Endogenous Nitric Oxide Protects Against Platelet Aggregation and Cyclic Flow Variations in Stenosed and Endothelium-Injured Arteries

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Background. This study was designed to test the hypothesis that endogenously produced nitric oxide protects against platelet aggregation and cyclic flow variations in stenosed and endothelium-injured arteries of mongrel dogs.

Methods and Results. N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide formation, was administered at 5 mg/kg to 15 dogs after the left anterior descending coronary artery was mechanically injured and narrowed by external constrictors and to nine dogs before endothelial injury of the femoral artery and after injury and moderate arterial constriction. Treatment with L-NMMA resulted in cyclic flow variations (as detected by external Doppler flow probes) in the left anterior descending artery of seven of 15 dogs and in the femoral artery of four of nine dogs after endothelial injury. L-Arginine, the precursor for nitric oxide synthesis, was administered at 60 mg/kg and abolished cyclic flow variations in each of the 11 dogs. D-Arginine did not change the L-NMMA-induced cyclic flow variations. Saline infusion did not induce change cyclic flow variations in any of the animals. Acetylcholine (1, 10, and 100 μg/min; n=9) was administered in the femoral artery of nine additional dogs before and after endothelial injury in moderately stenosed femoral arteries. Acetylcholine did not induce cyclic flow variations in any animal; however, it did increase the severity of cyclic flow variations that developed in severely stenosed arteries. The diameter of the femoral artery was measured by intravascular ultrasound imaging. L-NMMA caused vasoconstriction of normal arteries, but no change was detected in endothelium-injured arteries. In contrast, L-arginine caused vasodilation of normal arteries, but, again, no change was noted in endothelium-injured arteries. Acetylcholine dilated normal femoral arteries but constricted arteries with endothelial injury. In both in vitro and ex vivo platelet studies, L-NMMA enhanced platelet aggregation, whereas L-arginine significantly reduced platelet aggregation. D-Arginine and acetylcholine showed no effect on platelet aggregation.

Conclusions. Promotion of nitric oxide production decreases platelet aggregation and may eliminate cyclic flow variations, whereas a reduction in nitric oxide formation enhances platelet aggregation and may induce cyclic flow variations. Acetylcholine causes vasoconstriction at the femoral arterial site of endothelial injury and may increase the severity of cyclic flow variations. (Circulation 1992;86:1302–1309)

Key Words • acetylcholine • D-arginine • endothelium-derived relaxing factor • L-arginine • N^G-monomethyl-L-arginine • platelet aggregation

The factors that initiate coronary artery disease are not well defined. Interactions between the vessel wall and blood elements may play important roles in converting chronic to acute coronary artery disease syndromes.1–3 Platelets contain vasoactive substances such as thromboxane and serotonin that upon release can cause vasoconstriction or vasodilation, depending on the environment.4,5 In turn, the vascular endothelium may synthesize and release factors that alter platelet function.6,7

Endothelium-derived relaxing factor (EDRF) is a potent vasodilator.8 A number of studies have shown that EDRF is nitric oxide.9–11 Endothelial cells synthesize nitric oxide from the terminal guanidino nitrogen atoms of L-arginine.11 Platelets have also been shown to synthesize and release nitric oxide from L-arginine.12 In vitro studies show that an increase in nitric oxide synthesis by L-arginine reduces platelet aggregation, whereas inhibition of nitric oxide synthesis by N^G-monomethyl-L-arginine (L-NMMA) enhances platelet aggregation.13

Experimental studies in the canine heart have shown that recurrent platelet aggregation and dislodgment in severely stenosed and endothelium-injured arteries re-
sult in cyclical reductions in coronary blood flow.\textsuperscript{14–16}
Clinically, cyclic flow variations have been observed before and after coronary angioplasty in patients with unstable angina.\textsuperscript{17}

In the present study, we assessed the effects of endogenously produced nitric oxide on platelet aggregation and cyclic flow variations in dogs with stenosed and endothelium-injured arteries.

\textbf{Methods}

All procedures used in this study were conducted according to the principles of the American Physiological Society and were approved by the Institutional Animal Care and Use Committee at the Texas Heart Institute, Houston, Tex.

\textbf{Surgical Preparation}

Mongrel dogs (\textit{n}=33) weighing 25–35 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v.), intubated, and placed on mechanical respirators (Harvard model 60, Natick, Mass.). Plastic catheters were placed in a carotid artery for monitoring aortic pressure and in a jugular vein for administering fluids. In 15 dogs, a thoracotomy was performed in the fifth left intercostal space, and the heart was suspended in a pericardial cradle. A 1–2-cm segment of the left anterior descending coronary artery was carefully exposed, and nearby branches were ligated. In an additional 18 dogs, the left femoral artery was exposed. A small plastic catheter was inserted in the left atrium or the proximal branch of the femoral artery for administration of drugs.

An ultrasonic Doppler flow probe (Hartley Instruments, Houston, Tex.) was placed around the proximal portion of the exposed left anterior descending artery or femoral artery to measure the velocity of blood flow. An intravascular ultrasonic imaging probe (InterTherapy Inc., Costa Mesa, Calif.) was placed in the femoral artery to measure the cross-sectional area of the inside of the vessel wall.\textsuperscript{18–21} The measurement was based on the computer program developed by InterTherapy Inc. The accuracy and reproducibility of the ultrasonic imaging probe in measuring vessel cross-sectional area was verified by several in vitro studies. First, the diameter of a plastic syringe was measured with a standard ruler. Then, the diameter of the same syringe was measured with the ultrasound imaging probe, and the values were compared with those obtained with the standard ruler. This procedure was repeated on five different days. Both the imaging probe and the standard ruler yielded a value of 9.5 mm for the diameter of the syringe, even when measurements were obtained on different days.

\textbf{Experimental Procedures}

Baseline hemodynamics including heart rate, systolic and diastolic aortic blood pressures, and phasic and mean blood flow velocity in the left anterior descending coronary or femoral artery were recorded on an eight-channel recorder (model 3000, Gould, Inc., Cleveland, Ohio). Animals then were randomly assigned to different groups.

\textbf{Group 1.} In nine dogs with exposed left anterior descending coronary arteries, cylindrical plastic constrictors were placed distal to the Doppler flow probe on the artery after the endothelium was injured by gently squeezing the artery with cushioned forceps. The constrictor was adjusted to narrow the diameter of the artery until the coronary blood flow velocity was reduced to 80\% of the baseline level. For 30 minutes, the coronary blood flow was monitored, and saline was continuously infused in the left atrium. If no cyclic flow variations were produced, L-NMMA (Calbiochem, La Jolla, Calif.),\textsuperscript{13} an inhibitor of nitric oxide synthesis, was infused into the left atrium at 5 mg/kg for 15 minutes. When cyclic flow variations developed, saline was infused for 30 minutes. L-Arginine free base (Sigma, St. Louis, Mo.),\textsuperscript{13} a precursor of nitric oxide, then was administered at 60 mg/kg for 15 minutes in the left atrium. If cyclic flow variations were abolished, the coronary blood flow velocity was monitored for 30 more minutes to ensure that the cyclic flow variations had been eliminated.

\textbf{Group 2.} In six dogs, stenosis and endothelial injury were induced in the left anterior descending coronary artery in the same manner as in group 1 dogs. The infusion of saline and L-NMMA were also the same as above. After the induction of cyclic flow variations, D-arginine, an analogue of L-arginine, was infused into the left atrium at 60 mg/kg for 15 minutes. If there was no change in cyclic flow variations in 30 minutes, L-arginine was administered as described in group 1 dogs.

\textbf{Group 3.} In nine dogs, saline was infused in the exposed femoral artery for 30 minutes followed by L-NMMA at 5 mg/kg for 15 minutes and then L-arginine at 60 mg/kg for 15 minutes. The cross-sectional area of the lumen of the femoral artery was measured by ultrasonic imaging before and after the infusion of saline, L-NMMA, and L-arginine. The dogs were monitored for a 1–2-hour period of recovery after discontinuation of the treatment. The endothelium of the femoral artery then was injured by squeezing the artery with cushioned forceps, and cylindrical plastic constrictors were placed around the artery to reduce the femoral blood flow velocity to 50\% of the baseline level. The degree of stenosis was more severe in the femoral artery because a larger volume of blood flows through the femoral artery than through the coronary artery. Saline was infused in the femoral artery for 30 minutes followed by infusion of L-NMMA at 5 mg/kg for 15 minutes. If cyclic flow variations developed, L-arginine was infused 30 minutes later at a concentration of 60 mg/kg for 15 minutes. The cross-sectional area of the arterial lumen at the site of endothelial injury proximal to the stenosis was measured with ultrasonic imaging before and after the infusion of saline, L-NMMA, and L-arginine.

\textbf{Group 4.} In nine dogs, saline was infused in the femoral artery for 30 minutes followed by the infusion of acetylcholine at 1, 10, and 100 \(\mu\text{g}/\text{min}\) (estimated blood concentration \(5.5\times10^{-10} \text{M}\), \(5.5\times10^{-9} \text{M}\), and \(5.5\times10^{-8} \text{M}\), respectively) each for 5 minutes. The cross-sectional area of the arterial lumen was measured before and after saline infusion and after each dose of acetylcholine. The dogs were allowed to recover for 2 hours after the infusion of acetylcholine. The endothelium of the artery then was injured by squeezing the artery with cushioned forceps, and cylindrical plastic constrictors placed around the artery reduced the blood flow velocity to 50\% of the baseline level. Saline was infused again followed by acetylcholine at 1, 10, and 100 \(\mu\text{g}/\text{min}\) (estimated blood concentration \(5.5\times10^{-10} \text{M}\), \(5.5\times10^{-9} \text{M}\), and \(5.5\times10^{-8} \text{M}\), respectively) each for 5 minutes. If cyclic flow variations were abolished, the coronary blood flow velocity was monitored for 30 more minutes to ensure that the cyclic flow variations had been eliminated.
Effects of cyclic flow variations did not develop, the size of the constrictor was reduced to adjust the arterial blood flow velocity until cyclic flow variations were established. Acetylcholine was infused again in 30 minutes at 10 μg/min for 30 minutes. Femoral arterial blood flow velocity was continuously monitored.

Platelet Aggregation Studies

Blood samples obtained before and after each treatment were collected in plastic tubes containing a 3.8% solution of sodium citrate (9 vol blood:1 vol sodium citrate). Platelet-rich plasma was obtained by centrifuging whole blood at 200g for 20 minutes at room temperature. Platelet-poor plasma was obtained by centrifuging whole blood at 2,000g for 10 minutes. The platelet count in platelet-rich plasma was adjusted to 300,000/mm². A four-channel platelet aggregometer (Bio Data, model PAP4, Horsham, Pa.) was used for all assays. Collagen was used as the agonist at final concentrations of 5, 10, and 20 μg/ml. The degree of platelet aggregation was reported as a percentage of maximal increase in light transmission in platelet-rich plasma as compared with platelet-poor plasma. In addition, platelet-rich plasma obtained from blood samples taken before drug treatment was incubated with saline, L-NMMA at a final concentration of 5, 10, and 15 μM, L-arginine or D-arginine at 10, 20, and 30 μM, or acetylcholine at 0.1, 1, and 10 μM. Collagen then was added at 10 μg/ml, and the degree of platelet aggregation was recorded in the same manner as above.

Statistical Analyses

All values are expressed as mean±SEM. Fisher's exact test was used to compare the frequency of induction or abolition of cyclic flow variations by different treatments. The values of vessel diameters and hemodynamics obtained at different time periods were compared by a one-way ANOVA with repeated measurements and a Student’s t test with Bonferroni's corrections. The values of platelet aggregation obtained after different treatments were compared with those obtained after saline treatment by Dunnett’s multiple-range test. A value of p<0.05 was considered significant.

Results

Effects of N⁰-Monomethyl-L-Arginine and L-Arginine on Arterial Blood Flow Velocity

In group 1 and 2 dogs, the presence of external constrictors reduced the mean left anterior descending coronary artery blood flow velocity to 81±4% and 84±4% of the baseline level, respectively. No cyclic flow variations developed at this time in any of the 15 dogs. Saline infusion did not significantly change coronary blood flow velocity. When L-NMMA was administered, cyclic flow variations developed in 10–15 minutes in the left anterior descending coronary artery in seven of the 15 dogs. These cyclic flow variations were consistent and were not affected by infusion of saline in group 1 dogs or D-arginine in group 2 dogs. L-Arginine abolished the cyclic flow variations in all seven dogs (Figures 1 and 2).

In group 3 dogs, before endothelial injury, administration of L-NMMA reduced the mean femoral artery flow velocity to 78±8% of the baseline level, which was reversed by treatment with L-arginine to 132±19% of the baseline level (Table 1). None of the dogs developed cyclic flow variations before endothelial injury. When the endothelium was injured and the femoral arteries were stenosed by external constrictors (reducing femoral flow velocity to 49±7% of the baseline level), L-NMMA induced cyclic flow variations in four of the nine dogs, and L-arginine abolished them in all four animals. Saline infusion did not induce or affect cyclic flow variations in any dog.

Aortic blood pressure increased after infusion of L-NMMA and decreased after infusion of L-arginine into the left atrium (Table 1). Intrafemoral infusion of L-NMMA also increased aortic pressure, and administration of L-arginine decreased the pressure (Table 1).

Effects of N⁰-Monomethyl-L-Arginine and L-Arginine on Femoral Artery Diameter

Before endothelial injury, administration of L-NMMA reduced the mean cross-sectional area of the femoral artery from 10.4±1.1 mm² to 8.9±1.1 mm² (p<0.05). After infusion of L-arginine, the mean cross-sectional area of the femoral artery increased to 12.9±1.2 mm² (compared with the baseline value, p<0.05) (Figures 3A and 4A). After endothelial injury, the mean cross-sectional area of the femoral artery increased to 16.6±2.1 mm². Infusion of L-NMMA and L-arginine did not significantly change the luminal cross-sectional area (16.4±2.3 mm² and 16.7±1.7 mm², respectively) (Figures 3B and 4B).

Effect of Acetylcholine on Diameter and Blood Flow Velocity of Femoral Artery

In group 3 dogs, administration of acetylcholine caused a dose-dependent vasodilation as illustrated by an incremental increase in the mean cross-sectional area of the femoral artery before endothelial injury (Figure 5). However, administration of acetylcholine after endothelial injury resulted in a dose-dependent vasoconstrictive effect (Figure 5). Acetylcholine did not induce cyclic flow variations in any of the nine dogs with injured endothelia and moderately stenosed (mean blood flow reduced to 50±4% of the baseline level) femoral arteries. An increase in the severity of constriction achieved by adjusting the external constrictor (reducing mean blood flow velocity to 38±7% of the baseline level) caused cyclic flow variations in all nine dogs. Saline infusion did not affect the cyclic flow variations, but infusion of acetylcholine increased their severity as shown by a reduction in the nadir flow velocity of the cyclic flow variations (from 26±6% of baseline level to 11±3%, p<0.01) (Table 1).

Effects of N⁰-Monomethyl-L-arginine, L-Arginine, and Acetylcholine on Platelet Aggregation

Ex Vivo and In Vitro

In control samples, collagen induced platelet aggregation in a dose-dependent manner (Figure 6A). Treatment with L-NMMA resulted in a higher percentage of platelet aggregation than that observed in control groups when low to moderate doses of collagen were used to stimulate platelets. D-Arginine administration
Yao et al. Endothelium-Derived Relaxing Factor

AORTIC PRESSURE (mmHg)
PHASIC FLOW VELOCITY (kHz)
MEAN FLOW VELOCITY (kHz)
AORTIC PRESSURE (mmHg)
PHASIC FLOW VELOCITY (kHz)
MEAN FLOW VELOCITY (kHz)

FIGURE 1. Representative recording of blood flow velocity of the left anterior descending coronary artery and aortic blood pressure before (panel A) and after treatment with N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) (panel B) and L-arginine (panel C). Administration of L-NMMA induced cyclic flow variations. L-Arginine abolished cyclic flow variations induced by L-NMMA.

did not change platelet aggregation significantly. L-Arginine significantly inhibited platelet aggregation induced by low to high levels of collagen (Figure 6A). In in vitro studies, L-NMMA significantly enhanced platelet aggregation in a dose-dependent manner, whereas L-arginine significantly decreased platelet aggregation (Figure 6B). Neither D-arginine nor acetylcholine affected platelet aggregation in vitro (Figure 6B).

Discussion

In this study, we have shown that inhibition of nitric oxide synthesis by L-NMMA induces cyclic flow variations in slightly stenosed, endothelium-injured arteries of dogs, whereas restoration of nitric oxide production eliminates these changes in blood flow.

Cyclic flow variations are caused by recurrent platelet aggregation and dislodgment in stenosed and endothelium-injured arteries.\textsuperscript{14-16} The ability of platelets to aggregate affects the development of cyclic flow variations. Antiplatelet reagents such as thromboxane and serotonin antagonists inhibit platelet aggregation and thereby abolish cyclic flow variations.\textsuperscript{22-25} However, infusion of epinephrine stimulates platelet aggregation and can restore abolished cyclic flow variations.\textsuperscript{26,27}

In this study, administration of L-NMMA induced cyclic flow variations. L-Arginine but not D-arginine abolished the L-NMMA–induced cyclic flow variations. L-Arginine is a precursor for nitric oxide synthesis,\textsuperscript{11,12} and L-NMMA competitively inhibits the synthesis of nitric oxide.\textsuperscript{11,13} Our data suggest that nitric oxide may protect against platelet aggregation and cyclic flow variations. When the protective effect is impaired by...
### TABLE 1. Hemodynamics Before and After Treatments

<table>
<thead>
<tr>
<th>Group 1 (LAD, n=9)</th>
<th>Heart rate (bpm)</th>
<th>Aortic pressure (mm Hg)</th>
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<th>Mean FV*</th>
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<td>Systolic</td>
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A, after stenosis; B, before stenosis; bpm, beats per minute; FV, flow velocity in coronary artery; LAD, left anterior descending coronary artery; L-NMMA, N⁶-monomethyl-L-arginine.

*Reported as a percentage of baseline.
†Compared with saline, p<0.05.
‡Compared with baseline, p<0.05.

Diminishing nitric oxide synthesis, platelets presumably accumulate on the arterial wall, and cyclic flow variations develop. The protective mechanism resumes when nitric oxide synthesis is restored by treatment with L-arginine. Accumulated platelets are then eliminated, and cyclic flow variations are abolished.

Radomski et al.¹³ have reported that in humans, an L-arginine/nitric oxide pathway present in platelets regulates platelet aggregation. In their studies, in vitro treatment with L-NMMA enhanced platelet aggregation, whereas L-arginine inhibited platelet aggregation. Increased levels of intracellular cyclic GMP are involved in inhibiting platelet aggregation by nitric oxide.¹³ In our studies with dogs, both in vivo administration and in vitro addition of L-NMMA enhanced platelet aggregation, and L-arginine inhibited platelet aggregation. Together, these data suggest that inhibition of nitric oxide synthesis in platelets may be responsible for the induction of cyclic flow variations, and restoration of nitric oxide production in platelets may eliminate cyclic flow variations.

The intermediate doses of L-NMMA and L-arginine used in our in vitro studies of platelet aggregation approximate the plasma concentration of these drugs after in vivo administration. However, the degree of enhancement or inhibition of 10 μg/ml of collagen-induced platelet aggregation achieved in vivo treatment with L-NMMA or L-arginine is similar to that observed in studies conducted in vitro with intermediate doses of L-NMMA and L-arginine. It appears that no
additional effect on platelet aggregation was achieved by in vivo treatment. Therefore, platelets may be a site of origin of the nitric oxide that provided protection against cyclic flow variations in our canine model. Arterial endothelium, another source of nitric oxide, is injured before the administration of either L-NMMA or L-arginine; therefore, the injured endothelium is unlikely as an origin of nitric oxide in this model. However, the endothelium proximal to the site of arterial injury may also contribute nitric oxide to the elimination of cyclic flow variations. These findings indicate that platelet-derived or endothelium-derived nitric oxide or both may protect against platelet aggregation and thrombus formation in endothelium-injured arteries.

Vasoconstriction may be involved in the pathogenesis of cyclic flow variations. In fact, some studies have shown vasoconstriction of coronary arteries with injured endothelium during cyclic flow variations. In the present study, L-NMMA caused vasoconstriction of the femoral arteries before endothelial injury. L-Arginine reversed the effect of L-NMMA and even caused vasoconstriction of the femoral arteries before endothelial injury. L-Arginine reversed the effect of L-NMMA and even caused vasoconstriction of the femoral arteries before endothelial injury.
dilation, which may have resulted from the excessive production of nitric oxide or from overcoming the initial vasoconstriction caused by mild endothelial dysfunction after surgical manipulation of the vessel wall. As expected, treatment with either L-NMMA or L-arginine did not alter the diameter of the arteries after endothelial injury. The effects of L-NMMA and L-arginine on the induction and abolition of cyclic flow variations therefore may not be related to vasoactivity. Alternatively, our study suggests that induction of cyclic flow variations by L-NMMA may result from enhanced platelet aggregation, and elimination of cyclic flow variations by L-arginine may result from decreased platelet aggregation.

To further address the relation between vasoconstriction and cyclic flow variations, the effect of acetylcholine on vasoactivity of the femoral artery was studied. Acetylcholine stimulates the release of EDRF from vascular endothelium. However, previous studies have shown that acetylcholine may exert divergent effects on vascular tone. When the endothelium is intact, acetylcholine dilates coronary vessels. In contrast, acetylcholine may actually cause constriction in an endothelium-injured artery. The data presented here are similar to these findings. In endothelium-injured femoral arteries, the vasoconstriction caused by acetylcholine did not induce cyclic flow variations; however, acetylcholine enhanced the severity of cyclic flow variations. This finding demonstrates that vasoconstriction at the endothelium-injured site may not initiate cyclic flow variations but may increase their severity.

**Conclusions**

We have demonstrated that endogenously produced nitric oxide protects against cyclic flow reductions in stenosed and endothelium-injured coronary and femoral arteries by inhibiting platelet aggregation. We have also shown that an endothelium-dependent vasodilator, acetylcholine, constricts femoral arteries with injured endothelium and enhances the severity of cyclic flow reductions.

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