Low-Density Lipoprotein Level Reduction by the 3-Hydroxy-3-Methylglutaryl Coenzyme-A Inhibitor Simvastatin Is Accompanied by a Related Reduction of F₂-Isoprostane Formation in Hypercholesterolemic Subjects

No Further Effect of Vitamin E

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Background—Both statins and vitamin E, by reducing the rate of lipid peroxidation, may interfere with oxidative stress, but the impact of their combination is unknown.

Methods and Results—We randomized 43 hypercholesterolemic patients (21 men, 22 women, age 63\(\pm\)11 years) to either simvastatin, to achieve \(>20\%\) reduction of total cholesterol, or simvastatin plus 600 mg/d vitamin E for 2 months. Patients were then crossed over to the alternative treatment. Lipid parameters documented patients’ compliance to simvastatin, whereas plasma levels of vitamin E documented compliance and absorption of vitamin E. We assessed urinary excretion of the isoprostane 8-iso-prostaglandin F₂(\(\alpha\)) (8-iso-PGF₂\(\alpha\)) as an in vivo index of oxidative stress at baseline and after each month of therapy. 8-iso-PGF₂\(\alpha\) was significantly reduced by simvastatin, from 361\(\pm\)148 pg/mg creatinine (mean\(\pm\)SD) at baseline to 239\(\pm\)124 pg/mg creatinine after 1 month. The addition of vitamin E did not reduce such levels any further (256\(\pm\)125 after 1 month). Linear regression analysis showed a weak inverse relationship of 8-iso-PGF₂\(\alpha\) with vitamin E levels but a much stronger relationship with LDL cholesterol (\(R^2=0.162; P<0.001\)).

Conclusions—In hypercholesterolemic patients, LDL cholesterol is a major correlate of oxidative stress. Concomitant with LDL cholesterol reduction, simvastatin causes a drastic reduction of oxidative stress to a level that is not further reduced by the addition of vitamin E. Results of clinical trials with vitamin E may have been hampered by inadequate knowledge of the background level of lipid peroxidation, which is a major determinant of vitamin E bioactivity. (Circulation. 2002; 106:2543-2549.)

Key Words: atherosclerosis ■ lipids ■ pharmacology ■ peroxidation ■ stress

Reactive oxygen species (ROS) have multiple roles in vascular disease. ROS may irreversibly modify LDL, rendering them recognizable by the macrophage scavenging receptor, thus allowing the formation of foam cells in early atherogenesis.\(^1\) In addition, ROS may transduce the extracellular signal by cytokines and other proatherogenic mediators, activating ROS-sensitive transcription factors, thus contributing to all phases of atherosclerosis.\(^2\)

Despite a clear rationale for their use, antioxidants have an uncertain role in vascular disease. Five recently published large randomized trials with vitamin E have failed to show favorable results with regard to cardiovascular events.\(^3\)–\(^7\)

Contrary to antioxidants, reduction of plasma LDL by lipid-lowering agents does reduce clinical events in vascular disease. Inhibitors of HMG-CoA reductase (statins) are the most widely used drugs for this purpose. Statins reduce vascular events both in patients with established coronary artery disease\(^8\)–\(^10\) and in persons at risk.\(^7\)–\(^11\),\(^12\) Statins may reduce oxidative stress by reducing enhanced plasma levels of LDL, which are more susceptible to peroxidation in hypercholesterolemia,\(^13\),\(^14\) and change the LDL structure, making them more resistant to peroxidation.\(^15\) Statins may also inhibit NAD(P)H oxidase, thus decreasing the generation of ROS.\(^16\),\(^17\) Statins may therefore add or synergize biological effects of antioxidants.

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F₂-isoprostanes are nonenzymatic products of the ROS-catalyzed attack on esterified arachidonic acid, followed by enzymatic release from cellular or lipoprotein phospholipids (reviewed in Lawson et al⑧). 8-Iso-prostaglandin F₉α (8-iso-PGF₉α; also referred to as iPF₉α-III⑦) is an abundant F₂-isoprostane formed in vivo in humans and endowed with vasoconstrictive and platelet-activating properties.⑧ Enhanced urinary excretion of F₂-isoprostanes has been reported in association with several cardiovascular risk factors, including diabetes,⑨ smoking,⑩,⑪ and hypercholesterolemia.⑫,⑬ In both diabetic⑨ and hypercholesterolemic⑫ patients, 2-week supplementation with pharmacological doses of vitamin E (600 mg/d) was associated with normalization of enhanced F₂-isoprostane formation. In contrast, the short-term administration of vitamin E (100 and 800 IU/d for 5 days)⑤ or a 3-week administration (300, 600, and 1200 mg/d)⑥ to healthy chronic smokers failed to suppress urinary 8-iso-PGF₂α excretion. This discrepancy might be accounted for not only by the doses and mode of administration of vitamin E but also by the different rates of lipid peroxidation associated with different risk conditions. It is not known whether the administration of vitamin E together with a reduction of elevated cholesterol levels by a statin produces an additive effect on lipid peroxidation. We therefore investigated the effects of a statin and the addition of a fixed dose of vitamin E on 8-iso-PGF₂α formation in vivo.

Methods

Subjects and Study Design
This was a prospective, randomized, open-label study assessing and comparing the effects of a statin with or without the addition of an antioxidant on indices of lipid peroxidation. The study specifically tested the hypotheses that (1) a decrease in plasma total and LDL cholesterol is accompanied by some reduction of urinary 8-iso-PGF₂α, and (2) the addition of the antioxidant vitamin E to simvastatin, because of the different site of action, would further decrease 8-iso-PGF₂α.

We included hypercholesterolemic subjects (Fredrickson IIA or IIB) with serum cholesterol >200 mg/dL and proven vascular (coronary, carotid, or peripheral arterial) disease. Forty-three such patients (21 men, 22 women) were recruited and randomized to 1 of 2 treatments: (1) simvastatin (Sinvacor, Merck Sharp and Dohme Italy) 10-20-40 mg/d, administered once daily in the evening, titrated to achieve 20% reduction of total cholesterol after 60 days; or (2) the same treatment plus vitamin E 600 mg/d (Ephynal soft-gel 300-mg capsules; Roche Pharmaceuticals, Basel, Switzerland) given twice daily with meals.

The sequence of treatments was randomized so that 22 patients received simvastatin alone as first treatment and 21 received simvastatin plus vitamin E as first treatment. Characteristics of the study population are detailed in Table 1.

Investigated parameters were assessed at baseline (twice, then averaged), after a ≥15-day withdrawal of any previous lipid-lowering medication, and at 1 and 2 months with treatment (for both treatment regimens 1 and 2).

The study protocol was approved by the ethics committees of the 2 recruiting centers at Pisa and Chieti. Patients were informed of the investigational nature of the study and gave written informed consent to participate.

Serum triglycerides and total and HDL cholesterol were measured by standard enzymatic colorimetric methods. LDL cholesterol was calculated with the Friedewald formula. Plasma vitamin E was measured by reverse-phase high-performance liquid chromatography④ and reported both as plasma levels and after adjustment for different

<table>
<thead>
<tr>
<th>TABLE 1. Clinical Characteristics of Study Patients</th>
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<td>Group A: Simvastatin + Vitamin E as First Treatment (n=21)</td>
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IHD indicates ischemic heart disease; CVD, cerebrovascular disease; and PVD, peripheral vascular disease.
antisera and with gas chromatography/mass spectrometry. Urinary measurements were corrected for recovery and creatinine excretion. Oxidized LDL (ox-LDL) were measured by ELISA with a murine monoclonal antibody obtained after immunization of Balb/c mice with ox-LDL according to Holvoet et al, with specificity tested by the inhibition of binding to immobilized ox-LDL with different competing soluble ligands, including native LDL, ox-LDL, and malonyldialdehyde-modified LDL. Copper-induced ox-LDL, controls without competing ligand, and blanks without antibody were included routinely.

Statistical Analysis and Data Presentation
Sample size was calculated for 21 patients for each treatment arm of the study, who were expected to be equally distributed for additional diseases, assuming a variability (coefficient of variation) of 40% for subjects with proven atherosclerosis with regard to 8-iso-PGF$_2$ (data on file), a type I error probability $\alpha=0.05$ for a 1-tailed test, a type II error probability $\beta=0.20$ (ie, a test power of 80%), and the hypothesis that the 2 treatments (simvastatin versus simvastatin plus vitamin E) could differ by $\geq 25\%$ as to levels of 8-iso-PGF$_2$. The calculation also allowed for a loss function. Each of the sequence-randomized groups was also stratified for sex and age.

Main characteristics of the 2 groups (according to treatment sequence) were compared by the Student $t$ test for unpaired data for continuous variables and by the $\chi^2$ test for dichotomous variables and found to be not significantly different (Table 1). Treatment sequence-specific and carryover effects were specifically ruled out. Data were then represented “on phase,” with patients receiving the same treatment in each phase of the study grouped as independent of the treatment sequence. Comparisons of treatment effects were then performed by repeated-measures ANOVA after verification of normality of distribution. Post hoc testing was performed by the Fisher protected least significance test. Linear regression analysis was performed by standard methods.

Results
Control of Compliance and Clinical Efficacy of Study Treatments
We monitored plasma lipid levels as a control for the effects of simvastatin and plasma levels of vitamin E as a measure of vitamin E intake. The effects of study treatments on lipid levels are shown in Figure 1. Simvastatin alone, at the dose used (26.5 ± 10.2 mg, mean ± SD), determined a 26.5% ($P=0.01$) reduction of total cholesterol after the first month, which remained stable at 2 months and was unchanged by vitamin E (Figure 1, top left). The reduction was, according to the prespecified aim, $>20\%$ in each patient. The reduction in total cholesterol was due to a 37.1% ($P=0.01$) reduction of LDL cholesterol after 1 month of simvastatin alone (Figure 1, top right), whereas HDL cholesterol was unchanged (from 51.8 ± 17.4 mg/dL at baseline to 54.0 ± 15.3 mg/dL at 1 month, $P=NS$; Figure 1, bottom left). Triglycerides were also reduced by simvastatin (by 28.5% at 1 month; Figure 1, bottom right). Neither LDL cholesterol nor triglycerides were affected by vitamin E (Figure 1).

The effects of study treatments on vitamin E levels are shown in Figure 2, with and without correction for cholesterol levels. Simvastatin alone caused no appreciable variation of uncorrected plasma vitamin E levels (Figure 2, top) and a trend toward increased corrected vitamin E, explained by the change in cholesterol (Figure 2, bottom). Conversely, the addition of vitamin E 600 mg/d to simvastatin was associated with a $>50\%$ increase in unadjusted vitamin E levels and a near doubling of cholesterol-corrected vitamin E levels (Figure 2, top and bottom, respectively).
Effects of Simvastatin With or Without Vitamin E on Urinary Levels of 8-Iso-PGF$_{2alpha}$

Urinary excretion of 8-iso-PGF$_{2alpha}$ throughout the various phases of the study is shown in Figure 3. Simvastatin alone caused a significant (33.5%) reduction of 8-iso-PGF$_{2alpha}$ at 1 month, which was sustained thereafter and unchanged by the addition of vitamin E (urinary excretion after 1 month of simvastatin alone, 240±124 pg/mg creatinine; after 1 month of simvastatin plus vitamin E, 237±122 pg/mg creatinine; $P$=NS). Excretion of 8-iso-PGF$_{2alpha}$ was significantly different from baseline at all study points during therapy administration and nonsignificantly different among all study time points, irrespective of time and type of therapy (Figure 3).

Figure 2. Effects of study treatments on plasma levels of vitamin E, expressed as plasma concentration (top) and as plasma concentration adjusted for total cholesterol (bottom). Study treatments are presented in phase, as detailed in legend for Figure 1. Simv indicates simvastatin; vit.E, vitamin E.

Effects of Simvastatin With or Without Vitamin E on Urinary Levels of 8-Iso-PGF$_{2alpha}$

At linear regression analysis, vitamin E levels were a poor correlate of 8-iso-PGF$_{2alpha}$ excretion. The relationship of unadjusted vitamin E levels with 8-iso-PGF$_{2alpha}$ excretion was nonstatistically significant (not shown), whereas a significant but extremely weak inverse relationship was apparent when vitamin E levels were expressed after adjustment for cholesterol ($R^2=0.024$, $P=0.03$; Figure 4, top). When only the data of patients who had 8-iso-PGF$_{2alpha}$ values at baseline >300 pg/mg creatinine (n=30) were taken into account, this relationship did not improve much ($R^2=0.029$, $P=0.06$). Conversely, total cholesterol (Figure 4, middle) and, even more, LDL cholesterol (Figure 4, bottom) directly and highly significantly correlated with 8-iso-PGF$_{2alpha}$ excretion. There were no significant relationships of 8-iso-PGF$_{2alpha}$ excretion with HDL levels ($R^2=0.002$, $P=0.5$, not shown), whereas a

Figure 3. Effects of study treatments on urinary levels of 8-iso-PGF$_{2alpha}$. Study treatments are presented in phase, as detailed in legend for Figure 1. Simv indicates simvastatin; vit.E, vitamin E.

Figure 4. Linear regression analysis of relationship between urinary levels of 8-iso-PGF$_{2alpha}$ (dependent variable, on ordinate) as function of vitamin E levels adjusted for total cholesterol (independent variable, on abscissa; top), of total cholesterol (middle), and of LDL cholesterol (bottom).

Contributions of Lipid and Vitamin E Levels to Explaining 8-Iso-PGF$_{2alpha}$ Variability

At linear regression analysis, vitamin E levels were a poor correlate of 8-iso-PGF$_{2alpha}$ excretion. The relationship of unadjusted vitamin E levels with 8-iso-PGF$_{2alpha}$ excretion was nonstatistically significant (not shown), whereas a significant but extremely weak inverse relationship was apparent when vitamin E levels were expressed after adjustment for cholesterol ($R^2=0.024$, $P=0.03$; Figure 4, top). When only the data of patients who had 8-iso-PGF$_{2alpha}$ values at baseline >300 pg/mg creatinine (n=30) were taken into account, this relationship did not improve much ($R^2=0.029$, $P=0.06$). Conversely, total cholesterol (Figure 4, middle) and, even more, LDL cholesterol (Figure 4, bottom) directly and highly significantly correlated with 8-iso-PGF$_{2alpha}$ excretion. There were no significant relationships of 8-iso-PGF$_{2alpha}$ excretion with HDL levels ($R^2=0.002$, $P=0.5$, not shown), whereas a
Effects of Simvastatin With or Without Vitamin E on Plasma Levels of ox-LDL

To assess whether a general reduction of lipid peroxidation, as reflected by 8-iso-PGF$_{2\alpha}$ excretion, could also be reflected in the oxidation of LDL, which is more directly involved in atherogenesis, we measured ox-LDL levels throughout the various phases of the study (Table 2). Simvastatin alone caused a significant reduction of ox-LDL levels, which were not further decreased by the addition of vitamin E. There was only a weak and nonsignificant direct relationship between plasma ox-LDL levels and urinary 8-iso-PGF$_{2\alpha}$ excretion ($y=2.426+0.001x$; $R^2=0.014$, $n=184$, $P=0.115$) when all data, mostly obtained under treatment, were pooled. However, this relationship was much stronger and highly significant when analysis was restricted to basal values ($y=1.768+0.004x$; $R^2=0.153$, $n=37$, $P=0.0165$), which suggests that these indices are related when an antioxidant treatment does not suppress values to background noise.

Discussion

This study shows that, in hypercholesterolemic subjects, (1) a common cholesterol-lowering regimen with simvastatin substantially reduces indices of enhanced lipid peroxidation in vivo; (2) a regimen with vitamin E, associated with substantial elevation of vitamin E levels, adds nothing to the effects of a statin on the same indices; and (3) similarly, simvastatin reduces plasma ox-LDL, but vitamin E induces no additional effect. These findings have practical implications for the understanding of the effects of statins and vitamin E in clinical trials.

Our patient population consisted of frankly hypercholesterolemic subjects with evidence of vascular disease, who were therefore candidates for lipid-lowering therapy, primarily with a statin. Here we confirm the increase in F$_2$-isoprostane excretion previously documented in hypercholesterolemia, as well as in other conditions characterized by enhanced lipid peroxidation, such as type 2 diabetes, unstable angina, or heavy smoking. Concomitant with the expected reductions in lipid levels, simvastatin reduced the elevated levels of 8-iso-PGF$_{2\alpha}$ by approximately one third, to levels found in healthy individuals, mild smokers who were healthy, or subjects with stable coronary artery disease.

In the present study, the addition of vitamin E did not cause any further suppression of 8-iso-PGF$_{2\alpha}$ excretion. These findings are paralleled by directionally similar changes in plasma ox-LDL, starting from levels consistent with recent literature. Because ox-LDL levels reflect oxidative events within a lipid pool that is highly relevant for atherosclerosis, these data complement data derived from peroxidative markers in the total lipid pool (F$_2$-isoprostanes). The lack of effects of vitamin E on 8-iso-PGF$_{2\alpha}$ excretion in the present study contrasts with the 36% reduction obtained by the same dose of vitamin E in a similar patient population when vitamin E was given in the absence of a statin treatment.

There are several possible explanations for these findings. The dose of vitamin E might have been insufficient because of poor planning of the study, insufficient compliance to prescribed dosage, or insufficient absorption. Despite the fact that a dose range was not explored, the dose of vitamin E used in the present study was greater than or equal to that used in most large clinical trials with vitamin E, including the recently reported Heart Protection Study, and which has been previously shown close to the plateau of vitamin E plasma concentration among doses of 300, 600, and 1200 mg. Previous studies showed that large doses of supplemental vitamin E do not increase circulating vitamin E concentrations more than 3-fold, probably because newly absorbed vitamin E in part replaces α-tocopherol in circulating lipoproteins. Also, the dose used in the present study (600 mg/d) was associated with a substantial reduction of enhanced 8-iso-PGF$_{2\alpha}$ formation in both hypercholesterolemic and diabetic patients in previous studies. Issues of compliance and variable bioavailability after vitamin E administration have been emphasized recently as possible explanations for variable results of clinical studies. The present study, however, included measurements of vitamin E plasma concentrations throughout all phases, showing a near doubling of vitamin E plasma concentrations correct for total plasma cholesterol, as recently reported. Correction for plasma lipids appears particularly relevant here because of ample variations in plasma lipoproteins that occur with statin treatment, which reduces the lipid pool that carries most of the vitamin E in plasma. Therefore, it appears unlikely that the failure of vitamin E to reduce 8-iso-PGF$_{2\alpha}$ or ox-LDL in the present study could be due to inadequate dosing, compliance, absorption, or study design.

The inability of vitamin E to lower 8-iso-PGF$_{2\alpha}$ or ox-LDL levels in the present study can be better ascribed to the fact that these data complement data derived from peroxidative markers in the total lipid pool (F$_2$-isoprostanes). The lack of effects of vitamin E on 8-iso-PGF$_{2\alpha}$ excretion in the present study contrasts with the 36% reduction obtained by the same dose of vitamin E in a similar patient population when vitamin E was given in the absence of a statin treatment. The inability of vitamin E to lower 8-iso-PGF$_{2\alpha}$ or ox-LDL levels in the present study can be better ascribed to the fact that these data complement data derived from peroxidative markers in the total lipid pool (F$_2$-isoprostanes). The lack of effects of vitamin E on 8-iso-PGF$_{2\alpha}$ excretion in the present study contrasts with the 36% reduction obtained by the same dose of vitamin E in a similar patient population when vitamin E was given in the absence of a statin treatment.
that vitamin E was given concomitantly with another medication (simvastatin) that by itself is able to reduce these indices. Previous studies have also shown the inability of a similar or higher (up to 1200 mg/d) dose of vitamin E to reduce F₂-isoprostane levels in mild smokers who were healthy, contrary to previous studies in heavy smokers that showed clearly increased F₂-isoprostane levels. These data suggest that the same dose of vitamin E may have variable antioxidant effects in different patient populations characterized by variable rates of lipid peroxidation and that the basal rate of lipid peroxidation is a major determinant of the response to vitamin E. Consistent with this hypothesis are our findings in the present study, where enhanced 8-iso-PGF₂α and ox-LDL levels were already reduced to near-normal levels by the sole administration of a statin, and where vitamin E produced no further reduction.

Analysis of the relationship between lipid variables and vitamin E levels on the one hand and 8-iso-PGF₂α on the other in the present study showed that vitamin E was a modest inverse correlate of 8-iso-PGF₂α levels. This is most likely due to the fact that 4 of 5 points for correlation in each subject were obtained in conditions of suppressed 8-iso-PGF₂α synthesis due to the administration of simvastatin, a condition clearly different from others, such as unstable angina, for which a much better correlation was found. On the other hand, the correlation was much better with total cholesterol, triglycerides, and especially LDL cholesterol. The variability in LDL cholesterol can actually explain almost 20% of the variability of 8-iso-PGF₂α excretion. Because this also includes the “background” production and excretion of isoprostanes, which are likely not affected by any treatment, these data suggest that LDL cholesterol levels are responsible for the enhanced rate of lipid peroxidation, modified by risk factors or disease processes, and their reduction is at least in part responsible for the presumably beneficial effect of simvastatin on lipid peroxidation and ox-LDL levels. Though “direct” antioxidant effects of (some) statins or effects mediated by simultaneous interference with other (Rac1) metabolites of the mevalonate pathway are plausible, their demonstration, which requires a formal comparison of statin and nonstatin modalities of lipid lowering, is beyond the scope of the present study.

These data have implications for the interpretation of results of various trials reported with the use of vitamin E in either primary or secondary prevention of coronary artery disease. In none of such trials is there information on baseline levels of lipid peroxidation, and all included a population of subjects with hypercholesterolemia, primarily already treated or treated concomitantly with a statin (46% at the end of follow-up in the GISSI-Prevenzione Study). Such treatment would likely obscure any possibility of detecting an effect of vitamin E in hypercholesterolemic subjects.

In summary, LDL cholesterol levels are a major correlate and possibly a determinant of enhanced F₂-isoprostane formation in hypercholesterolemic subjects. Statin treatment is a powerful means to reduce enhanced lipid peroxidation in these subjects, which calls into question the validity of the antioxidant approach with vitamin E given concomitantly with the effective use of a statin.
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