Statins Promote Potent Systemic Antioxidant Effects Through Specific Inflammatory Pathways

Mehdi H. Shishehbor, DO*; Marie-Luise Brennan, PhD*; Ronnier J. Aviles, MD; Xiaoming Fu, MS; Marc S. Penn, MD, PhD; Dennis L. Sprecher, MD; Stanley L. Hazen MD, PhD

Background—The pleiotropic actions of hydroxymethylglutaryl CoA reductase inhibitors (statins) include antiinflammatory and antioxidant actions. We recently reported that statins induce reductions in plasma protein levels of nitrotyrosine (NO2Tyr), a modification generated by nitric oxide–derived oxidants. Whether alternative oxidative pathways are suppressed in vivo after statin administration has not yet been reported.

Methods and Results—As an extension of our prior study, hypercholesterolemic subjects with no known coronary artery disease were evaluated at baseline and after 12 weeks of atorvastatin therapy (10 mg/d). Plasma levels of protein-bound chlorotyrosine, NO2Tyr, dityrosine, and orthotyrosine, specific molecular fingerprints for distinct oxidative pathways upregulated in atheroma, were determined by mass spectrometry. In parallel, alterations in lipoproteins and C-reactive protein were determined. Statin therapy caused significant reductions in chlorotyrosine, NO2Tyr, and dityrosine (30%, 25%, and 32%, respectively; P<0.02 each) that were similar in magnitude to reductions in total cholesterol and apolipoprotein B-100 (25% and 29%, P<0.001 each). Nonsignificant decreases in orthotyrosine and C-reactive protein levels were observed (9% and 11%, respectively; P>0.10 each). Statin-induced reductions in oxidation markers were independent of decreases in lipids and lipoproteins.

Conclusions—Statins promote potent systemic antioxidant effects through suppression of distinct oxidation pathways. The major pathways inhibited include formation of myeloperoxidase-derived and nitric oxide–derived oxidants, species implicated in atherogenesis. The present results suggest potential mechanisms that may contribute to the beneficial actions of statins. They also have important implications for monitoring the antiinflammatory and antioxidant actions of these agents. (Circulation. 2003;108:426-431.)

Key Words: statins ■ antioxidants ■ hypercholesterolemia ■ atherosclerosis ■ inflammation

Hydroxymethylglutaryl CoA reductase inhibitors (statins) promote reductions in plasma levels of LDL cholesterol, a primary risk factor in coronary artery disease. Numerous primary and secondary prevention trials confirm clinical benefits with this class of agents.1,2 The mechanisms involved have largely been attributed to the ability of these agents to inhibit cholesterol synthesis, leading to upregulation of hepatic LDL receptors and corresponding reductions in circulating levels of LDL particles. However, a growing body of evidence suggests that some of the clinical benefits of statin therapy may be attributed to mechanisms independent of their cholesterol-lowering effects.3-5 These so-called pleiotropic effects are believed to include antiinflammatory and antioxidant actions. Statin-induced reductions in acute-phase reactant proteins such as C-reactive protein (CRP) provide strong evidence for an overall antiinflammatory effect of these agents.6,7 We recently reported that statin therapy lowers systemic levels of protein-bound nitrotyrosine (NO2Tyr), a marker for oxidant stress mediated via pathways involving nitric oxide (·NO)-derived oxidants.8 A systematic approach to both quantify and define the specific oxidative pathways suppressed in vivo after statin administration has not yet been reported.

Enhanced oxidant stress occurs within the artery wall of atherosclerotic vessels.9-11 Multiple distinct oxidation products are enriched within human atherosclerotic plaques, as well as LDL recovered from diseased versus normal human aorta.12,13 However, the role of oxidation in the pathogenesis of coronary artery disease has recently been questioned because of the failures of multiple prospective interventional trials with antioxidant supplements.9,14,15 However, none of the major antioxidant trials to date concomitantly measured systemic markers of oxidant stress to ensure an effect on the process targeted for intervention (ie, oxidation). This is
potentially leading to endothelial dysfunction,\textsuperscript{28} and activation of matrix metalloprotease and alternative protease cascades, potentially leading to development of the vulnerable plaque.\textsuperscript{29,31} Remarkably, \( \alpha \) tocopherol is relatively ineffective in blocking these oxidation pathways.\textsuperscript{10,15–17}

The ability of statins to inhibit isoprenylation of key proteins involved in oxidant/antioxidant-generating machinery within the vessel wall suggests that these agents may promote systemic antioxidant effects. Cell culture studies demonstrate that statin therapy suppresses superoxide formation and enhances \( \cdot \text{NO} \) generation by vascular endothelial cells via inhibition of isoprenylation of Rac and Rho.\textsuperscript{32,33} Rac is a component of the NAD(P)H oxidase complex of both leukocytes and vascular cells. Statin-induced inhibition of Rac isoprenylation impairs its translocation to membranes, leading to suppression in superoxide formation from cultured cells.\textsuperscript{32} Rho is a small GTPase involved in cell signaling. Inhibition in Rho isoprenylation in endothelial cells has been shown to result in enhanced \( \cdot \text{NO} \) production, an effect likely to produce overall antioxidant action.\textsuperscript{13}

The present study was designed to test the hypothesis that statins promote systemic antioxidant effects via multiple distinct oxidation pathways implicated in the atherosclerotic process. It is based on the recognition that superoxide formation from leukocyte and vascular cell NAD(P)H oxidases is a critical proximal step in oxidant formation by pathways implicated in generation of reactive oxidant species within the artery wall (Figure). Using mass spectrometry-based methods for the quantification of plasma protein levels of distinct molecular markers, we now both quantify and define specific oxidative pathways suppressed in vivo after statin administration to hypercholesterolemic subjects.

**Methods**

**Study Protocol**

Samples used were from subjects enrolled in a recently described prospective, open-label interventional trial with atorvastatin.\textsuperscript{8} Consecutive patients (n = 35) meeting entry criteria were recruited from the Preventive Cardiology Clinic at the Cleveland Clinic Foundation. Patients 21 years of age and older without clinical evidence of coronary artery disease and with LDL cholesterol (LDL-C) levels \( \geq 130 \text{ mg/dL} \) or greater, despite at least 8 weeks of therapeutic lifestyle interventions, were eligible to participate in the study. Patients received treatment with atorvastatin at a dose of 10 mg orally per day. Fasting morning plasma samples were collected before initiation of therapy (baseline) and after 12 weeks of therapy. Patients with active liver disease or renal insufficiency defined as a serum creatinine level \( \geq 1.8 \text{ mg/dL} \) were excluded. Patients were followed through clinic visits at weeks 2, 4, 6, 8, and 12. All patients gave written informed consent, and the Institutional Review Board at the Cleveland Clinic Foundation approved the study protocol.

**Blood Samples**

Fasting blood was collected into EDTA tubes. Plasma was recovered after centrifugation at 3500 rpm for 10 minutes at \( 4^\circ \text{C} \). Aliquots were stored under conditions to minimize artificial oxidation by addition of antioxidant cocktail (100 \( \mu \text{mol/L} \) butylated hydroxytoluene and 100 \( \mu \text{mol/L} \) diethylentriaminepentaacetic acid) overlaid with argon, stored at \( -80^\circ \text{C} \) until analysis. Standard methods were used to measure lipid levels and high-sensitivity CRP.
Nitrotyrosine, Dityrosine, Chlorotyrosine, and ortho-Tyrosine Analyses

Protein-bound NO$_2$Tyr was determined by stable isotope dilution liquid chromatography–tandem mass spectrometry on an ion trap mass spectrometer (LCQ Deca, ThermoFinigann), as previously described. Synthetic [$^{13}$C$_9$,$^{15}$N$_1$]-labeled standards (in cases of NO$_2$Tyr, ClTyr, and diTyr) or [$^{13}$C$_9$,$^{15}$N$_1$]-labeled standards (in case of diTyr) were added to plasma protein pellets and used as internal standards for quantification of natural abundance analytes. Simultaneously, universal labeled precursor amino acids [13C$_9$,15N$_1$]tyrosine (for NO$_2$Tyr, ClTyr, and diTyr) or [13C$_9$,15N$_1$]-labeled phenylalanine (for o-Tyr) were added to plasma protein pellets. Proteins were hydrolyzed under argon atmosphere in a column; 3 mL; Supelco, Inc) before mass spectrometry analysis. Methane sulfonic acid, and then samples were passed over mini stands (in cases of NO$_2$Tyr, ClTyr, and o-Tyr) were monitored within the same injection of each oxidized analyte. Simultaneously, universal labeled precursor amino acids [13C$_9$,15N$_1$]tyrosine (for NO$_2$Tyr, ClTyr, and diTyr) or [13C$_9$,15N$_1$]-labeled phenylalanine (for o-Tyr) were added to plasma protein pellets. Proteins were hydrolyzed under argon atmosphere in a column; 3 mL; Supelco, Inc) before mass spectrometry analysis. Results are normalized to the content of the precursor amino acid tyrosine (for NO$_2$Tyr, ClTyr, or diTyr) or phenylalanine (for o-Tyr), which were monitored within the same injection of each oxidized amino acid. Intrapreparative formation of nitro[13C$_9$,15N]tyrosine, chloro[13C$_9$,15N]tyrosine, di[13C$_9$,15N]tyrosine, and ortho[13C$_9$,15N] tyrosine was routinely monitored and was negligible (ie, <5% of the level of the natural abundance product observed) under the conditions used.

Statistical Analysis

Data are presented as mean±SD, and significance level was set at $P<0.05$. Wilcoxon rank-sum test was used to analyze the differences between NO$_2$Tyr, diTyr, and CRP at baseline and 12 weeks, because they were not normally distributed. The differences between baseline and 12 weeks for lipid parameters, ClTyr, and o-Tyr levels were performed using paired Student’s $t$ test. Spearman-rank correlation was used to assess the association between baseline NO$_2$Tyr, diTyr, ClTyr, o-Tyr, CRP, and lipid parameters. Multiple regression analyses were performed to determine factors associated with changes in NO$_2$Tyr, diTyr, and ClTyr. Statistical analyses were performed using SPSS version 11.0.

Results

Baseline characteristics of the patients are shown in Table 1. Follow-up data were available for all 35 patients at 12 weeks. In general, other than hypercholesterolemia, the patients were a healthy cohort without any known coronary artery disease or diabetes. Absolute and percentage change of baseline and 12-week measurements of total cholesterol (TC), LDL-C, HDL-C, triglycerides, CRP, CITyr, diTyr, NO$_2$Tyr, and o-Tyr are shown in Table 2. As expected, treatment with atorvastatin led to a significant reduction in TC, LDL-C, and apolipoprotein (apo) B-100 levels (25%, 39%, and 29%, respectively). Atorvastatin caused comparable significant reductions in the levels of oxidation products produced by myeloperoxidase and ·NO-derived oxidants (reductions in CITyr, diTyr, and NO$_2$Tyr of 30%, 32%, and 25%, respectively; Table 2). In contrast, the reduction in o-Tyr and CRP were modest (9% and 11%, respectively) and failed to reach statistical significance (Table 2).

Additional analyses were performed to determine if either baseline levels or observed changes in oxidation markers (NO$_2$Tyr, diTyr, ClTyr, and o-Tyr) were associated with baseline levels or observed changes in either lipid parameters or CRP. Baseline NO$_2$Tyr levels, a specific molecular fingerprint for protein modification by ·NO-derived oxidants, were correlated with fasting triglyceride levels ($r=-0.36$, $P=0.033$; Table 3). No other significant correlations were found between baseline levels of oxidation markers and either lipid parameters or CRP (Table 3). Significant correlations were noted between statin-induced changes in CITyr, a specific molecular fingerprint of myeloperoxidase-catalyzed oxidation, and changes in both NO$_2$Tyr and HDL-C levels ($r=0.37, P=0.028$ and $r=0.36, P=0.036$, respectively; Table 4). Changes in o-Tyr, a product of protein oxidation by metal

| TABLE 2. Lipid and Inflammatory Markers at Baseline and After Atorvastatin Therapy |
|---------------------------------|-------------|-------------|--------------------|------------------|
| Characteristics                | Baseline ($n=35$) | 12 Weeks ($n=35$) | Absolute (%) Change | $P$ |
| diTyr, μmol/mol tyrosine        | 34±11        | 23±8        | -11 (32)          | <0.001 |
| CITyr, μmol/mol tyrosine        | 19±10        | 13±4        | -6 (30)           | 0.01  |
| NO$_2$Tyr, μmol/mol tyrosine    | 15±7         | 11±5        | -4 (25)           | 0.02  |
| o-Tyr, μmol/mol tyrosine        | 89±54        | 81±40       | -8 (9)            | 0.49  |
| CRP, mg/dL                      | 2.6±3.2      | 2.3±3.3     | -2.0 (11)         | 0.10  |
| Total cholesterol, mg/dL        | 255±27       | 190±28      | -63 (25)          | <0.001 |
| HDL-C, mg/dL                    | 56±12        | 58±12       | 2 (4)             | 0.21  |
| LDL-C, mg/dL                    | 169±22       | 103±29      | -66 (39)          | <0.001 |
| Triglycerides, mg/dL            | 146±90       | 132±81      | -14 (10)          | 0.22  |
| Apo B-100, mg/dL                | 135±17       | 96±21       | -39 (29)          | <0.001 |
catalyzed hydroxyl radical-like species, was associated with changes in fasting triglycerides ($r = -0.38$, $P = 0.026$; Table 4). In multiple regression analyses that included changes in lipid parameters and oxidation markers, the only significant correlation noted was between changes in ClTyr and NO2Tyr, ($P = 0.002$).

Discussion

The present studies demonstrate significant reductions in levels of specific molecular footprints of distinct oxidative pathways after atorvastatin therapy. Marked reductions in systemic markers specific for protein oxidative modification by myeloperoxidase-derived and -NO-derived oxidants were observed that were largely independent of statin-induced changes in lipid parameters and CRP. Furthermore, the magnitude of reductions in oxidation markers on statin therapy was comparable in size to the reductions observed in fasting TC and apo B100. The mechanisms underlying the overall systemic antioxidant effects are likely class effects for these agents (ie, inhibition in isoprenylation). It is thus tempting to speculate that statin trials may in fact represent not only lipid-lowering interventions but also antioxidant trials. Additional studies are needed to determine if the systemic antioxidant effects promoted by atorvastatin therapy are similarly seen with other agents in this class. Furthermore, it will be of interest to determine if the clinical benefits observed with statins are independently predicted by reductions in specific oxidation markers, particularly because reductions in oxidation products were independent of statin-induced alterations in TC, LDL-C, and apo B100, lipid and lipoprotein parameters presently used in monitoring efficacy and dosing of statins.

The present studies not only suggest a reappraisal of the oxidation hypothesis of atherosclerosis but also invite the overhaul of the design and monitoring of future antioxidant intervention trials. Common sense dictates that to claim antioxidant effect, one needs to demonstrate reductions in oxidation products. Incorporation of this approach in future antioxidant studies is required. The oxidation markers chosen for the present study provide mechanistic information with regards to the pathways responsible for their formation. Furthermore, unlike lipid oxidation products, which are readily generated during sample storage and archiving, many of the molecular markers monitored are stable and not readily formed during storage. These characteristics make them potentially useful and practical tools for both defining oxidative pathways operative in cardiovascular syndromes and for assessing the efficacy of antioxidant and antiinflammatory interventions. They also are required for the meaningful analysis of archival specimens for correlation with clinical outcomes, because significant measures are rarely taken during sample collection and storage to prevent or minimize lipid oxidation. The sophisticated and labor-intensive methods required for accurate determination of oxidative markers,

### Table 3. Baseline Spearman Correlations*

<table>
<thead>
<tr>
<th></th>
<th>TG</th>
<th>HDL-C</th>
<th>LDL-C</th>
<th>CRP</th>
<th>dITyr</th>
<th>NO2Tyr</th>
<th>ClTyr</th>
<th>o-Tyr</th>
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<tbody>
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<td>TC</td>
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<td>0.18</td>
<td>0.76‡</td>
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<tr>
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<td></td>
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<td>0.29</td>
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<tr>
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<tr>
<td>o-Tyr</td>
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</tbody>
</table>

TC indicates total cholesterol; and TG, triglycerides.

*P values shown only for significant correlations ($P < 0.05$).
†$P = 0.014$; †$P = 0.001$; §$P = 0.017$; ||$P = 0.033$.

### Table 4. Spearman Correlations for Changes in Oxidative Markers and Lipid Parameters

<table>
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<tr>
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<th>HDL-C</th>
<th>LDL-C</th>
<th>CRP</th>
<th>dITyr</th>
<th>NO2Tyr</th>
<th>ClTyr</th>
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</tr>
</thead>
<tbody>
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<td>TC</td>
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<td>0.04</td>
<td>0.18</td>
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<td>0.36</td>
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<tr>
<td>LDL-C</td>
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<td>-0.04</td>
<td>0.19</td>
<td>0.09</td>
<td>0.21</td>
<td></td>
<td></td>
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<tr>
<td>CRP</td>
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<td>0.15</td>
<td>-0.18</td>
<td>0.23</td>
<td></td>
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<tr>
<td>dITyr</td>
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<td>0.02</td>
<td>-0.11</td>
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<tr>
<td>NO2Tyr</td>
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<tr>
<td>ClTyr</td>
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</tbody>
</table>

TC indicates total cholesterol; and TG, triglycerides.

*P values shown only for significant correlations ($P < 0.05$).
†$P = 0.04$; †$P = 0.00004$; §$P = 0.026$; ||$P = 0.036$; ¶$P = 0.028$. 

## Table 3. Baseline Spearman Correlations*
which typically involve mass spectrometry, have delayed their widespread use in clinical studies. However, these very same methods illustrate the necessity of using such techniques, because simultaneous monitoring of assay methods to ensure no significant artifactual formation of the oxidation markers during sample handling and processing for analyses has proven to be critical in method development and accurate quantitative assessment of these markers.

Oxidative consumption of NO, such as through interaction with superoxide, both suppresses NO bioavailability and produces a potent nitrating oxidant, peroxynitrite (ONOO·; Figure). We recently reported that systemic NO2Tyr levels serve as an independent predictor of cardiovascular risk and burden and are modulated by statin therapy. The present studies confirm and extend these observations by showing that multiple alternative oxidation pathways, particularly those catalyzed by myeloperoxidase, demonstrate comparable reductions. Myeloperoxidase is a leukocyte-derived heme protein that is enriched in human atheroma. Normally playing a role in innate host defenses, myeloperoxidase-generated reactive nitrogen species, chlorinating oxidants, and tyrosyl radical have each been linked to potential pathogenic mechanisms, including conversion of LDL into a high-uptake form, activation of matrix metalloproteases and other protease cascades, and initiation of lipid peroxidation in vivo. Autopsy studies of subjects with sudden death reveal intense immunostaining for myeloperoxidase within culprit lesions that have undergone fissuring or plaque rupture. Myeloperoxidase has also recently been shown to serve as an independent predictor of atherosclerotic risk in subjects undergoing coronary angiography. Myeloperoxidase deficiency is associated with decreased frequency of cardiovascular events, and functional polymorphisms in the myeloperoxidase gene that lead to decreased enzyme expression confer cardioprotection. The present studies thus provide additional support, albeit indirect, for the hypothesis that myeloperoxidase-generated oxidants are involved in the pathogenesis of cardiovascular disease.

Another intriguing finding of the present studies was the significant association between statin-elicited reductions in levels of protein-bound NO2Tyr and CITyr in plasma (r=0.37, P=0.028; Table 4). Such a finding is consistent with myeloperoxidase playing a significant role in formation of NO-derived oxidants in humans (Figure). A role for myeloperoxidase in the generation of NO-derived oxidants is supported by studies using leukocytes isolated from subjects with myeloperoxidase deficiency, animal models of inflammation using myeloperoxidase knockout mice, and the discovery that myeloperoxidase and other members of the mammalian heme peroxidase superfamily catalytically consume NO as a physiological substrate.

In summary, by using molecular footprints of specific oxidative pathways, we have shown that statins promote potent systemic antioxidant effects independent of changes seen in lipid, lipoprotein, and CRP levels. Furthermore, the amino acid oxidation products CITyr, diTyr, and NO2Tyr demonstrate significant reductions even when presented as a product to precursor ratio, indicating a true decrease in oxidant stress after atorvastatin therapy. These data support the hypothesis that statins induce potent systemic antiinflammatory and antioxidant effects and have important implications for the monitoring of nonlipid-related, or so-called pleiotropic actions, of this important class of drug.

Acknowledgments

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References


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