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CLINICAL RESEARCH

Early decrease of oxidative stress by atorvastatin in hypercholesterolaemic patients: effect on circulating vitamin E

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Aims Statins inhibit oxidative stress, but the interplay between cholesterol lowering and antioxidant vitamins is still unclear. Aims of the study were to assess if statins inhibit oxidative stress independently from cholesterol lowering, to assess the behaviour of vitamin E simultaneously with the changes of oxidative stress, to determine *in vitro* if atorvastatin was able to directly influence platelet-mediated LDL oxidation and vitamin E consumption.

Methods and results In 30 hypercholesterolaemic patients (HC) and 20 healthy subjects (HS), urinary isoprostanes and plasma vitamin E were determined. The HC were randomized to diet or diet plus atorvastatin 10 mg/day. Compared with HS, HC had higher isoprostanes and lower vitamin E levels. The statin-allocated group showed a reduction of isoprostanes after only 3 days (-18.8%, $P < 0.01$); after 30 days, a stronger reduction of isoprostanes was noted (-37.1%, $P < 0.01$) whereas an increase of vitamin E ($+42\%$, $P < 0.01$) and a reduction of cholesterol (-24.9%, $P < 0.01$) were observed. The diet-allocated group showed a weak decrease of cholesterol after 30 days. *In vitro* study showed that atorvastatin dose-dependently inhibited platelet-mediated LDL oxidation and isoprostane formation with a mechanism involving NADPH-oxidase.

Conclusion The study provides the first evidence that atorvastatin exerts an early antioxidant effect that could contribute to enhancing circulating vitamin E.

Keywords Statins • Oxidative stress • Vitamin E • Hypercholesterolaemia

Introduction

Statins reduce cardiovascular events in patients with average and high cholesterol levels.[1–3] Such effect is attributed to statins principal mechanism of action, i.e. cholesterol lowering property. However, other so-called pleiotropic effects have been taken into account as components of statins antiatherosclerotic effect.[4] Among such pleiotropic effects, inhibition of oxidative stress is believed to play an important role, as oxidative stress is implicated in initiation and progression of atherosclerotic disease.[5,6] Statins have been shown to reduce several markers of oxidative stress, such as nitrotyrosine and isoprostanes,[7,8] in patients with hypercholesterolaemia, but it is still unclear if such effect is related to the cholesterol lowering action or to pleiotropic effects involving specific oxidant species-generating pathways. This issue is of relevance taking into account that an experimental study on an animal model of atherosclerosis showed that cholesterol lowering *per se* is a major determinant of oxidative stress inhibition and endothelial dysfunction amelioration *in vivo*.[9]

Another issue that deserves careful investigation is if statins influence antioxidant vitamins. Antioxidant vitamins, such as vitamin E, may positively counteract atherosclerotic lesion as shown by the fact that, in apoE$^{-/-}$ mice,[10] the higher its circulating levels are the lower the atherosclerotic progression is. An *in vitro* study showed that vitamin E is consumed in the presence of enhanced oxidative stress;[11] therefore, one would expect that

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statin increases vitamin E in the human body as a consequence of its antioxidant effect. Although previous human studies provided equivocal results,[12–14] more recent data seem to suggest that the statins enhance antioxidant vitamins.[15,16] However, it is still unclear if such effect is related to the inhibition of oxidative stress.

On the basis of these data, the first aim of the study was to assess if statins inhibit oxidative stress independently from cholesterol lowering. For this purpose, oxidative stress was measured as early as 3 days after atorvastatin treatment (that is not associated with changes of lipid profile)[17] in patients with hypercholesterolaemia. The second aim was to assess the behaviour of vitamin E simultaneously with the changes of oxidative stress. The third aim was to determine *in vitro* if atorvastatin was able to directly influence platelet-mediated LDL oxidation and vitamin E consumption (in a milieu) requiring activation of NADPH oxidase.[11]

Methods

The study has been divided into two parts. In the first part, we performed a cross-sectional study comparing urinary isoprostanes, serum cholesterol, and plasma vitamin E/cholesterol ratio in a population of hypercholesterolaemic (HC) patients and healthy subjects.

In the second part, we performed an interventional trial in patients to assess if diet alone or diet plus atorvastatin were able to influence plasma vitamin E/cholesterol ratio, LDL oxidation, and urinary isoprostanes concentration.

Cross-sectional study

In this study, we compared 30 consecutive HC patients and 20 healthy subjects, presenting for metabolic screenings in the ambulatory of our Division between September 2005 and December 2005. Both patients and controls were recruited from the same geographic area and they were all Caucasians.

All subjects underwent a full medical history, physical examination, 12-lead ECG, and echocardiography. Subjects were excluded from the study if they had liver insufficiency, serious renal disorders (serum creatinine > 2.8 mg/dL), myocardial infarction, unstable angina, coronary revascularization, clinical history of cardiovascular disease, peripheral vascular surgery or percutaneous intervention procedure, acute cerebrovascular disease, deep venous thrombosis, or were in treatment with statins or antioxidant vitamins.

Body mass index (BMI) was calculated as weight (kg) divided by height^2 (m^2).

Written informed consent was obtained from all subjects: the study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee.

The number of patients initially assessed for inclusion into the study was 71 (*Figure 1*); after initial assessments, 36 patients were excluded from the study because they were treated with statins, one patient for serum creatinine >2.8 mg/dL, and four patients for coronary heart disease. All the patients eligible for the study gave their consent.

Interventional study with atorvastatin

Thirty HC patients participating to the cross-sectional study were openly randomized to a treatment with diet or diet plus atorvastatin (10 mg/day). Serum cholesterol, urinary isoprostanes, and plasma vitamin E/cholesterol ratio were measured at baseline and after 3 and 30 days. During the study, the participants followed low-fat diets with mean macronutrient profiles that were close to the current ATP III guidelines (7% energy from saturated fat and <200 mg dietary cholesterol/day).[18]

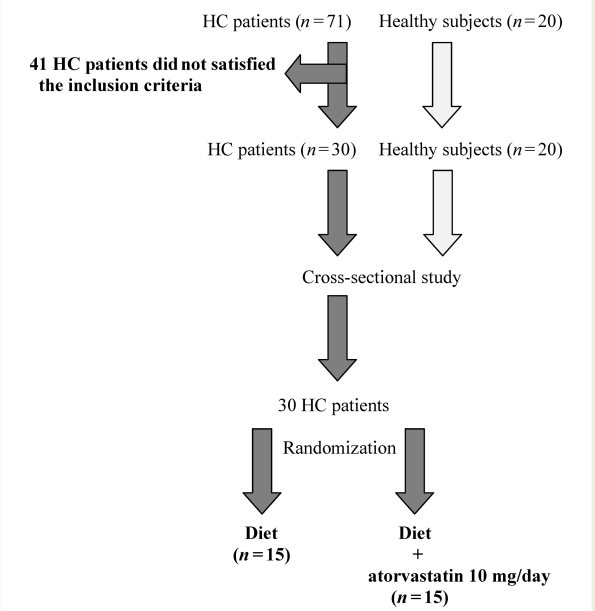

Figure 1 Patient flow diagram of the study

Randomization and blinding

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 unblinding of the treatment allocation only after the study had ended and laboratory analyses were completed.

Lipid profile

Fasting serum levels of total cholesterol and triglycerides were determined with enzyme-based methods. HDL cholesterol was measured after phosphotungstic acid/$MgCl_2$ precipitation of fresh plasma. LDL cholesterol was calculated according to the Friedewald formula.

Eicosanoid assays

Urinary 8-iso prostaglandin F2α, (PGF2α-III) was measured by previously described and validated EIA assay method.[19] Ten millilitre urine aliquots were extracted on a C-18 SPE column; the purification was tested for recovery by adding a radioactive tracer (tritiated PGF2α-III) (Cayman chemical). The eluates 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 picagramme per milligramme of creatinine.

Analysis of vitamin E

Blood samples were taken into tubes containing EDTA and centrifuged at 3000 r.p.m. for 15 min to obtain plasma, which was stored at -80°C.

Plasma concentration of vitamin E (α-tocopherol-alcohol) was measured by HPLC[20] using tocopheryl acetate as internal standard. Reagents included HPLC-grade ethanol, methanol and hexane (E. Merck, Darmstad, Germany), and tocopheryl acetate (Sigma Chemical, St Louis, MO, USA). A flow rate of 2.0 mL/min was used with a LC/233 Diodine Array Detector (Restek Corporation, PA, USA) set at 0.02–0.1 attenuation. Levels were expressed as micromole vitamin E per millimole serum cholesterol.[21]

oxLDL assays

Plasma concentration of oxLDL was measured by a commercially available competitive sandwich ELISA (Mercodia; interassay coefficient of variation, 15.6%) with the same specific murine monoclonal antibody, mAb-4E6, as in the assay described by Holvoet *et al.*[22] It has been shown that oxLDL remains stable in stored samples and that the aforementioned assay has good reproducibility.[23] Values are expressed as Units per millilitre (U/mL).

In vitro experiments

Sample preparation

We have previously shown that agonist-stimulated platelets produce reactive oxidant species (ROS) that elicit LDL oxidation via activation of NADPH oxidase.[11] In this experimental model, we analysed if atorvastatin was able to affect platelet-mediated LDL oxidation. Briefly, experiments were performed by incubating platelets (5×10^8/mL) taken from healthy subjects with or without LDL (50 μg protein/mL) at 37°C for 30 min as previously described.[11] Then, platelet samples were stimulated with collagen (6 μg/mL, 37C°, 10 min) in the presence or not of scalar concentration of atorvastatin (0.1–10 μM) or apocynin (100 μM), an inhibitor of NADPH oxidase.[24] The supernatant was treated to measure conjugated dienes production as a marker of LDL oxidation, vitamin E consumption, and isoprostane formation (as below reported). Platelet suspension was fixed with paraformaldehyde to evaluate p38 map-kinase phosphorylation (as below reported).

Collagen was selected as agonist because it elicits much more production of ROS than other agonists such as thrombin.[25]

Conjugated dienes

The standard oxidation assay was performed on sample supernatant using a Perkin Elmer Lambda 4B UV/VIS spectrometer.

The measurement of the 234 nm absorption was read at intervals of 2 min for a period of 2 h as previously described and expressed as micromoles of conjugated dienes formation.[23]

Vitamin E estimation

Sample supernatant was added with tocopheryl acetate (internal standard) and deproteinized by the addition of ethanol, and extracted with hexane. Phase separation was achieved by centrifugation. The collected upper phase was evaporated and analysed by HPLC[20] as above reported.

α-Tocopherol was expressed as μg/50 μg protein/mL of LDL.

Isoprostane formation

PGF2α-III content in the supernatant was measured by previously described and validated EIA assay method.[19] One millilitre of each sample was extracted on a C-18 SPE column; PGF2α-III evaluation was performed as previously reported. PGF2α-III concentration was expressed as picagramme per milligramme platelet protein.

Flow cytometry analysis of p38 MAP-kinase phosphorylation

p38MAP-kinase (p38MAPK) phosphorylation was analysed using the specific polyclonal antibodies anti p38MAPK (Mab) (Sigma Aldrich) followed by incubation with the secondary specific FITC-labelled anti-mouse IgG. All assays included samples to which an irrelevant primary isotype-matched polyclonal antibody was added.

Platelets were fixed with (2%) paraformaldehyde (0.1% BSA) for 60 min at room temperature; the suspension was treated with Mab (10 μL) for 60 min at room temperature. The unbound Mab was removed by centrifugation at 300 *g* for 3 min (twice) after the addition of phosphate buffer saline (0.1% bovine serum albumin). For primary non-FITC-labelled antibody, the incubation was followed by treatment with a secondary specific FITC-labelled antibody (30 min, room temperature).

Fluorescence intensity was analysed on an Epics XL-MCL Cytometer (Coulter Electronics, FL, USA) equipped with an argon laser at 488 nM. For every histogram, 50 000 platelets were counted to evaluate the percentage of positive platelets. Antibody reactivity is reported as mean fluorescence intensity.

Platelet NADPH oxidase activity

Measurement of platelets NADPH oxidase activity was performed in platelet homogenates according to Seno *et al.*[26] Washed platelets were suspended in a homogenate buffer containing 50 mM Tris/HCl (pH 7.4), 1.0 mM EDTA, 2.0 mM leupeptin, and 2.0 mM pepstatin A, incubated with or without LDL 30 min at 37°C and then homogenized. Platelet homogenates were then incubated 10 min at 37°C with 25 μM NADPH and added with or without atorvastatin (0.1–10 μM). The assay solution contained 400 μL Tyrode buffer and 5 μM lucigenin. After preincubation at 37°C for 3 min, the reaction was started by adding 100 μL of platelet homogenates in the presence or less of 0.5 mM arachidonic acid (AA) as a trigger of the enzymatic reaction.[27]

The chemiluminescent signal was expressed as counts per minute (c.p.m.) for an average of 10 min corrected by protein concentration (c.p.m./mg) and expressed as RLU (relative chemiluminescence units). Protein concentrations were determined by the method of Lowry *et al.*[28]

Statistical analysis

Sample size determination

As above reported, for the cross-sectional study we recruited all the patients ($n = 30$) attending the ambulatory of our Division between September 2005 and December 2005, who respected the inclusion/exclusion criteria. The number of controls ($n = 20$) was computed with respect to a two-tailed Student's *t*-test for independent groups, considering as (i) clinically relevant difference in vitamin E/total cholesterol levels to be detected between patients and controls $|\delta| \geq 1$ μmol/mmol, (ii) standard deviations homogeneous between the groups, SDs = 0.9 μmol/mmol, (iii) type-I error probability $\alpha = 0.05$ and power $1 - \beta = 0.90$; this resulted in $n = 19$/group.

As regards the interventional cross-over study, we computed the minimum sample size with respect to a two-tailed one-sample Student's *t*-test, considering as (i) clinically relevant difference in vitamin E/total cholesterol levels to be detected between diet plus atorvastatin and control treatment $|\delta| \geq 1$ μmol/mmol, (ii) standard deviation of the paired differences SD = 0.8 μmol/mmol, (iii) type-I error probability $\alpha = 0.05$ and power $1 - \beta = 0.90$; this resulted in $n = 15$/group. To account for the inflation of the experimentwise type-I error due to multiple testing, Bonferroni correction was used.

Table 1 Baseline characteristics of hypercholesterolaemic patients and healthy subjects

Variables	Hypercholesterolaemic patients ($n = 30$)	Healthy subjects ($n = 20$)	*P*-value
Age (years)[a]	52.5 ± 3.8	52 ± 3	0.277
BMI (kg/m^2)[a]	25.4 ± 2.5	25.7 ± 2.4	0.628
Males/females	16/14	10/10	0.954
Smokers	3	2	0.630
Total cholesterol (mg/dL)[a]	278 ± 39	187 ± 11	<0.001
LDL cholesterol (mg/dL)[a]	187 ± 13	98 ± 14	<0.001
HDL cholesterol (mg(dL)[a]	62 ± 11	50 ± 11	<0.001
Triglycerides (mg/dL)[a]	103 ± 21	73 ± 15	<0.001
Fasting blood glucose levels (mg/dL)[a]	84 ± 12	84 ± 12	0.961
Systolic blood pressure (mmHg)[a]	127 ± 12	125 ± 11	0.924
Diastolic blood pressure (mmHg)[a]	75 ± 9	75 ± 10	0.928
Vitamin E unadjusted (μmol/L)[a]	30.5 ± 8.3	26.2 ± 2.9	0.01
Vitamin E/total cholesterol (μmol/mmol)[a]	4.21 ± 0.90	5.43 ± 0.57	<0.001
Isoprostanes (pg/mg creatinine)[a]	366 ± 63	210 ± 38	<0.001

[a]Data are expressed as mean ± SD.

Statistical methods

Categorical variables are 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 healthy subjects were carried out by Student's *t*-test and were replicated as appropriate with non-parametric test [Kolmogorov–Smirnov (*z*) test] in the case of non-homogeneous variances as verified by Levene's test. The correlation analysis was done with Pearson's test. $P < 0.05$ was considered as statistically significant.

Interventional study data were analysed for the assessment of treatment effect on Vitamin E plasma levels, total cholesterol, OxLDL, and urinary isoprostanes performing a MANOVA with one between-subject factor (treatment group) and one within-subject factor (time at three levels: baseline, 3 days; 30 days after the beginning of the treatment).

As covariates, we considered the possible random differences in age, sex, BMI, systolic and diastolic blood pressure between the two groups (the one allocated to diet and atorvastatin and the other allocated to diet alone).

To assess the effect of the treatment on the above variables after only 3 days, the same analysis was performed using a two levels within-subject factor (time: baseline and 3 days after the beginning of treatment).

The statistical analysis was performed using the SPSS 13.0 software for Windows.

Results

Cross-sectional study

Clinical characteristics of HC patients and healthy subjects are reported in *Table 1*.

Compared with healthy subjects, HC patients had a lower plasma vitamin E/cholesterol ratio (*Table 1*); also, HC patients had enhanced oxidative stress, as documented by elevated urinary excretion of isoprostanes, compared with controls (*Table 1*). At bivariate analysis, plasma vitamin E/cholesterol ratio and isoprostanes were inversely correlated ($r = -0.43$, $P < 0.05$).

Interventional study

At baseline, patients randomized to diet alone (Group A) (eight males and seven females, mean age 52.2 ± 4.1 years) and those randomized to diet plus atorvastatin (10 mg/day, Group B) (eight males and seven females, mean age 52.8 ± 3.7 years) had similar values of total cholesterol, vitamin E/cholesterol ratio urinary isoprostanes, and oxLDL (*Table 2* and *Figure 2*).

From the MANOVA performed on interventional study data, we found a significant effect of the interaction between time × group, showing a significant effect of the different treatments on vitamin E plasma/cholesterol ratio [$F(1.36,29.9) = 47.6$, $P < 0.001$], urinary isoprostanes [$F(2,44) = 49.9$, $P < 0.001$], oxLDL [$F(2,44) = 17.4$, $P < 0.001$], and total cholesterol [$F(1.42,31.3) = 8.3$, $P < 0.005$]. On the contrary, we did not found any significant effect of time and its interaction with covariates, such as age, sex, BMI, smoke, diastolic and systolic blood pressure, on the above-mentioned variables.

The MANOVA performed for the assessment of the effect of treatment on the two groups after 3 days showed a significant effect of the interaction of time × group only on urinary isoprostanes [$F(1,22) = 28.2$, $P < 0.001$] and oxLDL [$F(1,22) = 4.6$, $P < 0.05$].

In particular, after 3 days, Group B showed a reduction of isoprostanes by 18.8% and of oxLDL by 31.9%; after 30 days, a stronger reduction of isoprostanes (−37.1%) and oxLDL (−58.9%) along with an increase of vitamin E/cholesterol ratio (+42%) and a reduction of cholesterol (−24.9%) were observed.

Group A showed a weak decrease of cholesterol after 30 days (−6.51%).

In Group B, before–after treatment changes (from baseline to day 30) in vitamin E/cholesterol ratio and isoprostanes

Table 2 Interventional study: baseline characteristics of hypercholesterolaemic patients randomized to diet alone (Group A) or diet plus atorvastatin (Group B)

Variables	Group A ($n = 15$)	Group B ($n = 15$)	*P*-value
Age (years)[a]	52.8 ± 3.7	52.2 ± 4.1	0.677
BMI (kg/m^2)[a]	25.1 ± 2.4	25.7 ± 2.6	0.502
Males/females	8/7	8/7	0.714
Smokers	1	2	1.000
Total cholesterol (mg/dL)[a]	280 ± 32	276 ± 46	0.796
Triglycerides (mg/dL)[a]	102 ± 19	103 ± 24	0.960
Fasting blood glucose levels (mg/dL)[a]	83 ± 12	85 ± 11	0.720
Systolic blood pressure (mmHg)[a]	128 ± 12	126 ± 12	0.661
Diastolic blood pressure (mmHg)[a]	76 ± 10	74 ± 7	0.660
Vitamin E unadjusted (μmol/L)[a]	31.9 ± 9.0	29.1 ± 7.4	0.360
Vitamin E/total cholesterol (μmol/mmol)[a]	4.38 ± 1.10	4.05 ± 0.64	0.329
Isoprostanes (pg/mg creatinine)[a]	348 ± 69	383 ± 51	0.129
OxLDL (U/L)[a]	36.8 ± 9.1	40.3 ± 8.2	0.288

[a]Data are expressed as mean ± SD.

($r = -0.707$, $P < 0.01$) and in isoprostanes and cholesterol ($r = 0.523$, $P < 0.01$) were significantly correlated.

No significant change in absolute values of vitamin E was found in both groups (Group A: from 31.9 ± 9.0 μmol/L before treatment to 29.0 ± 8.8 μmol/L after 30 days of treatment; Group B: from 29.1 ± 7.5 μmol/L before treatment to 31.2 ± 10.5 μmol/L after 30 days of treatment).

In vitro study

Compared with platelets incubated with LDL alone, collagen-stimulated platelets incubated with LDL showed a significant increase in conjugated dienes (*Figure 3A*). Incubation of platelet suspension with atorvastatin resulted in a dose-dependent reduction of conjugated dienes when compared with LDL treated with collagen-stimulated platelets. The NADPH oxidase inhibitor apocynin significantly reduced conjugated dienes formation induced by collagen-stimulated platelets (*Figure 3A*).

Compared with collagen-stimulated platelets, those added with LDL showed a marked increase in isoprostanes formation (*Figure 3B*). Such effect was significantly inhibited by apocynin and dose-dependently by atorvastatin (*Figure 3B*).

Analysis of vitamin E consumption in LDL treated with collagen-stimulated platelets showed a marked decrease of vitamin E compared with LDL added with un-stimulated platelets. Platelet incubation with apocynin or atorvastatin significantly reduced vitamin E consumption (*Figure 3C*).

LDL-treated platelets added to the substrate of NADPH oxidase induced a significant increase of superoxide anion formation (*Figure 4A*). This effect was dose-dependently inhibited by scalar concentrations of atorvastatin (0.1–10 μM) and completely abolished by the NADPH oxidase inhibitor apocynin (*Figure 4A*).

LDL induced a marked enhancement of p38MAPK phosphorylation in collagen-stimulated platelets compared with collage-stimulated platelets alone (*Figure 4B*). Preincubation of platelets with atorvastatin or apocynin inhibited p38MAPK phosphorylation induced by LDL.

Discussion

This study provides evidence that atorvastatin exerts an early antioxidant effect that seems to be independent from its cholesterol lowering property and likely involves inhibition of NADPH oxidase activation.

Previous studies have already demonstrated that statins possess antioxidant properties, but it was unclear if such effect reflected inhibition of specific oxidant species-generating pathways or was a reflection of cholesterol lowering. Shishehbor *et al.*[7] demonstrated that in HC patients 12 weeks of 10 mg/day atorvastatin therapy reduced plasma levels of nitrotyrosine, suggesting a mechanism related to myeloperoxidase inhibition. In multiple regression analysis, reduction of oxidative stress was not associated with cholesterol lowering. Rueckschloss *et al.*[29] showed that 1 month of statin treatment was associated with lower expression of gp91phox, the central core of NADPH oxidase, in the mammary artery of patients undergoing CABG.

We approached this issue by analysing oxidative stress as early as after only 3 days of statin therapy, which is usually associated with scarce changes of lipid profile in humans.[17] We observed a significant reduction of urinary excretion of isoprostanes whereas no changes of serum cholesterol were detected. The early decrease of oxidative stress was confirmed by the behaviour of oxLDL serum levels, that, in fact, significant lowered 3 days after atorvastatin treatment. The inhibition of oxLDL after statin therapy is consistent with most, but not all, interventional trials that investigated the effect of statins on oxLDL.[13,30,31]

Assuming that atorvastatin reduced urinary isoprostanes independently from its lipid lowering action, we should expect that it exerted a direct antioxidant effect by interfering with specific oxidant species-generating pathways.

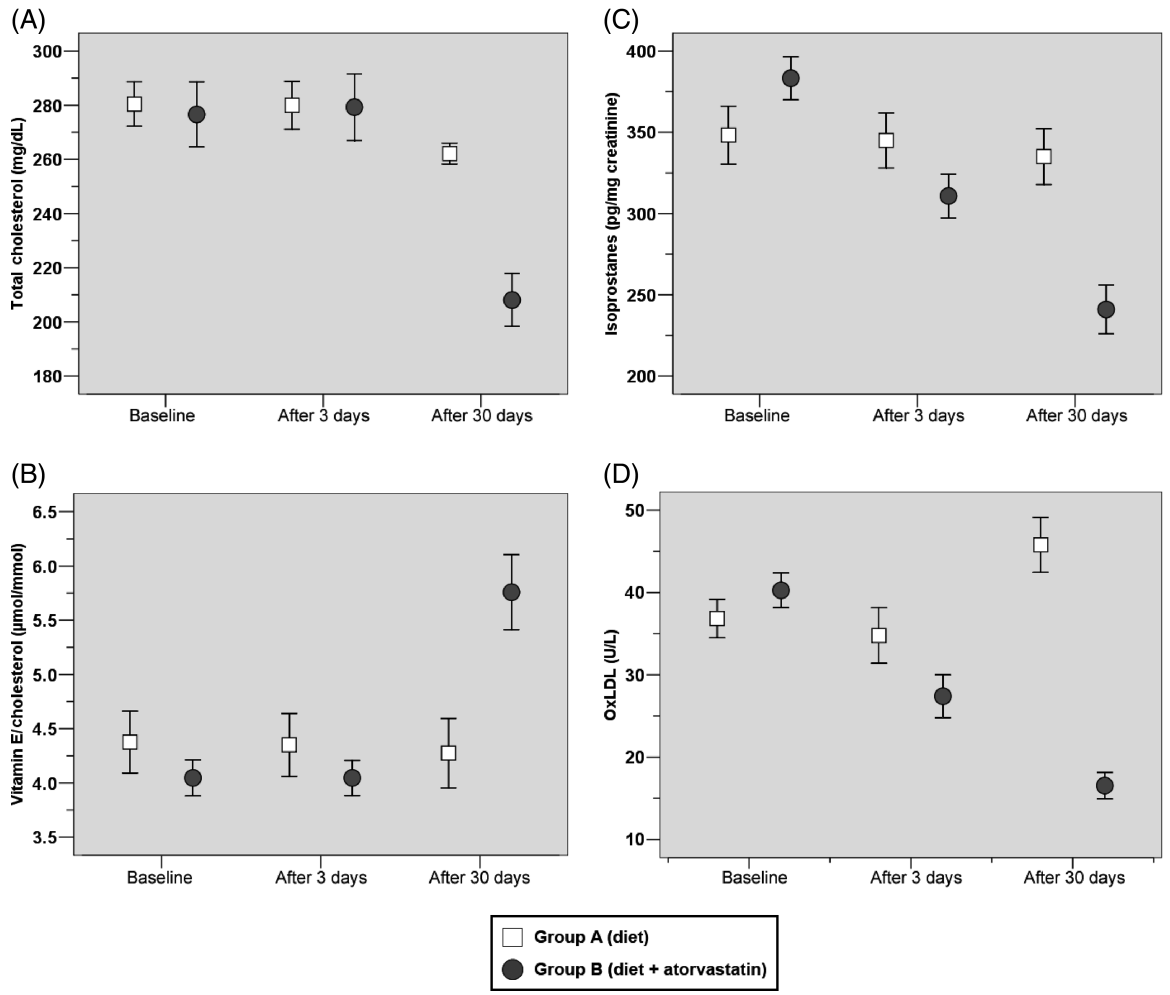

Figure 2 Total cholesterol (*A*), vitamin E/cholesterol (*B*), urinary isoprostanes (*C*), and oxLDL (*D*) levels in hypercholesterolaemic patients randomized to diet alone (Group A) or diet plus atorvastatin (Group B) at baseline and after 3 and 30 days of treatment. Boxes and T-bars represent means ± SE

Several studies have shown that statins down-regulate NADPH oxidase, which is one of the most important cellular sources of superoxide anion production.[25] In particular, statins have been shown *in vitro* and in animal models to reduce several NADPH oxidase subunits such as rac 1, gp22phox, and gp91phox, suggesting that such effect may be relevant in the antioxidant property of statins.[29,32,33] We speculated that this pathway may be implicated in the isoprostanes formation inhibition elicited by atorvastatin. Thus, we have recently shown that in patients with hereditary deficiency of gp91phox, urinary isoprostanes were reduced compared with controls.[34] Also, this catalytic subunit of NADPH oxidase is relevant for the oxidation of LDL[11] and could therefore play an important role in generating isoprostanes.

To explore this hypothesis, we performed *in vitro* experiments using platelets as a tool to oxidize LDL[11] and observed that LDL oxidation by platelets was associated with formation of isoprostanes. In a previous study that employed other cells to oxidize LDL, such as human monocytes, LDL oxidation was accompanied by isoprostane formation, an effect inhibited by the superoxide scavenger SOD.[35] Our findings support and extend these data as they show that NADPH activation is likely to play an important role in the generation of isoprostanes occurring during platelet-induced LDL oxidation. In this experimental model, atorvastatin, at concentrations achievable *in vivo* after supplementation,[36] inhibited the activation of NADPH oxidase and the phosphorylation of p38MAPK, an activator of NADPH oxidase,[37] so indicating that it could directly interfere with isoprostane formation via inhibition of NADPH oxidase.

Thirty days after atorvastatin treatment, a further decrease of isoprostanes and oxLDL, vis-à-vis to significant reduction of cholesterol, was observed. The direct correlation observed between these two variables would suggest that cholesterol lowering may also be implicated in the reduction of oxidative stress by atorvastatin. Thus, more than one mechanism eliciting an antioxidant effect could coexist: one being related to inhibition of NADPH oxidase and the other to cholesterol lowering. Further

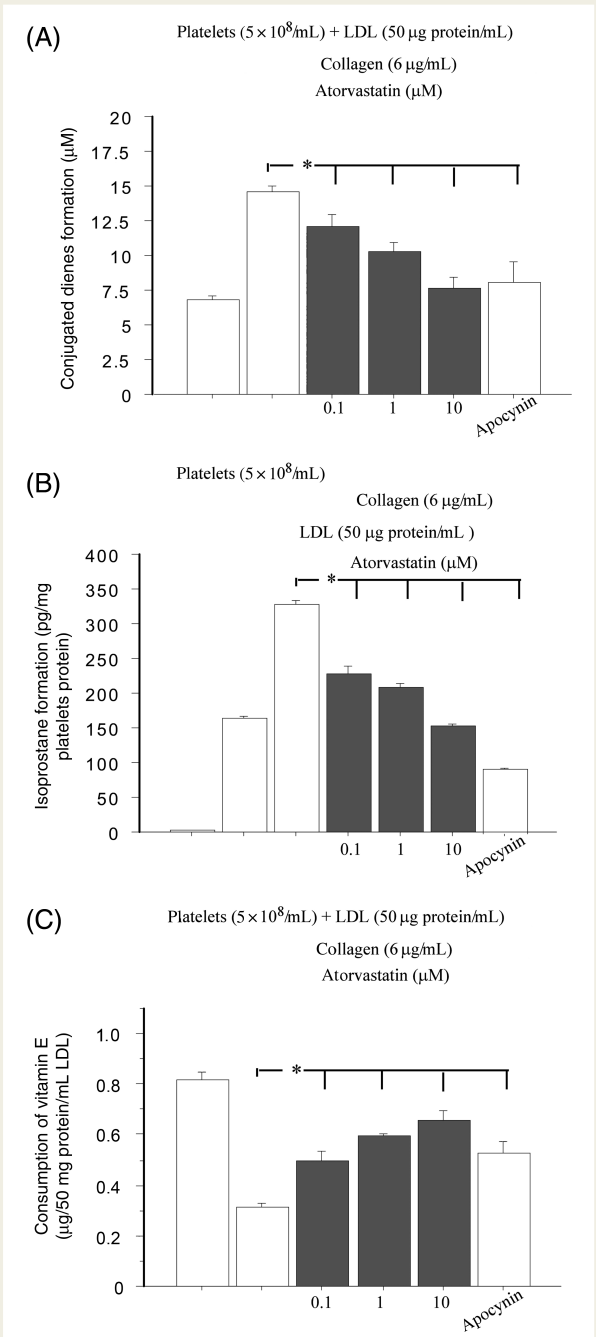

Figure 3 Conjugated dienes production (*A*), isoprostanes formation (*B*), vitamin E consumption (*C*) in platelets from healthy subjects ($n = 5$) treated with or without apocynin or scalar concentration of atorvastatin. *$P < 0.001$

study is necessary to investigate if there is some interplay between these two mechanisms and to quantify their different impact on the antioxidant effect of atorvastatin.

Although previous studies provided equivocal results on the effect of statins on the antioxidant status,[12–14] more recent reports demonstrated that statins enhance antioxidant status, including antioxidant vitamins such as vitamin E[15,16] and serum

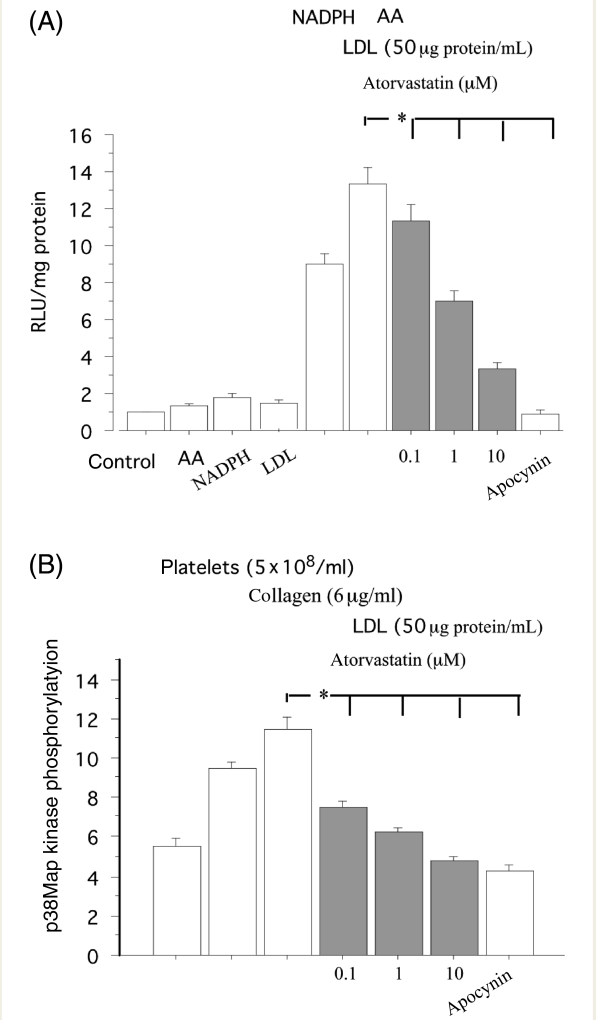

Figure 4 NADPH oxidase activation (*A*) and p38MAPK phosphorylation (*B*) in platelets from healthy subjects ($n = 5$) treated with or without apocynin or scalar concentration of atorvastatin. *$P < 0.001$

activity of PON1, the antioxidant enzyme associated with HDL.[38] The increase of antioxidant vitamins in statin-treated patients has been suggested to reflect a lower consumption of antioxidant molecules.[15,16] To the best of our knowledge, there is only one report that simultaneously evaluated isoprostanes and circulating vitamin E in statin-treated patients. In this study, De Caterina *et al.*[8] found a significant decrease of isoprostanes and a trend to an increase of vitamin E plasma levels after 2 months of statin therapy. In our study, vitamin E plasma levels did not change after 3 days but significantly increased after 30 days of treatment. The absence of vitamin E changes after 3 days of statin treatment may depend on its long half-life (about 50 h)[39] or on the need of a marked decrease of oxidative stress before the increase of vitamin E may be detectable in the circulation. Consistent with this hypothesis, the reduction of isoprostanes was more marked at 30 days of treatment compared with that observed after 3 days and inversely

correlated with circulating vitamin E. Such significant inverse correlation would imply that the increase of vitamin E is a result of its reduced consumption. We sought to explore this hypothesis *in vitro* by measuring the behaviour of vitamin E in LDL oxidized by activated platelets. Consistent with our previous report,[11] we demonstrated that vitamin E was markedly reduced in platelet-mediated LDL oxidation and that atorvastatin was able to inhibit vitamin E consumption.

These data may have potential pathophysiological and clinical implications. Uptake of oxidized LDL by macrophages via scavenger receptors is believed to represent the early phase of atherosclerotic lesion.[40] The mechanisms that determine LDL oxidation *in vivo* are likely to involve several oxidant species-generating pathways. In this context, our data provide evidence that NADPH oxidase may represent an important mechanism eliciting LDL oxidation and isoprostane formation and that atorvastatin is able to counteract such phenomenon. At the moment, however, we cannot elucidate if isoprostane formation inhibition is a result of atorvastatin interference with one or more cellular lines also because the contribution of resident and circulating cells, platelets included, on circulating isoprostanes is still undefined.

Another important implication of our study relates to the levels of vitamin E achieved after statin therapy. Thus, the cross-sectional study showed that HC patients have enhanced oxidative stress and low levels of vitamin E compared with controls. After statin treatment, HC patients had plasma values of vitamin E comparable with those of healthy subjects, indicating that statins normalize the circulating levels of this antioxidant vitamin. This finding might have several implications. First, the rise of vitamin E is a relevant property that could further enhance the antiatherosclerotic property of statins; in fact, in an animal model of atherosclerosis, circulating levels of vitamin E are strictly correlated with the progression of atherosclerotic lesion.[10] Secondly, statin-treated patients should not be supplemented with antioxidant vitamins as no further decrease of oxidative stress could be expected. This speculation is consistent with previous study showing no further decrease of isoprostanes in statin-treated patients also given vitamin E.[8]

Finally, our data indicate that the results of interventional trials with vitamin E should be re-analysed. In the HOPE trial, for instance, about one-third of patients allocated to vitamin E were also given statins.[41] On the basis of the present study, the probable increase of vitamin E in such patients could have precluded the possibility to adequately check the clinical efficacy of vitamin E.

The study has some limitation that must be acknowledged. Even if analysis of lipid profile as early as 3 days after statin treatment has been previously used to investigate biological effects that could be independent from cholesterol lowering,[17,42] such interpretation should be wisely considered Thus, taking into account that half-life of LDL is in the order of 3–4 days,[43] we cannot exclude that intracellular synthesis of cholesterol is already reduced as early as 3 days after statin therapy and that the long half-life of LDL precludes to observe early systemic changes of LDL. However, the fact that atorvastatin inhibited isoprostanes in cell lines such as platelets, which do not synthesize cholesterol, could suggest a direct effect on specific oxidant species-generating pathways.

In conclusion, we show that atorvastatin elicits an antioxidant effect that is partly independent from its lipid lowering action and ultimately enhances circulating vitamin E via a mechanism involving NADPH oxidase. Both these effects could concur in retarding atherosclerotic progression in statin-treated patients.

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