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### Research Article

The role of nitric oxide in basal vasomotor tone and stimulated endothelium-dependent dilations in the coronary arteries in chronically instrumented awake dogs was studied by examining the consequences of inhibiting endogenous nitric oxide formation with the specific inhibitor of nitric oxide formation, NG-monomethyl-L-arginine (L-NMMA). In four awake dogs, coronary dimension crystals were chronically implanted on the circumflex artery for the measurement of epicardial coronary diameter, and Doppler flow probes were implanted for quantitation of phasic coronary blood flow (vasomotion of distal regulatory resistance vessels). Basal epicardial coronary diameter, acetylcholine-stimulated endothelium-dependent dilation, and flow-induced endothelium-dependent dilation of the epicardial arteries and phasic blood flow were recorded before, and after 5, 15, 50, and 120 mg/kg of L-NMMA. L-NMMA induced a dose-related increase in basal epicardial coronary vasomotor tone. There was an accompanying increase in aortic pressure and a decrease in heart rate. At doses greater than or equal to 50 mg/kg, rest phasic coronary blood flow was also decreased. Left ventricular end-diastolic pressure and contractility were not significantly changed. In contrast, the flow-induced or acetylcholine-stimulated endothelium-dependent responses were attenuated only after infusion of the highest doses of L-NMMA (120 mg/kg). The changes in the basal vasomotor tone and acetylcholine-stimulated endothelium-dependent responses returned towards the control states in the presence of L-arginine (660 mg/kg). These data support the view that nitric oxide [...]

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# Effects of Inhibition of Nitric Oxide Formation on Basal Vasomotion and Endothelium-dependent Responses of the Coronary Arteries in Awake Dogs

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## Abstract

The role of nitric oxide in basal vasomotor tone and stimulated endothelium-dependent dilations in the coronary arteries in chronically instrumented awake dogs was studied by examining the consequences of inhibiting endogenous nitric oxide formation with the specific inhibitor of nitric oxide formation, *N*<sup>G</sup>-monomethyl-L-arginine (L-NMMA). In four awake dogs, coronary dimension crystals were chronically implanted on the circumflex artery for the measurement of epicardial coronary diameter, and Doppler flow probes were implanted for quantitation of phasic coronary blood flow (vasomotion of distal regulatory resistance vessels). Basal epicardial coronary diameter, acetylcholine-stimulated endothelium-dependent dilation, and flow-induced endothelium-dependent dilation of the epicardial arteries and phasic blood flow were recorded before, and after 5, 15, 50, and 120 mg/kg of L-NMMA. L-NMMA induced a dose-related increase in basal epicardial coronary vasomotor tone. There was an accompanying increase in aortic pressure and a decrease in heart rate. At doses  $\geq 50$  mg/kg, rest phasic coronary blood flow was also decreased. Left ventricular end-diastolic pressure and contractility were not significantly changed. In contrast, the flow-induced or acetylcholine-stimulated endothelium-dependent responses were attenuated only after infusion of the highest doses of L-NMMA (120 mg/kg). The changes in the basal vasomotor tone and acetylcholine-stimulated endothelium-dependent responses returned towards the control states in the presence of L-arginine (660 mg/kg). These data support the view that nitric oxide plays a significant role in modulating basal vasomotion and endothelial-dependent dilation stimulated by acetylcholine or increase in blood flow in epicardial coronary arteries and also influence the regulation of coronary blood flow during physiologic conditions. (*J. Clin. Invest.* 1991; 87:1964–1968.) Key words: nitric oxide • coronary vasomotion • awake dogs

## Introduction

Since the description by Furchgott and co-workers (1) of endothelium-derived relaxing factor (EDRF),<sup>1</sup> intensive efforts

have been made to characterize and identify the nature of this biologic mediator (2–5). Increasing evidence suggests that nitric oxide or a closely related compound represents at least one type (if not the only type) of EDRF because both nitric oxide and EDRF have the same biologic and pharmacologic properties (3, 6–8) and nitric oxide is released in sufficient quantities to explain the biological actions of EDRF (3). Other investigators have provided data that suggest nitric oxide may not account for all the actions of EDRF, and that, depending on the vascular bed and the activator, there may be more than one type of EDRF (9–13).

Nitric oxide is synthesized by endothelial cells from the terminal guanidino nitrogen atom of the amino acid L-arginine (14) and *N*<sup>G</sup>-monomethyl-L-arginine (L-NMMA) has been shown to inhibit its formation in a concentration-dependent and enantiomerically specific manner (15). In addition, L-NMMA increases basal tone in rings of the rabbit aorta (15, 16), guinea pig pulmonary artery (17), and Langendorff perfused rabbit heart preparation (18). Acetylcholine-induced relaxation is also attenuated by L-NMMA in these preparations (16–18). Furthermore, L-NMMA increases mean arterial pressure in anesthetized rabbits and this is associated with reduced release of NO from the perfused aorta of treated animals (19). A similar rise in blood pressure induced by L-NMMA has been reported in guinea pigs (20). More recently, the rise in blood pressure induced by L-NMMA in conscious rats has been shown to be accompanied by a substantial fall in regional blood flow in the renal, mesenteric hindquarters and internal carotid vascular beds (21). The relevance of these findings to man has recently been highlighted by the observation that infusion of L-NMMA into the brachial artery causes vasoconstriction and inhibition of the vasodilation induced by acetylcholine (22). All these effects of L-NMMA are reversed by an excess of L-arginine.

This study was designed to examine the role of endogenous nitric oxide in coronary vasomotion in chronically instrumented awake dogs by evaluating the effects of a wide range of inhibition of endogenous nitric oxide formation by L-NMMA on basal coronary vasomotion and endothelium-dependent stimulated vasodilation by acetylcholine and increases in blood flow.

## Methods

Mongrel dogs (30–35 kg) ( $n = 4$ ), primarily screened for the absence of anemia and infection, were subjected to left thoracotomy under general anesthesia with intravenous thiamylal sodium (60–80 mg/kg). Heparin-filled polyvinyl catheters were inserted into the ascending aorta via the left internal thoracic artery, in the left atrium via the atrial appendage and in the left ventricular chamber via the apex. A proximal segment (0.5–1 cm) of the left circumflex artery just distal to the atrial appendage was dissected minimally. Miniature 7 MHz piezoelectric crystals (1.5  $\times$  2.5 mm, 15–20 mg), attached to a Dacron backing, were

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1. Abbreviations used in this paper: EDRF, endothelium-derived relaxing factor; L-NMMA, *N*<sup>G</sup>-monomethyl-L-arginine.

sutured to the adventitia on opposite surfaces of the vessel segment with 6-0 prolene (Ethicon, Inc., Somerville, NJ). Oscilloscope monitoring and on-line sonomicrometry (Sonomicrometer 120-2; Triton Technology, Inc., San Diego, CA) were used to verify proper crystal alignment. A pulse Doppler flow probe (10 MHz, cuff type) was implanted distal to the crystals. An inflatable balloon occluder was also placed distal to the flow probe. All arterial branches between the crystals and the occluder were carefully ligated. The catheters and electrode wires were tunneled to a subcutaneous pouch at the base of the neck.

The dogs were allowed to recover for 10–14 d. The catheters and wires were then exteriorized under lidocaine infiltration anesthesia. On a day before subjecting the dogs to the study protocol, each animal was given a bolus injection of nitroglycerin (0.04 mg) to ensure a responsive vasculature (>3% dilation). On the study day, the dogs were loosely restrained and lying awake on their right side. Aortic pressure, left ventricular end-diastolic pressure,  $dp/dt$ , external coronary diameter, coronary flow, and electrocardiograms were continuously recorded. Pressure tracings from the fluid-filled catheters were optimally damped with a Corrector device (Norton Health Care Products, Akron, OH).

After obtaining the baseline coronary and systemic hemodynamic parameters each dog was subjected to the following two interventions: (a) a bolus injection (left atrial catheter) of acetylcholine (4  $\mu$ g) during constant phasic coronary flow maintained by partial inflation of the pneumatic occluder, and (b) 20-s transient coronary occlusion followed by complete release of the occluder. The 20-s occlusion below the crystal site was used to cause a transient increase in coronary blood flow, the reactive hyperemic response, which in turn was followed by flow-mediated vasodilation of the epicardial coronary artery. At least 15 min were allowed between interventions and the order was randomized. L-NMMA acetate salt (Wellcome Research Laboratories, Beckenham, UK) (5 mg/kg dissolved in saline at a concentration of 30–50 mg/ml), was infused slowly into the left atrium at a rate of < 100 mg/min to minimize acute hemodynamic changes. At 5 min after the hemodynamic parameters reached a new steady state, the stimuli for the endothelium-dependent responses (20-s transient occlusion, flow-induced dilation, and acetylcholine injection) were repeated. Subsequently, higher doses of L-NMMA (15, 50, 120 mg/kg) were administered in an increasing order. Each administration was followed by a repeat measurement of the basal and the stimulated endothelium-dependent responses. After the stimulated responses after the highest dose of L-NMMA, the nonendothelium-dependent dilator nitroglycerin (0.04 mg) was given as a bolus in the left atrium. After 20–30 minutes, when the basal coronary diameter had returned to the level before nitroglycerin injection, L-arginine (660 mg/kg dissolved in saline at 100 mg/ml) (Sigma Chemical Co., St. Louis, MO) was infused slowly over 20–30 min into the left atrium. The basal and stimulated endothelium-dependent responses were again repeated. Nitroglycerin (0.04 mg) was given at the end of the study day.

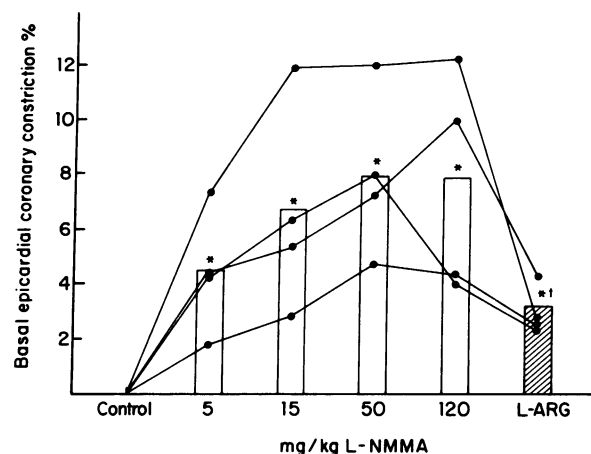


Figure 1. Effects of increasing doses of L-NMMA on basal epicardial coronary vasomotor tone. Individual (circles) and mean (bars) data are plotted as percent change from control. Coronary dimensions decreased in a dose-related fashion after infusion of L-NMMA. L-Arginine partially reversed the response. \*Significant when compared with control. †Significant when compared with 120 mg/kg L-NMMA.

All hemodynamic measurements were compared with measurements obtained before infusion of L-NMMA using the same dog as its own control. Statistical analyses were performed using an analysis of variance for repeated measures.

## Results

Table I summarizes the baseline hemodynamic changes before and after the infusion of each dose of L-NMMA. L-NMMA (5–120 mg/kg) caused dose-related significant increases in mean aortic pressure from a control of  $94 \pm 6$  to  $129 \pm 15$  mmHg after the highest dose of 120 mg/kg. The largest effects of inhibition of endogenous NO production on aortic pressure occurred after the lowest dosages of L-NMMA 5–15 mg/kg, with minimal further increase despite a > 10-fold increase in L-NMMA dosage. Heart rate decreased from  $62 \pm 2$  to  $43 \pm 4$  beats/min. At > 15 mg/kg, L-NMMA caused intermittent second- or third-degree atrioventricular block. Changes in aortic pressure and heart rate were reversed with L-arginine. Left ventricular end-diastolic pressure tended to increase from  $10 \pm 1$  to  $14 \pm 2$

Table I. Resting Hemodynamic Measurements after Increasing Inhibition of Nitric Oxide Formation

	Control	L-NMMA (mg/kg)				L-Arg
		5	15	50	120	
Aortic pressure (mmHg)	94±6	114±4*	120±7*	121±11*	129±15*	104±5‡
Heart rate (bpm)	62±2	57±2*	46±3*	41±3*	43±4*	62±6‡
LVEDP (mmHg)	10±1	13±1	14±1	15±1	14±2	11±1
LV $dp/dt$ (mmHg/s)	2483±306	2500±153	2533±167	2467±213	2633±120	2817±117

Results expressed in mean±SEM. LV, left ventricular. EDP, end-diastolic pressure. \* Significant when compared with control. ‡ Significant when compared with measurement at 120 mg/kg L-NMMA.

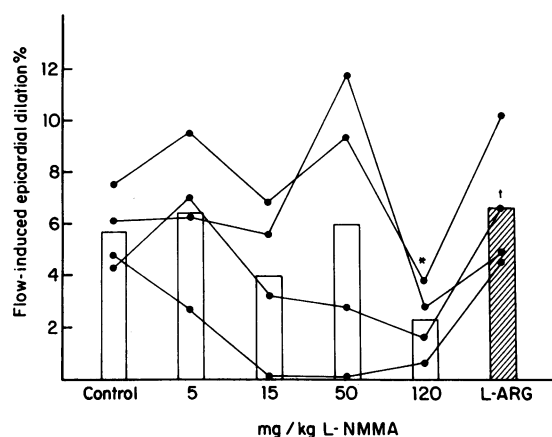


Figure 2. Effects of increasing doses of L-NMMA on flow-induced dilation. Individual (circles) and mean (bars) data are plotted as percent dilation. Although the effects of L-NMMA were variable at lower doses, the reductions were consistent at the 120 mg/kg dose (\*) when compared with control. L-Arginine reversed the reduction in Ach-induced dilation. \*Significant when compared with 120 mg/kg L-NMMA.

mmHg but the change was not statistically significant. Left ventricular  $dP/dt$  was not significantly altered after L-NMMA.

The effects of increasing doses of L-NMMA on basal epicardial coronary dimensions in individual dogs are illustrated in Fig. 1. Although the magnitude of the response was variable in individual animals, L-NMMA-induced dose-related vasoconstriction in each animal. The epicardial diameter decreased from  $3.67 \pm 0.10$  to  $3.38 \pm 0.06$  mm after the highest dose of L-NMMA representing an 8% decrease in vessel diameter or 15% decrease in cross-sectional area. As with aortic pressure, the largest effect of inhibition of endogenous NO production on coronary dimensions occurred after the lower dosages of L-NMMA, with only small changes occurring with dosages  $> 15$  mg/kg. Again, the epicardial vasoconstriction was partially reversed toward control levels with L-arginine.

In contrast to the effect on aortic pressure and basal epicardial dimension flow-induced, endothelium-dependent dilation was not significantly altered after infusions of up to 50 mg/kg L-NMMA, but was significantly reduced after 120 mg/kg L-NMMA from  $0.209 \pm 0.024$  mm ( $5.71 \pm 0.70\%$ ) to  $0.076 \pm 0.024$  mm ( $2.26 \pm 0.73\%$ ) (Fig. 2). The response, however, was variable in individual animals; one animal was particularly sensitive to inhibition by L-NMMA. The flow-induced, endothelium-dependent dilation was reversed to control levels after infusion of L-arginine. The maximal epicardial coronary diameter after nitroglycerin was not significantly changed in the presence of L-NMMA.

A similar pattern was seen for the acetylcholine-induced, endothelium-dependent dilation which was not significantly inhibited until after infusion of 120 mg/kg of L-NMMA (control,  $0.154 \pm 0.016$  mm or  $4.20 \pm 0.48\%$ ; L-NMMA,  $0.71 \pm 0.008$  or  $2.11 \pm 0.21\%$ ) (Fig. 3). The response also was variable in individual animals. The same animal that demonstrated greater sensitivity to L-NMMA inhibition of flow-induced vasodilation also demonstrated greater sensitivity to L-NMMA inhibition of acetylcholine induced vasodilation. The acetylcholine-induced dilation response was reversed to control levels after infusion of L-arginine.

The effects of L-NMMA on rest phasic coronary blood flow (vasomotion of distal resistance coronary vessels) were modest; maximum decrease was 19%. Although there was a tendency for blood flow to decrease at the 15 mg/kg dose, because of the variable responses, significance was not achieved until the 50 mg/kg dose (Table II). Peak reactive hyperemic flow-induced after 20 s transient coronary occlusion was, however, not significantly different even after the infusion of the highest dose of L-NMMA. Therefore, the reduction of flow-induced epicardial coronary dilation seen after L-NMMA resulted from a direct effect of inhibition of nitric oxide formation rather than secondary to reduced peak flow. L-Arginine reversed the resting flow toward control levels.

## Discussion

The present study is the first to assess the effects of a wide range of inhibition of endogenous nitric oxide production on basal or rest vasomotion and acetylcholine and flow-stimulated endothelium-dependent vasodilation of coronary epicardial conductance vessels and systemic hemodynamics in an intact awake physiologic model. In addition, effects of a wide range of inhibition of endogenous NO production on basal or rest phasic coronary blood, a measure of resistance vessel vasomotion was also assessed. L-NMMA has been demonstrated to inhibit endogenous nitric oxide formation from L-arginine in vitro and in anesthetized preparations (15, 16, 18, 19). Using increasing doses of L-NMMA, the present study demonstrated dose-related decreases in basal epicardial coronary dimensions. The changes in epicardial dimensions were accompanied by increases in aortic pressure and decreases in heart rate. The largest change in epicardial dimensions and aortic pressure occurred with the lower doses of L-NMMA, 5 and 15 mg/kg, with only minimal further change in dimensions or pressure despite  $> 10$ -fold further increase in L-NMMA. At doses of L-NMMA  $\geq 50$  mg/kg, rest coronary flow was decreased also; the maximum decrease was  $\sim 19\%$ . In contrast to the effects on basal vasomotion, the flow-induced or acetylcholine-stimulated, endothelium-dependent dilation responses in the coronary arteries were attenuated only after the highest dose of L-NMMA

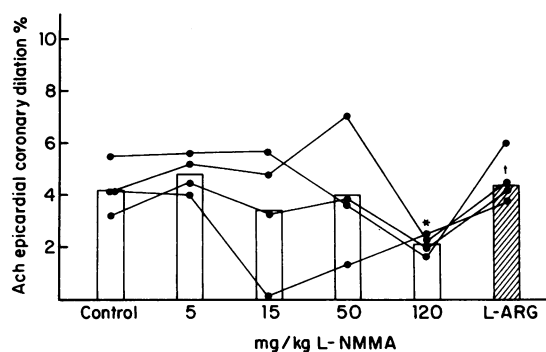


Figure 3. Effects of increasing doses of L-NMMA on acetylcholine-induced dilation. Individual (circles) and mean (bars) data are plotted as percent dilation. Although the effects of L-NMMA were variable at lower doses, the reductions were consistent at the 120 mg/kg dose (\*) when compared with control. L-Arginine reversed the reduction in Ach-induced dilation. \*Significant when compared with 120 mg/kg L-NMMA.

Table II. Effects of Increasing Inhibition of Nitric Oxide Formation on Rest and Peak Reactive Hyperemic Flow after a 20-s Occlusion

	Control	L-NMMA (mg/kg)				L-Arg
		5	15	50	120	
Rest flow (KHz)	2.18±0.42	2.09±0.34	1.76±0.36	1.78±0.34*	1.78±0.30*	1.92±0.28
Peak reactive hyperemic flow (KHz)	8.37±0.63	8.68±0.25	7.91±0.56	7.21±0.17	7.32±0.48	8.92±0.61

Results expressed in mean±SEM. \* Significant when compared with control.

(120 mg/kg). Thus there was a > 20-fold differential effect of L-NMMA on basal as compared with stimulated epicardial vasomotion. The effects of L-NMMA on rest coronary flow, and flow-induced or acetylcholine-stimulated, endothelium-dependent dilation responses were variable in individual animals; certain animals were affected by lower doses of L-NMMA. It is possible that with larger number of animals, a statistically significant effect of L-NMMA on these parameters may be achieved at doses < 120 mg/kg. Left ventricular end-diastolic pressure and  $dP/dt$  were not significantly altered after L-NMMA. The changes in basal coronary vasomotion and stimulated endothelium-dependent responses were reversed by a high dose of L-arginine. In an earlier study, in a different group of animals, we described the effects of a low dose of L-NMMA, 5 mg/kg on basal coronary vasomotion (23). The present study extends this initial observation to a wide range of inhibition of NO production and assess effects on stimulated as well as basal vasomotion for the first time.

Previous studies by Amezcua et al. (18) demonstrated that L-NMMA, at a dose between 10 and 100  $\mu$ M, produced a dose-related sustained increase in coronary perfusion pressure (resistance coronary artery vasomotion) in a Langendorff-perfused rabbit heart preparation. In the Langendorff preparation, acetylcholine (0.3  $\mu$ M) caused a decrease (vasodilation) before and an increase (vasoconstriction) in coronary perfusion pressure after inhibition of nitric oxide formation. In our present study, we observed that as compared to basal coronary vasomotion, the flow-induced and acetylcholine-stimulated coronary endothelium-dependent responses required a 20-fold higher dose of L-NMMA for significant inhibition. Rees et al. (16) also demonstrated similar effects in *in vitro* rabbit aortic ring preparations after inhibition of nitric oxide formation on endothelium-dependent relaxations induced by acetylcholine, calcium ionophore, or substance P. In that study, the concentration of L-NMMA that produced 50% contraction of the rings was six times lower than that required to achieve 50% inhibition of acetylcholine-induced relaxation (16). Although the reason for this difference between basal and stimulated responses is not known, several possibilities may be considered. The stimulated as compared to basal responses may increase mobilization of the stored substrate within the endothelial cells that required higher concentrations of inhibitor for blockade (16). An alternative explanation may be the presence of more than one EDRF unrelated to nitric oxide: nitric oxide may be the EDRF primarily responsible for modulating basal coronary and peripheral vasomotor tone whereas other EDRFs may contribute to stimulated responses. The fact that a substantial portion (> 50%) of the stimulated responses was inhibited at the higher doses of L-NMMA argues against this possibility.

Coronary blood flow was decreased when the dose of L-NMMA was 50 mg/kg or greater. Because aortic pressure increased with the lowest dose of L-NMMA and coronary flow was unchanged, distal coronary vascular resistance was elevated even after the initial dose of L-NMMA. Previous studies from our laboratory have demonstrated that cyclic guanosine monophosphate-mediated dilators such as EDRF, nitrates, and atrial natriuretic peptide preferentially dilate proximal conductance arteries as compared to distal regulatory resistance coronary arteries in awake dogs (24–27). The vasomotor state of the distal resistance vasculature is influenced by the dominant effects of local myocardial metabolic demands (28). Because nitric oxide has been demonstrated to mediate its vasodilator effects through activation of guanylate cyclase (29, 30), it is reasonable to speculate that the increased vasomotor tone of the distal regulatory resistance arteries (as a result of inhibition of nitric oxide formation) may have been overridden by the dominant vasodilating stimuli mediated by increased myocardial metabolic demands. The lack of significant change in peak hyperemic flow also indicates that the coronary reserve for increasing blood flow was present and that the reduction in flow-induced dilation of the proximal epicardial arteries after L-NMMA is a direct effect of inhibition of nitric oxide formation and not secondary to a difference in the flow stimuli. In recent studies, Vallance et al. (22) observed that infusion of L-NMMA into the brachial artery of healthy volunteers caused a 50% decrease in basal blood flow and attenuation of the dilator response to acetylcholine but not nitrates suggesting that endothelial-derived nitric oxide influences basal and stimulated limb blood flow in man.

The persistent dose-dependent hypertensive effect seen after L-NMMA in the present study is consistent with the observations of Rees et al. (19) who reported a sustained (15–90 min) dose-dependent increase in mean systemic arterial pressure after infusion of L-NMMA (3–100 mg/kg) in anesthetized rabbits. Indomethacin, prazosin, or vagotomy did not alter the hypertensive response to L-NMMA (19), whereas it was reversed by L-arginine, as in our present study. The hypertensive response to L-NMMA probably contributed to the decrease in heart rate seen after the lower doses used in our study (Table I). Second- and third-degree atrioventricular block was observed after the 15–50 mg/kg dose which may have been influenced by the increase in blood pressure and/or a direct effect of inhibition of nitric oxide production or L-NMMA on the AV node. The effects on pressure, heart rate, and atrioventricular conduction were reversed by L-arginine.

In summary, the results from the present study support the view that endogenous nitric oxide plays a significant physiologic role in modulating basal vasomotor tone in the proximal

epicardial coronary arteries and distal regulatory resistance coronary vessels. These data also demonstrate that endogenous nitric oxide plays a role in the endothelium-dependent dilation induced by increases in flow or acetylcholine in the epicardial coronary vessels. The effects of endogenous NO on epicardial dimension and aortic pressure were more sensitive to inhibition by L-NMMA with largest change occurring at the lower dosage, whereas the effect on endothelium-dependent stimulated vasodilation occurred consistently at a >20-fold higher dose of L-NMMA.

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