Immediate Antioxidant and Antiplatelet Effect of Atorvastatin via Inhibition of Nox2

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Background—Statins exert an antithrombotic effect in patients at risk of or with acute thrombosis, but no study has investigated whether this effect is immediate and whether there is an underline mechanism.

Methods and Results—Patients with hypercholesterolemia were randomly allocated to a Mediterranean diet with low cholesterol intake (<300 mg/d; n = 15) or atorvastatin (40 mg/d; n = 15). Oxidative stress, as assessed by serum Nox2 and urinary isoprostanes, and platelet activation, as assessed by platelet recruitment, platelet isoprostanes, and thromboxane A2, platelet Nox2, Rac1, p47phox, protein kinase C, vasodilator-stimulated phosphoprotein, nitric oxide, and phospholipase A2, were determined at baseline and after 2, 24, and 72 hours and 7 days of follow-up. An in vitro study was also performed to see whether atorvastatin affects platelet oxidative stress and activation. The atorvastatin-assigned group showed a significant and progressive reduction of urinary isoprostanes and serum Nox2, along with inhibition of platelet recruitment, platelet isoprostanes, Nox2, Rac1, p47phox, and protein kinase C, starting 2 hours after administration. Platelet phospholipase A2 and thromboxane A2 significantly decreased and vasodilator-stimulated phosphoprotein and nitric oxide increased after 24 hours. Low-density lipoprotein cholesterol decreased significantly after 72 hours and further declined after 7 days. No changes were observed in the Mediterranean diet group. In vitro experiments demonstrated that atorvastatin dose-dependently inhibited platelet Nox2 and phospholipase A2 activation, along with inhibition of platelet recruitment, platelet isoprostanes, and thromboxane A2, and increased vasodilator-stimulated phosphoprotein and nitric oxide.

Conclusions—The study provides the first evidence that atorvastatin acutely and simultaneously decreases oxidative stress and platelet activation by directly inhibiting platelet Nox2 and ultimately platelet isoprostanes and thromboxane A2. These findings provide a rationale for the use of statins to prevent or modulate coronary thrombosis.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT01322711.

Key Words: antioxidants ■ blood platelets ■ hydroxymethylglutaryl-CoA reductase inhibitors ■ hypercholesterolemia ■ statins ■ thromboxanes

Statins are lipid-lowering drugs that lower serum cholesterol via inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase.1 Interventional trials with statins demonstrated a significant reduction of cardiovascular events in patients with both high and average serum cholesterol.2,3 Moreover, statins were shown to reduce cardiovascular events in patients with acute coronary syndrome, suggesting that they could also exert an antithrombotic effect.2 A recent meta-analysis reinforced the concept that statins may possess antithrombogenic property because these drugs were reported to reduce periprocedural infarction in patients undergoing percutaneous coronary intervention.4 Of note, the clinical benefit was detected after a short treatment with statins (median, 0.5 days), indicating that statins could potentially exert an antithrombotic effect even earlier than supposed from pharmacological studies. Inhibition of platelet activation and thromboxane (Tx) synthesis has been detected 2 to 3 days after statin treatment,5,6 but no study has been done so far to see whether statins possess a rapid antiplatelet effect, ie, within 24 hours of administration. Also of relevance is the fact that it is not fully defined whether the antiplatelet property of statins is a pleiotropic effect and/or is dependent on low-density lipoprotein (LDL) lowering. Addressing these issues may be of potential relevance for providing further mechanistic support to the early use of statins in clinical settings associated with platelet-related uneventful cardiovascular events.

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There is a growing body of evidence of a mechanistic link between oxidant stress and platelet activation. An experimental study in animals provided support for such interplay by

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showing that animals with knockout of glutathione peroxidase, an enzyme with antioxidant property, are prone to thrombosis via a mechanism involving platelet overactivation.7 Agonist-stimulated platelets release reactive oxidant species, which are implicated in the propagation of platelet activation by inactivating nitric oxide, releasing platelet agonists such as ADP, or forming isoprostanes.5,9 Isoprostanes are a family of eicosanoids that, unlike TXA2, are chemically stable and greatly contribute to propagate platelet agonists such as ADP, or forming isoprostanes.8,9 Isoprostanes, which are implicated in the propagation of platelet activation, were detected by ELISA as previously described.12 The peptide was recognized by the specific monoclonal antibody against the amino acid sequence (224–268) of the extramembrane portion of Nox2 (catalytic core of NADPH oxidase), which was released in the medium on platelet activation. Values were expressed as picograms per milliliter; intra-assay and interassay coefficients of variation were 5.2% and 6%, respectively.

Platelet isoprostane (8-iso-PGF2α-III) was measured by the enzyme immunoassay method (Amersham Pharmacia, Biotech, Little Chalfont, UK) and expressed as picomoles per liter. Intra-assay and interassay coefficients of variation were 4.0% and 3.6%, respectively.

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Platelet Soluble Nox2-Derived Peptide, TxA2, and Platelet Isoprostane
Platelet suspension was activated with arachidonic acid (AA; 0.5 mmol/L), and the supernatant was stored at ~80°C. Extracellular levels of soluble Nox2-derived peptide (sNOX2-dp), a marker of NADPH oxidase activation, were detected by ELISA as previously described.12 The peptide was recognized by the specific monoclonal antibody against the amino acid sequence (224–268) of the extramembrane portion of Nox2 (catalytic core of NADPH oxidase), which was released in the medium on platelet activation. Values were expressed as picograms per milliliter; intra-assay and interassay coefficients of variation were 5.2% and 6%, respectively.

Platelet TxB2 was measured by ELISA (Amersham Pharmacia, Biotech, Little Chalfont, UK) and expressed as picomoles per liter. Intra-assay and interassay coefficients of variation were 4.0% and 3.6%, respectively.

Platelet Nitric Oxide Metabolites Nitrite and Nitrate Measurement
A colorimetric assay kit (Tema Ricerca, Italy) was used to determine the nitric oxide metabolites nitrite and nitrate (NOx) in the supernatant of AA (0.5 mmol/L)-stimulated platelets. Intra-assay and interassay coefficients of variation were 2.9% and 1.7%, respectively.

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Platelet Recruitment
Platelet recruitment was performed with a method modified from that described by Krotz et al.9 Collagen (2 μg/mL)-induced platelet aggregation was measured for 10 minutes. Then, an equal portion of untreated platelets was added to each tube, which caused a reduction in light transmission. Aggregation of the newly added platelet portion in the presence of an existing aggregate was then measured for 5 minutes and expressed as a percentage of the aggregation that had been initially reached, according to Pignatelli et al.9

Flow Cytometry Analysis of Platelet Protein Kinase C, Rac1, and Phospholipase A2 Phosphorylation
Platelet protein kinase C (PKC) phosphorylation was analyzed by flow cytometry and expressed as mean fluorescence as previously reported by Carnevale et al.13 Platelet Rac1 and phospholipase A2 (PLA2) phosphorylation in resting or AA (0.5 mmol/L)-stimulated washed platelets was analyzed by flow cytometry with the use of specific antibodies (anti-Rac1 IgG goat polyclonal, monoclonal anti–phosphorylated Circulating PLA2 [cPLA2]). In all assays, an irrelevant isotype-matched antibody was used as a negative control. Antibodies (1 μg/mL) were added to 200 μL platelet suspension (2×10^8), previously fixed with (2%) paraformaldehyde (0.1% BSA) for 60 minutes at room temperature, and then permeabilized with digitonin (100 μmol/L) for 30 minutes at room temperature. Unbound antibody was removed by adding 0.1% BSA PBS and subsequently centrifuging at 3000g for 3 minutes (twice). Fluorescence intensity was analyzed on an Epics XL-MCL cytometer (Coulter Electronics) equipped with an argon laser at 488 nmol/L. For every histogram, 50,000 platelets were counted to determine the proportion of positive platelets. We reported antibody reactivity in arbitrary units obtained by multiplying the number of positive events resulting from platelet stimulation by the mean values of the fluorescence observed when the specific antibody was used and by correcting for the values obtained in unstimulated samples treated with the same antibody.

Western Blot Analysis of Platelet PLA2, Rac1, PKC, p47phox, and Vasodilator-Stimulated Phosphoprotein Phosphorylation
Platelets (2×10^9/mL in HEPES buffer) were activated with AA (0.5 mmol/L) and then washed and suspended in a 2× Lysis buffer

Laboratory Analyses
All blood samples were taken after a 12-hour fast. Blood analyses were performed in a blinded manner. All materials were from Sigma Aldrich unless otherwise specified.

Platelet Preparation
To obtain platelet-rich plasma, samples were centrifuged for 15 minutes at 180g. To avoid leukocyte contamination, only the top 75% of the platelet-rich plasma was collected. Platelet pellets were suspended in HEPES buffer, pH 7.4 (2×10^9 platelets per 1 mL unless otherwise noted). In some experiments, samples were treated with atorvastatin (1–10 μmol/L for 10 minutes at 37°C) or control buffer before activation with the agonist.
(5 mmol/L EDTA, 0.15 mol NaCl, 0.1 mol Tris, pH 8.0, 1% triton and protease inhibitor cocktail). Equal amounts of protein (30 μg per lane) estimated by Bradford assay were solubilized in a 2× Laemmli buffer containing 2-mercaptoethanol and loaded in a denaturing SDS/10% polyacrylamide gel. Western blot analysis was performed with monoclonal anti–phosphorylated cPLA₂ (1 μg/ml), anti-Rac1 (2 μg/ml), anti-PKC (2 μg/ml), anti-p47phox (2 μg/ml), or anti-phosphorylated vasodilator-stimulated phosphoprotein (VASP) (2 μg/ml) incubated overnight at 4°C. Immune complexes were detected by enhanced chemiluminescence. The phosphorylation rate of Rac1, PKC, p47phox, VASP, and anti–phosphorylated cPLA₂ was analyzed by autoradiography. The developed spots were calculated by densitometric analysis on an NIHimage 1.62f analyzer, and the value was expressed in arbitrary units.

Flow Cytometry Analysis of Glycoprotein IIb/IIIa

PAC1 is an antibody that recognizes an epitope on the glycoprotein IIb/IIIa of activated platelets at or near the platelet fibrinogen receptor. PAC1 binding on AA (0.5 mmol/L)-activated platelets membrane was analyzed by use of the specific FITC-labeled monoclonal antibodies anti-PAC1 (BD International) as previously reported. All assays included samples to which an irrelevant isotype-matched antibody (FITC-labeled IgM) was added. Fluorescence intensity was analyzed on an Epics XL-MCL Cytometer (Coulter Electronics, Hialeah, FL) equipped with an argon laser at 488 nm. For every histogram, 5 × 10⁵ platelets were counted to evaluate the percentage of positive platelets. Antibody reactivity is reported as mean fluorescence intensity.

Cytoplasmic Free Calcium Mobilization

Cytoplasmic free calcium mobilization was evaluated according to Labiós et al. Briefly, CD61PE (specific monoclonal antibody to platelet glycoprotein IIIa; Pharmingen, San Diego, CA) and 250 AM fluo-3-acetoxymethyl ester (FLUO 3-AM; calcium probe; Molecular Probes, Leiden, the Netherlands) were added to the sample. The basal fluorescence intensity corresponding to FLUO 3-AM (FL1 log; FLUO 3-AM-FITC) was measured for 15 seconds in the platelet population identified in the gate FS log/SS log as CD61-positive events. Platelet stimulation with thrombin (0.1 U/ml) was used as positive control. Changes in fluorescence were evaluated in arbitrary units as fold increase in calcium mobilization. Samples were incubated 30 minutes at 37°C with or without atorvastatin (1 and 10 μmol/L). For every histogram, 50,000 platelets per event were counted to determine the proportion of positive platelets.

Serum Nox2

Serum levels of sNOX2-dp were detected by ELISA as previously described by Pignatelli et al. Blood samples were kept for 60 minutes at 37°C and centrifuged at 300g; serum was stored at −80°C. Values were expressed as picograms per milliliter; intra-assay and interassay coefficients of variation were 5.2% and 6%, respectively.

Urinary 8-IsopGF2α-III Assays

Urinary 8-iso-PGF2α-III was measured by the enzyme immunoassay method. Urinary 8-iso-PGF2α-III concentration was corrected for recovery and creatinine excretion and expressed as picograms per milligram of creatinine. Intra-assay and interassay coefficients of variation were 2.1% and 4.5%, respectively.

Sample Preparation and Atorvastatin Evaluation in High-Performance Liquid Chromatography

The stock solution of atorvastatin was prepared by dissolving an appropriate amount corresponding to a 0.5 mg/mL concentration of working standard in methanol. The stock solution of atorvastatin was further diluted with the mobile phase methanol-water (68:32, vol/vol; pH adjusted to 3.0 with trifluoroacetic acid) to give a series of standards having a final concentration in the range of 0.01 to 2 μmol/L.

A simple 2-step liquid-liquid extraction procedure was carried out for the extraction of atorvastatin from serum samples. A volume (50 μL) of internal standard (atorvastatin 400 ng/mL) was added to 200 μl of serum and mixed for 10 seconds. Then, 600 μL ethanol was added and vortex-mixed for 2 minutes for deproteination. In step 1, 1.0 mL diethyl ether (extraction solvent 1) was added, vortex-mixed for 5 minutes, and centrifuged at 3500 rpm at 0°C for 5 minutes. The supernatant organic layer was separated in a test tube. In step 2, 0.5 mL dichloromethane (extraction solvent 2) was added, vortexed for 5 minutes, and then centrifuged (3500 rpm) at 0°C for 5 minutes. The organic layer was separated, collected in the same tube, and evaporated to complete dryness under nitrogen. After drying, the residue was reconstituted in 500 μL of the mobile phase and vortex-mixed for 2 minutes, and 20 μL of the sample was injected into the high-performance liquid chromatography system.

Chromatographic separation was performed with different proportions of acetonitrile-water and methanol-water as a mobile phase with flow rates of 1.0 mL/min in an isocratic mode. The injection volume was 50 μL. The column oven temperature was 25°C, and the eluate was monitored with ultraviolet detection at a wavelength of 241 nm.

Statistical Analysis

The minimum sample size was computed with respect to a 2-tailed Student t test considering (1) relevant difference in platelet isoprostanes levels to be detected between treatment groups δ ≥30 pmol/L, (2) standard deviations homogeneous between the groups (SD = 25 pmol/L), and (3) type I error probability α = 0.05 and power 1 − β = 0.90. This resulted in n = 15 per group. Categorical variables are reported as counts (percentage) and continuous variables as mean ± SD unless otherwise indicated. Differences between percentages were assessed by the χ² test or Fisher exact test. A Student unpaired t test and Pearson correlation analysis were used for normally distributed continuous variables. Appropriate nonparametric tests (Mann-Whitney U test or Spearman rank correlation test) were used for all the other variables. Interventional study data were analyzed for the assessment of treatment effect on platelet recruitment, platelet and urinary isoprostanes, platelet and serum sNOX2-dp, and platelet TxB₂ by performing an ANOVA for
repeated measurements with 1 between-subject factor (treatment group) and 1 within-subject factor (time at 5 levels: baseline and 2 hours, 24 hours, 3 days, and 7 days after the beginning of the treatment). As covariates, we considered the possible random differences in age, sex, body mass index, blood pressure, and blood lipid profile between the 2 groups (the group allocated to diet and atorvastatin and the other group allocated to diet alone). Pairwise comparisons were performed with the Bonferroni correction. Values of $P<0.05$ were regarded as statistically significant. All calculations were made with Statistica 7 software for Windows (StatSoft, Tulsa, OK).

**Results**

**Interventional Study**

The study design is shown in Figure 1. At baseline, patients randomized to diet alone (group A) and those randomized to diet plus atorvastatin (40 mg/d; group B) had similar clinical and anthropometric characteristics (the Table). Platelet (263±54 versus 236±31 mm$^3$), red blood cell (5.1±0.38×10$^6$/μL), and white blood cell (7524±1221 versus 7631±1196 mm$^3$) counts were similar in the 2 groups.

Lipid profile, urinary isoprostanes, serum sNOX2-dp, platelet recruitment, and platelet isoprostanes, TxB2, Nox2, p47 phox, Rac1, PKC, PLA2, VASP, NOx, and serum atorvastatin were not different in the 2 groups (the Table and Figures 2–5).

ANOVA for repeated measurements showed significant time-dependent differences between the 2 treatment groups for platelet recruitment ($F=4.9; P=0.0011$), platelet ($F=5.1; P=0.00084$), and urinary ($F=2.9; P=0.0253$) isoprostanes, platelet TxB$_2$ ($F=4.1; P=0.0038$), platelet ($F=2.4; P<0.05$) and serum ($F=2.5; P=0.04562$) sNOX2-dp, platelet phosphorylation of p47$^{phox}$ ($F=6.1; P=0.00018$), Rac1 ($F=2.8; P=0.0285$), PKC ($F=4.1; P=0.00361$), VASP ($F=3.13; P=0.0176$), PLA$_2$ ($F=6.6; P=0.0001$), platelet NOx ($F=2.6; P=0.0367$), and LDL cholesterol ($F=6.5; P=0.0001$).

The diet-assigned group (group A) did not show any difference in platelet recruitment, platelet and urinary isoprostanes, platelet and serum sNOX2-dp, platelet TxB$_2$, platelet p47$^{phox}$, sNOX2-dp, and LDL cholesterol.

**Table. Baseline Characteristics of Hypercholesterolemic Patients and Control Subjects**

<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, y</td>
<td>67.5±14.4</td>
<td>63.4±13</td>
<td>0.434</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>7 (47)</td>
<td>7 (47)</td>
<td>0.948</td>
</tr>
<tr>
<td>BMI, kg/m$^2$</td>
<td>26.8±5.8</td>
<td>26.7±3.1</td>
<td>0.955</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>124±21</td>
<td>128±11</td>
<td>0.510</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>76±12</td>
<td>80±9</td>
<td>0.415</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>6 (40)</td>
<td>5 (33)</td>
<td>0.320</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>231±28</td>
<td>232±19</td>
<td>0.956</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>161±25</td>
<td>165±25</td>
<td>0.657</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>60±13</td>
<td>49±17</td>
<td>0.143</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>99±30</td>
<td>112±30</td>
<td>0.108</td>
</tr>
<tr>
<td>Fasting glycemia, mg/dL</td>
<td>94±14</td>
<td>94±9</td>
<td>0.946</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.8±0.2</td>
<td>0.8±0.1</td>
<td>0.746</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; BP, blood pressure; LDL, low-density lipoprotein; and HDL, high-density lipoprotein. Group A is composed of hypercholesterolemic patients assigned to diet alone; group B, hypercholesterolemic patients assigned to diet plus atorvastatin (40 mg/d). Data are expressed as mean±SD when appropriate.

*$P$ for comparison between groups A and B.
Rac1, PKC, VASP, PLA2, NOx, LDL cholesterol, and serum atorvastatin at any study point (Figures 2–5).

Systemic oxidative stress as assessed by serum sNOX2-dp (−26%; P<0.01) and urinary isoprostanes (−35%; P<0.001) was significantly reduced 2 hours after atorvastatin administration and further declined after 24 hours, reaching a plateau after 72 hours (Figure 2); there was a parallel decrease of the 2 markers of oxidative stress (R=0.836, P<0.001). LDL cholesterol did not change 2 and 24 hours after atorvastatin administration (1% and 2%, respectively; P=NS) but was significantly reduced after 3 and 7 days (P<0.001; Figure 2).

Changes of platelet activation were simultaneous to those of oxidative stress. Thus, platelet recruitment (−30%; P<0.001), platelet isoprostanes (−29%; P<0.001), and platelet Nox2 activation (−32%; P<0.001) decreased significantly 2 hours after atorvastatin administration with a further reduction after until 72 hours (Figure 3).

Coincident with platelet Nox2 activation, we observed impaired phosphorylation of platelet p47phox (−28%; P<0.001), Rac1 (−28%; P<0.001), and PKC (−27%; P<0.001) 2 hours after atorvastatin administration with a further reduction up to 72 hours (Figure 4). Conversely, platelet TxB2 and PLA2 phosphorylation did not change after 2 hours, significantly decreased after 24 hours, and continued to decline until 7 days from atorvastatin administration (Figures 3B and Figure 5B). In addition, platelet VASP phosphorylation and platelet NOx did not change after 2 hours, were significantly enhanced after 24 hours, and continued to increase concomitantly thereafter (r=0.63, P=0.011; Figure 5A and 5C).

To explore the relationship among time-dependent reduction of TBX2, LDL cholesterol, and PLA2 phosphorylation, ANOVAs were repeated to assess the effect of treatment from 2 hours to 1, 3 and 7 days. Significant time-dependent differences between the 2 treatment groups were shown for platelet TxB2 (P=0.02375), PLA2 phosphorylation (P=0.00203), and LDL cholesterol (P=0.0287). Time-related changes between LDL cholesterol and TxB2 (R=0.505, P<0.001) and PLA2 phosphorylation (R=0.25, P<0.03) were significantly correlated. A significant correlation was also observed between time-related changes of PLA2 and TxB2 (R=0.26, P<0.025).

Serum atorvastatin significantly increased as early as 2 hours after the first administration of the drug in group B, reaching a plateau at 24 hours (0.104±0.03 μmol/L at 2 hours versus 0.178±0.02 μmol/L at 24 hours) and was unchanged in group A (Figure 5D).

**In Vitro Study**

To investigate whether atorvastatin directly influences platelet formation of eicosanoids, experiments were conducted in vitro with different concentrations of atorvastatin, which were close to that found in human circulation after atorvastatin intake.
Effect of Atorvastatin on Platelet Oxidative Stress
Isoprostane formation and Nox2 activation were significantly and dose-dependently inhibited by atorvastatin (Figure 6A and 6B). To analyze the pathway involved in atorvastatin-dependent Nox2 inhibition, we studied PKC phosphorylation, an upstream signaling for activation of Rac1, a key unit for NADPH oxidase activation.18 Atorvastatin (1–10 μmol/L) dose-dependently decreased AA-induced PKC (Figure 6C) and Rac1 phosphorylation (Figure 6D).

Effect of Atorvastatin on Platelet TX
PLA2 phosphorylation has a pivotal role in platelet Tx formation via release of AA from platelet membrane.19 The incubation of platelets with atorvastatin (1–10 μmol/L) dose-dependently decreased AA-induced PKC (Figure 6C) and Rac1 phosphorylation (Figure 6D).

Effect of Atorvastatin on Platelet Recruitment
Incubation of platelets with atorvastatin (1–10 μmol/L) showed a dose-dependent decrease of platelet recruitment (Figure 8A).

Calcium mobilization and glycoprotein IIb/IIIa activation are 2 fundamental steps for isoprostane- and TxB2-mediated platelet recruitment. We found that atorvastatin dose-dependently inhibited calcium mobilization and glycoprotein IIb/IIIa activation (Figure 8B and 8C).

Discussion
The study provides the first evidence that atorvastatin exerts an antioxidant effect as early as 2 hours after its administration via

![Figure 4](https://example.com/image4)
inhibition of Nox2. A simultaneous antiplatelet effect was also observed, including inhibition of platelet isoprostane and TxB2, suggesting an interplay between oxidative stress and platelet activation inhibition. This hypothesis was corroborated by an in vitro study showing that atorvastatin directly inhibits platelet Nox2 and ultimately platelet eicosanoids.

**Antioxidant Effect by Statins**

Experimental and clinical studies demonstrated that statins exert an antioxidant effect with a mechanism potentially involving downregulation of Nox2 and upregulation of nitric oxide synthase coupling. A reduction of urinary isoprostanes or an increase of circulating vitamin E has been observed after short-term (3 days) or long-term treatment with statins. In the present study, using 2 markers of oxidative stress, ie, urinary isoprostanes and sNOX2-dp, we demonstrated that high-dose atorvastatin acutely decreases both markers, indicating that it exerts a rapid antioxidant effect. The parallel decrease of Nox2 activity and urinary isoprostanes reinforces data from previous studies suggesting a major role for Nox2 in the production of isoprostanes. Oxidative stress lowering by atorvastatin was not associated with changes in LDL cholesterol, indicating a direct inhibitory mechanism of statin on the NADPH oxidase enzyme. Using platelets as a tool to investigate this issue, we could demonstrate that atorvastatin, at dosages higher but relatively close to those found in the human circulation after 40-mg administration, inhibited in vitro Nox2 activation with a mechanism involving PKC-induced p47phox and Rac1 phosphorylation. These data support and extend previous data showing that the same dosage of atorvastatin exerts an antioxidant effect at endothelial levels within 3 days from its administration.

**Antiplatelet Effect by Statins**

Previous studies using several markers of platelet activation in patients with stable or unstable cardiovascular disease have already shown that statins exert an antiplatelet effect. In a few studies, such an antiplatelet effect was early and apparently independent from the cholesterol-lowering property of statins. In one of these studies, the antiplatelet effect was examined 24 hours after 10-mg atorvastatin administration, and no changes were observed. In our study, the dose of
Atorvastatin was higher (40 mg) and the effect was examined immediately, i.e., 2 hours after the administration of statin. Here, we report that atorvastatin exerts an immediate antiplatelet effect, as assessed by platelet recruitment, that is independent of cholesterol lowering. The immediate inhibition of platelet isoprostane production seems to be the mechanism more implicated in the early antiplatelet effect of atorvastatin. Thus, the reduction of TxB2 was slower and almost evident only 24 hours after the administration of statin. This effect paralleled platelet downregulation of PLA2, suggesting that impaired AA release from platelet membrane was implicated in the reduced formation of TxB2. It is interesting to note, however, that although the early reduction of TxB2 was also independent of cholesterol lowering, the later TxB2 reduction was coincident with LDL lowering, suggesting a time-related interplay between LDL lowering and platelet TxB2 inhibition.

The study has implications and limitations. Atorvastatin exerts an early and a late antiplatelet effect that is independent of its lipid-lowering property and is related to 2 different intraplatelet signalings. The early effect (2 hours) involves NADPH oxidase; the late effect involves PLA2. Thus, the early effect was related to NADPH oxidase downregulation via reduced phosphorylation of Rac1 and p47phox and resulted in platelet isoprostane formation reduction, whereas TxB2 lowering was related to PLA2 inhibition. The in vitro study was in agreement with this hypothesis in that atorvastatin directly and dose-dependently inhibited both platelet Nox2 activation and platelet isoprostane formation. The antioxidant effect of atorvastatin was likely to explain the inhibition of platelet

Figure 6. In vitro study: platelet (PLT) isoprostanes (A), platelet soluble Nox2-derived peptide (sNOX2-dp; B), platelet protein kinase C (PKC) phosphorylation (C), and platelet Rac1 (D) in arachidonic acid (AA)-stimulated samples treated or not treated with atorvastatin (1–10 μmol/L). Experiments were done on 6 subjects. M.F. indicates mean fluorescence. *P<0.001.
isoprostanes and ultimately platelet activation as indicated by the reduced platelet calcium mobilization and glycoprotein IIb/IIIa activation elicited by platelet incubation with atorvastatin.

The antioxidant effect of atorvastatin could also be implicated in PLA2 downregulation in that reactive oxidant species exert a proaggregatory activity by releasing AA from platelet membrane via platelet PLA2 activation.29 Accordingly, platelet incubation with atorvastatin downregulated PLA2 activation, thus providing mechanistic insight into the in vitro inhibition of platelet TxB2, which was detected in the present and previous studies.5

Finally, the inhibition of reactive oxidant species could also explain the increase of platelet NOx observed 24 hours after atorvastatin administration. This is in accordance with previous studies showing that antioxidants enhance VASP phosphorylation and nitric oxide biosynthesis.30,31 This study has pharmacological implications in that it suggests platelet NADPH oxidase as a novel target to accomplish an antiplatelet effect. The inhibition of platelet isoprostanes by atorvastatin is particularly intriguing, taking into account the fact that aspirin does not affect platelet isoprostanes.32 Combining statins with aspirin could provide a deeper inhibition of platelet function via downregulation of platelet NADPH oxidase and COX1, respectively.

The early inhibition of oxidative stress and platelet function provides a rationale for the immediate use of statins in patients with acute coronary syndrome or at high risk of thrombosis such

Figure 7. In vitro study: platelet phospholipase A2 (PLA2) phosphorylation (A), platelet thromboxane B2 (TxB2) production (B), platelet vasodilator-stimulated phosphoprotein (VASP) phosphorylation (C), and platelet nitrite and nitrate formation (D) in arachidonic acid (AA)–stimulated samples treated or not treated with atorvastatin (1–10 μmol/L). Experiments were done on 6 subjects. *P<0.001.
as patients undergoing percutaneous coronary intervention. However, our study should be repeated in these clinical settings to confirm the early antiplatelet effect by statins.

The inhibition of platelet TxB2 seems to occur via at least two mechanisms, 1 involving the inhibition of platelet PLA2 and the other involving LDL lowering. The mechanism through which LDL lowering reduced platelet TxB2 needs to be better evaluated in the future.

An important issue is whether the antiplatelet effect of atorvastatin is comparable to that of other antiplatelet drugs. Analysis of platelet TxB2 could help with the comparison because the inhibition of platelet TxB2 7 days after atorvastatin treatment was \( \approx 50\% \), which is less than that achieved by aspirin \( (<95\%) \). However, aspirin does not affect platelet isoprostanes, whereas atorvastatin reduced it by \( \approx 50\% \) (see Figure 3).

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**Figure 8.** In vitro study: platelet (PLT) recruitment (A), platelet \( \mathrm{Ca}^{2+} \) mobilization (B), and platelet IIb/IIIa activation (C) in arachidonic acid (AA)–stimulated samples treated or not treated with atorvastatin (1–10 \( \mu \)mol/L). Experiments were done on 6 subjects. M.F. indicates mean fluorescence. *\( P<0.001 \).
We acknowledge the potential weakness of the open design of the study; however, randomization and blind analysis of laboratory variables were likely to limit this bias.

Conclusions

This study shows an immediate antioxidant and antiplatelet effect of atorvastatin in patients with hypercholesterolemia. This effect occurs via inhibition of Nox2 activation and results in downregulation of platelet isoprostane and TxB2. Further study is necessary to see whether a similar effect can be achieved in patients with acute coronary syndrome and in those undergoing percutaneous coronary intervention.

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References


**CLINICAL PERSPECTIVE**

There is evidence that statins exert an antithrombotic effect in patients at risk of or with acute thrombosis, but the underlying mechanism is still to be clarified. Because platelets play a key role in artery thrombosis, the present study addressed the question of whether statins possess a direct antiplatelet property. Patients with hypercholesterolemia were randomly allocated to a Mediterranean diet with low cholesterol intake (n=15) or atorvastatin (40 mg/d; n=15). Laboratory variables of platelet activation such as platelet recruitment, platelet isoprostanes, and thromboxane A2 were determined at baseline and after 2, 24, and 72 hours and 7 days of follow-up. A significant reduction of platelet recruitment and platelet isoprostanes was observed as early as 2 hours after atorvastatin administration. This change was coincident with downregulation of platelet and systemic oxidative stress, including Nox2 activation and urinary isoprostanes, suggesting that atorvastatin exerts a direct antiplatelet effect that is mediated by Nox2 downregulation and eventually platelet isoprostane lowering. Platelet thromboxane A2 was inhibited after 24 hours with a further decline up to 7 days of follow-up; this late change was associated with cholesterol lowering. An in vitro study supported a direct effect of atorvastatin on intraplatelet signaling, showing that it dose-dependently inhibited platelet Nox2 and phospholipase A2 activation, along with inhibition of platelet recruitment, platelet isoprostanes, and thromboxane A2, and increased vasodilator-stimulated phosphoprotein and nitric oxide. Together, these data show that atorvastatin exerts a direct antiplatelet effect and suggest its potential usefulness as an antiplatelet drug in patients at risk of thrombosis.
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