Brief Antecedent Ischemia Enhances Recombinant Tissue Plasminogen Activator–Induced Coronary Thrombolysis by Adenosine-Mediated Mechanism

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Background—Clinical studies have implicated preinfarct angina (brief antecedent ischemia/reperfusion [I/R]) as a predictor of more rapid thrombolysis and lower rates of reocclusion. However, the effects of antecedent ischemia on the efficacy of thrombolysis have not been rigorously assessed. Using a canine model of coronary thrombosis, we aimed to (1) reproduce these clinical findings and (2) determine whether release of adenosine (a potent inhibitor of platelet aggregation via stimulation of platelet A2 receptors) during brief I/R contributes to this improved patency.

Methods and Results—To address our first objective, we compared the time required to achieve lysis with recombinant tissue plasminogen activator and patency during the first 2 hours after lysis in dogs in which 1-hour thrombotic occlusion was preceded by brief I/R (10-minute coronary occlusion/10-minute reperfusion) versus 20-minute uninterrupted perfusion (controls). Time to lysis was accelerated in the I/R group versus the control group (11 ± 1 versus 35 ± 6 minutes, P = 0.004). In addition, the duration of subsequent reocclusion was reduced (17 ± 12 versus 30 ± 11 minutes), and the area of the flow-time profile (normalized to baseline flow × 120 minutes) was increased (64 ± 12% versus 35 ± 7%, P = 0.04) in the I/R cohort. The protocol was then repeated, but all dogs were pretreated with the adenosine A2/A1 antagonist CGS 15943 (CGS, 1.5 mg/kg). Time to lysis (38 versus 39 minutes) and subsequent patency were comparable in the CGS control group versus the CGS I/R group.

Conclusions—Brief antecedent I/R enhances the efficacy of coronary thrombolysis in this canine model, which is due, at least in part, to an adenosine-mediated mechanism. (Circulation. 2000;102:88-95.)

Key Words: ischemia □ thrombolysis □ adenosine □ myocardial infarction

Brief “preconditioning” (PC) ischemia in addition to its ability to render myocytes resistant to infarction1,2 may also have favorable effects on arterial patency.3–6 This is supported by our recent observation that 10 minutes of coronary artery occlusion attenuated the subsequent spontaneous formation/dislodgment of platelet thrombi in damaged and stenotic canine coronary arteries, which is due largely to the release of adenosine (a potent inhibitor of platelet aggregation7,8) during the PC stimulus.9 Clinical reports further suggest that the benefits of antecedent ischemia on coronary patency may extend to the setting of persistent thrombotic occlusion: in patients with acute myocardial infarction, preinfarct angina was associated with more rapid thrombolysis6 and lower rates of reocclusion after initial lysis.5 However, the effect of antecedent ischemia on the efficacy of coronary thrombolysis has, to date, not been rigorously assessed. By use of a canine model of occlusive coronary thrombosis, our objectives were to (1) establish whether brief PC ischemia is associated with accelerated recombinant tissue plasminogen activator (rtPA)-induced thrombolysis and better maintenance of patency after initial restoration of blood flow and (2) determine whether, as in models of recurrent platelet-mediated occlusion,3,4 adenosine receptor stimulation may play a role.

Methods

The present study conforms to the principles endorsed in the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996)

Surgical Preparation

Forty mongrel dogs (20±4 kg) were anesthetized with sodium pentobarbital (30 mg/kg IV), intubated, and ventilated with room air. After cannulation of the left jugular vein (for administration of drugs and fluids) and the left carotid artery (for measurement of systemic hemodynamics), the heart was exposed through a left lateral thoracotomy. Two or 3 adjacent segments of the mid left anterior descending coronary artery (LAD) were isolated. The proximal segment served as the site of later thrombosis; the second was instrumented with a Doppler flow probe (Transonic Systems Inc) for monitoring of mean coronary blood flow (CBF); and in animals enrolled in protocols 1, 2, and 3, the third segment was used

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Protocol 1: Thrombus Morphology

Our first aim was to document the consistent formation of an occlusive platelet- and fibrin-rich thrombus in our model (Figure 1). Ten dogs received 10 minutes of mechanically induced LAD occlusion (achieved by placing vascular clamps at the site of later thrombotic occlusion) and 10 minutes of reperfusion (PC group, n=5) or 20 minutes of uninterrupted perfusion (control group, n=5). Coronary thrombosis was then initiated by compressing the proximal LAD segment with hemostats to remove the endothelium, tear the tunica media, and expose the underlying (and highly thrombogenic) tunica adventitia. A micrometerometer constrictor was applied at the site of injury and tightened such that CBF was reduced to 30% to 40% of its baseline value (stenosis), thereby precipitating an immediate decline in CBF due to adhesion and aggregation of platelets at the site of injury. To facilitate formation of a fully occlusive thrombus, stasis was then induced by placing a vascular clamp on the third (distal) LAD segment.

Fifty-five minutes after the onset of thrombosis, the distal clamp was removed, and occlusive thrombosis was confirmed by maintenance of CBF at 0 mL/min. Five minutes later, the animals were euthanized under deep anesthesia by intracardiac injection of KCl, whereas platelets and erythrocytes are not birefringent and thus appear dark.

Protocol 2: Effect of Brief Antecedent Ischemia on Thrombolysis

To address our primary objective (whether brief antecedent ischemia enhances the efficacy of thrombolysis), 14 dogs received 10 minutes of PC occlusion followed by 10 minutes of reperfusion (n=7) or a 20-minute control period (n=7, Figure 1). Coronary thrombosis was then initiated as in protocol 1.

At 50 minutes after the onset of thrombosis, the distal clamp was removed. After confirming thrombotic occlusion, RMBF (ie, collateral perfusion to the ischemic territory) was assessed by injection of radiolabeled microspheres. At 1 hour after occlusion, all dogs received rtPA (1.3 mg/kg IV infused over 1 hour, a gift from Genentech Inc (South San Francisco, Calif)), and the time required to achieve reperfusion (defined below) was noted. CBF was then monitored for 2 hours after the initial reflow.

To confirm that 10 minutes of antecedent ischemia was also effective in eliciting cardioprotection, infarct size was measured by use of routine methods. At the end of the protocol, the LAD was ligated at the site of thrombotic occlusion, Unisperse Blue pigment (CIBA; 0.5 mL/kg) was injected via the left atrial catheter to delineate the area of myocardium at risk of infarction (AR), and the dogs were euthanized as in protocol 1. The hearts were then excised, cut into 3 to 8 transverse slices (10- to 12-mm thickness), and photographed. The slices were incubated in triphenyltetrazolium chloride (10 minutes at 37°C) to delineate necrotic from viable myocardium and photographed again.

Protocol 3: Effect of Adenosine Receptor Antagonist on Thrombolysis

to evaluate our second hypothesis (whether improvements in the efficacy of thrombolysis with PC are mediated by adenosine), protocol 2 was repeated in 13 dogs. In this case, however, all animals received the adenosine A_{1}/A_{2} receptor antagonist CGS 15943 (CGS, 1.5 mg/kg IV bolus, a gift from Novartis Inc, Summit, New Jersey) 10 minutes before the PC (n=6) or control period (n=7, Figure 1). Time to lysis, LAD patency during the 2 hours after lysis, and infarct size were quantified as in protocol 2.

Protocol 4: Effect of Adenosine Receptor Antagonist on Platelet Aggregation

To confirm in vitro findings that CGS has no intrinsic effect on platelet aggregation, 3 dogs underwent LAD injury plus stenosis. However, rather than causing deep arterial injury, we damaged the tunica media without adventitial exposure by compressing the LAD with cushioned hemostats, thereby initiating cyclic variations in coronary flow (CFVs) caused by formation/dislodgment of platelet thrombi rather than persistent thrombotic occlusion.

CBF was monitored for 2 hours after injury plus stenosis. All dogs then received CGS (1.5 mg/kg IV bolus), and LAD patency was monitored for an additional 2 hours. The dogs were then euthanized, and the damaged LAD segment was excised for histological assessment.

Analysis

Histological evaluation of LAD segments (all protocols) was conducted from cross sections (5-μm thickness, 4 to 10 per sample) stained with hematoxylin-eosin and picrosirius red (to visualize adventitial collagen) and viewed at magnifications of ×10 to ×25. All samples were evaluated by using bright-field illumination to confirm the expected loss of endothelium and tearing of the tunica media and to document the presence or absence of adventitial exposure. Identification of thrombotic components was facilitated by viewing picrosirius red–stained sections with polarized light; fibrin fibers are birefringent and appear green when this method is used, whereas platelets and erythrocytes are not birefringent and thus appear dark.

Heart rate and arterial pressure (all protocols) were measured and averaged over 5 cardiac cycles for each sample period. Mean CBF (all protocols) was determined at discrete time points during which coronary flow was stable, ie, at baseline, after CGS treatment (protocol 3), after the PC/control period (protocols 1, 2, and 3), and immediately after application of the stenosis. Time to lysis (protocols 2 and 3) was defined as the time from the onset of the thrombus to the reappearance of the desired coronary flow.
of rtPA infusion until CBF was restored to its stenotic value. Coronary patency after lysis (protocols 2 and 3) and after injury plus stenosis (protocol 4) was assessed by measuring the duration (in minutes) of reocclusion (CBF=0) and percent flow-time area (defined as the area of the flow-time profile throughout each 2-hour observation period) and normalized for each dog to the baseline flow×120 minutes. Total duration of thrombotic occlusion (protocols 2 and 3) was calculated as Σ (1-hour test occlusion+time to achieve reperfusion+duration of reocclusion after initial lysis). RMBF (protocols 2 and 3) was measured in subendocardial and subepicardial tissue blocks cut from the center of the LAD territory with the use of routine methods.1,11 Risk region and infarct size (protocols 2 and 3) were measured from photographic images of the heart slices traced at magnifications of ×2 to ×4. AR and area of necrosis (AN) in each slice were quantified by computerized planimetry, corrected for tissue weight, expressed in grams, and summed for each heart.11

Statistics
CBF and hemodynamics (protocols 1, 2, and 3) were compared by 2-factor ANOVA with replication, followed by the Tukey test. For protocols 2 and 3, the time to lysis, patency after lysis, total duration of LAD occlusion, RMBF, and risk region were compared between matched control and PC groups by t test. Infarct size was compared by t test and by incorporating RMBF and the duration of occlusion as covariates in the analysis. For protocol 4, hemodynamics and coronary patency were qualitatively compared before versus after CGS treatment. All data are reported as mean±SEM.

Results
Protocol 1

CBF and Hemodynamics
Baseline CBF was 10.0±0.9 and 12.0±1.9 mL/min in the control and PC cohorts, respectively, and was reduced to 3.7±0.4 and 3.7±0.3 mL/min on application of the stenosis. Heart rate and arterial pressure did not differ between groups at any time (not shown).

Arterial Injury and Thrombus Morphology
Histological evaluation revealed (1) the absence of endothelium, tearing of the tunica media, and exposure of the adventitia and (2) platelet- and fibrin-rich thrombus within the lumen, with no differences in the severity of arterial injury or thrombus composition between groups (Figure 2).

Protocol 2

CBF and Hemodynamics
CBF was comparable between control and PC groups at baseline, before application of the stenosis (after the transient hyperemia in the PC group), and immediately after the stenosis (flow reduced to 3.3 to 3.5 mL/min, or 39% to 45% of baseline). There were no significant group differences in heart rate or arterial pressure during the protocol (Table 1).

Efficacy of Thrombolysis
The time required to achieve thrombolysis was 35±6 minutes in the control group versus 11±1 minutes in the PC group (P=0.004, Figure 3A). All controls exhibited multiple episodes of reocclusion/reperfusion (CFVs) after initial lysis, with the duration of reocclusion and flow-time area averaging 30±11 minutes and 35±7%, respectively. Although reocclusion was not prevented in the PC group, the duration of reocclusion tended to be reduced (17±12 minutes, Figure 3B) and flow-time area was increased (64±12%, P=0.04; Figure 3C) compared with control values. As a result, the total duration of thrombotic occlusion was 1.5±0.2 versus 2.1±0.2 hours in the PC versus control groups (P=0.07, Figure 3D).

RMBF and Infarct Size
Both RMBF and AR were comparable between groups (Table 2). However, infarct size was smaller in PC dogs versus controls, with AN/AR averaging 16±5% versus 47±8% (P=0.02; Table 2).

Protocol 3

CBF and Hemodynamics
CBF did not differ between groups at baseline and showed the same temporal profile during the PC/control period and
after stenosis as described for protocol 2. CGS had no effect on hemodynamics, and there were no significant group differences in heart rate or arterial pressure (Table 1).

Efficacy of Thrombolysis
Time required to achieve thrombolysis was similar in both CGS-control and CGS-PC groups, averaging 38 ± 6 and 39 ± 4 minutes, respectively (Figure 4A), and there were no differences in subsequent patency or total duration of thrombotic occlusion between the 2 cohorts (Figures 4B to 4D).

RMBF and Infarct Size
RMBF and infarct size did not differ between CGS-control and CGS-PC groups: AN/AR averaged 48 ± 8% and 49 ± 7% (Table 2).

Protocol 4
CBF and Hemodynamics
CBF averaged 13.2 ± 3.1 mL/min at baseline and was reduced to 4.6 ± 0.2 mL/min (39% of baseline) on application of the stenosis. Hemodynamics remained stable throughout the protocol and were not altered by CGS treatment (not shown).

Arterial Injury and Thrombus Morphology
All arteries exhibited endothelial denudation and medial damage, without adventitial exposure (Figure 5A). Remnants of platelet-rich (rather than fibrin-containing) thrombus were present in the lumen.

Coronary Patency
All dogs developed CFVs on application of the stenosis. Neither the duration of total thrombotic occlusion nor flow-time area deteriorated after CGS treatment (Figures 5B and 5C).

Discussion
Antecedent Ischemia Enhances Thrombolysis
We report a significant improvement in the efficacy of rtPA-induced thrombolysis in dogs in which thrombotic occlusion was preceded by a brief 10-minute episode of PC ischemia. These results are consistent with clinical observations of accelerated reperfusion and lower rates of reclosure in patients with versus patients without preinfarct angina. Moreover, these benefits of antecedent ischemia on

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Values are mean ± SEM. CO indicates coronary occlusion; post-CGS, 10 min after CGS administration; post-PC, immediately after PC ischemia; and NA, not applicable. *P < 0.05 vs control; †P < 0.05 vs baseline.
vessel patency may, together with the well-described protective effects of PC on the myocardium, contribute to the favorable association between preinfarct angina and outcome after infarction reported in patients receiving thrombolytic therapy.2,5,6,15

Role of Adenosine

We further found that pretreatment with CGS abrogated the effects of antecedent ischemia on rtPA-induced thrombolysis. These results implicate adenosine released during the PC stimulus and adenosine receptor stimulation in eliciting the more rapid reflow and better maintenance of subsequent patency seen in the PC group.

How might adenosine improve the efficacy of thrombolysis? Numerous studies have documented massive release of adenosine from myocytes after relief of a brief ischemic episode.2,16 Moreover, adenosine, despite its highly labile nature,4,16 initiates a potent and sustained (>3-hour) inhibition of platelet aggregation via A2 receptor-mediated signaling.7,8 Thus, the improved patency observed in the PC group after thrombolysis may reflect an adenosine-mediated attenuation of recurrent platelet-mediated thrombosis analogous to that seen in models of CFVs.3,4

The mechanisms by which PC ischemia accelerated thrombolysis are unclear. One possibility is that PC ischemia, by attenuating initial platelet aggregation, impaired the formation of a fully occlusive thrombus. However, protocol 1 revealed that PC ischemia did not impair the evolution of a persistent platelet- and fibrin-containing clot.

Although thrombus morphology appeared comparable between groups, we speculate that 3 factors might contribute to the more rapid lysis. First, the porosity of the thrombus (determined by the architecture of the fibrin fibers17) might differ, such that penetration of rtPA might be facilitated in the PC cohort. Second, accretion of platelets onto the evolving thrombus may have been inhibited in the PC group, thereby altering thrombus stability, an explanation invoked previously to explain the accelerated reflow reported with administration of platelet glycoprotein IIb/IIIa receptor inhibitors.18 Finally, thrombus stability might be compromised in the PC cohort via adenosine-mediated inhibition of platelet adhesion molecule expression, possibly P-selectin.8

Deleterious Effect of CGS on Coronary Patency?

In protocol 3, the time required to achieve thrombolysis and patency after reflow were comparable in the CGS+PC...
and CGS control groups. However, qualitative comparison of protocols 2 and 3 suggests that reocclusion after thrombolysis was exacerbated by CGS treatment, suggesting that CGS may have had a direct proaggregatory effect.

To address this issue, vessel patency was assessed before versus after CGS treatment in a model of CFVs (protocol 4). We observed no deleterious changes in patency, consistent with ex vivo evidence showing CGS to be devoid of intrinsic effects on platelet aggregation.12,13

An explanation for the poor patency in CGS-treated dogs may be derived from evidence from a canine model of hypoperfusion, which showed that maintenance of stable coronary blood flow is dependent on endogenous adenosine; ie, infusion of a nonselective adenosine receptor antagonist initiated a progressive deterioration in coronary perfusion that was due to the formation of platelet thrombi.7,8 Thus, even in non-PC controls, adenosine may participate in the regulation of platelet activity and coronary patency.

Infarct Size Reduction With Antecedent Ischemia: PC or Accelerated Lysis?

In addition to the enhanced efficacy of thrombolysis, we also observed, in protocol 2, a significant reduction in infarct size in dogs that received antecedent ischemia. Does this represent cardioprotection conferred by brief PC ischemia,1,2 or is it a secondary consequence of the more rapid reflow in the PC group?

To resolve this issue, infarct size was compared between control and PC groups by incorporating, as covariates, the 2 major determinants of infarction in the dog: total duration of occlusion and collateral perfusion (severity of ischemia)11 (Figure 6). Multiple regression analysis confirmed a significant correlation between these variables and infarct size for both control and PC cohorts ($r^2=0.85$, $P<0.02$). However, there was a significant downward shift in the regression plane for the PC group versus controls.
with respect to both collateral perfusion and occlusion time ($P=0.003$ and $P=0.05$ by ANCOVA), indicating that dogs in the PC group developed smaller infarcts over the range of collateral flow values and total occlusion times obtained in the present study. Similar results were obtained when the duration of persistent occlusion (1-hour thrombotic occlusion+time to lysis) rather than the total occlusion time was used as the covariate (not shown). Thus, although the shorter ischemic duration was a contributing factor, the smaller infarct sizes in the PC group were not uniquely due to differences in the duration of ischemia. Rather, classic ischemia-induced cardioprotection was manifest in the PC cohort.

**Loss of Cardioprotection With CGS: Poor Patency or $A_1$ Inhibition?**

The efficacy of infarct size reduction with PC is dependent on ischemic duration and, in the canine model, wanes with occlusions $>90$ minutes.$^{1,2}$ As a result, we propose that the absence of protection seen in PC dogs treated with CGS is a consequence of poor patency (prolonged duration of ischemia) that is due to the inhibition of platelet $A_2$ receptors. However, this conclusion is confounded by the fact that PC-induced cardioprotection is initiated by stimulation of adenosine $A_2$/A$1$ receptors on myocyte membranes; and CGS, despite its nanomolar affinity for the $A_1$ receptor, is not subtype specific$^{12,13}$; ie, the loss of protection seen in PC dogs that received CGS could be due in part to residual $A_1$ receptor antagonism on myocytes.

To distinguish between these possibilities, we conducted supplemental experiments using ZM 241385 (ZM, Tocris-Cookson Inc), a potent and highly selective (1000-fold) $A_2$ antagonist. Five dogs received ZM (1.5 mg/kg IV bolus) and, 10 minutes after treatment, underwent 10-minute PC ischemia plus 10-minute reflow followed by 1 hour of thrombotic occlusion (same design as protocol 3). The time required to achieve thrombolysis (35±3 minutes) and patency after initial lysis (flow-time area 16±2%, mean duration of reocclusion 75 minutes, and total duration of occlusion 2.8±0.4 hours) seen in ZM-treated dogs were comparable to results obtained in CGS+control and CGS+PC groups in protocol 3. Moreover, the infarct size in PC dogs that received ZM (AN/AR=46±9%) was also similar to the value of 49% obtained in the PC+CGS group. These data support the hypothesis that the improved efficacy of thrombolysis achieved with PC ischemia is mediated by an $A_2$ mechanism. Furthermore, although residual $A_1$ effects remain possible, the results suggest that the large infarct sizes seen in antagonist-treated PC dogs are due predominantly to $A_2$ receptor inhibition (presumably on platelets) and the resultant prolonged duration of ischemia rather than to $A_1$ receptor inhibition on myocytes.

Finally, it is perhaps surprising that infarct size was not increased in the CGS+control group of protocol 3 versus the control group of protocol 2, given that the duration of total thrombotic occlusion was 2.9 versus 2.1 hours. This increase in total ischemic burden was due to exacerbated reocclusion after initial lysis (68 versus 30 minutes) rather than differences in the time to achieve reflow (38 versus 35 minutes), suggesting that “stuttering” flow (ie, CFVs with short individual episodes of recurrent ischemia) does not exacerbate necrosis.$^{19}$

**Summary**

Brief antecedent ischemia, in addition to protecting myocytes and limiting infarct size, significantly accelerates rtPA-induced thrombolysis and improves patency during the first 2 hours after reflow in the canine model. Pretreatment with CGS and ZM rendered PC ineffective in augmenting the efficacy of thrombolysis, thereby implicating adenosine release during the PC stimulus and the resultant adenosine receptor stimulation as important contributors to this ischemia-induced improvement in patency.

Our results suggest that stimulation of platelet $A_2$ receptors “triggers” the improved patency achieved with PC ischemia. However, local vasodilation via $A_2$ and/or $A_1$ receptor stimulation on the coronary vasculature or $A_2$/A$1$ stimulation on neutrophils and attenuation of neutrophil “plugging” may also play a role.$^{20,21}$ Moreover, platelet adhesion and aggregation are complex, and other metabolites liberated from ischemic myocardium (eg, nitric oxide and prostaglandins) might also be involved. Finally, the time course of PC-induced adenosine release on vessel patency and the downstream mechanism(s) by which adenosine receptor stimulation elicits an increase in the efficacy of thrombolysis remain to be elucidated.

**References**


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