Thrombin-Induced Endothelium-Dependent Inhibition and Direct Activation of Platelet–Vessel Wall Interaction
Role of Prostacyclin, Nitric Oxide, and Thromboxane A₂

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Background Platelet–vessel wall interaction plays an important role in acute cardiovascular disorders. Thrombin is a potent platelet activator but also has profound effects on the endothelium. Endothelial cells possess antithrombotic activity by releasing nitric oxide and prostacyclin, both potent vasodilators and platelet inhibitors. We studied the role of thrombin as a regulator of platelet–vessel wall interaction in isolated human arteries suspended in organ chambers for isometric tension recording.

Methods and Results In arteries with endothelium, thrombin (0.01 to 1 U/mL) induced endothelium-dependent relaxations, which were reduced by the nitric oxide synthase inhibitor N⁶-nitro-L-arginine methyl ester (L-NAME; 10⁻⁴ mol/L) and/or indomethacin (10⁻⁵ mol/L). Human platelets (75 000/μL) evoked only marginal contractions in arteries with endothelium (3±5% of the contraction to KCl 100 mmol/L; NS), which were markedly enhanced by endothelial removal (22±4%; P<.05). Thrombin (1 U/mL) did not affect the response to platelets in arteries with (6±5%; NS) but induced a huge contraction in rings without endothelium (53±6%; P<.01 versus control without endothelium). The potent contraction to thrombin-activated platelets (1000 to 75 000/μL) in arteries without endothelium was markedly inhibited by the thromboxane A₂ synthetase/receptor antagonist ridogrel (10⁻⁵ mol/L; P<.005 versus control) and the single-acting thromboxane receptor blocker SQ-30741 (10⁻⁷ mol/L; P<.01 versus control).

Conclusions Thus, thrombin directly stimulates platelets to release thromboxane A₂, inducing potent vasoconstriction, which is prevented by the simultaneous thrombin-induced release of prostacyclin and nitric oxide from endothelial cells. In arteries devoid of functional endothelial cells, as occurs in patients with coronary artery disease, a combined inhibition of thromboxane production and action provides a potent therapeutic tool to interfere with the thrombin-induced activation of platelet–vessel wall interaction. (Circulation. 1994;89:2266-2272.)

Key Words • arteries • N⁶-nitro-L-arginine methyl ester • ridogrel • SQ-30741

Platelets play an essential role in acute coronary syndromes such as unstable angina and myocardial infarction.¹⁻⁴ In addition, platelet inhibitory trials suggest that activation of platelets plays an important role in the natural history of coronary artery disease in general as well as in coronary bypass graft disease.⁵⁻⁸ Although under normal conditions platelets remain inactivated in the circulation, under pathological conditions the cells can be stimulated to release a variety of substances such as serotonin and thromboxane A₂, which evoke potent vasoconstriction and further activation of platelets.⁹ In patients with coronary artery disease, local platelet activation commonly occurs and contributes to local vasospasm, thrombus formation, and eventually vascular occlusion.¹⁻³ Thrombin is a potent platelet activator and has been implicated in thrombotic coronary artery occlusion and in particular in vascular reocclusion after coronary thrombolysis and angioplasty.¹⁰⁻¹² Antithrombin therapy appears to prevent vascular reocclusion after thrombolysis in the animal model.¹¹,¹² In addition to platelets, thrombin specifically activates endothelial cells of human arteries.¹³,¹⁴ Endothelial cells manifest antithrombotic activity by releasing vasoactive substances with antiplatelet activities such as the endothelium-derived nitric oxide¹⁵⁻¹⁸ and prostacyclin.¹⁹ Platelet-derived substances (ie, adenine nucleotides, serotonin) and thrombin, on the other hand, cause endothelium-dependent relaxation, at least in certain blood vessels.²⁰⁻²⁵ Hence, platelet-derived substances such as serotonin, ADP, and ATP as well as thrombin can activate platelets and stimulate the release of nitric oxide and prostacyclin from the endothelium. Thus, depending on the functional status of the endothelium, these substances should differentially regulate platelet–vessel wall interaction. Since endothelial dysfunction, and in particular a decreased release of nitric oxide and prostacyclin, occurs in coronary artery disease,²⁶⁻²⁷ these mechanisms may have important implications in unstable angina and myocardial infarction.

This study was designed to investigate the potential role of thrombin as a regulator of platelet–vessel wall
interaction as well as the effects of antiplatelet drugs in human arteries.

Methods

Preparation of Blood Vessels

Internal mammary artery preparations were obtained intraoperatively as previously described. Coronary arteries were obtained from patients with cardiomyopathy who underwent heart transplantation. After removal, vessels were placed into modified Krebs-Ringer bicarbonate solution at 4°C (mM/L): NaCl 118; KCl 4.7; CaCl₂ 2.5; MgSO₄ 1.2; KH₂PO₄ 1.2; NaHCO₃ 25.0; edetate calcium disodium 0.026; and glucose 11.1 (control solution). Then the blood vessels were dissected free, cut into rings 5 mm long, and suspended in organ chambers filled with control solution (37°C; 95% O₂/5% CO₂).

Experimental Setup

Vascular rings were suspended in organ chambers, and changes in isometric tension were recorded with force transducers (Statham Universal UC2). Before the experiment, the rings were stretched and repeatedly exposed to norepinephrine (3 × 10⁻⁷ mol/L) or prostaglandin F₂α (10⁻⁶ mol/L); until a passive tension of 4 g was reached; then they were allowed to equilibrate for 45 minutes.

Endothelium Removal

The endothelium was removed from some rings by gentle rubbing of the intraluminal surface with a cotton swab. The presence or absence of the endothelium was confirmed by the presence or absence of a relaxation to acetylcholine (10⁻⁴ mol/L, internal mammary artery) or bradykinin (10⁻⁸ mol/L, coronary artery).

Preparation of Platelets

Platelets were prepared as previously described. Briefly, blood was obtained from healthy blood donors (Blutspendezentrum, University Hospital Basel). Blood was drawn into 9.3 mM/L sodium citrate, 0.7 mM/L citric acid, and 14 mM/L glucose and centrifuged for 20 minutes at 120g in 20°C. Platelet-rich plasma was pipetted off, and an equal volume of cold citrate anticoagulant solution (mM/L: sodium citrate 93, citric acid 7, glucose 105, potassium chloride 5; pH 6.3) was added. The platelet pellet produced by centrifugation at 570g for 20 minutes was resuspended in a small volume of the second citrate solution. A platelet count of this suspension was obtained (Thrombocounter-C; Coulter Electronics Ltd). Platelets readily aggregate at the concentrations used once injected into the organ chambers.

Drugs

The following drugs were used (unless otherwise indicated, from Sigma Chemical Co): acetylcholine hydrochloride; indomethacin; N⁴-nitro-monomethyl-L-arginine (L-NMA; Calbiochem); N⁵-nitro-L-arginine methyl ester (L-NAME); n-noradrenaline bitartrate; human thrombin; ridogrel (Janssen Pharmaceutica); SQ-30741 (Bristol Myer Squibb); and U-46619 (Cayman Chemical Co). The concentrations of the drugs are expressed as final molar concentrations (mol/L) in the bath solution. All drugs were dissolved in distilled water except ridogrel, which was dissolved in DMSO and diluted in distilled water, and indomethacin, which was dissolved in Na₂CO₃ (10⁻⁴ mol/L); SQ-30741 was dissolved in ethanol and diluted in distilled water.

Protocols and Statistics

To study the effects of thrombin on platelet–vessel wall interaction, arteries with and without endothelium were exposed to platelets (75 000/μL). Previous studies have demonstrated that platelets readily aggregate in organ chambers as well as the effects of antiplatelet drugs in human arteries.

Preparation of Platelets

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Results

Vascular Effects of Aggregating Platelets

In the internal mammary artery with endothelium pretreated with indomethacin (10⁻⁵ mol/L), platelets (75 000/μL) evoked only a marginal contraction (6±2% of the response to 100 mM/L KCl), which was markedly enhanced by the specific inhibitor of nitric oxide formation L-NMMA (10⁻⁵ mol/L; 20±6%, P<0.05; n=4; Fig 1). Removal of the endothelium unmasked a contraction to platelets comparable to that of L-NMMA (from 3±3% to 22±4%; P<0.05; n=4).

Vascular Effects of Thrombin

In internal mammary and coronary arteries, thrombin (0.01 to 1 U/mL) induced a marked concentration-dependent and endothelium-dependent relaxation (maximal relaxation, 53±7% and 75±9%, respectively; n=4; Fig 2). In coronary arteries, concentration-response curves to thrombin (0.01 to 1 U/mL) were somewhat reduced by the addition of L-NMMA (10⁻⁵ mol/L to inhibit nitric oxide production; NS; n=6) and particularly with L-NAME (10⁻⁴ mol/L; P<0.01; n=4) or indomethacin (10⁻⁵ mol/L; to inhibit vascular prostacyclin production; P<0.01; n=6; Fig 3). The combination of indomethacin and L-NMMA caused a further slight inhibition of the relaxations to thrombin (Fig 3; n=5; NS).
In mammmary arteries, the relaxations to a single concentration of thrombin (1 U/mL; 53±7%) were not affected by the cyclooxygenase inhibitor indomethacin (10⁻⁵ mol/L; 47±7%) or by the inhibitor of nitric oxide production L-NMMA (10⁻³ mol/L; 51±13%) alone but were abolished with combined treatment with the two inhibitors (P<.001 versus control; Table).

### Thrombin and Platelet–Vessel Wall Interaction

Under control conditions, platelets (75 000/μL) evoked no significant change in tension (3±3%) in quiescent arteries with endothelium but marked contractions after removal of the endothelium (Fig 4; 22±4%; P<.05; n=4). In arteries with endothelium, the addition of thrombin (1 U/mL) did not significantly affect the response to platelets (change in tension, 6±5%; NS; n=4) but caused a prominent further platelet-induced contraction in vessels without endothelium (Fig 4; 53±6%; P<.01 versus control without endothelium; n=4).

### Effects of Thromboxane Inhibitors

Increasing concentrations of platelets (1000 to 75 000/μL) activated by thrombin (1 U/mL) were added to internal mammmary arteries without endothelium. Under these conditions, the activated platelets induced marked and concentration-dependent contractions (Fig 5, left). These contractions were markedly antagonized by the combined thromboxane A₂ synthetase inhibitor/receptor antagonist ridogrel (Fig 5, left; 10⁻³ mol/L; n=5 and 10, respectively; P<.005 versus control) or the single-acting thromboxane receptor antagonist SQ-30741 (Fig 5, left; 10⁻⁷ mol/L; n=5 and 10, respectively; P<.01 versus control). Ridogrel (10⁻³ mol/L) caused both a shift to the right of the concentration-response curve and a pronounced inhibition of the maximal response to aggregating platelets (88±7% versus 24±8%; P<.01).

Ridogrel tended to have more pronounced effects than the single-acting thromboxane receptor antagonist SQ-30741 (Fig 5, left; 10⁻⁷ mol/L; 44±9%), although this did not reach statistical significance.

In parallel experiments, the effects of ridogrel (10⁻⁵ mol/L) and SQ-30741 (10⁻⁷ mol/L) on the contractions induced by the thromboxane analogue U-46619 were tested (Fig 5, right). Both drugs at the concentrations used to inhibit platelet-induced contractions exhibited equal potency in inhibiting contractions evoked by U-46619 (10-fold shift of the control EC₅₀; P<.0001) without reducing the maximal response (SQ-30741, 154±8% and ridogrel, 133±7% versus control, 150±6%; NS).

### Discussion

The present study demonstrates the protective role of the endothelium as an inhibitor of platelet–vessel wall interactions, in particular during activation with thrombin. In arteries with functionally intact endothelium, thrombin releases enough prostaglandins and nitric oxide from the human mammmary and coronary artery...
endothelium to prevent platelet-induced contractions resulting from the direct activation of platelets by thrombin. In contrast, in arteries devoid of functional endothelium, thrombin exclusively stimulates platelets to release large amounts of thromboxane A₂, which in turn cause potent contractions and further platelet activation. Treatment with the combined thromboxane A₂ synthetase inhibitor/receptor antagonist ridogrel²⁹,³⁰ as well as with the single-acting thromboxane A₂ receptor antagonist SQ-30741²² markedly reduced platelet-induced contractions in arteries without endothelium.

Previous studies in human arteries have shown that aggregating platelets release enough adenine nucleotides to activate purinergic receptors on endothelial cells linked to the release of nitric oxide.²²,²⁶ In precontracted arteries, aggregating platelets cause endothelium-dependent relaxations, despite the concomitant release of potent vasoconstrictors such as serotonin and thromboxane A₂ from platelets.²² In quiescent arteries used in this study, endothelial removal as well as the inhibitor of nitric oxide production L-NMMA³⁴,³⁵ unmasked platelet-induced contractions. This suggests that in arteries with endothelium, platelet-induced contractions are prevented by the release of nitric oxide in response to platelet-derived products. The fact that even a high concentration of aggregating platelets was unable to induce significant vasoconstriction in arteries with endothelium demonstrates the potency of the endothelial L-arginine/nitric oxide pathway as an inhibitor of platelet-vessel wall interaction.

At sites at which platelets are activated in vivo, the coagulation cascade is activated as well, which in turn leads to the formation of thrombin. Thrombin can stimulate both the endothelium (but not vascular smooth muscle of human arteries)³³,³⁴ and the platelets themselves. Indeed, in the human internal mammary and coronary arteries, thrombin caused pronounced endothelium-dependent relaxations. When concentration-response curves to thrombin were constructed, L-NMMA tended to inhibit that response,³⁹ whereas L-NAME or indomethacin was more effective. Complete inhibition of the effect was achieved with the combination of the two drugs. With a single high concentration of thrombin, neither indomethacin nor L-NMMA was effective alone, and only the combination was able to fully prevent the response. These slightly different results under both experimental conditions could be due to a more important contribution of prostacyclin over the entire concentration range of thrombin studied, whereas nitric oxide may be more important at higher concentrations. Alternatively, since the response to thrombin is associated with tachyphylaxis,³⁹ tolerance of the response may affect mechanisms linked to nitric oxide production more than those to prostacyclin. Hence, at a single high concentration of thrombin, activation of either pathway may be efficient enough to retain a response, whereas with repeated application of increasing concentrations of thrombin, the production of nitric oxide may be downregulated. In line with this interpretation, the maximal relaxation to thrombin was slightly more pronounced with a single concentration of the enzyme compared with a cumulative concentration-response curve. An interaction between nitric oxide and prostacyclin also has been demonstrated in human platelets,¹⁸ in intact porcine coronary⁵⁷ and mammary arteries,²⁸ and in endothelial cells in culture; under the latter conditions, nitric oxide inhibits the production of prostacyclin; hence, most likely after blockade of nitric oxide, more prostacyclin is formed to substitute for the effects of the endogenous nitrovasodilator.

In arteries with endothelium, thrombin did not stimulate platelet-induced contractions, although platelets were further stimulated to release potent vasoconstrictors. This suggests that thrombin-induced stimulation of prostacyclin and nitric oxide within the endothelium acts as a negative feedback preventing platelet-induced vasoconstriction in arteries with endothelium. Under
Fig 5. Graphs showing effects of the thromboxane A2 synthetase/receptor inhibitor ridogrel and the single-acting thromboxane receptor blocker SQ-30741 on thrombin-stimulated platelet-vasculature interaction in human internal mammary arteries without endothelium. In the absence of endothelium, platelets (in the presence of thrombin 1 U/mL) evoked potent vasoconstrictions (c). Ridogrel (10−5 mol/L, △, left) tended to inhibit platelet-induced contractions more potently than SQ-30741 (10−7 mol/L, ●, left), but this did not reach statistical significance, whereas both ridogrel and SQ-30741 at the concentrations used are equally potent to inhibit the vasoconstriction induced by thromboxane analogue U-46619 (right; shift at EC50: 10-fold for both drugs; *P<.005 vs control).

our experimental conditions, the relevant targets for these endothelium-derived inhibitory mediators must be primarily smooth muscle cells rather than platelets, since a minute endothelial surface of the arterial segments studied (∼25 mm2) is faced with a huge number of aggregated platelets (19×10^8 in 25 mL) that are stimulated with a high concentration of thrombin. Indeed, it is unlikely that platelet aggregation is inhibited by thromboxane antagonists under our experimental conditions, since thromboxane antagonists do not affect platelet activation induced by agonists other than thromboxane A2, such as thrombin or collagen. 39

In contrast, in preparations without endothelium, platelets alone caused a moderate contraction, 22 whereas thrombin markedly potentiated platelet-induced contractions. Hence, under these conditions, thrombin alone further activates platelets, whereas the inhibitory effects of endothelium-derived prostacyclin and nitric oxide at the level of vascular smooth muscle are lacking. The platelet-induced contraction must be mediated primarily

by platelet-derived thromboxane A2, since the response was markedly reduced by the thromboxane A2 synthetase/receptor antagonist ridogrel 29,30,40 or SQ-30741, a single-acting thromboxane A2 receptor antagonist. Indeed, thromboxane A2 is formed from the phospholipids of the cell membrane after stimulation with agonists such as thrombin and collagen. 19 Other substances such as serotonin are released from dense bodies of platelets that are already emptied after aggregation has occurred. Furthermore, ridogrel does not influence the release of other platelet-derived substances such as serotonin after stimulation by thrombin (References 40 and 41; F. De Clerck, PhD, written communication, November 19, 1993). Both ridogrel and SQ-30741 at the concentrations used were equally potent in inhibiting U-46619-induced vasoconstriction. With platelets, ridogrel tended to exhibit slightly more pronounced inhibitory effects than SQ-30741, but this did not reach statistical significance. This suggests that the receptor-blocking effects of ridogrel must be of primary importance to antagonize platelet-induced vasoconstriction, whereas the inhibition of thromboxane production only slightly contributes to its antiplatelet effects.

The present observation may have important clinical implications in acute coronary syndromes and bypass graft occlusion. In unstable angina, activation of platelets and the coagulation cascade play an important role. 34 In the coronary sinus of such patients, serotonin and thromboxane or its metabolites and fibrinopeptide A (an index of thrombin formation) are increased. 3,42,43 Whereas normally, endothelium-derived mediators such as prostacyclin and nitric oxide prevent platelet-vessel wall interaction, functional endothelial changes, such as occur in hyperlipidemia and atherosclerosis, 25,27,44,45 favor platelet-vessel wall interaction, vaso- spasm, thrombus formation, and vascular occlusion.

### Relaxations Induced by a Single Concentration (1 U/mL) of Thrombin in the Human Internal Mammary Artery With Endothelium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Relaxation</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>53±7% relaxation*</td>
</tr>
<tr>
<td>Indomethacin (10−5 mol/L)</td>
<td>47±15% relaxation*</td>
</tr>
<tr>
<td>L-NMMA (10−3 mol/L)</td>
<td>51±13% relaxation*</td>
</tr>
<tr>
<td>L-NMMA plus indomethacin</td>
<td>6.6±6.5% contraction†</td>
</tr>
</tbody>
</table>

The experiments were performed under control conditions (control) or in the presence of an inhibitor of cyclooxygenase (indomethacin) or that of nitric oxide production (N⁰-monomethyl-L-arginine, L-NMMA).

*Relaxations or contractions are expressed as percent of the contraction induced by norepinephrine.

†P<.005 vs other conditions.
This study suggests that thrombin is an important amplifier of platelet–vessel wall interactions in arteries without normal endothelium. Since thromboxane A₂ plays a major role, a combined inhibitor of thromboxane formation and action may represent an important therapeutic tool. It remains to be shown whether such a drug provides a greater benefit than aspirin.  

Acknowledgments

This study was supported by grants from the Swiss National Research Foundation (grant 32-32541.91) and by an educational grant from Janssen Pharmaceutica, Baar, Switzerland.

Dr. Yang is the recipient of a stipend from the Hartmann Müller Foundation, Zürich, Switzerland. The authors wish to thank Sabine Bohnert and Bernadette Lübsig, Department of Research, University Hospitals Basel for their invaluable help during the preparation of the manuscript and its figures and Draguljč Popovic and Silvia Distel, Operating Room Laboratory, Department of Cardiovascular Surgery, University Hospital Zürich for technical assistance.

References


Thrombin-induced endothelium-dependent inhibition and direct activation of platelet-vessel wall interaction. Role of prostacyclin, nitric oxide, and thromboxane A2.
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Circulation. 1994;89:2266-2272
doi: 10.1161/01.CIR.89.5.2266

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