Immediate Antioxidant and Antiplatelet Effect of Atorvastatin

via Inhibition of NOX2

Running title: Pignatelli et al.; Antioxidant and antiplatelet effect by atorvastin

Pasquale Pignatelli, MD, PhD; Roberto Carnevale, PhD; Daniele Pastori, MD;
Roberto Cangemi, MD, PhD; Laura Napoleone, MD; Simona Bartimoccia, PhD;
Cristina Nocella, PhD; Stefania Basili, MD; Francesco Violi, MD

I Clinica Medica, Sapienza, University of Rome, Italy

Correspondence:
Francesco Violi, MD
I Clinica Medica, Sapienza
University of Rome
Viale del Policlinico 155
Roma 00161, Italy
Tel: +39064461933
Fax: +390649970103
E-mail: francesco.violi@uniroma1.it

Journal Subject Codes: [118] Cardiovascular Pharmacology; [91] Oxidant stress; [92] Platelets; [186] Platelet function inhibitors
Abstract:

**Background**—Statins exert an antithrombotic effect in patients at risk of or with acute thrombosis; it has never been investigated if such effect is immediate and if there is an underline mechanism.

**Methods and Results**—Patients with hypercholesterolemia were randomly allocated to a Mediterranean diet with low cholesterol intake (<300 mg/day; n=15) or atorvastatin (40 mg/day; n=15). Oxidative stress, as assessed by serum NOX2 and urinary isoprostanes, and platelet activation, as assessed by platelet recruitment (PR), platelet isoprostanes and thromboxane (Tx) A₂, platelet NOX2, Rac1, p47phox, PKC, VASP, nitric oxide (NO) and PLA₂, were determined at baseline and after 2, 24, 72 hours and 7 days of follow-up. In vitro study was also performed to see if atorvastatin affects platelet oxidative stress and activation. Atorvastatin-assigned group showed a significant and progressive reduction of urinary isoprostanes and serum NOX2 along with inhibition of PR, platelet isoprostanes, NOX2, Rac1, p47phox and PKC starting 2 hours from administration. Platelet PLA₂ and TxA₂ significantly decreased while VASP and NO increased after 24 hours. LDL-cholesterol significantly decreased 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 PLA₂ activation along with inhibition of PR, platelet isoprostanes and TxA₂ while increased VASP and NO.

**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 TxA₂. These findings provide a rationale for the use of statins to prevent or modulate coronary thrombosis.

**Clinical Trial Registration Information**—clinicaltrials.gov; Identifier: NCT01322711.

**Key words:** antioxidants; hypercholesterolemia; platelets; statins; thromboxane
Introduction

Statins are lipid lowering drugs that lower serum cholesterol via inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. Interventional trials with statins demonstrated a significant reduction of cardiovascular events in patients with both high and average serum cholesterol. Moreover, statins were shown to reduce cardiovascular events in patients with acute coronary syndrome suggesting that they could also exert an antithrombotic effect. A recent meta-analysis reinforced the concept that statins may possess antithrombotic property as these drugs were reported to reduce peri-procedural infarction in patients undergoing PCI. 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 that supposed from pharmacologic studies. An inhibition of platelet activation and thromboxane (Tx) synthesis has been detected 2-3 days after statin treatment, but no study has been done so far to see if statins possess a rapid antiplatelet effect, i.e. within 24 hours from administration. Also of relevance is the fact that it is not fully defined if the antiplatelet property of statins is a pleiotropic effect and/or is dependent on LDL lowering. Addressing these issues may be of potential relevance in order to provide further mechanistic support to the early use of statins in clinical settings associated with platelet-related uneventful cardiovascular events.

There is a growing body of evidence of mechanistic link between oxidant stress and platelet activation. Experimental study in animals provided support to such interplay showing that animals knock-out of Glutathione Peroxidase, an enzyme with antioxidant property, are prone to thrombosis via a mechanism involving platelet over-activation. Agonist-stimulated platelets release reactive oxidant species (ROS), that are implicated in the propagation of platelet activation by inactivating nitric oxide, releasing platelet agonists such as ADP or giving
formation of isoprostanes. Isoprostanes are a family of eicosanoids, that, differently from TxA₂, are chemically stable and greatly contribute to propagate platelet aggregation via activation of the glycoprotein IIb/IIIa. Experimental and clinical studies consistently showed that statins possess antioxidant activity that could eventually result in lowering platelet activation. Based on this, we tested the hypothesis that statins may directly inhibit platelet activation via reduction of ROS generation and eventually platelet isoprostanes independently from its cholesterol lowering effect. Therefore we performed a proof-of-concept study to analyze if atorvastatin exerts an early inhibitory effect on oxidative stress and platelet activation immediately after its administration. Also, we performed an in vitro study to see the interplay between atorvastatin and platelet oxidative stress and activation.

Materials and Methods

Study design

From December 2010 to June 2011, we studied 30 patients with polygenic hypercholesterolemia (patients, LDL-C>160 mg/dL), who signed a written informed consent to participate to the interventional study. Subjects were excluded if they had liver insufficiency, serious renal disorders (serum creatinine >2.5 mg/dL), diabetes mellitus, history of alcohol or drug abuse, arterial hypertension, a history or evidence of atherothrombotic diseases, cardiac arrhythmias, congestive heart failure, any autoimmune diseases, cancer or recent infections. Patients on treatment with non-steroidal anti-inflammatory drugs, drugs interfering with cholesterol metabolism, antioxidant supplements or antiplatelet drugs in the previous 30 days were also excluded from the study.

Patients were recruited from the same geographic area, were of white race and
followed a typical Mediterranean diet. During the study, all participants followed a low-fat diet with mean macronutrient profiles that were close to the Adult Treatment Panel (ATP) III guidelines (7% energy from saturated fat and 200 mg dietary cholesterol per day)\textsuperscript{11}.

Patients were randomized to a Mediterranean diet alone (Group A, N=15) or Mediterranean diet plus atorvastatin 40 mg/day (Group B, N=15) for 7 days. Blood and urine samples were collected at the baseline and after 2, 24, 72 and 168 hours of follow-up.

The study protocol was approved by the Sapienza University institutional review board and was in accordance to the declaration of Helsinki (ClinicalTrials.gov Identifier: NCT01322711).

**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 (PRP), samples were centrifuged for 15 minutes at 180g. To avoid leukocyte contamination, only the top 75% of the PRP was collected. Platelet pellets were suspended in HEPES buffer, pH 7.4 (2x10\textsuperscript{5} platelets/mL, unless otherwise noted). In some experiments samples were treated with Atorvastatin (1-10 \textmu M) (10 min 37 C\textdegree) or control before activation with the agonist.

**Platelet sNOX2-dp, TxA\textsubscript{2} and 8-iso-PGF2a-IIIa**

Platelet suspension was activated with arachidonic acid (AA) (0.5 mM) and the supernatant was stored at -80 C\textdegree.

Extracellular levels of soluble NOX2-derived peptide (sNOX2-dp), a marker of NADPH oxidase activation, were detected by ELISA method as previously described by
Pignatelli et al. The peptide was recognized by the specific monoclonal antibody against the amino acidic sequence (224-268) of the extra membrane portion of NOX2 (catalytic core of NADPH oxidase), which was released in the medium upon platelet activation. Values were expressed as pg/ml; intra-assay and inter-assay coefficients of variation were 5.2% and 6%, respectively.

Platelet TxB2 was measured by ELISA method (Amersham Pharmacia, Biotech, Little Chalfont, UK) and expressed as pmol/L. Intra- and inter-assay coefficients of variation were 4.0% and 3.6%, respectively.

Platelet isoprostane (8-iso-PGF2α-III) was measured by EIA assay method (Amersham Pharmacia, Biotech, Little Chalfont, UK) and expressed as pmol/L. Intra- and inter-assay coefficients of variation were 5.8% and 5.0%, respectively.

**Platelet nitric oxide metabolites nitrite and nitrate (NOx) 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 mM)-stimulated platelets. Intra-assay and inter-assay coefficients of variation were 2.9% and 1.7% respectively.

**Platelet recruitment**

Platelet recruitment was performed with a method modified from that described by Krotz et al. Collagen (2μg/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 five minutes and expressed as a percentage of the aggregation that had been initially reached, according to Pignatelli et al.

**Flow cytometry analysis of Platelet PKC, Rac 1 and PLA2 phosphorylation**
Platelet PKC phosphorylation was analyzed by flow cytometry and expressed as mean fluorescence as previously reported by Carnevale et al. Platelet Rac1 and PLA2 phosphorylation in resting or AA- (0.5 mM) stimulated washed platelets was analyzed by flow cytometry using specific antibodies (anti-Rac1 IgG goat polyclonal, monoclonal anti-phosphorylated cPLA2 (p-c PLA2)). In all assays, an irrelevant isotype matched antibody was used as a negative control. Antibodies (1 μg/mL) were added to 200 μL of platelet suspension (2x10^5), previously fixed with (2%) paraformaldehyde (0.1% BSA) for 60 min at room temperature and then permeabilized with digitonin (100 mmol/L) for 30 min at room temperature. Unbound antibody was removed by adding 0.1% BSA PBS and subsequent centrifugation at 3000 g for 3 min (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, p47^phox and VASP phosphorylation**

Platelets (2x10^8/ml in HEPES buffer) were activated with AA (0.5 mM), then washed and suspended in a 2X Lysis buffer (5 mM EDTA, 0.15 mol NaCl, 0.1 mol Tris pH 8.0, 1% triton and protease inhibitor cocktail). Equal amounts of protein (30 μg/lane) estimated by Bradford assay were solubilized in a 2X Laemml buffer containing 2-mercaptoethanol and loaded in a denaturing SDS/10% polyacrylamide gel. Western blot analysis was performed with monoclonal anti-phosphorylated cPLA2 (p-c PLA2) (1 μg/mL) or anti-Rac1 (2 μg/mL) or anti-PKC (2 μg/mL) or...
anti-p47\textsuperscript{phox} (2μg/ml) or anti-VASP-P (2μg/ml) incubated overnight at 4°C. Immune complexes were detected by enhanced chemiluminescence. The phosphorylation rate of Rac1, PKC, p47\textsuperscript{phox}, VASP and p-c PLA\textsubscript{2} was analyzed by autoradiography. The developed spots were calculated by densitometric analysis on a NIHimage 1.62f analyzer and the value was expressed in arbitrary unit.

**Flow cytometry analysis of gpIIb/IIIa**

PAC 1 is an antibody that recognizes an epitope on the glycoprotein IIb/IIIa (gpIIb/IIIa) of activated platelets, at/or near the platelet fibrinogen receptor. PAC1 binding on AA (0.5 mM)-activated platelets membrane was analyzed using the specific FITC-labeled monoclonal antibodies anti PAC1 (Mab) (BD International) as previously reported\textsuperscript{15}. 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, FL, USA) equipped with an argon laser at 488 nM. For every histogram, 5x10\textsuperscript{5} 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 M et al\textsuperscript{16}. Briefly, CD61PE (specific monoclonal antibody to platelet GPIIIa, Pharmingen, CA, USA) 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 15sec. in the platelet population identified in the gate FS log/SS log as CD61-positive events (CD61-PE). Platelet stimulation with thrombin (0.1U/mL) was used as positive control. Changes in Fluorescence was evaluated
in arbitrary units as fold increase in calcium mobilization. Samples were incubated 30 min. at
37°C with or without atorvastatin (1 and 10µM). For every histogram, 50,000 platelets/events
were counted to determine the proportion of positive platelets.

**Serum NOX2**

Serum levels of soluble NOX2-derived peptide (sNOX2-dp) were detected by ELISA as
previously described by Pignatelli et al\(^\text{12}\). Blood samples were kept for 60 min at 37°C and
centrifuged at 300 g; serum was stored at -80°C. Values were expressed as pg/ml; intra-assay
and inter-assay coefficients of variation were 5.2% and 6%, respectively.

**Urinary 8-iso-PGF2α-III assays**

Urinary 8-iso-PGF2α-III was measured by EIA assay method. Urinary 8-iso-PGF2α-III
concentration was corrected for recovery and creatinine excretion and expressed as pg/mg of
creatinine. Intra-assay and inter-assay coefficients of variation were 2.1% and 4.5%
respectively.

**Sample preparation and atorvastatin evaluation in HPLC**

The stock solution of atorvastatin was prepared by dissolving appropriate amount corresponding
to 0.1mg/ml concentration of working standard in methanol. The stock solution of atorvastatin
was further diluted with the mobil phase methanol-water (68:32, v/v; pH adjusted to 3.0 with
trifluoroacetic acid) to give a series of standards having a final concentration in the range of
0.01µM-2µM.

A simple two step liquid-liquid extraction procedure was carried out for the extraction of
atorvastatin from serum samples. A volume (50µl) of internal standard (atorvastatin 400ng/ml)
was added to 200µl of serum and mixed for ten seconds. Then 600µl of ethanol was added and
vortex-mixed for two minutes for deproteination. In step one, 1.0ml of diethyl ether (extraction
solvent one) was added, vortex-mixed for five minutes and centrifuged at 3500rpm at 0°C for five minutes. The supernatant organic layer was separated in a test tube.

In step two 0.5 ml of dichloromethane (extraction solvent two) was added, vortexed for five minutes followed by centrifugation (3500 rpm) at 0°C for five 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 500μl of mobile phase, vortex-mixed for two minutes and 20μl sample was injected into HPLC 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 50μl. The column oven temperature was of 25°C and the eluate was monitored using UV detection at wavelength of 241 nm.

**Statistical analysis**

The minimum sample size was computed with respect to a two-tailed Student’s t-test considering (i) relevant difference in platelet isoprostanes levels to be detected between treatment groups \( \delta \geq 30 \text{ pmol/L} \), (ii) standard deviations homogeneous between the groups, SD=25 pmol/L, and (iii) type-I error probability \( \alpha=0.05 \) and power \( 1-\beta=0.90 \). 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 chi-square test or Fisher exact test. Student unpaired t test and Pearson’s correlation analysis were used for normally distributed continuous variables. Appropriate nonparametric tests [Mann Whitney U test or Spearman Rank correlation Test] were employed for all the other variables. Interventional study data were analysed for the assessment of treatment effect on platelet recruitment, platelet and urinary isoprostanes, platelet and serum sNOX2-dp and platelet TxB2
performing a analysis of variance for repeated measurements with one between subject factor (treatment group) and one 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, BMI, blood pressure and blood lipid profile between the two groups (the one allocated to diet and atorvastatin and the other allocated to diet alone). Pairwise comparisons were performed using Bonferroni's correction. Probability values <0.05 were regarded as statistically significant. All calculations were made with Statistica 7 software for Windows (StatSoft, Tulsa, USA). Probability values <0.05 were regarded as statistically significant. All calculations were made with Statistica 7 software for Windows (StatSoft, Tulsa, USA).

Results

Interventional study

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 daily, Group B) had similar clinical and anthropometric characteristics (Table 1). Platelet (263.000±54.000 mm³ vs 236.000±49.000 mm³), red cell (5.1±0.41-10⁶/μl vs 5.3±0.38-10⁶/μl) and white cell (7524±1201 mm³ vs 7631±1196 mm³) counts were similar in the two groups. Lipid profile, urinary isoprostanes, serum sNOX2-dp, platelet recruitment and platelet isoprostanes, TxB2, NOX2, p47phox, Rac1, PKC, PLA2, VASP, NOx and serum atorvastatin did not differentiate the two groups (Table 1 and Figures 2-5).

Analyses of variance for repeated measurements showed significant time-dependent differences between the two treatment groups for platelet recruitment (F=4.9; p=0.0011),
platelet (F=5.1; p=0.00084) and urinary isoprostanes (F=2.9; p=0.0253), platelet TxB\(_2\) (F=4.1; p=0.0038), platelet (F=2.4; p<0.05) and serum sNOX2-dp (F=2.5; p=0.04562), 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\(^{phox}\), Rac1, PKC, VASP, PLA\(_2\), 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 after 2 hours from atorvastatin administration and further declined after 24 hours reaching a plateau after 72 hours (Figure 2); there was a parallel decrease of the two 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 after 2 hours from atorvastatin administration with a further reduction after till 72 hours (Figure 3).

Coincidentally with platelet NOX2 activation we observed impaired phosphorylation of platelet p47\(^{phox}\) (-28%, p<0.001), Rac1 (-28%, p<0.001) and PKC (-27%, p<0.001) 2 hours from atorvastatin administration with a further reduction up to 72 hours (Figure 4).
Conversely, platelet TxB$_2$ and PLA$_2$ phosphorylation did not change after 2 hours, significantly lowered after 24 hours and continued to decline till 7 days from atorvastatin administration (Figure 3 panel B and Figure 5 panel B). Also, platelet VASP phosphorylation and platelet NOx, did not change after 2 hours, significantly enhanced after 24 hours and continued to increase concomitantly thereafter (r=0.63, p=0.011). (Figure 5 panel A and C).

To explore the relationship among time-dependent reduction of TBX$_2$, LDL-cholesterol and PLA$_2$ phosphorylation, analyses of variance were repeated to assess the effect of treatment from 2 hours to 1, 3 and 7 days. Significant time-dependent differences between the two treatment groups were shown for platelet TxB$_2$ (p=0.02375), PLA$_2$ phosphorylation (p=0.00203) and LDL-cholesterol (p=0.0287). Time-related changes between LDL-cholesterol and TxB$_2$ (R=0.505 p<0.001) and PLA$_2$ phosphorylation (R=0.25 p<0.03) significantly correlated. A significant correlation was also observed between time related changes of PLA$_2$ and TxB$_2$ (R=0.26 p=0.025).

Serum atorvastatin significantly increased as early as after two hours from the first administration of the drug in group B reaching a plateau at 24 hours (0.104±0.03 μM at 2h vs 0.178±0.02 μM at 24h) and was unchanged in group A (Figure 5 panel D).

**In vitro study**

In order to investigate if 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**

Isoprostanes formation and NOX2 activation were significantly and dose-dependently
inhibited by atorvastatin (Figure 6 panels A and B). To analyse the pathway involved in atorvastatin-dependent NOX2 inhibition we studied PKC phosphorylation, an up-stream signaling for activation of Rac1, a key unit for NADPH oxidase activation\(^{18}\); atorvastatin (1-10\(\mu\)M) dose-dependently decreased AA-induced PKC (Figure 6 panel C) and Rac1 phosphorylation (Figure 6 panel D).

**Effect of atorvastatin on platelet thromboxane**

PLA\(_2\) 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\(\mu\)M) dose-dependently decreased AA-induced PLA\(_2\) phosphorylation and TxB\(_2\) (Figure 7 panels A and B). VASP phosphorylation is associated with NO formation\(^{20}\); atorvastatin (1-10\(\mu\)M) dose-dependently increased VASP phosphorylation and NOx formation (Figure 7 panels C and D).

**Effect of atorvastatin on platelet recruitment**

Incubation of platelets with atorvastatin (1-10\(\mu\)M) showed a dose-dependent decrease of platelet recruitment (Figure 8 panel A).

Calcium mobilization and GpIIb/IIIa activation are two fundamental steps for isoprostane- and TxB\(_2\)-mediated platelet recruitment. We found that atorvastatin dose-dependently inhibited calcium mobilization and GpIIb/IIIa activation (Figure 8 panels B and C).

**Discussion**

The study provides the first evidence that atorvastatin exerts an antioxidant effect as early as 2 hours after its administration via inhibition of NOX2. A simultaneous antiplatelet effect
was also observed including inhibition of platelet isoprostane and TxB₂ so suggesting an interplay between oxidative stress and platelet activation inhibition. This hypothesis was corroborated by 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 down-regulation of NOX2 and up-regulation of NO synthase coupling²¹,²². Reduction of urinary isoprostanes or increase of circulating vitamin E have been observed after short (3 days)- or long-term treatment with statins²³-²⁵. In the present study, using two markers of oxidative stress, i.e. 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 isoprostanes⁸. Oxidative stress lowering by atorvastatin was not associated with changes of 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 administration²⁶, inhibited in vitro NOX2 activation with a mechanism involving PKC-induced p47phansox and Rac1 phosphorylation¹⁴,²⁷. 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 administration²².

**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 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 from 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. after 2 hours from statin’s administration. Here we report that atorvastatin exerts an immediate antiplatelet effect, as assessed by platelet recruitment, that is independent from 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 from statin’s administration. Such effect paralleled platelet down-regulation 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 while the early reduction of TxB₂ was also independent from cholesterol lowering, later TxB₂ reduction was coincidental 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 late antiplatelet effect which is independent from its lipid lowering property and is related to two different intraplatelet signaling. The early effect (2 hours) involves NADPH oxidase, the late effect involves PLA₂. Thus, the early effect was related to NADPH oxidase down-regulation via reduced phosphorylation of rac1 and p47phox and resulted in platelet isoprostane formation reduction while TxB₂ lowering was related to PLA₂ inhibition. In vitro study was in agreement with this hypothesis as 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 isoprostanes and ultimately platelet activation as indicated by the reduced platelet calcium mobilization and GPIIb/IIIa activation elicited by platelet incubation with atorvastatin.

The antioxidant effect of atorvastatin could also be implicated in PLA₂ down-regulation as ROS exert a pro-aggregatory activity by releasing AA from platelet membrane via platelet PLA₂ activation²⁹. Accordingly, platelet incubation with atorvastatin down-regulated PLA₂ activation so providing a mechanistic insight into the in vitro inhibition of platelet TxB₂, which was detected in the present and previous studies⁵.

Finally, the inhibition of ROS could also explain the increase of platelet NOₓ observed 24 hours form atorvastatin administration. This is in accordance with previous studies showing that antioxidants enhance VASP phosphorylation and NO biosynthesis³⁰,³¹.

The study has pharmacologic implication as 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 that aspirin does not affect platelet isoprostanes³². Combination of statins with aspirin could provide a deeper inhibition of platelet function via down-regulation 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 as patients undergoing PCI. 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, one 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 if the antiplatelet effect of atorvastatin is comparable with that of other antiplatelet drugs. Analysis of platelet TxB₂ could help to make a comparison as the inhibition of platelet TxB₂ seven days after atorvastatin treatment was about 50%, which is less than that achieved by aspirin (<95%) \(^\text{33}\). Of note, however, aspirin does not affect platelet isoprostanes \(^\text{33}\), while atorvastatin reduced it by about 50% (see Figure 3).

We acknowledge the potential weakness of an open design of the study; however randomization as well as blind analysis of laboratory variables were likely to limit this bias.

Conclusions

The study shows an immediate antioxidant and antiplatelet effect of atorvastatin in patients with hypercholesterolemia. Such effect occurs via inhibition of NOX2 activation and results in down-regulation of platelet isoprostane and TxB₂. Further study is necessary to see if similar effect can be achieved in patients with acute coronary syndrome and in those undergoing PCI.

Funding Sources: Study support: Ateneo Federato 2009 to FV, Sapienza, University of Rome.

Conflict of Interest Disclosures: FV received fee for advisory board from Pfizer, BMS and Bayer.

References:


Table 1. Baseline characteristics of Hypercholesterolemic Patients and Controls.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group A (n=15)</th>
<th>P values**</th>
<th>Group B (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>67.5±14.4</td>
<td>0.434</td>
<td>63.4±13</td>
</tr>
<tr>
<td>Males n (%)</td>
<td>7 (47)</td>
<td>0.948</td>
<td>7 (47)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.8±5.8</td>
<td>0.955</td>
<td>26.7±3.1</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>124±21</td>
<td>0.510</td>
<td>128±11</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>76±12</td>
<td>0.415</td>
<td>80±9</td>
</tr>
<tr>
<td>Smokers n (%)</td>
<td>6 (40)</td>
<td>0.320</td>
<td>5 (33)</td>
</tr>
<tr>
<td>Total-Ch (mg/dl)</td>
<td>231±28</td>
<td>0.956</td>
<td>232±19</td>
</tr>
<tr>
<td>LDL-Ch (mg/dl)</td>
<td>161±25</td>
<td>0.657</td>
<td>165±25</td>
</tr>
<tr>
<td>HDL-Ch (mg/dl)</td>
<td>60±13</td>
<td>0.143</td>
<td>49±17</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>99±30</td>
<td>0.108</td>
<td>112±30</td>
</tr>
<tr>
<td>Fasting glycaemia (mg/dl)</td>
<td>94±14</td>
<td>0.946</td>
<td>94±9</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.8±0.2</td>
<td>0.746</td>
<td>0.8±0.1</td>
</tr>
</tbody>
</table>

Group A= hypercholesterolemic patients assigned to Diet alone; Group B= hypercholesterolemic patients assigned to diet plus atorvastatin (40 mg daily). Data are expressed as mean+/−SD or as counts (n) and percentages (%). Ch=Cholesterol; BP= blood pressure. *, P value for comparison between group A and group B.

Figure Legends:

Figure 1. Study design

Figure 2. Interventional study: Urinary isoprostanes (panel A), serum sNOX2-dp (panel B) and serum LDL cholesterol (panel C) in diet (group A)- and Atorvastatin- assigned (group B) subjects. *p<0.005.

Figure 3. Interventional study: platelet isoprostanes (panel A), platelet TxB2 (panel B),
platelet sNOX2-dp (panel C) and platelet recruitment (panel D), in diet (group A-) and Atorvastatin-assigned (group B) subjects. *p<0.005.

**Figure 4.** Interventional study: phosphorylation analysis in platelet p47\textsuperscript{phox} (panel A), Rac1 (panel B) and PKC (panel C), in diet (group A-) and Atorvastatin-assigned (group B) subjects. *p<0.005. In each blots were reported the β-actin controls.

**Figure 5.** Interventional study: platelet VASP (panel A) and PLA\textsubscript{2} (panel B) phosphorylation and platelet NOx formation (panel C) in diet (group A-) and Atorvastatin-assigned (group B) subjects. Serum levels of atorvastatin (panel D) in Atorvastatin-assigned (group B) subjects. **p<0.005, *p<0.001. In each blots were reported the β-actin controls.

**Figure 6.** In vitro study: platelet isoprostanes (panel A), platelet sNOX2-dp (panel B), platelet PKC phosphorylation (panel C) and platelet Rac1 (panel D), in AA- stimulated samples treated or not with atorvastatin (1-10μM). Experiments were led on 6 subjects. *p<0.001.

**Figure 7.** In vitro study: platelet PLA\textsubscript{2} phosphorylation (panel A), platelet TxB\textsubscript{2} production (panel B), platelet VASP phosphorylation (panel C) and platelet NOx formation (panel D) in AA- stimulated samples treated or not with atorvastatin (1-10μM). Experiments were led on 6 subjects. *p<0.001.

**Figure 8.** In vitro study: platelet recruitment (panel A), platelet Ca\textsuperscript{2+} mobilization (panel B) and platelet IIb/IIIa activation (panel C), in AA- stimulated samples treated or not with atorvastatin (1-10μM). Experiments were led on 6 subjects. *p<0.001.
Study design

Diet

Hypercholesterolemic patients (n=30)

N=15

Diet + Atorvastatin 40 mg/day

Hours 2 24 72 168

blood and urine sampling
Immediate Antioxidant and Antiplatelet Effect of Atorvastatin via Inhibition of NOX2
Pasquale Pignatelli, Roberto Carnevale, Daniele Pastori, Roberto Cangemi, Laura Napoleone, Simona Bartimoccia, Cristina Nocella, Stefania Basili and Francesco Violi

Circulation. published online May 21, 2012;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2012/05/21/CIRCULATIONAHA.112.095554

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/