Long-term Impairment of Endothelium-Dependent Relaxations to Aggregating Platelets After Reperfusion Injury in Canine Coronary Arteries

Paul J. Pearson, Hartzell V. Schaff, MD, and Paul M. Vanhoutte, MD, PhD

Experiments were designed and performed to determine whether endothelial function remained chronically impaired after coronary artery reperfusion. Canine left anterior descending coronary arteries were exposed to ischemia (60 minutes) followed by reperfusion (12 weeks). Rings (3–4 mm wide) of the reperfused artery and of normal left circumflex (control) coronary artery segments were suspended in organ chambers containing physiological saline solution (37°C, gassed with 95% O₂-5%CO₂) for isometric force measurement. Endothelium-independent contractions to KCl or prostaglandin F₂α and endothelium-independent relaxations to nitric oxide or isoproterenol were comparable in control and chronically reperfused arteries. However, chronically reperfused coronary arteries exhibited impaired endothelium-dependent relaxations to aggregating platelets. In addition, the reperfused coronary arteries exhibited impaired endothelium-dependent relaxations to the platelet-derived compounds adenosine diphosphate, serotonin, and thrombin. However, the endothelium-dependent relaxations to acetylcholine were comparable between control and reperfused arteries. Thus, after 12 weeks of reperfusion, previously occluded coronary arteries exhibited a selective impairment of endothelium-dependent relaxation evoked by aggregating platelets. In vivo, this phenomenon could favor platelet adhesion, aggregation, and platelet-induced contraction of coronary smooth muscle and thus facilitate ischemic events such as vasospasm and coronary thrombosis. (Circulation 1990;81:1921–1927)

Short-term reperfusion after coronary artery occlusion impairs endothelium-dependent relaxations in response to aggregating platelets. This altered endothelium-dependent response to platelets is probably due to decreased production or release of endothelium-derived relaxing factor in the reperfused vessel. Endothelium-derived relaxing factor not only relaxes vascular smooth muscle but also reduces platelet adhesion and is itself a potent antiaggregatory substance. If production of endothelium-derived relaxing factor were chronically impaired after reperfusion, this impairment would favor the occurrence of platelet adhesion, aggregation, and unopposed platelet-induced contraction of coronary smooth muscle.

Such an occurrence could lead to ischemic events such as vasospasm and coronary thrombosis. The present study was designed to examine the long-term effects of reperfusion injury to the canine coronary artery on endothelium-dependent responses to aggregating platelets and platelet products. Indeed, in other long-term models of coronary endothelial cell injury and regeneration, endothelium-dependent relaxations to aggregating platelets are still impaired 4 weeks after the initial injury.

Methods

Animal Preparation

Heartworm-free mongrel dogs (25–30 kg) of either sex were anesthetized with intravenous sodium pentobarbital (30 mg/kg bolus injection; Fort Dodge Labs, Inc., Fort Dodge, Iowa), intubated with a cuffed endotracheal tube, ventilated with 100% O₂, and continuously monitored electrocardiographically. A left lateral thoracotomy was performed in the fourth intercostal space to expose the heart. A 4-mm-long segment of the left anterior descending (LAD) coronary artery was carefully dissected free.

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immediately distal to its first diagonal branch. After dissection, the LAD coronary artery was occluded with a small vascular clamp. Cessation of blood flow was confirmed by darkening of the myocardium subtended by the artery, paradoxical wall motion of the ischemic myocardium, and electrocardiographic evidence of myocardial ischemia (typically, ST segment depression in leads II and III). After 60 minutes of ischemia, the occlusion clip was gently removed, and the artery was reperfused for 60 minutes. Reperfusion was confirmed by vascular and myocardial hyperemia, electrocardiographic changes, and resumption of normal myocardial wall motion. If a dog had extensive collateral blood flow and did not exhibit the previously mentioned ischemic changes 15 minutes after arterial occlusion, the dog was excluded from the study (n = 6). The chest was then closed, and the dog was allowed to recover from surgery. During the 24-hour recovery period, the dogs were housed indoors at the animal care facility of the Mayo Clinic. Postoperative and long-term animal care was supervised by two doctors of veterinary medicine directing a staff of licensed veterinary technicians.

Twelve weeks after surgery, the dogs were anesthetized (sodium pentobarbital, 30 mg/kg i.v.) and exsanguinated, and the beating hearts were quickly removed and immersed in cold, oxygenated physiological saline solution of the following composition (mM): NaCl 118.3, KCl 4.7, MgSO4 1.2, KH2PO4 1.22, CaCl2 2.5, NaHCO3 25.0, Ca-EDTA 0.016, and glucose 11.1 (control solution). Blood was also collected with acid-citrate-dextrose anticoagulant for preparation of autologous platelets. The procedures and handling of the animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Mayo Foundation.

**In Vitro Experiments**

The LAD and left circumflex (LCx) coronary arteries were carefully dissected free and placed in control solution. A maximum of four rings (4–5 mm wide) of the reperfused LAD coronary artery were taken at least 10 mm distal to the occlusion site. Rings of middle to distal LCx coronary arteries were used as controls. Previous organ chamber experiments demonstrated no difference in endothelium-dependent responses between these vessels (data not shown). In some rings, the endothelium was removed by gently rubbing the intimal surface with the tip of a pair of watchmakers’ forceps.

The rings were suspended in 25-ml organ chambers filled with control solution maintained at 37°C and aerated with 95% O2-5% CO2, pH 7.4. Each ring was suspended by two stainless steel clips passed through the lumen. One clip was anchored to the bottom of the organ chamber; the other was connected to a strain gauge (Statham-Gould model UC2, Glen Burnie, Maryland) for measurement of isometric force. The rings were placed at the optimal point of their length–tension relation by progressively stretching them until their contraction to KCl (20 mM given at each level of distension) was maximal.9

In all experiments, the presence or absence of endothelium was confirmed by determining the response to acetylcholine (10−6 M) in rings contracted with K+ (20 mM).10,11 After optimal tension was achieved, the coronary artery rings were allowed to equilibrate for 45 minutes before administration of drugs.

**Protocols**

Rings of reperfused and control arteries (with and without endothelium) from the same dog were stud-
ied in parallel. The rings were used for one of four protocols outlined below.

Protocol 1. Rings contracted with prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) ($2\times10^{-6}$ M) were exposed to acetylcholine ($10^{-9}$--$10^{-4}$ M) and then to platelets (10,000--100,000 platelets/µl final bath concentration).

Protocol 2. Rings contracted with PGF$_{2\alpha}$ ($2\times10^{-6}$ M) were exposed to adenosine diphosphate (ADP) ($10^{-9}$--$10^{-4}$ M), 5-hydroxytryptamine ($10^{-9}$--$10^{-4}$ M), and sodium nitroprusside ($10^{-9}$--$10^{-4}$ M).

Protocol 3. Rings precontracted with PGF$_{2\alpha}$ ($2\times10^{-6}$ M) were exposed to thrombin (0.01--10 units/ml final bath concentration), and then noncontracted rings were exposed to PGF$_{2\alpha}$ ($10^{-9}$--$10^{-3}$ M).

Protocol 4. Rings precontracted with PGF$_{2\alpha}$ ($2\times10^{-5}$ M) were exposed to nitric oxide ($10^{-5}$--$10^{-6}$ M) and isoproterenol ($10^{-9}$--$10^{-4}$ M), and then noncontracted rings were exposed to KCl (5--50 mM).

In all experiments, indomethacin ($10^{-5}$ M) was added to the organ chambers to prevent synthesis of endogenous prostanoids. An equilibration period of at least 30 minutes occurred between each experiment in the specific protocols.

Drugs and Platelets

The following drugs were used: acetylcholine chloride, ADP, 5-hydroxytryptamine (serotonin; as creatinine sulfate complex), (+)-isoproterenol hydrochloride, indomethacin, PGF$_{2\alpha}$, thrombin (bovine) (all from Sigma, St. Louis, Missouri), and nitric oxide (Union Carbide, Chicago, Illinois). All drugs were prepared daily with distilled water except for indomethacin, which was dissolved in Na$_2$CO$_3$ ($10^{-3}$ M). The concentrations are expressed as final molar concentration in the organ chamber. A platelet-rich solution from individual dogs was prepared by centrifugation as reported previously.8,9,12

Nitric oxide from a cylinder was used to fill a glass bulb filled with a silicon injection septum. With a glass syringe, gas was removed from the bulb and injected into another glass bulb that had been filled with 100 ml distilled water (which had been bubbled with He for 3 hours) to give stock solutions of nitric oxide.13

Data Analysis

Results are expressed as mean±SEM. In all experiments, $n$ refers to the number of animals from which rings were taken. In rings precontracted with PGF$_{2\alpha}$,

![Figure 3](image3.png) **FIGURE 3.** Concentration–response curves to 5-hydroxytryptamine (serotonin, $-\log$ M) in control (•, ○) and chronically reperfused (■, □) coronary arterial rings with (●, ■) and without (○, □) endothelium. Rings were contracted with prostaglandin F$_{2\alpha}$ ($2\times10^{-6}$ M). Values shown are % change in tension and are mean±SEM ($n=6$). LCX, left circumflex coronary artery; LAD, left anterior descending coronary artery.

![Figure 4](image4.png) **FIGURE 4.** Concentration–response curves to thrombin (units/ml) in control (●, ○) and chronically reperfused (■, □) coronary arterial rings with (●, ■) and without (○, □) endothelium. Rings were contracted with prostaglandin F$_{2\alpha}$ ($2\times10^{-6}$ M). Values shown are % relaxation and are mean±SEM ($n=6$). LCX, left circumflex coronary artery; LAD, left anterior descending coronary artery.

<table>
<thead>
<tr>
<th>Table 1.</th>
<th>$-\log$ M IC$_{50}$ Values for Control and Chronically Reperfused Rings With and Without Endothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>With endothelium</td>
<td></td>
</tr>
<tr>
<td>Acetylcholine ($n=9$)</td>
<td>7.65±0.12</td>
</tr>
<tr>
<td>Adenosine diphosphate ($n=8$)</td>
<td>6.78±0.08</td>
</tr>
<tr>
<td>Without endothelium</td>
<td></td>
</tr>
<tr>
<td>Nitric oxide ($n=5$)</td>
<td>7.41±0.07</td>
</tr>
<tr>
<td>Isoproterenol ($n=6$)</td>
<td>8.25±0.07</td>
</tr>
</tbody>
</table>

Values shown are percent of the response to prostaglandin F$_{2\alpha}$ ($2\times10^{-6}$ M) and are mean±SEM. *Significantly different from control rings by Student’s $t$ test for paired observation ($p<0.05$).
responses are expressed as percent changes from the contracted levels, and in quiescent rings, responses are expressed as percent changes of the responses to KCl (20 mM) unless otherwise stated. For relaxations, the negative logarithm of the effective molar concentration of agonist causing 50% inhibition (IC50) of the contractions to PGF2α was calculated for concentration–response curves, and the means of these values are presented. Statistical evaluation of data was performed by Student’s t test for either paired or unpaired observations. Values were considered to be statistically significant when p was smaller than 0.05.

**Results**

**Platelets**

In control and reperfused rings without endothelium, there was no significant difference in response to aggregating platelets (10–100,000 platelets/ml final organ bath concentration). In control and reperfused rings with endothelium, platelets caused concentration-dependent relaxations that were significantly greater than in rings without endothelium (Figure 1). However, in reperfused rings with endothelium, the concentration–response curve to platelets was shifted to the right. Thus, at the highest platelet concentration, reperfused rings with endothelium only relaxed to 42±11% of the initial contraction to PGF2α, as opposed to 4±3% for control rings.

**Platelet Products**

*Adenosine diphosphate.* In control and reperfused rings without endothelium, ADP caused comparable concentration-dependent relaxations that did not reach baseline value. In control and reperfused rings with endothelium, ADP caused concentration-dependent relaxations to baseline that were significantly greater than in rings without endothelium. However, in the reperfused rings with endothelium, there was a significant shift of the concentration–response curve to the right compared with control rings with endothelium (Figure 2 and Table 1).

*Serotonin.* There was no significant difference between control and reperfused rings without endothelium in response to serotonin. In control rings with endothelium, serotonin caused concentration-dependent relaxations that were significantly greater than in rings without endothelium. In reperfused rings with endothelium, serotonin-induced relaxations were not significantly different from rings without endothelium.

*Thrombin.* Thrombin (0.01–10 units/ml) caused endothelium-dependent, concentration-dependent relaxations in control and reperfused arteries with endothelium (Figure 4). In reperfused rings, the concentration–response curves to this agonist were shifted to the right.

*Acetylcholine.* Acetylcholine (10−9–10−4 M) caused comparable endothelium-dependent, concentration-dependent relaxations in control and reperfused arteries with endothelium (Figure 5 and Table 1).

*Endothelium-independent dilators.* Nitric oxide (10−9–10−5 M) and isoproterenol (10−9–10−7 M) caused comparable concentration-dependent relax-
Concentration-dependent contractions in control and reperfused rings without endothelium (Figures 6 and 7 and Table 1).

**Contractions.** KCl (5-40 mM) caused comparable concentration-dependent contractions in control and reperfused rings with and without endothelium (Table 2). There was no difference in maximal response to the agonist between the two groups. Within each group, there was no difference between rings with and without endothelium. PGF$_{2a}$ ($10^{-5}$-10$^{-3}$ M) caused comparable concentration-dependent contractions in control and reperfused rings with and without endothelium (Table 3). There was no difference in maximal response to the agonist between the two groups. Within each group, there was no difference between rings with and without endothelium.

**Discussion**

The present study was performed to examine endothelium-dependent responses to aggregating platelets and vasoactive drugs in the canine coronary artery 12 weeks after short-term occlusion and reperfusion. The major findings of this study were 1) reperfused coronary arteries still exhibit impaired endothelium-dependent relaxations to aggregating platelets; 2) reperfused coronary arteries recover their ability to generate normal endothelium-dependent relaxations to acetylcholine; 3) endothelium-dependent responses to ADP, serotonin, and thrombin are still impaired 12 weeks after reperfusion; 4) the ability of the reperfused vascular smooth muscle to contract remains unaltered; and 5) the ability of the

![FIGURE 7. Concentration–response curves to isoproterenol (−log M) in control (○) and chronically reperfused (□) coronary arterial rings without endothelium. Rings were contracted with prostaglandin F$_{2a}$ (2×10$^{-6}$ M). Values shown are % relaxation and are mean±SEM (n=6). LCX, left circumflex coronary artery; LAD, left anterior descending coronary artery.](image)

**TABLE 2. Concentration-Dependent Contractions to Potassium Chloride in Control and Chronically Reperfused Canine Coronary Arterial Rings**

<table>
<thead>
<tr>
<th>[K$^+$] (mM)</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>Maximal response (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With endothelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12±3</td>
<td>40±4</td>
<td>83±3</td>
<td>98±1</td>
<td>100</td>
<td>100</td>
<td>10±1</td>
</tr>
<tr>
<td>Reperfused</td>
<td>10±3</td>
<td>37±7</td>
<td>80±5</td>
<td>95±3</td>
<td>99±1</td>
<td>100</td>
<td>12±1</td>
</tr>
<tr>
<td>Without endothelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14±3</td>
<td>44±6</td>
<td>84±4</td>
<td>97±2</td>
<td>99±1</td>
<td>100</td>
<td>9±1</td>
</tr>
<tr>
<td>Reperfused</td>
<td>10±3</td>
<td>35±8</td>
<td>77±7</td>
<td>95±3</td>
<td>98±1</td>
<td>100</td>
<td>8±1</td>
</tr>
</tbody>
</table>

Values shown are percent of maximal response to K$^+$ (40 mM) and are mean±SEM (n=6).

*Increase in concentration of K$^+$ above control solution.

**TABLE 3. Concentration-Dependent Contractions to Prostaglandin F$_{2a}$ in Control and Chronically Reperfused Canine Coronary Arterial Rings**

<table>
<thead>
<tr>
<th>Prostaglandin F$_{2a}$ (−log M)</th>
<th>7.5</th>
<th>7</th>
<th>6.5</th>
<th>6</th>
<th>6.5</th>
<th>5</th>
<th>Maximal response (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With endothelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>3±2</td>
<td>20±4</td>
<td>53±5</td>
<td>85±3</td>
<td>100</td>
<td>8±1</td>
</tr>
<tr>
<td>Reperfused</td>
<td>0</td>
<td>3±1</td>
<td>17±2</td>
<td>50±3</td>
<td>85±2</td>
<td>100</td>
<td>8±1</td>
</tr>
<tr>
<td>Without endothelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>3±1</td>
<td>18±3</td>
<td>52±5</td>
<td>8±2</td>
<td>100</td>
<td>8±1</td>
</tr>
<tr>
<td>Reperfused</td>
<td>0</td>
<td>3±1</td>
<td>18±4</td>
<td>56±7</td>
<td>81±4</td>
<td>100</td>
<td>7±1</td>
</tr>
</tbody>
</table>

Values shown are percent of maximal response to prostaglandin F$_{2a}$ (10$^{-7}$ M) and are mean±SEM (n=5).
reperfused artery to relax to either one endothelium-derived relaxing factor (nitric oxide) or isoproterenol remains unaltered.

Short-term ischemia followed by 60 minutes of reperfusion results in general impairment of endothelium-dependent responses,1,14,15 similar to that seen in atherosclerosis.16,17 However, in the weeks after the reperfusion episode, the endothelial dysfunction changes from a general to a selective impairment. This profile is similar to that with regenerated endothelium.8 As documented by electron microscopy, it has been reported that immediately after reperfusion, widespread injury of the coronary endothelium occurs, which results in the general impairment of endothelial function while leaving smooth muscle function unaltered.15 However, in the following weeks, the injured endothelial cells are most likely replaced by regenerated endothelium. Regenerated endothelium typically exhibits impaired relaxation responses to aggregating platelets because of a decreased release of endothelium-derived relaxing factor to platelet products.8

In the canine coronary artery, ADP (and ATP) and serotonin9,18 are platelet products that mediate endothelium-dependent relaxation. In this species, the adenosine nucleotides apparently exert the key role in mediating endothelium-dependent relaxation to aggregating platelets.12 Twelve weeks after reperfusion, endothelium-dependent relaxations mediated by both platelet products are impaired, which most likely explains the altered response to aggregating platelets. This finding differs from those observed in porcine arteries with regenerated endothelium, in which the altered endothelium-dependent responses to serotonin are the primary abnormality that underlies the abnormal response to platelets.8

The impaired endothelium-dependent relaxations to ADP and serotonin in the chronically reperfused coronary arteries could be due to altered properties of the serotonergic and purinergic receptors, respectively, on the endothelial cell. These effects would have to involve selective dysfunction of receptors, as endothelium-dependent relaxations to acetylcholine remain unaltered. A decreased ability to produce and/or release endothelium-derived relaxing factor is unlikely, as the relaxations to acetylcholine are unaltered in chronically reperfused vessels. The altered response cannot be attributed to changed sensitivity to endothelium-derived relaxing factor. Indeed, the vascular smooth muscle of the reperfused arteries relaxed normally to acetylcholine and to exogenous nitric oxide (which has been identified as the major component of endothelium-derived relaxing factor.13,19-21) In addition, relaxations to isoproterenol are also unaltered, while contractions to KCl (voltage dependent) and PGI2 (receptor operated) remain normal.

Clinical Implications

Aorta-coronary bypass surgery, percutaneous transluminal balloon angioplasty, and thrombolytic therapy are currently used to restore blood flow to ischemic myocardium. If these interventions were to cause reperfusion injury to the coronary endothelium, the present study indicates that the reperfused vessel would retain "memory" of the initial insult by a decreased release of endothelium-derived relaxing factor in response to aggregating platelets. This could favor platelet adhesion in the reperfused vessel, leading to aggregation and platelet-induced contraction of the coronary smooth muscle, favoring coronary thrombosis.

Acknowledgments

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**KEY WORDS**  •  thrombins  •  vasospasm  •  thrombosis  •  ischemia
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