Vasoconstrictor Potential of Coronary Aspirate From Patients Undergoing Stenting of Saphenous Vein Aortocoronary Bypass Grafts and Its Pharmacological Attenuation

Petra Kleinbongard, Dirk Böse, Theodor Baars, Stefan Möhlenkamp, Thomas Konorza, Sandra Schöner, Miriam Elter-Schulz, Holger Eggebrecht, Hubertus Degen, Michael Haude, Bodo Levkau, Rainer Schulz, Raimund Erbel, Gerd Heusch

Rationale: Stent implantation into atherosclerotic plaques releases, apart from particulate debris, soluble substances that contribute to impaired microvascular perfusion.

Objective: To quantify the release of vasoconstrictors and to determine the efficacy of coronary dilators to attenuate their action.

Methods and Results: Using a distal protection/aspiration device, coronary arterial blood was retrieved before and during stenting in 22 patients with severe saphenous vein aorto-coronary bypass stenoses. The release of catecholamines, endothelin, serotonin, thromboxane B2, and tumor necrosis factor (TNF)α was measured. The response of rat mesenteric arteries with intact (+E) and denuded (−E) endothelium to aspirate plasma was normalized to that by KCl. Responses to selective receptor blockade, adenosine, nitroprusside, and verapamil against the aspirate-induced constriction were determined. The coronary arterial plasma withdrawn before stenting induced 21.5% and the aspirate plasma after stenting induced 95.8% of maximum KCl-induced vasoconstriction. Serotonin, thromboxane B2, and TNFα release into aspirate plasma increased by 1.9±0.1 μmol/L, 25.6±3.1 pg/mL, and 19.7±6.1 pg/mL, respectively, during stenting. The aspirate-induced vasoconstriction was largely antagonized by selective serotonin receptor blockade, with little further antagonism by additional thromboxane receptor blockade. TNFα did not induce constriction per se but potentiated the constriction with serotonin and the thromboxane-analog U-46619 in arteries +E. The concentrations to induce half-maximal vasodilation were comparable for nitroprusside (+E, 3.9×10⁻⁸; −E, 1.9×10⁻⁸ mol/L) and verapamil (+E, 8.3×10⁻⁸; −E, 7.8×10⁻⁸ mol/L), and the vasoconstriction was eventually eliminated. The vasodilator response to adenosine was dependent on functional endothelium and weaker.

Conclusion: Serotonin is the main coronary vasoconstrictor after stenting, and thromboxane and TNFα somewhat potentiate the serotonin response. Nitroprusside and verapamil are more potent than adenosine to attenuate the aspirate plasma-induced vasoconstriction, and they are not dependent on functional endothelium. (Circ Res. 2011;108:00-00.)

Key Words: coronary disease ▪ ischemia ▪ reperfusion ▪ vasoconstriction ▪ vasodilation

Early reperfusion is mandatory to salvage ischemic myocardium from infarction but also imposes additional reperfusion injury.1 The no-reflow phenomenon is a characteristic manifestation of reperfusion injury.2,3 A no-reflow phenomenon is observed in 30% of patients with an acute myocardial infarction and carries an adverse prognosis.4–6 A no-reflow phenomenon is also seen in 0.2% to 6% of patients undergoing an elective percutaneous coronary intervention (PCI) and again carries an adverse prognosis.5,7 With PCI for a saphenous vein aortocoronary bypass graft stenosis without protection, a no-reflow phenomenon is even seen in 14% to 42% of patients.8–10

The causal relation between infarction and the no-reflow phenomenon is not entirely clear. No-reflow within infarcted myocardium can result from structural and functional damage to the microcirculation.3 Vice versa, physical and functional...
obstruction of the coronary microcirculation after microembolization of debris and release of soluble vasoconstrictor substances from the ruptured atherosclerotic plaque contribute to the loss of viable myocardium. Protection devices are used to prevent debris and vasoconstrictor substances from being washed into the coronary microcirculation during PCI, but apart from the reduction of thrombus burden by thrombolysis or aspiration in acute myocardial infarction, their clinical benefit has been modest at best. Therefore, the prevention of coronary microcirculatory impairment by vasodilator drugs is still an attractive target.

We have now taken advantage of the use of an aspiration device during stenting of saphenous vein aortocoronary bypass grafts and analyzed the aspirate plasma, which in-stent bioassay, for its soluble vasoconstrictor, thrombogenic, bypass grafts and analyzed the aspirate plasma, which in-stent bioassay, for its soluble vasoconstrictor, thrombogenic, and inflammatory constituents. Of note, we have also studied the potential pharmacological antagonism of the observed vasoconstriction with the aim of developing a more rational treatment of the no-reflow phenomenon.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Patients**

Twenty-two symptomatic patients (20 men, 2 women; age, 68 ± 4 years) with a significant diameter stenosis (>75%) in a saphenous vein aortocoronary bypass graft were studied. All patients were on aspirin (100 mg/d) and received 10 000 IU of heparin intravenously. Coronary angiography was performed via the femoral approach. Quantification of stenosis severity was performed with the use of off-line caliper measurements (QCA-MEDIS, Leiden, The Netherlands). Thrombolysis in myocardial infarction (TIMI) flow was determined before and after each intervention. Saphenous vein graft age, target vessels, laboratory blood analysis, comorbidities, and current medications are presented in Online Table I. The investigation conforms to the principles outlined in the Declaration of Helsinki. The study protocol was approved by the local Institutional Review Board of the University of Essen School of Medicine (GZ.: 05-2807). Full informed consent was obtained from all patients before participating in the study.

**Interventional Procedure**

The implantation of balloon-expandable bare-metal stents (Bx Sonic, Cordis Corp, Miami, FL; Genius Magic, Eurocor GmbH, Bonn, Germany; Lekton, Biotronic SE & CoKG, Berlin, Germany) was performed using maximum balloon pressures of >14 atm and a balloon-to-vessel diameter ratio of 1:1. To prevent microembolization, a distal balloon occlusion flushing/extraction device (TriAktiv SVG/3.5-FX-catheter; Kensey Nash, Exton, PA) was used. The balloon guide wire was placed distal to the lesion and inflated at 2 atm with carbon dioxide. After stent implantation the interventional catheter was removed, and the LFX catheter was loaded on the guide wire. During slow withdrawal of this catheter, the stented area was flushed with saline and the blood–saline mixture was retrieved. Then, the balloon was deflated.

**Blood Samples**

Arterial blood was taken via the flushing/extraction catheter (10 mL into potassium EDTA S-Monovette, SARSTEDT AG & Co, Nümbrecht, Germany) distal to the lesion before the intervention. Aspirate (20 mL) was obtained during the intervention, diluted with saline to an approximately blood/saline-ratio ~1:2 vol/vol and filtered ex vivo through a 40-μm mesh filter. The aspirate dilution was corrected for by reference to the hematocrit. In each instance, visible particulate debris was retained on the filter. The filtered aspirate and the arterial blood were immediately centrifuged (600g, 10 minutes, 4°C), and the plasma was removed, quickly frozen in liquid nitrogen, and stored at ~80°C until further use. Troponin I was determined in venous plasma before the intervention and at 6, 12, 24, 36, and 48 hours after the intervention using a specific 2-side immunoassay (Dimension Flex, Dade Behring GmbH, Marburg, Germany). The detection range of troponin I was 0.04 to 40 ng/mL; the upper limit of normal was 0.1 ng/mL. Plasma aliquots from healthy volunteers were also frozen in liquid nitrogen and stored at ~80°C.

**Plasma Concentration of Vasoconstrictors, Tissue Factor, and Tumor Necrosis Factor α**

The plasma concentration of several vasoconstrictors in coronary arterial blood and aspirate was measured: epinephrine, norepinephrine, and serotonin were determined using high-performance liquid chromatography and electrochemical detection (Chromsystems, München, Germany). Endothelin and thromboxane (Tx)B2 were measured using angiotensin-converting enzyme immunoassays (Cayman Chemical Company, Ann Arbor, MI). Tissue factor was measured using IMUBIND Tissue Factor Elisa Kit (American Diagnostica Inc, Stamford, CT). Tumor necrosis factor (TNF)-α was determined using a sandwich enzyme immunoassay (Quantikine Human TNF-α/TNFSF1A, R&D Systems, Abingdon, UK). For details, see the Online Data Supplement.

**Vasomotor Bioassay**

Human coronary arteries and rat mesenteric arteries are characterized by a comparable receptor arrangement for adenosine, epinephrine, norepinephrine, serotonin, and TxA2. Therefore, we used rat mesenteric arteries to characterize the vasomotor response to calcium and aspirate plasma and to subsequent vasodilators.

Male Lewis rats were euthanized after enflurane-inhalation anesthesia by rapidly removing the heart, in accordance with the German laws for animal welfare and approved by the local review committee. The mesenteric arteries were dissected and perfused with saline at 60 mm Hg; their diameter was 152 ± 15 μm on light microscopic examination (n = 16). Segments of 2-mm length were mounted with intact endothelium (+E) and after mechanical removal of the endothelium (−E) into the Mulvany myograph (Danish Myo Technology, Aarhus, Denmark). Maximal vasoconstriction was defined by the response to potassium chloride (KCl; 1.2 × 10−3 mol/L). Subsequently, arteries were washed and rechallenged with norepinephrine (10−5 mol/L) and carbobachol (10−4 mol/L) to verify a strong agonist response and endothelial functionality, as previously described. For details, see the Online Data Supplement.
Experimental Protocols

Rat mesenteric arteries (+E and −E) were incubated with coronary arterial and aspirate plasma. Coronary arterial and aspirate plasma were diluted to a final ratio of 1:10 vol/vol each. The contribution of serotonin and/or TxA2 to the vasoconstriction by aspirate plasma was determined by use of selective blockade of serotonin receptors by ketanserin (5-hydroxytryptamine [5-HT]2A/2C receptor blocker with α1-adrenoceptor blocker effects) and pindolol (β-adrenoceptor blocker with 5-HT1A/1B receptor blocker effects) and of TxA2 receptors by ICI 185,282 (TxA2 receptor blocker) and their combination (each 10−5 to 10−3 mol/L).20,21

To characterize the concentration-dependent actions of serotonin and thromboxane, arteries were exposed to plasma of healthy volunteers and vasoconstriction was then determined after addition of serotonin and U-46619 (the metabolically stable analog of thromboxane A2) (each 10−8 to 10−4 mol/L). In a subset of experiments, we determined the impact of TNAα on the vasoconstriction by serotonin and U-46619. Arteries were exposed to plasma of healthy controls supplemented with a combination of serotonin (3.5 pg/mL) and U-46619 (50 pg/mL) with and without TNAα (50 pg/mL).

Table 1. Baseline and Postinterventional Concentrations of Vasoconstrictors, Tissue Factor, and TNAα

<table>
<thead>
<tr>
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<th>n</th>
<th>Coronary Arterial Plasma</th>
<th>Coronary Aspirate Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelin (pg/mL)</td>
<td>17</td>
<td>1.4±0.3</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>Catecholamines (pg/mL)</td>
<td>10</td>
<td>100.5±28.8</td>
<td>89.8±19.5</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>10</td>
<td>451.7±64.6</td>
<td>479.8±71.3</td>
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<tr>
<td>Norepinephrine</td>
<td>15</td>
<td>7.9±1.7</td>
<td>33.4±3.4*</td>
</tr>
<tr>
<td>Tissue factor (pg/mL)</td>
<td>15</td>
<td>351.4±24.7</td>
<td>367.6±27.5</td>
</tr>
<tr>
<td>TNAα (pg/mL)</td>
<td>10</td>
<td>9.0±1.4</td>
<td>28.7±7.1*</td>
</tr>
</tbody>
</table>

Values are means±SEM. Statistical comparison by paired t test. *P=0.05 coronary arterial plasma vs coronary aspirate plasma.

Statistical Analysis

All data are given as the means±SEM. Troponin I data and the release of substances into the coronary aspirate were evaluated with a paired t test. Statistical comparisons of concentration–response curves were made by 2-way ANOVA followed by Bonferroni post hoc tests to assess differences between treatment groups (SigmaStat 2.03, SPSS Inc, Chicago, IL). In addition, concentration–response curves were analyzed in terms half-maximal effective concentration (EC50) after least-square sigmoidal curve fitting of individual curves using Origin 7G SR2 (Northampton, MA). Probability values of less than 0.05 were considered statistically significant.

Results

In all patients, we retrieved an aspirate of 77±4 mL (after correction for dilution by the hematocrit). In each instance, particulate debris was retained on the ex vivo filter. TIMI flow was I in 3 and II in 19 patients before the intervention, and was II in 3 and III in 19 patients after the intervention. Troponin I was 0.049±0.02 ng/mL before and 0.16±0.02 ng/mL (P<0.05) at its maximum after the intervention. Troponin I exceeded the proposed cutoff level of 0.15 ng/mL23 to reflect myonecrosis in 6 of 22 patients. No patient experienced no-reflow; without protection, 14% to 42% of patients undergoing PCI of a saphenous vein graft are expected to develop no-reflow.8–10

Kleinbongard et al Vasoconstrictor Potential of Coronary Aspirate 3

The dilator responses of mesenteric arteries to adenosine, nitroprusside, and verapamil were determined after constriction by aspirate plasma. When a plateau of constriction had been reached, dilation was induced by adding cumulatively increasing concentrations of adenosine, nitroprusside, or verapamil (each 10−9 to 10−4 mol/L). Dilator responses were expressed as percentage of the maximum vasoconstriction induced by the coronary aspirate.
Release of Vasoconstrictors, Tissue Factor, and TNFα Into the Aspirate

The concentrations of endothelin, epinephrine, norepinephrine, and tissue factor were not different before and after stent implantation (Table 1). Coronary aspirate plasma levels of serotonin, TxB2, and TNFα increased by 1.9 ± 0.2 μmol/L, 25.6 ± 3.1 pg/mL, and 19.7 ± 6.1 pg/mL, respectively, during stent implantation (Table 1).

Vasoconstriction by Coronary Arterial and Aspirate Plasma

Coronary arterial plasma withdrawn distal to the lesion before stent implantation induced a vasoconstriction which amounted to 21 ± 5% (+E) and 34 ± 8% (−E) of that by KCl (100%). The vasoconstriction of aspirate plasma, however, was 95 ± 8% (+E) and 101 ± 9% (−E) of that by KCl (100%). The extent of vasoconstriction was independent from the integrity of the endothelium (Figure 1). Arteries constricted immediately after the exposure to aspirate plasma, and a stable maximum was reached within 2 to 4 minutes. Representative original registrations of constriction in response to coronary arterial and aspirate plasma are presented in Figure 1A and 1B.

Potentiation of Vasoconstriction by TNFα

The rat mesenteric arteries ex vivo constricte in response to plasma supplemented with serotonin and the TxA2 analog U-46619 (Figure 2; Online Figure III); this vasoconstriction was weaker in vessels with (+E, 85 ± 4%) than without (−E, 124 ± 5%) endothelium. The vasoconstriction in vessels +E was potentiated by TNFα (111 ± 8%) but not that in vessels −E (118 ± 3%), indicating that TNFα per se does not induce vasoconstriction (Figure 2).

Antagonism of Vasoconstriction by Selective Blockade of Serotonin and Thromboxane Receptors

In aspirate plasma-constricted arteries, selective serotonin receptor blockade dose-dependently attenuated vasoconstriction in the presence and absence of endothelium and abolished vasoconstriction at the highest concentration. In contrast, selective thromboxane receptor blockade only somewhat attenuated the aspirate plasma-induced vasoconstriction. Combined blockade of serotonin and thromboxane receptors was somewhat more potent than serotonin receptor blockade alone (Figure 3 and Table 2). Likewise, exogenous serotonin at the concentration observed in the retrieved aspirate plasma induced maximum vasoconstriction (118% +E and 141% −E of KCl-induced vasoconstriction); the TxA2 analog U-46619 at the concentration of TxB2 observed
in aspirate plasma induced no vasoconstriction (Online Figure III).

Antagonism of Vasoconstriction by Adenosine, Nitroprusside, and Verapamil
In aspirate plasma-constricted arteries, both nitroprusside and verapamil concentration-dependently and endothelium-independently attenuated vasoconstriction and virtually abolished it at their highest concentrations. In contrast, the effect of adenosine was endothelium-dependent and weaker than that for nitroprusside and verapamil (Figures 4 and 5 and Table 3). Representative original registrations of constriction in response to aspirate plasma and the following attenuation of the vasoconstriction by adenosine, nitroprusside, and verapamil are presented in Online Figure IV.

Discussion
The present study confirms prior studies by others24–26 and ourselves13 on the release of serotonin from stented coronary vessels, but extends those studies and provides significant novel information:

- We measured biochemically the release of a number of mediators; excluded a role for catecholamines, endothelin, and tissue factor in the observed vasoconstriction; defined the quantitative contribution of serotonin and thromboxane (which only potentiates the serotonin response) by selective pharmacological blockade; and demonstrated a facilitatory role for TNFα through impairment of endothelial function.

- Of note, and of clinical relevance, we report the largely different potency of prototype pharmacological vasodilators, notably the inability of adenosine, to attenuate the coronary aspirate-induced vasoconstriction.

Release of Vasoconstrictor, Thrombogenic, and Inflammatory Factors During Plaque Rupture
In the present study, we have taken advantage of the clinical situation of patients undergoing interventional atherosclerotic plaque rupture. During stenting of stenotic saphenous vein aortocoronal bypass grafts, we retrieved the aspirate from a protection device and identified the release of vasoconstrictor (serotonin, thromboxane), thrombogenic (thromboxane), and inflammatory (TNFα) substances. The atherosclerotic plaque composition of saphenous vein grafts differs from that of native vessels,27–29 and saphenous vein grafts are particularly vulnerable to plaque rupture.30 Also, we do not know to what extent the traumatic PCI-induced plaque rupture mirrors the spontaneous plaque rupture during acute myocardial infarction. Still, we identified the release of substances that impair myocardial perfusion, and the no-reflow phenomenon is a characteristic complication of

<table>
<thead>
<tr>
<th>Receptor Blocker and Endothelium</th>
<th>EC50 (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketanserin + pindolol + E</td>
<td>2.5×10^-8 ± 2.2×10^-9</td>
</tr>
<tr>
<td>Ketanserin + pindolol - E</td>
<td>1.3×10^-7 ± 1.6×10^-8</td>
</tr>
<tr>
<td>ICI 185,282 + E</td>
<td>1.1×10^-7 ± 4.5×10^-8</td>
</tr>
<tr>
<td>ICI 185,282 - E</td>
<td>1.0×10^-7 ± 1.4×10^-7</td>
</tr>
<tr>
<td>Ketanserin + pindolol + ICI 185,282 + E</td>
<td>8.3×10^-9 ± 1.6×10^-9</td>
</tr>
<tr>
<td>Ketanserin + pindolol - ICI 185,282 - E</td>
<td>7.2×10^-9 ± 2.5×10^-9</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
both PCI (particularly of saphenous vein grafts) and acute myocardial infarction.

**Vasoconstrctor Potential of Coronary Aspirate**
Baseline concentrations of arterial plasma catecholamines were elevated, probably reflecting the stress situation of PCI. However, there was no net release of catecholamines into the aspirate during PCI. Also, there was no net release of endothelin and tissue factor during the elective PCI in our study. In contrast, net endothelin release into the aspirate has been reported in primary PCI after acute myocardial infarction.31,32 Also, net release of tissue factor into the aspirate has been reported during acute coronary syndromes.33,34 It is unclear to what extent the different underlying atherosclerotic substrates (saphenous vein graft vs native vessel), the nature of plaque rupture (traumatic vs spontaneous), or the interaction with thrombotic processes in the acute coronary syndromes contributes to such differences in endothelin and tissue factor release.

We confirmed the net release of serotonin and thromboxane into the aspirate as in our prior study, where we demonstrated that the aspirate-induced vasoconstriction is eliminated by combined pharmacological antagonism of serotonin and thromboxane, respectively, to the vasoconstriction by the aspirate. Serotonin was largely responsible for the observed vasoconstriction, and thromboxane only contributed by potentiating the serotonin-induced response, as evidenced by the slight leftward shift of the concentration–response curve with combined serotonin and thromboxane blockade as compared to serotonin blockade alone.

Somewhat surprisingly, there was net thromboxane release, although all patients received 100 mg of aspirin daily. However, thromboxane is not only synthesized in platelets, but also in monocytes/macrophages and in vascular cells via cyclooxygenase (COX)-2. Although platelets are persistently activated by aspirin, the nucleated cells can rapidly recover from the aspirin-dependent irreversible inhibition of COX-2 activity through de novo synthesis of COX-2 in response to inflammatory and mitogenic stimuli, especially in symptomatic atherosclerotic plaques.35–37 Also, we cannot exclude an insufficient suppression of platelet TXA2 production in some of our patients.38

We also confirmed the release of the TNFα into the aspirate.14 TNFα is a proinflammatory cytokine that impairs endothelium-dependent vasodilation.39 Such impairment of endothelium-dependent vasodilation is of particular importance in the coronary circulation during myocardial ischemia/reperfusion.40–42 In the present study, we demonstrated that TNFα potentiates the vasoconstriction induced by the combination of serotonin and thromboxane. Because the potentiation of serotonin/thromboxane-induced vasoconstriction by TNFα was only seen in the presence of intact endothelium, such apparent potentiation most likely results from the elimination of the endothelium-dependent dilation by serotonin after 5HT1B receptor stimulation.43

**Pharmacological Attenuation of Vasoconstriction**
European Society of Cardiology guidelines for acute myocardial infarction44 and PCI45 recommend the use of vasodilators to treat no-reflow, notably that of adenosine, nitroprusside, and verapamil. However, in the present study, adenosine was not as potent to attenuate the aspirate-induced vasoconstriction as nitroprusside or verapamil. The failure of adenosine to

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**Table 3. EC50 of Vasodilators to Attenuate Coronary Aspirate Plasma-Induced Vasoconstriction**

<table>
<thead>
<tr>
<th>Vasodilators and Endothelium</th>
<th>EC50 (mol/L)</th>
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<tbody>
<tr>
<td>Adenosine</td>
<td></td>
</tr>
<tr>
<td>+E</td>
<td>5.4×10^{-8} ± 2.4×10^{-8}</td>
</tr>
<tr>
<td>-E</td>
<td>1.3×10^{-8} ± 6.3×10^{-6}</td>
</tr>
<tr>
<td>Nitroprusside</td>
<td></td>
</tr>
<tr>
<td>+E</td>
<td>3.3×10^{-8} ± 4.8×10^{-9}</td>
</tr>
<tr>
<td>-E</td>
<td>1.9×10^{-8} ± 2.6×10^{-9}</td>
</tr>
<tr>
<td>Verapamil</td>
<td></td>
</tr>
<tr>
<td>+E</td>
<td>8.3×10^{-8} ± 4.6×10^{-9}</td>
</tr>
<tr>
<td>-E</td>
<td>7.8×10^{-8} ± 4.2×10^{-9}</td>
</tr>
</tbody>
</table>

Values are means±SEM. Statistical comparison by 2-way ANOVA with Bonferroni correction. *P<0.05 vs adenosine+endothelium.
fully antagonize the aspirate-induced vasoconstriction in our study is not related to species, organ of origin, or size of our bioassay vessels, because human coronary small arteries/arterioles of the same diameter range (140 μm) have quantitatively similar dilator responses to adenosine as our rat mesenteric bioassay vessels. Adenosine often fails to induce maximal coronary dilation also in patients with coronary artery disease. Very recently, intracoronary high-dose adenosine also failed to attenuate microvascular obstruction in patients with acute myocardial infarction undergoing primary PCI.

In contrast to adenosine, both nitroprusside and verapamil dose-dependently and eventually fully attenuated the aspirate-induced vasoconstriction, and the dilator action was not endothelium-dependent. The functional antagonism of the aspirate-induced vasoconstriction by nitroprusside and verapamil was as potent as the competitive antagonism for both serotonin at 5HT2A/C receptors and 5HT1A/B receptors and TxA2 at thromboxane–prostanoid receptors in our prior study. Both nitroprusside and verapamil have been clinically used to potentially reverse no-reflow. Intracoronary nitroprusside has the advantage of having no hemodynamic side-effects. In contrast, intracoronary verapamil can cause bradyarrhythmias, but it may also attenuate stunning and contribute to a reduction of infarct size. Also, the half-life of verapamil is longer, and it does not require continuous intracoronary infusion.

In conclusion, we have identified the release of serotonin, thromboxane, and TNFα during stenting of saphenous vein aortocoronary bypass grafts. The vasconstrictor activity of serotonin and thromboxane is fully attenuated by nitroprusside or verapamil but not by adenosine. The action of nitroprusside and verapamil is not dependent on functional endothelium and therefore expected to functionally antagonize microvascular constriction also in patients with endothelial dysfunction.

Study Limitations

Our study is limited to a small number of patients undergoing elective PCI of saphenous vein bypass grafts, and further studies on a larger scale and with a focus on the coronary microcirculation are needed. Also, our study used rat mesenteric microvessels as bioassay. Although rat mesenteric microvessels share essential properties with human coronary microvessels, further studies with an isolated heart bioassay system are warranted to study the impact of coronary aspirate on the coronary microcirculation and on cardiac contraction.

Sources of Funding

None.

Disclosures

None.

References


Novelty and Significance

What Is Known?

- Particulate debris from ruptured coronary atherosclerotic plaques embolizes into the microcirculation.
- Release of vasoconstrictor, thrombogenic, and inflammatory substances also contributes to coronary microvascular flow impairment.

What New Information Does This Article Contribute?

- Stent implantation in patients with saphenous vein graft stenosis causes release of serotonin, thromboxane and tumor necrosis factor (TNFα) into the coronary arterial blood.
- Serotonin causes vasoconstriction in a rat mesenteric bioassay, which is potentiated by thromboxane and TNFα.
- Nitroprusside and verapamil, but not adenosine, effectively antagonize such vasoconstriction.

The spontaneous or periinterventional rupture of an atherosclerotic plaque in an epicardial coronary artery does not always result in complete thrombotic occlusion and impending myocardial infarction, but often some residual blood flow is retained and the atherosclerotic debris, together with superimposed thrombotic material, is dislodged into the coronary microcirculation, where it causes microinfarcts, along with an inflammatory reaction. Not only particulate debris but also soluble vasoconstrictor, thrombogenic, and inflammatory mediators are released during plaque rupture and contribute to microcirculatory impairment. The present study takes advantage of stent implantation in patients with severe saphenous vein bypass graft stenoses and analyzes the coronary aspirate retrieved with a protection device during stent implantation for its vasoconstrictor, thrombogenic, and inflammatory mediators. Serotonin is quantified as the main coronary vasoconstrictor, and its constrictor action is potentiated by thromboxane and TNFα in a rat mesenteric bioassay. Nitroprusside and verapamil, but not adenosine, can effectively antagonize the observed vasoconstriction. These data suggest use of nitroprusside or verapamil rather than adenosine to prevent/attenuate a no-reflow phenomenon.
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Circ Res. published online December 23, 2010;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplemental Data

Online Table I

Patient Characteristics

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<tr>
<td>obesity</td>
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<table>
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<th>laboratory analysis</th>
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<td>HDL cholesterol [mg/dl]</td>
<td>48 ± 6</td>
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<tr>
<td>LDL cholesterol [mg/dl]</td>
<td>101 ± 10 *</td>
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<tr>
<td>triglycerides [mg/dl]</td>
<td>212 ± 77 *</td>
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<tr>
<td>creatinine [mg/dl]</td>
<td>1 ± 1    *</td>
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<tr>
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<td>beta-blockers</td>
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<td>calcium antagonists</td>
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<td>phenprocoumon</td>
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<td>graft-age [years]</td>
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<tr>
<td>left anterior descending coronary artery</td>
<td>7</td>
</tr>
<tr>
<td>right coronary artery</td>
<td>9</td>
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mean±SEM

Chemicals and Drugs

Adenosine, carbachol, [-]-norepinephrine bitartrate, serotonin, sodium nitroferricyanide (III) dehydrate, and verapamil hydrochloride were purchased from Sigma, Deisenhofen, Germany. Ketanserin, pindolol and ICI 185,282 were purchased from Tocris Bioscience, Ellisville, USA. U-46619 was purchased from Merck, Darmstadt, Germany. Recombinant porcine TNFα was purchased from Thermo Fisher Scientific, Rockford, USA. All chemicals were of the purest grade commercially available.

Plasma Concentration of Vasoconstrictors, Tissue Factor, and TNFα

Epinephrine and Norepinephrine

Plasma concentrations of epinephrine and norepinephrine were determined by HPLC with electrochemical detection (EC 41,000 Chromsystems, München, Germany) using a kit and a reverse phase analytical column (Chromsystems, München, Germany) according to the manufacturer’s instructions. The internal standard 3,4-dihydroxybenzylamine was added to the plasma before analysis. After pretreatment with a sample clean-up column,
catecholamines were separated and quantified. This commercial method eluted norepinephrine, epinephrine, 3,4-dihydroxybenzylamine, and dopamine at 8, 9, 12–13, and 17–19 min, respectively. The working electrode potential was set at +0.5 V. The peaks were evaluated by integration (32 Karat™ Software 5.0, Beckmann Coulter, Krefeld, Germany). Sensitivity was <10 ng/l, the absolute recovery for epinephrine and norepinephrine was about 60–65% (Chromsystems, München, Germany).

**Serotonin**

The serotonin concentration in the plasma was measured by HPLC with electrochemical detection. To 200 µl plasma, 220 µl urea-saturated methanol containing 20 ng 2-methyl serotonin were added as an internal standard. The precipitated proteins were removed by centrifugation (15,000 g, 15 min, 4 °C), residual proteins were removed from the supernatant by ultrafiltration (AMicon Centricron YM, 30; Millipore Corporation, Billerica, USA ; 1,000 g, 60 min, 20 °C). Ultrafiltrated plasma (50 µl) was injected into a Nova Pac C18 column (3.69x150mm; Waters, Milford, USA). The mobile phase consisted of 150 mmol/l H₃PO₄/NaH₂PO₄, pH 3.5, 1 mmol/l EDTA, 5 mmol/l octane sulfonic acid sodium and 15% methanol, the flow was 1.2 ml/min. The potential of the detector (BAS, Bioanalytical Systems Warwickshire, UK) was set to 600 mV against Ag/AgCl, and the peaks were evaluated by integration (Maxima 200; Waters, Milford, USA). The serotonin recovery was determined from serotonin-spiked plasma samples (n=7) and was 97.7±10.8%.

**Endothelin**

The sum of the three known endothelin isopeptides in the plasma was detected using the immunometric endothelin assay kit (ACE™ enzyme immunoassay, Cayman Chemical Company, Ann Arbor, USA) following the manufacturer’s instructions. The immunometric assay is based on a double-antibody sandwich technique. The specific monoclonal antibody against endothelin 1, 2, and 3 is coated on the microtiter plate. An acetylcholinesterase:Fab’ conjugate binds selectively to an epitope of the endothelin molecule, which binds to the capture antibody and consequently to the microtiter plate. The concentration in the sample is determined by measuring the enzymatic activity of acetylcholinesterase by adding Ellmans reagent [acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid)]. Hydrolysis of acetylthiocholine by acetylcholinesterase produces thiocholine. The non-enzymatic reaction of thiocholine with 5,5'-dithio-bis-(2-nitrobenzoic acid) results in the product 2-nitro-5-thiobenzoate, which was determined spectrophotometrically at 412 nm (Microplate Reader 680, BIORAD, München, Germany). The absorption is directly proportional to the amount of bound conjugate, which in turn is proportional to the endothelin concentration. The plasma endothelin concentration was quantified by comparison to a standard curve.

**Tissue Factor**

To determine tissue factor in the plasma the IMUBIND Tissue Factor Elisa Kit was used, as described by the manufacturer (American diagnostica inc, Stanford, USA). Plasma samples were incubated in a microtiter plate precoated with a murine anti-human tissue factor monoclonal antibody. After binding of the tissue factor to this capture antibody, streptavidin-conjugated horseradish peroxidase was added and bound specifically to the antibody/antigen complex. After addition of the enzyme substrate 3,3',5,5'-tetramethylbenzidine, the substrate solution produced a blue byproduct. The color intensity is proportional to the amount of horseradish peroxidase activity, which in turn is related to the levels of bound tissue factor. After addition of sulfuric acid solution the color changes to yellow, enabling an accurate measurement of the color intensity at 450 nm using a spectrophotometer (Microplate Reader 680, BIORAD, München, Germany). The plasma tissue factor concentration was quantified by comparison to a standard curve.

**Thromboxane B₂**

TxA₂ is rapidly non-enzymatically hydrolyzed to form the stable and inactive metabolite TxB₂. Plasma TxB₂ thus gives an estimate of in vivo TxA₂ formation. TxB₂ was determined using the ACE™ enzyme immunoassay (Cayman Chemical Company, Ann Arbor, USA). In the fist step, TxB₂ binds specifically to an acetylcholinesterase conjugate, this conjugate then binds to TxB₂–specific rabbit antiserum binding sites. The complex of acetylcholinesterase–TxB₂–rabbit antiserum is finally bound to a mouse monoclonal anti-rabbit IgG which has been previously attached to the well. After washing and removing of any unbound reagents, Ellmans reagent [acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid)] was added.
Hydrolysis of acetylthiocholine by acetylcholinesterase produces thiocholine. The non-enzymatic reaction of thiocholine with 5,5′-dithio-bis-(2-nitrobenzoic acid) results in the product 2-nitro-5-thiobenzoate, which was determined spectrophotometrically at 412nm (Microplate Reader 680, BIORAD, München, Germany). The absorption is proportional to the amount of TxB2. The plasma TxB2 concentration was quantified by comparison to a standard curve.

**TNFα**

The concentration of TNFα in the plasma was determined using a sandwich enzyme immunoassay (Quantikine® Human TNF-α/TNFSF1A, R&D systems, Abingdon, UK). Standards and samples were added to a monoclonal anti-TNFα IgG which has been previously attached to the microplates. Following a wash to remove unbound substances, a horseradish peroxidase-linked polyclonal antibody was added. After addition of the enzyme substrate 3,3′,5,5′-tetramethylbenzidine, the substrate solution produces a blue byproduct. The color intensity is proportional to the amount of horseradish peroxidase activity, which in turn is related to the levels of bound TNFα. After addition of sulfuric acid solution the color changes to yellow, enabling an accurate measurement of the color intensity at 450 nm using a spectrophotometer (Microplate Reader 680, BIORAD, München, Germany). The plasma TNFα concentration was quantified by comparison to a standard curve.

**Vasomotor Bioassay**

Male Lewis rats (250 to 300 g body weight) were sacrificed by rapidly removing the heart during anesthesia with enflurane. Inhalation anesthesia was performed over 2 min in a glass chamber of 6 liter capacity with 5ml enflurane. The mesentery was immediately removed and transferred into carbogenated (5% CO2/95% O2) Krebs-Henseleit buffer (mmol/l: 119 NaCl, 4.7 KCl, 2.5 CaCl₂·x2 H₂O, 1.17 MgSO₄·x7H₂O, 25 NaHCO₃, 1.18 KH₂PO₄, 0.027 EDTA, 5.5 glucose). Arterial segments of 2mm length were carefully dissected and mounted in an isometric small vessel myograph (Danish Myo Technology, Aarhus, Denmark). Each preparation was measured with intact endothelium (+E) versus denuded endothelium (-E). The endothelium was mechanically removed by passing a cat whisker through the lumen. The vessels were threaded on two stainless steel wires, 40 µm in diameter, attached to a force transducer and a micrometer, respectively. In this way the myograph permitted the measurement of force and calculation of vessel wall tension while its internal diameter was controlled. Arteries were equilibrated for 20 min in oxygenated Krebs-Henseleit buffer warmed to 37 °C before an automated normalization procedure was performed. This normalization is controlled from the interface using a standardized procedure according to the manufacturer’s protocol. The normalization uses an approximation of the lumen diameter (d₁₀₀) which the artery would have had in vivo, when relaxed and subject to a transmural pressure of 100 mmHg, and the Laplace law for vessels with infinitely thin walls: P=2T/d, where P is transmural pressure, T is wall tension and d is lumen diameter. The arteries were then set to a lumen diameter d=0.9 x d₁₀₀ where active force development is maximal. Contraction was measured as active wall tension (mN), the active force was divided by twice the vessel segment length (AD converter: PowerLab8/30, software: LabChart6, ADInstruments GmbH, Spechbach, Germany). The vessels were equilibrated for a further 30 min with frequent buffer changes before challenge with KCl (1.2*10⁻¹ mol/l) to define maximum vasoconstriction as that by depolarization of the vascular smooth muscle cells. The vasoconstrictor response to KCl was comparable for all experimental groups (Online Figure I).
Supplemental Data CIRCRESAHA/2010/235713/R2

**Online Figure I.** Mean±SEM of maximal vasoconstrictor response to KCl of rat mesenteric arteries, which were subsequently used to characterize vasoconstriction by serotonin (S), the TxA2 analogue U-46619 (U), and the combination of serotonin + U-46619 with and without TNFα (T) added to plasma of healthy volunteers, to determine serotonin-receptor blockade (ketanserin and pindolol, K/P), thromboxane-receptor blockade (ICI 185,282, I) and the combination of both (K/P/I), and to determine the dilator responses to adenosine (A), nitroprusside (N), and verapamil (V) after constriction by aspirate plasma, respectively.

Subsequently, arteries were washed (3 times over 15 min) and re-challenged with norepinephrine (10⁻⁵ mol/l) and carbachol (10⁻⁴ mol/l) to verify a strong agonist response and endothelial functionality (differentiation of vessels with intact endothelium (+E) versus denuded endothelium (-E)). Endothelial integrity was defined by vasodilation to carbachol by ≥60% of the norepinephrine-induced preconstriction amplitude. Successful endothelial removal was confirmed by lack of vasodilator response (by ≤10% of the preconstriction amplitude) to carbachol. Vessels with endothelium, but with a partial relaxation or no relaxation (by <60% of the preconstriction amplitude), or vessels with an incomplete denudation of the endothelium (dilation by >10% of the preconstriction amplitude preserved) were discarded. Endothelial functionality was comparable between the experimental groups (Online Figure II).
Online Figure II. Mean±SEM of dilator response to carbachol of rat mesenteric arteries, which were subsequently used to characterize vasoconstriction by serotonin (S), the TxA2 analogue U-46619 (U), and the combination of serotonin + U-46619 with and without TNFα (T) added to plasma of healthy volunteers, to determine serotonin-receptor blockade (ketanserin and pindolol, K/P), thromboxane-receptor blockade (ICI 185,282, I) and the combination of both (K/P/I), and to determine the dilator responses to adenosine (A), nitroprusside (N), and verapamil (V) after constriction by aspirate plasma, respectively.

Experimental Protocols
After this stimulation procedure, the vessels were rinsed 3 times with fresh buffer and left to recover at baseline for about 15 min.

In a subset of experiments, rat mesenteric arteries were exposed to plasma of healthy volunteers. Concentration-dependent actions of serotonin and U-46619 (the metabolically stable analogue of TxA2) (each 10^{-10} to 10^{-5} mol/l) were then determined (n=4) (Online Figure III).

Online Figure III. Vasoconstriction of rat mesenteric arteries with intact (+E) and denuded endothelium (-E) induced by serotonin (A) and the TxA2 analogue U-46619 (B). Highlighted are the serotonin and TxB2 concentrations observed in aspirate plasma. Values are mean±SEM for n=4 each.
Preconstriction of mesenteric arteries by aspirate plasma after stent implantation was used for the determination of dilator responses to adenosine, nitroprusside, and verapamil, subsequently. Representative original registrations of constriction by aspirate plasma and the following concentration-dependent attenuation of the vasoconstriction by adenosine, nitroprusside, and verapamil are presented in Online Figure IV.

**Adenosine**

![Adenosine graph]

**Nitroprusside**

![Nitroprusside graph]

**Verapamil**

![Verapamil graph]

**Online Figure IV.** Original registrations of rat mesenteric artery (with and without endothelium): Vasodilator responses to adenosine, nitroprusside and verapamil against the constriction induced by coronary aspirate plasma.

After each experimental setup, arteries were washed and re-challenged with norepinephrine ($10^{-5}$ mol/l) and carbachol ($10^{-4}$ mol/l) to again verify the agonist response and endothelial functionality. The arterial responses to norepinephrine and carbachol were comparable between the begin and the end of all experiments.

**References**