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Effects of Atorvastatin Treatment on sICAM-1 and Plasma Nitric Oxide Levels in Hypercholesterolemic Subjects

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Summary: This study investigated the behavior of soluble intercellular adhesion molecule-1 (sICAM-1) and serum nitric oxide (NO) products, nitrite/nitrate (NO_2^-/NO_3^-), in subjects with primary hypercholesterolemia (HCh) without other risk factors and atherosclerosis. The effect of a short-term cholesterol-lowering treatment with atorvastatin, an HMG-CoA reductase inhibitor, on the levels of sICAM-1 and NO_2^-/NO_3^- were also investigated. After 4 weeks of placebo administration, 40 HCh (15 males and 25 females) were randomized in 2 groups: 20 subjects (atorvastatin group) received 10 mg/day of atorvastatin and the remaining 20 (placebo group) continued to take placebo. At baseline and after 4 and 12 weeks of atorvastatin or placebo administration, serum sICAM-1 and NO_2^-/NO_3^- levels were evaluated. The basal levels of these parameters were compared with those of 20 healthy subjects (C), matched for sex and age. Hypercholesterolemic subjects showed sICAM-1 and NO_2^-/NO_3^- basal values that were higher (331.7 ± 60.3 ng/mL vs. 202.3 ± 32.3 ng/mL, $p<0.001$) and lower (10.4 ± 2.5 μmol/L vs. 20.7 ± 4.4 μmol/L, $p<0.01$) than controls. No correlation between sICAM-1 or NO products and plasma cholesterol values was found, whereas there was an inverse correlation between sICAM-1 and NO_2^-/NO_3^- levels. Atorvastatin administration significantly decreased sICAM-1 and increased NO_2^-/NO_3^- levels, however these changes were not correlated with the reduction of plasma cholesterol. These data support the hypothesize that patients with HCh with no signs of arterial lesions, may have latent atherosclerosis, expressed as an increase of sICAM-1 and decrease in NO product levels. An improvement in the levels of these parameters after a short-time treatment with atorvastatin was also demonstrated.

Key Words: Hypercholesterolemia—sICAM-1—Nitric oxide—Atorvastatin—Endothelial activation—Atherosclerosis.

Disorders in the release and in the function of endothelial mediators regulating vascular homeostasis result in the expression of an endothelium's dysfunctional phenotype (1,2). The increased adhesion of circulating leukocytes to the endothelium and impairment in endothelium-dependent vasorelaxation are the first events of such dysfunction. Asymptomatic subjects with conventional risk factors for atherosclerosis, such as hypercholesterolemia, exhibited signs of endothelial dysfunction at very early stage, before the formation of arterial intimal lesion (3,4).

The interaction between leukocytes and endothelial cells is mediated by adhesion molecules (CAMs), such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), which are expressed by a wide variety of cell types including the endothelial cells. Circulating forms of CAMs (sCAMs), deriving from enzymatic cleavage of the membrane-bound molecules and reflecting the expression of CAMs on the cell surface, were increased in the serum of patients with inflammatory conditions, such as atherosclerosis. Thus the concentration of sCAMs may serve as marker of the endothelial state. In hypercholesterolemia, sCAMs levels are precociously increased (5) and correlated with the degree of endothelial dysfunction (6).

Endothelium-dependent vasorelaxation is mediated by nitric oxide (NO). The defect of this mediator, which has multiple antiatherogenetic properties (7,8) impairs the vascular function by promoting vasoconstriction, platelet aggregation

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(9), cell proliferation (10), and expression of proatherosclerotic gene products such as adhesion molecules and chemokines (11,12).

An estimation of NO-dependent endothelial dysfunction is difficult to obtain because NO is a labile factor readily inactivated by O_2 radicals to form NO_2^-/NO_3^-. The relaxation of brachial and coronary arteries in response to pharmacologic or physiologic stimuli is considered an efficacious index to evaluate the endothelium's ability to release NO. The levels of stable molecules NO_2^-/NO_3^-, reflecting the amount of endogenously NO-derived production, have been used as an in vivo indicator of endothelial dysfunction as well (13,14). Patients with increased levels of cholesterol presented a decline in the levels of plasma NO_2^-/NO_3^- and more recently, it has been suggested a mechanism of cholesterol-induced impairment of NO production (15), that together with the increased degeneration of NO (16) may be responsible for endothelial dysfunction in hypercholesterolemia.

Although the administration of HMG-COA reductase inhibitors has demonstrated rapidly improved endothelial dysfunction (17) and increased NO_2^-/NO_3^- levels (18,19), recently it has reported that the cholesterol-lowering therapy may not be effective in decreasing sCAMs levels, suggesting that factors other than cholesterol may sustain endothelial activation (5,20).

In the present study we show that atorvastatin administration significantly diminishes the levels of sICAM-1 and increase those of NO_2^-/NO_3^- in HCh patients who presented with no other risk factors and atherosclerosis. Furthermore we report that a significant correlation between the decrease of sICAM-1 and NO_2^-/NO_3^- levels occurs in these subjects.

METHODS

Forty HCh (15 males and 25 females, mean age 45.25 ± 9.25 years), displaying fasting levels of total cholesterol (TC) greater than 7 mmol/L, low-density lipoprotein cholesterol (LDL-C) greater than 4.1 mmol/L, and triglycerides (TG) less than 2.0 mmol/L, were consecutively recruited from our center. Twenty healthy subjects (8 males and 12 females, mean age 42.6 ± 8.2 years) matched for body mass index, and systolic and diastolic blood pressure were selected as a control group (C) (Table 1). Exclusion criteria were arterial hypertension; body mass index greater than > 27 kg/m^2; thyroid, liver, or kidney diseases; cigarette smoking; diabetes mellitus; infectious, inflammatory or autoimmune diseases; and arterial and cardiovascular diseases. During the run-in period (4–6 weeks), all the patients underwent complete clinical and instru-

TABLE 1. Clinical Characteristics of Subjects Admitted to the Study

Characteristic	Control Subjects	Hypercholesterolemic Subjects: Placebo Group	Hypercholesterolemic Subjects: Atorvastatin Group
Number	20	20	20
Sex (m/f)	8/12	7/13	8/12
Age (yr)	42.6±8.2	45.1±9.4	45.4±9.1
Body mass index (kg/m^2)	25.4±1.3	26.2±1.7	26.1±1.5
Systolic blood pressure (mmHg)	121±12	122±9	123±8
Diastolic blood pressure (mmHg)	79±5	79±7	80±6
Serum glucose (mmol/L)	4.62±0.27	4.67±0.33	4.73±0.38
Plasma cholesterol (mmol/L)	5.16±0.30	7.61±0.23*	7.52±0.25*
Plasma triglycerides (mmol/L)	1.07±0.39	1.14±0.47	1.17±0.38
HDL-cholesterol (mmol/L)	1.35±0.22	1.38±0.31	1.35±0.36
LDL-cholesterol (mmol/L)	3.17±0.68	5.34±0.79*	5.41±0.86*

Data are expressed as mean values ± standard deviation. Statistical analysis with unpaired student *t* test.

HDL: high-density lipoprotein; LDL: low-density lipoprotein.

*$p < 0.001$ vs. control subjects.

mental examination, including electrocardiography and echo-color doppler of the heart, carotid, and peripheral vessels (Vingmed 750 CFM, Norway). Furthermore they were on a step I diet and received treatment with placebo for 4 weeks, then were randomized into 2 groups. Twenty HCh patients (atorvastatin group) received 10 mg of atorvastatin once daily before dinner and the other 20 continued to be treated with placebo (placebo group). None of them took other drugs. At baseline and after 4 and 12 weeks of treatment with placebo or atorvastatin, a sample of venous blood was taken from fasting subjects. Serum TC, HDL-C, and TG were measured enzymatically with standard laboratory methods. LDL-C levels were calculated with the Friedewald formula (21). Apoproteins AI and B were assayed by immunonephelometry, using commercial antibodies (OSAN 14/15 for Apo B and OLIED 14/15 for Apo AI, both from Behring Lab, Milan, Italy). The levels of sICAM-1 were determined on frozen serum by using monoclonal antibody-based enzyme-linked immunoasorbent assay (ELISA) (British Biotechnology Products). Briefly 100 μL of a biotinylated antibody raised against human sICAM-1 was added to each well. After washing with buffer, 100 μL of streptavidin-conjugated horseradish peroxidase were added. The sample were incubated for 1 hour and again washed with buffer. Then 100 μL of tetramethylbenzidine was added and each sample was further incubated at 37°C for 10 minutes, in the presence of acidic solution. The optical density was determined within 30 minutes using a microtiter plate reader set at 450 nm with a correction wavelength of 620 nm. The samples were assayed in duplicate and the levels were expressed in nanograms per milliliter.

Serum NO_2^-/NO_3^- levels were measured by using Greiss reagent as previously described (22). Briefly 250 μL of serum were diluted with 500 μL of ultrapure water and incubated at room temperature with 250 μL of substrate buffer (0.1 mol/L imidazole, 210 μmol/L NADPH, 3.8 μmol/L flavine adenine dinucleotide, pH 7.6) in presence of nitrate-reductase (*Aspergillus niger,* 70 IU/L, Boehringer Mannheim) for 45 minutes to convert NO_3^- to NO_2^-. The levels of total NO ($NO_2^- + NO_3^-$) were measured by reacting with Greiss reagent (58 mmol/L sulphanilamide and 3.8 mmol/L naphthalene-ethylene diamine dihydrochloride in 0.5 mol/L H_3PO_4, Merck). Each samples were then treated with 200 μL trichloroacetic acid (1.2 mol/L) and centrifuged for 5 minutes at 8000 g. The absorbance of the supernatant was measured at 525 nm and the amounts of NO_2^- were estimated from a standard curve of $NaNO_2$ obtained by enzymatic conversion of $NaNO_3$ (0 to 32 μmol/L, Merck). Because NO_2^- is limitedly present in the plasma, it was enzymatically converted by NO_3^-. Then the results were reported as $NO_3^- + NO_2^-$ and expressed in micromoles per liter.

The protocol study was approved by the local institutional committee on human research and conducted in observance of the declaration of Helsinki and successives revision. All the patients signed an informed consensus before to be included in the study.

STATISTICAL ANALYSIS

Data are expressed as mean values ± standard deviation. Differences between 2 means were analyzed using the paired or unpaired student *t* tests with Bonferroni correction for multiple comparisons. Variations of sICAM-1 and NO_2^-/NO_3^- values during placebo or atorvastatin treatment were analyzed by one-way ANOVA and ANCOVA for repeated measures. The relationship between sICAM-1, NO products, and the other variables was evaluated by the Spearman rank correlation test. Simple regression analysis was used to examine the relationship between sICAM-1 and $NO_2^- + NO_3^-$ levels. Statistical analysis was performed using statgraphics package running on an IBM-compatible Pentium 300 computer (Olivetti, Ivrea, Italy).

RESULTS

Serum sICAM-1 levels were significantly ($p<0.001$) higher in HCh (mean values, 329.4 ± 51.9 ng/mL; range, 204–585 ng/mL) compared with the C (mean values, 202.3 ± 39.6 ng/mL; range, 161–298 ng/mL) (Fig. 1). The sICAM-1 elevated levels were not correlate with TC ($r = 0.10$, p:ns), LDL-C ($r = 0.09$), or HDL-C ($r = 0.09$) levels.

The values of NO_2^-/NO_3^- were significantly ($p<0.001$) lower in HCh (mean values, 10.4 ± 2.3 μmol/L; range, 6.6–19.4 μmol/L) than C (mean values, 20.7 μmol/L; range, 12.5–32.6 μmol/L) (Fig. 1). The NO_2^-/NO_3^- values did not correlate with the levels of TC, LDL-C, HDL-C, TG or apoproteins. In contrast there was a significant inverse ($p<0.02$) correlation between sICAM-1 and NO_2^-/NO_3^- levels (Fig. 2).

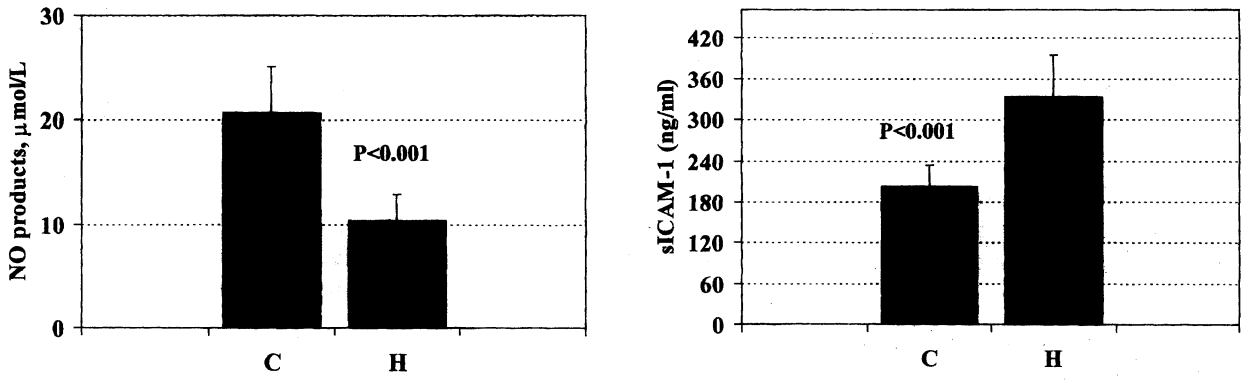

FIG. 1. Plasma NO products (*left panel*) and sICAM-1 (*right panel*) levels in 40 hypercholesterolemic patients (HCh) and in 20 control subject (C).

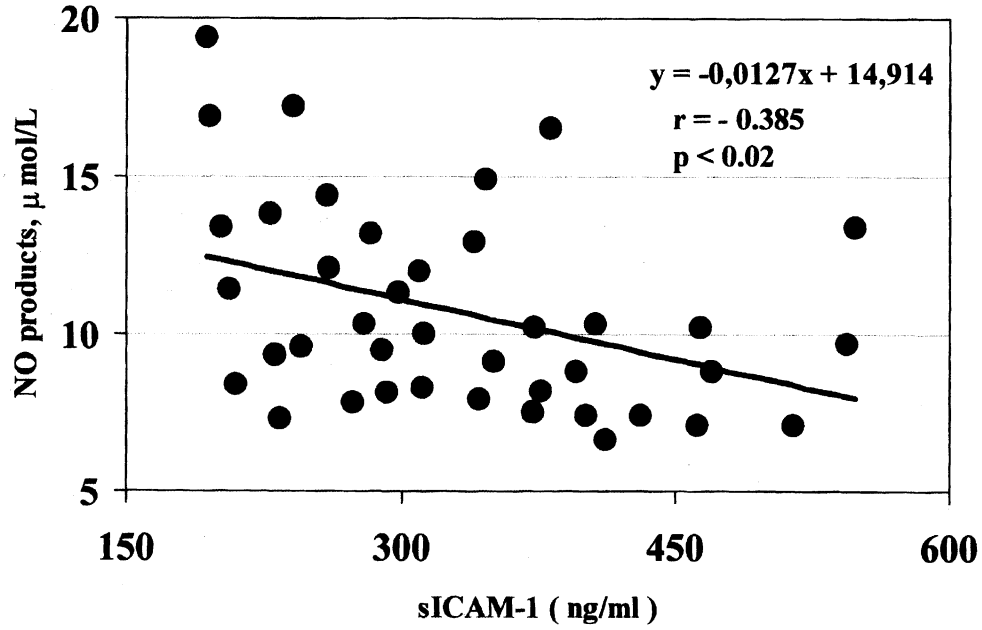

FIG. 2. Correlation between sICAM-1 values and plasma NO products levels in HCh.

Atorvastatin group displayed, at each time point, levels of TC (25.4% and 28.6% after 4 and 12 weeks), LDL-C (37.7% and 48.4% after 4 and 12 weeks), Apo B (23.4% and 31.6% after 4 and 12 weeks), TC/HDL-C ratio (33.0% and 35.7% after 4 and 12 weeks), and apo B/AI ratio (26.7% and 30.5% after 4 and 12 weeks) significantly lower in comparison with the baseline levels and placebo group values. No significant variation in the concentration of triglycerides, HDL-C, apo AI was observed (Table 2).

Atorvastatin significantly decreased serum sICAM-1 (from 333.6 ± 50.3 ng/mL of basal values to 298.6 ± 52.9 after 4 weeks and to 297.2 ± 51.3 after 12 weeks, $p<0.05$) and significantly increase NO_2^-/NO_3^- levels (from 10.4 ± 2.3 μmol/L of basal values to 14.2 ± 3.1 after 4 weeks and to 14.6 ± 2.9 after 12 weeks, $p<0.01$) (Fig. 3). There was a significant correlation between the reduction of sICAM-1 levels and the increase of NO_2^-/NO_3^- values during atorvastatin treatment ($r = 0.462$, $p<0.01$) (Fig. 4), but there was no

TABLE 2. Changes in the Levels of Lipoproteins After 4 and 12 Weeks of Atorvastatin or Placebo Treatment in HCh

Parameter		Week 0	4 Weeks	12 Weeks
Total cholesterol (mmol/L)	p	7.61±0.23	7.94±0.29	7.71±0.25
	a	7.52±0.25	5.61±0.65*	537±0.84*
Triglycerides (mmol/L)	p	1.14±0.47	1.11±0.54	1.24±0.42
	a	1.17±0.38	0.99±0.37	1.16±0.28
LDL-cholesterol (mmol/L)	p	5.34±0.79	5.72±0.53	5.64±0.43
	a	5.41±0.86	3.37±0.74*	2.79±0.83*
HDL-cholesterol (mmol/L)	p	1.38±0.31	1.43±0.27	1.41±0.24
	a	1.35±0.36	1.48±0.34	1.50±0.49
TC/HDL-c ratio	p	5.51±1.02	5.40±1.04	5.39±0.98
	a	5.57±1.44	3.73±1.09†	3.58±1.81†
Apoprotein B (mg/dL)	p	157.3±29.8	161.4±31.4	159.8±30.7
	a	155.7±24.9	119.3±23.1‡	106.4±17.5‡
Apoprotein AI (mg/dL)	p	159.2±39.8	158.9±43.7	165.6±49.3
	a	155.4±34.1	159.1±24.4	150.2±30.0
Apo B/Apo AI ratio	p	0.99±0.36	1.01±0.31	0.96±0.29
	a	1.05±0.27	0.77±0.29‡	0.73±0.21‡

Data are expressed as mean values ± standard deviation.

p: placebo group (20 subjects); a: atorvastatin group (20 subjects); LDL: low-density lipoprotein; HDL: high-density lipoprotein; TC: total cholesterol.

*$p<0.001$; †$p<0.01$; ‡$p<0.04$ vs. week 0 and placebo.

correlation between the decline in the cholesterol (TC and LDL-C and the serum sICAM-1 decrease or NO_2^-/NO_3^- increase.

DISCUSSION

We evaluated the effects of atorvastatin treatment on the serum sICAM-1 and NO_2^-/NO_3^- levels in HCh patients with no other risk factors and clinical and instrumental evidence of atherosclerosis. We also investigate whether a correlation exists between the decrease of plasma cholesterol and sICAM-1 or NO_2^-/NO_3^- levels.

Previously it has been already demonstrated that in HCh the administration of HMG-CoA reductase inhibitors increased the levels of NO_2^-/NO_3^- (18). Experimental studies recently provide new evidence that the statins may promote endothelial NO expression (19,23). Although these findings indicate that the cholesterol lowering may lead to an endothelium's restoration, such decreases may have no significant influence on the sICAM-1 levels (5,20). This suggests that mechanisms in addition to the elevated cholesterol levels may contribute to keep the endothelium active.

After 12 weeks of treatment with 10 mg/day of atorvastatin, our HCh patients had CT, LDL-C and sICAM-1 levels that were significantly reduced and NO_2^-/NO_3^- levels that were significantly increased. These variations were not correlated with the decline in cholesterol levels.

In apparent discordance with previous studies (5,20), our results indicate that a marked decrease in the cholesterol levels affects sICAM-1 and NO_2^-/NO_3^- values. However, we failed to find any correlation between the reduction of cholesterol and the changes in the values of sICAM-1 and NO products. This suggests that the cholesterol lowering may not be sufficient to normalize the endothelial function and that, in addition to the cholesterol, other factors may concur to maintain activated the endothelium. Accordingly, the endothelial improvement, achieved by using statins, may be due to different factors than the cholesterol lowering (including the direct statins' effects on the endothelium), which may contribute to restore the endothelial function.

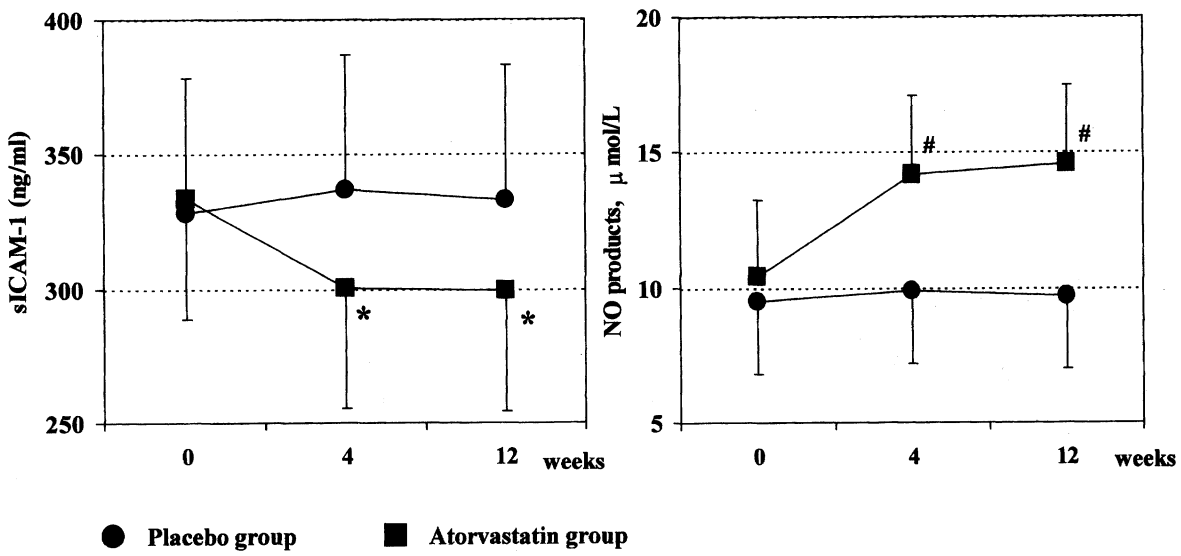

FIG. 3. Variations of sICAM-1 and plasma NO products levels during placebo (circles, 20 subjects) and 10 mg/day of atorvastatin (squares, 20 subjects) treatment. *p<0.05 and †p<0.01 vs. placebo group and basal values.

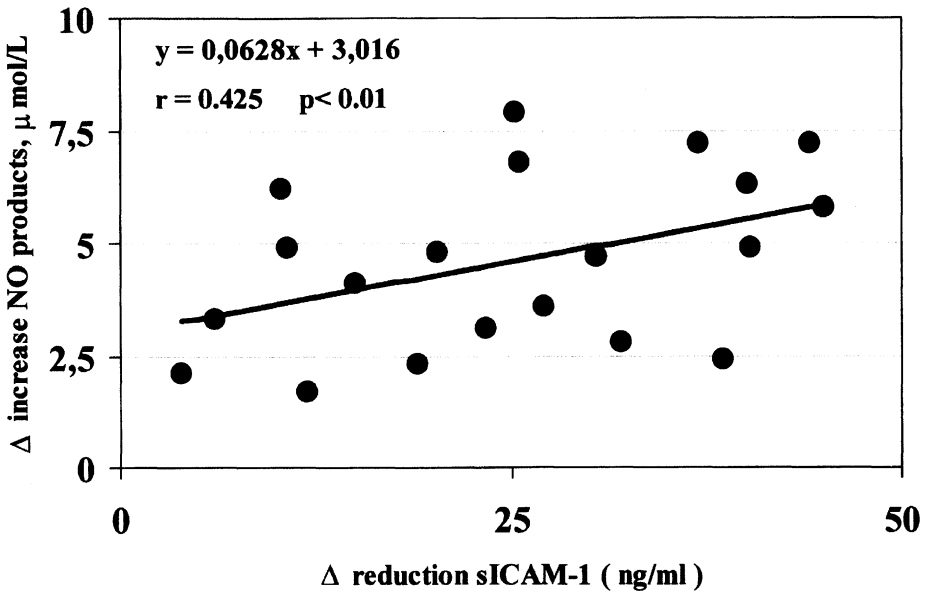

FIG. 4. Correlation between the reduction of sICAM-1 values (Δ: difference versus basal values) and the increase of plasma NO products levels (Δ: difference versus basal values) in 20 HCh after 4 weeks of atorvastatin treatment.

A significant correlation between the reduction of sICAM-1 and increase of plasma NO levels was observed in HCh patients. That confirms the studies showing that the statins increase endothelial NO synthesis (24) and inhibit endothelial O_2 formation, shifting the NO/O_2 balance toward NO. The increased NO production inhibits in turn, nuclear binding protein NF-kB, by scavenging and inactivating superoxide anions (25,26), limiting the expression of proatherosclerotic gene products, such as adhesion molecules (27).

In conclusion, our data show that in isolated hypercholesterolemia with no evidence of arterial lesions, the decrease in plasma cholesterol, even if it ameliorates the endothelial state, may not be the only goal to obtain normalization of endothelial ftmction. Further studies should be designed to investigate factors that may sustain endothelial activation.

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