Interaction of a Thrombin Inhibitor and a Platelet GP IIb/IIIa Antagonist In Vivo: Evidence That Thrombin Mediates Platelet Aggregation and Subsequent Thromboxane A$_2$ Formation During Coronary Thrombolysis$^1$

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Accepted for publication February 24, 1997

ABSTRACT

We examined the effect of a specific thrombin inhibitor, Ro 46-6240, alone and combined with an antagonist of the platelet GP IIb/IIIa, Ro44-9883, on the response to tissue-type plasminogen activator in a canine model of thrombolysis. Platelet activity was determined by measuring the excretion of 2,3-dinor-thromboxane (TX)B$_2$, an enzymatic metabolite of TXA$_2$. Ro 46-6240 administered before tissue-type plasminogen activator induced a dose-dependent prolongation of the activated partial thromboplastin time and prothrombin time. The time to reperfusion decreased dose-dependently (P < .01) to 10 ± 6 min vs. 52 ± 5 min in controls. Ro 46-6240 also prevented reocclusion, which occurred in every case in control experiments. Urinary excretion of 2,3-dinor-TXB$_2$ increased from 3 ± 1 to 37 ± 9 ng/mg creatinine in controls after reperfusion. This increase was reduced in a dose-dependent fashion by Ro 46-6240, such that at the highest dose, urinary 2,3-dinor-TXB$_2$ after reperfusion was 5.6 ± 1 ng/mg creatinine. Similar functional and biochemical effects were seen when a subthreshold dose of Ro 46-6240 was combined with Ro 44-9883. At the dose used, Ro 44-9883 alone abolished platelet aggregation ex vivo but failed to modify the response to tissue-type plasminogen activator or the excretion of 2,3-dinor-TXB$_2$ after reperfusion (51 ± 6 ng/mg creatinine, n = 3). However, the combination of Ro 44-9883 and Ro 46-6240 reduced the time to reperfusion (40 ± 8 vs. 68 ± 15 min; n = 7, P < .05), prevented reocclusion and abolished the rise in urinary 2,3-dinor-TXB$_2$ (5 ± 1 ng/mg creatinine, n = 4). These findings suggest that thrombin mediates platelet activation during coronary thrombolysis. The increased platelet activity results in platelet aggregation and a subsequent increase in TXA$_2$ formation.

Platelet activation is increased in patients undergoing coronary thrombolysis and may delay reperfusion and induce acute reocclusion (Fitzgerald et al., 1991). The increase in platelet activation has a number of consequences. There is an enhanced formation of TXA$_2$ (Fitzgerald et al., 1988; Kerins et al., 1989), the major cyclooxygenase product of arachidonic acid in platelets (Hamberg et al., 1975). TXA$_2$ is a potent platelet activator, acting to amplify the response to weak agonists such as ADP and low concentrations of thrombin and epinephrine (Hamberg et al., 1975). That TXA$_2$ is functionally important is demonstrated by the response to TXA$_2$ receptor antagonists in experimental models of thrombolysis, where they accelerate reperfusion and inhibit reocclusion (Fitzgerald et al., 1989; Golino et al., 1988). Moreover, aspirin reduces mortality in patients with acute myocardial infarction treated with streptokinase (ISIS-2, 1988).

In addition to generating TXA$_2$, the activation of platelets results in their aggregation. Platelet aggregation reflects the activation of an adhesion receptor, GP IIb/IIIa, to bind fibrinogen (Coller, 1990). Antagonists of GP IIb/IIIa have profound effects in experimental models of thrombolysis, enhancing the response to plasminogen activators and abolishing reocclusion (Fitzgerald and FitzGerald, 1989; Nicolini et al., 1994; Gold et al., 1988; Mickelson et al., 1990).

A possible mediator of the increased platelet activity and aggregation during coronary thrombolysis is thrombin. There is strong biochemical evidence, based on measurement of thrombin-antithrombin complexes, for the de novo generation of thrombin during coronary thrombolysis (Merlini et al., 1995; Galvani et al., 1994). There is also evidence of increased thrombin activity during coronary thrombolysis (Fitzgerald and FitzGerald, 1989; Merlini et al., 1995; Galvani et al., 1994; Eisenberg et al., 1987; Jang et al., 1990; Yao

ABBREVIATIONS: TX, thromboxane; t-PA, tissue-type plasminogen activator; GP, glycoprotein; aPTT, activated partial thromboplastin time; PT, prothrombin time.

Received for publication October 9, 1996.

$^1$ The work was supported by grants from the Wellcome Trust and the Irish Heart Foundation.

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JPET 281:1178–1185, 1997

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The Journal of Pharmacology and Experimental Therapeutics Vol. 281, No. 3

0022-3565/97/2813-1178$03.00/0

Received for publication October 9, 1996.

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et al., 1992). It is unclear, however, whether thrombin is responsible for the increased platelet activity and thromboxane formation that occurs during coronary thrombosis. In this study, we examine the response to a novel and specific thrombin inhibitor, Ro 46-6240 (Rudd et al., 1992), in a model of coronary thrombosis that is associated with marked platelet activation and wherein the functional response is highly platelet-dependent (Fitzgerald et al., 1989; Fitzgerald and FitzGerald, 1989).

Materials and Methods

**Materials.** ADP and thrombin were obtained from Sigma Chemical Co. (St. Louis, MO). Human recombinant t-PA, Ro 46-6240 (napsagatan, N-[N-[4-[[[(S)-1-amidino-3-piperidinyl]methyl]-N(2-naphthalenesulfonyl)-1-asparaginyl]-N-cyclopropylglycine; Carteaux et al., 1995) and Ro 44-9883 (lamifiban, [[1-(N-p-aminobenzoyl)-4-tiroyl]-4-piperidinyl]oxy]acetic acid; Carteaux et al., 1993) were kind gifts from Dr. Sebastien Roux (F. Hoffmann-La Roche Ltd., Basel, Switzerland).

**Animal studies.** All animal studies were reviewed and approved by the Animal Committee at University College Dublin, Ireland. Mongrel dogs, weighing 13 to 30 kg, were studied using the previously described protocol (Fitzgerald et al., 1989). Briefly, after anesthesia with pentobarbitone (30 mg/kg), the animal was intubated with an Apple MacLab multichannel recorder. A 200-gauge, 1.5-mm needle electrode was inserted through the needle electrode to induce endothelial injury. The wires from the electrode and the flow probe were brought to the surface and placed in a subcutaneous pouch. The dog was allowed to recover for a period of 5 to 7 days, by which time biochemical indices of platelet activation had returned to baseline (Fitzgerald et al., 1989). Postoperatively, heparin (200 IU/kg s.c.) was administered every 8 hr for the first 48 hr.

**Experimental procedures.** On the day of the study, the dogs were sedated with acepromazine (91 mg/kg) and morphine sulphate (1–2 mg/kg), and the wires were retrieved via a small skin incision. The flow probe was connected to a directional pulsed Doppler flowmeter (545C-4 Bioengineering, University of Iowa City, IA). Coronary blood flow velocity was recorded throughout the experiment with an Apple MacLab multichannel recorder. A 200-μA current was passed through the needle electrode to induce endothelial injury. This resulted in coronary thrombosis and subsequent occlusion, detected as abolition of the signal from the Doppler flow probe. The thrombotic occlusion occurred in 1 to 2 hr, and the current was discontinued 30 min later. Two hours after complete coronary occlusion, t-PA 10 μg/kg/min was administered as an infusion through a peripheral vein; the infusion was continued until 10 min after reperfusion. This results, in about 60 min, in reperfusion that is always complicated by acute reocclusion in control experiments (Fitzgerald et al., 1989; Fitzgerald and FitzGerald, 1989). Ro 46-6240 and Ro 44-9883 were administered either alone or in combination as a bolus 30 min before t-PA. This was immediately followed by a continuous infusion that was calculated from a standard curve constructed with canine plasma containing known amounts of drug. The detection limit of the assay was 20 ng/ml.

Reperfusion was defined as a return of velocity of flow in the coronary artery to at least 70% of base line. Reperfusion time was defined as the time from t-PA administration to reperfusion. After reperfusion, coronary flow velocity was monitored until the artery reoccluded or for a period of 120 min after reperfusion. Reocclusion time was defined as the time from reperfusion to complete cessation of flow. Dogs without reocclusion after 120 min of reperfusion were considered to have a reocclusion time of 120 min. Dogs that developed reocclusion were monitored for an additional 30 min to document persistent reocclusion.

**Thromboxane biosynthesis.** Urine was collected throughout in hourly aliquots by catheter for measurement of 2,3-dinor-TXB₂, the major enzymatic metabolite of TXA₂ in vivo. 2,3-Dinor-TXB₂ was determined by isotope dilution using a deuterated internal standard with quantitation by negative ion–chemical ionization, gas chromatography-mass spectrometry, as previously described (Fitzgerald et al., 1989; Fitzgerald and FitzGerald, 1989).

**Coagulation assays.** Plasma fibrinogen concentration was determined by the Clauss method as thrombin clottable protein using a fibrinometer (BBL Fibrotest, Becton Dickinson, Cockeysville, MD). The coefficient of variation between measurements on the same sample was 3%, and each sample was run in triplicate. The aPTT was determined in citrated plasma as the clotting time after the addition of Actin FS and CaCl₂ 25 mM. The PT was determined in citrated plasma as the clotting time after the addition of Dade thromboplastin C (Baxter, Miami, FL).

**Platelet aggregation studies.** Ex vivo platelet aggregation was analyzed by light transmission (Bio Data PAP-4, Horsham, PA) before, 15 min after and 1 hr after the administration of drugs (Fitzgerald et al., 1989; Fitzgerald and FitzGerald, 1989). Blood samples were collected into plastic tubes containing 3.8% solution of sodium citrate (9 vol blood to 1 vol sodium citrate). Platelet-rich plasma was obtained by centrifuging blood samples at 900 g for 45 sec, and platelet-poor plasma was obtained by centrifuging the residual blood at 900 × g for 10 min. The platelet agonists used were ADP at a concentration of 1 to 5 μM and thrombin at a final concentration of 0.1 to 0.5 U/ml. Agonists were added in a volume of 50 μl or less to 500-μl aliquots of platelet-rich plasma. The degree of platelet aggregation was reported as a percentage of maximal increase in light transmission in platelet-rich plasma as compared with platelet-poor plasma.

**Bleeding rate analysis.** The bleeding rate was assessed from the incision on the chest wall used to retrieve the flow probe and the electrode terminal. The wound was standardized so that it was half an inch in length and involved the full thickness of the subcutaneous tissues. Bleeding rate was determined by packing the incision with gauze and determining the increase in weight of the gauze hourly throughout the experiment (Fitzgerald et al., 1991).

**Plasma drug measurements.** Plasma concentrations of Ro 46-6240 were determined functionally using a bioassay (Tschopp TB, F. Hoffmann-La Roche, Basel, Switzerland). Two hundred microliters of citrated plasma was mixed with 400 μl of acetone and incubated for 5 min at room temperature. After centrifugation at 12,000 × g for 2 min at room temperature, 500 μl of the supernatant was evaporated under nitrogen and resuspended in 167 μl of 0.05 M Tris, 0.1 M NaCl, 0.1% (w/v) PEG 6000 and 0.02% (v/v) Tween 80, pH 7.8. The thrombin inhibitory activity was measured with a chromogenic substrate kinetic assay run on a Cobas Bio spectrophotometric centrifugal analyzer (F. Hoffmann-La Roche). The final concentration of human thrombin was 12 nM, and that of the substrate (methoxysulfonyl-d-Leu-Gly-Arg-paranitroanilide) was 100 μM. The concentration of Ro 46-6240 in the samples was calculated from a standard curve constructed with canine plasma containing known amounts of drug. The detection limit of the assay was 20 ng/ml.

Plasma concentrations of Ro 44-9883 were determined using a competitive, solid-phase receptor assay (Kouns et al., 1992). Briefly, citrated plasma samples were mixed with ice-cold 10% trichloroacetic acid solution (1:1, v/v) in buffer (150 mM NaCl, 1 mM CaCl₂, 1 mM
MgCl₂, 20 mM Tris/HCl). The solution was vortexed vigorously and centrifuged at 15,000 x g for 15 min at 4°C. To 1 vol of supernatant, 1.5 vol of 84 mM sodium carbonate buffer was added. Fifty microliters of serial dilutions of this solution was added to microtiter wells containing purified GP IIb/IIIa followed by 50 μl of fibrinogen 0.2 μg/ml. The plate was incubated at room temperature overnight, and bound fibrinogen was detected by enzyme-linked immunosorbent assay (ELISA) (Kouns et al., 1992).

Plasma t-PA was measured by ELISA (American Diagnostica Inc., Greenwich, CT), as previously described (Bergsdorf et al., 1983).

**Statistical analysis.** The data were analyzed by Kruskal-Wallis one-way analysis of variance with a subsequent Mann-Whitney test for comparison between groups. These analyses make no assumptions as to the distribution of the data. Chi-square analysis was also used where appropriate. Values are expressed as mean ± S.E.M. P < .05 was considered statistically significant.

**Results**

Occlusive thrombi developed in all dogs within 82 ± 10 min after application of the current. After the administration of t-PA, reperfusion occurred in all control animals, with a mean time to reperfusion of 52 ± 5 min. Complete reocclusion occurred in every case at 38 ± 8 min (n = 10), and no further reperfusion was detected. Ro 46-6240, the selective thrombin inhibitor, induced a dose-dependent prolongation of the aPTT and PT that was evident 15 min after administration of the bolus of drug (table 1). There was no effect on plasma fibrinogen before or after the infusion of t-PA in control animals or in those receiving Ro 46-6240 (table 2). Ro 46-6240 also reduced the time to reperfusion in a dose-dependent manner (P < .01) and delayed or prevented reocclusion (table 3). At the lowest dose of Ro 46-6240 (5 μg/kg/min), complete reocclusion was not prevented, but cyclical episodes of reocclusion/reperfusion were seen for up to 2 hr after reperfusion. At 20 μg/kg/min and 40 μg/kg/min, reocclusion was prevented in all but one experiment, where reocclusion occurred at 115 min despite prolongation of the aPTT and high plasma drug levels.

Ro 44-9883, the antagonist of the platelet GP IIb/IIIa, at 2 μg/kg/min did not alter the time to reperfusion or the rate of or time to reocclusion when it was administered alone. How-

<p>| TABLE 1 |
| Effects of increasing doses of Ro 46-6240 on prothrombin time (PT) and activated partial thromboplastin time (aPTT) |</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>PT (secs)</th>
<th>Ro 46-6240</th>
<th>aPTT (secs)</th>
<th>Ro 46-6240</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Base</td>
<td>15 Min</td>
<td>Reperfusion 2 Hrs</td>
<td>Base</td>
</tr>
<tr>
<td>Ro46-6240 (5 μg/kg/min)</td>
<td>9 ± 0.2</td>
<td>11 ± 0.2*</td>
<td>11 ± 0.4*</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>Ro46-6240 (10 μg/kg/min)</td>
<td>10 ± 0.3</td>
<td>13 ± 0.5*</td>
<td>14 ± 1*</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Ro46-6240 (20 μg/kg/min)</td>
<td>9 ± 1</td>
<td>16 ± 2*</td>
<td>18 ± 2*</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Ro46-6240 (40 μg/kg/min)</td>
<td>9 ± 0.3</td>
<td>20 ± 1*</td>
<td>26 ± 2*</td>
<td>12 ± 1</td>
</tr>
</tbody>
</table>

* P < .01 compared to base.

<p>| TABLE 2 |
| Effect of increasing doses of Ro 46-6240 on plasma fibrinogen levels |</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fibrinogen mg/dl</th>
<th>Ro 46-6240</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Base</td>
<td>15 min</td>
</tr>
<tr>
<td>Control</td>
<td>270 ± 55</td>
<td>ND</td>
</tr>
<tr>
<td>Ro 46-6240 5 μg/kg/min</td>
<td>222 ± 52</td>
<td>216 ± 47</td>
</tr>
<tr>
<td>Ro 46-6240 10 μg/kg/min</td>
<td>302 ± 68</td>
<td>302 ± 60</td>
</tr>
<tr>
<td>Ro 46-6240 20 μg/kg/min</td>
<td>256 ± 32</td>
<td>243 ± 28</td>
</tr>
<tr>
<td>Ro 46-6240 40 μg/kg/min</td>
<td>215 ± 30</td>
<td>222 ± 37</td>
</tr>
<tr>
<td>ND = not determined.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| TABLE 3 |
| The effect of increasing doses of Ro 46-6240 and Ro 46-6240 plus Ro 44-9883 on time to reperfusion and time and rate of reocclusion in a canine model of coronary thrombolysis induced by t-PA |</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Reperfusion Time (mins)</th>
<th>Reocclusion Time (mins)</th>
<th>Rate of Reocclusion %</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PA (10 μg/kg/min)</td>
<td>10</td>
<td>52 ± 5</td>
<td>38 ± 8</td>
<td>100</td>
</tr>
<tr>
<td>+Ro 46-6240 (5 μg/kg/min)</td>
<td>5</td>
<td>68 ± 15</td>
<td>44 ± 5*</td>
<td>100</td>
</tr>
<tr>
<td>+Ro 46-6240 (10 μg/kg/min)</td>
<td>4</td>
<td>55 ± 13</td>
<td>100 ± 10</td>
<td>25*</td>
</tr>
<tr>
<td>+Ro 46-6240 (20 μg/kg/min)</td>
<td>3</td>
<td>48 ± 10</td>
<td>&gt;120</td>
<td>0*</td>
</tr>
<tr>
<td>+Ro 46-6240 (40 μg/kg/min)</td>
<td>5</td>
<td>10 ± 6*</td>
<td>&gt;120</td>
<td>20*</td>
</tr>
<tr>
<td>+Ro 44-9883 (2 μg/kg/min)</td>
<td>4</td>
<td>65 ± 16</td>
<td>49 ± 22</td>
<td>75</td>
</tr>
<tr>
<td>+Ro 46-6240 (5 μg/kg/min)</td>
<td>7</td>
<td>40 ± 8</td>
<td>&gt;120</td>
<td>0*</td>
</tr>
</tbody>
</table>

* P < .01 compared to t-PA control.

* Cyclic reocclusion/reperfusion.

* Reocclusion at 115 minutes.
ever, when Ro 44-9883 was combined with the lowest dose of Ro 46-6240 (5 μg/kg/min), which alone had little effect, the time to reperfusion was reduced, and reocclusion was prevented in every case (table 3). It is worth noting that the cycles of reocclusion seen after treatment with Ro 46-6240 5 μg/kg/min were abolished by Ro 44-9883, which indicates that they reflected activation of the platelet GP IIb/IIIa.

**Platelet aggregation studies.** At baseline, the platelets of all animals responded to ADP with an EC₅₀ of 2 ± 1 μM and to thrombin with an EC₅₀ of 0.3 ± 0.1 U/mL. Ro 46-6240 abolished thrombin-induced platelet aggregation at even the lowest dose of 5 μg/kg/min. Suppression of aggregation was similar at 15 min after administration of Ro 46-6240 and 1 hr after reperfusion. In contrast, ADP-induced platelet aggregation was not influenced by Ro 46-6240 at any dose used, a result that confirms its specific antithrombin activity. When the GP IIb/IIIa antagonist Ro 44-9883 was added, ADP-induced platelet aggregation was also prevented (fig. 1).

**Urinary 2,3-dinor TXB₂.** Urinary excretion of 2,3-dinor TXB₂, the major metabolite of TXA₂ in dogs and an index of in vivo TXA₂ formation, increased during the induction of thrombolysis and after reperfusion (fig. 2). In control animals, urinary excretion of 2,3-dinor TXB₂ increased from 3 ± 1 to 33 ± 5 and 37 ± 9 ng/mg creatinine at 30 min and 2 hr after reperfusion, respectively (n = 7). Excretion of the metabolite was suppressed by Ro 46-6240 in a dose-dependent manner (P < 0.01). However, at the lowest dose of Ro 46-6240 (5 μg/kg/min), urinary 2,3-dinor TXB₂ was unaltered (6 ± 1 ng/mg creatinine at base line rose to 45 ± 4 ng/mg creatinine at 2 hr of reperfusion; n = 5). Similarly, Ro 44-9883 at a dose of 2 μg/kg/min did not alter excretion of 2,3-dinor-TXB₂ (3 ± 3 ng/mg creatinine at base line rose to 51 ± 6 ng/mg creatinine at 2 hr of reperfusion; n = 3). However, combining Ro 46-6240 and Ro 44-9883 abolished the increase in this metabolite (4 ± 1 ng/mg creatinine at base line rose to 5 ± 1 ng/mg creatinine at 2 hr of reperfusion, n = 4).

**Bleeding rate.** Bleeding from the skin incision was minimal before administration of thrombolytic therapy. The infusion of t-PA resulted in a small increase in bleeding in control animals. At the lowest concentrations of Ro46-6240 (5 and 10 μg/kg/min), there was no increase in bleeding compared with control. However, there was a 3 ± 2.4-fold increase in bleeding at 20 μg/kg/min, and a 6.4 ± 4-fold increase in bleeding at 40 μg/kg/min, compared with control (fig. 3). The increase in bleeding persisted over the period of observation after reperfusion (2 hr) despite the discontinuation of t-PA. The combination of the lowest dose of Ro 46-6240 with Ro 44-9883 increased the bleeding rate by 5.4 ± 2-fold compared with control (fig. 4).

**Plasma drug levels.** The mean plasma t-PA level at reperfusion was 660 ± 100 ng/ml; it returned to base-line levels at 2 hr of reperfusion in control animals (fig. 5). Plasma t-PA was unaltered by coinfusion of the thrombin inhibitor Ro 46-6240 alone or in combination with the GP IIb/IIIa antagonist Ro 44-9883. Plasma concentrations of Ro 46-6240 reached steady state 15 min after the bolus and infusion of drug and increased in a dose-dependent manner (fig. 6). The plasma concentration of Ro 46-6240 at 5 μg/kg/min (294 ± 49 nM, n = 5) was not altered when it was combined with the GP IIb/IIIa antagonist Ro 44-9883 (380 ± 60 nM, n = 7). Likewise, plasma concentrations of Ro 44-9883 were similar when it was infused alone and when it was combined with Ro 46-6240 (fig. 7).

**Discussion**

There is compelling evidence of increased platelet activation during coronary thrombolysis (Fitzgerald et al., 1991). Thus the formation of TXA₂, determined as excretion or plasma levels of its stable enzymatic metabolites, is increased markedly in patients treated with either t-PA or streptokinase. In addition, antiplatelet agents greatly enhance the response to plasminogen activators in the human (ISIS-2, 1988) and in experimental models of coronary thrombosis (Fitzgerald et al., 1989; Fitzgerald and FitzGerald, 1989; Nicolini et al., 1994; Gold et al., 1988; Mickelson et al., 1990). These agents include antagonists of the platelet GP IIb/IIIa, which suggests that platelet aggregation is a major consequence of the enhanced platelet activation. What triggers this intense platelet activity during thrombolytic therapy is unclear. Several agonists have been implicated, including serotonin and TXA₂ (Fitzgerald et al., 1989; Golino et al., 1988). However, their role may be secondary, because they are released upon platelet activation and serve to amplify the response to primary agonists, such as thrombin, collagen and epinephrine. It is consistent with this hypothesis that inhibition of serotonin or of TXA₂ alone does not prevent reocclusion, which suggests that some other agonist is involved.

In this study, we examined the relationship between thrombin and platelet activation in a well-characterized model of coronary thrombolysis (Fitzgerald et al., 1989; Fitzgerald and FitzGerald, 1989). Several lines of evidence implicate thrombin as a platelet agonist in this setting. First, clinical and experimental studies demonstrate increased thrombin activity during coronary thrombolysis in that plasma levels of fibrinopeptide A, a peptide cleaved by thrombin from the Aα chain of fibrinogen, are elevated (Merlini et al., 1995; Galvani et al., 1994; Eisenberg et al., 1987). In addition, inhibitors of factor Xa enhance the response to thrombolytic therapy, which implies de novo thrombin generation (Sitko et al., 1992). Second, in experimental models of coronary thrombolysis, thrombin inhibition evokes a response similar to that seen with platelet inhibitors (Fitzgerald and FitzGerald, 1989; Jang et al., 1990; Yao et al., 1992; Rudd et al., 1997). Thrombin in Thrombolysis (1997, Thrombin in Thrombolysis) 1181.
et al., 1992). In this model of coronary thrombolysis, the coronary thrombus formed is platelet-rich, and there is marked platelet activation during and after thrombolysis, detected as an increase in TX formation (Fitzgerald et al., 1989).

We examined the response to Ro 46-6240, a competitive inhibitor of thrombin with an apparent $K_i$ of 34 nM. Moreover, Ro 46-6240 is a potent inhibitor of clot-bound thrombin (Gast et al., 1994). The thrombin-inhibitory activity is highly specific, the $K_i$ for other serine proteases being several orders of magnitude higher (Carteaux et al., 1995). In our experiments, Ro-46-6240 inhibited reocclusion in a dose-dependent manner and, at the highest doses, accelerated reperfusion. As an index of platelet activation in vivo, we measured the formation of TXA$_2$. TXA$_2$ is the major cyclooxygenase product of arachidonic acid in platelets and is formed in response to most platelet activators, including thrombin (Offermans et al., 1994, Brune and Ullrich, 1991). It is rapidly inactivated in aqueous medium by hydrolysis to the inactive metabolite TXB$_2$. Measurement of TXB$_2$ is fraught with difficulties, because it is generated during blood sampling and its excretion in urine reflects renal production (FitzGerald et al., 1983). TXB$_2$ is further metabolized enzymatically to several products, including 2,3-dinor-TXB$_2$. Consequently, we monitored platelet activation by determining the excretion of this stable enzymatic metabolite.

Previous studies in this model of thrombolysis have shown a marked increase in TXA$_2$ formation coincident with coronary reperfusion (Fitzgerald et al., 1983). The increase in TXA$_2$ biosynthesis persists unabated for at least 4 hr after reperfusion, long after the discontinuation of t-PA. In this study, the specific thrombin inhibitor Ro 46-6240 suppressed urinary 2,3-dinor-TXB$_2$ in a dose-dependent manner, and little increase in the thromboxane metabolite was detected at the highest doses. The suppression of 2,3-dinor-TXB$_2$ coincided with an acceleration of reperfusion and inhibition of
reocclusion, which have been shown previously to be platelet-dependent events (Fitzgerald et al., 1989; Fitzgerald and FitzGerald, 1989). Thus Ro 46-6240 markedly suppressed functional and biochemical evidence of platelet activation in vivo.

Similar effects were seen when a subthreshold dose of Ro 46-6240 was administered in combination with Ro 44-9883. Ro 44-9883 is a potent peptidomimetic antagonist of the platelet GP IIb/IIIa (Carteaux et al., 1993). At high dose, Ro 44-9883 abolishes coronary reocclusion (Murphy, Pratico and Fitzgerald, unpublished observations), as has been reported with other antagonists of the platelet GP IIb/IIIa. At the dose used, Ro 44-9883 inhibited platelet aggregation in response to ADP ex vivo. Alone, it had no discernible effect on the response to t-PA or on biochemical evidence of platelet activation in vivo. However, when Ro 44-9883 was combined with a subthreshold dose of the thrombin inhibitor, reocclusion was prevented. Similar findings have been reported with a structurally distinct GP IIb/IIIa antagonist, integrulin, combined with the thrombin inhibitor hirudin (Nicolini et al., 1994). Moreover, the combination of Ro 44-9883 and Ro 46-6240 abolished the increase in urinary 2,3-dinor-TXB₂. It is worth emphasizing that this synergistic interaction did not result from an alteration in the plasma concentration of either drug.

These data suggest that one of the consequences of throm-

Fig. 3. Bleeding rate after reperfusion in animals treated with increasing doses of the thrombin inhibitor Ro 46-6240. * P < 0.05 vs. control, ** P < 0.01 vs. control.

Fig. 4. Bleeding rate after reperfusion in animals treated with the GP IIb/IIIa antagonist Ro 44-9883 2 μg/kg/min alone and combined with the thrombin inhibitor Ro 46-6240 5 μg/kg/min.
bin generation during coronary thrombolysis is activation of the platelet GP IIb/IIIa. The findings of this study also suggest that the increase in TXA2 formation in this model is due largely to platelet aggregation. TXA2 is formed upon platelet activation by agonists through G protein-linked activation of phospholipases (Brass et al., 1993). However, the largest component of TXA2 formation upon platelet stimulation occurs coincident with platelet aggregation (Shattil et al., 1994). This reflects occupancy of the platelet GP IIb/IIIa by fibrinogen, which triggers “inside-out” transmembrane signaling and further TXA2-dependent cell activation.

In conclusion, thrombin is the primary agonist mediating reocclusion in a model of coronary thrombolysis. Thrombin induces platelet activation and aggregation, which in turn result in a marked increase in TXA2 formation. The increase in TXA2 biosynthesis largely reflects platelet aggregation (indeed, this may be a useful marker of aggregation in vivo). Coadministration of a platelet GP IIb/IIIa antagonist and a thrombin inhibitor reduces the dose required of either drug alone, and the combination may prove useful in enhancing the response to thrombolytic therapy of acute myocardial infarction.

Fig. 5. Plasma t-PA levels at steady state during infusion of the plasminogen activator. There were no significant differences between groups.

Fig. 6. Plasma concentrations of Ro 46-6240 at steady-state infusion.

Fig. 7. Plasma concentrations of Ro 44-9883 and Ro 46-6240 when infused alone or in combination.

References


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