Endothelium-Derived Relaxing Factor Modulates Platelet Aggregation in an In Vivo Model of Recurrent Platelet Activation

Paolo Golino, Maurizio Cappelli-Bigazzi, Giuseppe Ambrosio, Massimo Ragni, Enrico Russolillo, Mario Condorelli, and Massimo Chiariello

It has been shown that endothelium-derived relaxing factor (EDRF) may inhibit platelet aggregation in vitro through activation of platelet-soluble guanylate cyclase. To assess whether EDRF may also affect platelet function in vivo, intravascular platelet aggregation was initiated by placing an external constrictor around endothelially injured rabbit carotid arteries. Carotid blood flow velocity was measured continuously by a Doppler flow probe placed proximal to the constrictor. After placement of the constrictor, cyclic flow reductions (CFRs), due to recurrent platelet aggregation, developed at the site of the stenosis. After CFRs were observed for 30 minutes, a solution of authentic nitric oxide (NO, n = 10) was infused into the carotid artery via a small catheter placed proximally to the stenosis. Before infusion of NO, CFR frequency averaged 18.3 ± 2.9 cycles per hour, and CFR severity (lowest carotid blood flow as percentage of baseline values) was 6 ± 1%. NO completely inhibited CFRs in all animals, as shown by the normal and constant pattern of carotid blood flow (CFR frequency, 0 cycles per hour, p < 0.001; carotid blood flow, 92 ± 5%, p = NS versus baseline). These effects were transient; CFRs were restored spontaneously within 10 minutes after cessation of NO infusion. After CFRs returned, S-nitroso-cysteine (S-NO-cys), a proposed form of EDRF, was infused into the carotid artery. S-NO-cys also abolished CFRs in all animals but at a significantly lower dose than NO (0.3 ± 0.1 versus 12 ± 4 nmol/min). The role of endogenously released EDRF in modulating in vivo platelet function was then tested in additional experiments. In 10 animals, endogenous release of EDRF was stimulated by infusing acetylcholine into the aortic root during CFRs. Infusion of acetylcholine was also associated with a complete inhibition of CFRs, similar to that observed during exogenous infusion of NO or S-NO-cys. These antithrombotic effects of acetylcholine were completely lost when EDRF synthesis was prevented by administration of the L-arginine analogue N^ω-monomethyl L-arginine (L-NMMA). Furthermore, in six additional rabbits the basal release of EDRF was blocked by L-NMMA after CFRs had been previously abolished with aspirin or the combination of aspirin and ketanserin, a serotonin S2 receptor antagonist. L-NMMA caused restoration of CFRs in all animals, indicating that even the basal release of EDRF is important in modulating platelet reactivity in vivo. Taken together, the data of the present study demonstrate that endogenous EDRF might importantly contribute to the modulation of platelet function in vivo. (Circulation Research 1992;71:1447–1456)

KEY WORDS • endothelium-derived relaxing factor • platelet aggregation • cyclic flow reductions

A growing body of evidence indicates that interactions between the coronary vessel wall and circulating platelets may be responsible for the sudden conversion from chronic to acute coronary artery disease syndromes, including sudden cardiac death, acute myocardial infarction, and unstable angina.1 In particular, clinical2–3 and epidemiological4–6 studies have indicated that platelet aggregation at sites of coronary artery stenosis and endothelial injury may lead to transient reductions in coronary blood flow, thus playing an important role in the pathophysiology of acute coronary artery disease syndromes.

Several chemical mediators have been shown to be involved in initiating and/or sustaining platelet aggregation after exposure of the subendothelial matrix to circulating blood. Indeed, some of these substances, such as thromboxane A2,7,8 serotonin,9,10 platelet activating factor,11,12 thrombin,13 and ADP,14 have been shown to be important mediators of coronary cyclic flow variations in an experimental model of recurrent platelet–vessel wall interaction at sites of coronary stenosis and endothelial injury.

Thrombosis is a dynamic process in which procoagulant activity, which comprises platelet activation and fibrin formation, is balanced by physiological anticoagulant, fibrinolytic, and antiplatelet activity. In particular, platelet response in vivo is highly dependent on the balance between activating and inhibiting substances. The normal endothelium greatly contributes to main-
taining circulating platelets in a quiescent inactive state by producing substances, such as prostacyclin and endothelium-derived relaxing factor (EDRF), capable of inhibiting platelet activity. Thus, it can be speculated that platelet activation at sites of coronary artery stenosis and endothelial injury could be facilitated by the relative or absolute decrease in local arterial concentrations of EDRF and/or prostacyclin.

EDRF is a labile nonprostanoid substance secreted by endothelial cells, which has been proposed to be nitric oxide (NO)\textsuperscript{13} or an organic molecule carrying NO, such as S-nitroso-cysteine (S-NO-cys), which may release NO at the cell membrane.\textsuperscript{16} Several studies clearly indicate that EDRF causes relaxation of vascular smooth muscle cells through the activation of soluble guanylate cyclase, leading to cGMP-dependent protein phosphorylation.\textsuperscript{17,18} The fact that a soluble guanylate cyclase is also present in platelets,\textsuperscript{19} that an increase in the intercellular cGMP levels is associated with an inhibition of in vitro platelet function,\textsuperscript{20} and that EDRF is also released toward the vascular lumen has led to the concept that EDRF may be involved in the control of platelet activity. In fact, several studies have provided evidence that both authentic NO and EDRF secreted by endothelial cells might inhibit platelet aggregation or deposition on vascular endothelium in vitro.\textsuperscript{21-27} In addition, stimulation of endogenous EDRF release has been associated with a decreased responsiveness of platelets when challenged in vitro with aggregating agents.\textsuperscript{28} Furthermore, a recent study has shown that nitroglycerin, an organic nitrate that may lead to NO release in vivo via formation of S-nitrosothiol intermediates, inhibits cyclic flow variations in endothelially injured canine coronary arteries with experimental stenosis.\textsuperscript{29} Altogether, these studies suggest a possible role for EDRF in modulating platelet function and prompted us to test this hypothesis in an in vivo model of intravascular platelet aggregation.

Thus, the aims of the present study were 1) to assess whether exogenously infused EDRF (as authentic NO or S-NO-cys) can abolish cyclic flow reduction (CFR) in an in vivo model of arterial stenosis and endothelial injury and 2) to determine whether endogenous EDRF may play a role in modulating platelet function in vivo. To accomplish this latter goal, we have determined the effects of stimulating the endogenous release of EDRF on CFRs as well as the effects of inhibiting the basal release of EDRF in animals in which CFRs had been previously eliminated by aspirin. Evidence is provided that EDRF is an important modulator of platelet function in vivo.

Materials and Methods

A rabbit model of intravascular platelet aggregation was used in the present study.\textsuperscript{30,31} This model represents a modification of the coronary canine model originally described by Foletts et al.\textsuperscript{32} in 1976. Briefly, New Zealand White rabbits of either sex were anesthetized with a mixture of ketamine (35 mg/kg) and xylazine (5 mg/kg) given intramuscularly. Anesthesia was maintained during the course of the experiment by an intravenous infusion of ketamine sufficient to abolish the corneal reflex. Through a median incision of the neck, the left or right common carotid artery was exposed and carefully isolated from the surrounding tissue. Polyethylene catheters were placed into a jugular vein and a femoral artery for drug administration and continuous blood pressure monitoring, respectively. Thereafter, a segment of the exposed carotid artery was deendothelialized by gently squeezing the artery between a pair of rubber-covered forceps. A plastic constrictor was then placed around the deendothelialized segment to create a stenosis of approximately 75% of the luminal diameter. Carotid blood flow velocity was measured continuously by a Doppler flow probe placed proximal to the constrictor. In this model, cyclic flow reductions due to recurrent platelet aggregation at the site of the stenosis usually develop within a few minutes after placement of the constrictor. Finally, a small polyethylene catheter was positioned proximal to the flow probe through the arterial wall and held in place with a 6-0 silk purse-string suture. This catheter was used to infuse nitric oxide (NO) or S-nitroso-cysteine (S-NO-Cys) during cyclic flow reductions.

![Figure 1. Schematic representation of the experimental preparation of intravascular platelet aggregation used in group 1 rabbits. Either the left or the right carotid artery was exposed surgically under general anesthesia. Endothelial injury was produced by gently squeezing the artery between a pair of rubber-covered forceps. A plastic constrictor was then placed around the deendothelialized segment to create a stenosis of approximately 75% of the luminal diameter. Carotid blood flow velocity was measured continuously by a Doppler flow probe placed proximal to the constrictor. In this model, cyclic flow reductions due to recurrent platelet aggregation at the site of the stenosis usually develop within a few minutes after placement of the constrictor. Finally, a small polyethylene catheter was positioned proximal to the flow probe through the arterial wall and held in place with a 6-0 silk purse-string suture. This catheter was used to infuse nitric oxide (NO) or S-nitroso-cysteine (S-NO-Cys) during cyclic flow reductions.](http://circres.ahajournals.org/doi/abs/10.1161/01.RES.71.6.1448?journalCode=circ)

**Experimental Protocol: Group 1**

**Effects of exogenous administration of NO or S-NO-cys.**

Ten rabbits entered this arm of the study. Once induced, CFRs were observed for 30 minutes, and their frequency and severity (nadir of carotid blood flow as a percentage of baseline flow) were measured. After 30 minutes of CFRs, the animals received through the small catheter positioned proximal to the stenosis an infusion of NO (4 × 10⁻⁷ M) dissolved in deoxygenated 0.9% NaCl (see below). The infusion was started at a
rate of 1 nmol/min (25 μl/min) and progressively increased until CFRs were abolished or until hemodynamic effects were observed. The volume of the NO solution infused into the carotid artery never exceeded 0.5 ml/min. After CFRs were abolished, NO infusion was maintained for 10 minutes and then replaced by the vehicle alone (deoxygenated 0.9% NaCl) at the same rate that was effective in abolishing CFRs. The time necessary for CFRs to be spontaneously restored was measured. After CFRs had returned, an S-NO-cys solution (10⁻⁵ M) was infused through the intracarotid catheter, starting at a rate of 0.1 nmol/min (100 μl/min), and progressively increased until CFRs were abolished or until hemodynamic effects were observed. Once abolished, the infusion was kept for 10 minutes and then suspended until CFRs returned.

Preparation of NO solutions. Authentic NO gas (25 μl) was dissolved in a gas-tight container into 25 ml of 0.9% NaCl previously deoxygenated by vigorous aeration with N₂ for at least 15 minutes. This resulted in a concentration of 4 x 10⁻⁴ M. Solutions prepared in this manner were found to remain stable for at least 1 hour; the in vitro relaxation of rabbit aortic rings in response to various concentrations of NO did not differ immediately after the solution was made and 1 hour later (n=3, M. Cappelli-Bigazzi, unpublished observations).

Synthesis of S-NO-cys. S-NO-cys was prepared as previously described.³³ Briefly, 25 ml oxygen and 50 ml NO at room temperature and pressure were mixed in a gas-tight syringe to form nitrogen dioxide gas. This gaseous mixture was injected into a vacutainer tube kept in dry ice until frozen (several seconds). L-Cysteine (1 mmol of the HCl salt) was dissolved in 1 ml methanol to produce a 1-M solution. The frozen nitrogen dioxide was dissolved into the cysteine methanol solution to produce a 1-M solution of S-NO-cys. The solution turned from clear to rose colored after completion of the reaction. This reaction was performed in a vial surrounded by dry ice. When this method is used, nearly 100% conversion of l-cysteine to S-NO-cys occurs, as assessed by a chromatographic separation by high-performance liquid chromatography.³³ The stock solution was stored at −20°C in the absence of light and appropriately diluted immediately before use. This stock solution was stable for at least 1 month, as assessed by measuring the relaxation of rabbit aortic rings as previously described (n=3, M. Cappelli-Bigazzi, unpublished observations).

Experimental Protocol: Group 2

Effects of endogenous EDRF on platelet function in vivo. To study the effects of endogenous EDRF in modulating platelet activity in vivo, 16 rabbits were included in this arm of the study; they were divided into two subgroups, group 2a and group 2b.

Group 2a. Intravascular platelet aggregation was initiated in the left carotid artery of 10 rabbits, as described above, with the exception that the intracarotid catheter was not positioned inside the artery. Instead, an additional polyethylene catheter was placed in the ascending aorta through the right carotid artery (Figure 3). This catheter was used to infuse acetylcholine into the aortic root to stimulate EDRF release from endothelial cells. Special care was taken to position the catheter above the coronary ostia. To accomplish this, the catheter was first advanced into the left ventricle and thereafter pulled back while connected to a pressure transducer. When an aortic pressure waveform was visualized, the catheter was pulled further for about one additional centimeter.

After positioning the plastic constrictor around the left carotid artery, CFRs developed, and they were
observed for 30 minutes. Then, a continuous infusion of acetylcholine was initiated through the intra-aortic catheter at an initial rate of 0.1 μg/kg per minute and increased progressively until CFRs were abolished or until a decrease in mean arterial pressure ≥50% of the baseline value was observed. Once CFRs were abolished, acetylcholine infusion was maintained for 10 minutes. The infusion was then stopped, and the time necessary for CFRs to return was measured. After CFRs had returned, they were observed for an additional 30 minutes. To test the hypothesis that the antiplatelet effects of acetylcholine are mediated through the release of EDRF, a second infusion of acetylcholine was repeated, at the same dose previously effective in abolishing CFRs, after administration of the l-arginine analogue Nω-monomethyl l-arginine (L-NMMA, 10 mg/kg i.v.). This arginine analogue is known to inhibit the synthesis of EDRF from l-arginine.34

Group 2b. Six rabbits were included in this group in which CFRs were obtained as previously described. After 30 minutes of CFRs, aspirin was administered as an intravenous bolus of 10 mg/kg. If CFRs were not abolished by aspirin, an intravenous bolus of ketanserin (0.25 mg/kg), a selective serotonin 5-HT receptor antagonist, which has been shown to abolish CFRs in all animals that do not respond to aspirin,35 was given. To test the hypothesis that the basal release of EDRF is also important in determining platelet reactivity in vivo, 30 minutes after CFRs were completely abolished, the basal release of EDRF was blocked by administering an intravenous bolus of L-NMMA (10 mg/kg).

In vitro platelet aggregation studies. To assess whether acetylcholine and L-NMMA per se had some effects on platelet function, platelet aggregation in response to ADP and arachidonic acid was performed in vitro under baseline conditions or in the presence of acetylcholine or L-NMMA. Nine milliliters of blood was drawn in a syringe containing 1 ml of 3.8% sodium citrate. Blood was centrifuged at 120g for 20 minutes at room temperature to obtain platelet-rich plasma. Platelet-rich plasma was removed and centrifuged at 1,000g for 5 minutes to obtain platelet-poor plasma. Platelet aggregation was measured turbidimetrically on an aggregometer (Chrono-Log Corp., Havertown, Pa.) and recorded on a linear recorder. The aggregometer was calibrated with the use of platelet-poor plasma, and the test was performed on 250 μl platelet-rich plasma in a siliconized cuvette with continuous stirring. The platelet count in the platelet-rich plasma was adjusted to 300,000/μl by dilution with platelet-poor plasma as needed. Aggregation was induced in platelet-rich plasma in response to various concentrations of ADP and arachidonic acid either under baseline conditions or in the presence of acetylcholine (10⁻³ M) or L-NMMA (5.5×10⁻⁴ M).

Statistical Analysis
All values are expressed as mean±SEM. CFR frequency and severity, as well as hemodynamic variables, were compared within groups by a one-way analysis of variance (ANOVA) with a design for repeated measurements that was followed, when an F value was found to be significant, by Student’s t test for paired observations with Bonferroni’s correction for comparison of different treatments. A two-way ANOVA with a design for repeated measurements was used to compare in vitro platelet aggregation data. When applicable, differences among groups were tested by Student’s t test for unpaired samples with Bonferroni’s correction. A value of p<0.05 defined significant differences between populations.

Results
Group 1: Effects of Exogenous Administration of NO or S-NO-cys
After placement of the constrictor around the carotid artery, CFRs promptly developed in 10 of 10 rabbits, with a mean frequency of 18.3±2.9 cycles per hour (Figure 4A). Severity of CFRs, expressed as the nadir of carotid blood flow velocity (the lowest blood flow velocity recorded before flow restoration), averaged 6±1% of baseline values (Figure 4B). After 30 minutes of CFRs, all animals received an intracarotid infusion of NO that started at a rate of 1 nmol/min and progressively increased every 10 minutes until CFRs were abolished. NO completely abolished CFRs in 10 of 10 rabbits (Figure 4A, p<0.001) at an average rate of 12±4 nmol/min and increased carotid blood flow velocity up to 92±5% of baseline values (Figure 4B, p=NS versus baseline). By assuming a carotid blood flow of approximately 20 ml/min, it can be calculated that NO reached a concentration of approximately 1.5×10⁻⁷ M in the carotid circulation. NO infusion, at the dose effective in abolishing CFRs, was not associated with significant changes in systemic blood pressure or heart rate (Table 1).

After cessation of NO infusion, CFRs were spontaneously restored in 7±2 minutes in all animals at a frequency and severity similar to those observed before infusing NO (Figure 4). After an additional 30 minutes of CFRs, S-NO-cys was infused through the intracarotid catheter. Again, CFRs were completely abolished in 10
of 10 animals (Figure 4A, p<0.001). Similarly, carotid blood flow velocity during S-NO-cys infusion returned to baseline values and actually averaged 111±8% of baseline values (Figure 4B). S-NO-cys was significantly more potent than NO in abolishing CFRs; they were completely abolished at an average infusion rate of 0.3±0.1 nmol/min. This would correspond to a concentration in the carotid circulation of approximately 4×10⁻⁹ M. Again, at the dose effective in abolishing CFRs, S-NO-cys did not cause significant changes in systemic blood pressure and heart rate (Table 1). This lack of hemodynamic changes by both NO and S-NO-cys was due to the fact that these compounds were injected locally, i.e., in the carotid circulation, and that they are short lived.

Taken together, these findings indicate that exogenously infused NO and S-NO-cys are powerful antiplatelet agents in this experimental model of in vivo platelet aggregation.

**Group 2: Effects of Endogenous EDRF on Platelet Function In Vivo**

**Group 2a.** Similar to group 1 rabbits, CFRs also developed in 10 of 10 rabbits in this group after placement of the constrictor around the left carotid artery. Baseline CFRs had a frequency of 15.4±1.9 cycles per hour and a nadir of carotid blood flow velocity (as a percentage of baseline values) of 5±2% (Figure 5). After CFRs were observed for 30 minutes, acetylcholine was infused at increasing doses through the catheter placed into the aortic root. Acetylcholine infusion resulted in a complete inhibition of CFRs in 10 of 10 rabbits (p<0.001) at an average infusion rate of 0.3±0.1 µg/kg per minute. Consequently, CFR frequency decreased to zero in all animals (Figure 5A, p<0.001), and carotid blood flow velocity increased to 92±4% of the baseline values (p=NS versus baseline, Figure 5B). After cessation of acetylcholine infusion, CFRs came back in 4±2 minutes with a frequency and severity similar to those observed before infusing acetylcholine (Figure 5). Administration of L-NMMA, an inhibitor of NO synthesis, was associated with a worsening of CFRs; their frequency increased to 19.1±1.7 cycles per hour (p<0.05 versus baseline, Figure 5A), thus suggesting a role for the basal release of EDRF in affecting platelet response in vivo. Furthermore, when acetylcholine, at the dose previously effective in abolishing CFRs, was repeated after administration of L-NMMA, CFRs were not abolished; their frequency and severity did not change significantly with respect to preacetylcholine values (Figure 5), thus suggesting that the in vivo antiplatelet effects of acetylcholine were mediated by the release of endogenous EDRF.

Infusion of acetylcholine, at the dose effective in abolishing CFRs, was associated with a significant decrease in arterial blood pressure to 71% of baseline values (Table 2). This effect is due to a release of EDRF from the whole circulation, since acetylcholine was administered systemically and promptly disappeared when the infusion was stopped. On the contrary, administration of L-NMMA (10 mg/kg i.v. bolus) caused a significant increase in arterial blood pressure (Table 2), which is thought to be related to the inhibition of the

**TABLE 1. Hemodynamic Variables in Rabbits Receiving Nitric Oxide and S-Nitroso-cysteine**

(1) Group 1

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<thead>
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<th>Baseline</th>
<th>CFRs</th>
<th>NO</th>
<th>S-NO-cys</th>
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<td>Mean arterial pressure (mm Hg)</td>
<td>67±6</td>
<td>70±5</td>
<td>72±6</td>
<td>68±7</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>124±8</td>
<td>129±6</td>
<td>123±5</td>
<td>126±6</td>
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CFRs, cyclic flow reductions; NO, nitric oxide; S-NO-cys, S-nitroso-cysteine; bpm, beats per minute. Values are mean±SEM.
basal release of endogenous NO. Finally, infusion of acetylcholine after L-NMMA administration caused a decrease in arterial blood pressure significantly less pronounced than that observed with acetylcholine alone (Table 2).

Group 2b. In this group, CFRs developed in six of six rabbits. After 30 minutes of CFRs, aspirin (10 mg/kg) abolished them in three of six animals, and in the remaining three, a subsequent ketanserin administration (0.25 mg/kg) resulted in a complete inhibition of CFRs (Figure 6). After CFRs had been abolished for 30 minutes, inhibition of endogenous EDRF synthesis by L-NMMA resulted in the restoration of CFRs in all animals (p<0.001, Figure 6).

These data outline the importance of endogenous EDRF in modulating in vivo platelet function.

**In vitro platelet aggregation studies.** To determine whether acetylcholine and L-NMMA had some effect on platelet function per se, i.e., independent of the release of EDRF, platelet aggregation was performed in vitro. Dose–response curves to ADP and arachidonic acid were performed under baseline conditions and in the presence of either acetylcholine (10⁻³ M) or L-NMMA (5.5×10⁻⁴ M). Acetylcholine did not cause any significant change in platelet response to both ADP and arachidonic acid, whereas L-NMMA significantly shifted upward the dose–response curve at the lowest concentrations of ADP and arachidonic acid (Figure 7), consistent with previous observations that this analogue of L-arginine may potentiate in vitro platelet aggregation.

**Discussion**

The data of the present study indicate that exogenously infused EDRF, in the form of authentic NO or the NO-containing molecule S-NO-cys, is a potent inhibitor of CFRs in rabbit stenotic carotid arteries with endothelial injury. S-NO-cys appeared to be approximately 40 times more potent than authentic NO in inhibiting platelet aggregation, a finding already described with respect to the vasorelaxant effect of

**Figure 5.** Bar graphs showing the effects of stimulating endogenous endothelium-derived relaxing factor (EDRF) release on cyclic flow reduction (CFR) frequency (panel A) and severity (panel B) expressed as carotid blood flow velocity. Stimulation of EDRF release by acetylcholine (Ach) administration completely abolished CFRs (0 cycles per hour) and significantly increased carotid blood flow velocity. However, when EDRF synthesis was blocked by N⁰-monomethyl L-arginine (L-NMMA), Ach completely lost its antithrombotic effects (Ach+L-NMMA). *p<0.05 vs. base 2; #p<0.001 vs. base 1.

**Figure 6.** Plot showing effects of inhibiting the endogenous basal release of endothelium-derived relaxing factor (EDRF) on in vivo platelet reactivity. After 30 minutes of cyclic flow reductions (CFRs), aspirin (ASA) abolished CFRs in three of six rabbits. In the remaining three rabbits, addition of ketanserin (KETA) to aspirin was effective in abolishing CFRs. Once inhibited, CFRs returned in all animals after administration of N⁰-monomethyl L-arginine (L-NMMA), an inhibitor of EDRF synthesis.

<table>
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<th>Table 2. Hemodynamic Changes in Rabbits Receiving Acetylcholine (Group 2a)</th>
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<tr>
<td>Mean arterial pressure (mm Hg)</td>
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<td>Heart rate (bpm)</td>
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CFRs, cyclic flow reductions; Ach, acetylcholine; L-NMMA, N⁰-monomethyl L-arginine; bpm, beats per minute. Values are mean±SEM.

*p<0.05 vs. baseline.
EDRF. This pronounced antiplatelet effect, according to the chemical and pharmacological properties of EDRF, was transient; CFRs were promptly restored within a few minutes after cessation of both NO and S-NO-cys infusions. Another major finding of the present study is that stimulating the synthesis of endogenous EDRF by acetylcholine resulted in a complete inhibition of CFRs in our experimental model. This antiplatelet effect of acetylcholine was completely lost when this substance was infused after the administration of L-NMMA, an L-arginine analogue that blocks EDRF synthesis from L-arginine. In addition, in rabbits in which CFRs had been previously abolished by aspirin or the combination of aspirin and ketanserin, a selective serotonin \(S\) receptor antagonist, inhibition of the basal release of EDRF by L-NMMA administration caused a restoration of CFRs in all animals. Altogether, these observations outline the pivotal role of endogenous EDRF in modulating platelet function in vivo.

In the present study we have used an experimental model of recurrent in vivo platelet aggregation in rabbits according to a modification of the canine model originally described in 1976. Both these models involve placement of an external constrictor around a coronary artery or a carotid artery such that there is a focal endothelial injury and an arterial stenosis without impairment of resting blood flow. However, the stenosed vessels subsequently develop cyclic changes in coronary blood flow characterized by progressive reductions in blood flow followed by sudden returns of flow to basal levels. Histological studies and studies with radiolabeled platelets have convincingly demonstrated that these CFRs correlate with the recurrent formation of platelet-rich thrombi in the stenosed artery. Early studies have suggested that thromboxane \(A_2\) is an important mediator in initiating and/or sustaining CFRs. Indeed, it has been demonstrated that aspirin, which blocks arachidonic acid metabolism at the cyclooxygenase level, or more selective thromboxane synthase inhibitors or receptor antagonists all abolish CFRs in the majority of animals. More recent studies, however, have indicated that other endogenous substances may act as chemical mediators of CFRs, including serotonin, thrombin, platelet activating factor, and possibly ADP.

Since the first report by Furchgott and Zawadzki of the existence of a labile endothelium-derived substance capable of relaxing smooth muscle cells, an impressive number of studies have been published describing the effects of this substance and the factors that regulate its synthesis and secretion. Different independent groups of investigators, studying the biological, pharmacological, and chemical properties of EDRF, have proposed that it may in fact be NO or \(\text{N}^\circ\)-monomethyl-L-arginine (L-NMMA, \(5.5 \times 10^{-4}\) M). Ach did not affect platelet response to ADP or arachidonic acid, whereas L-NMMA significantly increased aggregation only at the lowest concentrations of these agonists. *p<0.05 vs. control and Ach.

**Figure 7.** Graphs showing maximum (MAX) in vitro platelet aggregation in response to increasing concentrations of ADP (left panel) or arachidonic acid (right panel) under baseline conditions and in the presence of either acetylcholine (Ach, \(10^{-5}\) M) or \(\text{N}^\circ\)-monomethyl-L-arginine (L-NMMA, \(5.5 \times 10^{-4}\) M). Ach did not affect platelet response to ADP or arachidonic acid, whereas L-NMMA significantly increased aggregation only at the lowest concentrations of these agonists. *p<0.05 vs. control and Ach.
recent preliminary study, Rovin et al.\(^5\) have shown that sodium nitroprusside (which, unlike nitroglycerin, directly releases NO) is able to inhibit CFRs in the canine model and that it does so by increasing intracellular cGMP levels. Taken together, these observations suggested a role of EDRF in modulating platelet function in vivo and prompted us to investigate the role of this substance in the regulation of platelet aggregation in vivo.

Indeed, our study provides direct evidence that endogenous EDRF is an important modulator of platelet function in vivo. This conclusion stems from the observation that CFRs, which are due to recurrent platelet aggregation at sites of arterial stenosis and endothelial injury, were completely abolished during the administration of acetylcholine, a substance known to stimulate EDRF release from endothelial cells. That the in vivo antiplatelet effects of acetylcholine are mediated by the release of EDRF is further supported by the observation that this substance per se, even at a very high concentration, is devoid of any effects on platelet aggregation in vitro. Furthermore, the finding that acetylcholine was unable to inhibit CFRs after the administration of L-NMMA, an arginine analogue that inhibits EDRF synthesis, also points toward an EDRF-mediated inhibition of platelet function by acetylcholine. Similar findings have been reported in a recent preliminary study.\(^5\)

Another major finding of the present study is that the basal release of EDRF also plays an important role in affecting platelet function. This conclusion arises from the finding that inhibiting the basal release of EDRF by L-NMMA was associated with a restoration of CFRs in animals in which CFRs had been previously abolished with aspirin or the combination of aspirin and ketanserin, a selective serotonin \(S\), receptor antagonist. This finding might be explained by viewing platelet activity in vivo as a balance of inhibiting and activating stimuli. In fact, platelets normally circulate in a nonactive resting state because of the relative prevalence of inhibitory stimuli (including the continuous basal release of EDRF). After endothelial damage, this balance is altered as activating stimuli prevail. As a consequence of the vascular damage, platelets adhere to the subendothelial tissue. Then, a number of chemical mediators, such as thromboxane \(A_2\), serotonin, platelet activating factor, and ADP, are released to recruit additional platelets and promote further aggregation. If one eliminates the effects of one or more of these mediators, as we did in our animals treated with aspirin and ketanserin, a new equilibrium between activating and inhibiting stimuli is achieved, and platelet aggregation in vivo is usually inhibited. However, if this equilibrium is altered again (for instance, by eliminating one or more inhibitory stimuli, as we did by blocking the basal release of EDRF), platelet activation at the site of the stenosis and endothelial injury may ensue again, because activating stimuli predominate again. In this regard, it seems important to note that the administration of L-NMMA was associated with a restoration of CFRs, even in those animals in which two different activating pathways, namely, thromboxane \(A_2\) and serotonin, had been blocked. Previous studies have in fact demonstrated that under these circumstances it is very difficult to restore CFRs with intravenous infusions of epinephrine, because very high plasma epinephrine concentrations are required.\(^1\) Thus, it is reasonable to conclude that the basal release of EDRF also importantly contributes to regulating the reactivity of circulating platelets, such that the inhibition of its synthesis makes platelets more reactive to external stimuli, even in the presence of a simultaneous blockade of thromboxane \(A_2\) and serotonin-activating pathways.

It is of note that previous studies have demonstrated that platelets contain NO synthase, the enzyme responsible for the synthesis of NO from L-arginine, and that this enzyme can be stimulated on aggregation in response to collagen and ADP, thus leading to an increase in intracellular cGMP levels.\(^5\) Considering that the inhibition of platelet NO synthase by L-NMMA is associated with a potentiation of in vitro platelet aggregation, some authors have suggested that platelet production of NO might represent a negative feedback regulatory process of platelet aggregation.\(^6\) Thus, in view of the observation in the present study that L-NMMA restored CFRs despite the administration of aspirin and ketanserin, we cannot rule out the possibility that these effects were due, at least in part, to the inhibition of platelet NO synthase, besides the inhibition of EDRF synthesis from endothelial cells. However, considering that the effects of L-NMMA on in vitro platelet aggregation were not pronounced, we find this hypothesis unlikely. In addition, the possibility that the lack of prostacyclin production by endothelial cells after aspirin administration might have facilitated platelet activation in the setting of simultaneous inhibition of EDRF synthesis should be mentioned also.

In conclusion, the present study emphasizes the role of endogenous EDRF in modulating platelet activity in vivo. Because endogenous EDRF synthesis seems to be altered both in atherosclerotic animals and in patients with atherosclerotic lesions\(^7\)–\(^9\), it is tempting to speculate that this relative absence of a naturally occurring platelet inhibitory substance may predispose platelets to aggregate at sites of coronary stenosis and endothelial injury. Whether interventions aimed at increasing the basal release of EDRF may be useful as an antiplatelet regimen\(^6\) in these patients is not clear and would need further clarification.

**Acknowledgment**

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