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Acute Intravenous L-Arginine Infusion Decreases Endothelin-1 Levels and Improves Endothelial Function in Patients With Angina Pectoris and Normal Coronary Arteriograms

Correlation With Asymmetric Dimethylarginine Levels

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Background—We tested the hypothesis that asymmetric dimethylarginine (ADMA) levels could be elevated and influence endothelin-1 and nitric oxide release and action in patients with cardiac syndrome X (CSX). In addition, we evaluated whether an intravenous infusion of L-arginine would improve endothelial function in these subjects.

Methods and Results—Nine patients with CSX and 14 control subjects underwent a continuous infusion of L-arginine (0.125 g/min) or saline for 120 minutes. Sixty minutes after L-arginine or saline infusions, an intravenous insulin bolus (0.1 U/kg) combined with a euglycemic clamp was performed. Basal ADMA and endothelin-1 levels were higher in patients with CSX than in controls. At the end of the first hour of infusion, compared with saline, L-arginine infusion increased basal forearm blood flow, nitrite and nitrate (NOx), and forearm cGMP release and decreased endothelin-1. After insulin bolus, during saline, insulin-induced NOx, endothelin-1, and forearm cGMP release was almost abolished. Conversely, L-arginine restored a physiological profile of all endothelial variables compared with control subjects. In control subjects, compared with saline infusion, L-arginine infusion did not modify any parameter. ADMA levels were positively correlated with basal endothelin-1 levels and negatively correlated with insulin-induced incremental levels of NOx and forearm cGMP release.

Conclusions—Plasma ADMA levels are increased in patients with CSX, and they are correlated with increases in endothelin-1 and reductions in insulin-induced increments in plasma NOx and cGMP, effects that are reversed by intravenous L-arginine. These data suggest that increased ADMA levels play a role in the abnormal vascular reactivity that is observed in patients with CSX. (*Circulation*. 2003;107:429-436.)

Key Words: endothelin ■ nitric oxide ■ amino acids ■ angina ■ arteries

The endothelium plays a pivotal role in maintaining homeostasis of the blood vessels. It synthesizes biologically active substances that regulate vascular tone, modulates blood cell-vessel wall interaction, prevents thrombosis, and influences smooth muscle cell growth.¹

In control subjects, Baron² and Steinberg et al³ demonstrated that insulin-mediated vasodilation is largely dependent on the action of insulin on nitric oxide (NO) activity. Conversely, patients with angina pectoris and angiographically normal coronary arteries (coronary syndrome X [CSX]) showed blunted NO and endothelin-1 responsiveness to intravenously infused insulin in the presence of high basal endothelin-1 levels and normal basal NO levels,⁴ which suggests a defect in NO synthesis.⁵ In agreement with this

hypothesis, Egashira et al⁶ showed that an acute intracoronary infusion of L-arginine was able to normalize NO-dependent intracoronary vasodilation in these subjects, whereas Botcher et al⁷ were unable to confirm these results. An elevation in levels of asymmetric dimethylarginine (ADMA), which is an endogenous inhibitor of NO synthase, might be another possible mechanism to explain the defect in vasodilation in these patients.⁸ In fact, previous studies documented that ADMA negatively influences NO-mediated vasorelaxation^{9,10} and is correlated with mononuclear cell adherence to the endothelium.¹¹

L-Arginine is a precursor of NO, and both in vitro and in vivo studies have demonstrated that it can augment vascular dilation under certain conditions.¹² In particular, in young

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hypercholesterolemic adults, the administration of L-arginine results in significant improvement in endothelium-dependent dilation after 4 weeks.¹³ In addition, Theilmeier et al¹⁴ showed that oral L-arginine administration was associated with a reduction in the adhesiveness of mononuclear cells, which suggests a reduction of the atherogenic processes in human. Interestingly, Boger et al⁹ have shown that administration of L-arginine reverses the endothelial vasodilator dysfunction associated with elevated ADMA levels. Similar results were found in cholesterol-fed rabbits treated with L-arginine dietary supplementation.^{15–17}

In the present study, we evaluated whether ADMA plasma concentrations were elevated in patients with CSX compared with control subjects and to what extent they were correlated with indexes of endothelial dysfunction. Moreover, the effects of an acute systemic intravenous infusion of L-arginine, at doses unable to stimulate insulin secretion, on basal endothelin-1 levels and on insulin-mediated nitrite and nitrate (NOx), endothelin-1, and forearm cGMP release were evaluated. It was previously demonstrated that the release of endothelial factors such as endothelin-1 and NO is modulated by an acute administration of supraphysiological doses of insulin.¹⁸ Thus, an intravenous insulin bolus combined with a euglycemic clamp was performed, which allowed us to evaluate simultaneously the dynamic effect of insulin on endothelin-1 and NO release and activity.

Methods

Patients

All subjects gave informed consent to participate in the study, which was approved by the local ethics committee. Nine patients with CSX with rest and/or effort angina pectoris, a reproducible positive exercise test (≥ 1 mm planar or downsloping ST-segment depression), and angiographically smooth epicardial coronary arteries were studied. In all, prolonged hyperventilation and/or ergonovine administration, performed during coronary angiography, failed to induce epicardial coronary spasm. In these patients, all treatments were withdrawn 15 days before the study, which was performed only after an angina-free period of at least 3 days. All patients were recruited in the Clinical Cardiology Unit, Department of Cardiology and Cardiovascular Science, H. San Raffaele Hospital.

Fourteen control subjects matched for age, sex, body mass index, lipids, and insulin levels were studied as controls. The clinical characteristics of patients and controls are reported in Table 1. In all subjects, physical examination, chest radiograph, and monodimensional and 2D echocardiographic and Doppler studies were normal. They had no diabetes, hypertension, left ventricular hypertrophy, pericardial or valve disease, or cardiomyopathy. All study subjects were nonsmokers.

Experimental Protocol

Patients with CSX and the control subjects underwent 2 different tests, in random order, with at least a 7-day interval in between. All subjects were admitted to the Metabolic Unit in the morning after an overnight fast. The tests consisted of a continuous infusion of L-arginine (0.125 g/min) or saline for 120 minutes. Sixty minutes later, an intravenous insulin bolus (0.1 U/kg) combined with a euglycemic clamp and forearm indirect calorimetry was performed. The dose of L-arginine was fixed at 0.125 g/min, according to preliminary pilot studies performed to find a dose of L-arginine unable to increase insulin secretion, as suggested by Van Haefen et al.¹⁹ The validation of our method is reported elsewhere.^{4,18}

Arterialized samples were withdrawn for ADMA, endothelin-1, nitrate and nitrite (end products of NO metabolism [NOx]), cGMP

(second messenger of NO), and insulin measurements, and deep venous samples to measure forearm cGMP release were also taken. The day after each test, patients and control subjects underwent a continuous infusion of L-arginine (0.125 g/min) or saline for 60 minutes. Basal and postischemic forearm blood flows were evaluated by the venous occlusion plethysmography technique, as reported previously.^{20,21}

Assays

Plasma glucose was measured with a glucose-oxidase-based analyzer (Beckman Glucose Analyser 2, Beckman Instruments). Serum insulin levels (intra-assay coefficient of variation [CV] 3.0%, inter-assay CV 5.0%) were assayed with a microparticle enzyme immunoassay (IMX, Abbott Laboratories, Diagnostics Division). Endothelin-1 samples were measured with a commercial radioimmunoassay kit (NEN Life Science Products) with an intra-assay CV of 3.0% and an interassay CV of 11.9%. cGMP was assayed with a radioimmunoassay kit (Amersham International).

NOx levels were evaluated through the measurement of metabolic end products, ie, nitrite and nitrate, by enzymatic catalysis coupled with Griess reaction, as reported previously.²² ADMA was extracted from plasma samples by cation-exchange Strata SCX 100-mg columns (Phenomenex) as reported by Pettersson et al²³ and assayed by high-performance liquid chromatography as described by Pi et al.²⁴

Calculations and Statistical Analysis

All values are expressed as mean \pm SEM at each time interval. Shaded areas in Figures 1, 2, and 3 represent mean \pm 1SD for observations in 14 control subjects during saline infusion. The net forearm balance of cGMP was calculated as $(A-dV)\times F$, where A and dV indicate arterial and deep venous cGMP concentrations and F is the forearm blood flow. Two-way ANOVA for repeated measures was applied to test differences during the test between groups (StatView, Abacus Concepts). When the F ratio was significant, to assess the effects of L-arginine, we used the Student's *t* test. A 2-tailed probability level of less than 0.05 was considered statistically significant.

Results

Effects of Saline or L-Arginine Infusions in Control Subjects

Compared with saline infusion, during the first hour of L-arginine infusion, there was a significant increase in forearm

TABLE 1. Clinical, Hormonal, and Metabolic Details of the Two Study Groups

	Patients With CSX	Control Subjects
Sex, male/female	4/5	6/8
Age, y	52 \pm 2	51 \pm 2
Weight, kg	66.4 \pm 2.4	66.7 \pm 3.0
Body mass index, kg/m ²	24.1 \pm 0.5	23.6 \pm 0.9
Waist/hip ratio	0.89 \pm 0.01	0.88 \pm 0.01
Systolic blood pressure, mm Hg	128 \pm 4	123 \pm 3
Diastolic blood pressure, mm Hg	75 \pm 2	76 \pm 2
Plasma glucose, mmol/L	5.12 \pm 0.16	4.59 \pm 0.10
Serum insulin, pmol/L	45.6 \pm 6.8	41.8 \pm 6.9
Serum triglycerides, mmol/L	1.22 \pm 0.11	1.19 \pm 0.08
Plasma free fatty acids, mmol/L	0.70 \pm 0.16	0.69 \pm 0.06
Serum cholesterol, mmol/L	5.78 \pm 0.20	5.80 \pm 0.45
Serum HDL cholesterol, mmol/L	1.38 \pm 0.34	1.54 \pm 0.33

Values are mean \pm SEM.

blood flow and forearm cGMP release (Table 2). Conversely, no changes were found during measurement of NOx and endothelin-1 (Table 2), insulin (4.5 ± 0.3 versus 4.6 ± 0.3 $\mu\text{U/mL}$; $P=\text{NS}$), C-peptide (1.0 ± 0.1 versus 1.1 ± 0.2 pg/mL ; $P=\text{NS}$), and glucose levels (4.5 ± 0.2 versus 4.3 ± 0.2 mmol/L ; $P=\text{NS}$).

During saline infusion, insulin bolus elicited a significant increase of circulating NOx and endothelin-1 levels, with results similar to those reported previously.⁴ Forearm cGMP release and forearm blood flow were significantly higher. Similar patterns were observed for all parameters after insulin bolus during L-arginine infusion (Table 2). No changes were observed in systolic and diastolic blood pressure (data not shown). Because no differences were observed in control subjects between saline and L-arginine infusion, endothelial parameters in patients affected by CSX were compared with shaded areas representing the mean \pm 1SD for observation in control subjects during saline infusion, as shown in Figures 1, 2, and 3.

Effects of Saline or L-Arginine Infusions in Patients Affected by CSX

Before the start of saline or L-arginine infusions, no differences were found for any of the variables studied in patients with CSX and control subjects (Figures 1, 2, and 3), except that endothelin-1 levels were significantly higher (8.1 ± 0.3 and 7.4 ± 0.5 versus 4.9 ± 1.2 pg/mL ; $P < 0.01$; Figure 2) and there was a significant increase in ADMA levels (0.70 ± 0.06 versus 0.44 ± 0.02 $\mu\text{mol/mL}$; $P < 0.001$) in patients with CSX compared with control subjects.

During the first hour of saline or L-arginine infusions, insulin and glucose levels remained unchanged in both tests

TABLE 2. Forearm Blood Flow and Endothelial Function During Saline and L-Arginine Infusions in 14 Normal Controls

	Saline	L-Arginine
Forearm blood flow, $\text{mL} \cdot 100 \text{ mL}^{-1} \cdot \text{min}^{-1}$		
-60 min	2.7 ± 0.2	2.2 ± 0.2
0 min	2.5 ± 0.2	$2.9 \pm 0.2^*$
60 min	$3.1 \pm 0.3^*$	$4.3 \pm 0.7^*$
NOx, $\mu\text{mol/L}$		
-60 min	24.3 ± 2.6	21.5 ± 2.0
0 min	24.6 ± 3.1	23.0 ± 2.1
Incremental area, $\mu\text{mol/L}$ 0–60 min	257.8 ± 114.4	256.2 ± 68.6
Endothelin-1, pg/mL		
-60 min	3.04 ± 0.61	3.57 ± 0.23
0 min	3.05 ± 0.61	2.51 ± 0.16
Incremental area, pg/mL 0–60 min	46.0 ± 23.5	22.3 ± 14.8
Forearm c-GMP release, $\mu\text{mol} \cdot 100 \text{ mL}^{-1} \cdot \text{min}^{-1}$		
-60 min	1.63 ± 0.21	1.64 ± 0.08
0 min	1.58 ± 0.26	$2.42 \pm 0.22^{\dagger}$
Incremental area, $\mu\text{mol} \cdot 100 \text{ mL}^{-1} \cdot \text{min}^{-1}$ 0–60 min	146.4 ± 35.5	174.6 ± 69.4

Intravenous insulin bolus (0.1 U/kg body weight) was infused at time 0. * $P < 0.05$ vs time -60 min; $\dagger P < 0.05$ vs saline infusion.

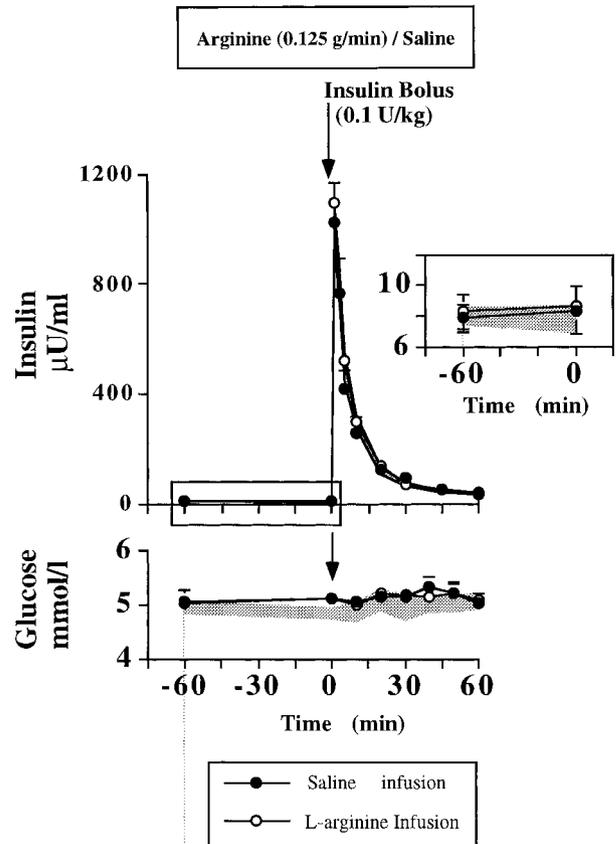


Figure 1. Insulin and glucose levels after intravenous administration of 0.1 U/kg of insulin in 9 patients with CSX during saline or L-arginine infusion. Data are mean \pm SEM. Shaded areas represent mean \pm 1SD for observations in 14 control subjects during saline infusion. Inset shows magnification of insulin data in first hour of saline or L-arginine infusions to demonstrate that L-arginine, at dosage administered, did not modify basal insulin levels.

(Figure 1). During the same period, saline infusion did not modify endothelin-1, NOx, or forearm cGMP release (Figure 2), with no changes in forearm blood flow or systolic and diastolic blood pressure (Figure 3).

Conversely, L-arginine infusion decreased endothelin-1 levels by 29.8%, increasing circulating NOx by 8% ($P < 0.05$) and forearm cGMP release by 63% ($P < 0.01$; Figure 2). This was accompanied by a 25% increase in forearm blood flow, whereas systolic and diastolic pressure decreased by 8% and 8.6%, respectively ($P < 0.01$; Figure 3). Maximal postischemic forearm blood flow significantly increased after L-arginine infusion (29.6 ± 3.8 versus 21.1 ± 1.7 $\text{mL} \cdot 100 \text{ mL}^{-1} \cdot \text{min}^{-1}$, L-arginine versus saline, respectively; $P < 0.01$), reaching values similar to those found in control subjects (33.7 ± 4.9 $\text{mL} \cdot 100 \text{ mL}^{-1} \cdot \text{min}^{-1}$; $P=\text{NS}$ versus L-arginine; $P < 0.01$ versus saline).

Insulin Effects on Blood Pressure and Endothelial Activity During Saline or L-Arginine Infusions in Patients Affected by CSX

One minute after the insulin bolus, insulin levels peaked at ≈ 1000 $\mu\text{U/mL}$ in all tests and rapidly declined to basal levels; plasma glucose was successfully clamped to baseline

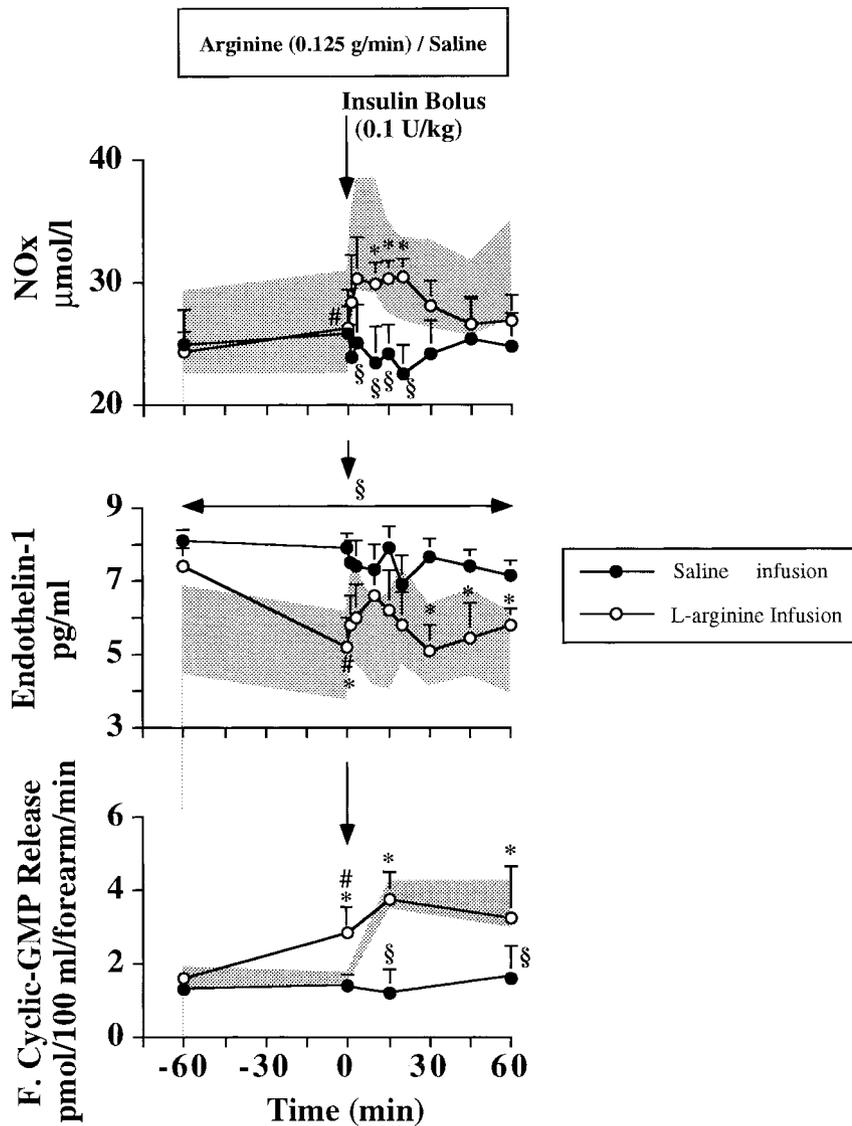


Figure 2. Circulating NO_x, endothelin-1, and forearm (F.) cGMP release in 9 patients with CSX after intravenous administration of 0.1 U/kg of insulin in 9 patients with CSX during saline or L-arginine infusion. Data are mean \pm SEM. Shaded areas represent mean \pm 1SD for observations in 14 control subjects during saline infusion. * P <0.05 vs saline infusion; # P <0.01 vs time -60 minutes; § P <0.05 vs control subjects.

levels with a CV <6% in all tests (Figure 1). In Figures 2 and 3, endothelin-1, NO_x, forearm cGMP release, forearm blood flow, and blood pressure levels after insulin bolus are shown. Endothelin-1 response was flat in patients affected by CSX, remaining higher with respect to our control range. In addition, NO_x levels did not significantly increase in patients affected by CSX, remaining at levels that were always lower than those in control subjects. Forearm cGMP release, forearm blood flow, and blood pressure remained unchanged compared with basal levels. Compared with saline, L-arginine was able to normalize endothelin-1, NO_x, forearm cGMP, and forearm blood flow patterns, with a slight decrease in systolic and diastolic blood pressure.

Correlation Between ADMA Levels and Basal and Insulin-Mediated Endothelial Function

When all subjects were pooled, ADMA levels were positively correlated with basal endothelin-1 levels ($r=0.57$, $P<0.005$; Figure 4) and negatively correlated with insulin-induced incremental levels of NO_x ($r=-0.44$, $P<0.05$; data not shown), forearm cGMP release ($r=-0.44$, $P<0.05$; Figure

4), and forearm blood flow at the end of the test ($r=-0.35$, $P<0.05$; Figure 4).

Discussion

We tested the hypothesis that ADMA levels could be elevated and influence endothelin-1 and NO release and action in patients with CSX. In addition, we evaluated whether an increment in NO availability by an acute L-arginine infusion might improve endothelial function in these subjects.

ADMA levels were elevated in patients with CSX, and this increment was strictly correlated with basal endothelin-1 levels and negatively correlated with insulin-induced NO_x and forearm cGMP release. In addition, an acute intravenous L-arginine administration, which was unable to stimulate insulin secretion, decreased endothelin-1 levels and enhanced NO_x and cGMP levels and release, normalizing insulin-stimulated endothelin-1 and NO_x release and activity.

Influence of ADMA Levels on Endothelial Function in Patients With CSX

Previous studies have shown that elevated ADMA levels are present in patients with vascular disease,²⁵ hypercholesterolemia,⁹

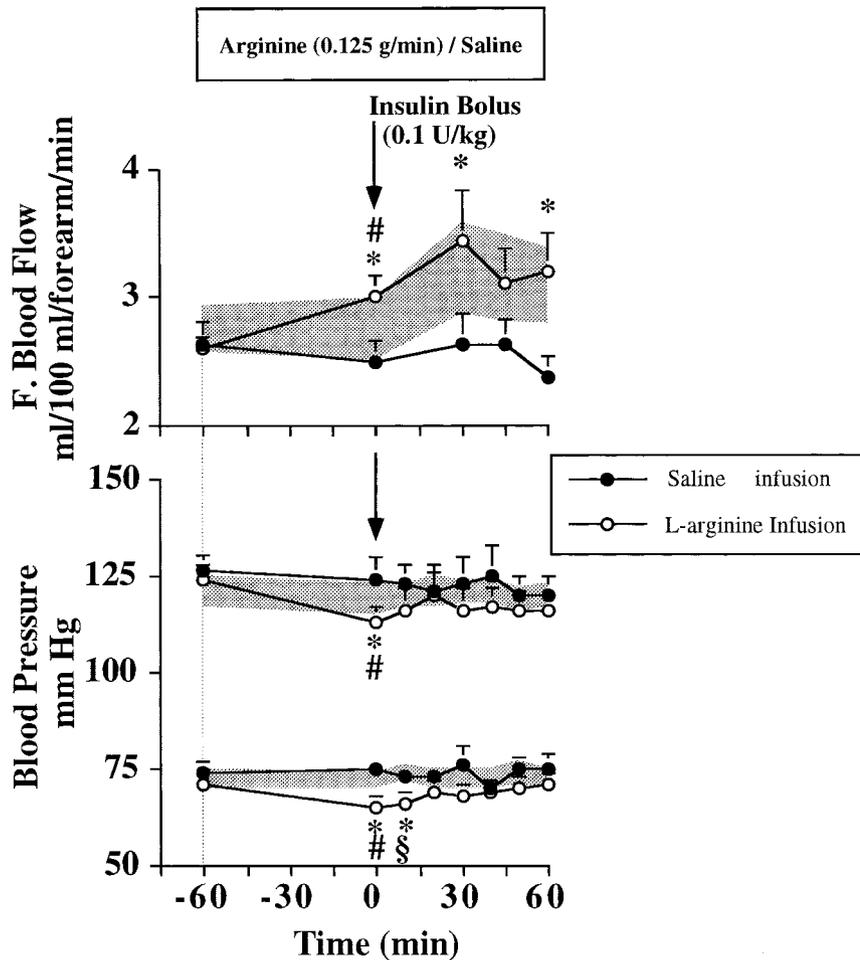


Figure 3. Forearm blood flow (F. Blood Flow) and systolic and diastolic blood pressure after an intravenous administration of 0.1 U/kg of insulin in 9 patients with CSX during saline or L-arginine infusion. Data are mean \pm SEM. Shaded areas represent mean \pm 1SD for observations in 14 control subjects during saline infusion. * P < 0.01 vs saline infusion; # P < 0.01 vs time -60 minutes; § P < 0.01 vs control subjects.

and insulin resistance syndrome.²⁶ Interestingly, for the first time, it was possible to show increased ADMA levels in patients with CSX. These diseases appear to have in common, in addition to increased ADMA levels, increased endothelin-1 concentrations and reduced NO-dependent vasodilation. Moreover, in the present study, ADMA levels significantly and positively correlated with basal endothelin-1 levels, whereas they were inversely correlated with insulin-induced NOx and forearm cGMP release. These in vivo data appear to confirm previous in vitro studies on endothelial cells that demonstrated a strict correlation between ADMA and endothelin-1 levels.²⁷

High ADMA concentrations can be related to increased synthesis or reduced degradation. Because increased oxidative stress is a common marker in the presence of elevated ADMA concentrations,²⁵ it could be postulated that ADMA levels are elevated in patients with CSX because of an increase in oxidative stress. In agreement with this hypothesis, a recent study demonstrated that patients with CSX showed enhanced concentrations of lipid hydroperoxides and conjugated dienes, 2 sensitive and independent markers of oxidative stress.²⁸ However, this hypothesis is highly speculative and deserves further research to better understand this important issue.

Influence of L-Arginine on Basal Endothelial Function

The evidence that L-arginine directly influenced NOx levels and activity was provided by the observed significant incre-

ment in NOx levels. The increase in the availability of NOx was also confirmed by a significant increase in forearm cGMP release. In addition, blood pressure significantly decreased and basal and postischemic forearm blood flows significantly increased compared with saline infusion. These changes observed after 1 hour of L-arginine infusion were similar to those found in control subjects during L-arginine stimulation of insulin secretion. In fact, Giuliano et al²⁹ showed that in control subjects, a systemic L-arginine infusion decreased systolic blood pressure by 10% and increased leg blood flow by 36% in the presence of L-arginine-induced insulin secretion. Conversely, the simultaneous infusion of L-arginine and octreotide to maintain basal insulin levels did not induce a significant decrease in systolic blood pressure and increase in forearm blood flow.

In agreement with a previous study after chronic L-arginine administration,³⁰ the most striking result of the present study is related to the possibility that an acute infusion of L-arginine can determine a near normalization of circulating endothelin-1 levels in these patients. Most studies have shown that circulating endothelin-1 levels are elevated in patients with CSX.^{17,31,32} In 1995, Kaski et al³¹ reported for the first time that circulating endothelin-1 levels were elevated, and Cox et al³² showed a direct relationship between endothelin-1 levels and impaired coronary flow reserve in these patients. Lerman et al³³ demonstrated that coronary

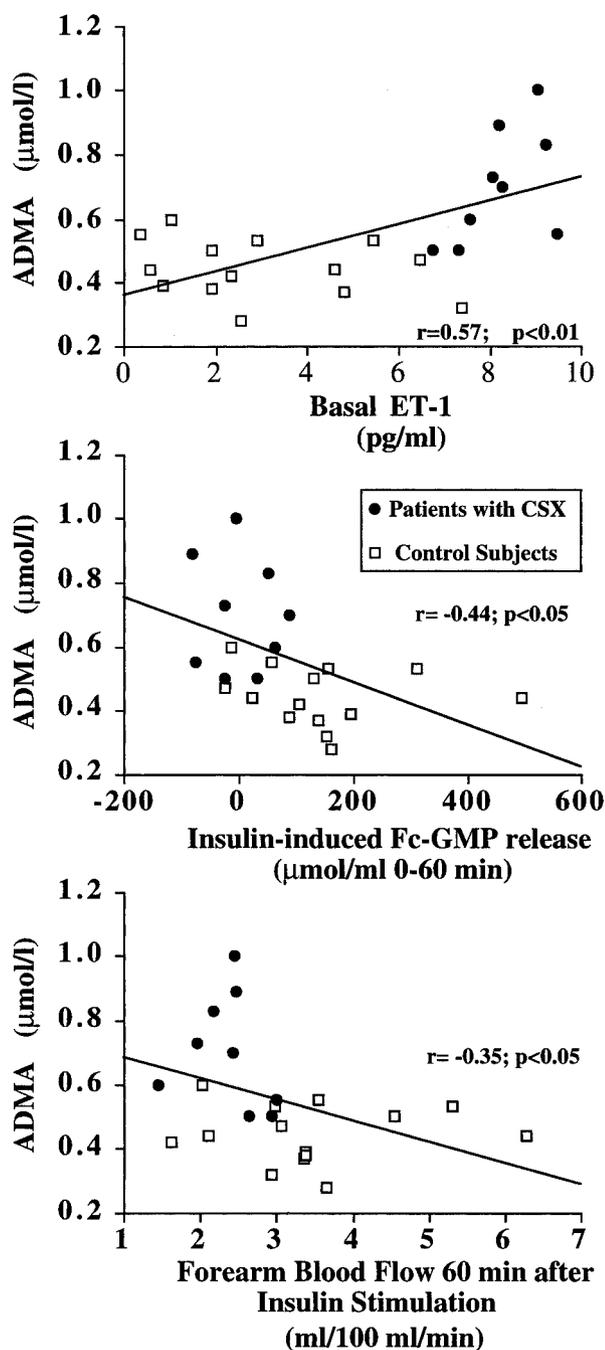


Figure 4. Relationship between basal endothelin-1 and ADMA levels (top), between incremental insulin-induced forearm cGMP (Fc-GMP) release and basal ADMA levels (middle), and between forearm blood flow 60 minutes after insulin stimulation and basal ADMA levels (bottom).

endothelial dysfunction in humans is characterized by increased coronary and circulating endothelin-1 and decreased production of the second messenger of NOx, cGMP.

In the present study, we confirmed that elevated endothelin-1 levels were associated with normal NOx levels, data previously reported by our group,⁴ and we found a significant and inverse correlation between the decrement in endothelin-1 levels and the increment in forearm cGMP

release. These data are not surprising, because endothelin-1 is not able to inhibit basal NOx production,³⁴ whereas NO can inhibit the vasoconstrictive effects of endothelin-1 on internal mammary arteries³⁵ and decrease endothelin-1 production in smooth muscle cells.³⁶ In addition, basal NO production can modulate endothelin-1 activity through endothelial endothelin-B receptor activity.³⁷

Influence of L-Arginine on Insulin-Stimulated Endothelial Function

L-Arginine infusion induced important changes in insulin-induced NOx, cGMP and endothelin-1 release in patients with CSX. To the best of our knowledge, this is the first description of the possibility of a near normalization of endothelin-1 levels and of insulin-induced endothelial function in these patients.

The decrement in endothelin-1 levels obtained during the first hour of L-arginine infusion elicited a near-normal insulin-induced endothelin-1 release. This finding suggests that *in vivo* endothelin-1 release is submitted to a negative feedback, as shown in previous *in vitro* studies.³⁸ More recently, Newby et al³⁹ demonstrated that there is a decrease in sensitivity of peripheral resistance vessels to exogenous endothelin-1 in patients with CSX compared with healthy age- and sex-matched control subjects. They showed that the presence of increased endothelin-1 levels induced a downregulation of the endothelin-A receptor in these patients.

Another important finding of the present study was that L-arginine infusion restored the blunted insulin-induced NOx release that is typically observed in these patients⁴ while increasing forearm cGMP release, a new and interesting finding. The possibility that a decrease in endothelin-1 levels, induced by L-arginine infusion, could be the cause of the restoration of insulin-induced NOx and cGMP release is supported by the evidence that endothelin-1 has been shown to significantly inhibit NOx release induced by different stimuli, such as interleukin-1 β , in vascular smooth muscle cells.³⁴

Clinical Implications

The presence of an imbalance between endothelin-1 and NO release appears to be typical of patients with CSX. The mechanism responsible for the increase in basal endothelin-1 levels in patients with CSX remains unknown. In fact, these patients are by definition not affected by hypertension,⁴⁰ hypertriglyceridemia and diabetes,^{14,41} or other diseases, such as ischemic heart disease⁴² and atherosclerosis,⁴³ that could determine or be caused by a sustained stimulation of endothelin-1 release. One putative mechanism might be related to the presence of increased ADMA levels, which, by inhibiting NO release, cause a defect in the negative feedback of NO on endothelin-1 release. An acute L-arginine infusion not only restored NO activity but also decreased endothelin-1 levels and almost normalized insulin-induced endothelial function.

The present study confirms that oral or intravenous L-arginine administration can improve coronary endothelial function and symptoms in patients with coronary artery

disease.^{29,44,45} This appears to be effective only in the presence of endothelial dysfunction, because in control subjects in the present study and in patients with coronary artery disease and normal endothelial function, L-arginine administration was not effective to further increase endothelial vasodilation in the brachial artery.⁴⁶ A limitation of the present study may be related to its small size with regard to the number of patients, and further studies to evaluate an increased number of patients are required, because the present results may not apply to the general population of patients with CSX.

Other possible mechanisms of L-arginine in ameliorating endothelial function might be by interference with the production of superoxide anion, as suggested in Pritchard et al,⁴⁷ or by an abnormality of cellular L-arginine transport that affects endothelial function,⁴⁸ but we have no data to support these hypotheses, which require further investigation.

Conclusions

ADMA levels are increased in patients with CSX and are positively correlated with basal endothelin-1 levels and negatively correlated with insulin-induced incremental levels of NO_x and forearm cGMP release. An increment in NO availability by an acute L-arginine infusion decreases endothelin-1 levels and therefore improves endothelial function in patients with CSX. These data suggest that increased ADMA levels play a role in the abnormal vascular reactivity that is observed in patients with CSX.

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