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.9,11,14,15 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

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From the Departments of Cell Biology, Cardiovascular Medicine, and the Center for Cardiovascular Diagnostics and Prevention, Cleveland Clinic Foundation, Cleveland, Ohio.

Dr Hazen is named as coinventor on pending patents filed by the Cleveland Clinic Foundation that relate to use of biomarkers for inflammatory and cardiovascular diseases.

*These authors contributed equally to this work.

Correspondence to Stanley L. Hazen, MD, PhD, Cleveland Clinic Foundation, 9500 Euclid Ave, NC10, Cleveland, OH 44195. E-mail hazens@ccf.org

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particularly relevant because many oxidation pathways known to occur within human atheroma (Figure) are not effectively inhibited by α-tocopherol (vitamin E),\(^\text{10,15–17}\) the major antioxidant supplement in these trials. Moreover, under certain conditions, pro-oxidant rather than antioxidant actions for species like α-tocopherol and ascorbate (vitamin C) are observed.\(^\text{18,19}\) Thus, it has been argued that the failures of antioxidant trials should not be perceived as an indictment of the “oxidation hypothesis” of atherosclerosis.\(^\text{9,10,15,20}\) Indeed, recent studies confirm no significant reductions in systemic levels of lipid oxidation products in subjects taking up to 2000 IU/d of α-tocopherol.\(^\text{14}\)

Much of what is known about the pathways responsible for oxidative injury within atherosclerotic vessels has been gained by detection of stable structurally informative oxidative products that convey information regarding the pathways responsible for their generation.\(^\text{10,12,21}\) The Figure is a model of major pathways for oxidative generation implicated in the pathogenesis of atherosclerosis and stable informative protein oxidation products produced by these pathways. A molecular marker for protein oxidative damage by NO-derived oxidants (NO\(_2\)-Tyr), myeloperoxidase-generated chlorinating species (nitrogen dioxide [NO\(_2\)], nitrite; NOX, NAD(P)H oxidase of vascular endothelial cells; O\(_2\)-, molecular oxygen; O\(_2\)-, superoxide anion; ·OH, hydroxyl radical; ONOO\(^-\), peroxy nitrite; Pr(M^2+), redox-active metal ion; and ·Tyr, tyrosyl radical.

### Methods

#### Study Protocol

Samples used were from subjects enrolled in a recently described prospective, open-label interventional trial with atorvastatin.\(^\text{8}\) 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 130 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 ≥1.8 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 by centrifugation at 3500 rpm for 10 minutes at 4°C. Aliquots were stored under conditions to minimize artificial oxidation by addition of antioxidant cocktail (100 μmol/L butylated hydroxytoluene and 100 μmol/L diethylenetriaminepentaacetic acid) overlaid with argon, stored at −80°C until analysis. Standard methods were used to measure lipid levels and high-sensitivity CRP.
Nitrotyrosine, Dityrosine, Chlorotyrosine, and ortho-Tyrosine Analyses

Protein-bound NO2Tyr was determined by stable isotope dilution liquid chromatography–tandem mass spectrometry on an ion trap mass spectrometer (LCQ Deca, Thermo Finigan), as previously described.34 Protein-bound CTyr, dITyr, and o-Tyr analyses were performed by gas chromatography/mass spectrometry after derivatization of amino acids to their n-propyl per heptafluorobutryl derivatives using a Finnigan Voyager GC/MS in the negative ion chemical ionization mode, as described.34 Synthetic [13C6,15N]-labeled standards (in cases of NO2Tyr, CTyr, and o-Tyr) or [13C6]-labeled standards (in cases 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 [13C6,15N]tyrosine (for NO2Tyr, CTyr, and dITyr) or [13C6,15N]phenylalanine (for o-Tyr) were added to plasma protein pellets.34 Proteins were hydrolyzed under argon atmosphere in methan sulfonic acid, and then samples were passed over mini solid-phase C18 extraction columns (Supelclean LC-C18-SPE Mini-column; 3 mL; Supelco, Inc) before mass spectrometry analysis.34 Results are normalized to the content of the precursor amino acid tyrosine (for NO2Tyr, CTyr, or dITyr) or phenylalanine (for o-Tyr), which were monitored within the same injection of each oxidized amino acid. Intrapreparative formation of nitro[13C6,15N]tyrosine, chloro[13C6,15N]tyrosine, di[13C6,15N]tyrosine, and ortho[13C6,15N]tyrosine were 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 NO2Tyr, diTyr, and CRP at baseline and 12 weeks, because they were not normally distributed. The differences between baseline and 12 weeks for lipid parameters, CTyr, and o-Tyr levels were performed using paired Student’s t test. Spearman-rank correlation was used to assess the association between baseline NO2Tyr, diTyr, CTyr, o-Tyr, CRP, and lipid parameters. Multiple regression analyses were performed to determine factors associated with changes in NO2Tyr, diTyr, and CTyr. 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, CTyr, diTyr, NO2Tyr, 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 NO2-derived oxidants (reductions in CTyr, diTyr, and NO2Tyr 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 (NO2Tyr, diTyr, CTyr, and o-Tyr) were associated with baseline levels or observed changes in either lipid parameters or CRP. Baseline NO2Tyr 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 CTyr, a specific molecular fingerprint of myeloperoxidase-catalyzed oxidation, and changes in both NO2Tyr 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 1. Baseline Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Primary Prevention (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>54±10</td>
</tr>
<tr>
<td>Female sex</td>
<td>18 (51)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>29±6</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>119±14</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>71±8</td>
</tr>
<tr>
<td>Aspirin treatment</td>
<td>10 (29)</td>
</tr>
<tr>
<td>Multivitamin use</td>
<td>22 (63)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>2 (6)</td>
</tr>
</tbody>
</table>

Values are No. (%) or mean±SD.

### Table 2. Lipid and Inflammatory Markers at Baseline and After Atorvastatin Therapy

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Baseline (n=35)</th>
<th>12 Weeks (n=35)</th>
<th>Absolute (%) Change</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>dITyr, μmol/mol tyrosine</td>
<td>34±11</td>
<td>23±8</td>
<td>−11 (32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CTyr, μmol/mol tyrosine</td>
<td>19±10</td>
<td>13±4</td>
<td>−6 (30)</td>
<td>0.01</td>
</tr>
<tr>
<td>NO2Tyr, μmol/mol tyrosine</td>
<td>15±7</td>
<td>11±5</td>
<td>−4 (25)</td>
<td>0.02</td>
</tr>
<tr>
<td>o-Tyr, μmol/mol tyrosine</td>
<td>89±54</td>
<td>81±40</td>
<td>−8 (9)</td>
<td>0.49</td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td>2.6±3.2</td>
<td>2.3±3.3</td>
<td>−2.0 (11)</td>
<td>0.10</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>255±27</td>
<td>190±28</td>
<td>−63 (25)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>56±12</td>
<td>58±12</td>
<td>2 (4)</td>
<td>0.21</td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>169±22</td>
<td>103±29</td>
<td>−66 (39)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>146±90</td>
<td>132±81</td>
<td>−14 (10)</td>
<td>0.22</td>
</tr>
<tr>
<td>Apo B-100, mg/dL</td>
<td>135±17</td>
<td>96±21</td>
<td>−39 (29)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
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 CTyr 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>CTyr</th>
<th>α-Tyr</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>0.41†</td>
<td>0.18</td>
<td>0.76‡</td>
<td>−0.16</td>
<td>0.02</td>
<td>−0.16</td>
<td>−0.22</td>
<td>−0.02</td>
</tr>
<tr>
<td>TG</td>
<td>−0.40§</td>
<td>0.07</td>
<td>−0.11</td>
<td>0.1</td>
<td>−0.36</td>
<td></td>
<td></td>
<td>−0.20</td>
</tr>
<tr>
<td>HDL-C</td>
<td>−0.01</td>
<td>0.12</td>
<td>0.08</td>
<td>0.27</td>
<td>0.06</td>
<td>−0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C</td>
<td>−0.20</td>
<td>−0.01</td>
<td>−0.07</td>
<td>−0.33</td>
<td>−0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>0.07</td>
<td>0.15</td>
<td>−0.07</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diTyr</td>
<td>0.06</td>
<td>0.03</td>
<td>−0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO2Tyr</td>
<td>0.29</td>
<td>−0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTyr</td>
<td>−0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</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>
<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>CTyr</th>
<th>α-Tyr</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>0.23</td>
<td>0.34†</td>
<td>0.64‡</td>
<td>0.11</td>
<td>0.03</td>
<td>0.10</td>
<td>−0.01</td>
<td>−0.02</td>
</tr>
<tr>
<td>TG</td>
<td>0.04</td>
<td>−0.12</td>
<td>−0.02</td>
<td>−0.03</td>
<td>0.04</td>
<td>0.18</td>
<td>0.38§</td>
<td></td>
</tr>
<tr>
<td>HDL-C</td>
<td>−0.02</td>
<td>0.16</td>
<td>−0.23</td>
<td>0.16</td>
<td>0.36</td>
<td></td>
<td></td>
<td>−0.05</td>
</tr>
<tr>
<td>LDL-C</td>
<td>−0.01</td>
<td>−0.04</td>
<td>0.19</td>
<td>0.09</td>
<td>−0.21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>0.10</td>
<td>0.15</td>
<td>−0.18</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diTyr</td>
<td>−0.18</td>
<td>0.02</td>
<td>−0.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO2Tyr</td>
<td>0.37¶</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTyr</td>
<td></td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

TC indicates total cholesterol; and TG, triglycerides.

*P values shown only for significant correlations ($P < 0.05$).

†$P = 0.04$; †$P = 0.00004$; §$P = 0.026$; ||$P = 0.036$; ¶$P = 0.028$. 

Tyr indicates tyrosine.
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\textsuperscript{-}; Figure). We recently reported that systemic NO\textsubscript{Tyr} levels serve as an independent predictor of cardiovascular risk and burden and are modulated by statin therapy.\textsuperscript{5} 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.\textsuperscript{10,35} Normally playing a role in innate host defenses, myeloperoxidasederived 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,\textsuperscript{10,25,26} activation of matrix metalloproteases and other protease cascades,\textsuperscript{19–31} and initiation of lipid peroxidation in vivo.\textsuperscript{27,36} Autopsy studies of subjects with sudden death reveal intense immunostaining for myeloperoxidase within culprit lesions that have undergone fissuring or plaque rupture.\textsuperscript{30} Myeloperoxidase has also recently been shown to serve as an independent predictor of atherosclerotic risk in subjects undergoing coronary angiography.\textsuperscript{37} Myeloperoxidase deficiency is associated with decreased levels of cardiovascular events,\textsuperscript{38} and functional polymorphisms in the myeloperoxidase gene that lead to decreased enzyme expression confer cardioprotection.\textsuperscript{39} 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 NO\textsubscript{Tyr} 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,\textsuperscript{27,34} and the discovery that myeloperoxidase and other members of the mammalian heme peroxidase superfamily catalytically consume NO as a physiological substrate.\textsuperscript{28}

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, dTyr, and NO\textsubscript{Tyr} 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.

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References
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