Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 557 (2007) 178–185
www.elsevier.com/locate/ejphar

Asymmetric dimethylarginine (ADMA) induces vascular endothelium impairment and aggravates post-ischemic ventricular dysfunction in rats

Vito De Gennaro Colonna [a,*], Sara Bonomo [a], Paolo Ferrario [a], Mauro Bianchi [a], Marco Berti [b], Marco Guazzi [c], Barbara Manfredi [a], Eugenio E. Muller [a], Ferruccio Berti [a], Giuseppe Rossoni [a]

[a] *Department of Pharmacology, Chemotherapy and Medical Toxicology, University of Milan, Via Vanvitelli 32, Italy*
[b] *Institute of Cardiology, University of Milan, Monzino Cardiology Center, IRCCS, Milan, Italy*
[c] *Cardiopulmonary Laboratory, Cardiology Division, University of Milan, San Paolo Hospital, Milan, Italy*

Received 15 September 2006; received in revised form 8 November 2006; accepted 9 November 2006
Available online 22 November 2006

Abstract

Asymmetric dimethylarginine (ADMA) is an endogenous nitric oxide (NO) inhibitor recognized as an independent risk factor for endothelial dysfunction and coronary heart diseases. This study investigated whether ADMA (10 mg/kg day for 14 days) affected endothelial function and aggravated post-ischemic ventricular dysfunction in the perfused rat heart. Systolic blood pressure and heart rate, plasma levels of ADMA and nitrite/nitrate were measured in vehicle- and ADMA-treated rats. Perfused hearts were submitted to global ischemia–reperfusion and vascular endothelial dysfunction was examined with angiotensin II in coronary vessels and aortic rings. Endothelial NO synthase (eNOS) and angiotensin-converting enzyme (ACE) mRNA expression in aortic and cardiac tissues were measured. ADMA-treated rats had higher systolic blood pressure (1.3-fold, $P<0.01$) and slower heart rate (16%, $P<0.05$) than controls. Plasma ADMA rose (1.9-fold, $P<0.01$) and nitrite/nitrate concentration decreased 59% ($P<0.001$). Ventricular contraction (stiffness) increased significantly, with worsening of post-ischemic ventricular dysfunction. In preparations from ADMA-treated rats the coronary vasculature's response to angiotensin II was almost doubled ($P<0.01$) and the maximal vasorelaxant effect of acetylcholine in aortic rings was significantly lower than in preparations from vehicle-treated rats. In cardiac and aortic tissues eNOS mRNA and ACE mRNA levels were similar in controls and ADMA-treated rats. The increased plasma levels of ADMA presumably cause endothelial dysfunction because of a deficiency in NO production, which also appears involved in the aggravation of myocardial ischemia–reperfusion injury.

Keywords: Asymmetric dimethylarginine; Myocardial ischemia–reperfusion; Endothelial dysfunction; (Rat)

1. Introduction

Asymmetric dimethylarginine (ADMA) is a naturally occurring inhibitor of nitric oxide (NO) synthesis that accumulates in a variety of diseases associated with endothelial dysfunction and enhanced atherosclerosis (Boger et al., 1998; Cooke, 2000; Leiper, 2005). Thus ADMA, which was identified as circulating in human plasma at concentration ten times that of N^G-monomethyl-L-arginine, is considered the most important regulator of the L-arginine/NO pathway *in vivo* (Vallance et al., 1992). ADMA and its biologically inactive stereoisomer, symmetrical dimethylarginine, may be released into the plasma after the breakdown of proteins containing arginine residues previously dimethylated by protein arginine methyltransferase, and protein arginine methyltransferase is now considered the control point for ADMA production (Brahms et al., 2000; Leiper and Vallance, 2006). With regard to the route of elimination, dimethylarginine dimethylaminohydrolase hydrolyzes ADMA and appears to have a major role in its metabolism (MacAllister et al., 1996). Several reports have indicated that ADMA concentrations in the pathophysiological range of 3–10 μM significantly inhibit vascular NO production (Kurose et al., 1995; Segarra et al., 2001). However, in spite of a number of studies demonstrating a correlation between plasma levels of ADMA and severity of the disease, its possible role in cardiovascular pathology is still debated (Kielstein and Zoccali, 2005). The main reason is the limited evidence of its pathophysiological role *in vivo*; in addition, only a few studies

* Corresponding author. Tel.: +39 02 50317017; fax: +39 02 50316956.
E-mail address: vito.colonna@unimi.it (V. De Gennaro Colonna).

0014-2999/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2006.11.034

have looked into the acute effects of ADMA. Vallance et al. (1992) reported that the infusion of ADMA (3 mg/kg) in guinea pigs raised systolic blood pressure by 15% while a bolus injection (3–30 mg/kg) led to a dose-dependent increase in mean blood pressure, up to 53 mmHg. Gardiner et al. (1993) confirmed these dose-dependent pressor and bradycardiac effects in rats, showing also that ADMA (1–100 mg/kg) caused vasoconstriction in renal, mesenteric and hindquarter districts. Suda et al. (2004), investigating the long-term effects of subcutaneous ADMA infusion in wild-type mice and endothelial NO synthase (eNOS) knockout mice, reported that it caused significant and comparable coronary microvascular lesions, which were not prevented by L-arginine. This suggested that ADMA might have affected the coronary vessels independently from eNOS, through different mechanisms, possibly related to activation of the renin–angiotensin system and oxidative stress.

This information prompted us to conduct *ex vivo* experiments in rats to investigate the effects on the cardiovascular system of ADMA given subcutaneously for 14 days, with particular focus on impairment of the vascular endothelium and aggravation of myocardial ischemia–reperfusion injury.

2. Materials and methods

2.1. Animals and experimental procedures

Male Wistar rats (Charles River Laboratories Italia, Calco, Lecco, Italy), initial weight 280±20 g body weight, were used. The animals were housed in a conditioned environment (22±1°C, 55±5% relative humidity, 12-h light/dark cycles) and were fed standard laboratory chow and water. This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1996). Experiments were conducted on 20 rats randomly divided into two groups. The animals were anesthetized with inhalation diethyl ether to allow the implantation of Alzet® osmotic minipumps (model 2ML2; Alza Corporation, Cupertino, CA, USA). The minipumps were filled with the test agents and placed in a Petri dish with sterile 0.9% saline at 37 °C for at least 4 h before implantation in order to prime them. The pumps were implanted subcutaneously through a small interscapular incision, using a sterile surgical technique. The first group of ten rats was fitted with ADMA-filled osmotic minipumps (10 mg/kg day for 14 days). A second group of ten (controls; vehicle) received saline-filled osmotic minipumps. During the 14-day treatment period the rats were weighed, and their systolic blood pressure and heart rate were measured weekly by the tail-cuff method. At the end of treatment, all the animals were anesthetized with 60 mg/kg i.p. thiopentone sodium (Pentothal®) and the blood, heart and thoracic aorta were immediately removed for biochemical determinations and *in vitro* studies (see later).

2.2. Indirect systolic blood pressure and heart rate measurements in conscious rats

Systolic blood pressure was measured by tail-cuff plethysmography (mod 8006; U. Basile, Comerio, Varese, Italy) in conscious rats that had been placed in a warm cupboard (30 °C) for 30 min. Systolic blood pressure for individual rats were the average of three consecutive measurements and were considered valid only when these readings did not differ by more than 5 mmHg. At the same time, heart rate was measured from the arterial pulse wave.

2.3. Plasma ADMA measurements

The concentration of ADMA was determined by the HPLC method of Teerlink et al. (2002), with minor modification. Briefly, a Millipore Waters model 590 liquid chromatograph (Waters Ass., Milford, MA, USA) equipped with an injection valve model 7125 Rheodyne (Cotati, CA, USA) and a Waters 474 Scanning Fluorescence Detector was employed. The system was connected to a D-2000 chromato-integrator Hitachi-Merck (Merck, Darmstadt, Germany). A Waters Symmetry C_{18} 3.5 μM (150×4.6 mm i.d.) coupled to a Waters Sentry Symmetry C_{18} guard column was operated at room temperature. The mobile phase was 9:91 (v/v) acetonitrile: 50 mM potassium phosphate-buffer (pH 6.5). The flow rate was 1.1 ml/min and the column effluent was monitored at excitation and emission wavelengths of 340 and 455 nm respectively. Plasma samples were purified by solid phase extraction. N^G-monomethyl-L-arginine (760 ng/100 μl) was added to the plasma samples (200 μl) as internal standard, together with 700 μl of phosphate-buffered saline (10 mM sodium phosphate, 140 mM NaCl, pH 7.0). The samples were extracted on disposable cartridges (Waters Oasis MCX SPE 1 ml/100 mg) positioned on a vacuum-manifold (Waters). After washing with one volume of HCl 100 mM and one volume of methanol, elution was carried out with two 0.5-ml portions of concentrated ammonia/water/methanol (0.1 M NaOH, 10/0.5/10/80). The eluate was evaporated to dryness at 40 °C under nitrogen flow. The residue was reconstituted with 100 μl of bidistilled water and 100 μl of the *ortho*-phthaldialdehyde diluted reagent was added. This reagent was prepared beforehand by dissolving 10 mg *ortho*-phthaldialdehyde in 0.2 ml methanol, followed by addition of 1.8 ml of 200 mM potassium borate buffer (pH 9.5) and 10 μl 3-mercaptopropionic acid, then diluted 1/5 with borate buffer. Five minutes after addition of the *ortho*-phthaldialdehyde reagent, 20-μl aliquots of the solution were injected into the high-performance liquid chromatography system. For ADMA, linearity was assessed in the range of 0.1–20 μM of ADMA, the mean correlation coefficient was >0.99, and the limit of quantitation was 0.01 μM. Analytical recovery was 98% and the interassay coefficient of variation was better than 3%.

2.4. Plasma concentrations of nitrite/nitrate and 8-isoprostane-prostaglandin $F_{2\alpha}$

The NO radical has a short half-life, so the plasma concentrations of NO stable end-products, nitrite/nitrate, were used as an indicator of vascular NO production. Combined plasma nitrite/nitrate concentrations were measured using a commercial colorimetric kit, according to the established

method based on the use of Griess reagent (Green et al., 1982). Plasma concentrations of 8-isoprostane-prostaglandin $F_{2\alpha}$, a sensitive and reliable measure of *in vivo* oxidative stress (Roberts and Morrow, 2000), were determined using a competitive enzyme immunoassay kit according to the manufacturer's instructions. The limit of quantification of the assay was 5 pg/ml of 8-isoprostane-prostaglandin $F_{2\alpha}$.

2.5. Isolated perfused rat heart experiments

2.5.1. Perfused rat heart preparations

Rat hearts ($n=10$ for group) were perfused as previously described (Rossoni et al., 1998). In brief, the heart was rapidly excised, and placed in cold (4 °C) Krebs Henseleit solution with the following composition (mM): NaCl 118, KCl 4.8, KH_2PO_4 1.2, $CaCl_2$ 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.5 mM. The heart was mounted on the experimental set-up within 2 min after thoracotomy and perfused at 15 ml/min (Minipuls-3 peristaltic pump; Gilson, Villiers-Le Bel, France) through the aorta with Krebs Henseleit solution, maintained at 37 °C and aerated with 95% O_2 +5% CO_2 to stabilize normal pH, pO_2 and pCO_2. Coronary perfusion pressure and left ventricular pressure were measured with two HP-1280C pressure transducers (Hewlett-Packard, Waltham, MA, USA) connected to a Hewlett-Packard dynograph (HP-7754A). Left ventricular pressure was recorded with a polyethylene catheter, with a small latex balloon on the tip (Hugo Sachs Elektronik, March-Hugstetten, Germany), inserted into the left ventricular cavity through the mitral valve opening. The volume of the balloon was adjusted to give peak left ventricular systolic pressure 90±5 mmHg with left ventricular end-diastolic pressure 5–7 mmHg. Hearts that could not achieve this contractile performance (8–10%) were excluded. Left ventricular developed pressure (peak left ventricular systolic pressure minus left ventricular end-diastolic pressure) was also calculated. After 15-min equilibration, hearts were paced at 300 beats/min with an electrical stimulator (S-88; Grass Instruments, Quincy, MA, USA) using two silver electrodes attached to the right atrium, and a further 20 min of perfusion was carried out (pre-ischemic period).

2.5.2. Effect of angiotensin II activity on coronary perfusion pressure

At the beginning of each experiment, coronary vasculature reactivity to angiotensin II was evaluated to assess the integrity of endothelium-dependent relaxant mechanisms. Angiotensin II (1 μg) was injected as a bolus into the perfusion system.

2.5.3. Ischemia–reperfusion experiments in isolated rat heart

Ischemia was induced by reducing the flow rate from 15 to 1 ml/min for 20 min (ischemic period). Normal flow rate (15 ml/min) was then restored and the perfusion was continued for another 30 min (reperfusion period). Throughout the experiment, a thermoregulated chamber held the heart at 37 °C to avoid hypothermia-induced cardioprotection. The total duration of each experiment did not exceed 90 min, during which the experimental preparation remained stable.

2.5.4. Creatine kinase and lactate dehydrogenase activities in heart perfusates

The effluent from the heart during the pre-ischemic and reperfusion periods was collected in an ice-cooled beaker as 2.5-min samples. Each sample was used for the determination of creatine kinase and lactate dehydrogenase activities according to the method of Bergmeyer et al. (1970) and Hohorst (1963), respectively. Total activity was measured spectrophotometrically (Lambda-16; Perkin Elmer Italia, Monza, Milan, Italy) at 37 °C using specific kits, according to the manufacturer's instructions.

2.6. Endothelial function in isolated rat aortic rings

Segments of thoracic aorta from the different groups of rats were cleaned of connective tissue in Krebs Henseleit solution and cut into rings (3–5 mm long). The rings were handled carefully to avoid damage to the inner surface and suspended in organ bath chambers (10 ml) containing Krebs Henseleit solution gassed with 95% O_2 +5% CO_2 and maintained at 37 °C (pH 7.4). Tissues were connected with silk sutures to force-displacement transducers (model 7004; U. Basile), and changes in isometric force were displayed on a Gemini chart recorder (model 7070; U. Basile). All rings were gradually stretched to a baseline resting tension of 1.5–1.7 g, which was maintained throughout the experiment, and the preparations were allowed to equilibrate for 60 min. To evaluate maximal contraction, the tissues were depolarized with 60 mM potassium chloride and washed with Krebs Henseleit solution. After 30 min, the rings were precontracted with norepinephrine (3×10^{-6} M), and when the contractile response was stable (steady-state phase, 12–15 min), endothelial-dependent relaxation was evaluated by cumulative addition of acetylcholine (from 10^{-11} to 10^{-4} M). The direct relaxant effect of the NO-donor sodium nitroprusside (10^{-10} to 10^{-3} M) was also recorded.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR): aortic and heart eNOS mRNA and angiotensin-converting enzyme (ACE) mRNA

Total RNA was isolated from aortic tissues by the single-step acid guanidium-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Reverse transcription-polymerase chain reaction was prepared by standard methods with 1 μg of total RNA. First-strand cDNA was synthesized with oligo dt and Molony murine leukemia virus reverse transcriptase. Reverse transcription was run at 37 °C for 50 min followed by initial denaturation at 70°C for 15 min. PCR amplification was then done with synthetic gene-specific primers for eNOS (forward primer, 5′-TGCACCCTTCCGGGGATTCT-3′; reverse primer, 5′-GGATCCCTGGAAAAGGCGGT-3′; product length, 189 bp) and ACE (forward primer, 5′-GTCAGCTTCATCATC-CAGTT-3′; reverse primer, 5′-AGGAAGAGCAGCAGC-CACTG-3′; product length, 409 bp). Amplification was done with 35 cycles of denaturation (95 °C for 30 s), annealing (60.5 °C for 30 s) and extension (72 °C for 30 s). Parallel

Table 1
Body weight, systolic blood pressure (SBP) and heart rate (HR) in vehicle- and ADMA-treated rats

	Vehicle		ADMA	
	Pre	Post	Pre	Post
Body weight (g)	293±16	365±32 [a]	282±15	412±38 [b]
SBP (mmHg)	124±8	122±6	120±7	151±7 [b,d]
HR (b/min)	321±10	325±13	329±16	277±12 [a,c]

Data are means±S.E.M. of 10 animals per group. [a]$P<0.05$ and [b]$P<0.01$ vs. the corresponding pre-treatment value; [c]$P<0.05$ and [d]$P<0.01$ vs. vehicle-treated rats.

amplification of rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was done. The reaction was linear to 30 cycles with the ethidium bromide detection method. PCR products were separated by electrophoresis on a 2% agarose gel containing ethidium bromide and were visualized by ultraviolet-induced fluorescence. The intensity of each band was quantified using a densitometer. The densities of the eNOS and ACE bands were expressed relative to the densities of the GAPDH bands from the same RNA sample (Hattori et al., 1997; Kobayashi et al., 1999).

2.8. Statistical analysis

Data are presented as mean±S.E.M. The differences between the treatment groups were compared by the unpaired *t* test or one-way analysis of variance (ANOVA), followed by the Student–Newman-Keuls *post hoc* test for multiple comparisons. A probability (P) value of <0.05 was considered significant. The area under the curve was assessed using the Microcal Origin 3.5 computer program (Microcal Software Inc., Northampton, MA, USA).

2.9. Drugs and chemicals

Angiotensin II, acetylcholine chloride, asymmetric dimethylarginine, diethyl ether, N^G-monomethyl-L-arginine, 3-mercaptopropionic acid, *ortho*-phthaldialdehyde and sodium nitroprusside were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade acetonitrile and methanol were obtained from BDH Italia (Milan, Italy). Oasis MCX cation-exchange SPE columns (30 μM, 30 mg, 1 ml) were purchased from Waters Ass. (Milford, MA, USA). Oligo dt and Molony murine leukemia virus reverse transcriptase were obtained from GIBCO (Milan, Italy). Rat glyceraldehyde-3-phosphate-dehydrogenase and synthetic gene-specific primers were obtained from Invitrogen Life Technologies (Milan, Italy). Thiopentone sodium (Pentothal®) was purchased from Abbott S.p.A. (Campoverde, Latina, Italy). The colorimetric kit for nitrite/nitrate and enzyme immunoassay kit for 8-isoprostane-prostaglandin $F_{2\alpha}$ determinations were obtained from Cayman Chemical Company (Ann Arbor, Michigan, USA). Kits for creatine kinase and lactate dehydrogenase determinations were purchased from Sentinel Diagnostic (Milan, Italy). All other chemicals were of analytical grade.

3. Results

3.1. Systolic blood pressure and heart rate in conscious rats

At the end of the ADMA treatment, all the animals appeared generally healthy, with no differences on gross examination from vehicle-treated rats. Growth showed a normal pattern and body weight was similar in the two groups (Table 1). The final tail-cuff measurements indicated no changes from the basal values in the vehicle-treated rats whereas in ADMA-treated animals the systolic blood pressure rose 1.3-fold ($P<0.01$) and heart rate decreased 16% ($P<0.05$) (Table 1).

3.2. Plasma ADMA, nitrite/nitrate and 8-isoprostane-prostaglandin $F_{2\alpha}$

Final concentrations of ADMA, the guanidine substituted arginine analogue that can potentially affect arginine handling and/or NOS in biological systems, are reported in Table 2. At the end of treatment ADMA plasma levels were 1.9 times higher ($P<0.001$) than in vehicle-treated rats (0.65±0.04 μM). The final plasma levels of nitrite/nitrate in ADMA-treated rats were 59% lower ($P<0.001$) than in vehicle-treated rats, indicating a general impairment of the NOS/NO pathway (Table 2). The plasma levels of 8-isoprostane-prostaglandin $F_{2\alpha}$ were not significantly different in the two groups. This may indicate that the ADMA dose was too low to trigger measurable oxidative stress (Table 2).

3.3. Isolated perfused rat heart

3.3.1. Angiotensin II activity on coronary perfusion pressure

A bolus injection of angiotensin II was made into the perfusion system of the isolated hearts at the end of ADMA or vehicle treatment to assess the reactivity of the coronary vasculature to this vasoconstrictor. In heart preparations from vehicle-treated rats, angiotensin II induced a prompt increase of coronary perfusion pressure which peaked at 21.5±2.7 mmHg above the basal value and faded away in 4–5 min. In heart preparations from ADMA-treated animals the vasoconstriction caused by angiotensin II increased 1.7-fold ($P<0.01$), indicating a certain degree of impairment of the endothelium-dependent relaxant function of the coronary vasculature (Fig. 1).

3.3.2. Ischemia–reperfusion experiments in isolated rat heart

The time-courses of left ventricular end-diastolic pressure and left ventricular developed pressure in ischemic-reperfused

Table 2
Plasma concentrations of asymmetric dimethylarginine (ADMA), nitrite/nitrate and 8-isoprostane-prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$) in vehicle- and ADMA-treated rats

	Vehicle	ADMA
ADMA (μM)	0.65±0.04	1.25±0.09 [a]
Nitrite/nitrate (μM)	15.8±1.9	6.4±1.1 [a]
8-*iso*-$PGF_{2\alpha}$ (pg/ml)	78.6±5.3	80.9±4.0

Data are means±S.E.M. of 10 animals per group. [a]$P<0.001$ vs. vehicle-treated rats.

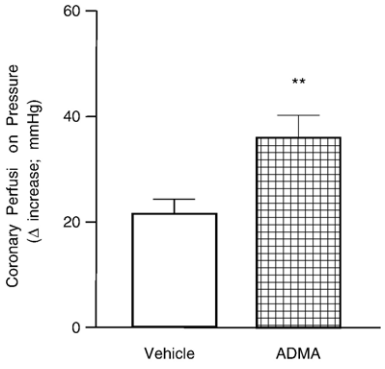

Fig. 1. Changes in coronary perfusion pressure induced by angiotensin II injected into perfused rat heart preparations during the pre-ischemic period. The hearts were obtained from vehicle- and ADMA-treated rats. Baseline coronary perfusion pressure-values: vehicle, 60.5±7 mmHg; ADMA, 68.3±5 mmHg (P=0.3765). Data are mean±S.E.M. of 10 hearts per group. **P<0.01 vs. vehicle-treated group.

hearts from vehicle- and ADMA-treated rats are depicted in Fig. 2. During the ischemic period, the left ventricular end-diastolic pressure of heart preparations from vehicle-treated animals began to rise after a standstill (ventricular contracture), peaking in 20 min (from 5±2 to 27±3 mmHg; P<0.001). Then left ventricular end-diastolic pressure dropped slightly during reperfusion but at the end of this period it was still significantly elevated (24±2 mmHg). Consequently in these heart preparations the left ventricular developed pressure was significantly depressed during reperfusion, and at the end of this period the strength of contraction had recovered only 45% (P<0.01) of the pre-ischemic value (87±7 mmHg) (Fig. 2). In preparations from ADMA-treated rats the ischemia–reperfusion insult was more marked than in controls. During the ischemic period, the left ventricular end-diastolic pressure peaked at 43±3 mmHg in 20 min, and at the end of reperfusion the left ventricular end-diastolic pressure was still significantly elevated (38±3 mmHg) compared to that obtained in preparations from vehicle-treated rats. The left ventricular developed pressure was further

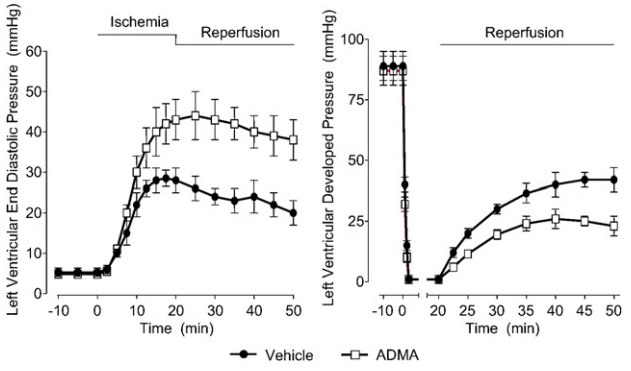

Fig. 2. Time-course of left ventricular end-diastolic pressure and left ventricular developed pressure in perfused rat heart preparations from vehicle- and ADMA-treated rats. Data are mean±S.E.M. of 10 hearts per group. The areas under the curves (from 0 to 50 min) related to left ventricular end-diastolic pressure were 791±126 and 1534±194 (P<0.01) for preparations from vehicle- and ADMA-treated rats, respectively. The areas under the curves (from 20 to 50 min) related to left ventricular developed pressure were 965±66 and 618±83 (P<0.01) for hearts from vehicle- and ADMA-treated rats, respectively.

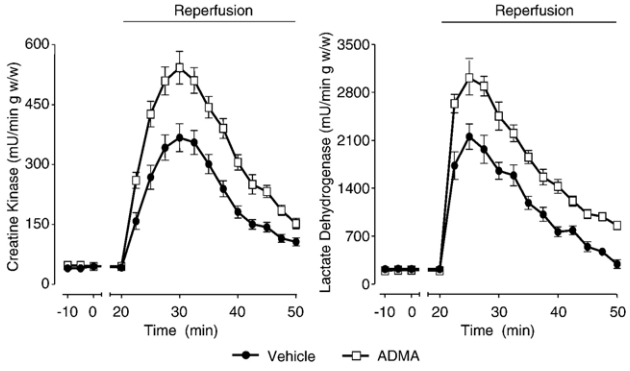

Fig. 3. Time-course of creatine kinase and lactate dehydrogenase release in perfused rat heart preparations from vehicle- and ADMA-treated rats. Data are means±S.E.M. of 10 hearts per group. The areas under the curves (from 20 to 50 min) related to creatine kinase were 6125±470 and 8592±508 (P<0.01) for preparations from vehicle- and ADMA-treated rats, respectively. The areas under the curves (from 20 to 50 min) related to lactate dehydrogenase were 17713±1244 and 23287±1820 (P<0.05) for preparations from vehicle- and ADMA-treated rats, respectively.

depressed and at the end of this period the heart contractility had recovered only 26% of the pre-ischemic values (Fig. 2).

3.3.3. Creatine kinase and lactate dehydrogenase activities in heart perfusates

Fig. 3 shows the creatine kinase and lactate dehydrogenase in coronary effluent collected during the pre-ischemic and reperfusion periods. There were no differences between the groups in either creatine kinase or lactate dehydrogenase released during the pre-ischemic period. However, during reperfusion, creatine kinase rose progressively in preparations from vehicle-treated rats with a peak increment 7.8 times the basal values (47±4 mU/min g w/w; P<0.001) whereas in preparations from ADMA-treated rats the increment for creatine kinase was 11.5-fold (P<0.001). The pattern was similar for lactate dehydrogenase activity which rose 9.4-fold (P<0.001) and 15.5-fold (P<0.001) over the basal values (208±19 mU/min g w/w) respectively in perfusates from vehicle- and ADMA-treated rats (Fig. 3).

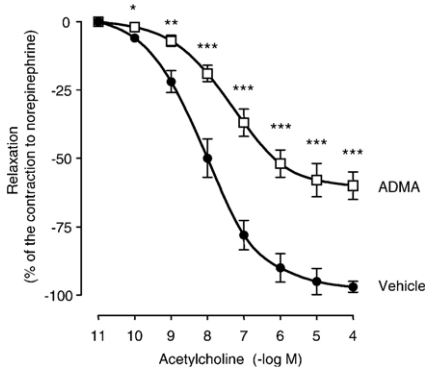

Fig. 4. Cumulative concentration-response curves of acetylcholine in norepinephrine-precontracted aortic rings from vehicle- and ADMA-treated rats. Data are mean±S.E.M. of 10 preparations per group. *P<0.05, **P<0.01 and ***P<0.001 vs. the tissues from vehicle-treated rats.

Table 3
Endothelial nitric oxide synthase (eNOS) and angiotensin-converting enzyme (ACE) mRNA expression in aortas and hearts from vehicle- and ADMA-treated rats

	Vehicle		ADMA	
	Aorta	Heart	Aorta	Heart
eNOS mRNA/ GAPDH mRNA	0.29±0.04	0.28±0.02	0.31±0.04	0.29±0.03
ACE mRNA/ GAPDH mRNA	0.39±0.09	0.40±0.06	0.45±0.07	0.37±0.06

Total RNA was assayed by reverse transcription-polymerase chain reaction with gene-specific primers for eNOS, ACE and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Data are means±S.E.M. of 10 animals per group.

3.4. Endothelial function in isolated rat aortic rings

Fig. 4 illustrates the endothelium-dependent relaxant effect of acetylcholine in norepinephrine-precontracted aortic rings. Norepinephrine caused the same degree of contraction in preparations from vehicle- and ADMA-treated rats (data not shown). When norepinephrine-precontracted aortic rings from vehicle-treated rats were exposed to cumulative concentrations of acetylcholine, marked vasorelaxation (E_{max} 94.3±3%) was obtained, whereas the response in preparations from ADMA-treated rats was significantly lower (E_{max} 57.4±6%; $P<0.001$). The dose-response curves for the relaxant activity of sodium nitroprusside were almost superimposable in preparations from vehicle- and ADMA-treated rats (data not shown).

3.5. RT-PCR: aortic and cardiac eNOS mRNA and ACE mRNA

As shown in Table 3, in cardiac and aortic tissues obtained from vehicle- and ADMA-treated rats the levels of eNOS mRNA were in the same range and statistically indistinguishable. The pattern was similar for ACE mRNA in the two tissues, where the levels were not different (Table 3).

4. Discussion

Dysfunction of the endothelium is a common mechanism by which several cardiovascular risk factors exert deleterious effects on the vascular wall (Panza et al., 1990; Hingorani et al., 2000). The endothelial L-arginine/NO pathway is thought to be the major effector of endothelial control of vascular homeostasis, and ADMA is now recognized as a regulator of NO generation and a potential marker of cardiovascular diseases (Boger et al., 1997, 2005). The present results in rats treated subcutaneously with ADMA for 14 days once more indicate that this compound, by affecting NO generation in vascular endothelial cells, significantly raises systolic blood pressure and slows heart rate, aggravating post-ischemic ventricular dysfunction in *ex vivo* perfused rat heart preparations. These effects correlate well with the elevated plasma ADMA levels and low plasma nitrite/nitrate concentrations found in ADMA-treated rats compared to that measured in vehicle-treated rats. These findings indicate that the dose regimen of ADMA was adequate to obtain plasma levels of this arginine analogue able to affect NO generation in the vascular wall. Plasma levels of ADMA in ADMA-treated rats (1.25±0.09 μM) were almost doubled as compared to those found in vehicle-treated animals (0.65±0.04 μM). In humans, two-fold increased ADMA levels versus controls were observed in patients affected by peripheral arterial occlusive disorders (Boger et al., 1997) and in hypercholesterolemic subjects (Boger et al., 1998).

In contrast with the report by Suda et al. (2004) relating to the activity of long-term ADMA treatment in eNOS knockout mice, we did not find any upregulation of ACE with the consequent rise in oxidative stress. In fact, ACE mRNA expression was not increased in aortic and cardiac tissues of ADMA-treated rats nor was there any increase in plasma 8-isoprostane-prostaglandin $F_{2\alpha}$ compared to vehicle-treated rats. This suggests that in our experimental conditions the renin–angiotensin system was not involved. The difference might be explained by species differences or, more important, by the dosage and duration of ADMA treatment in the present study (14 days) which might have been too short in comparison with the 28-day schedule used by Suda et al. (2004).

Furthermore, multiple mechanisms besides simple inhibition of eNOS and renin–angiotensin system activation are likely to be operative in the long-term vascular effect of L-arginine analogues. These include endothelial generation of superoxide anions (Heim et al., 1991), antagonism of muscarinic receptors (Buxton et al., 1993), inhibition of endothelium-independent relaxation by amiloride (an inhibitor of Na^+–H^+ exchange) and by dibutyril cyclic adenosine monophosphate, a membrane-permeable cyclic adenosine monophosphate analogue (Thomas and Ramwell, 1991). However, even if eNOS gene expression in aortic tissue was not reduced, the present results seem to imply that a primary target of ADMA treatment was the eNOS/NO pathway in vascular endothelium. This is supported by the results in norepinephrine-precontracted aortic rings from ADMA-treated rats, where acetylcholine's relaxant effect was significantly lower than in preparations from vehicle-treated rats. In addition, in perfused heart preparations from ADMA-treated rats the vasopressor effect of angiotensin II was almost doubled that in preparations from vehicle-treated rats, indicating that at the periphery too (coronary vasculature), there was a certain degree of endothelial-dependent relaxant dysfunction. The reduced capacity to regulate vascular tone, very likely due to insufficient NO generation, might also be a reasonable explanation for the increase in systolic blood pressure in ADMA-treated rats. In studies with arterial segments *in vitro* ADMA inhibited vascular NO production at concentrations from 10 to 300 μM (Kurose et al., 1995; Segarra et al., 1999, 2001).

There is also indirect evidence that ADMA has a role as an endogenous modulator of NO activity. Inhibition of dimethylarginine dimethylaminohydrolase, the enzyme that inactivates ADMA, caused vasoconstriction of isolated arterial rings (MacAllister et al., 1996) and enhanced endothelial superoxide radical formation in human endothelial cells (McCarty, 2004). In the present study, the indirect evidence of impaired NO generation (decrease of nitrite/nitrate in plasma and hyperresponsiveness to angiotensin II) in coronary vessels from

ADMA-treated rats may have some importance in explaining the worsening of post-ischemic ventricular dysfunction in perfused hearts after ischemia–reperfusion. This was consistent with a further increase of left ventricular end-diastolic pressure during ischemia and a marked depression of left ventricular developed pressure at reperfusion, compared to control preparations. These cardio-mechanical alterations were accompanied by significant increases of both creatine kinase and lactate dehydrogenase during reperfusion, indicating loss of sarcolemmal integrity which characterizes irreversibly injured myocardial cells.

According to Henry et al. (1977) the accumulation of Ca^{2+} in the mitochondrial fraction of cardiac myocytes and the increase of undissociated cross-bridges (actin-ADP-myosin complex) are responsible for the mechanical changes, such as incomplete or delayed myocardial relaxation and ventricular contraction (stiffness), typical of the ischemia–reperfusion model used in this study. Therefore, it is tempting to speculate that reduced generation of NO in the cardiac endothelium of ADMA-treated rats may have intensified the depletion of energy stores in ischemic cells, increasing undissociated cross-bridges and worsening ventricular stiffness. In this respect, agents that raise intracellular cyclic guanosine monophosphate through NO formation may have profound effect on the cytoplasmic Ca^{2+} concentration, thus mediating relaxation (Kai et al., 1987). Aggravation of ischemia–reperfusion damage with another well-known synthetic inhibitor of the eNOS/NO pathway, N^G-monomethyl-L-arginine, has been amply reported and discussed in isolated rabbit hearts where NO-donors were highly protective (Rossoni et al., 1995, 2000, 2004).

In conclusion, this study provides a further demonstration that increased plasma levels of ADMA in the rat are responsible for endothelial vasodilator dysfunction, very likely due to inadequate production of NO by the endothelium. This event may also be involved in the aggravation of cardiac ischemic insult, thus supporting the concept that action on ADMA concentrations constitutes a new area of intervention for cardiovascular diseases.

References

Bergmeyer, H.U., Rich, W., Butter, H., Schmidt, E., Hillman, G., Kreuz, F.H., 1970. Standardization of methods for estimation of enzyme activity in biological fluids. Z. Klin. Chem. Klin. Biochem. 8, 658–660.

Boger, R.H., Bode-Boger, S.M., Thiele, W., Junker, W., Alexander, K., Frolich, J.C., 1997. Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. Circulation 95, 2068–2074.

Boger, R.H., Bode-Boger, S.M., Szuba, A., Tsao, P.S., Chan, J.R., Tangphao, O., Blaschke, T.F., Cooke, J.P., 1998. Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. Circulation 98, 1842–1847.

Boger, R.H., Maas, R., Schulze, F., Schwedhelm, E., 2005. Elevated levels of asymmetric dimethylarginine (ADMA) as a marker of cardiovascular disease and mortality. Clin. Chem. Lab. Med. 43, 1124–1129.

Brahms, H., Raymackers, J., Union, A., de Keyser, F., Meheus, L., Luhrmann, R., 2000. The C-terminal RG dipeptide repeats of the spliceosomal Sm proteins D1 and D3 contain symmetrical dimethylarginines, which form a major B-cell epitope for anti-Sm autoantibodies. J. Biol. Chem. 275, 17122–17129.

Buxton, I.L., Cheek, D.J., Eckman, D., Westfall, D.P., Sanders, K.M., Keef, K.D., 1993. N^G-nitro L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. Circ. Res. 72, 387–395.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Cooke, J.P., 2000. Does ADMA cause endothelial dysfunction? Arterioscler. Thromb. Vasc. Biol. 20, 2032–2037.

Gardiner, S.M., Kemp, P.A., Bennett, T., Palmer, R.M., Moncada, S., 1993. Regional and cardiac haemodynamic effects of N^G, N^G,dimethyl-L-arginine and their reversibility by vasodilators in conscious rats. Br. J. Pharmacol. 110, 1457–1464.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and ^{15}N nitrate in biological fluids. Anal. Biochem. 126, 131–138.

Hattori, Y., Akimoto, K., Murakami, Y., Kasai, K., 1997. Pyrrolidine dithiocarbamate inhibits cytokine-induced VCAM-1 gene expression in rat cardiac myocytes. Mol. Cell. Biochem. 177, 177–181.

Heim, K.F., Thomas, G., Ramwell, P.W., 1991. Effect of substituted arginine compounds on superoxide production in the rabbit aorta. J. Pharmacol. Exp. Ther. 257, 1130–1135.

Henry, P.D., Schuchleib, R., Davis, J., Weiss, E.S., Sobel, B.E., 1977. Myocardial contracture and accumulation of mitochondrial calcium in ischemic rabbit heart. Am. J. Physiol. 233, H677–H684.

Hingorani, A.D., Cross, J., Kharbanda, R.K., Mullen, M.J., Bhagat, K., Taylor, M., Donald, A.E., Palacios, M., Griffin, G.E., Deanfield, J.E., MacAllister, R.J., Vallance, P., 2000. Acute systemic inflammation impairs endothelium-dependent dilatation in humans. Circulation 102, 994–999.

Hohorst, H.J., 1963. L-(+)-lactate. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press Inc., New York, NY, pp. 215–219.

Kai, H., Kanaide, H., Matsumoto, T., Nakamura, M., 1987. 8-bromoguanosine 3′:5′-cyclic monophosphate decreases intracellular free calcium concentrations in cultured vascular smooth muscle cells from rat aorta. FEBS Lett. 221, 284–288.

Kielstein, J.T., Zoccali, C., 2005. Asymmetric dimethylarginine: a cardiovascular risk factor and a uremic toxin coming of age? Am. J. Kidney Dis. 46, 186–202.

Kobayashi, N., Kobayashi, K., Hara, K., Higashi, T., Yanaka, H., Yagi, S., Matsuoka, H., 1999. Benidipine stimulates nitric oxide synthase and improves coronary circulation in hypertensive rats. Am. J. Hypertens. 12, 483–491.

Kurose, I., Wolf, R., Grisham, M.B., Granger, D.N., 1995. Effects of an endogenous inhibitor of nitric oxide synthesis on postcapillary venules. Am. J. Physiol. 268, H2224–H2231.

Leiper, J.M., 2005. The DDAH-ADMA-NOS pathway. Ther. Drug Monit. 27, 744–746.

Leiper, J.M., Vallance, P., 2006. The synthesis and metabolism of asymmetric dimethylarginine (ADMA). Eur. J. Clin. Pharmacol. 62, 33–38.

MacAllister, R.J., Parry, H., Kimoto, M., Ogawa, T., Russell, R., Hodson, H., Whitley, G., Vallance, P., 1996. Regulation of nitric oxide synthesis by dimethylarginine dimethylaminohydrolase. Br. J. Pharmacol. 119, 1533–1540.

McCarty, M.F., 2004. Vascular endothelium in the organ chiefly responsible for the catabolism of plasma asymmetric dimethylarginine— an explanation for the elevation of plasma ADMA in disorders characterized by endothelial dysfunction. Med. Hypotheses 63, 699–708.

Panza, J.A., Quyyumi, A.A., Brush, J.E., Epstein, S.E., 1990. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. N. Engl. J. Med. 323, 22–27.

Roberts, L.J., Morrow, J.D., 2000. Measurements of F2-isoprostanes as an index of oxidative stress in vivo. Free Radic. Biol. Med. 28, 505–513.

Rossoni, G., Berti, F., Bernareggi, M., Villa, L., Agozzino, S., Cereda, R., Giuliani, P., Mizrahi, J., 1995. Protective effects of ITF 296 in the isolated rabbit heart subjected to global ischemia. J. Cardiovasc. Pharmacol. 26, S44–S52.

Rossoni, G., De Gennaro Colonna, V., Bernareggi, M., Polvani, G.L., Müller, E.E., Berti, F., 1998. Protectant activity of hexarelin or growth hormone against postischemic ventricular dysfunction in hearts from aged rats. J. Cardiovasc. Pharmacol. 32, 260–265.

Rossoni, G., Berti, M., De Gennaro Colonna, V., Bernareggi, M., Del Soldato, P., Berti, F., 2000. Myocardial protection by the nitroderivative of aspirin, NCX 4016: in vivo and in vitro experiments in the rabbit. Ital. Heart J. 1, 146–155.

Rossoni, G., Manfredi, B., Del Soldato, P., Berti, F., 2004. The nitric oxide-releasing naproxen derivative displays cardioprotection in perfused rabbit heart submitted to ischemia–reperfusion. J. Pharmacol. Exp. Ther. 310, 555–562.

Segarra, G., Medina, P., Ballester, R.M., Lluch, P., Aldasoro, M., Vila, J.M., Lluch, S., Pelligrino, D.A., 1999. Effects of some guanidino compounds on human cerebral arteries. Stroke 30, 2206–2210.

Segarra, G., Medina, P., Vila, J.M., Chuan, P., Domenech, C., Torondel, B., Lluch, A., 2001. Inhibition of nitric oxide activity by arginine analogs in human renal arteries. Am. J. Hypertens. 14, 1142–1148.

Suda, O., Tsutsui, M., Morishita, T., Tasaki, H., Ueno, S., Nakata, S., Tsujimoto, T., Toyohira, Y., Hayashida, Y., Sasaguri, Y., Ueta, Y., Nakashima, Y., Yanagihara, N., 2004. Asymmetric dimethylarginine produces vascular lesions in endothelial nitric oxide synthase-deficient mice: involvement of renin–angiotensin system and oxidative stress. Arterioscler. Thromb. Vasc. Biol. 24, 1682–1688.

Teerlink, T., Nijveldt, R.J., de Jong, S., van Leeuwen, P.A., 2002. Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography. Anal. Biochem. 303, 131–137.

Thomas, G., Ramwell, P.W., 1991. N^{ω}-nitro L-arginine benzyl ester, a potent irreversible inhibitor of endothelium dependent relaxation. Biochem. Biophys. Res. Commun. 179, 1677–1682.

Vallance, P., Leone, A., Calver, A., Collier, J., Moncada, S., 1992. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet 339, 572–575.