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Effects of Simvastatin and L-arginine on Vasodilation, Nitric Oxide Metabolites and Endogenous NOS Inhibitors in Hypercholesterolemic Subjects

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Hypercholesterolemia is linked to endothelial dysfunction and enhancement of the endogenous inhibitor of NO synthase. The statins have lipid-lowering and pleiotropic properties, which could exert protective effects on the endothelium in hypercholesterolemia. The association of L-arginine with simvastatin could promote a further improvement on endothelial function in this condition. Thus, we investigated whether simvastatin, with or without supplementation with L-arginine, could improve endothelium-dependent vasodilation. In this study, 25 hypercholesterolemic subjects were treated according to the following protocol: washout period of 1 month; simvastatin (20 mg/day) for 2 months; simvastatin (20 mg/day) + L-arginine (7 g/day) for 2 months. From these patients, 10 were chosen at random for evaluation of vascular function by high resolution ultrassonography of the brachial artery. In subjects treated with simvastatin plus L-arginine, an increase of L-arginine levels (68%) and L-arginine/asymmetric dimethylarginine (ADMA) ratio (67%) were observed. Simvastatin reduced the plasma concentrations of NO metabolites nitrite + nitrate (NOx: 34%), *S*-nitrosothiols (RSNO: 42%), total cholesterol (25%), low density lipoprotein (LDL)-cholesterol (36%) and the LDL-cholesterol/high density lipoprotein (HDL)-cholesterol ratio (34%). Simvastatin, associated or not to L-arginine, did not affect ADMA levels and endothelium-dependent vasodilation. Our data showed that simvastatin reduced the plasma concentrations of NOx and RSNO without affecting either the levels of ADMA or endothelium-dependent vasodilation in hypercholesterolemia.

Keywords: Simvastatin; L-arginine; Endothelial function; Endogenous NOS inhibitors; Nitric oxide; Hypercholesterolemia

Abbreviations: ADMA, asymmetric dimethylarginine; BMI, body mass index; CAPS, 3-cyclohexylamino 1-propanesulfonic acid; EDTA, ethylenediamine-tetraacetic acid; eNOS, endothelial nitric oxide synthase; FITC, fluorescein isothiocyanate; HDL, high density lipoprotein; HMG-CoA, hydroxymethylglutaryl coenzyme A; HPCE, high performance capillary electrophoresis; IL-1, interleukin-1; iNOS: inducible nitric oxide synthase, K_m, Michaelis Menten constant; LDL, low density lipoprotein; NOx, nitrite+nitrate; RONS, reactive oxygen and nitrogen species; RSNO, S-nitrosothiol; SDMA, symmetric dimethylarginine; VLDL, very low density lipoprotein

INTRODUCTION

Hypercholesterolemia impairs endothelium-dependent vascular relaxation which occurs long before the formation of atherosclerotic lesion.[1] Suggestions as to how hypercholesterolemia reduces endothelial function include a decrease in the synthesis and release of endothelium-derived vascular relaxing factors[2] or a higher inactivation of nitric oxide ($^{\bullet}$NO) after its release from endothelial cells by its reaction with superoxide radical.[3] *S*-nitrosothiols (RSNO) formed by reaction of $^{\bullet}$NO and thiols are considered as a $^{\bullet}$NO reservoir, buffering the level of $^{\bullet}$NO, that is important for its storage and transport. The release of $^{\bullet}$NO from RSNO may occur via reduction by transition metal ions, thiols, ascorbate, as well as by xanthine

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oxidase.[4] Thus, the formation of RSNO would preserve $^{\bullet}NO$ biological functions contributing to improve vasorelaxation in hypercholesterolemia.[5]

L-arginine is the main substrate for nitric-oxide synthase to produce $^{\bullet}NO$ which is essential for the maintenance of vascular function. Dietary supplementation with L-arginine improves endothelium-dependent vasodilation, decreases platelet aggregation and monocyte adhesion to the endothelium, and reduces the development of atherosclerosis.[6] Moreover, L-arginine may act as an antioxidant contributing to the improvement of endothelial function in hypercholesterolemic subjects.[7] It has been shown that chronic L-arginine administration reduces vascular superoxide ($O_2^{\bullet -}$) production in isolated aortic segments and increases the urinary nitrate excretion rate in hypercholesterolemic rabbits.[8] L-arginine can also react with $O_2^{\bullet -}$ increasing the $^{\bullet}NO/O_2^{\bullet -}$ ratio, thus, avoiding the inactivation of $^{\bullet}NO$ by its reaction with $O_2^{\bullet -}$ leading to the formation of peroxynitrite ($ONOO^-$).[9]

Dietary supplementation with L-arginine has been shown to restore the biological activity of vascular $^{\bullet}NO$ and reduce the progression of atheromatous lesions in cholesterol-fed rabbit aorta.[10] Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase formed by post-translational methylation of arginine residues, followed by hydrolysis of methylated proteins.[11] The plasma concentrations of ADMA are elevated in hypercholesterolemia and related with endothelial dysfunction.[12] Moreover, it has been reported that chronic oral administration of L-arginine increases the plasma L-arginine/ADMA ratios and improves the endothelial dysfunction.[12]

Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase that limits cholesterol biosynthesis rate. Moreover, it has been shown that statins substantially reduce cardiovascular morbidity and mortality in clinical primary and secondary prevention trials.[13] Beyond the effect on low density lipoprotein (LDL) cholesterol reduction, other mechanisms, the so-called pleiotropic effects, may be involved in the protective effect of statins. In fact, it has been reported that statins may be involved in prevention of intimal thickening through induction of vascular smooth muscle cell apoptosis and inhibition of vascular smooth muscle cell migration and proliferation,[14] downregulation of monocyte chemotaxis and neutrophil-endothelial interaction,[15] increase in fibrinolytic activity,[16] plaque stabilization and upregulation of endothelial $^{\bullet}NO$ synthase (eNOS) expression and/or activity.[17]

The goal of this study was to investigate the effects of simvastatin and its association with L-arginine on endothelial function and on endogenous nitric oxide inhibitors in hypercholesterolemic subjects.

MATERIAL AND METHODS

Chemicals

Sodium nitrate ($NaNO_3$), sodium nitrite ($NaNO_2$), potassium iodide (KI), human albumin, sodium carbonate, sodium bicarbonate, sodium hydroxide (NaOH), sodium chlorite (NaCl), ethylenediaminetetraacetic acid (EDTA), L-arginine, L-homoarginine, fluorescein isothiocyanate (FITC), 3-cyclohexylamino 1-propanesulfonic acid (CAPS), boric acid, *n*-ethylmaliemide, sulfanylamide and vanadium chlorite (VCl_3) were obtained from Sigma Chemical Co. (St. Louis, MO, USA); glacial acetic acid and decanol were obtained from Merck (Darmstadt, Germany); total cholesterol, triglyceride and high density lipoprotein (HDL) cholesterol commercial kits were obtained from Biosystem (Barcelona, Spain); potassium EDTA-containing tubes (1 mg/ml final concentration, Vacutainer) were obtained from Becton Dickinson (Mountain View, CA, USA); N^G, $N^{G'}$, dimethyl-L-arginine and N^G, $N^{G'}$, dimethyl-L-arginine were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA); the simvastatin 20 mg (*Sinvascor*®) and L-arginine monohydrochloride were obtained from Laboratório Baldacci S/A, São Paulo, Brazil.

Study Subjects

The study was group comprised of 25 hypercholesterolemic patients (mean age, 46.2 ± 7.2 years; 12 male and 13 female) screened at the Instituto Dante Pazzanese de Cardiologia, São Paulo, Brazil. Among these patients, 13 were hypertensive (systolic blood pressure ≥140 mmHg) who were under antihypertensive medication (ACE inhibitors). Exclusion criteria included smoking, obesity body mass index (BMI ≥30 kg/m^2), diabetes, alcohol intake, coronary disease, kidney disease, liver disease or other serious diseases and inclusion criteria included total cholesterol >200 mg/dl, LDL-cholesterol >130 mg/dl and triglycerides <350 mg/dl. All subjects were asymptomatic. The study group was analyzed, according to the following three phase protocol: (1) a washout period (without medication) of 1 month (Basal); (2) simvastatin (20 mg/day) for 2 months (Simvastatin); (3) simvastatin (20 mg/day) + L-arginine (7 g/day) for 2 months (Simvastatin + L-arginine). After each phase of the protocol, blood was obtained from all patients in EDTA (1 mg/ml of blood). From those 25 patients, 10 were chosen at random to make the evaluation of vascular function (Fig. 1). The study was approved by the Ethics Committees of the Instituto Dante Pazzanese de Cardiologia and Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, São Paulo, Brazil. The procedures used in the study were in

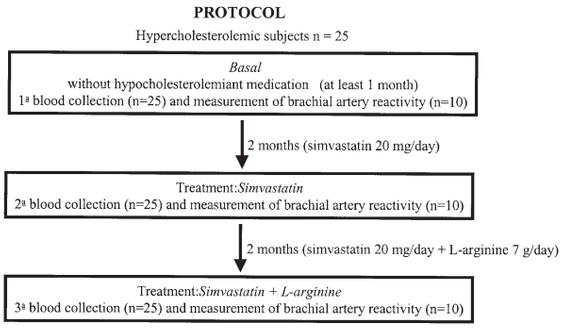

FIGURE 1 Study protocol. The 25 hypercholesterolemic subjects were kept without medication by 1 month (basal). After, they were treated with simvastatin (20 mg/day) for 2 months and then with simvastatin (20 mg/day) plus L-arginine (7 g/day) for more 2 months. Blood samples were collected and brachial artery reactivity was evaluated at basal and after each treatment.

accordance with the guidelines approved by the Ethics Committees of both institutions.

Biochemical Analysis

Blood samples were collected in EDTA-coated tubes and blood plasma was immediately separated by low speed centrifugation. The concentrations of total cholesterol, triglyceride, very low density lipoprotein (VLDL)-cholesterol and HDL-cholesterol were determined by enzymatic analyses using commercial kits. LDL cholesterol was calculated by the Friedwald equation.

ADMA (Asymmetric Dimethyl-L-arginine), SDMA (Symmetric Dimethyl-L-arginine) and L-arginine

Blood was collected by venipuncture in sterile Vacutainer tubes containing EDTA. Platelet-free plasma was obtained by centrifugation (2000*g* for 10 min at 4°C). An aliquot of plasma (200 µl), supplemented with internal standard (L-homoarginine), was treated with cold ethanol, vigorously vortexed and centrifuged at 13000*g* for 5 min at 4°C. The supernatant was then derivatized with fluorescein-5-isothiocyanate (FITC) and the reaction was performed in the dark for one night at room temperature. ADMA and SDMA were separated on a 85 cm (effective length 75 cm) × 75 µm I.D. fused-silica capillary (Polymicro Technology, Phoenix, AS, USA). The derivatized sample (50-fold diluted) was injected automatically into the high performance capillary electrophoresis (HPCE) equipment (BioFocus 2000 - Bio-Rad Laboratories, Inc.). Samples were injected by hydrodynamic injection (1 psi. sec). Separation conditions were 50 mM boric acid and 20 mM CAPS adjusted to pH 10.8. The total migration time was of 25 min at separation voltage of +30 kV. The laser-induced fluorescence detector (BioFocus LIF^2 - Bio-Rad Laboratories, Inc.) was set at 488 nm (excitation) and the peaks were monitored at 520 nm (emission).[18]

Plasma Nitrate + Nitrite (NOx) Levels

The concentrations of NOx in blood plasma were determined by chemiluminescence in gaseous phase, elicited by the reaction of $^{\bullet}$NO with ozone, after nitrate and nitrite reduction with VCl_3-saturated solution in 1 M HCl, at 90°C, by using an $^{\bullet}$NO analyzer (NOA^{TM280}; Sievers Instruments Inc.; Boulder, CO, USA). The nitrate concentrations were calculated from a $NaNO_3$ standard curve using the Bag program software 2.2 (Sievers Instruments Inc.).[44]

S-nitrosothiols

Samples (plasma) were treated with *n*-ethylmaliemide (500 nM) and sulfanilamide (1%) and were injected (500 µM) into the analyzer (NOA^{TM280}), which contained 16 ml glacial acetic acid, 4 ml KI (50 mg/ml), 600 µl decanol and 400 µl $CuSO_4$ (200 nM) at 70°C. Nitric oxide released from plasma was measured by chemiluminescence in gaseous phase and post-reaction with ozone. A standard of *S*-nitrosoalbumin was obtained by the reaction between human albumin and *S*-nitrosocysteine. The standard curve and samples were injected in triplicate. The standard curve was made by least-squares linear-regression analysis of the response of the analyzer (peak area vs. nM *S*-nitrosoalbumim).[45]

Evaluation of the Vascular Function

The vasodilation response to hyperemia of the brachial arteries was measured by high resolution ultrasound technique (SYSTEM-FIVE, General, 7.5 MHz linear array transducer). The measurements of blood flow and diameter of the brachial artery were performed initially in patients at rest. Increased forearm blood flow (reactive hyperemia) was induced by inflating a blood pressure tourniquet around the widest part of the forearm to a systolic blood pressure of 250 mm Hg for 5 min. After 30 s, the brachial artery diameters and blood flow were measured. After 15 min of reactive hyperemia, sublingual 400 µg nitroglycerin (NTG) was administered and final scans were performed after 5 min. Vessel diameters and blood flows were measured in triplicate. The brachial artery diameter and blood flow were measured at end-diastole, using intima-media interfaces or, if not well-visualized, media-adventitia interfaces as landmarks. Flow-mediated vasodilation (FMD) was calculated as the ratio of the brachial artery diameter after reactive hyperemia to

TABLE I Plasma lipids of hypercholesterolemic patients before treatment (basal) and after treatment with simvastatin (20 mg/day for 2 months) and simvastatin (20 mg/day) plus L-arginine (7 g/day) for 2 months

	Basal	Simvastatin	Simvastain plus L-arginine
Cholesterol (mg/dl)	271.5 ± 42.2	202.5 ± 26.7*	207.3 ± 30.0*
VLDL-cholesterol (mg/dl)	30.3 ± 11.7	27.0 ± 9.2	28.1 ± 9.0
LDL-cholesterol (mg/dl)	184.0 ± 42.9	118.4 ± 25.8*	122.4 ± 31.0*
HDL-cholesterol (mg/dl)	57.2 ± 9.7	56.1 ± 8.2	56.8 ± 9.2
Triglycerides (mg/dl)	151.6 ± 58.5	135.3 ± 47.1	140.4 ± 47.6
LDL-cholesterol/HDL-cholesterol	3.21 ± 0.81	2.11 ± 0.51*	2.15 ± 0.72*

Results are mean ± SEM. *Statistically significant in comparison to the basal ($p < 0.001$).

the baseline diameter, expressed as percent change. Nitroglycerin-mediated vasodilation (NTGMD) was calculated in an analogous fashion. All studies were performed in a quiet and temperature-controlled room (22–23°C).

Statistical Analysis

Data are represented as mean ± SEM. The statistical analysis was done using a parametric test (Bonferroni) with $p < 0.05$ considered significant. The correlation was analyzed by Spearman's test (Sigma Stat Software).

RESULTS

The methodologies used in this study presented adequate accuracy and replication. In all the calibration curves the linear regression coefficient (R) presented was higher than 0.97. The quantitation limit (signal–noise 10:1) for detection of L-arginine, ADMA and SDMA by HPCE was 25 nM and for detection of NOx and RSNO by chemiluminescence in gaseous phase was 10 nM.

Table I shows that the treatment with simvastatin, alone and associated to L-arginine, resulted in a reduction of total cholesterol (25%), LDL-cholesterol (36%) and LDL-cholesterol/HDL-cholesterol ratio (34%). The levels of triglycerides and VLDL-cholesterol did not change with the use of simvastatin. The supplementation with L-arginine increased 68% the L-arginine concentration (Fig. 2) and 67% the L-arginine/ADMA ratio (Fig. 3A) in relation to the treatment with simvastatin alone. In contrast, the plasma concentrations of ADMA (endogenous inhibitor of NOS) and SDMA (inactive isomer of ADMA) were not modified by both treatments (Fig. 2). Although a significant reduction of NOx (Fig. 3B) and RSNO (Fig. 3C) levels was observed with the use of simvastatin, the flow-mediated endothelium-dependent vasodilation did not change with the use of simvastatin alone or associated with L-arginine (Table II).

The data obtained after treating hypercholesterolemic subjects with simvastatin plus L-arginine indicated a positive correlation of the plasma concentrations of NOx with those of RSNO ($p = 0.033$, $r = 0.444$) and a negative correlation of NOx with the brachial artery diameter in response to hyperemia ($p = 0.015$, $r = -0.807$) (Fig. 4).

DISCUSSION

An impairment of endothelium-dependent vasodilation is an early event in atherosclerosis and occurs in hypercholesterolemia.[1] Endothelial dysfunction is generally defined as impaired endothelium-dependent vasodilation, related to reduced $^{\bullet}NO$ production or bioavailability. Reactive oxygen and nitrogen species (RONS), such as $O_2^{\bullet -}$, $^{\bullet}NO$, H_2O_2 and $ONOO^-$, can be produced in blood vessels by certain drugs and pathological conditions and induce or

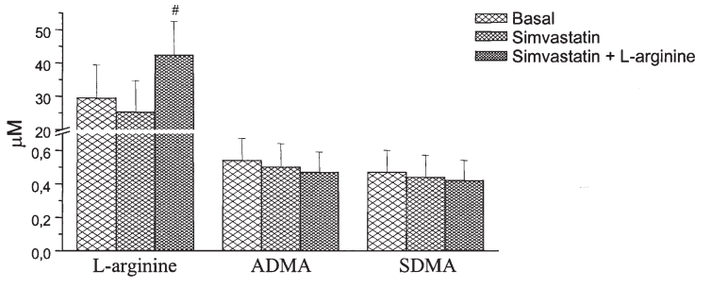

FIGURE 2 Concentrations of L-arginine, ADMA and SDMA in blood plasma of hypercholesterolemic patients before treatment (basal) and after treatment with simvastatin (20 mg/day for 2 months) and simvastatin (20 mg/day) plus L-arginine (7 g/day) for 2 months. # Statistically significant in comparison to the basal and simvastatin ($p < 0.001$).

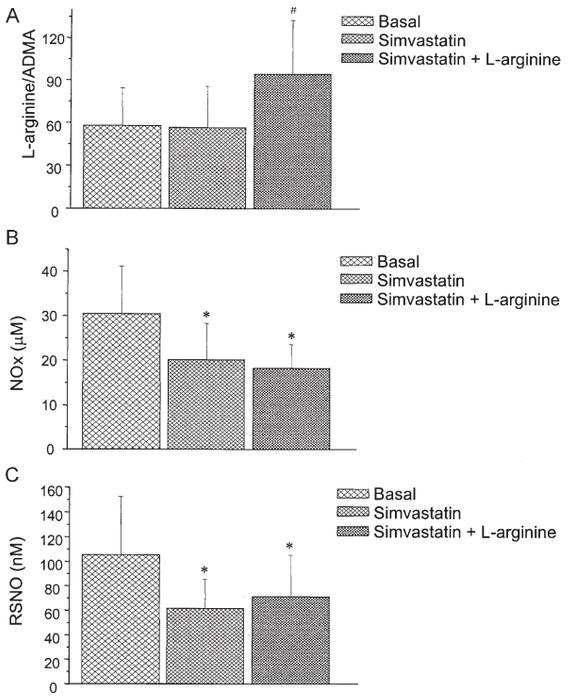

FIGURE 3 L-arginine/ADMA ratio (A), NOx (nitrite plus nitrate) (B) and RSNO (*S*-nitrosothiols) (C) in blood plasma of hypercholesterolemic patients before treatment (basal) and after treatment with simvastatin (20 mg/day for 2 months) and simvastatin (20 mg/day) plus L-arginine (7 g/day) for 2 months. # Statistically significant in comparison to the basal and simvastatin treatment ($p < 0.001$). * Statistically significant in comparison to the basal ($p < 0.001$).

accentuate endothelial dysfunction.[19] RONS may reduce •NO bioactivity through formation of peroxynitrite or by decreasing enzyme or transporter activities by oxidation of their thiol groups.[20] Endothelial dysfunction contributes to the initiation, progression and clinical manifestations of coronary heart disease. Normally, blood flow promotes a shear stress on endothelial cells, leading to nitric oxide-dependent arterial dilation. However, in patients with atherosclerosis or coronary heart disease risk factors, endothelial dysfunction leads to impaired flow-mediated endothelium dependent vasodilation or paradoxical vasoconstriction in response to increased blood flow. Flow-mediated vasodilation of the brachial and coronary arteries is correlated strongly and predict future adverse cardiovascular events.[21]

Recent studies have shown the vasculoprotective actions of statins by modulation of eNOS, independent of their lipid-lowering effects.[17] Treatment with statins has two major beneficial effects on endothelial function: (i) statins increase eNOS expression and (ii) post-translationally modulate this enzyme via a decrease in caveolin and enhancement of HSP90 recruitment promoting Akt-dependent phosphorylation. These effects facilitate •NO release, maintain a healthier endothelium and might have important implications for the impact of statins on atherosclerosis.[22]

In the present study, simvastatin (20 mg/day) reduced the concentration of total cholesterol, LDL-cholesterol and the LDL-cholesterol/HDL-cholesterol ratio, as it would be expected as statins inhibit cholesterol biosynthesis and enhances the clearance of LDL from plasma.[23] However, simvastatin did not change flow-mediated endothelium-dependent vasodilation in hypercholesterolemic subjects. Our data are in agreement with a recent report showing that six months of simvastatin therapy (40 mg/day) did not improve coronary endothelial function,[47] although are in contrast with other reports. Thus, it has been reported that the use of 10 mg/day of simvastatin by 8 weeks,[24] or 20 mg/day simvastatin during 4 weeks,[25] resulted in an improvement on vasodilation in hypercholesterolemic patients. One possible explanation for these apparently discrepant findings is the difference in the study population as it is notable that total and LDL-cholesterol levels at baseline were higher in the latter studies.

The association of simvastatin with L-arginine did not improve vasodilation in hypercholesterolemic subjects. There is not a consensus in relation to the L-arginine dose which should be used in chronic oral treatment in subjects with endothelial dysfunction. When L-arginine levels are depleted, exogenous L-arginine increases •NO and enhances endothelium-dependent vasodilation, whereas in normal nondepleted states, exogenous L-arginine does not change either •NO or endothelium-dependent

TABLE II Evaluation of vascular function (brachial artery reactivity) on hypercholesterolemic patients before treatment (basal) and after treatment with simvastatin (20 mg/day for 2 months) and simvastatin (20 mg/day) plus L-arginine (7 g/day) for 2 months

	Basal	Simvastatin	Simvastatin plus L-arginine
Blood flow (ml/min)			
Baseline	82.6 ± 26.3	77.4 ± 25.7	86.2 ± 44.9
Reactive hyperemia flow	215.5 ± 77.7	231.0 ± 90.7	220.8 ± 54.3
Post-NTG flow	112.1 ± 38.8	106.7 ± 25.9	130.1 ± 41.9
Brachial artery diameter			
Baseline (mm)	3.69 ± 0.36	3.63 ± 0.48	3.48 ± 0.42
FMD (%)	10.4 ± 12.2	13.7 ± 4.6	16.2 ± 9.5
NTGMD (%)	20.8 ± 9.2	22.4 ± 9.5	10.3 ± 13.5

FMD = flow-mediated dilation; NTGMD = nitroglycerin-mediated vasodilation. Results are mean ± SEM.

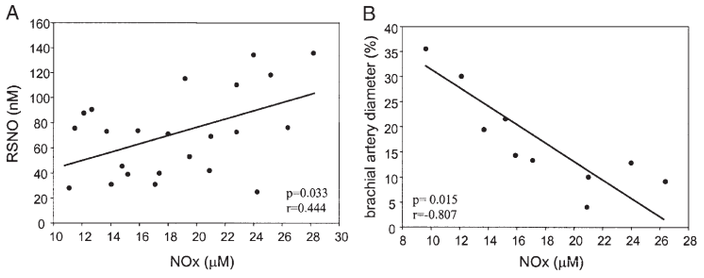

FIGURE 4 Correlation of blood plasma concentrations of NOx (nitrite plus nitrate) with those of *S*-nitrosothiols (RSNO) (A) and of NOx concentrations with brachial artery diameter (B) analyzed in the hypercholesterolemic subjects after treatment with simvastatin (20 mg/day) plus L-arginine (7 g/day). Correlation analysis was done by Pearson's test.

vasodilation.[26] It has also been demonstrated that either normal or hypercholesterolemic vessels have sufficient L-arginine levels to saturate nitric oxide synthase.[27] Furthermore, the nitric oxide synthase activity in hypercholesterolemic vessels is reported to be normal or even up-regulated rather than impaired.[28] One of the important roles of endothelium-derived $^{\bullet}NO$ is scavenging superoxide radical and a large quantity of $^{\bullet}NO$ may be expended to scavenge superoxide in hypercholesterolemia.[29] Thus, in the hypercholesterolemic subjects a relative deficiency in intracellular L-arginine could be induced by oxidative stress. However, in our study, the supplementation with L-arginine (7 g/day) did not add further benefits on endothelial function in hypercholesterolemic subjects receiving simvastatin (20 mg/day). A possible explanation for this finding may be related to the fact that some of the hypercholesterolemic subjects were also mildly hypertensive, which could mask improvements in flow-mediated vasodilation.

Several years ago the term "arginine paradox" was coined to describe the discordance between *in vitro* pharmacokinetic studies, which indicated that the Michaelis Menten constant (K_m) of nitric oxide synthase for L-arginine was in a micromolar range and that L-arginine should not be rate limiting, and the *in vivo* studies, which demonstrated that under certain conditions, e.g. hypercholesterolemia, L-arginine could enhance endothelium-dependent vasodilation and nitric oxide synthesis.[30] This arginine paradox may be explained in part by the existence of endogenous inhibitors of nitric oxide synthase and/or reduced L-arginine transport. The majority of L-arginine transport into endothelial cells occurs via the cationic amino acid transporter directly into the site where nitric oxide synthase is colocalized. Many factors can modify the activity of this transporter. In higher concentrations, L-arginine can enter into cells via other transport systems.[31] Moreover, the half-life of extracellular L-arginine is determined by the activity of argininases.[32] Several studies have shown an improvement of endothelial function by rising L-arginine/ADMA ratio.[12] The source of ADMA in hypercholesterolemia is unclear. ADMA is likely the result of the hydrolysis of methylated proteins.[11] *In vivo* lipid peroxidation results in peroxidative damage to tissue proteins and may accelerate the rate of proteolysis. Alternatively, there may be a downregulation or dysfunction of dimethylarginine dimethylaminodydrolase, the enzyme that degrades ADMA to L-citrulline.[33] Hypercholesterolemia may disturb the function or regulation of di-methylarginine dimethylaminohydrolase, thereby leading to intracellular accumulation of ADMA. Indeed, regenerating endothelial cells exhibit vasodilation dysfunction and produce more ADMA.[34] It is clear from the present study that chronic L-arginine administration (7 g/day) did not affect circulating ADMA levels. Although the reduction of total cholesterol could contribute for decreasing O_2^- production by arteries, avoiding the degradation of $^{\bullet}NO$,[3] the use of lipid-lowering therapy (simvastatin 20 mg/day) did not affect the ADMA levels. As far as we know, there are not other studies evaluating the effect of statins on ADMA.

The reduction of the plasma levels of NOx and RSNO was observed under simvastatin treatment independent of L-arginine supplementation. This may be due to a lower production of $^{\bullet}NO$ by inducible nitric oxide synthase (iNOS). The expression of iNOS as well as its functional activity has recently been reported in atherosclerotic lesions. Esaki *et al.*[35] reported the presence of iNOS in advanced atherosclerotic plaques, but not in normal vessel or in the early stages of atherosclerosis. The antiproliferative and/or cytoprotective effects of nitric NO have been associated with the persistent production of high levels of $^{\bullet}NO$ that occurs after the activation of the inducible form of NOS.[36] The expression of this enzyme in various cell types is known to be transcriptionally regulated and to be activated by a combination of pro-inflammatory signals such as ligands that activate receptors and/or cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α, and interferon-γ. The local overproduction of $^{\bullet}NO$ due to hypercholesterolemia could desensitize smooth muscle reactivity, thus, causing general

vascular hyporesponsiveness to vasoactive agents. For example, iNOS expression, enhanced cGMP levels and elevated •NO have been demonstrated in the vasculature of cholesterol-fed rabbits.[37] Rupin *et al.*[38] reported a 5-fold increase of tissue cyclic GMP concentrations (a second messenger for •NO-induced vasorelaxation) in advanced atherosclerotic lesions in the aorta from hypercholesterolemic rabbits, an evidence of the activation of •NO-guanylate cyclase pathway. Verbeuren *et al.*[39] demonstrated that the overproduction of •NO is not endothelium dependent, since it also exists in the absence of endothelium, suggesting that non-endothelial NOS has been induced in the aortas of the hypercholesterolemic rabbits. Moreover, significant expression of iNOS in the human heart was shown to be associated with a pronounced reduction of eNOS.[46] Therefore, an enhanced production of •NO by iNOS is causally related to the observed contractile dysfunction in cardiovascular diseases. Moreover, the intracellular concentration of L-arginine was drastically reduced in iNOS-over-expressing hearts.[40]

The *S*-nitrosoproteins represent 96% of the total RSNO content in human plasma, 82% of which is accounted for *S*-nitrosoalbumin, that are not normally excreted by kidneys.[41] The release of NO• from RSNO may occur via reduction by transition metal ions, thiols, ascorbate, as well as by xanthine oxidase.[42] Thus, the formation of RSNO would preserve •NO biological functions including its vasorelaxing effect.[43] Data of the present study suggest that the reduction of RSNO is due to the reduction of NOx, considering that the generation of RSNO is dependent on •NO. This fact is reinforced by the positive correlation of the plasma concentrations of NOx with those of RSNO ($p = 0.033$, $r = 0.444$). Recently, Rassaf *et al.*[48] detected the concomitant presence of *N*-nitroso and *S*-nitroso proteins in human plasma using chemiluminescence-based techniques. Both *N*- and *S*-nitroso moieties appear to be associated with the albumin fraction. Therefore, the concentration of RSNO in this study may be overestimated considering that *N*-nitroso compounds could have being included in this fraction.

In conclusion, our data showed that simvastatin reduced the plasma concentrations of NOx and RSNO without affecting either the levels of endogenous NOS inhibitors or endothelium-dependent vasodilation in the studied hypercholesterolemic subjects. The association with L-arginine did not add further benefits on endothelial function.

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