Evidence for an Independent and Cumulative Effect of Postprandial Hypertriglyceridemia and Hyperglycemia on Endothelial Dysfunction and Oxidative Stress Generation
Effects of Short- and Long-Term Simvastatin Treatment

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Background—Postprandial hypertriglyceridemia and hyperglycemia are considered risk factors for cardiovascular disease. Evidence suggests that postprandial hypertriglyceridemia and hyperglycemia induce endothelial dysfunction through oxidative stress; however, the distinct role of these two factors is a matter of debate.

Methods and Results—Thirty type 2 diabetic patients and 20 normal subjects ate 3 different meals: a high-fat meal; 75 g glucose alone; and high-fat meal plus glucose. Glycemia, triglyceridemia, nitrotyrosine, and endothelial function were assayed during the tests. Subsequently, diabetics took 40 mg/d simvastatin or placebo for 12 weeks. The 3 tests were performed again at baseline, between 3 to 6 days after the start, and at the end of each study. High-fat load and glucose alone produced a decrease of endothelial function and an increase of nitrotyrosine in normal and diabetic subjects. These effects were more pronounced when high fat and glucose were combined. Short-term simvastatin treatment had no effect on lipid parameters but reduced the effect on endothelial function and nitrotyrosine observed during each different test. Long-term simvastatin treatment was accompanied by a lower increase in postprandial triglycerides, which was followed by smaller variations of endothelial function and nitrotyrosine during the tests.

Conclusions—This study shows an independent and cumulative effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial function, suggesting oxidative stress as common mediator of such effect. Simvastatin shows a beneficial effect on oxidative stress and endothelial dysfunction, which may be ascribed to a direct effect as well as the lipid-lowering action of the drug. (***Circulation***. 2002;106:1211-1218.)

Key Words: lipids ■ cardiovascular diseases ■ endothelium ■ stress ■ diabetes mellitus

Patients with diabetes have an increased risk of cardiovascular disease (CVD). Recently, much attention has been paid to evidence that abnormalities of the postprandial state are important contributing factors to the development of atherosclerosis, even in diabetes mellitus.[1] In nondiabetic subjects, there is evidence that postprandial hypertriglyceridemia is a risk factor for CVD,[2] whereas in diabetic subjects, postprandial hyperglycemia has been recently proposed as an independent risk factor for CVD.[3] Since in diabetic patients the postprandial phase is characterized by the simultaneous increase of both plasma triglycerides and glucose,[1] the distinct role and the relative importance of these two factors in the pathogenesis of CVD in diabetes is a matter of debate.

The response-to-injury hypothesis of atherosclerosis states that the initial damage affects the arterial endothelium, leading to endothelial dysfunction.[4] Indeed, endothelial dysfunction has been demonstrated in patients with diabetes,[5]

and hyperglycemia has been implicated as a cause of endothelial dysfunction in normal as well as diabetic subjects.[6,7] Similarly, a negative influence of postprandial hypertriglyceridemia on endothelial function has been reported in both normal and diabetic subjects.[8,9]

It has been suggested that hyperglycemia[6-7] and hypertriglyceridemia[8-9] induce an endothelial dysfunction through the production of an oxidative stress. The process may involve the overgeneration of superoxide anion (O_2^-), which in turn inactivates nitric oxide (NO).[10] NO and O_2^- react by producing peroxynitrite, a potent, long-lived oxidant.[10] The peroxynitrite anion is cytotoxic because it oxidizes sulfhydryl groups in proteins, initiates lipid peroxidation, and nitrates amino acids such as tyrosine, which affects many signal transduction pathways.[10] The production of peroxynitrite can be indirectly inferred by the presence of nitrotyrosine (NT),[11] and NT has been found in the plasma of diabetic patients.[12]

TABLE 1.   Baseline Characteristics of Normal and Diabetic Subjects

|	Control Subjects (n=20)	Diabetics (n=30)
Sex, male/female	12/8	22/18
Age	53.5±2.5	54.3±2.6
BMI, kg/m^2	28.4±2.1	29.7±2.3
Fasting glucose, mmol/L	4.8±0.2	11.1±2.2*
HbA1c, %	5.8±0.2	7.8±0.3*
Resting systolic blood pressure, mm Hg	118.3±7.5	122.4±6.5
Resting diastolic blood pressure, mm Hg	78.4±2.1	82.2±3.1
Total cholesterol, mmol/L	4.5±0.6	7.5±0.8*
Triglycerides, mmol/L	0.9±0.2	3.7±0.4*
HDL-C, mmol/L	1.4±0.2	0.9±0.3*
LDL-C, mmol/L	2.5±0.3	3.6±0.4*
FMD, %	12.3±0.9	4.9±0.8*
NT, μmol/L	0.23±0.5	0.56±0.3*

Data are mean±SEM.

*$P<0.001$ vs control subjects.

There is also evidence that an acute increase of glycemia simultaneously induces an increase of NT and endothelial dysfunction in healthy subjects, suggesting that peroxynitrite may be involved in the generation of endothelial abnormalities during hyperglycemia.[13] On the other hand, a nitrosylation of VLDL has been found in experimental atherosclerosis, suggesting that increased production of peroxynitrite may also be associated with altered lipid metabolism.[14] However, data concerning NT in the postprandial state and the possible contribution of hyperglycemia and/or hypertriglyceridemia to the phenomenon are not yet available.

The aim of this study was to evaluate whether postprandial hypertriglyceridemia and hyperglycemia play a distinct role in producing endothelial dysfunction and whether this phenomenon is accompanied by NT generation. To demonstrate this hypothesis, endothelial function was measured after a fat-rich meal associated or not associated with simultaneous administration of an oral glucose tolerance test (OGTT), both in diabetic patients with moderate hyperlipidemia and in normal subjects. In addition, the diabetic patients were retested after 3 to 6 days or 3 months of simvastatin treatment. The objective of the short-term simvastatin treatment was to evaluate a possible direct effect of the drug on endothelial function, possibly through an inhibition of oxidative stress,[15,16] whereas the long-term treatment was aimed to also evaluate the effect of decreased postprandial hypertriglyceridemia.[17]

Methods

Thirty type 2 diabetic patients and 20 healthy subjects were recruited. The clinical characteristics of the groups are reported in Table 1. All subjects were nonsmokers and had a normal resting ECG and no history of vascular disease.

None of the subjects were taking aspirin, lipid-lowering agents, or supplemental vitamins. Diabetes was treated in 18 patients with diet alone and with sulfonylureas in the remaining 12 patients. All subjects were recommended to consume the habitual diet during the
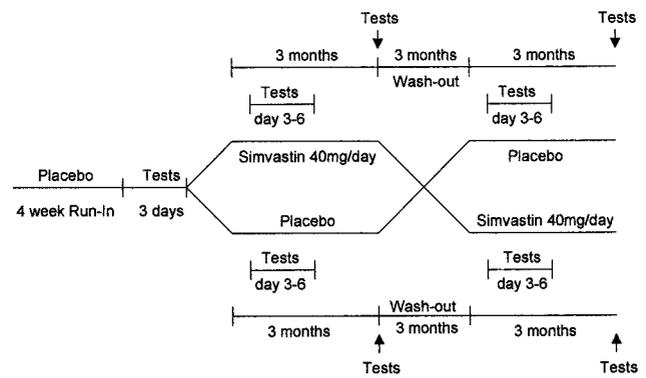
Figure 1. Scheme of the simvastatin trial.

entire period of the study. Written consent was obtained from all subjects, with local ethics committee approval.

Study Design

At the end of a 4-week placebo run-in period, blood samples were taken and fasting glycemia, total cholesterol, LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), triglycerides, NT, and HbA1c were measured. Therefore, diabetic patients and controls ate, in randomized order, on different days, 3 different menus: (1) a high-fat meal; (2) 75 g glucose alone; (3) a high-fat meal plus 75 g glucose. Blood samples were drawn at 0, 1, 2, 3, and 4 hours, and the following variables were assayed: glycemia, triglyceridemia, NT, and endothelial function. Studies were begun at 8 AM after a 12- to 14-hour overnight fast.

Subsequently, a double-blind, crossover, placebo-controlled study was started in diabetic patients. Diabetics were randomly assigned to take 40 mg/d simvastatin or placebo for 12 weeks. The washout period between the simvastatin treatment and placebo period was 3 months. At baseline and at the end of each period, the following parameters were measured: fasting glycemia, serum total cholesterol, LDL-C, HDL-C, triglycerides, HbA1c, NT, and endothelial function. The 3 meal tests were performed at baseline, between 3 to 6 days after the start of both placebo and simvastatin treatment, and at the end of each study. The scheme of the protocol is illustrated in Figure 1.

High-Fat Load

The standardized high-fat meal consisted of whipping cream and contained 75 g of fat, 5 g of carbohydrates, and 6 g of protein per m^2 body surface area. The corresponding caloric intake was 700 kcal/m^2.

Biochemical Measurements

Cholesterol and triglycerides were measured enzymatically (Roche Diagnostics, Basel, Switzerland). HDL-C was estimated after precipitation of apolipoprotein B with phosphotungstate/magnesium.[18] LDL-C was calculated after lipoprotein separation.[19] Plasma glucose was measured by the glucose-oxidase method and HbA1c by high-performance liquid chromatography. Nitrotyrosine plasma concentration was assayed by ELISA.[12]

Endothelial Function

Endothelial function was evaluated by measuring flow-mediated vasodilation (FMD) of the brachial artery. Vasodilation responses of the brachial arteries were measured by ultrasound technique by two skillful examiners. The validity of this method has been confirmed in previous studies.[20,21] Briefly, the diameter of the brachial artery was measured from B-mode ultrasound images with a 7.5-MHz linear array transducer (SSH-160A, Toshiba). Flow velocity in the brachial artery was measured with a pulsed Doppler signal at a 70° angle to the vessel, with the range gate (1.5 mm) in the center of the artery. The brachial artery was scanned in the antecubital fossa in a longitudinal fashion. Depth and gain settings were optimized at the
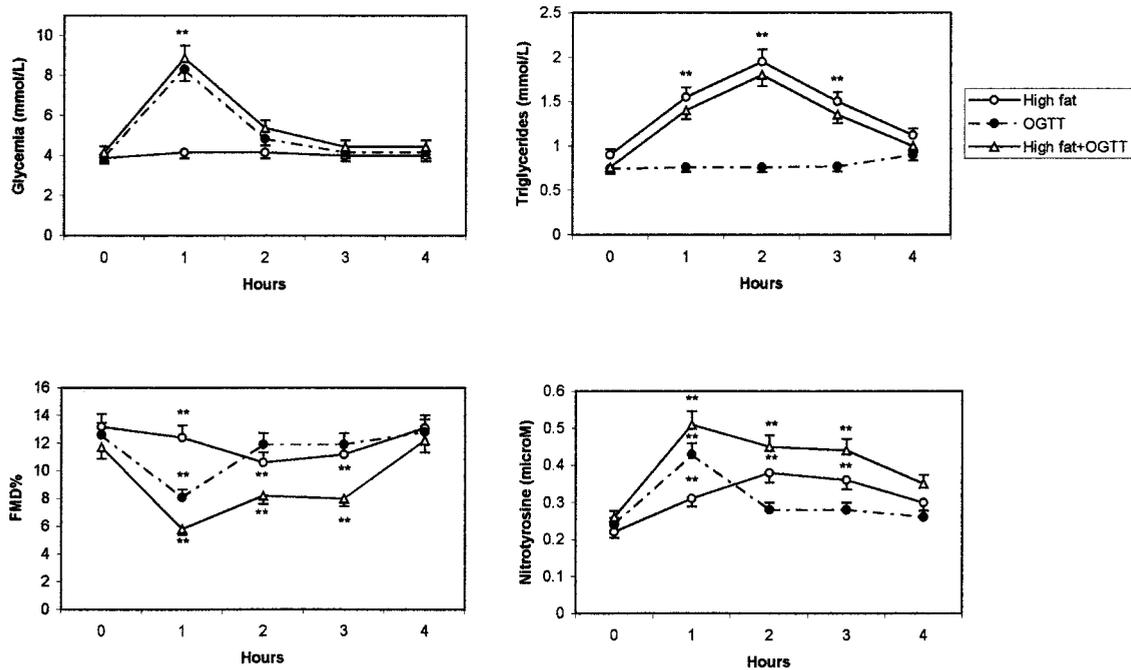
Figure 2. Effects of high-fat load, OGTT, and high-fat load plus OGTT on glycemia, triglycerides, FMD, and NT in healthy normal control subjects. Bars indicate SEM. **$P<0.001$ vs baseline.

beginning of the study and were kept constant throughout the recording period. When a satisfactory transducer position was found, the surface of the skin was marked, and the arm remained in the same position throughout the study. Each subject lay quietly for 10 minutes before the first scan.

At the end of each test, the subjects lay quietly for 15 minutes. Then, sublingual nitroglycerin (0.3 mg) was administered and 3 minutes later the last measurements were performed. Response to nitroglycerin was used as a measure of endothelium-independent vasodilation. All studies were performed in a quiet and temperature-controlled room (22°C to 23°C).

After baseline measurements of the diameter and flow velocity in the brachial artery, a blood pressure cuff placed around the forearm was inflated with a pressure of 250 to 300 mm Hg and was released after 5 minutes. The measurements of diameter and flow velocity were continuously performed from cuff inflation to due time after cuff deflation. The ultrasound images were recorded on a super VHS videocassette recorder (BR-S601 mol/L, Victor), and the arterial diameter was measured at a fixed distance from an anatomical marker with ultrasonic calipers by two independent observers. Measurements were taken from the anterior to the posterior interface between the media and adventitia ("m" line) at the end of diastole, coincident with the R wave on a continuously recorded ECG.[20] The diameters of 4 cardiac cycles were analyzed for each scan, and the measurements were averaged. Diameter measurements for the reactive hyperemia were taken 45 to 90 seconds after cuff deflation to measure peak diameter.[20] Responses of the vessel diameters to the reactive hyperemia and nitroglycerin were expressed as the percent increase above the baseline value of the diameter. Blood flow was calculated by multiplying the velocity-time integral of the Doppler flow signal by heart rate and the vessel cross-sectional area. The increase in the blood flow was calculated by dividing the maximum flow within the first 15 seconds after the cuff deflation by the flow at baseline.[20]

In our study, interobserver variability for repeated measurements of resting arterial diameter was 0.04±0.02 mm. The intraobserver variability for repeated measurements of resting arterial diameter was 0.02±0.02 mm.

Statistical Analysis

The Kolmogorov-Smirnov algorithm was used to determine whether each variable had a normal distribution. Comparisons of baseline data among the groups were performed with the unpaired Student's *t* test. The paired Student's *t* test was used for comparison of the various parameters before and after simvastatin or placebo treatment. The changes in variables during the tests were assessed by 2-way ANOVA with repeated measures. If differences reached statistical significance, post hoc analyses with a 2-tailed paired *t* test was used to assess differences at individual time periods in the study, with Bonferroni correction used for multiple comparisons. Statistical significance was defined as $P<0.05$.

Results

Baseline cholesterol, LDL-C, triglycerides, and NT were increased in diabetic patients, whereas HDL-C and FMD were reduced (Table 1).

As compared with preprandial values, serum triglyceride levels were increased from 1 to 3 hours during tests 1 and 3 in normal subjects ($P<0.001$ versus baseline; Figure 2) and from 1 to 4 hours in diabetics ($P<0.001$ versus baseline; Figure 3). Glycemia increased at 1 hour in normal subjects ($P<0.001$ versus baseline; Figure 2) and at 1 hour, 2 hours, and 3 hours in diabetics during tests 2 and 3 ($P<0.001$ versus baseline; Figure 3), whereas triglycerides remained unchanged during test 2 (Figures 2 and 3).

High-fat load alone produced a decrease of FMD and an increase of NT from 1 to 3 hours in normal subjects ($P<0.001$ versus baseline; Figure 2) and from 1 to 4 hours diabetic patients ($P<0.001$ versus baseline; Figure 3). The increase of glycemia during the OGTT was accompanied by a significant decrease of FMD at 1 hour both in normal and diabetic subjects ($P<0.001$ versus baseline; Figures 2 and 3), whereas FMD returned to basal values within 2 hours in normal subjects and remained low at 2 and 3 hours in diabetic patients ($P<0.001$ versus baseline; Figure 3), returning to basal values only at 4 hours (Figures 2 to 3). NT concentration changed during the studies, and its variations were
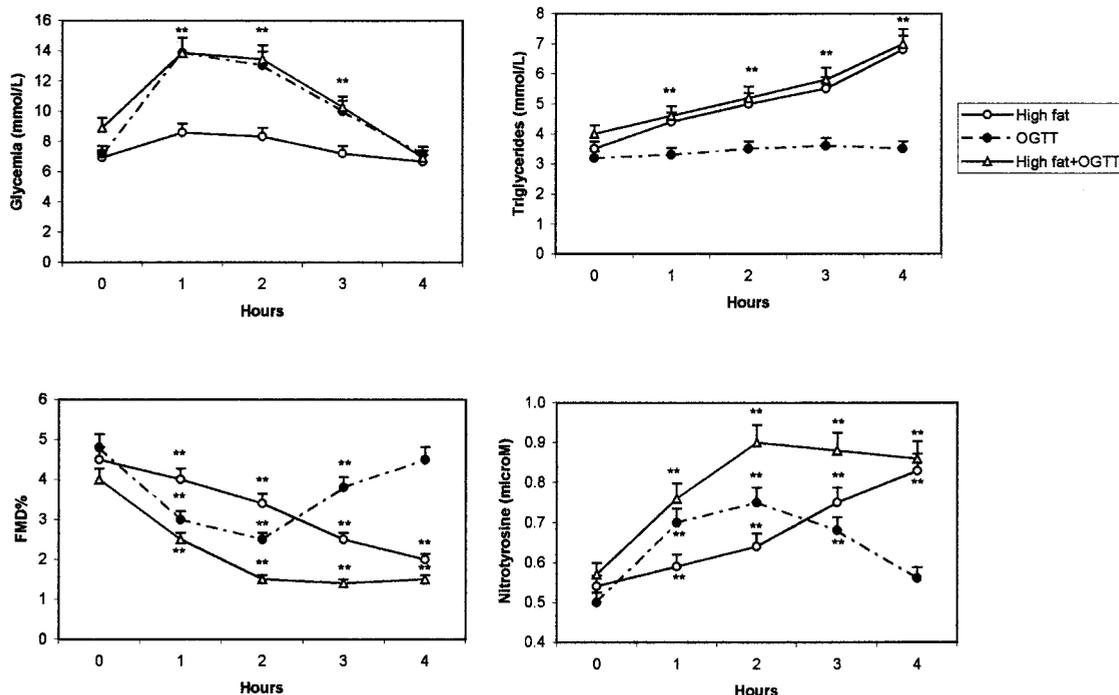
Figure 3. Effects of high-fat load, OGTT, and high-fat load plus OGTT on glycemia, triglycerides, FMD, and NT in type 2 diabetic patients. Bars indicate SEM. **$P<0.001$ vs baseline.

opposite to those of FMD ($P<0.001$ versus baseline; Figures 2 and 3).

The combination of high-fat and glucose load, test 3, produced a decrease of FMD and an increase of NT ($P<0.001$ versus baseline; Figures 2 and 3) even more pronounced than with either nutrient taken alone ($P<0.01$; Figures 2 and 3), both in normal and diabetic subjects. The decrease of FMD and increase of NT were maximal at 1 hour in normal subjects (Figure 2), whereas in diabetic patients, such variations were observed throughout the test, with a maximum at 2 hours (Figure 3).

Short-term simvastatin treatment had no effect on lipid parameters in diabetic patients (Table 2). However, a significant improvement of both basal FMD and NT was observed (Table 2). Such treatment was able to reduce the effect on endothelial function and NT observed during each different test, even though postprandial hypertriglyceridemia was not affected ($P<0.001$ versus placebo; Figures 4, 5, and 6).

Long-term simvastatin treatment reduced fasting total cholesterol, LDL-C, and triglycerides, and raised HDL-C in diabetic patients, whereas body mass index and HbA1c remained unchanged throughout the study (Table 2).

TABLE 2. Effects of Short- and Long-Term Simvastatin Treatment in Diabetic Patients

	Placebo 3–6 d	Simvastatin 3–6 d	Placebo 3 mo	Simvastatin 3 mo
BMI, kg/m^2	29.8±2.2	29.7±2.1	29.5±2.2	29.4±2.3
Fasting glucose, mmol/L	11.3±2.0	11.4±2.3	11.2±3.2	10.8±2.4
HbA1c, %	7.8±0.2	7.8±0.3	7.8±0.5	7.9±0.5
Resting systolic blood pressure, mm Hg	119.1±7.0	120.4±6.5	125.3±7.1	123.2±7.5
Resting diastolic blood pressure, mm Hg	80.4±2.1	81.2±3.1	84.6±2.1	82.4±3.7
Total cholesterol, mmol/L	7.4±0.6	7.5±0.8	7.5±0.9	5.5±0.6*
Triglycerides, mmol/L	3.5±0.5	3.7±0.4	3.6±0.5	2.3±0.4*
HDL-C, mmol/L	0.9±0.3	0.9±0.3	0.9±0.4	1.2±0.2†
LDL-C, mmol/L	3.6±0.5	3.5±0.4	3.5±0.4	2.6±0.6†
FMD, %	4.8±0.8	7.3±0.5*	4.9±0.7	9.2±0.8*‡
NT, μmol/L	0.55±0.3	0.41±0.2†	0.57±0.4	0.32±0.3*‡

Data are mean±SEM.

*$P<0.001$ vs placebo.

†$P<0.05$ vs placebo.

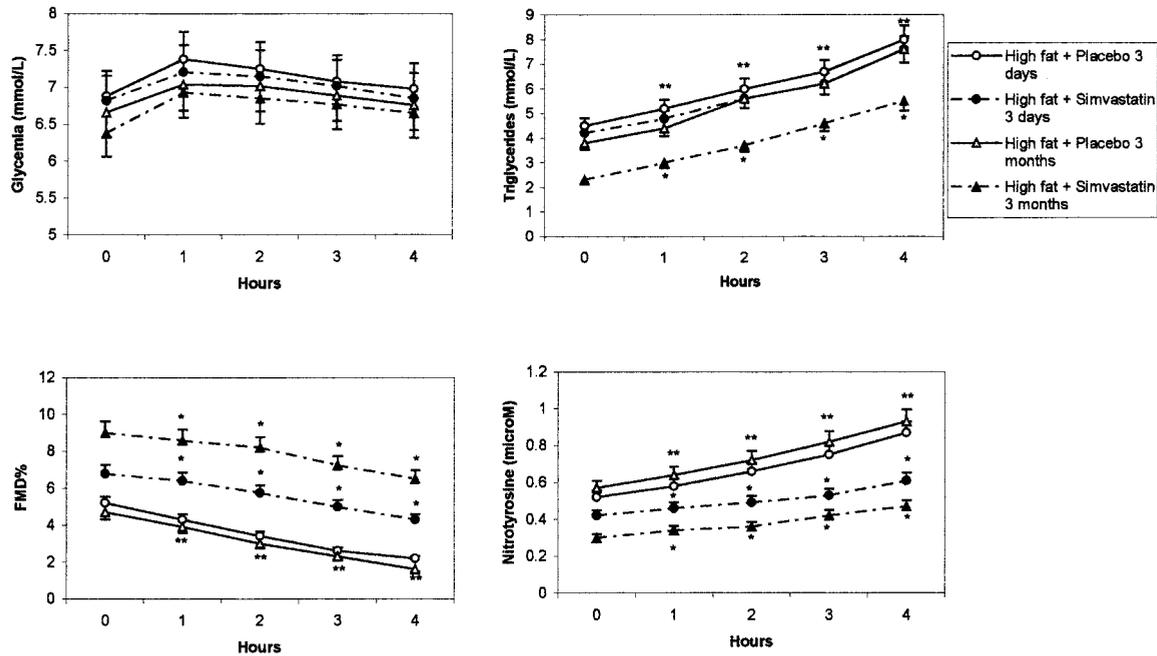‡$P<0.001$ vs simvastatin short-term treatment.

Figure 4. Effects of high-fat load on glycemia, triglycerides, FMD, and NT in type 2 diabetes after short- and long-term simvastatin treatment. Bars indicate SEM. *$P<0.0$, **$P<0.001$ vs baseline.

Long-term simvastatin treatment was accompanied by a significant improvement of fasting FMD and NT level (Table 2). When the high-fat load test was performed, the increase in postprandial triglycerides compared with placebo was lower ($P<0.01$; Figures 4 and 6), and FMD decrease as well as NT increase were still significant but significantly smaller ($P<0.01$; Figures 4 and 6). During the glucose challenge, triglycerides remained unchanged; FMD decrease and NT rise were smaller than in the

placebo period ($P<0.01$; Figure 5) but were still significant. The combined load of a high-fat meal and glucose resulted in a smaller increase of triglycerides after simvastatin treatment ($P<0.01$; Figures 4, 5, and 6). Again, FMD decreased significantly and NT increased in this test, the observed variations being smaller than at the end of the placebo period ($P<0.01$; Figures 4, 5, and 6).

Baseline arterial diameter, endothelium-independent vasodilation, blood pressure, and heart rate were not affected by
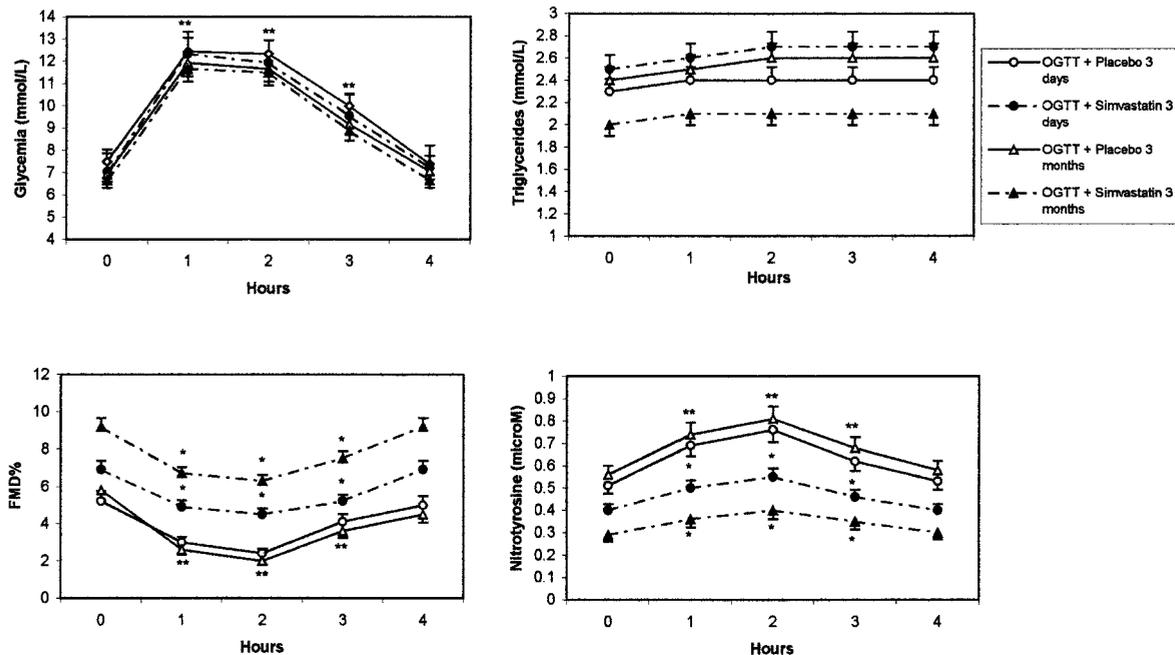
Figure 5. Effects of OGTT on glycemia, triglycerides, FMD, and NT in type 2 diabetes after short- and long-term simvastatin treatment. Bars indicate SEM. *$P<0.0$, **$P<0.001$ vs baseline.

Figure 6. Effects of high-fat load plus OGTT on glycemia, triglycerides, FMD, and NT in type 2 diabetes after short- and long-term simvastatin treatment. Bars indicate SEM. *$P<0.0$, **$P<0.001$ vs baseline.
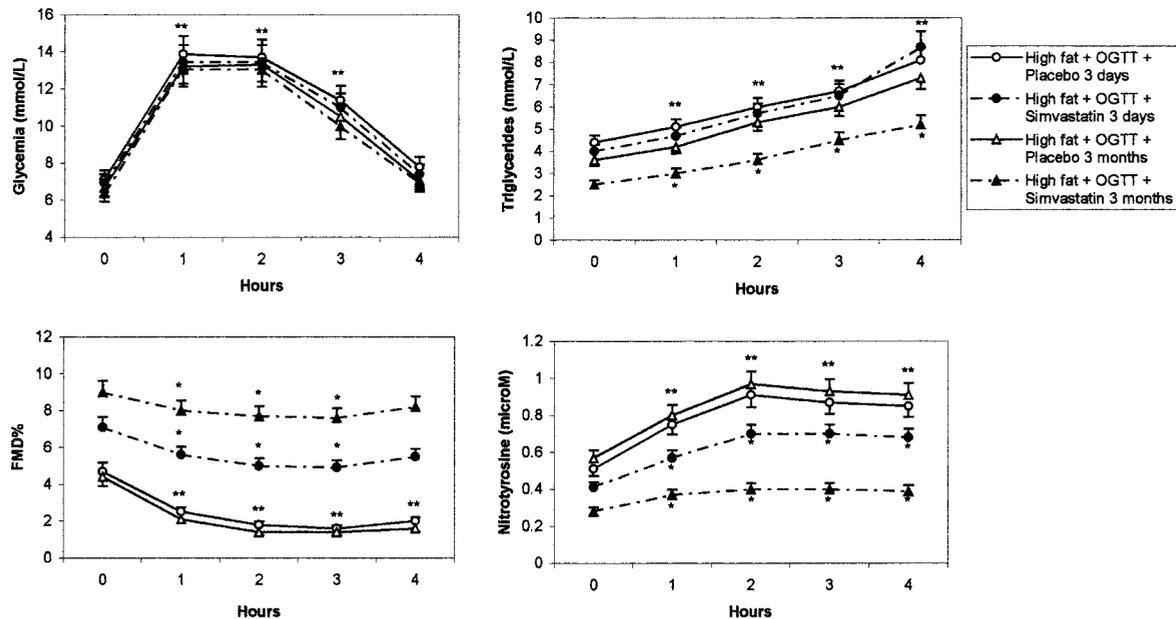
any of the different tests performed and did not change during simvastatin study (data not shown).

Discussion

This study confirms that both postprandial hypertriglyceridemia and postprandial hyperglycemia produce an endothelial dysfunction in both diabetic and normal subjects. However, for the first time, our data show that postprandial hyperglycemia and hypertriglyceridemia have an independent and cumulative effect in determining endothelial dysfunction. Our data also suggest that oxidative stress may be the common mediator of this phenomenon, since endothelial dysfunction during both postprandial hyperglycemia and hypertriglyceridemia is accompanied by a significant NT increase.

Postprandial hypertriglyceridemia may represent an independent predictor of CVD in nondiabetic patients[2] and may be a predictor of carotid intima-media thickness in patients with type 2 diabetes.[22] However, recent studies support the hypothesis that postprandial hyperglycemia also is a risk factor for CVD.[23] The concept that postprandial hypertriglyceridemia and hyperglycemia may be important factors for the development of CVD is supported by evidence that both induce endothelial dysfunction,[6–9] whereas there is a broad consensus that abnormalities of endothelium-dependent arterial relaxation are an early marker of atherosclerosis[4] and may predict CVD.[24]

Meal absorption is a complex phenomenon, and postprandial hyperlipidemia and hyperglycemia are simultaneously present in the postabsorptive phase, particularly in diabetics and in subjects with impaired glucose tolerance. Therefore, a specific and direct role of hyperglycemia, independent of the concomitant hyperlipidemia, has been frequently questioned. In our study, when hyperglycemia and hypertriglyceridemia were simultaneously present, there was a greater impairment of endothelial function compared with that observed during

either hyperglycemia or hypertriglyceridemia alone, suggesting that they have an independent but cumulative effect on endothelial cells.

The independent role of hyperglycemia is also supported by our long-term simvastatin study in diabetic patients. As expected after that treatment, endothelial function is ameliorated in basal conditions[25] and the level of postprandial triglycerides after the high fat load is significantly reduced.[26] Consequently, there is a smaller reduction of endothelial function during the fat load. In this condition, the independent role of hyperglycemia is pointed out by the demonstration that even in the presence of diminished postprandial hypertriglyceridemia and endothelial dysfunction, during the OGTT the FMD decreases.

It has been suggested that statins may ameliorate endothelial function independent of their lipid-lowering effect, and a short-term (3-day) statin treatment has been reported to protect endothelial function and reduce oxidative stress in aged diabetic patients.[27] To examine this effect as well as its influence on the results of long-term simvastatin treatment, in which postprandial hypertriglyceridemia is expected to be decreased by treatment, we also performed a short-term trial. We found that 3- to 6-day simvastatin treatment can ameliorate endothelial function in diabetic patients, a phenomenon independent of the lipid-lowering effect of the drug, as the plasma lipid concentration was unchanged after this brief treatment. Such effect is conceivably related to the reduction of oxidative stress because NT was significantly decreased, supporting the recent finding that simvastatin may work as intracellular antioxidant.[28] However, in this condition, simvastatin did not completely preserve the endothelium from the damage induced by both postprandial hyperglycemia and hypertriglyceridemia, whereas after long-term treatment, when postprandial hypertriglyceridemia was reduced, the effect of the high-fat load was smaller than after short-term

treatment, suggesting that even in presence of a direct effect of simvastatin, the level of postprandial triglycerides remains very important in determining the endothelial dysfunction. This concept is further supported by the finding that after long-term treatment, endothelial response to OGTT and high-fat load plus OGTT was significantly reduced compared with short-term treatment.

The mechanism through which postprandial hyperglycemia and hypertriglyceridemia produce endothelial dysfunction has been proposed to be the production of an oxidative stress. This hypothesis is supported by our finding that hyperglycemia and hypertriglyceridemia, alone or in combination, produce an increase of NT. Interestingly, NT plasma levels increase even more during combined hyperglycemia and hypertriglyceridemia. This finding suggests that oxidative stress may represent at least one of the mechanisms by which the additive effect of hyperglycemia and hypertriglyceridemia on endothelial function is achieved.

Our findings also support the hypothesis of increased NO inactivation by O_2^- as being an important mechanism for the impairment of endothelial function in postprandial conditions and possibly for CVD development. The interaction of O_2^- with NO is very rapid and leads to inactivation of NO and production of the potent oxidant peroxynitrite.[10] The finding that peroxynitrite production in the postprandial phase appears to be increased may have important clinical implications.

Peroxynitrite is a potent oxidant and nitrating agent that leads to a host of potentially injurious events including VLDL peroxidation,[14] depletion of antioxidant defenses,[29] and inactivation of enzymes.[30] In addition, it can be directly cytotoxic for endothelial cells.[31] All these events may convincingly be involved in the pathogenesis of CVD complications. This hypothesis is strongly supported by the recent demonstration that increased apoptosis of myocytes, endothelial cells, and fibroblasts in heart biopsy specimens from diabetic patients[32] and in hearts from streptozotocin diabetic rats,[33] even during acute hyperglycemia,[34] is selectively associated with levels of NT found in those cells. Furthermore, evidence that NT may induce endothelial dysfunction by itself[35] and that it is present in atherosclerotic lesions in humans and in diabetic cynomolgus mokeys[36–37] suggests that peroxynitrite production may be strongly involved in atherogenesis.

Many abnormalities of postprandial lipid metabolism other than hypertriglyceridemia, such as altered remnant particle clearance or increased LDL oxidation and size, have been involved in the phenomenon of postprandial endothelial dysfunction[38]; of these, we evaluated only hypertriglyceridemia, and our purpose was specifically to define the respective influence of postprandial hypertriglyceridemia and hyperglycemia on endothelium and oxidative stress generation. This kind of evaluation deserves attention from a clinical point of view: The finding that postprandial hypertriglyceridemia and hyperglycemia play an independent and cumulative role in favoring the postprandial endothelial dysfunction implies that both of them should be addressed by treatment to prevent the development of CVD, at least in patients with diabetes and impaired glucose tolerance.

The present study, although confirming that postprandial hypertriglyceridemia and hyperglycemia induce an endothe-

lial dysfunction, shows for the first time an independent and cumulative deleterious effect of both of these cardiovascular risk factors on endothelial function and suggests that oxidative stress is the common mediator through which they exert such effect. Simvastatin appears to have a beneficial effect on oxidative stress and endothelial dysfunction, at least in diabetic patients, which may be ascribed to a direct effect as well as the lipid-lowering action of the drug.

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