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Functional and Analytical Evidence for Scavenging of Oxygen Radicals by L-Arginine

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ABSTRACT

L-Arginine, the substrate of nitric oxide synthase, is known to exert favorable effects in the prevention and treatment of cardiovascular diseases. In several conditions, including atherosclerosis and ischemia/reperfusion, where oxygen metabolites are thought to mediate endothelial and myocardial injury, L-arginine has protective effects. Here we studied the mechanisms by which L-arginine protects against oxygen radical-induced myocardial injury. Buffer-perfused rat hearts were subjected to oxygen radicals generated by electrolysis or to hypoxanthine and xanthine oxidase, which generates superoxide anions ($O_2^{\cdot-}$). Both sources of radicals impaired myocardial contractility, whereas L-arginine prevented the impairment. The observation that D-arginine as well as nitric oxide synthase inhibitors, such as N^G-nitro-L-arginine but not glycine, had similar cardioprotective effects indicated that the protection might be due to a direct chemical interaction of L-arginine and its derivatives with oxygen radicals. In support, L-arginine and the derivatives prevented the formation of $O_2^{\cdot-}$ as determined by sensitive standard methods, whereas glycine did not. The radical scavenging activity of L-arginine and derivatives was dose-dependent, with an apparent rate constant of approximately 4.8×10^3 M s^{-1} for the reaction of L-arginine with $O_2^{\cdot-}$ as determined by electron paramagnetic resonance spectroscopy using 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine (TEMPONE-H) as spin trap. In summary, the results of this study demonstrate protective effects of L-arginine against oxygen radical-induced cardiac injury by free radical scavenging.

L-Arginine is a semiessential amino acid with a terminal guanidinium group that serves as natural substrate for the synthesis of nitric oxide (NO) by different NO synthases (NOS) (Palmer et al., 1988; Andrew and Mayer, 1999). NO is a potent endothelium-dependent vasodilator involved in the regulation of vascular function and blood pressure homeostasis (Moncada et al., 1991). L-Arginine is a vascular protectant that alleviates endothelial injury or corrects endothelial dysfunction. Dietary supplementation of cholesterol-fed animals with L-arginine reduced the extent of atheromatous lesions and restored endothelium-dependent arteriolar vasodilation (Cooke et al., 1992; Kuo et al., 1992). In hypercholesterolemic humans, L-arginine supplementation improved agonist-stimulated arterial dilatation and blood flow and normalized monocyte and platelet adhesiveness, all of which contribute to the initiation and progression of atherosclerosis (Creager et al., 1990; Drexler et al., 1991; Clarkson et al., 1996). L-Arginine also alleviates myocardial reperfusion injury (Weyrich et al., 1992; Sato et al., 1995; Brunner et al., 1997). Hence, it was suggested that L-arginine may serve as cost-effective therapy in human diseases associated with endothelial dysfunction (Velianou et al., 1999). However, detrimental effects of L-arginine have been reported as well (Takeuchi et al., 1995; Mori et al., 1998; Kronon et al., 1999).

The mechanism by which L-arginine exerts its protective effects is unclear. The abnormal endothelial functions in hypercholesterolemia or congestive heart failure appear to be related to a reduced ability of the endothelium to produce or release biologically active NO (Lefer and Ma, 1993; Katz et al., 1999). This is generally attributed to reduced biosynthesis of NO and/or to an increased oxidative degradation of NO. In support, L-arginine was shown to restore endothelial function in hypercholesterolemic rabbits by increasing NO production and protecting NO from inactivation by superoxide anions ($O_2^{\cdot-}$) (Böger et al., 1995). However, the intracellular concentration of L-arginine is ~0.5 to 0.8 mM (Baydoun et al., 1990) [i.e., far above the K_m for NOS (2–4 μM)], making it unlikely that additional L-arginine will stimulate NO synthesis. In a model of intestinal ischemia-reperfusion, in which

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ABBREVIATIONS: NO, nitric oxide; NOS, nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; EPR, electron paramagnetic resonance; TEMPONE-H, 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine; CLA, *Cypridina* luciferin analog; L-NMMA, N^G-monomethyl-L-arginine; SOD, superoxide dismutase; DTPA, diethylenetriaminepentaacetic acid; L-NNA, N^G-nitro-L-arginine; $O_2^{\cdot-}$, superoxide anion radical; $+dP/dt_{max}$, maximal rate of rise of left ventricular pressure; PBS, phosphate-buffered saline; ROI, reactive oxygen species; TEMPONE, 2,2,6,6-tetramethyl-4-oxo-piperidinoxyl; CPP, coronary perfusion pressure.

O_2^-, NO, and other reactive oxygen species were increased, both D-arginine and NOS inhibitors such as N^G-nitro-L-arginine methyl ester (L-NAME), which do not serve as substrate for NOS, were effective in reducing oxidative damage (Haklar et al., 1998), suggesting that besides serving as NO precursor, L-arginine may be an antioxidant by scavenging oxygen-derived free radicals.

The aim of this study was to elucidate the mechanism by which L-arginine protects against oxygen radical-mediated cardiac injury. We used three different approaches. First, rat hearts were exposed to oxygen radicals generated by electrolysis or to O_2^- generated enzymatically by hypoxanthine/xanthine oxidase, and the protective effects of L-arginine, D-arginine, derivatives of L-arginine, and structurally related compounds were tested. Second, because the protective effect of L-arginine appeared to be largely related to radical scavenging rather than to increased generation of NO, the radical-scavenging properties of L-arginine were also examined in vitro using established methods (reduction of cytochrome *c* and a chemiluminescence assay). Finally, EPR spectroscopic measurements, using 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine (TEMPONE-H) as spin trap (Dikalov et al., 1997), further demonstrated distinct antioxidative properties of L-arginine.

Experimental Procedures

Materials. *Cypridina* (*Vargula*) luciferin analog (CLA, 2-methyl-6-phenyl-3,7-dihydroimidazol[1,2-a]pyrazine-3-one) was purchased from Nanolight Technology (Pittsburgh, PA). TEMPONE-H and N^G-monomethyl-L-arginine (L-NMMA) was obtained from Alexis Corporation (Lausen, Switzerland). Bovine erythrocyte superoxide dismutase (SOD), horse heart cytochrome *c* (type VI), diethylenetriaminepentaacetic acid (DTPA), glycine, methionine, urate, N^G-nitro-L-arginine (L-NNA), L-NAME, *N*-bathocuproinedisulfonic acid, hypoxanthine, xanthine oxidase, and catalase (from bovine liver) were all purchased from Sigma-Aldrich (Vienna, Austria).

Animals and Heart Perfusion. Male Sprague-Dawley rats (270–360 g) were anesthetized with diethyl ether, heparinized (1000 U kg^{-1}, i.p.), the hearts excised, arrested in ice-cold buffer and mounted for retrograde perfusion (Langendorff mode) at 10 ml min^{-1} per gram of heart wet weight as previously described (Brunner et al., 1997). The perfusion medium was a modified Krebs-Henseleit bicarbonate buffer of the following composition: 118.4 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.25 mM $CaCl_2$ (2 H_2O), 1.2 mM $MgCl_2$ (6 H_2O), 10.1 mM D-glucose (H_2O). Cardiac parameters were monitored continuously and included the positive and negative first derivative of pressure over time ($+dP/dt_{max}$, $-dP/dt_{max}$) obtained via a left ventricular fluid-filled latex balloon; measure of contractility), coronary perfusion pressure measured at the aortic root (measure of coronary function), and heart rate.

Generation of Oxygen Radicals by Electrolysis. Electrolysis was performed essentially as previously described (Jackson et al., 1986). Two platinum wire electrodes were attached to the bottom of the bubble trap of the perfusion apparatus (Harvard Instruments/Hugo Sachs Elektronik, March-Hugstetten, Germany), directly neighboring the aortic cannula. The distance between anode and cathode was 7 mm, and they were localized 3 cm above the heart. A constant 15 mA direct current generated by a power supply was applied to the perfusion buffer in the bubble trap (~4.5 ml) for 10 s. The applied current was continuously monitored with an ampere meter. This setup enabled the generation of oxygen radicals in the perfusion buffer at a very short distance from the heart.

Generation of O_2^- by Hypoxanthine/Xanthine Oxidase. O_2^- was generated from hypoxanthine (1 mM) and xanthine oxidase (60 mU ml^{-1}). The two reactants were added to the perfusion buffer and the heart was perfused for 10 min in recirculation mode (total volume ~50 ml). Catalase (100 U ml^{-1}) was included to prevent the accumulation of H_2O_2 (Southworth et al., 1998).

Protection against Electrolysis-Generated Oxygen Radicals (for Protocol, see Fig. 1A). Hearts were equilibrated to establish stable perfusion conditions (15 min) and exposed to test compounds for 15 min by recirculating the perfusion buffer together with the test compound, followed by electrolysis for 10 s, during which the heart was still exposed to the test compound, and a final perfusion period of 15 min (in nonrecirculating mode), during which the oxygen radicals and test compounds were gradually washed out. The following compounds were used: SOD (50 U ml^{-1}), L-arginine, glycine, aminoguanidine (each 1 mM), and L-NNA (200 μM). SOD was previously used mostly at 100 to 200 U ml^{-1} (Wang and Zweier, 1996). The concentration of L-arginine was about 5 times higher than the plasma concentration in adult humans or experimental animals (~0.2 mM) (Wu and Morris, 1998). Glycine and aminoguanidine were used at the same concentration as L-arginine to allow direct comparisons, and 100 to 200 μM of L-NNA is known to fully antag-

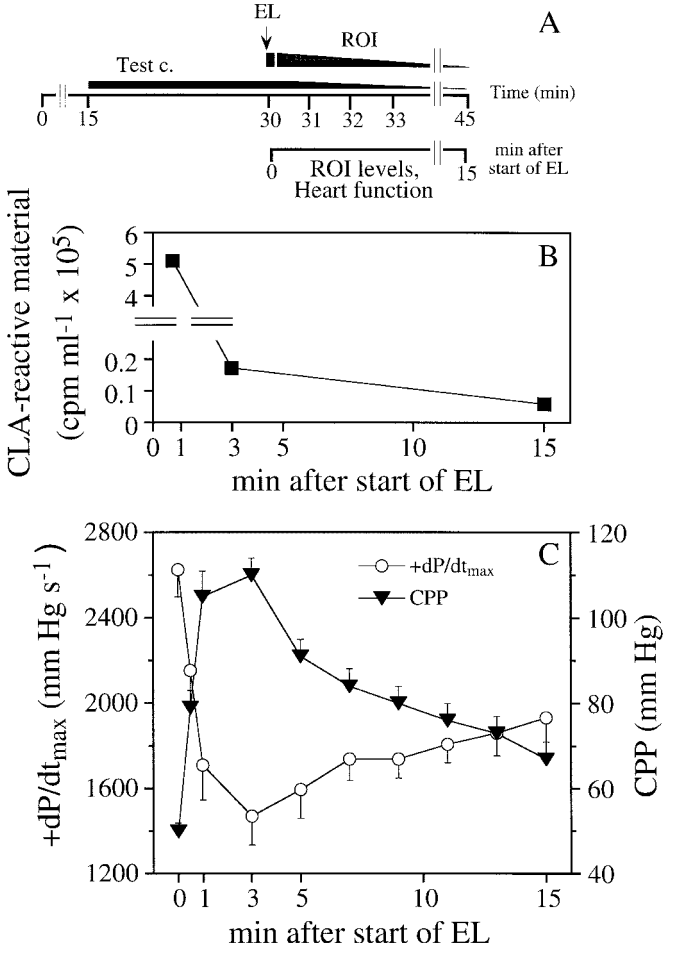

Fig. 1. Effect of electrolysis-generated oxygen free radicals on myocardial function. A, experimental protocol. Hearts were equilibrated (15 min) and perfused with test compound (Test c.) for 15 min by recirculation, followed by electrolysis (EL) of the perfusion medium within the bubble trap for 10 s, and 15 min of perfusion with fresh medium in nonrecirculating mode, which leads to gradual washout of the generated oxygen radicals (ROI) and the test compound. B, concentration of oxygen radicals (ROI) reaching the heart. Perfusion medium was taken at the aortic cannula and CLA-based chemiluminescence was measured immediately. C, maximal rate of rise of left ventricular pressure ($+dP/dt_{max}$, circles) and coronary perfusion pressure (CPP; triangles). Data are mean ± S.E.M. of seven hearts.

onize NOS activity (Klatt et al., 1994). "Control" refers to the effects of electrolysis in the absence of test compounds. The oxygen radical content of electrolyzed buffer and cardiac functional parameters were monitored over 15 min after the start of electrolysis.

Protection against $O_2^{\cdot-}$ Generated by Hypoxanthine/Xanthine Oxidase. Hearts were equilibrated (15 min), followed by perfusion with test compound to the end of the protocol (40 min) (for protocol, see Fig. 3A). At 28 min, hypoxanthine and catalase were added, followed by xanthine oxidase at 30 min. The generation of $O_2^{\cdot-}$ was then monitored over 10 min (Fig. 3B). Functional parameters were recorded just before the start of $O_2^{\cdot-}$ generation (at 30 min; baseline) and during $O_2^{\cdot-}$ generation. Test compounds were SOD (500 U ml^{-1}; 10 times more than in the first protocol, as a precaution), L-arginine, *N*-α-acetyl-L-arginine, aminoguanidine, and glycine (each 1 mM). Control perfusions were performed in the absence of test compounds. In additional experiments, SOD (500 U ml^{-1}) that had been inactivated as described previously (Hodgson and Fridovich, 1975), and the peroxynitrite scavengers methionine (1 mM) and urate (400 μM) were used (Hooper et al., 1998).

Determination of Electrolysis-Generated Oxygen Radical Scavenging by CLA Assay. Oxygen radicals generated by electrolysis were quantified using CLA-enhanced chemiluminescence as previously described (Skatchkov et al., 1998). Briefly, 45 s after the start of electrolysis ~120 μl of the perfusion buffer were collected from the aortic cannula (i.e., in absence of a heart), 100 μl of it were added to 10 μl of CLA solution (final concentration, 50 μM in 50 mM Tris buffer, containing 1 mM $MgCl_2$, 100 μM DTPA, and 50 μM *N*-bathocuproinedisulfonic acid), mixed and placed into a liquid scintillation vial. Chemiluminescence was counted for 30 s in a liquid scintillation counter. The chemiluminescence measured in an aliquot of perfusion buffer before electrolysis was defined as background signal and subtracted. All measurements were performed within 15 s after sample collection.

Determination of $O_2^{\cdot-}$ Scavenging by Cytochrome *c* Assay. Superoxide anion radicals were generated enzymatically by 1 mM hypoxanthine and 5 mU ml^{-1} xanthine oxidase in PBS (10 mM, pH 7.4) at 25°C. Detection of $O_2^{\cdot-}$ generation was based on the reduction of cytochrome *c* (10 μM) recorded at 550 nm (van Gelder and Slater, 1962). The reduction of cytochrome *c* in the absence and presence of L- or D-arginine, derivatives of L-arginine (L-NNA, L-NAME, L-NMMA), *N*-α-acetyl-L-arginine, glycine, and aminoguanidine (concentration range 100 nM to 10 mM) were recorded for 5 min against blank samples containing 50 U ml^{-1} SOD. The rates were calculated by linear regression. The inhibition of cytochrome *c* reduction by test compounds is expressed as percentage of the rate obtained in the absence of test drug (=100%).

Determination of $O_2^{\cdot-}$ Scavenging by EPR Spectroscopy. The scavenging activity of L-arginine, glycine, and aminoguanidine was assessed by the inhibition of the reaction of $O_2^{\cdot-}$ with the spin trap TEMPONE-H using EPR spectroscopy (Dikalov et al., 1997). The assay mixture consisted of 1 mM hypoxanthine, 5 mU ml^{-1} xanthine oxidase, 30 μM TEMPONE-H (10 mM in PBS) and 100 μM of the transition metal chelator DTPA in 10 mM PBS, pH 7.4. L-Arginine, glycine, aminoguanidine, and the combination of glycine and aminoguanidine in the concentration range of 100 nM to 10 mM were tested. Samples were placed in 50-μl micropipettes (Brand, Wertheim, Germany) and EPR spectra were recorded at room temperature using a MiniScope MS 100 EPR spectrometer (Magnettech, Berlin, Germany). The instrument settings were as follows: center field 3370 G, sweep width 100 G, sweep time 50 G, modulation amplitude 1.5 G, power attenuation 7 dB, receiver gain 9×10^2. Spectra were recorded in 1-min intervals for 5 min. The intensity of each spectrum (amplitude) was determined, and the rate of the increase in the intensity calculated by linear regression. Inhibition of the rate by the test compounds is expressed as percentage of the rate obtained in the absence of test compound (=100%).

Rate Constant for the Reaction of $O_2^{\cdot-}$ with L-Arginine. The calculation of the rate constant for the reaction of $O_2^{\cdot-}$ with L-arginine was based on the results of EPR measurements described above. $O_2^{\cdot-}$ was generated enzymatically by hypoxanthine/xanthine oxidase (1 μM min^{-1}) in the presence of TEMPONE-H (30 μM) as spin trap and varying concentrations of L-arginine (between 0.1 and 10,000 μM). Rates of TEMPONE formation were determined by EPR and plotted against the different L-arginine concentrations. The EC_{50} value of L-arginine was calculated using the Hill equation. Assuming that L-arginine solely competes with TEMPONE-H in the reaction with $O_2^{\cdot-}$, the EC_{50} concentration of L-arginine is proportional to the TEMPONE-H concentration and rate of reaction of TEMPONE-H with $O_2^{\cdot-}$ and inversely proportional to its rate of reaction with $O_2^{\cdot-}$. At the given TEMPONE-H concentration (30 μM) and the rate constant for the reaction of TEMPONE-H with $O_2^{\cdot-}$ ($k = 1.2 \times 10^4$ M s^{-1}) (Dikalov et al., 1997), the apparent rate constant for L-arginine is given by k_{app} = [TEMPONE-H]/[EC_{50} of L-arginine] × k (TEMPONE-H with $O_2^{\cdot-}$).

Statistical Analysis. Data are presented as arithmetic mean ± S.E.M. Differences were analyzed for statistical significance using Student's t test. EC_{50} values were computed by the Hill equation, asterisk indicates statistical significance at $P < 0.05$.

Results

Protective Effects of L-Arginine against Myocardial Injury Induced by Oxygen Radicals. First, we studied the cardiac effects of oxygen radicals that were generated by electrolysis of the perfusion buffer. Figure 1A shows the experimental protocol and Fig. 1B shows the amounts of oxygen radicals (reactive oxygen intermediates, ROI) generated. Immediately after electrolysis, there was a burst of free radicals that prompted a strong signal at 1 min in the CLA-based chemiluminescence assay, followed by quick decomposition and washout of radicals within 3 min. Between 3 and 15 min, the oxygen radical level was less than 5% of the initial amounts (Fig. 1B). The functional effects of electrolysis are shown in Fig. 1C. Before electrolysis, $+dP/dt_{max}$ was 2626 ± 126 mm Hg s^{-1}, and coronary perfusion pressure was 50 ± 2 mm Hg (n = 7). Electrolysis (15 mA, 10 s) of the perfusion medium resulted in an immediate decline in left ventricular $+dP/dt_{max}$, with a minimum of 1471 ± 136 mm Hg s^{-1} after 3 min (56% of baseline), followed by gradual recovery to 1934 ± 113 mm Hg s^{-1} (74% of baseline) at 15 min ($P < 0.05$ versus baseline; circles in Fig. 1C). Coronary perfusion pressure increased to 110 ± 4 mm Hg after 3 min (2.2-fold), indicating endothelial dysfunction, and partially recovered after 15 min (67 ± 4 mm Hg, 1.3-fold baseline; $P < 0.05$). A similar pattern was observed for the maximal rate of left ventricular pressure decline ($-dP/dt_{max}$; not shown).

To test the hypothesis that electrolysis-induced myocardial injury is mediated by $O_2^{\cdot-}$, hearts were perfused in the presence of SOD or other potential radical scavengers, followed by electrolysis and determination of cardiac function over 15 min. The effect of the test compounds on left ventricular $+dP/dt_{max}$ and coronary perfusion pressure at 3 min are shown in Fig. 2, A and B, respectively. All compounds were highly protective. In the presence of SOD, $+dP/dt_{max}$ was restored to 95% of baseline, indicating that $O_2^{\cdot-}$ is generated by electrolysis and is the major cause for the observed cardiac injury (Fig. 2A). Exposure of hearts to L-arginine similarly prevented the myocardial injury (97% of baseline). However, the observation that D-arginine and the NOS inhibitor L-NNA were as protective as L-arginine (97 and 95% of baseline), indicated that the protective effect of L-arginine might result from a direct reaction with oxygen radicals, rather than stimulated NO synthesis. Therefore, we tested glycine

and aminoguanidine, which contains the guanidinium group of L-arginine. Whereas aminoguanidine completely prevented oxygen radical-induced myocardial injury (95% of baseline), $+dP/dt_{max}$ was still significantly lower than baseline after glycine, suggesting that the guanidinium group of L-arginine is essential to its protective effects. With respect to coronary function, all test compounds, including glycine, were highly protective in that they inhibited the electrolysis-induced increase in coronary perfusion pressure (Fig. 2B).

Although generation of oxygen radicals by electrolysis is a convenient and elegant method it lacks specificity of the generated reactive oxygen species. To further test whether L-arginine does, indeed, prevent $O_2^{\cdot-}$-induced myocardial injury by radical scavenging, we used hypoxanthine/xanthine oxidase to generate $O_2^{\cdot-}$. Catalase was included to prevent the accumulation of H_2O_2, which by itself or via secondary reactions might contribute to myocardial injury (Fig. 3A). The rate of $O_2^{\cdot-}$ generation, determined as SOD-inhibitable reduction of cytochrome *c*, was maximal within 2 min and remained virtually constant for the duration of the protocol (means, 28–29 $\mu M\ min^{-1}$, $P > 0.05$, 10 versus 2 min) (Fig. 3B). The effect of $O_2^{\cdot-}$, generated in this manner, on $+dP/dt_{max}$ is shown in Fig. 3C. In the absence of test compounds (=control), $+dP/dt_{max}$ rapidly decreased within the first 5 min of perfusion (~55% of baseline), followed by a further decrease to ~40% of baseline after 10 min. The presence of SOD in the perfusion medium almost completely prevented the decrease in $+dP/dt_{max}$ (94% of baseline). Similarly, L-arginine, *N*-α-acetyl-L-arginine (87% of baseline; $n = 4$, not shown), and to a lesser extent, aminoguanidine (80% of baseline) prevented $O_2^{\cdot-}$-induced injury. In contrast, glycine was ineffective (47% of baseline), and inactivated SOD was very weakly effective

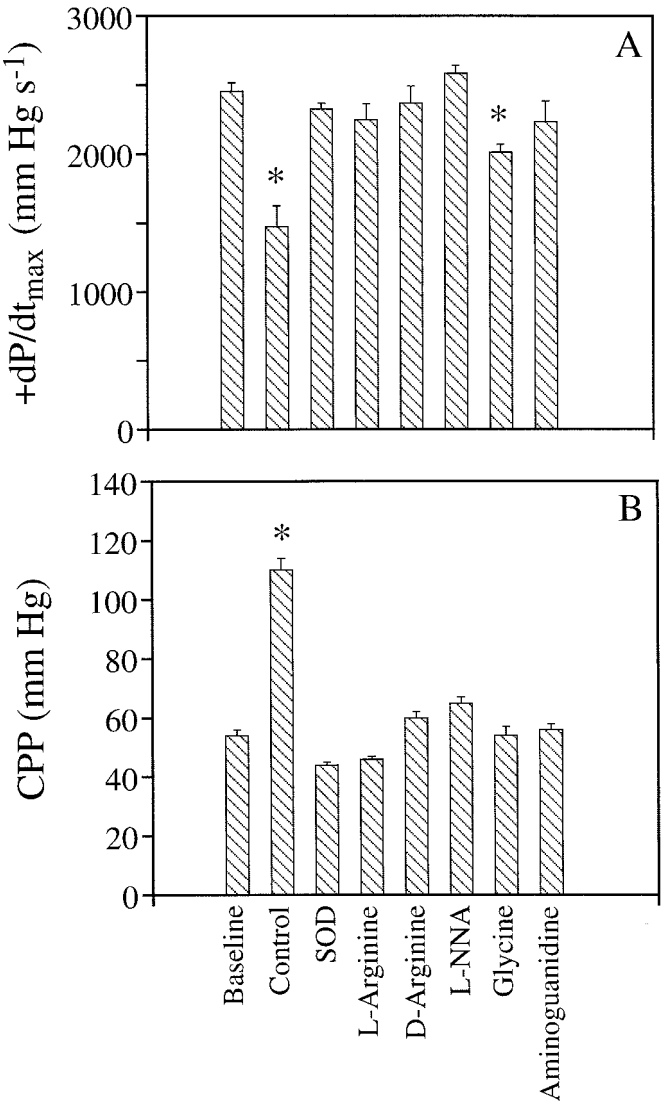

Fig. 2. Protective effects of L-arginine and other test compounds against oxygen radical-induced myocardial injury caused by electrolysis of the perfusion medium. Measurements show $+dP/dt_{max}$ (A) and CPP (B) at 3 min after start of electrolysis (maximal effect; see Fig. 1) in the absence of test compound (Control) or presence of SOD (50 U ml^{-1}), L-arginine, D-arginine, glycine, aminoguanidine (each 1 mM), or L-NNA (200 μM). Data are mean ± S.E.M. of four (test compounds) or seven (control) hearts. For comparison, the averaged values for the parameters before electrolysis (baseline) are also shown ($n = 31$). *, $P < 0.05$ compared with baseline.

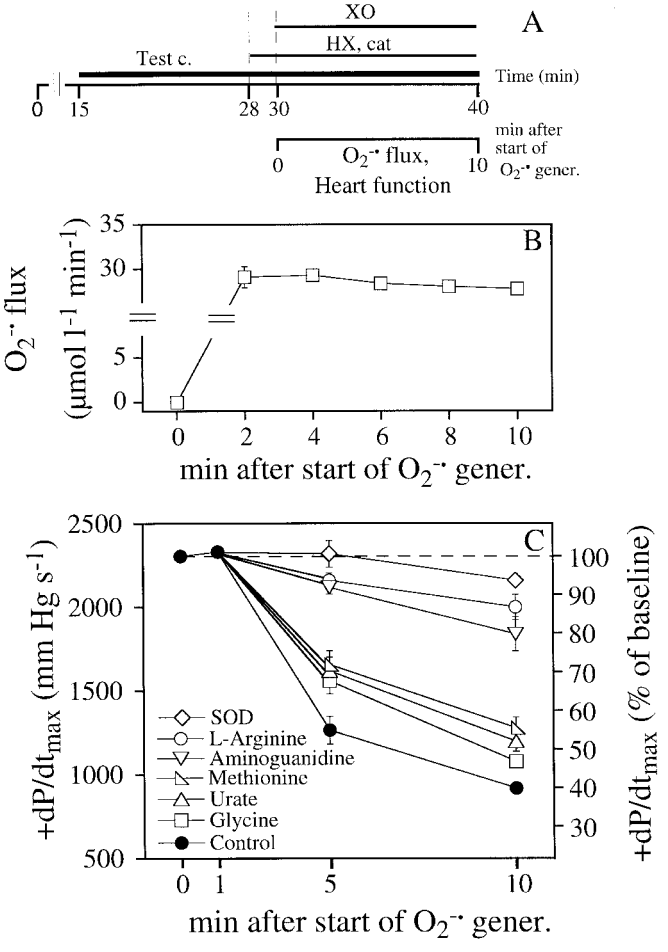

Fig. 3. Protective effects of L-arginine and other test compounds against oxygen free radical-induced myocardial injury caused by perfusing hearts with hypoxanthine/xanthine oxidase. A, experimental protocol. Hearts were equilibrated (15 min) and perfused with test compound (Test c.) by recirculation. At the indicated time points, hypoxanthine (HX, 1 mM), catalase (cat, 100 U ml^{-1}), and xanthine oxidase (60 mU ml^{-1}) were added. B, resulting $O_2^{\cdot-}$ flux rate as determined in separate experiments using reduction of cytochrome *c*. C, effect of $O_2^{\cdot-}$ generation on $+dP/dt_{max}$ in absence (Control) or presence of SOD (500 U ml^{-1}), L-arginine, glycine, aminoguanidine, methionine (each 1 mM), or urate (400 μM). The effects of *N*-α-acetyl-L-arginine and inactivated SOD are not shown for the sake of clarity (see text). The right ordinate shows $+dP/dt_{max}$ as percentage of baseline pressure. Data are mean ± S.E.M. of four (test compounds) or six (control) hearts.

(59% of baseline; $n = 4$, not shown). These results supported the hypothesis that L-arginine may, indeed, prevent myocardial injury by scavenging $O_2^{\cdot-}$.

A concern in this experimental setup was the formation of peroxynitrite, which could have been formed by the reaction of $O_2^{\cdot-}$ with endogenous NO from the coronary endothelium. Therefore, we also perfused hearts in the presence of urate or methionine, which are known to scavenge peroxynitrite (Fig. 3C). We found that neither compound significantly affected the $O_2^{\cdot-}$-induced myocardial injury, suggesting that peroxynitrite was not involved. In contrast to effects of electrolysis on coronary perfusion pressure where an increase was observed (see Fig. 1C), $O_2^{\cdot-}$ generated by hypoxanthine/xanthine oxidase caused a significant decline in coronary perfusion pressure, which was not affected by the various test compounds (Fig. 4A). Inactivated SOD and *N*-α-acetyl-L-arginine were likewise without effect ($n = 4$, not shown for the sake of clarity). The coronary relaxant was neither H_2O_2 nor peroxynitrite, because catalase was present during the experiment and neither urate nor methionine prevented the decrease in coronary perfusion pressure. We also tested whether urate itself, which is stoichiometrically formed by the hypoxanthine/xanthine oxidase reaction, was responsible for the decline in coronary perfusion pressure, but this was not the case (concentration, 400 μM; $n = 4$, not shown). However, oxygen radicals have been shown to stimulate cardiac norepinephrine release, and the release was inhibitable by different radical scavengers (Chahine et al., 1991). Therefore, we tested whether blocking vascular adrenergic receptors with phentolamine (10 μM) and propranolol (1 μM) would affect the oxygen radical-induced decline in coronary perfusion pressure, which was the case (51 ± 3 mm Hg, 88% of baseline; $P < 0.05$; Fig. 4B). Thus, the coronary relaxation observed in the hypoxanthine/xanthine oxidase protocol was largely due to endogenous norepinephrine. Clearly, the coronary vessels were not irreversibly damaged by the free radicals because they constricted in response to α-receptor stimulation with phenylephrine (Fig. 4C).

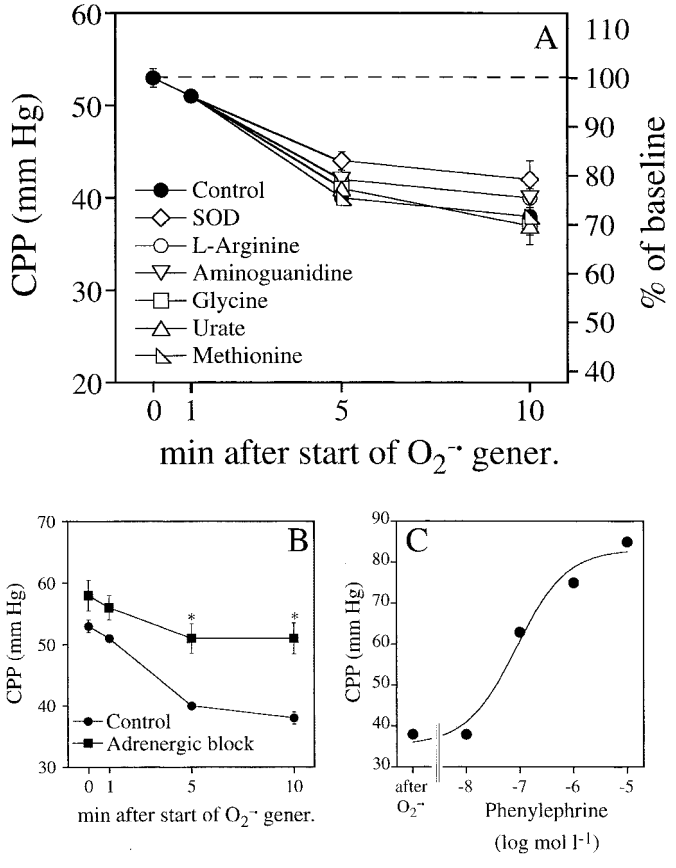

Fig. 4. A, effect of L-arginine and other test compounds on CPP in hearts perfused with hypoxanthine/xanthine oxidase (for protocol, see Fig. 3). Hearts were perfused as above and CPP was monitored for 10 min. For concentrations of test compounds and number of hearts, see Fig. 3. None of the test compounds significantly affected the decline in perfusion pressure. The right ordinate shows the CPP as percentage of baseline pressure. B, effect of adrenergic receptor blockade with a combination of phentolamine (10 μM) and propranolol (1 μM). The blockers were added 20 min before the start of $O_2^{\cdot-}$ generation and significantly reduced the decline in CPP ($P < 0.05$ versus control, $n = 4$). C, coronary constriction in response to exogenous phenylephrine (0.01–10 μM) starting after cessation of $O_2^{\cdot-}$ production (individual representative experiment). The EC_{50} value was 0.09 μM.

In Vitro $O_2^{\cdot-}$ Scavenging Activity of L-Arginine Determined by CLA-Enhanced Chemiluminescence and Cytochrome *c* Assays. In a complementary series of experiments, we determined the ability of the various agents to interact with $O_2^{\cdot-}$ in vitro. Oxygen radicals generated by electrolysis of the perfusion buffer were quantified using the CLA-enhanced chemiluminescence method (Fig. 5) and those generated in the hypoxanthine/xanthine oxidase reaction with the cytochrome *c* assay (Fig. 6). CLA was chosen as the chemiluminescence probe because we found that it reacts 1.7×10^6 times faster with $O_2^{\cdot-}$ than with H_2O_2 (also formed during electrolysis by autodismutation of $O_2^{\cdot-}$), thus combining high specificity with sensitivity for $O_2^{\cdot-}$ rather than H_2O_2.

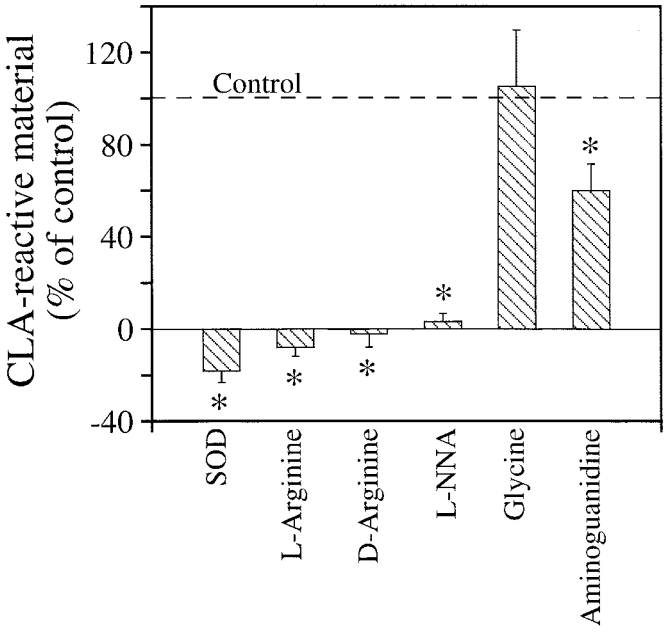

Fig. 5. $O_2^{\cdot-}$ scavenging activity of L-arginine and other test compounds as determined by CLA-enhanced chemiluminescence assay. Perfusion buffer without (Control) or with test compounds was collected 45 s after onset of electrolysis directly from the aortic cannula, mixed with CLA solution, and chemiluminescence was counted. The chemiluminescence signal measured in buffer before electrolysis (background) was subtracted. For concentrations of test compounds, see Fig. 2. Data are expressed as percentage of control, i.e., the chemiluminescence obtained for electrolyzed buffer (100 μl) in the absence of test compounds (51,004 ± 5,149 cpm = 100%). Data are mean ± S.E.M. of four (test compounds) or eight (control) independent measurements. *, $P < 0.05$ compared with baseline.

The concentration of CLA-reactive material (initially mostly $O_2^{\cdot-}$) in the perfusion medium 45 s after start of electrolysis was 510,040 ± 23,890 cpm ml^{-1} (control, Fig. 5). As expected, no signal above background was detectable in the perfusion medium in the presence of SOD. Interestingly, however, L-arginine, D-arginine, and L-NNA were equally effective scavengers, suggesting that these compounds are, indeed, capable of scavenging $O_2^{\cdot-}$ and possibly other reactive oxygen species. In contrast, aminoguanidine was only moderately effective ($P < 0.05$), and glycine was ineffective.

The $O_2^{\cdot-}$ scavenging potential of the various test agents in the hypoxanthine/xanthine oxidase model, expressed as inhibition of the reduction of cytochrome *c*, is shown in Fig. 6. At 0.1 μM, none of the compounds significantly affected cytochrome *c* reduction, whereas at higher concentrations the reduction of cytochrome *c* was significantly reduced. Specifically, at 10 mM, the respective rates of cytochrome *c* reduction were only 27 ± 8% of control (i.e., ~73% inhibition) for L-arginine, 37 ± 2% of control (i.e., 63% inhibition) for *N*-α-acetyl-L-arginine, ~51% of control (i.e., ~49% inhibition) for D-arginine and derivatives of L-arginine (L-NAME, L-NNA, L-NMMA), and 72 ± 7% of control (i.e., 28% inhibition) for aminoguanidine. Glycine had no effect on the rate of cytochrome *c* reduction at concentrations up to 10 mM. Thus, the results again indicated that L-arginine exhibits $O_2^{\cdot-}$ scavenging activity, which appeared to relate to the guanidinium group rather than to the α-amino/carboxyl moieties (*N*-α-acetyl-L-arginine and aminoguanidine, but not glycine, exhibited scavenging activity).

$O_2^{\cdot-}$ Scavenging Activity of L-Arginine Determined by EPR Spectroscopy. In a final set of experiments, we used EPR spectroscopy to study the radical scavenging activity of L-arginine. The $O_2^{\cdot-}$ radicals generated by hypoxanthine/xanthine oxidase were trapped by TEMPONE-H, resulting in the formation of 2,2,6,6-tetramethyl-4-oxo-piperidinoxyl (TEMPONE), which exhibits a prominent EPR spectrum (Fig. 7). The effect of L-arginine, aminoguanidine and glycine on the formation of TEMPONE is shown in Fig. 8. Clearly, all three compounds significantly inhibited the formation of TEMPONE, with L-arginine as the most effective compound (EC_{50}, 75.1 μM). Aminoguanidine also scavenged $O_2^{\cdot-}$ at 1 and 10 mM, whereas glycine was not effective below 10 mM. The apparent rate constant (k_{app}) for the reaction of L-arginine with $O_2^{\cdot-}$ was 4.8×10^3 M s^{-1}.

Discussion

The objective of the present study was to investigate whether L-arginine exerts protective effects against oxygen-mediated myocardial injury by directly scavenging radicals. This was studied in isolated perfused hearts in combination with in vitro quantification of free radicals using established methods. We have made three new observations: 1) L-arginine clearly prevents oxygen radical-induced cardiac contractile dysfunction by diminishing the generation of oxygen radicals; 2) the radical scavenging activity of L-arginine is concentration-dependent; and 3) the scavenging activity can be demonstrated using EPR spectroscopy. These data suggest that a free radical-scavenging action cannot be excluded from explanations of the protective effect of L-arginine.

We chose the crystalloid perfusion method to "isolate" the scavenging activity of the test compounds from that of other potential scavengers (proteins, thiols), which might be present in more complex media. Furthermore, for determination of oxygen radical flux rates it was important to avoid any interference from plasma components or blood cells that might obscure the relation to the observed cardiac dysfunction. Exposure of hearts to oxygen radicals generated by

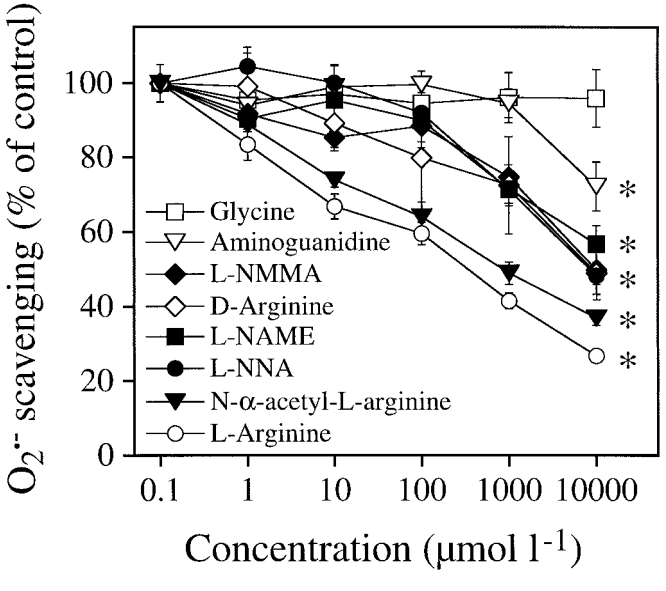

Fig. 6. $O_2^{\cdot-}$ scavenging activity of L-arginine and other test compounds as determined by cytochrome *c* assay. For composition of assay mixture, see *Experimental Procedures*. The final concentrations of the test compounds were between 100 nM and 10 mM. The reduction of cytochrome *c* was recorded at 550 nm over 1 min, the rates were determined by linear regression and expressed in percentage of the control rate (3.32 ± 0.01 μmol of $O_2^{\cdot-}$ l^{-1} min^{-1} = 100%). Data are mean ± S.E.M. of four independent measurements. *, $P < 0.05$ versus control.

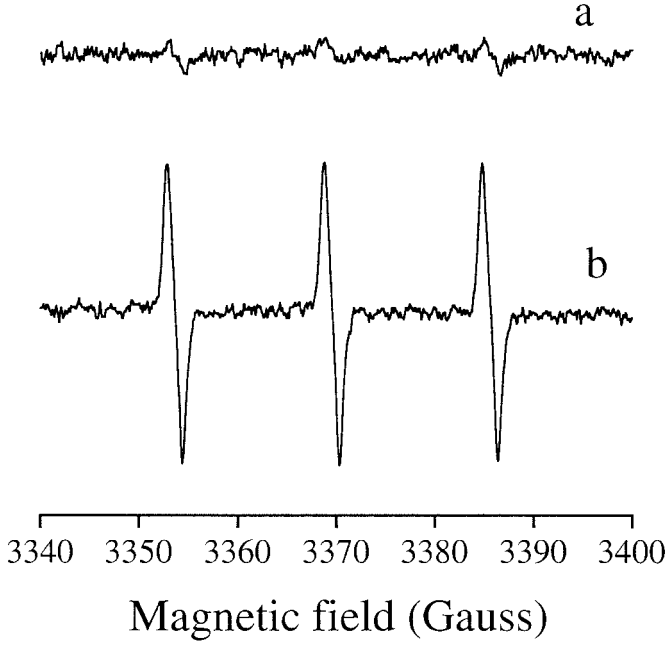

Fig. 7. Electron paramagnetic resonance spectra of samples containing 1 mM hypoxanthine, 100 μM DTPA, and 30 μM TEMPONE-H in 10 mM PBS, pH 7.4 before (trace a) and after addition of 5 mU ml^{-1} xanthine oxidase and incubation for 5 min at ambient temperature (trace b). The hyperfine splitting constant of the triplet signal of spectrum b (a_N) was 16.1 G.

electrolysis, or to O_2^- generated by hypoxanthine/xanthine oxidase, resulted in a rapid decline in the rate parameters $+dP/dt_{max}$ and $-dP/dt_{max}$, which are sensitive indices of left ventricular contractility. Previous authors have mostly used the left ventricular developed pressure as index of myocardial function (Jackson et al., 1986; Lecour et al., 1998). None of the two radical sources we used was arrhythmogenic, in agreement with previous observations in normoxic perfused hearts (Svendsen and Bjerrum, 1992). Perfusion of hearts in the presence of L-arginine prevented the decline in left ventricular contractility caused by electrolysis of the buffer or the addition of hypoxanthine/xanthine oxidase, presumably due to scavenging of O_2^- and/or other reactive oxygen species. This protection agrees with the improvement in myocardial and coronary function previously observed with L-arginine in regional and global models of cardiac ischemia/reperfusion injury (Weyrich et al., 1992; Brunner et al., 1997).

The protective effect of L-arginine against oxygen radical-induced myocardial injury is usually attributed to stimulated NOS activity resulting from increased substrate availability. However, the results of this study clearly indicate that the protective effect of L-arginine is related to its potential to scavenge oxygen radicals. This conclusion is based on the following evidence. First, NOS inhibitors and other derivatives of L-arginine were similarly effective in protecting hearts and in scavenging radicals (see Figs. 2, 5, and 6). Second, D-arginine, which does not serve as NOS substrate, mimicked L-arginine in protecting the hearts and scavenging radicals. Third, the amino acid glycine neither prevented the O_2^--induced myocardial contractile dysfunction (Fig. 3) nor exhibited O_2^- scavenging potential (Figs. 5 and 6). Finally, the radical scavenging features of L-arginine were also demonstrated by EPR spectroscopy using a spin trap that is regarded as highly specific for O_2^- (Dikalov et al., 1997).

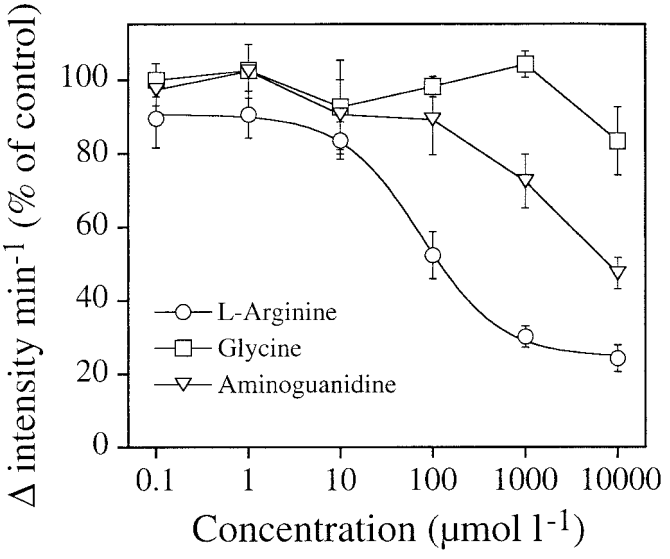

Fig. 8. O_2^- scavenging activity of L-arginine, glycine, and aminoguanidine as determined by EPR spectroscopy. The assay mixture consisted of 1 mM hypoxanthine, 5 mU ml^{-1} xanthine oxidase, 100 μM DTPA, 30 μM TEMPONE-H in 10 mM PBS, pH 7.4, at ambient temperature. The final concentrations of the test compounds were between 100 nM and 10 mM. The spectra were recorded in 1-min intervals for 5 min. The intensity of each spectrum was determined, and the rates of increase in intensity calculated by linear regression. The rates obtained in the presence of test compounds are expressed as percentage of control (100% = rates obtained in the absence of test compound). Data are mean ± S.E.M. of four to six measurements. The EC_{50} value for L-arginine was 75.1 μM. For L-arginine the least-squares curve fit is presented.

The apparent rate constant for the reaction of L-arginine with O_2^- was approximately 4.8×10^3 M s^{-1} [i.e., of similar magnitude as the reaction of O_2^- with α-tocopherol (4.5×10^3 M s^{-1}), but two orders of magnitude smaller than the reaction of O_2^- with ascorbate in aqueous solution (3.3×10^5 M s^{-1}) (Gotoh and Niki, 1992)]. These constants are given for comparison, but they allow as yet no conclusion as to the antioxidant potential of L-arginine, because we presently know neither the underlying mechanism for the reaction between O_2^- and the guanidinium group nor the local environment where the reaction takes place. Indirect evidence, however, suggests that the action of L-arginine is extracellular, because both L- and D-arginine were similarly cardioprotective (Fig. 2), which would not be expected in the case of an intracellular action since only L-arginine but not D-arginine is transported into the cells (Schmidt et al., 1993). It is for this reason that D-arginine is no useful control in in vivo experiments to show that L-arginine effects are due to stimulation of NOS activity. Despite this, it is conceivable that L-arginine is taken up by cells and exerts its action intracellularly, whereas D-arginine has an extracellular protective effect. Irrespective of this, the present results clearly strengthen the view that L-arginine may act as radical scavenger in biological systems (Wascher et al., 1997). This action may at least partly account for the antiatherosclerotic effects of L-arginine (Böger et al., 1995; Clarkson et al., 1996) and provide an additional rationale for its use as a dietary supplement in different diseases (Velianou et al., 1999).

We compared the radical scavenging potential of L-arginine with that of glycine and aminoguanidine, because glycine represents a simple α-amino carboxylic acid, whereas the aminoguanidine is expected to mimic the guanidinium group of L-arginine. The findings with glycine were consistent in the hypoxanthine/xanthine oxidase model where it was neither protective nor a radical scavenger. Glycine is an efficient scavenger of hypochlorous acid, which is formed during electrolysis from H_2O_2 and chlorine (+ electrons) (Jackson et al., 1986). Therefore, the partial protection in the electrolysis model was probably due to scavenging of hypochlorous acid, without significantly affecting CLA-based chemiluminescence (Fig. 5), which is rather specific for O_2^-. Aminoguanidine, on the other hand, was protective and scavenged electrolysis-generated oxygen radicals, although to a lesser extent. This suggested that the antioxidative activity of L-arginine is based on the guanidinium group rather than on the α-amino carboxylic acid structure. This finding was further corroborated using *N*-α-acetyl-L-arginine which was cardioprotective and similarly potent in scavenging O_2^- as L-arginine (Fig. 6). This is in contrast to a recent report by another group (also from Graz), which attributed the antioxidative effect of L-arginine to the α-amino group (Wallner et al., 2001). These authors used copper or 2,2′-azo-bis(2-amidinopropane) hydrochloride to generate free radicals, and the inhibition of serum lipoprotein oxidation as a measure of antioxidant effects. Although this latter method has been widely used, it is rather indirect and less specific than our approach in which the antioxidant effect of the test compound can be attributed to a chemically defined radical (O_2^-) and compared with resulting functional alterations.

Exposure of hearts to electrolyzed buffer resulted in an

increase in coronary perfusion pressure, which is in agreement with previous reports (Jackson et al., 1986; Lecour et al., 1998). This is probably largely due to oxidative consumption of NO released from the vascular endothelium. In addition, hypochlorous acid, which may be formed during electrolysis, exerts coronary constrictor effects (Eley et al., 1991). On the other hand, exposure to hypoxanthine/xanthine oxidase resulted in a considerable decrease of perfusion pressure, indicating substantial reduction in coronary resistance. Because of the presence of catalase, H_2O_2 was probably not involved. This also excluded the possibility that the relaxation was caused by increased availability of NO after activation of NOS by H_2O_2 (Zembowicz et al., 1993). Urate, which is stoichiometrically formed by the hypoxanthine/xanthine oxidase reaction, was also excluded because it had no vascular or myocardial effects. Finally, peroxynitrite was excluded by the lack of effect of the scavengers urate and methionine. Irreparable vascular damage was also unlikely to be involved because exogenous phenylephrine elevated coronary perfusion pressure in concentration-dependent fashion far above the baseline level (Fig. 4C). However, the combined blockade of α- and β-adrenergic receptors largely prevented the reduction in perfusion pressure, indicating that endogenous norepinephrine caused the relaxation (Fig. 4B). This is in line with the stimulated cardiac norepinephrine release in response to free radical exposure (Chahine et al., 1991) and the sustained coronary dilator effect of norepinephrine, which follows a brief and transient constricting phase (unpublished observations).

In conclusion, besides possibly increasing the formation of NO, L-arginine has substantial concentration-dependent antioxidant properties, suggesting that its well known vascular and myocardial protective effects occur partly through this mechanism. Future experiments will show to what extent this mechanism plays a role in pathophysiological situations in vivo, where other competing antioxidant systems may be present.

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