Active Metabolite of Atorvastatin Inhibits Membrane Cholesterol Domain Formation by an Antioxidant Mechanism*

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The advanced atherosclerotic lesion is characterized by the formation of microscopic cholesterol crystals that contribute to mechanisms of inflammation and apoptotic cell death. These crystals develop from membrane cholesterol domains, a process that is accelerated under conditions of hyperlipidemia and oxidative stress. In this study, the comparative effects of hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins) on oxidative stress-induced cholesterol domain formation were tested in model membranes containing physiologic levels of cholesterol using small angle x-ray diffraction approaches. In the absence of HMG-CoA reductase, only the atorvastatin active o-hydroxy metabolite (ATM) blocked membrane cholesterol domain formation as a function of oxidative stress. This effect of ATM is attributed to electron donation and proton stabilization mechanisms associated with its phe-noxy group located in the membrane hydrocarbon core. ATM inhibited lipid peroxidation in human low density lipoprotein and phospholipid vesicles in a dose-dependent manner, unlike its parent and other statins (pravastatin, rosuvastatin, simvastatin). These findings indicate an atheroprotective effect of ATM on membrane lipid organization through a potent antioxidant mechanism.

The unstable atherosclerotic lesion is characterized by extracellular lipid deposits consisting of cholesterol (both free and esterified), phospholipids, and lesser amounts of triacylglycerol (1). Free cholesterol is associated with phospholipid membranes and insoluble, extracellular crystals in the lipid core of the plaque. Membrane-associated cholesterol crystals have been characterized in cell culture systems and tissue explants from animal models of atherosclerosis using electron microscopy and x-ray diffraction approaches (2, 3). Microscopic cholesterol crystalline structures have also been observed in macropage foam cells following treatment with LDL2 or by inhibition of acyl-coenzyme A:cholesterol acyltransferase (4–6). These crystalline structures contribute to mechanisms of cell death and inflammation (3–5). Although non-crystalline membrane cholesterol can readily exchange from the plaque with plasma lipoprotein particles, cholesterol in the crystalline state is insoluble and does not respond to pharmacologic intervention or reverse cholesterol transport mechanisms (1).

We have recently reported that oxidative stress, a pathologic process associated with cardiovascular risk factors (e.g. hypertension, diabetes, hypercholesterolemia) (7), contributes directly to the formation of cholesterol crystalline microdomains in membranes (8). Domain formation as a function of lipid peroxidation was observed in lipid vesicles containing physiologic levels of cholesterol using small angle x-ray diffraction approaches (2, 9). These observations have led to our current hypothesis that the active o-hydroxy metabolite of atorvastatin (ATM) may interfere with cholesterol domain formation without altering membrane cholesterol content. ATM was selected as it has potent antioxidant activity, independent of HMG-CoA reductase inhibition (10, 11). In randomized clinical trials, atorvastatin treatment has been shown to have anti-inflammatory and antioxidant benefits (12–15) and is associated with reduced risk and progression of cardiovascular disease (16–18). In this study, the activity of ATM was compared with other statins (pravastatin, rosuvastatin, simvastatin) and known antioxidants (probucol, Trolox) using membranes enriched with polyunsaturated fatty acids (PUFAs) and physiologic levels of cholesterol (17–37.5 mol %) (19, 20). The results of this study indicate that ATM inhibits cholesterol domain formation by a potent antioxidant mechanism.

EXPERIMENTAL PROCEDURES

Materials—1,2-diarylchidonoyl-sn-glycero-3-phosphocholine (DAPC), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1-palmitoyl–2-o-leyo–sn-glycero-3-phosphocholine, and cholesterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and stored at −80 °C in high-performance liquid chromatography grade chloroform until use. Other chemical reagents and drugs were purchased from Sigma, Acros Organics (Morris Plains, NJ), and Calbiochem. Atorvastatin and its active o-hydroxy metabolite (ATM) were provided by Pfizer, Inc. The chemical structures of ATM and the other compounds used in this study are shown in Fig. 1.

Preparation of Human LDL, for Peroxidation Analysis—Human LDL was obtained from Calbiochem and preserved by EDTA. The EDTA was removed prior to peroxidation experiments by gel filtration with PD-10 Sephadex G-25 M columns equilibrated with 25 ml of phosphate-buffered saline. Following the loading of LDL, the column was washed with phosphate-buffered saline, and the eluant was collected and analyzed for protein content. The relevant fraction was diluted with phosphate-buffered saline to obtain a final protein concentration of 50 μg/ml for the peroxidation assays.

Determination of Malondialdehyde Levels in LDL.—Malondialdehyde (MDA), a reactive aldehyde produced by LDL lipid peroxidation, was quantitated by measuring its reaction with thiobarbituric acid to
form a stable chromogen referred to as thiobarbituric acid-reactive substances (TBARS) (21). The concentration of MDA was confirmed based on a standard curve for MDA/TBA complex using 1,1,3,3-tetramethoxypropane after acid hydrolysis. In these assays, LDL was incubated for 30 min with vehicle or ATM. The oxidation reaction was initiated by the addition of 10 μM CuSO4 at 37 °C. For measurement of TBARS, 100 μl of sample was removed after 2 h and combined with 1 ml of 0.5% TBA, 10 μl of 5% trichloroacetic acid, and aliquots of EDTA and butylated hydroxytoluene to produce a final concentration of 20 μM for each. After incubation at 100 °C for 30 min, the samples were cooled, and the absorbances were measured at 532 nm to calculate TBARS levels. The molar extinction coefficient for TBARS is 1.56 × 10^5 M^-1 cm^-1.

Preparation of Multilamellar Lipid Vesicles (MLVs) for Lipid Peroxidation and X-ray Diffraction Analyses—For lipid peroxidation and x-ray diffraction analyses, MLVs were prepared from phospholipid and cholesterol at specific mole ratios (0.2–0.6). Aliquots of lipid (1.0–5.0 mg) were transferred to 13 mm test tubes and dried down under a steady stream of N2 gas while vortexing vigorously for 3 min. Residual solvent was removed under vacuum in low light conditions. Lipid samples were then resuspended in diffraction buffer (0.5 mM HEPES, 154.0 mM NaCl, pH, 7.3), and vortex was mixed at ambient temperature to form MLVs, as described previously (22).

Determination of F2-Isoprostanes by Gas Chromatography (GC) with Negative Chemical Ionization Mass Spectroscopy (MS)—F2-Isoprostanes are derived principally from the formation of positioned peroxyl radical isomers of arachidonic acid, endocyclization to prostaglandin H2-like structures, and reduction to prostaglandin F2-like compounds. Total levels of F2-isoprostanes were measured in MLVs reconstituted from DAPC prepared in the presence of vehicle or various statins and Trolox (100 nM), using GC-MS with negative chemical ionization as described by Walter et al. (23). MLVs were allowed to autoxidize for 48 h at 37 °C, and peroxidation was terminated by the addition of 25 μl of 5.0 mM EDTA and 20 μl of 35.0 mM butylated hydroxytoluene. F2-Isoprostane formation was independently analyzed in blinded samples by mass spectroscopy in the Antioxidant Research Laboratory, Tufts University, Boston, MA.

Lipid Hydroperoxide Measurements in MLVs—For these experiments, 500 μl of MLVs reconstituted from DLPC (1.0 mg/ml) was freshly prepared in diffraction buffer in the absence and presence of freshly prepared ATM or Trolox at various concentrations. The vesicles were immediately placed in a shaking water bath at 37 °C and allowed to undergo gradual peroxidation without the use of exogenous initiators. After a 48-h incubation period to allow for autoxidation, 100-μl aliquots of the samples were removed, and the peroxidation reaction was terminated by the addition of 25 μl of 5.0 mM EDTA and 20 μl of 35.0 mM butylated hydroxytoluene. The extent of lipid peroxidation in the samples was measured by the CHOD-iiodide assay (24). The CHOD colorimetric assay is based on the spectrophotometric measurement of triiodide (I3^-), which has a molar absorptivity value (ε) of 2.46 × 10^4 M^-1 cm^-1 at 365 nm. The molar quantity of I3^- is directly proportional to the quantity of lipid hydroperoxides (LOOH) that is formed in the process of lipid peroxidation. One ml of CHOD color reagent was added to each aliquot followed by incubation in the absence of light for 4 h. Sample absorbances were then measured at 365 nm. Lipid peroxide concentration values were expressed as mean ± S.D. (n = 6).

Preparation of Oriented Membranes for Small Angle X-ray Diffraction Analysis—The effect of lipid peroxidation on cholesterol crystalline formation was measured in MLVs reconstituted from DAPC and cholesterol at specific mole ratios. The final phospholipid concentration was 2.5 mg/ml, and the mole ratio of cholesterol-to-phospholipid was 0, 0.2, and 0.6. The mole ratio of drug-to-phospholipid was 1:100–1:15, resulting in low final concentrations that range between 0.1 and 5.0%, by
Although a typical phospholipid bilayer has a molecular width of ~50 Å (depending on acyl chain length, degree of saturation, cholesterol content, temperature), the pure cholesterol crystalline domain has a fixed width of 34 Å, indicating a sterol bilayer (a single cholesterol molecule has a long dimension of 17 Å) (27, 28).

As an additional control experiment, we evaluated the effects of oxidative stress on membrane lipid vesicles (DAPC) prepared in the absence of cholesterol domains, lipid peroxidation produced a marked reduction in the width of the surrounding sterol-poor membrane as a function of time. Over a 12-h period, for example, the membrane width was reduced from 53 to 46 Å following oxidative stress. This reduction in membrane width was accompanied by a progressive increase in cholesterol domain formation. By 16 h, there was no further evidence of phospholipid bilayer structure due to extensive damage to the phospholipid acyl chains (Fig. 3). The amount of cholesterol in the membranes also affected domain formation. After 4 h, clear evidence for cholesterol domains was observed at a 0.2 C/P mole ratio (Fig. 3); at a 0.6 C/P mole ratio, cholesterol domains were observed as early as 2.5 h (data not shown).

As an additional control experiment, we evaluated the effects of oxidative stress on membrane lipid vesicles (DAPC) prepared in the absence and presence of increasing cholesterol levels. The membrane samples prepared without cholesterol were inherently more disordered than those prepared with cholesterol, as evidenced by a membrane width of only 48 Å. As expected, these samples did not show evidence of domains of 34 Å width, even after 16 h of lipid peroxidation. Despite the absence of cholesterol domains, there was still a marked reduction in membrane width of these samples (48–41 Å) due to extensive oxidative damage to the phospholipid acyl chains (data not shown).

Comparative Effect of ATM, Statins, and Trolox on Cholesterol Domain Development—As the formation of separate cholesterol domains was attributed to oxidative stress, we tested the effect of syn-

**RESULTS**

**Effect of Oxidative Stress on Organization of Membrane Lipids**—X-ray diffraction analysis of membrane vesicles consisting of phospholipid and cholesterol produced strong and reproducible diffraction orders that corresponded to distinct structures in the membrane (e.g. cholesterol domains, phospholipid-rich regions), as shown in Fig. 2. The d-space measurement refers to the average molecular width of the membrane, including surface hydration, with a resolution of 1 Å or 0.1 nm. Although a typical phospholipid bilayer has a molecular width of
Atorvastatin Inhibits Cholesterol Domain Formation

FIGURE 3. Representative x-ray diffraction patterns of oriented membrane lipid bilayers as a function of peroxidation. A, in the absence of lipid peroxidation, the x-ray diffraction pattern of oriented DAPC membranes indicated a single phase lipid bilayer with a molecular width of 53 Å for membranes with a cholesterol-to-phospholipid mole ratio of 0.2:1. B, at 4 h, samples showed both a sterol-poor phospholipid bilayer (peaks 1, 2, and 4) with a width of 51 Å and cholesterol crystalline domain of 34 Å (peaks 1’ and 2’). A schematic illustration of the cholesterol crystalline domain in the membrane lipid bilayer is shown to the right. Cholesterol molecules are illustrated in red. C, after 12 h, the cholesterol crystalline domains dominated the samples, and there was still evidence of the phospholipid bilayer but with a reduced width (46 Å). D, by 16 h, only cholesterol crystalline domains were observed with little evidence of any remaining intact phospholipid bilayer.

Membrane Location of Statins—Fourier analysis of the diffraction data was used to produce a one-dimensional electron density profile (Å electrons/Å^3) of the centrosymmetric membrane lipid bilayer. Two peaks of electron density of the electron density profile correspond to the center-of-mass positions of the phospholipid headgroups (Fig. 5A). A nadir of electron density associated with the terminal methylene segments of the phospholipid acyl chains was also observed, due to the high ratio of hydrogen to carbon and low molecular density. Sample electron density profiles were directly subtracted from control samples to calculate the equilibrium location of drug molecules in the membrane.

In membranes reconstituted from 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine and cholesterol, the addition of ATM produced a well defined increase in electron density in the membrane hydrocarbon core with a center-of-mass distribution of ~16 Å from the hydrocarbon core center (Fig. 5B). This location is consistent with strong lipophilic interactions between ATM and the phospholipid acyl chains. Specifically, these findings suggest that the long axis of ATM intercalates into the upper hydrocarbon core, adjacent to the headgroup region. This equilibrium membrane location of ATM may be necessary for access to the HMG-CoA reductase enzyme. Additionally, this membrane location places the o-hydroxy moiety in the region of the conjugated double bonds of the phospholipid bilayer, where it may participate in proton donation mechanisms to attenuate the propagation of free radicals. By contrast, the equilibrium membrane location for the hydrophilic statin, pravastatin, was restricted to the hydrated surface of the membrane with a center-of-mass location ~10 Å from ATM (Fig. 5C). Based on these data, we propose a membrane orientation for ATM that places its phenoxy group in close proximity to the conjugated double bonds of adjacent phospholipids (Fig. 5D); however, given the specific interaction of aromatic structures with lipid bilayers (29), other orientations may be possible.

**ATM Inhibits Isoprostane Formation in MLVs**—Isoprostanes are prostaglandin isomers that can be generated non-enzymatically by free radical modification of arachidonic acid associated with phospholipid in LDL and cellular membranes. These products of oxidative modification contribute to inflammatory processes and are associated with the atherosclerotic plaque (30, 31). We tested the effect of ATM on peroxidation of lipid vesicles enriched with arachidonic acid (DAPC), the substrate for non-enzymatic formation of isoprostanes (Fig. 6). The results of this experiment demonstrated that the addition of ATM at 100 nM significantly (p < 0.01, n = 3) reduced isoprostane levels by 30%, as compared with vehicle-treated controls (14.00 ± 2.46 ng/ml to 9.87 ± 1.70 ng/ml). By contrast, there was not a significant change in isoprostane levels in samples treated with simvastatin (14.42 ± 0.96 ng/ml; p =
0.75, n = 3), rosuvastatin (14.20 ± 3.51 ng/ml; p = 0.90, n = 3), or Trolox (12.97 ± 1.68 ng/ml; p = 0.22, n = 3). There was a reduction in isoprostane levels with atorvastatin parent by 11%, but it was not statistically significant (12.35 ± 1.68 ng/ml; p = 0.22, n = 3) (Fig. 6). Additionally, levels of the reactive aldehyde, MDA, were significantly (p < 0.01, n = 3) reduced in the presence of ATM by 20% from 1.29 ± 0.11 to 1.03 ± 0.07 μM at 37 °C, whereas significant inhibition was not observed for atorvastatin parent and other statins (data not shown).

ATM Inhibits Lipid Peroxidation in LDL and MLVs—We tested the dose-dependent effects of ATM on peroxidation in MLVs enriched with PUFAs (linoleic acid) and human LDL. ATM inhibited oxidation in lipid vesicles over a broad range of concentrations (100 nM through 10.0 μM) to reproduce pharmacologic conditions. There was a dose-dependent decrease in LOOH levels, an early modification of PUFAs, as a function of ATM treatment. The EC50 for ATM in the MLV samples was 1.0 μM, with significant reductions at a concentration as low as 100 nM (Fig. 7A). Over a range of concentrations that reproduce pharmacologic conditions (100 nM through 2.0 μM), ATM caused a pronounced and dose-dependent reduction in TBARS levels in human LDL (Fig. 7B). TBARS levels indicate the generation of reactive aldehydes such as MDA, an atherogenic product of LDL oxidation. At the highest dose tested (2.0 μM), ATM produced more than an 80% reduction in LDL oxidation, as compared with vehicle-treated samples.

Antioxidant Activity of Vitamin E Reduced in Cholesterol-enriched Membranes—The comparative antioxidant effects of vitamin E and ATM were tested in DAPC-enriched MLVs as a function of increasing cholesterol content. We have previously shown in animal feeding studies that the C/P mole ratio in animal vascular tissue increases dramatically under conditions of hyperlipidemia (2). This increase in cell plasma membrane cholesterol content may have important effects on protein function and drug interactions. To directly test the effect of cholesterol content on antioxidant effects, we examined the activity of vitamin E in lipid vesicles prepared with increasing ratios of cholesterol and phospholipid. As shown in Fig. 8, there was a strong inverse relationship
FIGURE 5. Membrane locations of ATM and pravastatin. A, a representative electron density (electrons/Å³) profile superimposed on a model of the membrane lipid bilayer. Peaks of electron density correspond to the phospholipid headgroups, whereas the nadir of density is associated with terminal methylene segments of the acyl chains. The shaded areas in subsequent panels indicate areas of positive or negative difference in electron density following direct subtraction of the profiles. B, superimposed electron density profiles for a lipid bilayer prepared in the absence and presence of ATM at a 1:15 drug:phospholipid mole ratio (<3% by mass). The data indicate a location for ATM in the upper hydrocarbon core/glycerol backbone of the membrane. C, superimposed electron density profiles for a lipid bilayer prepared in the absence and presence of pravastatin. D, schematic illustration of the membrane lipid bilayer location of ATM based on the x-ray diffraction analyses. The model has the drug with an orientation that places the phenoxy group in the membrane hydrocarbon core, near the polyunsaturated fatty acids.

FIGURE 6. Comparative effects of statins and Trolox on isoprostane formation from lipid vesicles enriched with DAPC. Peroxidation of lipids occurred over time in the absence of any exogenous initiators at 37 °C. Total levels of F2-isoprostanes were measured by GC-MS with negative chemical ionization in DAPC lipid vesicles (0.25 mg/ml) prepared in the presence of vehicle or drug at 100 nM. Values are mean ± S.D. (n = 4), * p < 0.01 versus vehicle treated samples.
between the ability of vitamin E (500 nM) to inhibit lipid hydroperoxide formation and the C/P ratio in the lipid vesicles. The inhibition of LOOH decreased from 26% at a 0.4:1 mole ratio to 19 and 6% at elevated C/P ratios of 0.8 and 1.2, respectively. By contrast, the inhibition of LOOH with ATM remained constant at 38% over the entire range of C/P mole ratios, even at a ratio as high as 1.6 (Fig. 9). The effects of the drugs on LOOH oxidation were compared with vehicle (control) based on TBARS formation measured at an absorbance of 532 nm. Values are mean ± S.D. (n = 6), *, p < 0.05 and **, p < 0.001 versus vehicle-treated samples.

FIGURE 7. Dose-dependent antioxidant effects of ATM in DLPC lipid vesicles and human LDL. A, DLPC-enriched MLVs (1.0 mg/ml) were incubated with ATM over a broad range of concentrations (100 nM through 10 µM). LOOH levels gradually increased in the absence of any exogenous initiators at 37 °C for 48 h. Total levels of LOOH were measured by the CHOD-iodide assay based on the spectrophotometric measurement of triiodide (I3⁻). The molar quantity of I3⁻ is directly proportional to the quantity of LOOH that is formed in the process. Values are mean ± S.D. (n = 6), *, p < 0.05 and **, p < 0.001 versus vehicle-treated samples. B, human LDL (50 µg protein/ml) was incubated with ATM for 30 min over a broad range of concentrations (100 nM through 2.0 µM). Oxidation of LDL was initiated with CuSO4 (10 µM) at 37 °C. The effects of the drugs on LDL oxidation were compared with vehicle (control) based on TBARS formation measured at an absorbance of 532 nm. Values are mean ± S.D. (n = 6), *, p < 0.05, **, p < 0.01, and ***, p < 0.001 versus vehicle-treated samples.

FIGURE 8. Effect of membrane cholesterol content on the antioxidant activity of vitamin E. At a concentration of 500 nM, vitamin E was incubated with DLPC lipid vesicles (1.0 mg/ml) containing various ratios of cholesterol-to-phospholipid. Peroxidation of lipids occurred gradually in the absence of any exogenous initiators at 37 °C for 48 h. Total levels of LOOHs were measured by the CHOD-iodide assay based on the spectrophotometric measurement of triiodide (I3⁻). The molar quantity of I3⁻ is directly proportional to the quantity of LOOH that is formed in the process. Values are mean ± S.D. (n = 3).

FIGURE 9. Comparative antioxidant effects of ATM and vitamin E in membranes enriched with cholesterol. ATM and vitamin E (each at 500 nM) were incubated with DLPC lipid vesicles (1.0 mg/ml) containing a ratio of cholesterol-to-phospholipid of 1.6:1. Peroxidation of lipids occurred over time in the absence of any exogenous initiators at 37 °C for 48 h. Total levels of LOOH were measured by the CHOD-iodide assay based on the spectrophotometric measurement of triiodide (I3⁻). The molar quantity of I3⁻ is directly proportional to the quantity of LOOH that is formed in the process. Values are mean ± S.D. (n = 3), *, p < 0.01 versus vehicle or vitamin E treated samples.

DISCUSSION

The essential observation from this study was that the ATM inhibited changes in membrane lipid structure and organization, including cholesterol domain formation, following oxidative stress. The effects of ATM were attributed to antioxidant activity and observed in membrane samples containing physiologic levels of sterol and phospholipid with various PUFAs. Oxidative modification of the membrane lipids caused the aggregation of unesterified membrane cholesterol into highly ordered, crystalline domains (width = 34 Å). In addition to interfering with cholesterol crystal development, ATM reduced changes in membrane width associated with lipid peroxidation. The antioxidant activity of ATM is attributed to electron donation and proton stabilization mechanisms associated with its phenoxy group located in the membrane hydrocarbon core. A similar effect was observed with probucol, a lipophilic molecule that overlaps the location of ATM in the membrane but not other statins that do not share this membrane location (e.g. pravastatin) or phenoxy constituent (atorvastatin parent). This may represent an atheroprotective property for ATM as formation of cholesterol crystals contributes to mechanisms of cell death and inflammation (3–5) and, once formed, these crystals do not respond to pharmacologic intervention or reverse cholesterol transport mechanisms.

These findings support earlier studies showing that oxidative modification of phospholipid acyl chains promote the organization of cholesterol into highly ordered, crystalline structures, even in lipid vesicles containing normal or low cholesterol levels (8). The basis for this effect on cholesterol aggregation is attributed to a loss in normal phospholipid-sterol interactions resulting from oxidative damage to PUFAs, a common and essential constituent of most biological membranes. Oxida-
tion of membrane phospholipid acyl chains is a free radical reaction by which molecular oxygen is incorporated into the PUFA moiety following abstraction of a hydrogen atom from the bisallylic methylene group. These chemical modifications alter the structure of the phospholipid molecule, disrupting their physico-chemical interactions with other lipid constituents, such as van der Waals interactions with neighboring phospholipids, especially those containing extended and saturated fatty acyl chains (32). Further studies of cholesterol domain formation in vesicles enriched with other phospholipid molecules, including those containing mixed acyl chains (e.g. sphingomyelin), are warranted as a function of oxidative stress.

In addition to the formation of cholesterol crystalline domains, we observed that oxidative damage to the membrane lipids causes a significant and irreversible reduction in membrane width. This decrease is attributed to chemical modification of the lipid acyl chains, including cleavage into reactive aldehydes. Additionally, as cholesterol was sequestered into these crystalline structures, the overall amount of cholesterol in the surrounding phospholipid bilayer decreased, resulting in increased trans-gauche isomerizations in the phospholipid acyl chains and reduced membrane width. These findings are consistent with previous studies that have demonstrated an important role for cholesterol in maintaining membrane width and lipid dynamics essential for function (2, 33). Such a reduction in membrane width would be expected to have pronounced effects on normal structure-function relationships for membrane-bound ion transport proteins and other proteins involved in signal transduction pathways (33, 34).

These findings suggest that the cardiovascular risk factors associated with oxidative stress (e.g. hypertension, diabetes) (7) could accelerate the abnormal deposition of cholesterol into crystalline domains, even in patients with normal cholesterol levels in the vessel wall. Levels of oxidized lipid, as measured by TBARS or monoclonal antibodies against oxidized LDL, correlate with the severity of acute coronary syndromes and plaque instability (35–37). In a longitudinal investigation of 634 patients, we found that elevated levels of oxidized lipid in the plasma were associated with a 3–4-fold increase in the relative risk for major vascular events and procedures over a 3-year period (37). The predictive effect of TBARS was observed in a multivariate model adjusted for inflammatory markers (C-reactive protein, soluble intercellular adhesion molecule-1 (sICAM-1), interleukin-6) and other risk factors (age, LDL high density lipoprotein, total cholesterol, triglycerides, body mass index, and blood pressure). This analysis showed an independent effect of oxidized lipids on major vascular events and procedures.

Despite this link between oxidative stress and cardiovascular risk, it has been demonstrated that antioxidant (e.g. vitamin E) supplementation does not appear to be beneficial in reducing events in prospective trials including patients with coronary artery disease (38, 39). Possible explanations for this apparent paradox may be due to trial design, baseline antioxidant status of participants, dosage and source of the antioxidants, and time of intervention relative to disease progression. An additional explanation is that vitamin E does not neutralize relevant oxidants, such as those produced by myeloperoxidase (40), and/or that there is reduced penetration of natural antioxidants into the atherosclerotic plaque (41). Our results support the later hypothesis as we observed a reduction in the activity of vitamin E as a function of increasing membrane cholesterol content (Fig. 8). By contrast, synthetic antioxidants (e.g. probucol), with superior scavenging activity and lipophilic properties, may have beneficial effects on relevant oxidative stress processes in the plaque and thereby beneficially influence the course of the disease.

The finding of a novel antioxidant effect for ATM may serve to explain why this statin may reduce clinical events in patients with otherwise normal levels of serum LDL, especially in the presence of other risk factors that contribute to oxidative stress pathways (e.g. hypertension, diabetes), as observed in recent clinical trials, such as the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) (18). As reported in the Physicians’ Desk Reference (42), 70% of the atorvastatin is present in the serum in the form of hydroxy metabolites that are active inhibitors of HMG-CoA reductase. Unlike other statins, ATM has the same enzymatic activity as its parent. Atorvastatin treatment has been successfully used to reduce the risk and progression of cardiovascular disease (16–18), but there is also clinical evidence that this agent has anti-inflammatory and antioxidant properties that may not be solely related to LDL reduction (12–15). A small clinical study that measured protein oxidation markers (e.g. dityrosine, nitrotyrosine) showed that treatment with atorvastatin caused a significant decrease in these oxidation products. Interestingly, reductions in oxidation markers were observed at its lowest dose (10 mg) over just 12 weeks (15). In a larger study of 2341 patients, treatment with a high dose of atorvastatin (80 mg) caused a significant reduction in levels of oxidized lipids on all apoB–100 particles after just a 16-week period (14). Comparative studies into the effects of atorvastatin versus other statins on biomarkers of oxidative stress are currently underway and will provide additional insight into this potential non-LDL mechanism of action.

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Atorvastatin Inhibits Cholesterol Domain Formation

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