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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-gp91^{phox}, the catalytic core of phagocyte NADPH oxidase. In a cross-sectional study performed in 30 hypercholesterolemic patients and in 20 controls, serum soluble-gp91^{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-gp91^{phox} (P<0.001) and urinary isoprostanes (P<0.001). After follow-up, the statin-allocated group showed a significant reduction of soluble-gp91^{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 gp91^{phox} expression. (*Arterioscler Thromb Vasc Biol.* 2010;30:00-00.)

Key Words: gp91^{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.[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.[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.[4] Reduction of several markers of oxidative stress including isoprostanes, 8-hydroxydeoxyguanosine (8-OHdG), and nitrotyrosine have been observed after statin treatment but the underlying mechanism is still unclear.[5–9]

In a pilot study performed in patients with chronic granulomatous disease (X-CGD), a very rare life-threatening disease secondary to hereditary deficiency of gp91^{phox} (the catalytic subunit of phagocyte NADPH oxidase), we found a significant reduction of urinary isoprostanes.[10] Also, in children with hypercholesterolemia, we observed a significant correlation between platelet gp91^{phox} expression and urinary isoprostanes.[11] These previous observations suggest a role for the phagocyte NADPH oxidase, one of the most important cellular producers of superoxide anion (O$_2^-$),[12] in the formation of this marker of oxidative stress. Because previous studies provided in vitro evidence that statins inhibit the expression and activation of NADPH oxidase,[6,13] we sought to analyze whether this occurs in vivo and ultimately contributes to the reduction of oxidative stress. Thus, we developed a method to measure gp91^{phox} in the circulation of patients affected by hypercholesterolemia that is characterized by accelerated atherosclerosis and enhanced oxidative stress.[14,15] Then, we undertook an interventional trial to see whether a statin was able to affect circulating gp91^{phox}. For this purpose, 30 hypercholesterolemic patients were randomized to 1 month of treatment with standard diet or standard diet plus atorvastatin. Herein, we provide the first evidence that statin reduces circulating gp91^{phox} in patients with hypercholesterolemia.

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

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 gp91[phox] that was diagnosed as previously described.[16] X-CGD is a rare (prevalence 1:1 000 000 individuals) primary immunodeficiency affecting the innate immunologic system; X-CGD is characterized by life-threatening bacterial and fungal infections.[17] It is caused by mutations in any of the 4 genes encoding subunits of the O_2^- generating NADPH oxidase, resulting in defective O_2^- generation and intracellular killing.[18]

Design of the Studies
In the first part of the study, we performed a cross-sectional analysis comparing urinary isoprostanes and serum soluble (s)gp91[phox] 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 openly randomized 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 sgp91[phox] 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).[19]

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). High-density lipoprotein (HDL) cholesterol was measured after phosphotungstic acid/MgCl2 precipitation of fresh serum (Dade Behring, Switzerland). Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula.

Urinary 8-isoprostaglandin F2α (PGF2α-III) was measured by previously described and validated EIA assay method.[20,21] Briefly, 10-mL urine aliquots were extracted on a C-18 SPE column; the purification was tested for recovery by adding a radioactive tracer (tritiated PGF2a-III) (Cayman Chemical, Ann Arbor, Mich). The elutes were dried under nitrogen, recovered with 1 mL of buffer, and assayed in a PGF2α-III–specific EIA kit (Cayman Chemical). PGF2α-III concentration was corrected for recovery and creatinine excretion and expressed as picograms per milligrams of creatinine. Intra- and interassay coefficients of variation were 2.1% and 4.5%, respectively.

Table 1.   ELISA Conditions for Serum sgp91[phox] Evaluation

Major Step	Material	Method
Coating Ab	Anti-gp91[phox] mAb (Santa Cruz Biotechnology sc-74514)	100 μL 4 μg/mL per well, RT 1 hour
Sample	Serum 100 μL	1 hour, RT
Detecting Ab	100 μL goat anti-mouse IgG1-HRP (Santa Cruz Biotechnology sc-2060)	100 μL dilute Ab 1:2000, 1 hour, RT
Signal	TMB (Santa Cruz Biotechnology sk-4400)	Absorbance 405 nm/450 nm, 15 minutes

Zero noise level was determined to be 0.015 pg/mL. Ab indicates antibody; mAb, monoclonal antibody; RT, room temperature.

Immunoprecipitation of sgp91[phox] From Human Serum
Gp91[phox] was immunoprecipitated from serum in denaturized conditions. Serum was incubated overnight at 4°C with gp91[phox] antibody sc-74514 (mouse monoclonal antibodies raised against amino acids 231 to 290 of gp91[phox] of human origin, Santa Cruz Biotechnology Inc, Santa Cruz, Calif). Immunocomplexes were bound to ImmunoPure-immobilized protein A (sc-2003, Santa Cruz Biotechnology Inc), boiled in sample buffer, and separated by SDS-PAGE (Bio-Rad, Hercules, Calif) following a described protocol. Negative controls involved a similar procedure using a goat IgG (Santa Cruz Biotechnology Inc).

After electroblotting of proteins to Immobilon membranes, the membranes were blocked and incubated with monoclonal mouse anti-gp91[phox] 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).

Electrophoresis and Immunoblotting of sgp91[phox] Patient Serum
Equal amounts of serum protein (130 μg/lane) estimated by Bradford assay were solubilized in a 2$\times$ Laemmli sample buffer containing 2-mercaptoethanol and loaded in a denaturing SDS/10% polyacrylamide gel. Western blot analysis was performed with monoclonal anti-gp91[phox] (2 μg/mL) incubated overnight at 4°C.

After incubation, the pure nitrocellulose membranes (0.45 μm) were washed and incubated with goat anti-mouse IgG1-HRP for 2 hours. Immune complexes were detected by enhanced chemiluminescence. The developed spots were calculated by densitometric analysis on a NIH Image 1.62f analyzer and the value was expressed as arbitrary units. Each sample was analyzed in triplicate.

ELISA of sgp91[phox] 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).

Procedure
ELISA conditions for serum sgp91[phox] evaluation are outlined in Table 1. Standard (50 μL; gp91[phox] peptide from the sequence LNFARKRIKNPEGGLC of gp91[phox]; 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.

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 H$_2$SO$_4$, 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 (2×10^8 platelets/mL, unless otherwise noted).[22] To evaluate the level of sgp91phox, platelet were stimulated with or without arachidonic acid (1 mmol/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 osmolarity (Lymphoprep; Nycomed, Oslo, Norway) at 800g 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 thawed to 37°C and then filtered using a microtiter 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 χ^2 test. Comparisons between HC patients and HSs were carried out by Student t test and were replicated as appropriate with nonparametric test (Kolmogorov–Smirnov [z] test) in the case of nonhomogeneous variances as verified by Levene's test.

To account for the inflation of the experimentwise type 1 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 $\delta \geq 10$ (pg/mL); (2) SDs between the groups, SD=17 (pg/mL); and (3) type 1 error probability $\alpha=0.05$ and power $1-\beta=0.90$; 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 between before and after treatments $\delta \geq 10$ (pg/mL); (2) standard deviation of the paired differences SD=8 (pg/mL); and (3) type 1 error probability $\alpha=0.05$ and power $1-\beta=0.90$; 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 sgp91^{phox} 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-gp91^{phox} (Figure 1A). We also showed the presence of sgp91^{phox} 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 sgp91^{phox}

An ELISA method was developed to simplify the methodology of sgp91^{phox} 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 gp91^{phox} peptide from the sequence LNFARKRIKNPEGGLC of sgp91^{phox}. 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 sgp91^{phox} detected by Western blot analysis significantly ($r=0.79$, $P<0.001$) correlated to that evaluated by ELISA.

Source of sgp91^{phox}

To evaluate the source of sgp91^{phox}, 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 sgp91^{phox} content was detected by ELISA. Sgp91^{phox} were 1.18 ± 0.84 pg/mL in unstimulated and 7.05 ± 2.04 pg/mL in
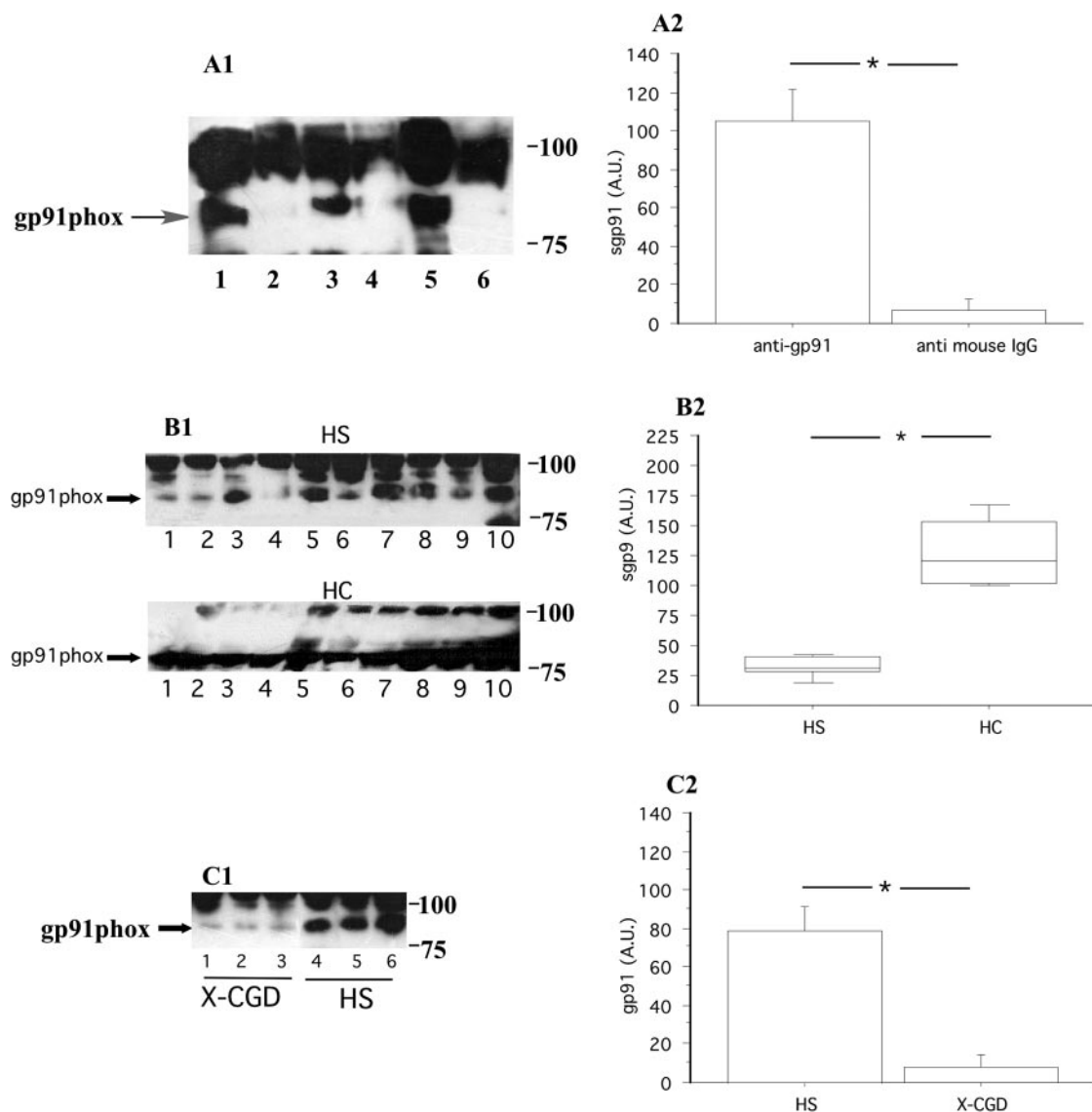
Figure 1. A1, Three representative gp91[phox] immunoprecipitations of 5 performed in HSs. Serum was precipitated with anti-gp91[phox] (lanes 1, 3, and 5) and aspecific goat IgG (lanes 2, 4, and 6). Molecular weight markers (kDa) are indicated. A2, Quantitative analysis of sgp91phox (n=5; *$P<0.001$). B1, Representative Western blots of 10 of 30 performed in HC and 10 of 20 performed in HSs. B2, Quantitative analysis of sgp91phox in HSs (n=20) and HC patients (n=30; *$P<0.001$). C1, Three Western blots of PMNs from HSs and X-CGD patients. C2, Quantitative analysis of sgp91phox in HSs (n=3) and X-CGD patients (n=3; *$P<0.001$).

arachidonic acid–stimulated platelets. In PMA-stimulated PMNs, sgp91[phox] were 11.85 ± 3.23 pg/mL versus 1.53 ± 0.66 pg/mL in unstimulated PMNs. In LPS-stimulated lymphocytes/monocytes, sgp91[phox] were 7.5 ± 2.64 pg/mL versus 1.11 ± 0.55 pg/mL in unstimulated samples (Figure 2B). The sum of sgp91[phox] released from activated platelets, PMNs, and monocytes was 31.8 pg/mL, which was >90% of the sgp91[phox] 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 sgp91[phox] serum levels compared to controls (Table 2 and Figure 2D). At bivariate analysis, serum sgp91[phox] 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;
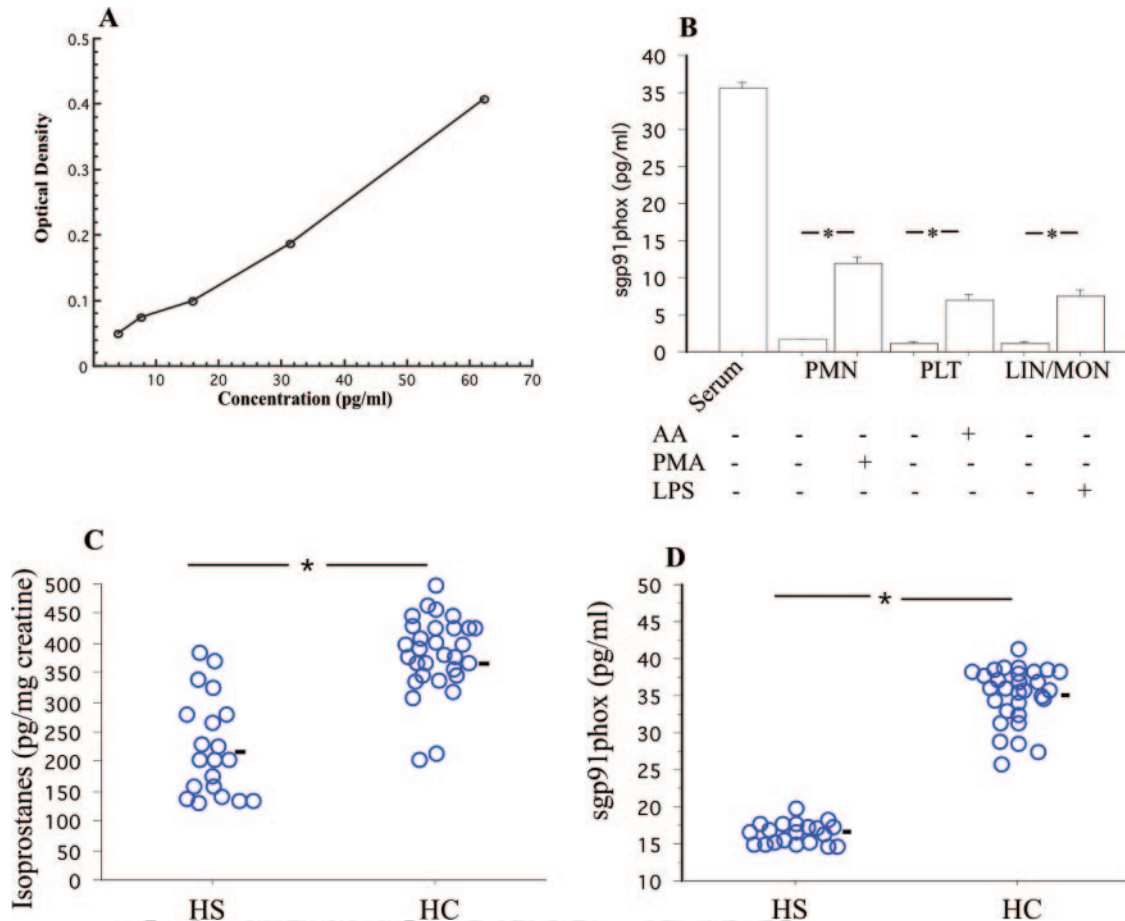
Figure 2. A, gp91^{phox} calibration curve. B, sgp91phox in serum, in arachidonic acid (AA)-stimulated and unstimulated platelets, in PMA-stimulated and unstimulated PMNs, and LPS-stimulated and unstimulated lymphocytes/monocytes (n=5; *$P<0.001$). C, Urinary isoprostanes levels in 30 HC patients and 20 HSs. D, Serum sgp91^{phox} in 30 HC patients and 20 HSs evaluated by ELISA.

also, no difference in markers of oxidative stress including serum sgp91^{phox}, microparticle-bounded gp91^{phox}, 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 sgp91^{phox} (-33%, from 36.6 ± 5.6 to 24.5 ± 7.7 pg/mL, $P<0.001$) and microparticle-bounded gp91^{phox} (-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 mm^3 preintervention versus 7462 ± 1620 mm^3 postintervention in group A and 7584 ± 1503 mm^3 preintervention versus 7407 ± 1459 mm^3 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 sgp91^{phox} ($F[1,21]=5.6$, $P=0.02$), 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 abovementioned variables.

Discussion

This study shows that an intervention with a statin is able to significantly reduce circulating sgp91^{phox}, 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

Variables	Hypercholesterolemic Patients (n=30)	Healthy Subjects (n=20)	P
Age (yr)*	52.5±3.8	52±3	0.277
Gender (male/female)	16/14	10/10	0.954
BMI (kg/m^2)*	25.4±2.5	25.7±2.4	0.628
Systolic blood pressure (mm Hg)*	127±12	125±11	0.924
Diastolic blood pressure (mm Hg)*	75±9	75±10	0.928
Smokers	3	2	0.630
Total cholesterol (mg/dL)*	278±39	187±11	0.001
LDL cholesterol (mg/dL)*	187±13	98±14	0.001
HDL cholesterol (mg/dL)*	62±11	50±11	0.001
Triglycerides (mg/dL)	103±21	73±15	0.001
Fasting blood glucose levels (mg/dL)*	84±12	84±12	0.961
Total leukocyte count (mm^3)	7549±1345	7396±1442	0.708
Urinary isoprostanes (pg/mg creatinine)*	366±63	210±38	0.001
sgp91^{phox} (pg/mL)*	35±5.9	16.3±3.5	0.001

*Data are expressed as means±SD.

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

Variables	Group A (n=15)	Group B (n=15)	P
Age (years)*	52.8±3.7	52.2±4.1	0.677
Gender (male/female)	8/7	8/7	0.714
BMI (kg/m^2)*	25.1±2.4	25.7±2.6	0.502
Systolic blood pressure (mm Hg)*	128±12	126+12	0.661
Diastolic blood pressure (mm Hg)*	75±9	75±10	0.660
Smokers	1	2	1.000
Fasting blood glucose levels (mg/dL)*	84±12	84±12	0.720
Triglycerides (mg/dL)*	102±19	103±24	0.965
Total cholesterol (mg/dL)*	280±32	276±46	0.796
Total leukocyte count (mm^3)	7513±1236	7584±1503	0.888
Urinary isoprostanes (pg/mg creatinine)*	348±69	383±51	0.129
sgp91^{phox} (pg/mL)*	35±7.3	36.6±5.6	0.491

*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 sgp91^{phox} in HC patients compared to controls. Also of note is that the circulating levels of sgp91^{phox} 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 gp91^{phox} 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 cholesterol lowering effect.[6] The mechanism accounting for such antioxidant effect is still unclear. Statins have been shown in vitro and in animal models to reduce the expression of several NADPH oxidase subunits such as rac1, p22^{phox}, and gp91^{phox}.[26–28] However, evidence indicating an interplay between statin treatment and NADPH oxidase expression is still lacking in humans. Herein, we demonstrated that after 1 month of atorvastatin treatment the circulating levels of sgp91^{phox} significantly reduced and paralleled the decrease of urinary isoprostanes. Such parallel behavior reinforce the data of the cross-sectional study strongly indicating that the activation of NADPH oxidase has a key role in the formation of isoprostanes in the human body. Also, the data suggest that downregulation of NADPH oxidase is likely to be a key element of the statins' antioxidant effect.

Even if in vitro studies suggested that statins directly downregulate NADPH oxidase,[6,13] we have no evidence in support of this hypothesis. Thus, inhibition of sgp91^{phox} circulating levels was parallel to serum cholesterol reduction,

indicating that the downregulation of gp91^{phox} expression was 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 sgp91^{phox} stemmed from platelets, PMNs, and monocytes. A small amount of sgp91^{phox} 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 sgp91^{phox}, but further study is necessary to explore this issue.

The reduction of sgp91^{phox} by atorvastatin was observed in both bound and unbound gp91^{phox} to microparticles, suggesting that such effect would prevalently reflect downregulation of gp91^{phox} 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 sgp91^{phox} 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 sgp91^{phox} in hypercholesterolemia needs to be determined in the future. Furthermore, it is
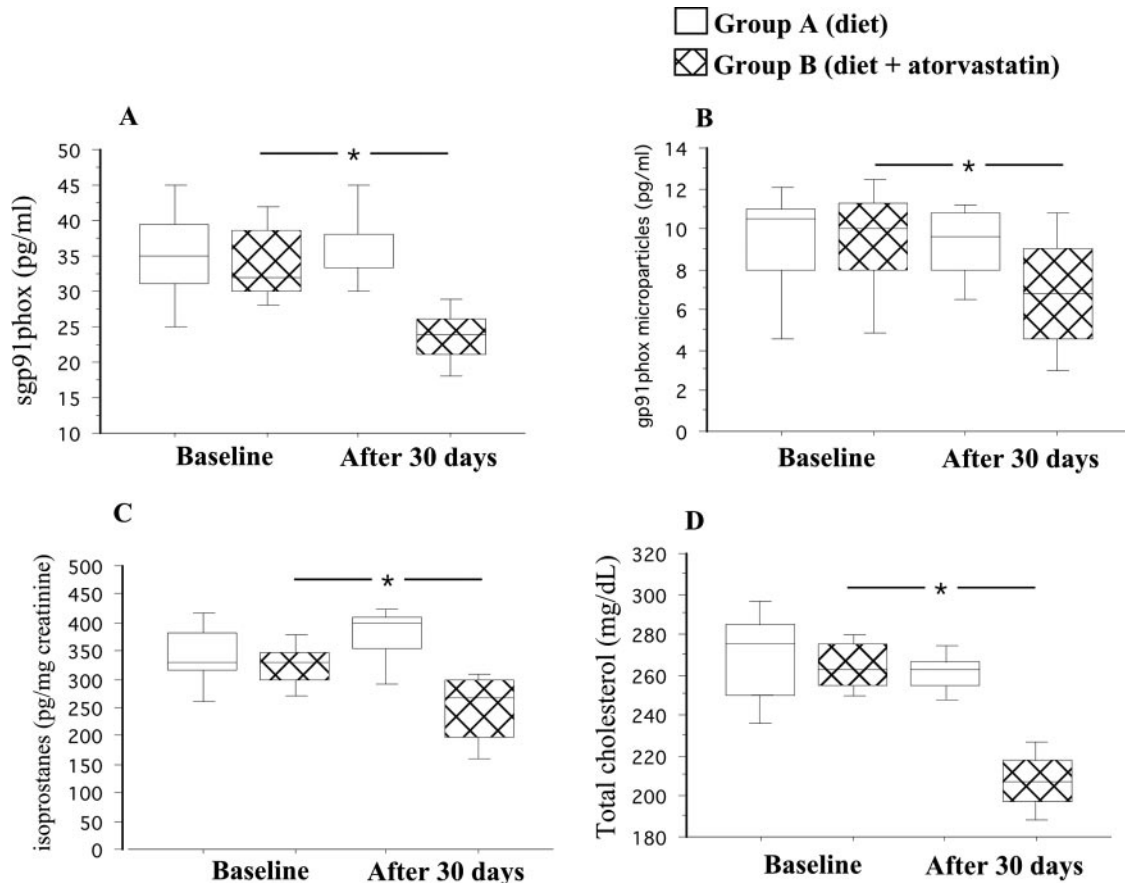
Figure 3. Serum sgp91phox (A), microparticle-bound gp91phox (B), urinary isoprostane (C), and total cholesterol (D) levels in hypercholesterolemic patients randomized to diet alone (group A) or diet plus atorvastatin (group B) at baseline and after 30 days of treatment. Box plots indicate median and 95% confidence intervals; whiskers, minimum and maximum values.

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.[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.[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 sgp91[phox] in vivo could be useful to explore the role of this ROS-generating pathway in the progression of atherosclerosis.

The fact that atorvastatin inhibits sgp91[phox] 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[35]; also, experimental studies demonstrated that the functional deficiency of NADPH oxidase is associated with reduced inflammation and atherosclerotic lesion.[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,[36] with a mechanism that seems to be independent from lowering cholesterol[37]; further study is necessary to determine whether gp91[phox] downregulation by statin is ROCK-mediated.

In conclusion, we provide evidence that in hypercholesterolemia, atorvastatin inhibits oxidative stress via gp91[phox] downregulation. The inhibition of circulating sgp91[phox] by atorvastatin represents a novel mechanism potentially accounting for the antiatherosclerotic effect of statins.

Disclosures

None.

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