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Nitric Oxide Synthase Inhibitors Decrease Coronary Sinus-Free Radical Concentration and Ameliorate Myocardial Stunning in an Ischemia-Reperfusion Model

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OBJECTIVES Our objective was to determine the effect of a nitric oxide synthase inhibitor, NG-nitro-L-arginine (L-NNA) on free radical generation and myocardial contractility after ischemia-reperfusion.

BACKGROUND Cardiotoxic free radicals are generated by ischemia-reperfusion sequences. Nitric oxide reacts with superoxide radical to form peroxynitrite, which generates additional free radicals. Our hypothesis was that by inhibiting NO production, free radical formation will be diminished, which should be cardioprotective.

METHODS We studied 32 dogs. Coronary occlusion-reperfusion (20 min each) sequences were created by intracoronary balloon angioplasty inflation-deflation. Using electron paramagnetic resonance, we monitored the coronary sinus concentration of ascorbate free radical ($Asc^{\cdot -}$), a measure of total oxidative flux. The L-NNA (4.8 mg/kg total) was infused intravenously during occlusion-reperfusion; control dogs received saline. Immunohistochemical staining demonstrated the peroxynitration product nitrotyrosine.

RESULTS In the control dogs $Asc^{\cdot -}$ rose from 3.2 ± SD 0.5 nmol/l to 4.8 ± 1.1 nmol/l with reperfusion, a 50% rise. With L-NNA the $Asc^{\cdot -}$ rose from 3.2 ± 0.9 nmol/l to 4.0 ± 1.2 nmol/l, a 25% rise ($p < 0.01$, L-NNA vs. control). Echocardiographic left ventricular fractional area shortening (FAS) in the control dogs declined from 38 ± 19% (baseline) to 26 ± 14% (ischemia), and to 22 ± 11% with reperfusion ($p < 0.01$ vs. baseline). With L-NNA, FAS declined from 36 ± 13% (baseline) to 27 ± 12% (ischemia) but then rose to 33 ± 14 with reperfusion (p = NS vs. baseline). Nitrotyrosine was present in the myocardium subjected to ischemia-reperfusion, but almost absent in dogs receiving L-NNA. Myocardial perfusion was not altered by L-NNA.

CONCLUSIONS The NO synthase inhibitors decrease coronary sinus free radical concentration and ameliorate myocardial stunning after ischemia-reperfusion. (J Am Coll Cardiol 2001;38:546–54)
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Ischemia-reperfusion injury is thought to be due to the generation of oxygen-derived free radicals such as the superoxide and hydroxyl radicals (1–5). Such free radicals lead to lipid peroxidation, cellular dysfunction and stunning of myocardium. If free radical generation can be limited during reperfusion, less myocardial injury and stunning may occur.

Various metabolic pathways lead to the formation of both oxygen and nitrogen free radicals. These species include reactive oxygen species such as $O_2^{\cdot -}$ (superoxide), $HO^{\cdot}$ (hydroxyl radical), and $LOO^{\cdot}$ (lipid peroxyl radicals) and reactive nitrogen species such as nitric oxide (NO) and nitrogen dioxide. Nitric oxide is synthesized from oxidative deamination of arginine by nitric oxide synthase (NOS), forming citrulline. Competitive inhibitors of NOS are usually derivatives of L-arginine with substitutions on the nitrogen atom of the guanidino group, such as NG-monomethyl-L-arginine, NG-nitro-L-arginine methyl ester (L-NAME) and NG-nitro-L-arginine (L-NNA). These inhibitors will decrease the production of nitric oxide (6). If NOS is active, the resulting formation of NO activates cyclic guanosine monophosphate (cGMP) synthesis.

Oxidation products of NO, namely nitrite and nitrate, are released by the hearts of patients undergoing acute myocardial infarction and percutaneous transluminal coronary angioplasty (7). Levels of NO in excess of that required to activate cell cGMP synthesis combine with reactive oxygen species, such as superoxide. Nitric oxide rapidly reacts with superoxide to form peroxynitrite anion in high yield (8–13). Peroxynitrite decays rapidly once it is protonated to form peroxynitrous acid, which is unstable and has a half-life of <1 s. In the presence of oxidative substrates, peroxynitrous acid-mediated oxidation of the substrate occurs via electron transfer, leading to the formation of other free radicals. In the absence of oxidative substrates, nitrate is formed.

The overall aim of this study was to demonstrate that the L-arginine-NO pathway contributes to free radical generation, which leads to ischemia-reperfusion injury. To accomplish this aim, we proposed a testable hypothesis: by inhibiting NO production in a closed-chest canine ischemia-reperfusion model, peroxynitrite and free radical formation should be diminished. Thus, NO synthase inhibitors may have cardioprotective effects. We utilized a previously

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Abbreviations and Acronyms

$Asc^{\cdot-}$	= ascorbate free radical
cGmp	= cyclic guanosine monophosphate
EPR	= electron paramagnetic resonance
$HO^{\cdot}$	= hydroxyl radical
IV	= intravenously
LAD	= left anterior descending
L-NAME	= NG-nitro-L-arginine methyl ester
L-NNA	= NG-nitro-L-arginine
NAD	= nicotinamide adenine dinucleotide
$LOO^{\cdot-}$	= lipid peroxyl radicals
NO	= nitric oxide [nitrogen monoxite]
NOS	= nitric oxide synthase
$O_2^{\cdot-}$	= superoxide

developed and validated technique, electron paramagnetic resonance (EPR) measurements of ascorbate free radicals, a real-time quantitative marker of free radical generation.

METHODS

Animal preparation. A closed-chest canine model was used; 32 dogs were studied in total. General anesthesia was achieved with combination of 400 mg of ketamine and 105 mg of xylazine intramuscularly, followed by 20 mg/kg of pentobarbital intravenously (IV) for continued sedation as needed. Ventilation was provided by a volume cycled respirator after endotracheal intubation; tidal volume, respiratory rate and Fio_2 were adjusted according to frequent blood gas measurements, to maintain physiologic pH (7.35 to 7.45), and Po_2 >100 torr.

The left femoral artery and vein were cannulated for administration of IV medications and blood pressure monitoring. The right carotid artery and jugular vein were cannulated. Then, under fluoroscopic guidance a 6F USCI Gensini catheter was placed via the jugular vein into the right atrium and advanced into the coronary sinus. Again under fluoroscopic guidance, a balloon dilation catheter (for coronary occlusion), with a balloon diameter of 2.5 mm and balloon length of 20 mm, was placed via the carotid artery into the proximal portion of the left anterior descending (LAD) coronary artery. A very brief balloon inflation was done under fluoroscopy, and coronary artery occlusion was confirmed with angiographic contrast injection.

EPR. The ascorbate free radical ($Asc^{\cdot-}$) is a resonance-stabilized tricarbonyl species that is readily formed from the one-electron oxidation of ascorbate, $AscH^-$. Because of the low potential of the $Asc^{\cdot-}/AscH^-$ couple, ascorbate is the terminal small molecule antioxidant (14–16). Nearly every oxidant, including peroxynitrous acid, that could be present in a biological system will bring about the one-electron oxidation of ascorbate (14). Thus, the concentration of ascorbate free radical, as monitored by EPR, is an excellent measure of the degree of free radical stress (total oxidative flux) in chemical, biochemical and biologic systems (17–19).

For this study we used a method we have previously described in detail (20). A brief description is given here. A Varian E-4 spectrometer with a TM_{110} cavity and an aqueous flat cell was used to monitor $Asc^{\cdot-}$. The lower end of the flat cell was connected to a Teflon tubing (OD 0.5 mm), which was connected to a manifold with multiple ports. The coronary sinus catheter and the femoral artery catheter were connected to different ports of the manifold. The upper end of the flat cell was connected to the femoral vein with a variable-speed infusion pump. Thus, coronary venous blood was circulated continuously through the spectrometer at a determined rate. To scan the arterial blood, the manifold was switched from coronary sinus to femoral artery. The blood sample was scanned within 4 s of leaving the vein or artery.

The EPR instrument settings used to acquire optimum $Asc^{\cdot-}$ spectra were as follows: nominal power = 40 mW; modulation amplitude = 0.63 Gauss; time constant 1 s; scan rate = 1 Gauss per 24 s. The spectra of $Asc^{\cdot-}$ (a^H = 1.8 Gauss) is typically a doublet. The peak-to-peak height (in mm) of the $Asc^{\cdot-}$ signal was measured and normalized to full instrument gain. The actual concentration of $Asc^{\cdot-}$ was determined from the signal height as previously described using 3-carboxyproxyl as a standard accounting for saturation effects (15,16). For our experimental conditions, 1 mm of normalized $Asc^{\cdot-}$ signal height corresponds to 0.073 nmol/liter $Asc^{\cdot-}$ in the blood being sampled as the volume of blood in the EPR flat cell remains constant.

Without ascorbic acid supplementation, a weak $Asc^{\cdot-}$ signal (about 1 nmol/l) can often be detected in the arterial blood, but not in the coronary sinus blood. To obtain an adequate EPR signal, 1 g of 1-ascorbic acid was infused IV as a bolus, followed by a slow infusion. The $Asc^{\cdot-}$ signal could then be obtained from arterial and coronary venous blood. The $Asc^{\cdot-}$ concentration varied from animal to animal; the arterial $Asc^{\cdot-}$ signal was usually about 14 nmol/l; the venous $Asc^{\cdot-}$ signal was usually 8 nmol/l. The negative arterial-venous gradient suggests that the ascorbate radical is repaired by the nicotinamide adenine dinucleotide (NAD) system as red blood cells pass through the myocardium. We maintained a steady-state level of arterial $Asc^{\cdot-}$ by adjusting the rate of ascorbic acid infusion (usually between 3.8 and 15.2 mg/ml); the arterial $Asc^{\cdot-}$ level was rechecked frequently. Arterial pH was maintained between 7.35 and 7.45 by respirator adjustments.

Left ventricular function. Left ventricular systolic contraction was measured by transthoracic echocardiography by a Hewlett-Packard (Andover, Massachusetts) Ultrasound Imaging System. Two-dimensional left ventricular cavity images in the parasternal short axis view were obtained at the papillary muscle level at baseline, after 15 min of coronary occlusion and after 15 min of reperfusion. Left ventricular end-diastolic and end-systolic areas were determined by planimetry by an observer blinded as to treatment group, and the percent area shortening was determined using the following formula: end-diastolic area minus end-systolic area divided by end-diastolic area.

Interobserver variability was assessed by having a second

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(independent) observer planimeter 20 randomly selected images to compare the area determinations to those of the initial observer. Intraobserver variability was determined by having the initial observer re-planimeter 17 images 1 month after the initial determination to compare the two data sets.

Measurement of myocardial blood flow by neutron activation assay. To investigate the effect of myocardial blood flow on this study, a new method using stable isotope-labeled microspheres for the measurement of myocardial blood flow was employed in 11 dogs (21). Five control dogs received saline injection and another six dogs received L-NNA before and during the occlusion-reperfusion sequence as above.

To gain access to the left atrium, a thoracotomy was performed and a catheter was inserted into the left atrium for injection of microspheres. Two additional catheters were inserted into the left carotid artery and the right femoral artery, respectively, to collect reference blood samples.

Three milliliters ($\approx 7 \times 10^6$) of 15 μm stable isotope-labeled microspheres (BioPAL, Wellesley Hills, Massachusetts) was injected through the left atrial catheter to measure myocardial blood flow in three conditions: baseline, occlusion and reperfusion. Simultaneously, two reference blood samples were drawn from carotid and femoral arteries using a withdrawal pump. The withdrawal rate was set at 2 ml/min, and blood was collected over a 3.5-min interval, resulting in a 7-ml reference blood collection.

After sacrificing the animal, the heart was removed and vigorously rinsed with distilled water to remove blood before being fixed for 48 h in formaldehyde. Two transmural myocardial tissue samples in each animal were taken from the area distal to the LAD occlusion (risk region). The blood samples were rinsed using a sodium-free solution (sansSaLine, BioPAL, Wellesley Hills, Massachusetts) and then centrifuged for 1 min at ~2,500 rpm. After discarding the supernatant, the blood samples and myocardial tissue were dried in a warming-oven (70°C overnight). The dried blood and tissue samples were sealed in vials and sent to BioPAL for neutron activation and measurement of regional myocardial perfusion, as described in detail by Reinhardt et al. (21).

Risk area assessment. In 14 dogs the degree of myocardium at risk was determined by direct staining. Five milliliters of 0.5% Evans blue dye was injected at the completion of the protocol through the distal tip of the balloon catheter, after inflation of the balloon. The area that was stained was the LAD risk area, as the dye was only distributed in the LAD distribution distal to the balloon inflation. The heart was removed and the entire left ventricle was weighed, followed by dissection and weighing of the stained risk area, allowing the percent of myocardium at risk to be calculated. This was done to show that our results were not influenced by variations in percent of risk area size between control and intervention groups.

Protocol. Once stable $Asc^{\cdot-}$ signals were obtained, a baseline echocardiogram was done. An initial bolus of L-NNA (Sigma Chemical, St. Louis, Missouri) 0.2 mg/kg/min IV was infused for 8 min. After this initial bolus, a maintenance infusion of 0.08 mg/kg/min IV was continued throughout 40 min of coronary artery occlusion and reperfusion. The balloon catheter was inflated to a pressure of 120 psi for 20 min. Verification of coronary artery occlusion was monitored by arterial blood pressure decline with occlusion, electrocardiogram changes and echocardiographic evidence of wall motion abnormality. After 20 min of occlusion, the balloon was deflated to accomplish reperfusion. The coronary sinus $Asc^{\cdot-}$ signal was monitored from the beginning of occlusion through 15 min of reperfusion. The femoral artery $Asc^{\cdot-}$ signal was rechecked to verify $Asc^{\cdot-}$ signal stability prior to occlusion, at 15 min of occlusion and at 15 min of reperfusion. Echocardiography was repeated after 15 min of coronary artery occlusion and 15 min of coronary artery reperfusion. Arterial blood gases were obtained at the time of coronary artery occlusion, 10 min after occlusion, and at 5 min of reperfusion. After 20 min of reperfusion, the balloon catheter was reinflated and Evans blue dye was injected through the distal tip of the balloon to determine the coronary risk area. Euthanasia was then achieved by massive IV barbiturate overdose.

Nitrotyrosine immunohistochemistry. In seven dogs, myocardium subjected to an occlusion-reperfusion sequence was prepared for immunohistochemistry using a method we recently described (22). After the occlusion-reperfusion experiment was completed the heart was removed and perfused with 4% formaldehyde buffer (500 ml). Left ventricular myocardial biopsies were obtained from the risk area, cut into 2-mm sections, and postfixed for 2 h in formaldehyde buffer. Tissue sections were subsequently processed through graded alcohols to paraffin blocks. Sections (4 μm) were cut from each block, mounted on Superfrost histology slides (Fisher Scientific, Pittsburgh, Pennsylvania), and deparaffinized in three changes of xylene (5 min each) followed by two changes in 100% ethanol. Endogenous peroxidase activity was blocked with a 30-min darkroom incubation in 3% H_2O_2-methanol. For antigen processing, sections were rehydrated and treated with 10 mmol/l citrate acid at 80°C for 10 min. After three changes of distilled water, sections were then treated with 10% normal goat serum for 40 min and incubated in antinitrotyrosine antibody (1:1000 dilution; Upstate Biotechnology, Lake Placid, New York) solution overnight.

The next day, tissue sections were rinsed with phosphate-buffered saline three times (5 min each) and processed for demonstration of immunoreactive protein with an immunoenzymatic staining kit (DAKO, Carpinteria, California) and an Immunopure Metal Enhanced diaminobenzidine substrate (Pierce, Rockford, Illinois) as directed by the manufacturers. The presence of immunoreactive protein was assessed microscopically by an independent reviewer.

Statistical analysis. The effect of L-NNA on percent change of $Asc^{\cdot-}$ and fractional area was tested using a repeated measures analysis. The two factors in the analysis

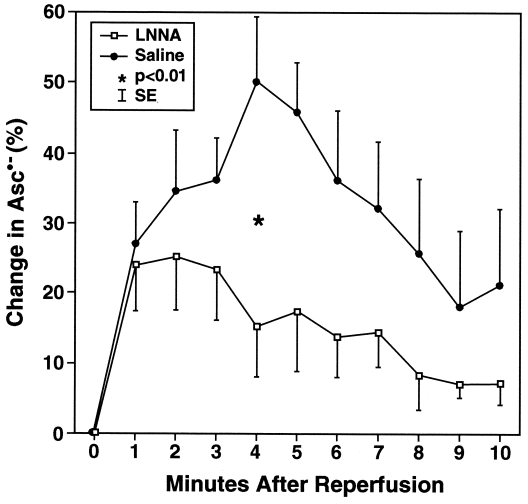

Figure 1. Coronary sinus concentration of ascorbate free radical ($Asc^{\cdot-}$) after ischemia-reperfusion sequences. The rise in $Asc^{\cdot-}$ concentration is significantly lower in the animals receiving the nitric oxide synthase inhibitor NG-nitro-L-arginine (L-NNA).

of $Asc^{\cdot-}$ were treatment (L-NNA and saline) and time (1 to 10 min). For fractional area, the two factors were treatment and condition (preocclusion, occlusion, and reperfusion). For each of these variables, mean contrasts and corresponding *t* test statistics were computed for the pairwise comparisons of interest based on estimates of mean differences and SE from the repeated measures analysis. Bonferroni's method was applied to adjust for the multiple number of statistical tests performed. A Bonferroni-adjusted p value <0.05 was considered statistically significant. The statistical analysis was done using the SAS/STAT procedure MIXED (23).

A repeated measures analysis was used to compare myocardial blood flow among three conditions (baseline, occlusion and reperfusion) for control and L-NNA dogs and also between control and L-NNA groups for all three conditions. The factors included in the repeated measures analysis were treatment (control vs. L-NNA) and condition (baseline, occlusion and reperfusion). Bonferroni's method was applied to the p values to adjust for the number of tests performed. A Bonferroni-adjusted $p < 0.05$ was considered statistically significant.

RESULTS

Changes in coronary venous $Asc^{\cdot-}$ concentration during ischemia-reperfusion in the control dogs and the L-NNA dogs are summarized in Figure 1 and Table 1. The prereperfusion $Asc^{\cdot-}$ concentrations were similar in the control (saline) dogs and the L-NNA dogs, 3.2 ± 0.5 nmol/liter and 3.2 ± 0.9 nmol/liter, respectively. In the control dogs the $Asc^{\cdot-}$ concentration rose 50% to 4.8 ± 1.1 nmol/liter. In comparison, $Asc^{\cdot-}$ concentration in the L-NNA dogs rose to 3.7 ± 1.3 nmol/liter, a 25% rise ($p < 0.01$ vs. control dogs at 4 min reperfusion) (Fig. 1). Compared to the baseline value, only the 1-, 2- and 3-min $Asc^{\cdot-}$ concentrations in the L-NNA dogs showed significant increases after reperfusion, whereas in the control dogs the $Asc^{\cdot-}$ increases were significant throughout the first 6 min after reperfusion compared to baseline. The area under the curve of percent change in $Asc^{\cdot-}$ signal was 107 (arbitrary units) for the control dogs versus 52 for the L-NNA dogs, a 52% decrease in area. The arterial concentrations of $Asc^{\cdot-}$ were similar in the L-NNA dogs and the

Table 1. Ascorbate Free Radical Data (nmol/l)

Dog no.	Baseline	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min
Saline Animals											
1	3.96	4.55	5.65	5.65	5.95	6.31	6.31	6.46	6.46	6.24	6.31
2	2.68	3.82	4.11	4.03	4.03	3.96	3.38	3.38	3.45	3.16	3.16
3	2.86	3.82	4.26	4.11	4.11	4.11	3.74	3.52	3.52	3.30	3.60
4	3.01	3.96	3.52	3.74	3.74	3.60	3.23	3.23	2.94	2.79	2.79
5	3.30	3.67	3.67	3.96	6.02	5.21	5.21	4.70	3.82	3.52	3.67
Mean	3.16	3.96	4.24	4.30	4.77	4.64	4.37	4.26	4.04	3.80	3.91
SD	0.50	0.34	0.84	0.77	1.11	1.11	1.34	1.36	1.39	1.39	1.39
L-NNA Animals											
1	1.61	1.61	1.76	1.76	1.47	1.39	1.54	1.61	1.76	1.61	1.61
2	4.44	4.92	4.84	4.62	4.62	4.55	4.55	4.70	4.70	4.70	4.92
3	3.82	4.04	3.23	3.52	3.52	3.60	3.67	3.67	3.30	3.82	3.67
4	4.18	4.84	5.36	5.21	5.28	5.28	5.28	4.70	4.70	4.84	4.84
5	3.34	5.58	5.58	5.58	5.43	5.43	4.92	4.92	4.70	3.67	3.67
6	3.01	3.74	4.04	3.89	3.30	3.30	3.45	3.52	3.45	3.45	3.89
7	2.97	3.93	3.93	3.60	3.60	3.60	3.67	3.45	3.08	3.16	3.16
8	3.08	4.18	4.18	4.18	3.67	3.23	3.23	3.52	3.16	3.16	3.23
9	2.31	2.86	2.94	2.94	2.57	3.45	2.64	2.79	2.28	2.50	2.35
Mean	3.20	3.97	3.98	3.92	3.72	3.76	3.66	3.65	3.46	3.43	3.48
SD	0.89	1.18	1.22	1.17	1.26	1.22	1.16	1.05	1.07	1.01	1.07

Times are minutes after reperfusion.
L-NNA = NG-nitro-L-arginine.

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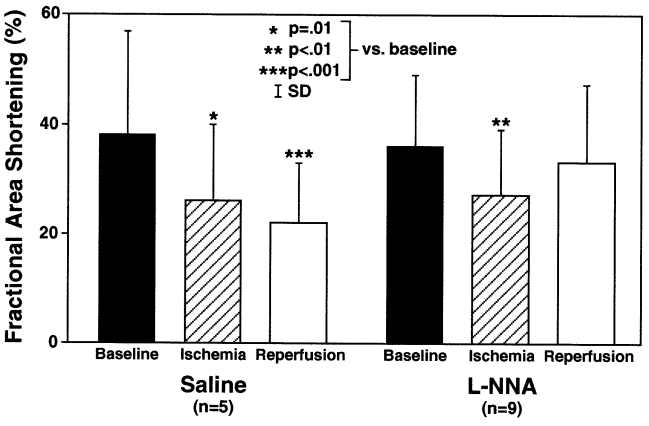

Figure 2. Fractional area shortening by echocardiography after ischemia and reperfusion. A significant decline in fractional area shortening by echocardiography occurs during ischemia in both animal groups. In animals receiving saline a further decline in fractional area shortening (FAS) is seen after reperfusion, indicating "stunning"; in the NG-nitro-L-arginine (L-NNA) animals, FAS after reperfusion returns to baseline levels—a cardioprotective effect.

control dogs just prior to reperfusion, (6.8 ± 2.0 nmol/liter vs. 8.1 ± 1.4 nmol/liter, respectively, p = NS) and showed no significant change during reperfusion.

Echocardiographic-measured fractional area shortening is shown in Figure 2. The control (saline) dogs showed a decline in fractional area shortening from 38 ± 19% to 26 ± 14% (p = 0.01 vs. baseline) during ischemia, and a further decline to 22 ± 11% (p < 0.001 vs. baseline) after reperfusion. In the L-NNA dogs the fractional area shortening was 36 ± 13% at baseline, fell to 27 ± 12% (p < 0.01) during ischemia, but then rose to 33 ± 14% (p = NS vs. baseline) postreperfusion. Intraobserver and interobserver variability correlations by regression analysis were y = 0.88x + 0.92; r = 0.97 and y = 0.93x + 3.09; r = 0.98, respectively.

The hemodynamic data are summarized in Table 2. None of the differences were statistically significant.

Changes in myocardial blood flow during ischemia-reperfusion for control and L-NNA–treated groups are summarized in Table 3. For the control (no L-NNA) dogs, myocardial perfusion during occlusion was significantly (p < 0.01) lower than during baseline, and it returned to baseline level during reperfusion. For the L-NNA–treated dogs, the pattern was similar: myocardial perfusion during occlusion fell from baseline and returned to baseline level with reperfusion. Furthermore, the repeated-measures analysis also demonstrated no differences in myocardial perfusion during baseline, occlusion and reperfusion between the control and L-NNA groups (p = NS for all three conditions).

Table 3. Myocardial Perfusion

Group	Baseline	Occlusion	Reperfusion
Control (n = 5)	0.52 ± 0.11	0.07 ± 0.03*	0.50 ± 0.12†
L-NNA (n = 6)	0.47 ± 0.05	0.08 ± 0.03*	0.40 ± 0.06†

*p < 0.01 vs. baseline for control and L-NNA dogs; †p < 0.05 vs. occlusion for control and L-NNA dogs. Mean ± SE, myocardial perfusion: ml/min/g.
L-NNA = NG-nitro-L-arginine.

The coronary risk areas stained by Evans blue dye were similar in the two groups of animals: 30.6 ± 6.6% (control) versus 32.0 ± 7.2% (L-NNA), p = NS.

Representative sections of the immunohistochemistry staining are shown in Figures 3, 4 and 5. In the absence of the antinitrotyrosine antibody, no nitrotyrosine is demonstrated (Fig. 3). Figure 4 shows a large quantity of nitrotyrosine (brown stain in cytoplasm) is present in myocardium subjected to an occlusion-reperfusion sequence without L-NNA. Myocardium from the nonischemic region indicated minimal nitrotyrosine (not shown). Figure 5 shows myocardium from dogs receiving L-NNA during the occlusion-reperfusion sequence; only minimum nitrotyrosine is present.

DISCUSSION

The major findings of this study are: 1) L-NNA decreases coronary sinus free radical concentration and tissue peroxynitrite formation in an ischemia-reperfusion canine model, and 2) the decrease in free radical concentration is accompanied by less postreperfusion myocardial stunning as measured by fractional area shortening with transthoracic echocardiography.

Previous studies of NO synthase inhibitors in ischemia-reperfusion injury. Numerous other studies have implicated the nitric oxide-peroxynitrite pathway in ischemia-reperfusion injury (24–41). Various competitive inhibitors of the NOS enzyme have been shown to reduce reperfusion injury in various settings. Patel et al. (25), in open-chest rabbits, showed that L-NAME reduced myocardial infarct size after 30 to 60 min of regional ischemia. Wang and Zweier (26) observed that L-NAME inhibited NO gener-

Table 2. Hemodynamic Data

	Systolic Arterial Pressure (mm Hg)		Diastolic Arterial Pressure (mm Hg)		Heart Rate (beats/min)	
	Saline	L-NNA	Saline	L-NNA	Saline	L-NNA
Baseline	133 ± 12	124 ± 44	97 ± 7	83 ± 24	99 ± 11	103 ± 21
Ischemia	110 ± 15	112 ± 38	83 ± 15	72 ± 25	118 ± 17	108 ± 18
Reperfusion	117 ± 15	116 ± 28	86 ± 13	79 ± 24	99 ± 21	99 ± 18

Data are mean ± SD.
L-NNA = NG-nitro-L-arginine.

Figure 3. Representative photomicrograph of myocardium subjected to an occlusion-reperfusion sequence without receiving NG-nitro-L-arginine and stained as described (see text), but not incubated in antinitrotyrosine antibody. No nitrotyrosine is demonstrated. Compare to Figure 4.

ation by over 80% and tripled the recovery of contractile function in isolated perfused rat hearts subjected to 30 min of global ischemia. A similar result was found by Naseem et al. (27), who used the NO synthase inhibitor L-NNA. Morita et al. (28) studied piglets rendered hypoxic while on cardiopulmonary bypass, and again showed that L-NAME had beneficial effects on recovery of left ventricular function. Xie and Wolin (30) demonstrated that reoxygenation-induced inhibition of cardiac muscle mitochondrial respiration was blocked by NO synthase inhibitors. Flogel et al. (31), using endothelial NO synthase knockout mice, found that endogeneously formed NO contributed to ischemia-reperfusion injury in the saline-perfused isolated mouse heart, most likely by peroxynitrite formation. Mori et al. (32) showed that intracoronary administration of L-arginine in dogs aggravated myocardial stunning through production of peroxynitrite; L-NAME improved myocardial contractile function in the ischemic region.

Figure 4. Representative photomicrograph of myocardium subjected to a coronary occlusion-reperfusion sequence and incubated with antinitrotyrosine antibody. Heavy nitrotyrosine staining of the cytoplasm **(brown stain)** is evident. Compare to Figure 3. This dog did not receive the nitric oxide synthase inhibitor NG-nitro-L-arginine.

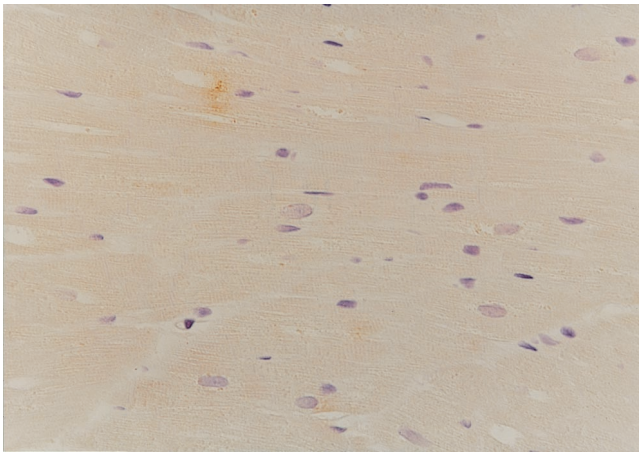

Figure 5. Representative photomicrograph of myocardium subjected to an occlusion-reperfusion sequence and incubated with antinitrotyrosine antibody. This dog received the nitricoxide synthase inhibitor NG-nitro-L-arginine during the occlusion-reperfusion sequence. Only minimal nitrotyrosine staining is present. Compare to Figure 4.

In contrast to the above studies, Engleman et al. (33) in an isolated working rat heart model found the NO precursor arginine ameliorated the endothelial dysfunction resulting from global ischemia-reperfusion sequences. A series of studies from Lefer's group found NO and peroxynitrite to be cardioprotective: after 5.5 h of ischemia-reperfusion in an open-chest dog model (34), in the isolated perfused rat heart undergoing global ischemia-reperfusion (35), and when peroxynitrite was administered directly into the left ventricle of open-chest cats during 90 min of ischemia and 4.5 h of reperfusion (36). In the latter study the administration of IV peroxynitrite was not cardioprotective. The cardioprotective effects of peroxynitrite may result from attenuation of polymorphonuclear leukocyte rolling and from adhesion to the coronary endothelium (32,37), although we found this mechanism did not reduce postischemic myocardial stunning (38). Hasebe et al. (39) found a deleterious effect of the NO synthase inhibitor L-NNA. Their study utilized a postthoracotomy canine model; the animals were subjected to three coronary occlusions (10 min each), and the L-NNA was administered intracoronary, not IV. The L-NNA caused a slower return of regional function (implanted sonomicrometers) compared to controls, although function did ultimately return to baseline. Jones et al. (40) showed a protective role of NO in the late phase of ischemic preconditioning in the conscious rabbit model. Kudejl et al. (41) showed adverse effects of NO inhibition in the conscious pig with coronary stenosis, whereas Gotto et al. (42) noted that L-NAME pretreatment did not affect protection of ischemic myocardium by ischemic preconditioning.

Thus, NO may have dual effects, beneficial in preserving endothelial function, preventing leukocyte adhesion, and promoting vasodilation, although harmful because it combines with superoxide after occlusion-reperfusion sequences to generate toxic peroxynitrite. The difference in results

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from the various studies discussed above may be due to the experimental models. Many of these studies have been done in isolated perfused rat hearts, often with prolonged ischemia resulting in irreversible necrosis and infarction, rather than potentially reversible postischemic dysfunction or stunning. We are unaware of any study in a closed-chest canine ischemia-reperfusion model that has evaluated simultaneously the effect of NO synthase inhibitors on free radical concentration and myocardial contractility.

Advantages of the ascorbate free radical technique. Important previous investigations have used spin-trapping techniques to demonstrate free radical generation in ischemia-reperfusion injury (43–45). Although valuable information has been gained, the spintrapping technique has some limitations. Spin traps when given intracoronary or at high concentrations systemically have significant cardiac effects (46). The spin-trap technique cannot provide on-line analysis of free radical concentration, as our ascorbate free radical technique allows. Also, during the processing of samples containing spin adducts, chain reactions cannot always be prevented, including lipid peroxidation.

Electron paramagnetic resonance using the ascorbate free radical technique allows immediate quantitative determination of free radical concentration. Previous studies in our laboratory have demonstrated that $Asc^{\cdot-}$ rises reproducibly after an ischemia-reperfusion sequence (20,47). The rise in $Asc^{\cdot-}$ elevation has been shown to be greatest after 20 min of occlusion (47). In the absence of ischemia, $Asc^{\cdot-}$ concentration over time has been demonstrated to be stable (20). The $Asc^{\cdot-}$ concentration is not altered by changes in coronary flow. Although ascorbate itself is an antioxidant, we have shown that exogenous ascorbate, administered as we did, does not alter or attenuate myocardial stunning (20). There is no known direct interaction between NO and ascorbate. Thus, the ascorbate EPR method is a unique, robust, real-time method of monitoring free radical stress (total oxidative stress) in a whole animal model (17–20).

A disadvantage of the ascorbate radical technique is that it cannot measure the concentration of other individual radicals such as the $O_2^{\cdot-}$ and $HO^{\cdot-}$; however, it yields information on total oxidative flux.

Myocardial perfusion. To assess the effect of L-NNA on regional myocardial perfusion during ischemia-reperfusion sequence, we measured myocardial perfusion in 11 dogs using a new isotope-labeled microsphere method, namely a neutron activation assay (21). Traditional methods for measuring myocardial blood flow, such as radiolabeled microspheres, which have been extensively employed, have some disadvantages: expensive, radioactive and time-consuming. The new neutron activation assay microsphere technique is an analytic method whereby neutrons penetrate the sample and activate elements within the sample (48,49). Reinhardt et al. (21) recently compared this technique with traditional radiolabeled microsphere method in an in vivo rabbit model of myocardial ischemia and reperfusion and demonstrated a linear relationship of myocardial blood flow measurements between the two methods over a wide detection range. Our present study showed that for both control and L-NNA–treated groups, risk area myocardial perfusion fell significantly during coronary occlusion and returned to baseline level during reperfusion. Importantly, L-NNA did not alter myocardial blood flow during the ischemia and reperfusion sequences compared to the control non-L-NNA dogs. This result rules out the possibility that differences in myocardial perfusion could be the cause of differences in ascorbate free radical production and contractile dysfunction between the groups. A similar conclusion was reached by Araki et al. (50), who used the colored microsphere technique to show that L-NAME did not alter regional myocardial perfusion in a porcine model of ischemia-reperfusion.

Study limitations. Anesthesia is known to depress left ventricular function, and it probably added to the cardiodepressant effect of the ischemia-reperfusion sequences. The dogs in the control and L-NNA groups were both given the same anesthetics.

Coronary artery occlusion in this study was limited to only 20 min, an interval known to produce stunning, but not infarction. Thus, our conclusions are only applicable to a 20-min (stunning) preparation. Shorter or longer coronary occlusion intervals might change the results. We have previously demonstrated that longer durations of coronary occlusion, >20 min (i.e., producing infarction), yielded smaller percentage increases in $Asc^{\cdot-}$ after reperfusion (47).

During baseline and reperfusion, the myocardial blood flows we measured using the neutron activation microsphere assay method were lower than measurements we have previously made using traditional radiolabeled microspheres (51). These differences from our earlier study may be due to the use of a different perfusion measurement method, and differences in anesthetics. Despite these differences, the decline in perfusion during occlusion and recovery during reperfusion followed the expected pattern and was not altered by L-NNA.

The dose of L-NNA we selected was arbitrary, and a dose-response study was not attempted. Higher doses of L-NNA might have different effects on free radical concentration after ischemia-reperfusion sequences.

Unlike L-NAME, L-NNA (used in our study) does not appear to inhibit the superoxide generated with uncoupling of endothelial nitric oxide synthase (52,53). However, we have not excluded the possibility that L-NNA may also decrease superoxide production.

Conclusions. Free radicals are generated from reperfusion injury, and free radicals are known to be toxic to the myocardium. Peroxynitrite, a product of NO and superoxide, is toxic, unstable, highly reactive and causes lipid peroxidation. We have demonstrated that by inhibiting NO production with an NOS inhibitor (L-NNA) during a myocardial ischemia-reperfusion sequence, coronary sinus free radical concentration was significantly diminished, and accumulation of the peroxynitration product nitrotyrosine was

reduced. The expected decrease in left ventricular fractional shortening was abolished, indicating protection against myocardial stunning. The NOS inhibitors deserve further evaluation as cardioprotective agents against reperfusion injury.

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