Atorvastatin Inhibits $\text{gp91}^{\text{phox}}$ Circulating Levels in Patients With Hypercholesterolemia

Pasquale Pignatelli, Roberto Carnevale, Roberto Cangemi, Lorenzo Loffredo, Valerio Sanguigni, Claudio Stefanutti, Stefania Basili, Francesco Violi

Objective—The inhibition of oxidative stress is among the most relevant pleiotropic effects of statins. The mechanism by which statins exert their antioxidant effect in vivo is still undefined. NADPH oxidase is among the most important sources of reactive oxygen species involved in atherosclerotic disease.

Methods/Results—We developed an ELISA to evaluate serum levels of soluble-$\text{gp91}^{\text{phox}}$, the catalytic core of phagocyte NADPH oxidase. In a cross-sectional study performed in 30 hypercholesterolemic patients and in 20 controls, serum soluble-$\text{gp91}^{\text{phox}}$ and urinary isoprostane, a marker of oxidative stress, were measured. The 2 variables were also measured in hypercholesterolemic patients, randomized to diet (n=15), or diet plus atorvastatin (10 mg daily, n=15) and followed for 30 days. Compared to controls, hypercholesterolemic patients had higher and significantly correlated ($R=0.71$; $P<0.001$) serum soluble-$\text{gp91}^{\text{phox}}$ ($P<0.001$) and urinary isoprostanes ($P<0.001$). After follow-up, the statin-allocated group showed a significant reduction of soluble-$\text{gp91}^{\text{phox}}$ ($-33\%$, $P<0.01$), that paralleled that of isoprostanes ($-37\%$, $P<0.01$) and cholesterol ($-25\%$, $P<0.01$). The diet-allocated group showed only a weak reduction of cholesterol.

Conclusion—Our study demonstrates that statins exert an antioxidant effect via inhibition of soluble-$\text{gp91}^{\text{phox}}$ expression. (Arterioscler Thromb Vasc Biol. 2010;30:360-367.)

Key Words: $\text{gp91}^{\text{phox}},$ oxidative stress, hypercholesterolemia, NADPH oxidase, statins

Primary and secondary prevention trials with statins clearly demonstrated that this drug category is able to reduce cardiovascular events.\textsuperscript{1,2} Even if the principal mechanism of action of statins is to lower cholesterol, other effects, the so-called pleiotropic effects, have been considered as adjunctive properties potentially accounting for the antiatherosclerotic effect of statins.\textsuperscript{3} Inhibition of oxidative stress may be considered an intriguing pleiotropic effect in view of the fact that oxidative stress is thought to be a key event in the initiation and progression of atherosclerotic disease.\textsuperscript{4} Reduction of several markers of oxidative stress including isoprostanes, 8-hydroxydeoxyguanosine (8-OHdG), and nitrotyrosine have been observed after statin treatment but the mechanism of action of statins is to lower cholesterol, other effects, have been considered as adjunctive properties potentially accounting for the antiatherosclerotic effect of statins.\textsuperscript{5} We studied 30 consecutive hypercholesterolemic (HC) patients (16 males and 14 females; 52±4 years of age) and 20 healthy subjects (HSs) who were screened in the ambulatory of our division between September 2005 and February 2006. Subjects were excluded if they

Materials and Methods

Study Participants

We studied 30 consecutive hypercholesterolemic (HC) patients (16 males and 14 females; 52±4 years of age) and 20 healthy subjects (HSs) who were screened in the ambulatory of our division between September 2005 and February 2006. Subjects were excluded if they

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had liver insufficiency, serious renal disorders (serum creatinine, >2.5 mg/dL), diabetes mellitus, arterial hypertension, a history or evidence of previous myocardial infarction or other atherothrombotic diseases, any autoimmune diseases, cancer, present or recent infections or were taking nonsteroidal antiinflammatory drugs, drugs interfering with cholesterol metabolism, or vitamin supplements. Both patients and controls were recruited from the same geographic area, and they were all of white race. Informed consent was obtained from each participating subject and the protocol was approved by the “Sapienza” University, Rome, Italy Ethic Committee.

X-Linked Chronic Granulomatous Disease Patients

We studied 3 recently identified male patients (age, 27 ± 2.5 years) with hereditary deficiency of gp91phox that was diagnosed as previously described. X-CGD is a rare (prevalence 1:1 000 000 individuals) primary immunodeficiency affecting the innate immune system; X-CGD is characterized by life-threatening bacterial and fungal infections. It is caused by mutations in any of the 4 genes encoding subunits of the O2 generating NADPH oxidase, resulting in defective O2 generation and intracellular killing.

Design of the Studies

In the first part of the study, we performed a cross-sectional analysis comparing urinary isoprostanes and serum soluble (sgp91phox) between population of HC patients and HSs. Then, we performed a 30 day interventional study with atorvastatin. For this purpose, the 30 HC patients were randomly assigned to a treatment with diet or diet plus atorvastatin (10 mg/d) to assess whether diet alone or diet plus atorvastatin were able to influence serum levels of sgp91phox and/or urinary isoprostanes excretion. During the study, all participants followed a low-fat diet with mean macronutrient profiles that were close to the present Adult Treatment Panel III guidelines (7% energy from saturated fat and, 200 mg dietary cholesterol per day).

A medical doctor not involved in the study assigned codes to the study treatments, randomly allocating the selected participants to a treatment with diet or diet plus atorvastatin, and kept the key in a sealed envelope. The randomization was carried out by a procedure based on a random numeric sequence. The authors were unaware of treatment allocation. The principal investigator performed the treatment allocation unblinded only after the study had ended and laboratory analyses were completed.

Blood Sampling and Laboratory Analysis

Between 8:00 and 9:00 AM, subjects underwent routine biochemical evaluations including fasting total cholesterol and glucose as well as WBC count. After overnight fasting (12 hours) and supine rest for at least 10 minutes, blood samples were collected in vacutainers (Vacutainer Systems, Belliver Industrial Estate) and centrifuged at 300g for 10 minutes to obtain supernatant, which was stored at −80°C until use. Fasting serum levels of total cholesterol and triglycerides were determined with enzyme-based methods (Dade Behring, Switzerland). Low-density lipoprotein (HDL) cholesterol and triglycerides were determined with enzyme-based methods (Dade Behring, Switzerland). High-density lipoprotein (HDL) cholesterol and triglycerides were determined with enzyme-based methods (Dade Behring, Switzerland).

Electrophoresis and Immunoblotting of sgp91phox

Patient Serum

Equal amounts of serum protein (130 µg/lane) estimated by Bradford assay were solubilized in a 2X Laemmlı sample buffer containing 2-mercaptoethanol and loaded in a denaturing SDS/10% polyacrylamide gel. Western blot analysis was performed with monoclonal anti-gp91phox antibody. After incubation, the pure nitrocellulose membranes (0.45 µm) were washed and incubated with goat anti-mouse IgG1–horseradish peroxidase (HRP) (sc-2004 Santa Cruz Biotechnology Inc) for 2 hours. Immune complexes were detected by enhanced chemiluminescence. (Bio-Rad, Hercules, Calif) following a described protocol. Negative controls involved a similar procedure using a goat IgG (Santa Cruz Biotechnology Inc).

ELISA of sgp91phox Patient Serum

Reagents

Reagents consisted of a coating buffer (0.05 mol/L carbonate-bicarbonate pH 9.6), a washing solution (0.05% Tween 20 in 50 mmol/L Tris-buffered saline at pH 8.0), a blocking buffer (50 mmol/L Tris-buffered saline at pH 8.0, 1% BSA), and a stopping solution for color development (1mol/L sulfuric acid).

Procedures

ELISA conditions for serum gp91phox evaluation are outlined in Table 1. Standard (50 µL; gp91phox peptide from the sequence LNFKRRKINPEGGLC of gp91phox; New England Peptide, Gardner, Mass) or sample was added into each antibody-coated well and incubated for 60 minutes at room temperature while shaking. After washing 3 times with the washing buffer, 100 µL of diluted HRP-conjugated detecting antibody (0.4 µg/mL) was added and incubated at room temperature for 60 minutes with gentle agitation.

Table 1. ELISA Conditions for Serum sgp91phox Evaluation

<table>
<thead>
<tr>
<th>Major Step</th>
<th>Material</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating Ab</td>
<td>Anti-gp91phox mAb</td>
<td>100 µL 4 µg/mL per well, RT 1 hour</td>
</tr>
<tr>
<td>Serum</td>
<td>Serum 100 µL</td>
<td>1 hour, RT</td>
</tr>
<tr>
<td>Detecting Ab</td>
<td>100 µL goat anti-mouse IgG1-HRP</td>
<td>1:2000, 1 hour, RT</td>
</tr>
<tr>
<td>Signal</td>
<td>TMB (Santa Cruz Biotechnology sk-4400)</td>
<td>Absorbance 405 nm/450 nm, 15 minutes</td>
</tr>
</tbody>
</table>

Zero noise level was determined to be 0.015 pg/mL. Ab indicates antibody; mAb, monoclonal antibody; RT, room temperature.
Wells were again washed 3 times with washing buffer and enzyme substrate was added. After incubation for 20 minutes at room temperature, the reaction was stopped with 100 µL of 1 mol/L H₂SO₄, and the absorbance was read at 405 nm/450 nm. Intra- and interassay coefficients of variation were 5.2% and 6%, respectively.

Platelet Preparation
To obtain platelet-rich plasma samples (n=5, HSs) were centrifuged 15 minutes at 180g to avoid leukocyte contamination, only the top 75% of the platelet-rich plasma was collected.²² Pelleted platelets were washed and suspended in HEPES buffer, pH 7.4 (2×10⁸ platelets/mL, unless otherwise noted).²² To evaluate the level of sgp91phox, platelet were stimulated with or without arachidonic acid (1 mM/L), and the samples were analyzed by ELISA method as above reported.

Human Polymorphonuclear Leukocyte Preparation
Polymorphonuclear leukocytes (PMNs) were isolated from freshly taken EDTA-blood from healthy volunteers (n=5, HSs) by dextran enhanced sedimentation of red blood cells, Ficoll-Histopaque density centrifugation, lysis of remaining erythrocytes with distilled water and washing of cells with Hank’s balanced salt solution (HBSS) in the absence of any divalent cations. Finally, the cell pellet was suspended in 1 mL of HBSS and stimulated with or without 10 µmol/L of phorbol 12-myristate 13-acetate (PMA). To evaluate sgp91phox in PMNs, the supernatant was analyzed by ELISA method as above reported.

Lymphocyte/Monocyte Preparation
Blood samples were collected in heparinized tubes (10 IU/mL). Lymphocytes/monocytes were isolated after centrifugation of the blood from healthy volunteers (n=5, HSs) with a polysucrose-sodium diatrizoate solution, 1.077 g/mL density and 280 mOsm osmolality (Lymphoprep; Nycomed, Oslo, Norway) at 80g at 20°C. The lymphocyte/monocyte cell layer was collected and the cells were thus washed 2 times in a solution of cold PBS (pH 7.2), supplemented with 1% FCS and 2 mmol/L EDTA (Sigma-Aldrich, Milano, Italy). The cell suspension was stimulated with or without lipopolysaccharide (LPS) (100 ng/mL); sgp91 content in the supernatant was evaluated by ELISA method as above reported.

Circulating Blood Microparticle Preparation
Microparticles were isolated from serum. An aliquot of serum was used to microparticle plate format 0.2-µm vacuum filtration device (Ceveron MFU-500, Technoclone, Dorking, UK) that is designed to remove microparticles.²³ Cellular microparticles were measured using a functional assay (Zymuphen MP-Activity, Hyphen BioMed, Neuville-sur-Oise, France). The samples, either microparticle-free or microparticle-rich, were analyzed by ELISA as above reported.

Statistical Analysis
Categorical variables were reported as counts (percentage) and continuous variables as means±SD unless otherwise indicated. Independence of categorical variables was tested by χ² test. Comparisons between HC patients and HSs were carried out by Student t test and were replicated as appropriate with nonparametric test.

To account for the inflation of the experimentwise type I error attributable to multiple testing, Bonferroni correction was used. The correlation analysis was performed with Spearman test. Interventional study data were analyzed for the assessment of treatment effect on sgp91phox, total cholesterol, and urinary isoprostanes performing a multivariate ANOVA with 1 between-subject factor (treatment group) and 1 within-subject factor (time at 2 levels: baseline, 30 days after the beginning of the treatment). As covariates, we considered the possible random differences in age, sex, body mass index (BMI), systolic, and diastolic blood pressure between the 2 groups (the one allocated to diet and atorvastatin and the other allocated to diet alone).

We recruited all the patients (n=30) who respected the inclusion/exclusion criteria for the cross-sectional study of sample size determination, as above reported. The number of controls (n=20) was computed with respect to a 2-tailed Student t test for independent groups, considering (1) as relevant difference in serum sgp91phox levels to be detected between patients and controls δ=10 (pg/mL); (2) SDs between the groups, SD=17 (pg/mL); and (3) type 1 error probability α=0.05 and power 1-β=0.90; this resulted in n=12/group. With regard to the interventional crossover study, we computed the minimum sample size with respect to a 2-tailed 1-sample Student t test, considering (1) relevant difference serum sgp91phox levels to be detected before and after treatments δ=10 (pg/mL); (2) standard deviation of the paired differences SD=8 (pg/mL); and (3) type 1 error probability α=0.05 and power 1-β=0.90; this resulted in n=9/group. For the in vitro experiments of cell stimulation, data were compared by paired Student t test. P<0.05 was considered as statistically significant. The statistical analysis was performed using the SPSS 13.0 software for Windows.

Results
Demonstration of sgp91phox in Human Serum by Immunoprecipitation and Immunoblotting
Immunoblots of the immunoprecipitates from sera from 5 HSs separated by SDS-PAGE showed bands of 91 and 105 kDa. A 105-kDa band was present in the background lane in all immunoblots of the electrophoresis, whereas the 91-kDa band was recognized by the specific monoclonal anti-sgp91phox (Figure 1A). We also showed the presence of sgp91phox by serum immunoblotting in HSs (n=20) and hypercholesterolemic patients (n=30) (Figure 1B). We also immunoprecipitated as a positive control human PMNs from HSs (n=3) (Figure 1C) and PMNs from X-CGD patients (n=3) (Figure 1C).

ELISA Detection of sgp91phox
An ELISA method was developed to simplify the methodology of sgp91phox detection. Serum samples were diluted 1:100 with coating solution. The standard curve was constructed by different concentrations (62.5, 31.25, 15.8, 7.6, 3.8 pg/mL) of a gp91phox peptide from the sequence LNFARKRKNPEGGLC of sgp91phox. The curve was constructed by plotting the mean absorbance for each concentration on the y axis against the concentration on the x axis, and a best fit curve through the points on the graph were drawn (Figure 2A). Intraassay was estimated from ten determinations in the same plate; the interassay was estimated from ten determinations in ten different plates.

The values of serum sgp91phox detected by Western blot analysis significantly (r=0.79, P<0.001) correlated to that evaluated by ELISA.

Source of sgp91phox
To evaluate the source of sgp91phox, we isolated platelets, PMNs, and lymphocytes/monocytes from the same blood sample. Cells suspension in PBS was stimulated with arachidonic acid for platelets, PMA for PMNs, and LPS for lymphocytes/monocytes as above reported; the supernatant sgp91phox content was detected by ELISA. Sgp91phox were 1.18±0.84 pg/mL in unstimulated and 7.05±2.04 pg/mL in
arachidonic acid–stimulated platelets. In PMA-stimulated PMNs, sgp91phox were 11.85 ± 3.23 pg/mL versus 1.53 ± 0.66 pg/mL in unstimulated PMNs. In LPS-stimulated lymphocytes/monocytes, sgp91phox were 7.5 ± 2.64 pg/mL versus 1.11 ± 0.55 pg/mL in unstimulated samples (Figure 2B). The sum of sgp91phox released from activated platelets, PMNs, and monocytes was 31.8 pg/mL, which was >90% of the sgp91phox in the whole serum sample (35.42 ± 2.87 pg/mL; Figure 2B).

**Cross-Sectional Study**

Table 2 shows demographic, laboratory, and clinical characteristics of subjects included in the cross-sectional study. As shown, age, sex, BMI, smoking habit, fasting blood glucose levels, WBC count, and systolic and diastolic blood pressure did not differ between HC and HSs. Serum total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides were significantly higher in patients with hypercholesterolemia compared to HSs ($P<0.001$).

HC patients had enhanced oxidative stress, as documented by elevated urinary excretion of isoprostanes (Table 2 and Figure 2C) and ELISA-evaluated sgp91phox serum levels compared to controls (Table 2 and Figure 2D). At bivariate analysis, serum sgp91phox levels significantly correlated with serum cholesterol ($R=0.52$, $P<0.001$) and isoprostane excretion ($R=0.71$, $P<0.001$); isoprostane excretion significantly correlated with serum cholesterol ($R=0.59$, $P<0.001$).

**Interventional Study**

At baseline, patients randomized to diet alone (group A) and those randomized to diet plus atorvastatin (10 mg daily) (group B) had similar clinical and laboratory characteristics;
also, no difference in markers of oxidative stress including serum sgp91phox, microparticle-bounded gp91phox, and urinary isoprostanes was observed (Table 3 and Figure 3A through 3C).

After the 30 days of treatment, group B showed a significant reduction of serum sgp91phox (−33%, from 36.6±5.6 to 24.5±7.7 pg/mL, P<0.001) and microparticle-bounded gp91phox (−27% from 9.3±2.1 to 6.8±2.8 pg/mL, P=0.01), in conjunction with a reduction of urinary isoprostanes (−37%, from 383±51 to 241±58 pg/mg creatinine, P<0.001) and total cholesterol (−25%, from 276±46 to 208±38 mg/dL, P<0.001). On the contrary, group A showed only a weak reduction in total cholesterol (−7%, from 280±32 to 262±15 mg/dL, P=0.045) (Figure 3D). We did not find any difference in the WBC count before and after the interventions (7513±1236 mm³ preintervention versus 7462±1620 mm³ postintervention in group A and 7584±1503 mm³ preintervention versus 7407±1459 mm³ postintervention in group B). No significant correlations were observed between serum total cholesterol and WBC count at baseline as well as after 30 days of atorvastatin treatment.

To further define the effect of treatment on the variable studied, we performed a multivariate ANOVA analysis that showed a significant effect of the interaction between time per group, indicating a significant effect of the different treatments on serum sgp91phox (F[1,21]=5.6, P=0.02), urinary isoprostanes (F[1,21]=66.1, P<0.01) and total cholesterol (F[1,21]=9.6, P<0.01).

Conversely, we did not find any significant effect of time and its interaction with covariates such as age, sex, BMI, smoke, blood pressure, and WBC count on the above-mentioned variables.

**Discussion**

This study shows that an intervention with a statin is able to significantly reduce circulating sgp91phox, indicating that this drug category may impair oxidative stress via inhibition of NADPH oxidase.

There is much evidence indicating that patients with hypercholesterolemia have enhanced oxidative stress. Experimental and clinical studies showed that hypercholesterolemia is associated with enhanced production of ROS in several cell lines including endothelial cells and platelets.24–27 Also, several circulating markers of oxidative stress, including 8-OHdG, lipid peroxides, nitrotyrosine, and urinary isoprostanes, are elevated in patients with either polygenic or familial hypercholesterolemia.5–9 The enzymatic pathway that may be potentially implicated in such a phenomenon has not been fully elucidated. Although we confirm that oxidative stress is enhanced in HC patients, as documented by the
Table 2. Baseline Characteristics of Hypercholesterolaemic Patients and Healthy Subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hypercholesterolic Patients (n=30)</th>
<th>Healthy Subjects (n=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)*</td>
<td>52.5±3.8</td>
<td>52±3</td>
<td>0.277</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>16/14</td>
<td>10/10</td>
<td>0.954</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>25.4±2.5</td>
<td>25.7±2.4</td>
<td>0.628</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)*</td>
<td>127±12</td>
<td>125±11</td>
<td>0.924</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)*</td>
<td>75±9</td>
<td>75±10</td>
<td>0.928</td>
</tr>
<tr>
<td>Smokers</td>
<td>3</td>
<td>2</td>
<td>0.630</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)*</td>
<td>278±39</td>
<td>187±11</td>
<td>0.001</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)*</td>
<td>187±13</td>
<td>98±14</td>
<td>0.001</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)*</td>
<td>62±11</td>
<td>50±11</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)*</td>
<td>103±21</td>
<td>73±15</td>
<td>0.001</td>
</tr>
<tr>
<td>Fasting blood glucose levels (mg/dL)*</td>
<td>84±12</td>
<td>84±12</td>
<td>0.961</td>
</tr>
<tr>
<td>Total leukocyte count (mm³)</td>
<td>7549±1345</td>
<td>7396±1442</td>
<td>0.708</td>
</tr>
<tr>
<td>Urinary isoprostanes (pg/mg creatinine)*</td>
<td>366±63</td>
<td>210±38</td>
<td>0.001</td>
</tr>
<tr>
<td>sgp91phox (pg/mL)*</td>
<td>35±5.9</td>
<td>16.3±3.5</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Data are expressed as means±SD.

Table 3. Interventional Study: Baseline Characteristics of Hypercholesterolaemic Patients Randomized to Diet Alone (Group A) or Diet Plus Atorvastatin (Group B)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group A (n=15)</th>
<th>Group B (n=15)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>52.8±3.7</td>
<td>52.2±4.1</td>
<td>0.677</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>8/7</td>
<td>8/7</td>
<td>0.714</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>25.1±2.4</td>
<td>25.7±2.6</td>
<td>0.502</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)*</td>
<td>128±12</td>
<td>126±12</td>
<td>0.661</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)*</td>
<td>75±9</td>
<td>75±10</td>
<td>0.660</td>
</tr>
<tr>
<td>Smokers</td>
<td>1</td>
<td>2</td>
<td>1.000</td>
</tr>
<tr>
<td>Fasting blood glucose levels (mg/dL)*</td>
<td>84±12</td>
<td>84±12</td>
<td>0.720</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)*</td>
<td>102±19</td>
<td>103±24</td>
<td>0.965</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)*</td>
<td>280±32</td>
<td>276±46</td>
<td>0.796</td>
</tr>
<tr>
<td>Total leukocyte count (mm³)</td>
<td>7513±1236</td>
<td>7584±1503</td>
<td>0.888</td>
</tr>
<tr>
<td>Urinary isoprostanes (pg/mg creatinine)*</td>
<td>348±69</td>
<td>383±51</td>
<td>0.129</td>
</tr>
<tr>
<td>sgp91phox (pg/mL)*</td>
<td>35±7.3</td>
<td>36.6±5.6</td>
<td>0.491</td>
</tr>
</tbody>
</table>

*Data are expressed as means±SD.

Elevated values of urinary isoprostanes, the novelty of the present study is the observation of high circulating serum values of sgp91phox in HC patients compared to controls. Also of note is that the circulating levels of sgp91phox correlated with urinary isoprostanes, suggesting that it may be responsible for the enhanced production of isoprostanes detected in patients with hypercholesterolemia. This finding further supports our previous report showing a correlation between platelet gp91phox upregulation and enhanced urinary isoprostanes in children with hypercholesterolemia.11

Previous studies have shown that statins exert an antioxidant effect that seems to be only partly related to the statin property of lowering cholesterol. Further study is necessary to see whether statins directly interfere with NADPH oxidase expression in vivo.

NADPH oxidase is a key enzyme of the innate immune system that is present not only in monocytes and leukocytes but also in platelets and endothelial cells.12,16,29 Several NADPH oxidases homologs, namely NOX1, NOX3, NOX4, and NOX5,30 that are relevant for the production of oxidant species have been recently detected in the cells of the arterial wall. Experimental study performed with blood cells allowed us to demonstrate that >90% serum sgp91phox stemmed from platelets, PMNs, and monocytes. A small amount of sgp91phox was also detected in microparticles, which is in accordance with previous studies showing that NADPH oxidase is expressed in microparticles.31 It is possible that vascular NADPH oxidase can also contribute to sgp91phox, but further study is necessary to explore this issue.

The reduction of sgp91phox by atorvastatin was observed in both bound and unbound gp91phox to microparticles, suggesting that such effect would prevalently reflect downregulation of gp91phox at level of blood cellular lines. We cannot exclude, however, that such downregulation can also occur at vascular cell lines such as endothelial cell; thus, the effect of atorvastatin at level of vascular cell lines should be explored in the future.

The study has potential implications and limitations. We have only indirect evidence that serum sgp91phox reflects the amount of the enzyme subunit released by blood cells; also, the cellular source contributing to sgp91phox bound to microparticles is unclear. Therefore, the contribution of each cell line in increasing serum sgp91phox in hypercholesterolemia needs to be determined in the future. Furthermore, it is
likely that analysis of gp91phox only partly reflects the NADPH oxidase expression because other subunits and/or catalytic cores of NADPH oxidase homologs could be detected in the human serum.\textsuperscript{32}

Analysis of oxidative stress in humans is essentially based on the measurement of markers of oxidative stress or molecules that modulate antioxidant status such as antioxidant vitamins or enzymes implicated in ROS scavenging or degradation.\textsuperscript{4,33,34} Analysis of molecules implicated in the generation of ROS such as NADPH oxidase may open new avenues in understanding the role of oxidative stress in several clinical settings including atherosclerosis. In this latter context, measurement of sgp91phox in vivo could be useful to explore the role of this ROS-generating pathway in the progression of atherosclerosis.

The fact that atorvastatin inhibits sgp91phox in vivo is novel and provides further insight in the mechanisms through which statins could halt the progression of atherosclerotic disease. Thus, studies conducted in human atherosclerotic plaque demonstrated that NADPH oxidase is overexpressed and predominantly contributes to vascular oxidative stress\textsuperscript{35}; also, experimental studies demonstrated that the functional deficiency of NADPH oxidase is associated with reduced inflammation and atherosclerotic lesion.\textsuperscript{12}

The exact mechanism by which atorvastatin reduces NADPH oxidase, however, is unclear. Recent study showed that statin treatment inhibits leukocyte ROCK activity, a protein kinase implicated in the activation of NADPH oxidase,\textsuperscript{36} with a mechanism that seems to be independent from lowering cholesterol\textsuperscript{37}; further study is necessary to determine whether gp91phox downregulation by statin is ROCK-mediated.

In conclusion, we provide evidence that in hypercholesterolemia, atorvastatin inhibits oxidative stress via gp91phox downregulation. The inhibition of circulating sgp91phox by atorvastatin represents a novel mechanism potentially accounting for the antiatherosclerotic effect of statins.

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Disclosures

None.

References


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In the article by Pignatelli et al, which appeared in the February 2010 issue of the journal (Arterioscler Thromb Vasc Biol. 2010;30:360–367. DOI: 10.1161/ATVBAHA.109.198622), corrections were needed.

In Table 1, text was added to the “Detecting Ab” line.

In the Materials and Methods section, under “Procedure,” 1 sequence was deleted (“LNFARKRiKNEGGGLC”) and another was added (“C+AERivRGQTEsLaAvHNtVC(Acm) EQKiseWgKIKeC(Acm)PiPQFAGNPPM-NH2”).

In the same paragraph, 1 sentence was expanded: “After washing 3 times with the washing buffer, we added a second antibody (anti-NOX2dp) (in patenting) for 60 minutes at room temperature while shaking. After washing 3 times with the washing buffer, 100 μL of diluted HRP-conjugated detecting antibody (0.4 μg/mL) was added and incubated at room temperature for 60 minutes with gentle agitation.”

In the Results section, under “ELISA Detection of sgp91phox,” 1 sequence was deleted (“LNFARKRKNEGGGLC”) and another was added (“C+AERivRGQTEsLaAvHNtVC(Acm) EQKiseWgKIKeC(Acm)PiPQFAGNPPM-NH2”).

The authors apologize for the errors.

The online version of the article has been corrected and is available at http://atvb.ahajournals.org/content/30/2/360.