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Role of thrombin and thromboxane A_2 in reocclusion following coronary thrombolysis with tissue-type plasminogen activator

(thrombolytic therapy/coronary thrombosis/platelet activation/reperfusion)

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ABSTRACT **Reocclusion of the coronary artery occurs after thrombolytic therapy of acute myocardial infarction despite routine use of the anticoagulant heparin. However, heparin is inhibited by platelet activation, which is greatly enhanced in this setting. Consequently, it is unclear whether thrombin induces acute reocclusion. To address this possibility, we examined the effect of argatroban {MCI9038, (2*R*,4*R*)-4-methyl-1-[N^{α}-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic acid}, a specific thrombin inhibitor, on the response to tissue-type plasminogen activator in a closed-chest canine model of coronary thrombosis. MCI9038 prolonged the thrombin time and shortened the time to reperfusion (28 ± 2 min vs. 59 ± 7 min in controls; mean ± SEM, $n = 5$, $P < 0.01$). At the highest dose, 2.5 mg/kg per hr, complete reocclusion was prevented in four of the five experimental animals, whereas reocclusion occurred in all five controls. However, reperfusion was complicated by cycles of decreased flow, which were abolished by the thromboxane A_2 antagonist, GR32191. GR32191 at 1 mg/kg combined with MCI9038 at 0.5 mg/kg per hr prevented reocclusion, whereas, at these doses, either drug alone was without effect. In addition, thromboxane A_2 biosynthesis, determined as excretion of its metabolite 2,3-dinor-thromboxane B_2, was increased after reperfusion at all doses of MCI9038. These data demonstrate that thrombin impairs thrombolysis induced by tissue-type plasminogen activator *in vivo* and induces acute reocclusion. Furthermore, the response to thrombin inhibition may be impaired by continued formation of thromboxane A_2.**

Thromobolytic therapy of acute myocardial infarction is limited by continued thrombosis, which may delay or prevent reperfusion and induce reocclusion (1, 2). Experimental models suggest that this is mediated by platelet aggregation that is partly thromboxane (Tx) A_2 dependent (3–6). It is less clear whether thrombin, a potent platelet activator and coagulant (7), also plays a role in this setting. Platelet activation increases prothrombinase activity (8, 9), which converts the inactive zymogen prothrombin to thrombin (10). Therefore, thrombin formation may be augmented in response to platelet activation during coronary thrombolysis. In addition, plasmin activates factor V and may directly induce thrombin formation during coronary thrombolysis (11). Consistent with these observations, Eisenberg and coworkers have demonstrated, in patients receiving thromobolytic therapy, increased plasma concentrations of fibrinopeptide A, a peptide cleaved from fibrinogen by thrombin (12). Studies with heparin, on the other hand, do not support a role for thrombin. Heparin fails to prevent reocclusion in experimental models of coronary thrombolysis despite *ex vivo* evidence of a marked anticoagulant effect (3–6). However, this may be due to the weak inhibitory activity of heparin-antithrombin against the prothrombinase formed on the platelet surface (13) and the neutralization of heparin by platelet factor 4 (14) and thrombospondin (15), proteins released by activated platelets.

To address the role of thrombin during coronary thrombolysis, we have examined the effect of a specific thrombin inhibitor, argatroban {MCI9038, (2*R*,4*R*)-4-methyl-1-[N^{α}-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic acid} on the response to tissue plasminogen activator (t-PA) in a closed-chest canine model of coronary thrombosis. MCI9038, an arginine derivative which binds to a hydrophobic pocket close to the active site of thrombin, is a competitive inhibitor of thrombin-induced cleavage of fibrinogen and platelet activation (16, 17), and it exhibits antithrombotic activity in experimental models and in humans (18, 19). In addition, we have examined the interaction between thrombin and TxA_2 to address the hypothesis that continued TxA_2 formation limits the response to thrombin inhibition in this setting.

MATERIALS AND METHODS

ADP, bovine thrombin, and arachidonic acid were purchased from Sigma, and D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK) was purchased from Calbiochem. We gratefully acknowledge gifts of (15*S*)-hydroxy-11,9-(epoxymethano)prostadienoic acid (U46619) from R. R. Gorman (Upjohn, Kalamazoo, MI), argatroban and t-PA (GR11044, Atlepase) from S. Bunting (Genentech, South San Francisco, CA), and {1*r*-[1α(*Z*), 2β, 3β, 5α]}-(+)-7-[5-{[(1,1′-biphenyl)-4-yl]methoxy}-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid hydrochloride (GR32191) from P. P. A. Humphrey (Glaxo Group Research, Ware, Herts, U.K.).

All animal studies were reviewed and approved by the Animal Care Committee at Vanderbilt University. In these studies we employed a chronic canine model of coronary thrombosis induced by electrical injury to the endothelium (20, 21). Briefly, through a left thoracotomy, a needle electrode was implanted in the circumflex coronary artery distal to an ultrasonic flow probe (Crystal Biotech, Havistan, MA) in the anesthetized animal. The terminals of the electrode and flow probe were brought to the surface in a subcutaneous pouch, the chest was closed, and the animal was allowed to recover.

Five to 7 days after surgery, the dog was lightly sedated with morphine sulfate at 1 mg/kg and acepromazine at 1–2 mg/kg. The electrode was connected in series with a potentiometer and a 9-V battery, and the circuit was grounded to

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Abbreviations: Tx, thromboxane; t-PA, tissue-type plasminogen activator; TT, thrombin time; aPTT, activated partial thromboplastin time; PT, prothrombin time; PPACK, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone.

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the subcutaneous tissues. Coronary flow velocity was recorded continuously by a directional pulsed Doppler flowmeter (545C-4, Biomedical Engineering, University of Iowa). The electrocardiogram, coronary flow velocity, and (in a subset) femoral artery pressure were recorded continuously by strip chart recorder. After 15 min of stable coronary flow, coronary thrombosis was induced by passing a 200-μA current through the electrode. This resulted in complete coronary occlusion in 1–2 hr by a thrombus that was rich in platelets (20, 21).

Two hours after complete coronary occlusion had been induced, t-PA was administered as a continuous infusion of 10 μg/kg per min through a peripheral vein until 10 min after complete reperfusion had occurred (see Figs. 5 and 6). Coronary blood flow was measured throughout the infusion and for 2 hr after reperfusion. In this model, reperfusion occurs in about 1 hr and is complicated in every case by acute reocclusion (6).

To examine the effect of thrombin inhibition on the response to t-PA, MCI9038 or vehicle was administered as a continuous intravenous infusion in doses of 0.2, 0.5, or 2.5 mg/kg per hr beginning 30 min prior to the infusion of t-PA and continued for 2 hr after reperfusion. The end points measured were the time to reperfusion, defined as a coronary blood flow equal to or greater than baseline, and the time from reperfusion to complete reocclusion. In some experiments, reperfusion failed to occur. In these and subsequent studies, this has been associated in every case with old, organized clot at the electrode site, which is easily recognized at the postmortem examination. This failure to reperfuse occurred in 7 of 39 experiments and was randomly distributed among treatment groups (1 in each group other than the highest dose of MCI9038, in which there were 2). These were excluded from subsequent analysis.

Coagulation studies [thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (aPTT)] and platelet aggregation studies were performed and plasma fibrinogen was determined at intervals throughout the experiment. Plasma for these studies was obtained from venous blood mixed with 3.8% sodium citrate (9:1, vol/vol) to prevent coagulation. Plasma t-PA was measured in a subset of experiments 30 min after the t-PA infusion was begun.

To determine the role of TxA_2 in limiting the response to the thrombin inhibitor, we examined the effect of GR32191, a specific and partially noncompetitive antagonist of the TxA_2/prostaglandin endoperoxide receptor (22), on the response to MCI9038. GR32191 at 1 mg/kg in normal saline was administered intravenously over 10 min immediately prior to MCI9038 at 0.5 mg/kg per hr or its vehicle, and the times to reperfusion and reocclusion were determined. In addition, TxA_2 biosynthesis was determined as excretion of a major enzymatic metabolite, 2,3-dinor-TxB_2 (23), at intervals throughout the experiment. Excretion of this metabolite reflects systemic formation of TxA_2, which is largely derived from platelets (24). Urinary 2,3-dinor-TxB_2 was determined by combined gas chromatography/mass spectrometry, using the deuterated molecule as internal standard as previously described (25).

Platelet aggregation due to ADP (5–10 μM), thrombin (0.2–0.4 unit/ml), U46619 (0.5–2 μM) or arachidonic acid (0.16–0.66 mM) was determined *ex vivo* by light transmission in platelet-rich plasma, as previously described (26). All coagulation assays were performed immediately on the day of study. Preliminary studies demonstrated similar results whether the sample was taken into PPACK (to prevent *ex vivo* plasminogen activation and fibrinogenolysis) or into citrate alone, with citrate alone used subsequently. Fibrinogen concentrations were determined as thrombin-clottable protein in citrated plasma (27) by using a fibrometer (Becton Dickinson). The TT was determined by measuring the time to clot formation after the addition of sufficient bovine thrombin to citrated plasma to achieve a baseline of 18–22 s. The aPTT, a measure of the intrinsic pathway of coagulation (28), was determined as the time to clot formation after the addition of activated rabbit brain cephalin (Actin, American Dade, Aguada, PR) and 0.025 M calcium chloride to citrated plasma in equal volumes. The PT, a measure of the extrinsic coagulation pathway (28), was determined by the addition of thromboplastin (Simplastin, General Diagnostics, Morris Plains, NJ). Plasma t-PA was determined by an enzyme-linked immunoabsorbent assay (American Diagnostica, New York). This assay detects both active t-PA and t-PA complexed to its physiological inhibitors (29).

Results are expressed as the mean ± SEM. The reperfusion and reocclusion times were compared between groups by nonparametric one-way analysis of variance (Kruskal–Wallis) (30). Reocclusion rates were compared by χ^2 analysis with correction for small sample size. Least squares analysis was used to measure correlation between TT and time to reperfusion.

RESULTS

MCI9038 induced a dose-dependent prolongation of the TT, the aPTT, and the PT (Fig. 1) and specifically inhibited thrombin-induced platelet aggregation (Fig. 2). The threshold for thrombin-induced aggregation was increased more than 10-fold at the highest dose. Peak anticoagulant effect was seen at 60 min into the MCI9038 infusion, 30 min after initiation of t-PA infusion. In contrast, plasma fibrinogen was unaltered by MCI9038 at any dose, either before or after t-PA administration (Table 1). GR32191 specifically inhibited platelet aggregation in response to U46619 and arachidonic acid (Fig. 2) and had no effect on coagulation parameters. In control experiments, plasma t-PA was 528 ± 35 ng/ml (mean ± SEM; n = 5) 30 min into the t-PA infusion and was unaltered by MCI9038 at 2.5 mg/kg per hr (499 ± 55 ng/ml, n = 3) or MCI9038 at 0.5 mg/kg per hr combined with GR32191 at 1 mg/kg (550 ± 51 ng/ml, n = 5).

In vehicle-treated controls, reperfusion occurred in 59 ± 7 min (n = 5). In every case reperfusion was complicated by cyclical flow and ultimately by complete reocclusion (at 37 ± 8 min). These results are similar to those previously reported in this model (6). Preliminary experiments demonstrated that MCI9038 did not alter blood pressure or heart rate even at the highest infusion rates. MCI9038 decreased the time to reper-

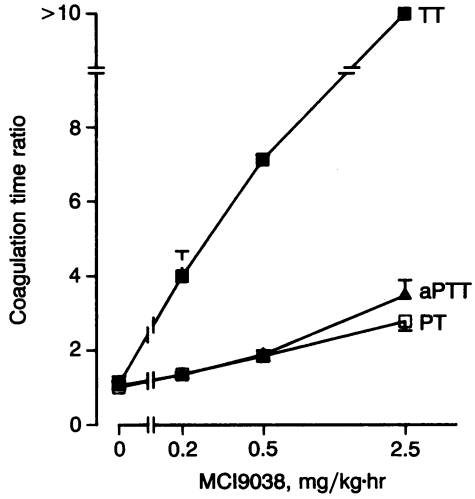

FIG. 1. Effect of MCI9038 on TT, aPTT, and PT. The coagulation times are expressed as the ratio of the measurements at 60 min of infusion and at baseline. At the highest dose of MCI9038, the TT was greater than 150 s. Results are presented as mean ± SEM.

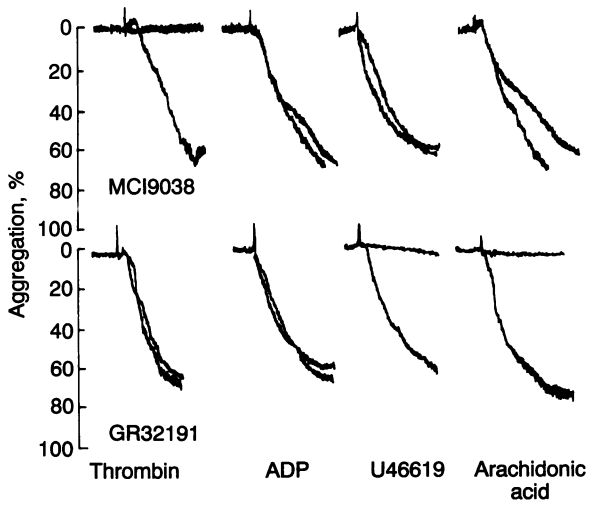

FIG. 2. Platelet aggregation in platelet-rich plasma before and 30 min after administration of MCI9038 at 2.5 mg/kg per hr (*Upper*) or GR32191 at 1 mg/kg (*Lower*). MCI9038 inhibited thrombin-induced platelet aggregation but had no effect on platelet aggregation induced by arachidonic acid, ADP, or the TxA_2/prostaglandin endoperoxide analog U46619. GR32191 prevented aggregation induced by activation of the TxA_2/prostaglandin endoperoxide receptor (U46619, arachidonic acid) but not by TxA_2-independent mechanisms (ADP, thrombin).

fusion dose dependently (Fig. 3). The peak effect occurred at a dose of 0.5 mg/kg per hr (28 ± 2 min, $n = 5$; $P < 0.01$ vs. controls), and no further effect was seen at a 5-fold higher dose (30 ± 7 min, $n = 5$; $P < 0.05$ vs. controls). In controls and at the two lower doses of MCI9038, the time to reperfusion was inversely related to the prolongation of the TT ($r = -0.76$; $n = 14$; $P < 0.002$).

Despite its effect on reperfusion and coagulation, MCI9038 at 0.2 and 0.5 mg/kg per hr had no effect on the time to or frequency of reocclusion (Fig. 4). In contrast, at the highest dose, 2.5 mg/kg per hr, reocclusion occurred in only one experiment over 2–4 hr of observation after reperfusion, and in that case reocclusion was delayed (97 min). However, in three of four experiments where reocclusion was prevented, reperfusion was complicated by cycles (3–7 per hr) of declining flow followed by abrupt increases that persisted throughout the observation period (Fig. 5). These cyclical changes in coronary flow were abolished by the TxA_2/prostaglandin endoperoxide receptor antagonist GR32191 at 1 mg/kg (Fig. 5). Furthermore, addition of GR32191 at 1 mg/kg to MCI9038 at a lower dose, 0.5 mg/kg per hr, prevented reocclusion in all cases ($n = 7$) without a further prolongation of the TT (Table 2). In none of these experiments was cyclical flow evident after reperfusion. In contrast, MCI9038 and GR32191, when used alone at these doses, did not prevent reocclusion ($n = 6$).

Urinary excretion of 2,3-dinor-TxB_2 increased after induction of coronary thrombosis and remained elevated during the

Table 1. Plasma fibrinogen before and 30 min after the initiation of MCI9038 infusion and after the administration of t-PA

MCI9038 infusion, mg/kg per hr	Plasma fibrinogen, mg/dl			
	Before MCI9038	After MCI9038	30 min after t-PA	2 hr after reperfusion
Vehicle	277 ± 48	279 ± 42	244 ± 28	238 ± 44
0.2	340 ± 36	307 ± 54	302 ± 43	304 ± 27
0.5	279 ± 17	237 ± 35	262 ± 18	259 ± 12
2.5	398 ± 60	368 ± 59	350 ± 64	338 ± 48

Results are mean ± SEM.

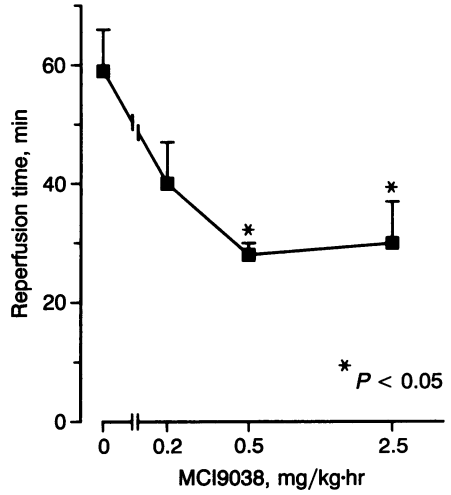

FIG. 3. Dose-dependent effect of MCI9038 on the time to reperfusion induced by t-PA. P is vs. vehicle-only control.

period of occlusion (Fig. 6). A further and more marked increase occurred during reperfusion and the subsequent reocclusion. The increase in 2,3-dinor-TxB_2 after reperfusion was unaltered by MCI9038 at any of the three doses used (Fig. 6, Table 3). Similarly, GR32191 alone or in combination with MCI9038 at 0.5 mg/kg per hr did not inhibit excretion of 2,3-dinor-TxB_2, consistent with its activity as a TxA_2/prostaglandin endoperoxide receptor antagonist.

DISCUSSION

The delay in achieving reperfusion and the reocclusion which complicates coronary thrombolysis have been thought largely to reflect platelet activation. Platelet activity is enhanced in patients with acute myocardial infarction treated with intravenous streptokinase (31) or t-PA (32). Possible mechanisms include release of active thrombin from the lysed clot (33) and the local formation of high concentrations of plasmin, which can activate platelets (34). Furthermore, prevention of platelet aggregation with an antibody to the platelet glycoprotein IIb/IIIa complex, the receptor for fibrinogen and other adhesive proteins (35), accelerates thrombolysis and prevents reocclusion in experimental models (3, 4, 6). Possible mediators of platelet activation in this setting

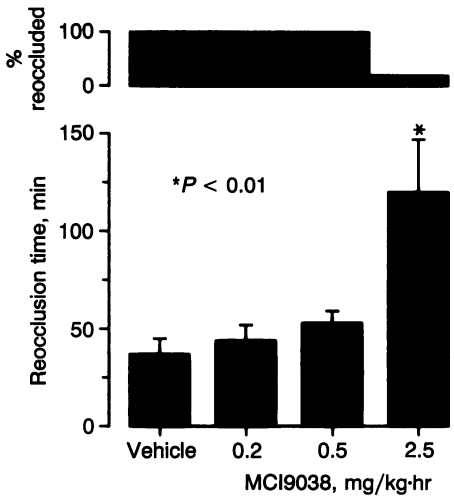

FIG. 4. Effect of MCI9038 on the reocclusion rate and time to reocclusion. At the highest dose, only one animal reoccluded in the observation period. The remainder were assigned a reocclusion time equal to the period of observation. P is vs. vehicle-only control.

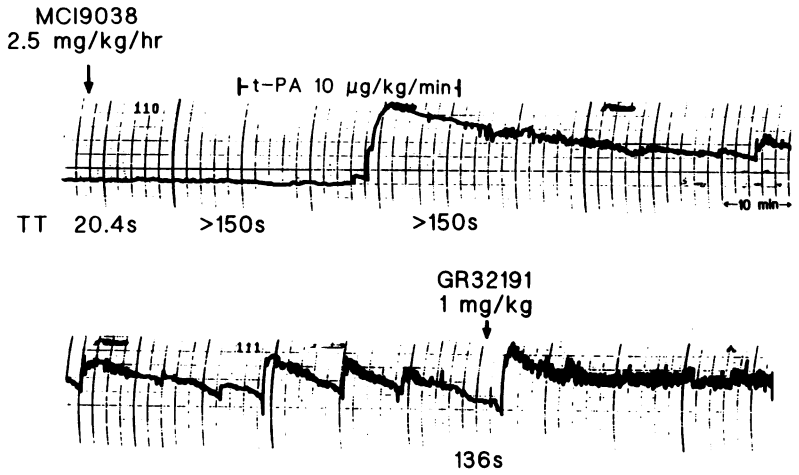

FIG. 5. Continuous recording of coronary blood flow from a single experiment. Coronary thrombosis was induced by passing a 200-μA current through the electrode implanted in the circumflex coronary artery. Two hours later, t-PA was administered intravenously until 10 min after reperfusion. The recording begins 90 min after induction of complete coronary occlusion. The vertical scale represents flow velocity and is in arbitrary units. MCI9038 at 2.5 mg/kg per hr was begun 30 min prior to t-PA and continued for the duration of the experiment. MCI9038 resulted in marked prolongation of the TT. Reperfusion induced by t-PA (indicated by the abrupt increase in flow) was followed by episodes of a gradual reduction in flow followed by an abrupt increase. These cycles continued for 2 hr and were abolished by the TxA_2/prostaglandin endoperoxide receptor antagonist GR32191 at 1 mg/kg, administered intravenously.

include TxA_2 (5, 6). TxA_2 is formed in platelets by cyclooxygenase metabolism of arachidonic acid and is an important mediator of platelet aggregation in response to weak agonists, including low-dose thrombin (36). In patients with acute myocardial infarction, biosynthesis of TxA_2 is increased (32, 33), and inhibition of TxA_2 biosynthesis enhances the response to thromobolytic therapy (37).

In contrast to the effects of platelet inhibition, anticoagulation with heparin exerts only a modest effect during coronary thrombolysis (38, 39). This has suggested that thrombin is not a major factor limiting the response to thromobolytic therapy. However, the activity of heparin may be limited in the setting of platelet activation (13–15). Furthermore, heparin is not specific for thrombin but inhibits the active form of other coagulation factors (40). Therefore, to address the role of thrombin more specifically, we examined the effect of a selective thrombin inhibitor, MCI9038, in a canine model of coronary thrombolysis. In this model, the coronary thrombus formed is rich in platelets (20, 21). Reocclusion consistently occurs after reperfusion with t-PA and is a platelet-dependent event (6). Thus, there is biochemical evidence of platelet activation after reperfusion, and platelet inhibition prevents reocclusion. In contrast, heparin, at a dose which prolongs the aPTT by more than 10-fold, has no effect on reocclusion (6).

MCI9038 prolonged the TT in a dose-dependent manner in this model and selectively inhibited thrombin-mediated platelet activation. Coincident with this, the time to reperfusion was shortened. This effect correlated with prolongation of the TT, suggesting that acceleration of reperfusion is due to inhibition of thrombin and is not a nonspecific effect of the drug. Furthermore, the accelerated reperfusion was not due to an alteration in plasma t-PA. These data are consistent with a previous study, which reported that MCI9038 enhanced thrombolysis by t-PA in a rabbit model of carotid thrombosis (41), and demonstrate that thrombin delays coronary reperfusion induced by t-PA.

In addition to accelerating reperfusion, MCI9038 prevented reocclusion. This effect occurred only at the highest dose which we employed and even then was incomplete. Thus, reperfusion flow was not constant but demonstrated cyclical variations. A similar cyclical flow pattern has been reported to occur at the site of a severe coronary stenosis, where it is platelet dependent (42, 43). Consistent with this mechanism in the present setting, inhibition of platelet aggregation by the addition of GR32191 stabilized coronary flow. GR32191 is a potent and specific TxA_2/prostaglandin

Table 2. TT and percent reocclusion in control experiments and in animals treated with MCI9038 or GR32191 alone or in combination

Treatment	n	TT ratio	% reocclusion
Vehicle	5	1.1 ± 0.1	100
MCI9038	5	7.1 ± 0.13*	100
GR32191 + MCI9038	7	6.8 ± 0.3*	0†
GR32191	6	1.1 ± 0.05	100

MCI9038 was infused at 0.5 mg/kg per hr and GR32191 was 1 mg/kg in all cases. The TT is expressed as a ratio of the measurements at 60 min of MCI9038 or vehicle and at baseline.
*$P < 0.001$ vs. vehicle-only control.
†$P < 0.01$ vs. vehicle-only control.

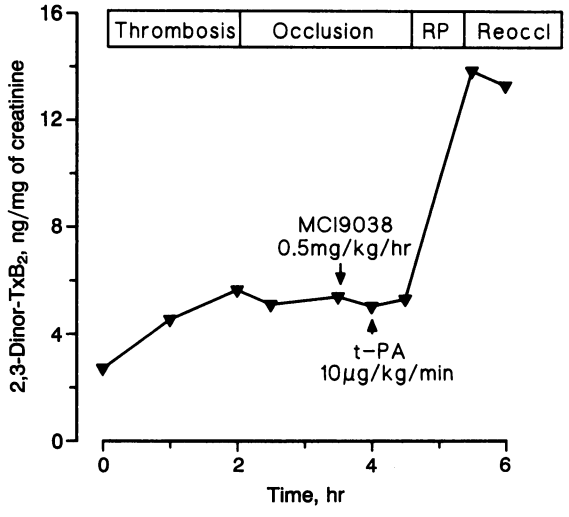

FIG. 6. Urinary excretion of 2,3-dinor-TxB_2 in a single experiment. Excretion of the metabolite increased during induction of coronary thrombosis. A further, marked increase occurred after reperfusion (RP) with t-PA despite pretreatment with MCI9038. Reocclusion (Reoccl) occurred soon after reperfusion.

Table 3. Urinary 2,3-dinor-TxB_2 after reperfusion relative to levels during coronary occlusion in control experiments and in animals pretreated with MCI9038 or GR32191 alone or in combination

Treatment	n	2,3-Dinor-TxB_2, %
Vehicle	4	238 ± 37
MCI9038 (0.2 mg/kg per hr)	4	254 ± 91
(0.5 mg/kg per hr)	5	177 ± 37
(2.5 mg/kg per hr)	5	394 ± 141
MCI9038 (0.5 mg/kg per hr) + GR32191 (1.0 mg/kg)	7	434 ± 179
GR32191 (1.0 mg/kg)	6	350 ± 54

endoperoxide receptor antagonist (22). Thus, GR32191 selectively inhibited arachidonate- and U46619-induced platelet aggregation *ex vivo*. Similarly, GR32191 had no effect on basal or stimulated TxA_2 biosynthesis, measured as excretion of its dinor metabolite. These data suggest that TxA_2-dependent platelet aggregation limited the effects of the thrombin inhibitor in preventing reocclusion.

This interpretation was supported by two additional findings. First, TxA_2 biosynthesis was markedly elevated after reperfusion at all doses of the thrombin inhibitor. Second, combination of MCI9038 and GR32191, at doses of each drug that had no effect when used alone, abolished reocclusion. Addition of GR32191 did not further prolong the TT, consistent with a lack of an effect on the disposition of MCI9038. Furthermore, even at 5 times the dose used in these experiments, GR32191 prevents reocclusion in only 50% of animals (D. M. Kerins, G.A.F., and D.J.F., unpublished observations). Therefore, the increased effect of the combination does not reflect a pharmacokinetic interaction but an additive, if not synergistic, functional interaction between the thrombin inhibitor and the TxA_2 receptor antagonist.

These observations suggest that combination of a TxA_2 inhibitor and an anticoagulant may be more effective than either alone in enhancing the outcome of thrombolytic therapy. Although there has been no randomized trial to address this hypothesis in humans, it is supported by the results of the ISIS-2 trial (37). This study examined the benefits of aspirin and streptokinase in over 17,000 patients with suspected myocardial infarction. Although the efficacy of heparin was not addressed *a priori*, a retrospective analysis demonstrates that the 5-week mortality was 6.4% in patients receiving intravenous heparin in addition to aspirin and streptokinase. In comparison, the mortality was 9.6% in patients treated with aspirin and streptokinase alone (37).

In conclusion, thrombin, a potent platelet activator and procoagulant, limits the response to coronary thrombolysis induced by t-PA, by both delaying reperfusion and inducing acute reocclusion. Furthermore, these studies demonstrate an interaction of potential therapeutic importance between a thrombin inhibitor and a TxA_2 receptor antagonist.

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