage accumulation. This study tested the hypothesis that lipid lowering decreases oxidative stress and improves EC functions related to inflammatory cell accumulation.

Methods and Results—Rabbits consumed an atherogenic diet for 4 months to produce atheroma, followed by a purified chow diet for 16 months. Atherosclerotic aortas from hypercholesterolemic rabbits produced high levels of reactive oxygen species. Oxidized LDL (oxLDL) accumulated in atheroma underlying ECs that overexpress VCAM-1. In contrast, few if any ECs in atheroma stained for endothelial NO synthase (eNOS). Lipid lowering reduced reactive oxygen species production, oxLDL accumulation, and plasma levels of anti-oxLDL IgG. After lipid lowering, VCAM-1 and MCP-1 expression decreased, eNOS expression increased, and ECs exhibited a more normal ultrastructure.

Conclusions—These results establish that lipid lowering can reduce oxidative stress and EC activation in vivo. These mechanisms may contribute to improvement in endothelial function and plaque stabilization observed clinically. (Circulation. 2002;106:1390-1396.)

Key Words: atherosclerosis ■ hypercholesterolemia ■ endothelium ■ cell adhesion molecules ■ nitric oxide synthase

Atherosclerotic plaques contain numerous inflammatory cells, including macrophages.1 These phagocytes produce various proteinases, procoagulant molecules, and reactive oxygen species (ROS), factors that likely contribute to endothelial dysfunction, plaque vulnerability, and thrombogenicity.2,3 Activated endothelial cells (ECs) in atheroma can overexpress adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and chemokines, including monocyte chemoattractant protein 1 (MCP-1), which contribute to monocyte recruitment.4,5 Oxidative stress can induce such EC activation.4 Atherosclerotic arteries produce excess ROS such as superoxide anion (O2•−), promoting oxidative modification of LDL.6 Oxidized LDL (oxLDL) accumulates in atheroma.7 Animal and clinical studies have also shown a strong correlation between extent of atherosclerosis and titers of autoantibodies to epitopes of oxLDL.8–10 ECs in apparently normal arteries constitutively express endothelial NO synthase (eNOS), an antiatherogenic factor,11,12 but its expression decreases in human atheroma.13 Moreover, O2•− can inactivate NO and form the injurious peroxynitrite anion (ONOO−). ROS can also activate nuclear factor κB, a central transcription factor that regulates expression of numerous proinflammatory mediators involved in atherogenesis. Recent clinical studies have established that lipid lowering by HMG-CoA reductase inhibitors (statins) reduces acute coronary events, probably by “stabilization” of plaques in a functional manner.3 Indeed, we demonstrated that lipid lowering diminishes macrophage number and reduces proteolytic and prothrombotic potential in atheroma of hypercholesterolemic rabbits.14–16 We also showed that a diminished number of macrophages expressing platelet-derived growth factor B chain may favor the accumulation of mature smooth muscle cells (SMCs) observed in atheroma after lipid lowering.17 However, the mechanisms by which lipid lowering reduces macrophage accumulation and other aspects of inflammation in atheroma remained obscure. This study therefore tested the hypothesis that lipid lowering can limit oxidative stress and functions of ECs involved in vascular inflammation.
Experimental Protocol

Figure 1. Experimental protocol. Thirty New Zealand White male rabbits consumed an atherogenic diet for 4 months to create atheroma. Balloon injury of the thoracic aortas by Fogarty embolectomy catheter was performed 1 week after initiation of the atherogenic diet. Fifteen rabbits euthanized at 4 months constituted the Baseline group. Five animals continued to consume the atherogenic diet for an additional 16 months (High group). The remaining animals consumed a chow diet with no added cholesterol and fat for 16 months (Low group).

Methods

Experimental Design

The experimental protocol used and other analyses of the animals studied here have been the subject of previous publications. Briefly, we created aortic atheroma by balloon injury followed by an atherogenic diet for 4 months. We switched 10 rabbits from the atherogenic diet to purified chow with no added cholesterol and fat and continued feeding for 16 months (Low group). The remaining 5 rabbits continued on the atherogenic diet for an additional 16 months (High group). All experiments conformed to a protocol approved by the Harvard Medical School Standing Committee on Animals. Tissues were harvested after intravenous administration of sodium pentobarbital (120 mg/kg). The proximal portion of the thoracic aorta (2 mm below the ligamentum arteriosum) was excised and snap-frozen with OCT compound (Sakura Finetek Inc). The rest of the thoracic aorta was obtained for electron microscopic study, lucigenin chemiluminescence, and nitroblue tetrazolium (NBT) staining.

Histological Assays

Mouse monoclonal antibodies used in this study include antibodies against rabbit VCAM-1 (Rb1/9), provided by Dr Michael A. Gimbrone, Jr, Brigham and Women’s Hospital, Boston, Mass), bovine eNOS (provided by Dr Guillermo Garcia-Cardena, Brigham and Women’s Hospital, Boston, Mass), human MCP-1 (Pharmingen), apolipoprotein B-100 (apoB-100) (MB47), MDA-LDL (MDA-2), and human CD31 (DAKO). Transmission electron microscopic study on aortic rings obtained from three animals in each group was performed as previously reported. The percentage of the intimal area immunopositive for oxLDL or apoB-100 and percentage positive endothelium for VCAM-1 or eNOS within CD31-positive endothelium were determined using a computer-based color image analysis system. Statistical testing used one-way ANOVA followed by post hoc testing (Fisher test).

Vascular ROS Production

ROS production by fresh rabbit aortic specimens was measured by lucigenin chemiluminescence. Lucigenin chemiluminescence is produced by O₂⁻ but not by myeloperoxidase (MPO) and only weakly by H₂O₂ or by the H₂O₂-MPO-CI⁻ system; autooxidation of lucigenin can also occur. First, background counts were obtained by measuring chemiluminescence in scintillation vials containing 2 mL of Krebs-HEPES buffer and 0.25 mmol/L of lucigenin for 15 minutes in the luminometer (Lumat LB9501, Berthold). Then, freshly isolated aortic rings from the normal rabbits and from rabbits of the Baseline, High, and Low groups were added. We used Tiron (1,3-benzene disulfonic acid, 10 mmol/L) (Sigma), a cell-permeable nonenzymatic scavenger of superoxide anions, and diphenyl iodonium (100 μmol/L), an inhibitor of flavoprotein enzymes including NADPH oxidase or NADH oxidase, and xanthine oxidase (Sigma) for the inhibition assay. Tiron-inhibitable lucigenin chemiluminescent counts served as the measure of ROS production. Because background counts might result in part from autoxidation of lucigenin, these counts were subtracted from each value obtained from aortic rings. Lucigenin chemiluminescent counts were adjusted on the basis of the dry weight of aortic rings. ROS production is expressed as relative light units (RLU); 19.0 RLU is equivalent to 1 pmol of O₂⁻. After freezing once, atherosclerotic aortic rings produced no or negligible ROS. An NBT-reducing activity assay in situ served to localize the production of ROS, including O₂⁻ in aortic specimens. NBT develops a blue color in the presence of ROS, including O₂⁻. Fresh aortic rings were incubated with NBT (1 mg/mL in Krebs-HEPES buffer) at 37°C for 4 hours and then snap-frozen with OCT compound in isopentane prechilled with liquid nitrogen. Frozen tissues were sectioned and counterstained with Nuclear Fast Red (DAKO), and then mounted. Tiron was used to demonstrate specificity.

Chemiluminescence Immunoassay for Antibodies Against oxLDL

White round-bottomed 96-well MicroFluor plates (Dynex Technologies Inc) were coated with MDA-LDL (5 μg/mL). Plasma samples were diluted 1:250 in PBS containing 1% BSA and incubated in wells for 1 hour at room temperature. Alkaline-phosphatase-labeled goat anti-rabbit IgG or IgM was added and the plates were incubated for 1 hour at room temperature. After 4 washings, 50% Lumi-Phos 530 (Lumigen Inc, Southfield, MI) was added, and the plates were incubated for 1.5 hours at room temperature in the dark. The light emissions were measured as RLU over 100 ms using a Dynex Luminometer.

Results

Lipid Profile

As previously reported in this cohort of animals, mean total cholesterol (TC) and triglyceride (TG) levels (mg/dL) rose after 4 months on the atherogenic diet, as follows: TC from 43±4 to 1562±123 and TG from 52±14 to 244±49. The TC and TG levels returned to baseline after 16 months on the control diet (Low group) lacking supplemental lipids (n=10; TC, 19±3; TG, 58±10) but remained elevated in the High group (n=5; TC, 1108±158; TG, 224±87).

Lipid Lowering Reduced apoB-100, oxLDL, VCAM-1, and MCP-1 and Increased eNOS in Atheroma

We examined levels of apoB-100, a major component of LDL and VLDL particles, by immunostaining using a specific monoclonal antibody, MB47. Immunoactive apoB-100 accumulated in atheroma of hypercholesterolemic rabbits (Figure 2A, Baseline group, top left; High group, data not shown). OxLDL epitopes (detected by the antibody MDA-2 against MDA-LDL, one epitope of oxidatively modified LDL) colocalized with apoB-100 (top right). Lipid lowering, however, reduced the amount of immunoreactive apoB-100 and oxLDL epitopes in lesions (bottom panels). Negative controls, applying nonimmune mouse IgG or PBS in place of the specific monoclonal antibodies, showed no staining (data not shown). Quantitative color image analysis substantiated significant reduction in apoB-100 and oxLDL accumulation (Figure 2B and 2C) in the intima during lipid lowering. Lipid lowering also produced a statisti-
cally significant reduction in oxLDL/apoB-100 ratio in atheroma (Figure 2D).

After 4 months on the atherogenic diet, oxLDL accumulated in the intima and the overlying ECs expressed VCAM-1 (Figure 3A, top panels). OxLDL accumulation and VCAM-1 expression persisted after 16 months of continued hypercholesterolemia (middle panels). In contrast, after 16 months of dietary lipid lowering, few if any ECs stained positively for VCAM-1 and oxLDL accumulation substantially decreased (bottom panels). Quantitative color image analysis showed that lipid lowering produced statistically significant reduction in VCAM-1 expression (Figure 3B) in the aortic intima.

MCP-1 localized in ECs as well as in SMCs and macrophages in atheroma of hypercholesterolemic rabbits after 4 months on the atherogenic diet (Figure 4). After 16 months of dietary lipid lowering, few if any ECs, SMCs, or macrophages expressed MCP-1, whereas these three cell types all expressed this monocyte chemoattractant after 16 months of continued hypercholesterolemia. We obtained similar results from 5 animals in each group.

ECs in rabbit atheroma showed less eNOS immunoreactivity than did ECs in normal rabbit aortae (99% eNOS [+], data not shown). CD31-positive aortic ECs from the aortas of the Baseline- and High-group animals displayed low levels of immunoreactivity for eNOS (Figure 5A, top and middle). However, Low-group rabbits that experienced long-term lipid lowering showed more eNOS-positive ECs than did the hypercholesterolemic rabbits (Figure 5A, bottom). Quantitative analysis demonstrated that lipid lowering yielded a statistically significant increase in eNOS-positive endothelium in rabbit atheroma (Figure 5B).
ECs After Lipid Lowering Exhibited More Normal Ultrastructure

Electron microscopy showed that ECs in the aortas of the Baseline and High groups displayed a cuboidal or rounded structure typical of an “activated” phenotype. However, the aortic ECs of the Low group had a more squamous morphology (Figure 6). The size, density, and number of cytoplasmic organelles of ECs in the atheroma of hypercholesterolemic rabbits exceeded that found in the Low-group animals. These morphological features are typical of ECs found in atherosclerotic arteries. Notably, a monocyteic cell (indicated by an arrowhead) appears to be entering the intima of the baseline lesion. The circular cells containing abundant lipid particles in the subendothelial space of the aortas in the Baseline and High groups are probably macrophage-derived foam cells. Consistent with our previous observations at the level of light microscopy, accumulation of organized collagen fibrils was more prominent in the intima of the Low-group animals than in atheroma of the high cholesterol–fed rabbits.

Lipid Lowering Reduced ROS Production and Plasma Levels of Autoantibodies to oxLDL

ROS production (detected as Tiron-inhibitable lucigenin chemiluminescence) in freshly isolated aortic segments from the Baseline- and High-group rabbits exceeded that of aortas from age-matched normal rabbits (Figure 7A). However, after 16 months of dietary lipid lowering, ROS production decreased to levels similar to those of the normal rabbits. Lucigenin chemiluminescence was inhibited 97.6% by diphenylidonium (100 μmol/L) (an inhibitor of flavoprotein enzymes including NADPH oxidase or NADH oxidase, and...
xanthine oxidase). Tiron-inhibitable NBT reducing activity revealed ROS production (Figure 7B). Development of the blue color indicates a reaction between ROS, produced by vascular cells, and its substrate NBT. Aortic ECs of the Baseline group had intense staining. Spindle-shaped cells (probably SMCs, top panel) and a cluster of circular cells (likely macrophages, middle panel) in the intima of the baseline lesion also stained with NBT. Tiron (10 mmol/L) inhibited this staining (bottom panel).

We measured plasma levels of autoantibodies against oxLDL epitopes (MDA-LDL) by immunoassay. Levels of anti–MDA-LDL IgG in the Baseline and High groups significantly exceeded those of the Low group (Figure 7C). Anti–MDA-LDL IgM levels did not differ significantly among the Baseline, High, and Low groups (data not shown).
**Discussion**

The present study demonstrates that dietary lipid lowering markedly decreased ROS production and oxLDL accumulation in rabbit atheroma. The reduction in oxidative stress, a proximal proinflammatory stimulus during atherogenesis, paralleled the recovery by ECs of a more normal phenotype. LDL lowering decreased oxLDL content within apoB-100–immunoreactive areas and likely limited the enhanced lipid peroxidation that occurs in hypercholesterolemia. Tsimikas et al demonstrated that dietary lipid lowering in hypercholesterolemic mice decreased in vivo uptake of the antibody for oxLDL (MDA2) to a greater degree than other features of atherosclerosis. These changes should mitigate EC dysfunction and inflammatory cell infiltration in atherosclerotic arteries.

In addition to increased monocyte recruitment, proliferation of macrophages may contribute to their accumulation in atheroma. OxLDL influences macrophage survival and growth in vitro. Hence, the reduced accumulation of oxLDL observed here might contribute to decreased numbers of macrophages in atheroma during lipid lowering. Recently, we demonstrated that administration of an HMG-CoA reductase inhibitor suppresses macrophage growth in rabbit atheroma. The current study shows that lipid lowering by diet alone can limit macrophage accumulation in lesions by mechanisms independent of any pharmacologic effects.

The sources of ROS in the vasculature and their importance in atherogenesis are not certain. Ohara et al previously demonstrated that ECs produce most of the excess O$_2^-$ (detected by the lucigenin chemiluminescence assay) at the early stage of rabbit atherosclerosis and that lipid lowering normalizes this endothelial O$_2^-$ production. The present observations show a similar reduction in ROS production in rabbits with more extensive fibro-fatty lesions at a later time point. The intensity of NBT staining of ECs in the present paper may indicate that ECs produce more ROS than do macrophages and SMCs.

Chemiluminescence measured by the lucigenin assay reflects in part autoxidation of lucigenin. However, we detected a considerable increase in Tiron-inhibitable lucigenin chemiluminescence counts generated by atherosclerotic aortic rings compared with those from normal aorta. Moreover, the counts of chemiluminescence on the aortas from treated rabbits decreased to levels similar to those from normal rabbit aortas, and atherosclerotic aortas frozen before the assay produced negligible signal, suggesting that lucigenin chemiluminescence measured in this study resulted from ROS produced by live cells in atheroma, not from artifactual O$_2^-$ generation due to lucigenin autoxidation.

Plasma levels of autoantibodies to epitopes of oxLDL, another index of oxidative stress, correlate in some studies with atherosclerotic lesion severity. In the present study, lipid lowering decreased plasma levels of IgG but not IgM antibody against oxLDL, consistent with the decreased extent of atherosclerosis. A similar finding was reported by Cyrus et al in atherosclerotic mice. Although the mechanism for this phenomenon remains unknown, the IgG antibody titer most likely reflects antigen-driven, T cell–dependent humoral immunity, whereas IgM titers may reflect T cell–independent factors such as natural antibodies.

Oemar et al have demonstrated decreased expression of eNOS, a possible atheroprotective factor, in human atheroma. Our study showed that dietary lipid lowering increased eNOS expression by aortic ECs in these atherosclerotic rabbits, whereas VCAM-1 and MCP-1 expression and oxidative stress decreased. Our in vivo results concur with in vitro findings that oxLDL decreases eNOS. Superoxide can react with NO, inactivating its endothelium-derived relaxing factor activity and producing peroxynitrite, a potentially proinflammatory and cytotoxic species. Therefore, reduced oxidative stress should heighten eNOS expression as well as augment NO activity and limit peroxynitrite formation. Increased NO production and availability represent additional mechanisms of decreased expression of VCAM-1 and reduced oxidative stress and should also promote vasodilation. The present observations provide further support for this mechanism of improved EC-dependent vasodilation after lipid lowering noted in human studies.

The interpretation of this study should consider several possible limitations. The degree of hypercholesterolemia produced in this rabbit preparation exceeds that usually encountered in human patients. Highly exaggerated levels of exogenous hypercholesterolemia in rabbits can yield a systemic cholesterol ester storage disease that caricatures rather than mimics human atherosclerosis. Therefore, we adjusted the cholesterol level in the diet of the animals subjected to prolonged hypercholesterolemia to limit this undesired aspect of experimental rabbit atherosclerosis. Lesions produced by cholesterol feeding and injury in these rabbits have several features typical of “vulnerable” atherosclerotic plaques of humans. Thus, despite these limitations, rabbit studies described above establish the concept that lipid lowering influences important aspects of atherogenesis. Indeed, our present observations demonstrate that dietary lipid lowering can improve several indices of oxidative stress and concomitantly normalize certain crucial EC functions involved in the progression of vulnerable and thrombogenic atherosclerotic lesions. Recent clinical studies have suggested that statin therapy reduces inflammation by mechanisms independent of their lipid lowering effect. However, the present study provides unambiguous evidence of the importance of lipid lowering itself in vascular inflammation and activation over and above any such “pleiotropic” effects of statins.

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