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Asymmetric Dimethylarginine Determines the Improvement of Endothelium-Dependent Vasodilation by Simvastatin

Effect of Combination With Oral L-Arginine

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Objectives We hypothesized that the level of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of endothelial nitric oxide (NO) synthase (eNOS), might determine the endothelial effects of statins.

Background Endothelial NO synthase is up-regulated by statins. However, statins failed to improve endothelial function in some studies. Asymmetric dimethylarginine inhibits eNOS by a mechanism that is reversible by L-arginine.

Methods Ninety-eight clinically asymptomatic elderly subjects had their plasma ADMA levels screened. Those in the highest (high ADMA, n = 15) and lowest quartiles of the ADMA distribution (low ADMA, n = 13) were eligible to receive, in a randomized order, simvastatin (40 mg/day), L-arginine (3 g/day), or a combination of both, each for 3 weeks. Endothelium-dependent vasodilation (EDD) was assessed by brachial artery ultrasound.

Results Simvastatin had no effect on EDD in subjects with high ADMA (6.2 ± 1.2% vs. 6.1 ± 0.9%), whereas simvastatin plus L-arginine significantly improved EDD (9.8 ± 1.5% vs. 5.3 ± 0.8%; $p < 0.01$). In subjects with low ADMA, simvastatin improved endothelial function when given alone (9.5 ± 3.2% vs. 6.1 ± 3.8%; $p < 0.001$) or in combination with L-arginine (9.0 ± 3.1% vs. 6.3 ± 3.3%; $p = 0.001$). L-arginine alone improved endothelial function in both groups. Endothelium-independent vasodilation was not affected.

Conclusions Simvastatin does not enhance endothelial function in subjects with elevated ADMA, whereas it does so in patients with low ADMA. Combination of simvastatin with oral L-arginine improves endothelial function in subjects with high ADMA, but has no additional effect in subjects with low ADMA. As NO-mediated effects may play a major role in the therapeutic effects of statins, ADMA concentration is an important factor that influences the "pleiotropic" effects of simvastatin. (J Am Coll Cardiol 2007;49:2274–82) © 2007 by the American College of Cardiology Foundation

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have been shown to enhance gene expression of endothelial nitric oxide (NO) synthase (eNOS), due to inhibition of post-translational modification of Rho proteins (1). This effect, which has also been observed with simvastatin (1), is suggested to mediate a major part of the statins' protective effects in the cardiovascular system (2). Up-regulation of eNOS messenger ribonucleic acid is the result of enhanced eNOS messenger ribonucleic acid stability in the presence of statins. This eNOS up-regulation has been suggested to also mediate the enhanced endothelium-dependent, NO-mediated vasodilation observed with statins in various animal models of hypercholesterolemia (3–5), as well as in hypercholesterolemic human subjects (6,7).

However, improvement of endothelium-dependent vasodilation has not been consistently observed in all studies performed with simvastatin. For example, Vita et al. (8) reported that 6 months of treatment with 40 mg/day of simvastatin did not improve endothelium-dependent coronary microvessel dilation despite a significant reduction in low-density lipoprotein (LDL) cholesterol. Failure to improve endothelium-dependent vasodilation was also ob-

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served with other statins (9). The reason why in some studies statins had no beneficial effect on the endothelium has remained poorly understood.

The eNOS is the key enzyme in the biosynthesis of NO in endothelial cells; it metabolizes L-arginine to NO and L-citrulline (10). Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of eNOS (11). Asymmetric dimethylarginine levels have been found to be elevated in patients with cardiovascular risk factors like hypertension, diabetes, hypercholesterolemia, and hyperhomocysteinemia (12–14). Moreover, high ADMA levels have been related to cardiovascular diseases such as peripheral artery disease, stroke, and congestive heart failure (15); both experimentally and clinically, high ADMA levels are associated with endothelial dysfunction (16). The inhibitory effect of ADMA on NOS activity can be overcome by this enzyme's natural substrate, L-arginine, suggesting that ADMA is a competitive inhibitor (17).

Recently, Janatuinen et al. (18) reported that improvement of myocardial blood flow by pravastatin was primarily dependent on the circulating ADMA concentration. In their study, subjects with ADMA levels below the median of the distribution (i.e., 0.3 μmol/l) experienced improvement of myocardial blood flow after statin therapy, whereas those with ADMA above the median did not benefit from statin treatment. This intriguing finding suggests that in subjects with elevated ADMA concentration, up-regulation of eNOS by statins may remain without the expected functional consequences (i.e., improvement of endothelium-dependent vasodilation), as the enzyme will be blocked by the endogenous inhibitor of its activity, ADMA (17). These observations led us to hypothesize that statins will not improve endothelium-dependent vasodilation in patients selected for the presence of high circulating ADMA concentrations, whereas this effect will be present in patients with low ADMA levels. In the former group of subjects, additional supplementation with L-arginine will uncover the statin's effect on endothelial function, whereas in the latter group there will be no additional effect. This hypothesis was investigated in 2 parallel studies including patients with high or low ADMA levels.

Methods

Screening for ADMA concentration. Ninety-eight clinically healthy elderly subjects were invited to participate in a screening blood sampling for circulating ADMA levels. All of these subjects filled a structured health questionnaire and had a peripheral venous blood sample drawn for analysis of ADMA. Due to the lack of reference ranges for ADMA at the time when this study was initiated, we predefined the highest quartile of the ADMA distribution to be "elevated ADMA," and the lowest quartile of the distribution to be "low ADMA."

Patients and study protocol. This trial was carried out at the Institute of Experimental and Clinical Pharmacology and Toxicology of the University Hospital Hamburg-Eppendorf in accordance with the principles outlined in the Declaration of Helsinki. The study protocol was approved by the local ethics committee, and all volunteers gave their written informed consent before enrollment.

Abbreviations and Acronyms

ADMA = asymmetric dimethylarginine
eNOS = endothelial nitric oxide synthase
FMD = flow-mediated dilation
HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A
LDL = low-density lipoprotein
NO = nitric oxide
SR = sustained release

Subjects who were allocated to the highest or lowest quartiles of the distribution of ADMA plasma levels in the screened population were consecutively invited into the study center until as many patients had agreed to participate as required by the sample size calculation, with a few additional subjects to account for potential drop-outs. The study consisted of 2 parts: in the first study, 15 subjects with ADMA levels within the highest quartile of the distribution participated. Clinically healthy male and female volunteers were included, age between 45 to 85 years. In the second study, 13 clinically healthy female and male volunteers age between 45 and 85 years with low plasma ADMA concentration were enrolled. Major exclusion criteria for all study participants were cardiovascular disease, diabetes mellitus, severe hypercholesterolemia, renal or liver insufficiency, regular intake of platelet aggregation inhibitors or HMG-CoA reductase inhibitors, hormone replacement therapy, elevation of creatinine kinase >1.5 times above the upper limit of the normal range, and all other acute or chronic diseases. All criteria were assessed by clinical examination, blood tests, and interview 1 week before the first study day. Demographic and anthropometric characteristics of both groups are given in Table 1.

Both studies were performed as observer-blinded, randomized, 3-period crossover trials.

Table 1 Demographic and Anthropometric Data

	High ADMA (n = 15)	Low ADMA (n = 13)
Gender (M/F)	11/4	5/8
Age (yrs)	59.6 (4.2)	54.5 (7.9)
Height (cm)	171.7 (6.7)	171.6 (9.7)
Weight (kg)	74.2 (11.6)	73.4 (12.7)
Current smokers (n)*	1	3
Diabetes mellitus (n)*	0	0
Hypertension (n)*	0	0
Hypercholesterolemia (n)*	12	6
Obesity (n)*	3	1
Systolic blood pressure (mm Hg)	140.5 (17.6)	118.1 (16.7)
Diastolic blood pressure (mm Hg)	83.3 (7.0)	76.5 (7.5)
Heart rate (1/min)	70.5 (10.9)	63.6 (7.0)

Data are mean (SEM) or absolute numbers, as indicated. *More than 1 risk factor may apply per subject.

After a 12-h overnight fast, each volunteer presented at the investigational clinic in the morning of the first study day. Blood and urine samples were taken.

Serum total cholesterol, LDL and high-density lipoprotein cholesterol, triglyceride levels, and serum creatinine, creatine kinase, as well as uric acid concentrations were determined immediately by standard laboratory methods using certified assays. Serum concentrations of high sensitive C-reactive protein were quantified by using a highly sensitive nephelometric assay method (Dade Behring, Eschborn, Germany). Urine samples were aliquoted and stored at −20°C until analysis.

After vascular function testing as described in the following text, volunteers received their medication for the first treatment period of 21 days. Medication was, in randomized order, either simvastatin (orally, every day, 40 mg/day; Merck, Sharp and Dohme, Munich, Germany), L-arginine sustained release (L-arginine SR; orally, 1.5 g twice a day; eNOS Pharmaceuticals, Inc., Cambridge, Massachusetts), or the combination of simvastatin plus L-arginine SR. After 21 days of drug intake, subjects returned to the clinic and underwent the same investigational procedures as on day 1. After at least 3 weeks of washout, patients received the second medication for 21 days, and after another washout period, patients received the third medication in an identical study design. Thus, each patient received each of the medications in randomized order. Repeat testing of all study parameters including ultrasound analysis of the brachial artery was performed before and after each treatment period, and treatment effects were always related to individual baseline data before each treatment period. The last dose of simvastatin medication was taken on the evening before study parameters were assessed; the last dose of L-arginine SR was taken in the early morning of the study day.

Endothelium-dependent vasodilation in the brachial artery. Endothelium-dependent vasodilation was determined by high-resolution ultrasound of the brachial artery according to the principles set by the international brachial artery reactivity task force (19). Brachial artery diameter was determined at baseline after at least 20 min of resting in the supine position arm in a quiet, temperature-controlled room (22 ± 2°C) by high-resolution ultrasound (12-MHz linear array transducer; Siena, Siemens, Germany). Longitudinal scans of the brachial artery were obtained approximately 5 cm proximal of the antecubital fossa. The transmit focus zone was set at the depth of the anterior wall. A view of a 5-cm longitudinal section of the brachial artery was recorded on S-VHS for 30 s at baseline and before and during peak (1 min) reactive hyperemia (induced by deflation of a blood pressure cuff previously inflated to 50 mm Hg above the subject's systolic blood pressure around the forearm for 5 min). Each 30-s recording was digitized (Vascular Imager 4.1.3, Medical Imaging Applications LLC, Coralville, Iowa) at a rate of 10 high-resolution frames per s (=300 frames per recording), using as specialized software (Brachial Analyzer 4.1.3, Medical Imaging Applications LLC). This allowed the averaging of vessel diameter from 25 to 40 heart beats.

Endothelium-dependent, flow-mediated dilation (FMD) was calculated as the percent change in diameter 1 min after cuff release relative to the baseline diameter before cuff release. Endothelium-independent vasodilation was determined as the difference between a second baseline diameter measurement and the maximum arterial diameter after 0.4 mg of sublingual glycerol trinitrate. Anatomical landmarks and snapshot images were used to assess FMD in the exact same vessel section on each study day and at each time point.

The mean intraindividual coefficient of variation of the baseline diameter of the brachial artery of the baseline measurements obtained on the 3 separate study days (representing the cumulative intraindividual day-to-day variation of the vessel diameter as well and the intra- and interobserver variation in measurement) was 4.20% and 4.75%, respectively, in the 2 studies.

Plasma concentrations of ADMA. Asymmetric dimethylarginine concentration was determined in plasma by means of a previously described, validated high-performance liquid chromatography method (14) after extraction of plasma samples on carboxylic acid solid phase extraction cartridges (Varian, Harbor City). Quality control sera were analyzed regularly throughout the course of this study; the coefficients of variation in this study were 1.65% intra-assay and 2.49% interassay for ADMA, 1.85% intra-assay and 3.91% interassay for symmetric dimethylarginine (SDMA), and 1.71% intra-assay and 2.97% interassay for L-arginine; the limit of quantitation was 0.04 μmol/l.

Calculations and statistical analyses. Data are given as mean ± SEM. All data were tested for normal distribution with the Kolmogorov-Smirnov test. Analysis of covariance was performed for the main efficacy parameters with treatment, period, sequence, and subject within sequence as main effects and pretreatment level as a covariate. Statistically significant differences between treatments were tested by analysis of variance followed by the Scheffé test for repeated measurements. An adjusted value of $p < 0.05$ was considered to be statistically significant. All calculations were performed with StatView version 12.0 (Cary, North Carolina) or SPSS (Chicago, Illinois) version 12.0.

Primary target variable was the intraindividual comparison of the study-medication-induced change in FMD before and after treatment with simvastatin, L-arginine, and a combination of simvastatin and L-arginine. We aimed to detect a minimal difference in the primary target variable of the mean study-medication-induced change in FMD between the treatment of 30% (in relative terms) with a 2-sided type I error protection of 0.05 and a power of 0.80. Based on previous trials and pilot data, we estimated the SD of the difference between the 2 values for the same patient to range from 20% to 50% (in relative terms), which gave a formal sample size requirement of ≥12 individuals for each group.

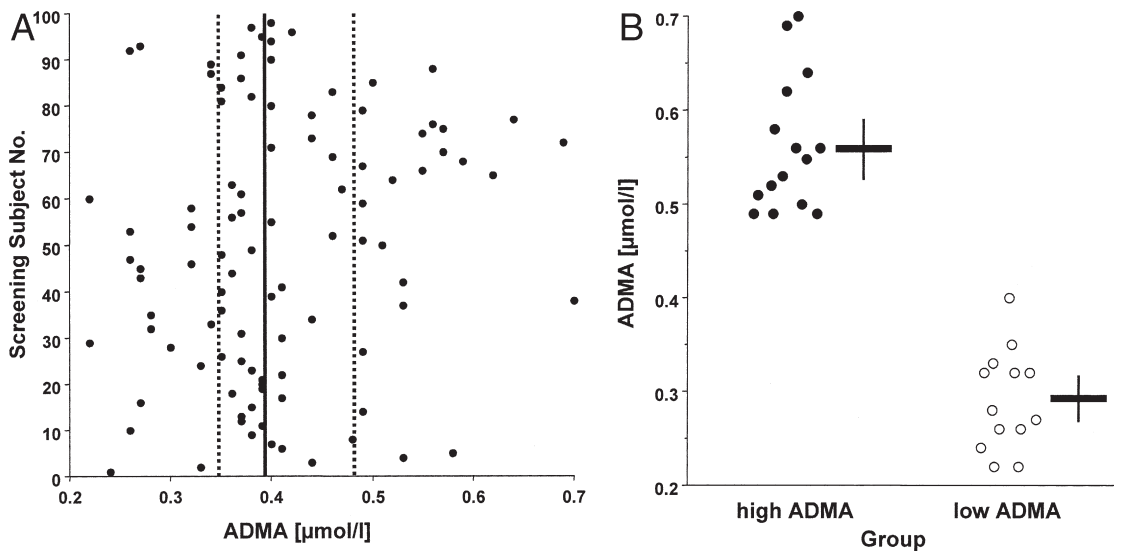

Figure 1 **Distribution of Circulating ADMA Concentrations**

(A) Distribution of circulating asymmetric dimethylarginine (ADMA) concentrations in a cohort of 98 clinically healthy ambulatory subjects. **Vertical lines** indicate the median **(solid line)**, which was 0.39 μmol/l, and the 25th and 75th percentiles (**dashed lines**; 0.35 μmol/l and 0.48 μmol/l, respectively). **(B)** Asymmetric dimethylarginine levels in study participants. Mean and SD in the 2 groups are marked by a **horizontal bar** and **vertical line**, respectively. Each subject is represented by a **circle**. In **panel B**, **filled circles** indicate subjects with high ADMA and **unfilled circles** indicate subjects with low ADMA.

Results

Distribution of ADMA plasma concentrations in the screened population. Figure 1A displays the distribution of ADMA levels in the population of 98 subjects that were screened; cutoff levels for the highest and lowest quartiles are also indicated. Mean ADMA in the total screened population was 0.41 ± 0.10 μmol/l; mean ADMA in the 15 subjects who participated in study A was 0.56 ± 0.06 μmol/l. By comparison, mean ADMA concentration in the subjects who participated in study B was 0.29 ± 0.05 μmol/l (Fig. 1B).

Effects of simvastatin, L-arginine, and their combination on endothelium-dependent vasodilation in subjects with high ADMA concentration (study A). Simvastatin alone had no significant effect on endothelium-dependent vasodilation (6.08 ± 0.94% vs. 6.16 ± 1.21% at baseline; p = NS). L-arginine SR enhanced endothelium-dependent vasodilation significantly (8.19 ± 0.87% vs. 4.88 ± 0.79% at baseline; $p < 0.05$). The greatest improvement in endothelium-dependent vasodilation, however, was induced by the combination of simvastatin plus L-arginine SR (9.89 ± 1.51% vs. 5.32 ± 0.84% at baseline; $p < 0.01$) (Fig. 2A). The effect of the combination of simvastatin plus L-arginine on endothelium-dependent vasodilation was significantly greater than that of either simvastatin alone (p = 0.024) or L-arginine SR alone (p = 0.048). Despite the variability of baseline FMD, there were no significant sequence effects between the treatment periods. There were no significant differences in brachial artery diameter before and after each treatment, or between groups (simvastatin 4.73 ± 0.17 mm to 4.79 ± 0.17 mm; L-arginine 4.60 ± 0.16 mm to 4.59 ± 0.14 mm; combination 4.75 ± 0.19 mm to 4.74 ± 0.17 mm; all p = NS). No significant carryover effects of the treatments were detected.

Endothelium-independent vasodilation induced by glyceryl trinitrate was not significantly affected by any of the treatments (Fig. 2B).

Effects of simvastatin, L-arginine, and their combination on endothelium-dependent vasodilation in subjects with low ADMA concentration (study B). Administration of simvastatin, L-arginine, as well as their combination each led to a significant increase in endothelium-dependent vasodilation (simvastatin 9.47 ± 3.16% vs. 6.08 ± 3.77% at baseline; $p < 0.001$; L-arginine 10.16 ± 2.85% vs. 5.69 ± 2.99% at baseline; p = 0.02; simvastatin + L-arginine 8.99 ± 3.06% vs. 6.34 ± 3.26% at baseline; p = 0.001) (Fig. 3A).

There were no significant differences in brachial artery diameter before and after each treatment, or between groups (simvastatin 4.43 ± 0.76 mm to 4.32 ± 0.61 mm; L-arginine 4.42 ± 0.75 mm to 4.33 ± 0.62 mm; simvastatin + L-arginine 4.29 ± 0.70 mm to 4.31 ± 0.77 mm; all p = NS). There were no significant changes of systolic or diastolic blood pressure or heart rate after any of the treatments. No significant carryover effects of the treatments were detected.

Endothelium-independent vasodilation induced by glyceryl trinitrate was not significantly affected by any of the treatments (Fig. 3B).

ADMA, SDMA, and L-arginine plasma concentrations. Asymmetric dimethylarginine concentration was neither significantly influenced by simvastatin treatment nor by

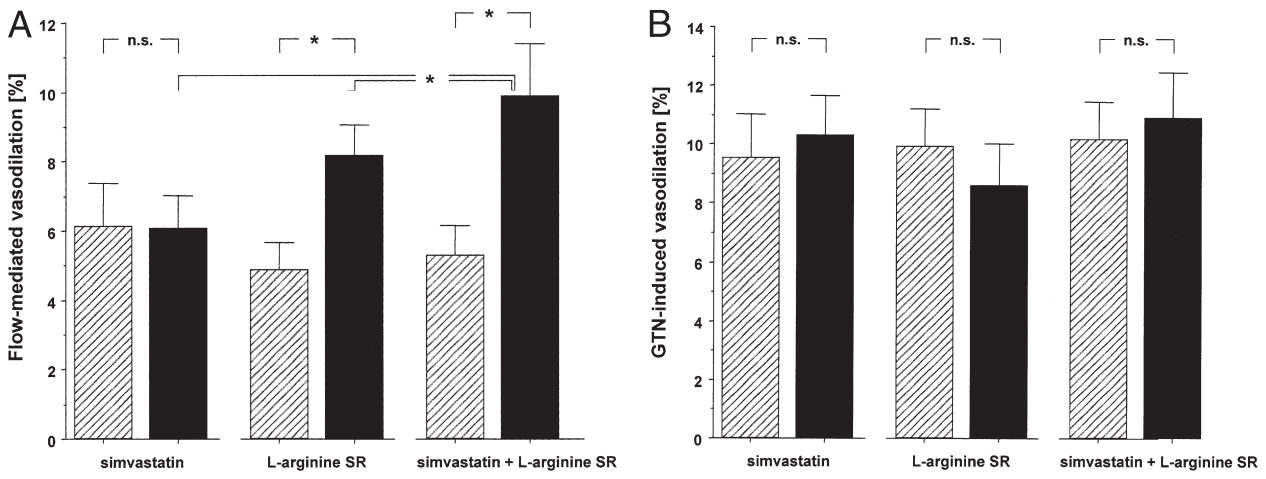

Figure 2 **Effects of Simvastatin, L-Arginine, and Their Combination on Endothelium-Dependent and -Independent Vasodilation in Subjects With High ADMA**

Endothelium-dependent **(A)** and endothelium-independent vasodilation **(B)** in the brachial artery as assessed by high-resolution ultrasound before and after 3 weeks of treatment with simvastatin, L-arginine sustained release (SR), or their combination, in subjects with elevated asymmetric dimethylarginine (ADMA) concentration. *$p <$ 0.05 versus baseline or between groups as indicated. **Hatched bars** = before treatment; **black bars** = after treatment. GTN = nitroglycerin; n.s. = not significant.

L-arginine SR supplementation in either of the groups (data not shown). During supplementation with L-arginine SR, L-arginine plasma concentration increased in both groups, resulting in significantly elevated L-arginine/ADMA ratio. SDMA plasma concentration remained stable during the study. There was a significant inverse relationship between ADMA plasma levels and the improvement of endothelium-dependent vasodilation by simvastatin (Fig. 4).

Lipoprotein and clinical chemistry profiles. Simvastatin significantly reduced LDL cholesterol by 42 ± 4% and 47 ± 2%, respectively, when it was given alone or in combination with L-arginine SR in subjects with high ADMA (Table 2). The respective ranges of reduction were 40 ± 3% and 35 ± 2% in subjects with low ADMA. L-arginine SR had no significant effect on the lipoprotein profile when given alone. There was a slight trend towards elevated serum creatine kinase, aspartate aminotransferase, and alanine aminotransferase activities during simvastatin treatment either given alone or in combination with L-arginine SR, but none of these serum enzyme activities reached a critical level

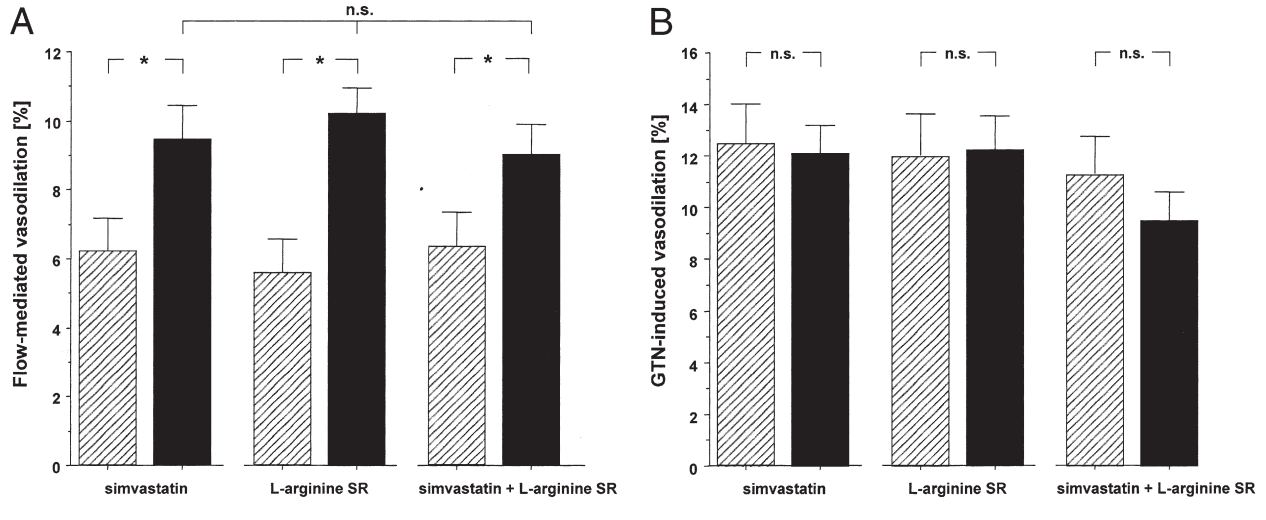

Figure 3 **Effects of Simvastatin, L-Arginine, and Their Combination on Endothelium-Dependent and -Independent Vasodilation in Subjects With Low ADMA**

Endothelium-dependent **(A)** and endothelium-independent vasodilation **(B)** in the brachial artery as assessed by high-resolution ultrasound before and after 3 weeks of treatment with simvastatin, L-arginine SR, or their combination, in subjects with low-normal ADMA concentration. *$p <$ 0.05 versus baseline or between groups as indicated. **Hatched bars** = before treatment; **black bars** = after treatment. Abbreviations as in Figure 2.

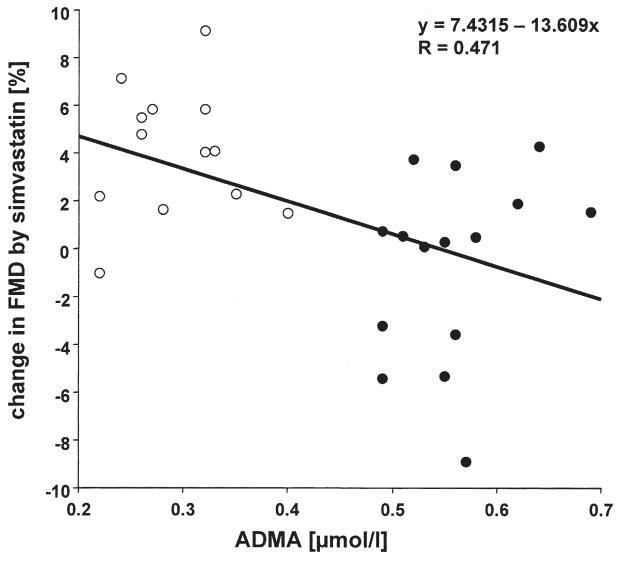

Figure 4 Relationship Between Plasma ADMA Concentration and the Endothelial Response to Simvastatin

Filled circles = subjects with high ADMA; **unfilled circles** = subjects with low ADMA. ADMA = asymmetric dimethylarginine; FMD = flow-mediated dilation.

in any of the subjects. Two subjects with high ADMA experienced muscle pain during simvastatin administered alone or in combination, and 5 subjects reported loose stools or flatulence during L-arginine SR administered either alone or in combination, but study medication was not interrupted in any of the participants. In subjects with low ADMA, no volunteer complained about side effects of simvastatin administered alone or in combination, 3 volunteers reported loose stools or flatulence, and 1 volunteer reported sleeping disturbance during L-arginine SR administered either alone or in combination, but study medication was not interrupted in any of the participants.

C-reactive protein. C-reactive protein concentration was not significantly affected by treatment with simvastatin or L-arginine SR in either of the groups. However, treatment with the combination of simvastatin and L-arginine SR significantly reduced C-reactive protein concentration in subjects with high ADMA (1.9 ± 0.3 vs. 2.7 ± 1.3 mg/l at baseline; $p < 0.05$), but not in subjects with low ADMA (Table 2).

Discussion

The main finding of the present study is that the addition of oral L-arginine SR to simvastatin improves endothelium-dependent vasodilation in patients with elevated ADMA concentration, whereas simvastatin alone had no significant effect. Moreover, endothelium-dependent vasodilation was significantly greater after combined treatment with simvastatin plus L-arginine than after L-arginine alone in this group. By contrast, in subjects with low ADMA, administration of simvastatin alone, L-arginine alone, or their combination all significantly improved endothelium-dependent vasodilation. There was no significant difference in the extent of improvement of endothelial function between the 3 treatments in this group of subjects.

Improvement in endothelium-mediated blood flow in response to statin treatment is believed to be mainly mediated by up-regulation of eNOS (1). This mechanism has been suggested to underlie the improvement in endothelial dysfunction by simvastatin or pravastatin in hypercholesterolemic rabbits (20), by simvastatin in hypercholesterolemic subjects (21) and heart transplant recipients (22), and the improvement in claudication distance induced by atorvastatin in patients with peripheral arterial occlusive disease (23).

However, studies have not unanimously shown improvement in endothelium-dependent vasodilation by statins: there have been several studies with different statins and in varying patient populations in which statin treatment did

Table 2 Lipoprotein and C-Reactive Protein Levels

	Simvastatin		L-Arginine SR		Combination	
Variable	Baseline	After	Baseline	After	Baseline	After
Subjects with high ADMA						
Total cholesterol (mg/dl)	258.1 (7.4)	181.7 (6.1)*	256.0 (6.2)	244.1 (6.9)	256.9 (6.1)	167.9 (3.8)*
LDL cholesterol (mg/dl)	168.5 (7.7)	94.5 (5.7)*	169.6 (4.9)	157.6 (6.8)	165.4 (5.5)	86.7 (4.0)*
HDL cholesterol (mg/dl)	56.8 (5.1)	58.0 (5.3)	54.3 (4.2)	54.1 (5.3)	55.7 (4.9)	58.5 (4.9)
Triglycerides (mg/dl)	165.1 (20.0)	140.2 (24.3)	161.1 (22.5)	162.0 (23.1)	178.2 (18.6)	113.7 (13.9)
C-reactive protein (U/l)	1.93 (1.35)	1.56 (0.80)	2.59 (3.22)	2.18 (2.76)	2.71 (4.87)	1.85 (1.2)
Subjects with low ADMA						
Total cholesterol (mg/dl)	254.4 (45.0)	182.2 (28.1)*	260.7 (36.5)	252.7 (45.5)	246.4 (50.3)	182.8 (24.7)*
LDL cholesterol (mg/dl)	162.6 (47.6)	90.8 (25.1)*	165.9 (40.7)	159.3 (46.5)	153.4 (53.6)	92.3 (30.5)*
HDL cholesterol (mg/dl)	62.9 (25.2)	71.6 (19.2)	68.8 (18.9)	69.3 (17.1)	68.7 (18.7)	69.5 (16.0)
Triglycerides (mg/dl)	122.5 (55.1)	99.2 (44.2)*	129.8 (83.9)	120.3 (50.8)	121.0 (73.6)	104.7 (52.2)
C-reactive protein (U/l)	1.56 (1.06)	1.18 (0.63)	1.18 (0.52)	1.05 (0.48)	1.33 (0.75)	1.27 (0.82)

Data are mean (SEM). *$p < 0.05$ versus baseline.
ADMA = asymmetric dimethylarginine; HDL = high-density lipoprotein; LDL = low-density lipoprotein; SR = sustained release.

not improve endothelial function (8,9,24,25). So far no plausible molecular explanation for these contradictory observations was proposed.

Interestingly, the notion that statin treatment leads to enhanced NO release via up-regulation of eNOS's gene expression was implicitly based on the assumption that eNOS, once its protein level is up-regulated during statin treatment, is enzymatically active. However, this may not necessarily be the case, as in contrast to in-vitro experimental studies and animal experiments, endogenous inhibitors of NO synthase have been shown to circulate in humans. Such endogenous NOS inhibitors, like monomethyl-L-arginine and ADMA, may block enzymatic activity of eNOS despite its up-regulation by statins and thereby annihilate the statins' effect on endothelial function. Indeed, Janatuinen et al. (18) recently demonstrated that treatment with 40 mg/day of pravastatin for 6 months resulted in improved myocardial blood flow only in those patients who had circulating ADMA concentrations below the median (0.3 μmol/l). Thus, ADMA (which is present at sufficiently high levels in humans, whereas monomethyl-L-arginine is only present in minor amounts in human plasma [26]) may represent one possible explanation for the contradictory findings of clinical studies on the statins' effect on endothelial function.

In our study, we have selected patients at the 2 extremes of the ADMA distribution in a population of 98 elderly, clinically asymptomatic screenees in order to test the therapeutic implication of the hypothesis outlined above: if ADMA, a competitive inhibitor of NO synthase, prevents the eNOS enzyme up-regulated by statins from being active, then displacement of the inhibitor by excess concentrations of the natural substrate, L-arginine, should result in a significant enhancement of the statin's effect on endothelium-mediated vasodilation in this group. Our results show that this is indeed the case. The combination of simvastatin plus L-arginine SR produced the strongest enhancement in endothelium-mediated vasodilation, whereas simvastatin alone had no significant effect at all in the study population selected for an elevated ADMA concentration. Furthermore, the combination of simvastatin plus L-arginine SR also induced a slightly, but significantly, greater improvement in endothelium-dependent vasodilation than L-arginine SR alone. This latter observation adds further evidence to confirm the hypothesis that up-regulation of eNOS gene expression plus supplementation of its natural substrate enable improvement of endothelial function in patients with elevated ADMA concentration.

By contrast, in subjects with low plasma ADMA levels that were investigated in the second part of our study, simvastatin alone significantly improved endothelium-dependent vasodilation in a manner that was not further enhanced by L-arginine. The extent of simvastatin's effect in the present study was comparable with that reported in previous studies (7,27). Again, these data suggest that ADMA plays an important role in the regulation of NOS activity and endothelial function in humans.

A previous experimental study has shown that L-arginine significantly enhanced cerebral blood flow in an eNOS-dependent manner in mice chronically treated with simvastatin (28). In this study, the combined effect of simvastatin plus L-arginine was absent in genetically eNOS-deficient mice, strongly supporting the important role of NO synthase in this effect. Furthermore, the combination of L-arginine (7 g/day) plus simvastatin (20 mg/day) was assessed in a clinical study in 25 hypercholesterolemic subjects (29). In the latter study, a tendency towards a stronger improvement of endothelium-dependent vasodilation by simvastatin plus L-arginine was shown as compared to simvastatin alone; however, this effect failed to reach statistical significance, probably because only 10 of 25 subjects underwent ultrasound investigation, and because vessel diameters were assessed manually. By contrast, in our study, a computer-aided image analysis software was used for determination of vessel diameter as recommended in current guidelines (19). Moreover, ADMA levels were not measured in the study by Pereira et al. (29).

Subjects were included in the first part of our study if they had ADMA levels within the upper quartile of the distribution of a group of 98 healthy elderly subjects. Thus, their ADMA levels were relatively high, but not necessarily elevated above what is now considered the normal range (30), and ADMA levels in subjects included in the second part of our study were clearly at the lower margin of the normal range. Mean ADMA levels in the 2 groups in our study were 0.29 ± 0.05 μmol/l and 0.56 ± 0.06 μmol/l, respectively. The corresponding mean ADMA levels in the 2 groups of patients investigated by Janatuinen et al. (18) were 0.28 ± 0.05 μmol/l and 0.50 ± 0.13 μmol/l, respectively (Dr. R. Laaksonen, personal communication, October 2006).

Asymmetric dimethylarginine levels comparable to those found in subjects in the first part of our study have been found to be associated with an increased risk of cardiovascular disease by various groups of investigators. Lu et al. (31) reported that high ADMA levels (median, 0.75 μmol/l) were prospectively associated with an increased risk of experiencing major adverse cardiovascular events in a group of 153 patients with stable coronary heart disease undergoing coronary angioplasty. In another large prospective clinical study including 1,874 patients with stable coronary heart disease, patients whose ADMA levels lay within the highest tertile of the distribution (ADMA concentration >0.70 μmol/l) had a 2.5-fold elevated risk of major adverse cardiovascular events or death (32). We found that ADMA levels above 0.86 μmol/l were associated with a 4-fold elevated risk of perioperative cardiac events in a population of mixed cardiovascular risk (33).

The biochemical mechanism leading to high ADMA levels in the present study has not been addressed. Reduced activity of dimethylarginine dimethylaminohydrolase, the

enzyme that inactivates ADMA (34), is one possible mechanism, as reduced renal excretory function was not detected according to calculation of creatinine clearances.

Our study has several limitations: simvastatin treatment was initiated based upon the hypothesis that ADMA may modulate the endothelial response to statins, despite the fact that elevated ADMA concentration confers no indication for statin treatment. We may, thus, have underestimated the effect of simvastatin on endothelium-dependent vasodilation, as subjects were not specifically selected for the presence of hypercholesterolemia in our study. However, 12 of 15 study participants with high ADMA had serum LDL cholesterol concentrations above 160 mg/dl, and none had a serum LDL cholesterol concentration below 130 mg/dl. By contrast, only 6 of 13 participants with low ADMA had hypercholesterolemia. This finding may relate to a frequent coincidence of elevated ADMA and cholesterol concentrations in the population, suggesting that many patients treated with statins for their high cholesterol levels may also display elevated ADMA concentration. We have reported earlier that hypercholesterolemic patients, even when clinically asymptomatic, are characterized by elevated ADMA concentration, which contributes to endothelial dysfunction in a manner that is reversible by L-arginine (14). Elevated ADMA concentration was unaffected by statin treatment in our study, as was reported by others (18,29). This finding may point to the fact that elevation of ADMA concentration occurs independently of LDL cholesterol and that it is not affected by molecular pathways modulated by statins, such as the Rho kinase pathway (35).

The relatively small sample size and the short duration of the study did not allow us to study clinical end points. Nevertheless, it was sufficient to significantly detect clinically relevant changes in endothelium-dependent vasodilation. Flow-induced vasodilation by using the method we applied (i.e., upper-arm cuff inflation) as a surrogate of endothelium-mediated vasodilation is not exclusively NO-dependent (19,36,37). Thus, the possible "dilution" of any effects on endothelial NO formation by pharmacologic intervention due to non–NO-mediated vasodilation may have reduced the sensitivity to detect changes in the main end point. The significant change in flow-mediated vasodilation that we observed after L-arginine alone or in combination with simvastatin shows that our method was sensitive enough to detect clinically relevant changes in endothelial function. Finally, we do not have data available from this study to show that exogenous L-arginine resulted in a significant elevation of intracellular L-arginine levels. However, in a recent study, we were able to show by means of stable isotope labelling that exogenously applied L-arginine is being taken up readily into cells with no difference between hypercholesterolemic and control animals (38), and that the ratio of L-arginine over ADMA may determine endothelial function.

Conclusions

Our study provides evidence that the endogenous inhibitor of NO synthase, ADMA, may not only be a novel, independent cardiovascular risk marker as demonstrated in other recent studies (31–33,39) and summarized in several reviews (15,16), but high ADMA levels may also diminish the ability of statins to improve endothelium-dependent vasodilation. Evidence in favor of our hypothesis is strengthened by a parallel study that shows that this effect of simvastatin is retained in subjects from the same population, but with low normal ADMA levels. As the ability of statins to improve endothelial function has been suggested to be a major contributor to this class of drugs' beneficial effects on morbidity and mortality, further studies should address the question whether ADMA concentration should be monitored before initiating statin treatment, and addition of L-arginine might be considered in patients with elevated ADMA levels.

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