JACC Vol. 21, No. 5
April 1993:1269–79

Combined Thromboxane A_2 Synthase Inhibition and Prostaglandin Endoperoxide Receptor Antagonism Limits Myocardial Infarct Size After Mechanical Coronary Occlusion and Reperfusion at Doses Enhancing Coronary Thrombolysis by Streptokinase

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Objectives. We sought to examine to what extent a combination of strong thromboxane A_2 synthase inhibition and moderate endoperoxide receptor blockade enhances streptokinase-induced coronary thrombolysis and provides anti-ischemic activity independent from its thrombolytic activity.

Methods. Coronary thrombi, induced by crush injury and stenosis of the coronary artery, were lysed with streptokinase, 10,000 IU/kg body weight over 90 min, in anesthetized dogs receiving solvent (n = 11), ridogrel, 0.31 mg/kg intravenously, for thromboxane A_2 synthase inhibition (n = 7) or ridogrel, 5 mg/kg, for additional prostaglandin endoperoxide receptor antagonism in addition to thromboxane A_2 synthase inhibition (n = 7) 10 min before the administration of streptokinase.

Results. Thrombolytic efficacy was greatest in animals receiving both dual-acting ridogrel, 5 mg/kg, intravenously, and streptokinase as evidenced by the highest incidence of high grade coronary reperfusion (solvent 3 of 11; ridogrel, 0.31 mg/kg, 5 of 7; ridogrel, 5 mg/kg, 7 of 7; $p < 0.05$ vs. solvent) within the shortest delay (solvent 210 min; ridogrel, 0.31 mg/kg, 85 min; ridogrel, 5 mg/kg, 37 min; $p < 0.05$ vs. solvent and ridogrel, 0.31 mg/kg) and the lowest incidence of reocclusion (solvent 5 of 7; ridogrel, 0.31 mg/kg, 2 of 7; ridogrel, 5 mg/kg, 1 of 7; $p < 0.05$ versus solvent).

Myocardial infarct size after coronary artery ligation (90 min) and subsequent reperfusion (150 min) in anesthetized dogs was 49.3 ± 4.5% versus 29 ± 3.9% ($p < 0.05$ vs. solvent) of the area of the left ventricle at risk in dogs receiving solvent (n = 9) or ridogrel, 5 mg/kg intravenously (n = 10), respectively, despite similar hemodynamic characteristics, collateral blood flow and area at risk in both groups.

Conclusions. Combined thromboxane A_2 synthase inhibition and endoperoxide receptor antagonism 1) upgrades thrombolysis with streptokinase in canine coronary arteries, 2) limits myocardial infarct size after nonthrombotic coronary occlusion and reperfusion, and 3) may preserve ventricular function compromised by coronary occlusion through dual manipulation of the arachidonic acid cascade in blood and myocardial tissue, respectively.

(J Am Coll Cardiol 1993;21:1269–79)

In humans, morbidity and mortality after acute myocardial infarction are related to the amount of necrotic cardiac tissue subsequent to the event (1). Therefore, treatment of acute myocardial infarction is designed to achieve the salvage of ischemic myocardium from eventual necrosis. Reducing the duration of the ischemic period by means of early coronary reperfusion with fibrinolytic agents is one way to improve ventricular function and to reduce the incidence of death after acute coronary thrombosis in humans (2,3). Limiting ischemic damage to the myocardium directly by reducing ischemic or reperfusion injury, or both, may represent an additional approach to reduce myocardial infarct size and functional impairment after coronary occlusion and reperfusion (4).

In experimental settings, adjunctive treatment with thromboxane A_2 synthase inhibition (5) or endoperoxide receptor antagonism (6,7) differentially attenuates some of the limitations of thrombolytic efficacy with streptokinase, such as delayed (6) or low grade (7) coronary reperfusion and early coronary reocclusion (5,6). In addition, thromboxane A_2 synthase inhibitors (8,9) and endoperoxide receptor antagonists (10–14), when given separately, have been demonstrated to reduce myocardial infarct size elicited by coronary occlusion and reperfusion in dogs (10,11), cats (8,12), rats (13) and monkeys (14). However, a combination of thromboxane A_2 synthase inhibition with endoperoxide receptor antagonism has a greater antiplatelet and antithrombotic effect than has either single intervention or aspirin in 1) preventing occlusive thrombosis in extensively damaged canine coronary arteries (15–17) and rat carotid arteries (18),

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Manuscript received May 11, 1992; revised manuscript received September 17, 1992, accepted September 22, 1992.

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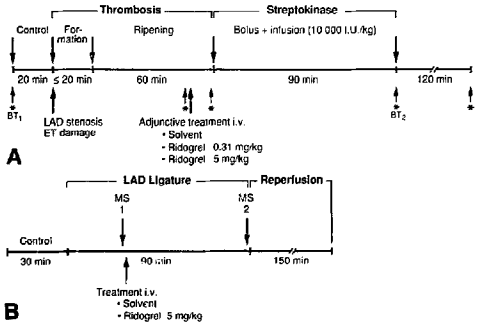

Figure 1. Experimental protocols. **A,** Coronary thrombolysis. **B,** Myocardial infarct size. *Blood sampling; BT_1 = bleeding time 1; BT_2 = bleeding time 2; BT = bleeding time; ET = endothelial; i.v. = intravenously; LAD = left anterior descending coronary artery; MS_1 = first injection of microspheres; MS_2 = second injection of microspheres.

2) epinephrine-induced exacerbations of cyclic flow reductions in stenosed canine coronary arteries (19), 3) enhancing thrombolysis with recombinant tissue-type plasminogen activator (rt-PA) and heparin in canine coronary arteries (20), and 4) reducing platelet hemostatic plug formation (21,22).

Therefore, we studied 1) to what extent a combination of strong thromboxane A_2 synthase inhibition combined with a modest degree of endoperoxide receptor blockade enhances streptokinase-induced coronary thrombolysis in dogs, and 2) whether this combined therapy also provides salvage of canine cardiac tissue after postischemic reperfusion. For that purpose, we used two compounds already used in humans: 1) streptokinase, producing fibrinolysis (23); and 2) ridogrel, providing thromboxane A_2 synthase inhibition at low doses and additional endoperoxide receptor antagonism at higher doses (21,22,24).

Methods

Surgical procedure. Male or female mongrel dogs (n = 54) weighing 18 to 28 kg were anesthetized with sodium pentobarbital (30 mg/kg intravenously). After endotracheal intubation, ventilation was performed with a mixture of 60% oxygen and 40% nitrous oxide using a Siemens servoventilator 900 c. The left jugular vein was cannulated for the administration of compounds. A high fidelity 7F catheter-tipped manometer (Philips) was inserted through the carotid artery in the left ventricle for measurement of left ventricular pressure, left ventricular end-diastolic pressure and the maximal and minimal first derivative of the left ventricular pressure (dP/dt). A catheter was inserted through the femoral artery and connected to a P23 pressure transducer for the measurement of systolic and diastolic blood pressure. Heart rate was derived from the electrocardiographic (ECG) lead II.

A thoracotomy was performed through the fifth intercostal space and the heart was suspended in a pericardial cradle. The left anterior descending coronary artery was dissected free over a length of ±1 cm at 1.5 to 2 cm from its origin. An electromagnetic flow probe was placed on the aorta (diameter 16 to 18 mm) and another on the anterior descending coronary artery (diameter ±2 mm); both were connected to electromagnetic blood flow meters (Skalar), calibrated in liters/min and ml/min, respectively.

Hemodynamic data were recorded on a multichannel recorder and, by means of transducer amplifiers, fed into the analog for digital conversion on a Micro-PDP 11 (Digital Equipment) computer system and displayed on a Macintosh computer (Apple Computer, Inc.).

Experimental protocols. The experimental protocols are summarized in Figure 1. Baseline recordings of the hemodynamic variables were obtained over a period of 20 min, during which bleeding time was measured and blood samples were taken for the determination of serum levels of thromboxane B_2, 6-keto-prostaglandin $F_{1\alpha}$ and prostaglandin E_2 and the assessment of coagulation and fibrinolysis variables.

In the thrombolysis experiments, a constrictor was placed on the left anterior descending coronary artery (diameter 1 to 1.3 mm) to induce a 50% reduction in flow. The constrictor was temporarily removed and the wall of the anterior descending coronary artery was damaged by crushing it with a forceps. Thereafter, the constrictor was replaced and the coronary artery was occluded for 10 min by a clamp placed distally from the constrictor. On release of the clamp in the presence of the constrictor, coronary blood flow decreased spontaneously to zero because of the formation of an occluding thrombus. Persistence of complete coronary obstruction was monitored for 60 min. Fifty minutes after occlusive thrombus formation, blood samples were taken for the measurement of serum prostanoid levels. Thereafter, the dogs were randomly assigned to one of the following groups: group I (n = 11) received saline solution, 0.2 ml/kg intravenously, to serve as control dogs and group II (n = 7) received

ridogrel, 0.31 mg/kg intravenously, for thromboxane A_2 synthase inhibition (17,21,25). Group III (n = 7) received ridogrel, 5 mg/kg intravenously, for thromboxane A_2 synthase inhibition combined with prostaglandin endoperoxide receptor antagonism (17,21,25). Group IV (n = 3) also received saline solution instead of active medication. Ten minutes after those treatments, another blood sample was taken for determination of serum prostanoid levels. Thereafter, thrombolysis was initiated with streptokinase, 1,000 IU/kg intravenously as a bolus injection, followed by a continuous infusion of 100 IU/kg/min during 90 min (total dose 10,000 IU/kg) in groups I to III. In contrast, group IV received saline solution instead of streptokinase. At the end of the streptokinase infusion, blood samples were taken for serum prostanoid determinations and bleeding times were measured. At the end of the experiment (210 min after initiation of thrombolysis), blood samples were again taken to determine the serum levels of prostanoids, coagulation and fibrinolysis variables and plasma levels of ridogrel.

Times required to achieve low grade restoration of coronary blood flow to >5% but <50% of the baseline value relative to the prethrombosis values or high grade coronary reperfusion (that is, restoration of coronary blood flow to >50% of the baseline value) were recorded; in case no coronary reperfusion occurred after the administration of streptokinase, the duration of the experimental period (210 min) was used for further data processing.

The maximal extent of coronary blood flow achieved on reperfusion as well as the flow at the end of the experiment at 210 min after exposure to streptokinase were recorded. Additionally, the incidence of 1) cyclic reperfusion and reocclusion patterns characterized by reductions in coronary blood flow to zero flow for <2 min before renewed reperfusion or permanent reocclusion; and 2) complete reocclusions, characterized by zero coronary blood flow for ≥10 min, were recorded in each adjunctive treatment group.

In experiments for the determination of myocardial infarct size, a small segment of the left anterior descending coronary artery, 1.5 to 2 cm from its origin, was dissected free. Coronary artery ligation was performed during 90 min, followed by 150 min of reperfusion.

Ridogrel, 5 mg/kg intravenously (n = 10), or solvent, 0.2 ml/kg intravenously (n = 9), was given intravenously 30 min after the onset of coronary occlusion. Regional myocardial blood flow was measured 25 min after left anterior descending coronary artery occlusion just before the administration of solvent or ridogrel and again at the end of the occlusion period; for that purpose, 15-μm radiolabeled microspheres (cesium-141, stannum-113, ruthenium-103 and niobium-95) were injected into the left atrium (Fig. 1B).

At the end of the experiment, the animals were killed with an overdose of sodium pentobarbital and myocardial infarct size and area at risk were measured using a combined Evans blue–triphenyltetrazolium chloride staining technique (26). The accuracy of the triphenyltetrazolium chloride method in identifying infarct size in the experimental conditions of the present study has been described elsewhere (26). The area at risk for myocardial infarction after mechanical coronary artery occlusion (90 min) and subsequent reperfusion (150 min) is expressed as a percent of the total mass of the left ventricle. Infarct size is then expressed as a percent of the area at risk in each animal (26,27).

Thromboxane A_2 synthase/prostaglandin endoperoxide receptor antagonism by ridogrel. The capacity of ridogrel to inhibit platelet thromboxane A_2 synthase was assessed by measuring its effect on serum prostanoid levels. For the determination of serum prostanoid levels, venous whole blood (1.6 ml) anticoagulated with sodium citrate (0.38%) was warmed to 37°C (10 min) and coagulated (1 h at 37°C) by the addition of calcium chloride (0.2 ml; 1×10^{-3} mol/liter final concentration) and thrombin (0.2 ml; 20 NIH U/ml final concentration). Thereafter, cell free serum was prepared by repeated centrifugations (15 min × 350 *g*; 5 min × 10,000 *g*) and stored at −25°C. Serum levels (ng/ml) of thromboxane B_2, 6-keto-prostaglandin $F_{1\alpha}$ and prostaglandin E_2 were assessed by radioimmunoassay using specific antisera and tritiated tracers (21,25).

Antagonism by ridogrel of canine platelet thromboxane A_2 and prostaglandin endoperoxide receptors at plasma levels of the compound achieved after its in vivo administration was assessed using platelet aggregation experiments. For that purpose, the inhibition by ridogrel, 1 to 100 μmol/liter, of canine platelet aggregation, triggered by U-46619 after presensitization with l-epinephrine, was measured in vitro as described previously (17,20). Briefly, 0.4 ml of citrated platelet-rich plasma was incubated (10 min at room temperature and 5 min at 37°C) with 0.05 ml of HEPES buffer (200 mmol/liter, pH 7.5) and 0.05 ml of solvent or 10 μl of ridogrel (1 to 100 μmol/liter) supplemented with 0.01 ml of l-epinephrine (10 μmol/liter, 2 min at 37°C) before the addition of U-46619 (1 μmol/liter) and the measurement of the reaction in an aggregometer. The extent of the platelet response was measured by calculation of the speed of increase of light transmission through the stirred sample (slope in % transmission/min). Percent inhibition of U46619-triggered platelet aggregation by ridogrel was calculated versus reactions in paired solvent-treated samples.

Plasma coagulation and fibrinolysis. To assess pharmacologic effects of treatment on plasma coagulation and fibrinogenolysis, the activated partial thromboplastin time and plasma fibrinogen levels were assayed on citrated platelet-poor plasma obtained from blood supplemented with aprotinin (1,000 IU/ml) using routine procedures (28). Plasma fibrinogen levels were expressed in mg/100 ml. Values after medication were compared with the premedication values in the same dogs.

A possible influence of ridogrel on the enzymatic pathways involved in streptokinase-induced fibrinolysis was checked using a diluted whole blood clot lysis test on canine blood. Briefly, canine citrated (sodium citrate 0.013%) blood was diluted 1/10 with ice-cold 0.07 mol/liter phosphate buffer pH 7.4, containing 0, 1, 2.5, 5 or 10 IU/ml of streptokinase

and 10 μmol/liter of ridogrel or its solvent (0.15 mol/liter of sodium chloride), incubated for 5 min at 37°C and thereafter coagulated with thrombin (2.5 NIH U/ml). After further incubation (1 h at 37°C), the residual clot was rinsed with 0.15 mol/liter of sodium chloride, dissolved in 0.1 N of potassium hydroxide and quantified by a photometric assessment of its hemoglobin content. The enzymatic fibrinolytic activity of streptokinase is reflected by the size of the residual clot and is expressed as a percent of a nonlysed control sample. Values in the presence of ridogrel were compared with those obtained in the presence of solvent (25).

Hemostasis. The effect of treatment on bleeding time was determined using a spring-loaded blade device (Simplate II, General Diagnostics) (29). Incisions were made on the dorsal side of the left shaved foreleg and blood was blotted until bleeding had stopped for ≥1 min or for 30 min when bleeding continued. Bleeding times were recorded in seconds; those >30 min were recorded as equal to 1,800 s. Values recorded after the administration of solvent or ridogrel were compared with the premedication values obtained in the same dog.

Morphologic study. Using light microscopy, damage to the vessel wall and the subsequent thrombus formation were evaluated in dogs in group IV (n = 3) receiving no active treatment. At the end of the experiment (270 min after initiation of thrombosis), a 2-cm long segment of the left anterior descending coronary artery with the constrictor in place was excised and immersed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer (pH 7.4) for ≥24 h. The areas of the left anterior descending coronary artery under, proximal and distal to the constrictor were separated, segmented and rinsed at 4°C in 2% osmium tetroxide in 0.05 mol/liter of Veronal acetate buffer (pH 7.4) containing 3.2% sucrose. After rinsing in 0.05 mol/liter of Veronal acetate buffer supplemented with 7% sucrose (pH 7.4, 4°C) for 5 min, the tissue was impregnated with 0.5% uranyl acetate buffer (pH 5.2) for 40 min at 4°C, dehydrated in graded series of ethanol and embedded in Epon. Transverse sections, 2 μm thick, were prepared and stained with toluidine blue for microscopic analysis (17).

Statistical analysis. Results are expressed as mean value ± SEM for continuous data. Baseline values of the hemodynamic variables in the experimental groups were compared using analysis of variance (ANOVA). Time to event measurements are represented as median values and 95% confidence limits based on the binomial distribution. Non-gaussian data were analyzed for intergroup comparisons by means of a two-tailed Mann-Whitney *U* test (30). Counted data were analyzed by the Fisher exact probability test. In all statistical procedures, Bonferroni's inequality was used to correct for multiple comparisons. Analysis of covariance with perfusion area as the covariate was used to assess the effect of treatment on myocardial infarct size (31). The time course of potential effects elicited by the administration of solvent or compounds on hemodynamic variables and myocardial blood flow was analyzed using a multivariate ANOVA for repeated measures (32).

Results

Morphologic study. Light microscopic analysis of left anterior descending coronary artery segments revealed extensive vessel wall damage associated with occlusive thrombus formation. In contrast to the rather normal appearance of distal segments without crush injury, disconnection of the elastica interna from the vessel wall, disruption of the media and focal insudation of red blood cells and polymorphonuclear white cells were prominent in arterial segments underneath the coronary constrictor. The occlusive thrombus adhering to the site of vessel damage consisted of a mixture of platelet aggregates, intermingled with fibrin threads entrapping red blood cells, and was infiltrated with polymorphonuclear cells (Fig. 2).

Coronary thrombolysis. Baseline hemodynamic variables (heart rate, systolic blood pressure, diastolic blood pressure, maximal and minimal first derivative of the left ventricular pressure (dP/dt), left ventricular end-diastolic pressure and flow values in the left anterior descending coronary artery before and after coronary stenosis) were not significantly different among the various experimental groups (Table 1).

In the control period, coronary blood flow showed a stable and constant pattern. Stenosis of the coronary artery at the site of crush injury to the vessel resulted in an occlusive thrombus within 20 min in all preparations. At this point in the experimental protocol, seven dogs were excluded from further analysis (in three dogs, development of a thrombotic occlusion persisting for 60 min did not occur; in another four dogs, death occurred shortly after coronary thrombosis as a result of ventricular fibrillation).

After the administration of solvent or ridogrel and subsequent infusion of streptokinase (groups I, II and III), heart rate remained constant and blood pressure decreased slightly and to a similar extent in all groups. A slight decrease in left ventricular performance (maximal and minimal left ventricular dP/dt) occurred in all groups during the total observation period relative to the value before thrombosis.

The pattern of coronary reperfusion (Fig. 3) and the overall efficacy of thrombolysis elicited by streptokinase were markedly influenced by the adjunctive treatment given in addition to the fibrinolytic agent; both doses of ridogrel were superior to solvent (Table 2, Fig. 3 to 5). Restoration of blood flow >5% but <50% of the prethrombosis values (low grade reperfusion) occurred in 7 (63%) of 11 solvent-treated dogs (group I) but in all animals receiving ridogrel in a low dose (group II) or a high dose (group III) ($p < 0.05$ vs. solvent). In contrast, high grade coronary reperfusion >50% of the prethrombosis level was achieved with streptokinase in 3 (33%) of 11 solvent-treated dogs, in 5 (71%) of 7 dogs receiving ridogrel at a low dose (0.31 mg/kg intravenously; $p < 0.05$ vs. solvent) and in all 7 (100%) of 7 dogs given the

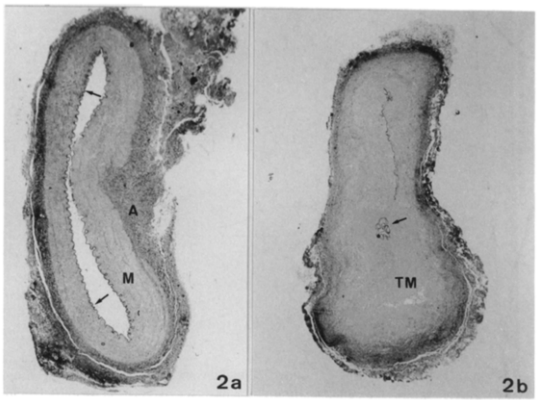

Figure 2. Transverse sections of the left anterior descending coronary artery outside **(a)** and under **(b)** the constrictor. **a,** Normal morphology of the vascular wall showing a noninterrupted internal elastic lamina **(arrows). b,** In this area under the constrictor, part of the internal elastic lamina **(arrow)** is disconnected from the vessel wall and surrounded by platelet-rich thrombus material (TM); the thrombotic occlusion of the artery is nearly complete. Magnification ×45, reduced by 20%. A = adventitia; M = media.

highest dose of ridogrel (5 mg/kg intravenously; $p < 0.05$ vs. solvent) in addition to streptokinase (Table 2).

The duration of streptokinase infusion required to achieve a low grade coronary reperfusion was significantly shorter with both doses of ridogrel as adjunctive medication in comparison with solvent (Fig. 4, upper left panel). However, the infusion period of streptokinase to reach high grade coronary reperfusion was significantly shorter in dogs treated with the highest dose of ridogrel (median 37 min) than in those receiving the lower dose of the compound (median 85 min) or solvent (median 210 min) (Fig. 4, upper right panel). The maximal extent of coronary reperfusion achieved during the experimental period (Fig. 4, lower left panel) as well as the eventual level of coronary blood flow at termination of the experiment (Fig. 4, lower right panel) were significantly higher with both doses of ridogrel relative to solvent.

The incidence of cyclic reperfusion and reocclusion was significantly lower in dogs receiving adjunctive treatment with either dose of ridogrel (four [57%] of seven dogs with 0.31 mg/kg and three [42%] of seven with 5 mg/kg) than with solvent (six [85%] of seven) (Table 2).

Moreover, in dogs treated with streptokinase, the incidence of permanent reocclusion within the experimental observation period was significantly lower with ridogrel, 0.31 mg/kg (two [28%] of seven) and ridogrel, 5 mg/kg (one [14%] of seven), than with solvent (five [71%] of seven) (Table 2).

Table 1. Baseline Hemodynamic Variables and Coronary Blood Flow in the Various Adjunctive Treatment Groups Before Induction of Occlusive Coronary Artery Thrombosis

	Solvent (ml/kg)	Ridogrel (mg/kg intravenously)	
	0.2 (n = 11)	0.31 (n = 7)	5 (n = 7)
SBP (mm Hg)	130 ± 10	125 ± 7	123 ± 8
DBP (mm Hg)	85 ± 5	95 ± 8	79 ± 6
HR (beats/min)	130 ± 10	122 ± 9	120 ± 14
LV dP/dt (mm Hg/s)			
Maximal	2,038 ± 1.15	2,024 ± 107	2,040 ± 128
Minimal	−1,193 ± 185	−2,178 ± 92	−1,909 ± 147
LVEDP (mm Hg)	5.8 ± 0.8	7.7 ± 0.3	7.4 ± 0.8
CBF (ml/min)			
Control	33.8 ± 2.0	30.4 ± 2.3	36.7 ± 2.3
Stenosis	17.9 ± 1.2	16.0 ± 1.7	18.9 ± 1.2

$p > 0.05$ by analysis of variance. CBF = coronary blood flow; DBP = diastolic blood pressure; dP/dt = first derivative of left ventricular pressure; HR = heart rate; LV = left ventricular; LVEDP = left ventricular end-diastolic pressure; SBP = systolic blood pressure.

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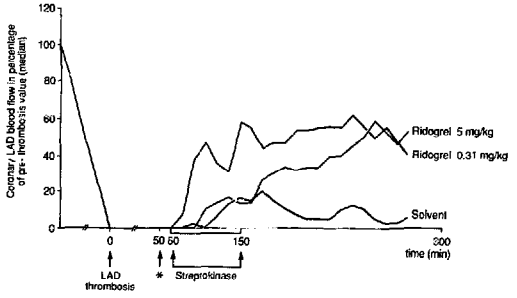


Figure 3. Effect of adjunctive medication on the time course of reperfusion during streptokinase-induced thrombolysis of canine coronary arteries. Occlusive thrombosis (60 min) was induced by crush injury stasis (10 min) in stenosed coronary arteries. Adjunctive intravenous (i.v.) medication (solvent, n = 11; ridogrel, 0.31 mg/kg, n = 7; ridogrel, 5 mg/kg, n = 7) was administered 50 min after initiation of thrombosis and 10 min before intravenous administration of streptokinase (1,000 IU/kg bolus injection; 100 IU/kg per min during 90 min). **Data lines** represent the mean values of seven or more experiments. $p < 0.05$ versus solvent for the total time course of coronary reperfusion with ridogrel, 0.31 and 5 mg/kg intravenously. LAD = left anterior descending coronary artery.

In the three dogs receiving solvent as adjunctive intervention and solvent instead of active thrombolytic treatment (group IV), no coronary reperfusion occurred during the experimental period.

Myocardial infarct size induced by mechanical coronary occlusion and reperfusion. In comparison with that of solvent (n = 9), the intravenous administration of ridogrel (5 mg/kg; n = 10) 30 min after coronary occlusion elicited no significant changes in hemodynamic variables recorded during mechanical coronary artery occlusion and subsequent reperfusion. Myocardial collateral blood flow in endocardial, midmyocardial and epicardial areas after 25 min of coronary occlusion, that is, before the administration of solvent (total collateral flow 14.2 ± 6.2 ml/100 g per min) or ridogrel (14 ± 8 ml/100 g per min), and at termination of the ischemic period (10 ± 9.2 ml/100 g per min in the solvent-treated group; 13.3 ± 7.3 ml/100 g per min in the ridogrel-treated group) were comparable in both groups. Areas at risk for myocardial infarction after coronary occlusion and reperfusion were similar in dogs receiving solvent (area at risk 33.2 ± 2.1% of the left ventricle, n = 9) or ridogrel (area at risk 36.3 ± 1.5% of the left ventricle, n = 10). In contrast, myocardial infarct size at the end of the reperfusion period was significantly ($p < 0.05$) smaller after ridogrel (infarct size 29 ± 3.9% of area at risk) than after solvent (infarct size 49.3 ± 4.3% of area at risk) (Fig. 5).

Table 2. Effects of Adjunctive Medication on the Thrombolytic Efficacy of Streptokinase in Thrombosed Canine Coronary Arteries

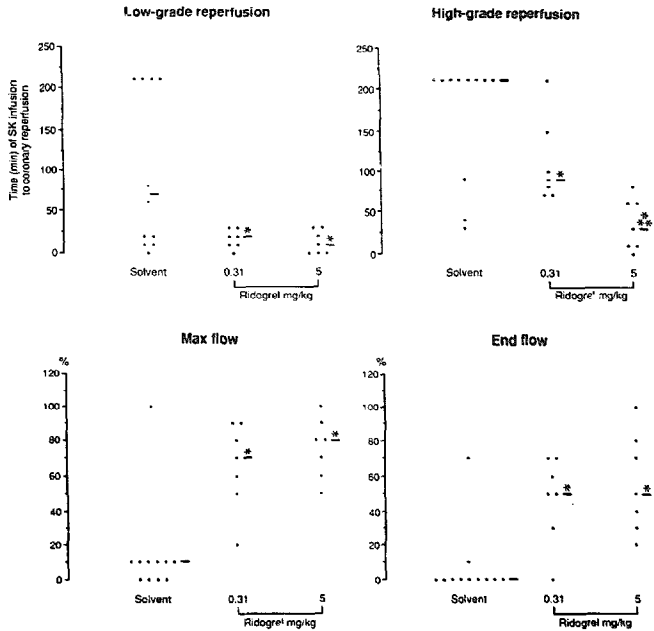
	Solvent (mg/kg)	Ridogrel (mg/kg intravenously)	
	0.2 (n = 11)	0.31 (n = 7)	5 (n = 7)
Low grade coronary reperfusion	7/11	7/7*	7/7*
High grade coronary reperfusion	3/11	5/7*	7/7*
Followed by cyclic reperfusion reocclusion	6/7	4/7	3/7*
Followed by permanent reocclusion	5/7	2/7*	1/7*

*$p < 0.05$ versus solvent by Fisher exact test. Data are presented as number of dogs.

Thromboxane A_2 synthase/prostaglandin endoperoxide antagonism by ridogrel. In dogs receiving solvent as adjunctive treatment before streptokinase (group I, n = 11), levels of thromboxane B_2 (before 244 ± 17 ng/ml; at 210 min 256 ± 24 ng/ml), prostaglandin $F_{2\alpha}$ (before 2.7 ± 0.2 ng/ml; at 210 min 3.7 ± 0.4 ng/ml) and 6-keto-prostaglandin $F_{1\alpha}$ (before 4 ± 1 ng/ml; at 210 min 9 ± 5 ng/ml) in serum of spontaneously coagulated whole blood remained comparable before and at various intervals up to 210 min after the initiation of fibrinolysis.

In contrast, serum levels of thromboxane B_2 were reduced significantly and to the same extent at various intervals after the low dose (0.31 mg/kg; group II) of ridogrel (before 196 ± 44 ng/ml; at 210 min 56 ± 23 ng/ml) and the high dose (5 mg/kg; group III) of the compound (before 253 ± 55 ng/ml; at 210 min 6 ± 2 ng/ml) in comparison with premedication values. Serum levels of prostaglandin E_2 were significantly and to the same extent higher after ridogrel, 0.31 mg/kg: before 5 ± 1 ng/ml; at 210 min 151 ± 30 ng/ml; and after ridogrel, 5 mg/kg: before 4 ± 1 ng/ml; at 210 min 218 ± 55 ng/ml. Likewise, serum levels of 6-keto-prostaglandin $F_{1\alpha}$ were significantly higher than pretreatment values after both doses of ridogrel (ridogrel, 0.31 mg/kg: before 3 ± 0.2 ng/ml; at 210 min 19 ± 5 ng/dl; ridogrel, 5 mg/kg: before 3 ± 0.3 ng/ml; at 210 min 18 ± 2.4 ng/ml).

Plasma levels of ridogrel were 1.65 ± 0.2 and 0.48 ± 0.1 μg/ml at 10 and 210 min, respectively, after the 0.31 mg/kg dose (group II) and reached values of 30.9 ± 2.3



and 27.5 ± 5.9 μg/ml at those intervals after the 5 mg/kg dose (group III) in dogs subsequently treated with streptokinase.

In vitro, concentrations of ridogrel matching the highest plasma level (1.65 μg/ml or 0.45 μmol/liter) obtained after the 0.31 mg/kg dose marginally reduced the extent of aggregation induced by U-46619 (1 μmol/liter) in citrated canine platelet-rich plasma presensitized with a subthreshold concentration of l-epinephrine (10 μmol/liter). In contrast, such a platelet reaction was substantially inhibited (27.1 ± 4.7%; $p < 0.05$ vs. solvent) by ridogrel in vitro at a concentration matching the highest level obtained after the 5 mg/kg dose in vivo (30.9 μg/ml or 8.5 μmol/liter).

Coagulation, fibrinolysis and hemostasis. In dogs receiving solvent (group I), low dose ridogrel (group II, 0.31 mg/kg) or high dose ridogrel (group III, 5 mg/kg), thrombolysis with streptokinase also produced a reduction in the plasma coagulation capacity as reflected by a significant prolongation of the activated partial thromboplastin time (Fig. 6A) and fibrinogenolysis as evidenced by a significant reduction in plasma fibrinogen levels (Fig. 6B). However, the extent of

Figure 4. Effect of adjunctive medication on reperfusion variables during streptokinase-induced thrombolysis of canine coronary arteries and occlusive thrombosis (60 min) induced by crush injury and stasis (10 min) in stenosed coronary arteries. Adjunctive intravenous medication (solvent, n = 11; ridogrel, 0.31 mg/kg, n = 7; ridogrel, 5 mg/kg, n = 7) was administered 50 min after initiation of thrombosis and 10 min before intravenous administration of streptokinase (SK, 1,000 IU/kg bolus injection; 100 IU/kg per min intravenously during 90 min). **Upper panel,** Time after initiation of streptokinase administration to reach reperfusion. **Lower panel,** Extent of maximal (Max) coronary reperfusion (left) and coronary flow at the end of the experiment (right) as a percent of prethrombosis values. *$p < 0.05$ versus solvent. · = individual values; – = median values.

such changes was similar in all groups. Template bleeding times were prolonged significantly after the administration of streptokinase in animals additionally treated with solvent (group I). Ridogrel in the low dose of 0.31 mg/kg (group II) produced no further statistically significant prolongation relative to solvent. By contrast, high dose ridogrel (group

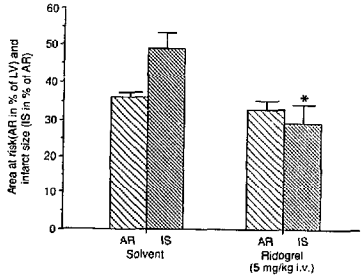


Figure 5. Reduction in myocardial infarct size (IS) after ridogrel without effect on the area at risk (AR) in comparison with solvent in dogs with mechanical left anterior descending coronary occlusion (90 min) followed by reperfusion (150 min). Solvent (n = 9) or ridogrel (5 mg/kg intravenously [i.v.], n = 10) was administered at 25 min of occlusion. *$p < 0.05$ versus solvent. LV = left ventricle.

III) resulted in template bleeding times significantly longer than those recorded after solvent or the low dose of the compound in dogs treated with streptokinase (Fig. 6C). In vitro, lysis within 1 h at 37°C by streptokinase (0 to 10 U/ml) of 1/10 diluted canine whole blood clotted with thrombin was identical in the presence of ridogrel (10 μmol/liter) or its solvent (results not shown).

Discussion

The present study demonstrates that pharmacologic manipulation of arachidonic acid metabolism and receptor effects, when appropriately balanced in terms of specific thromboxane A_2 synthase inhibition and additional endoperoxide receptor antagonism has two important actions. 1) It substantially enhances the thrombolytic efficacy of streptokinase; and 2), as a complementary effect, provides salvage of cardiac tissue from reperfusion damage subsequent to mechanically induced myocardial ischemia at the dose upgrading the thrombolytic efficacy of streptokinase in an optimal fashion.

Indeed, the overall efficacy of thrombolysis achieved with streptokinase against occlusive platelet-rich coronary thrombi is enhanced when ridogrel is used as an adjunctive medication to the fibrinolytic agent. Such an upgrading of thrombolysis is evidenced by the shorter period of fibrinolytic treatment required to reach high grade reperfusion, the increased incidence and extent of maximal coronary reperfusion as well as the reduced occurrence of reocclusion in animals receiving ridogrel compared with solvent in addition to streptokinase. Moreover, the substantial difference in efficacy in that respect between the low and high dose of ridogrel as adjunctive interventions points to a positive

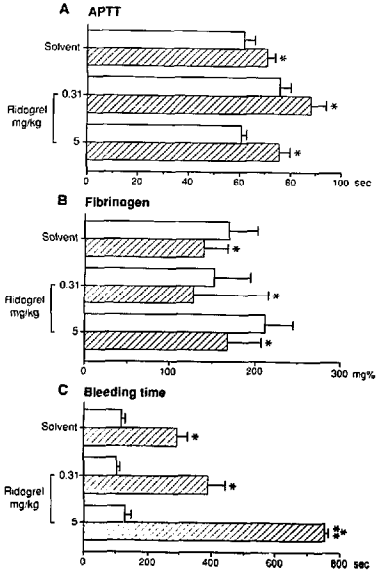


Figure 6. Effect of streptokinase and adjunctive medication on plasma coagulation, fibrinogenolysis and bleeding time in dogs subjected to coronary thrombolysis and occlusive thrombosis (60 min) induced by crush injury stasis (10 min) in stenosed coronary arteries. Adjunctive intravenous medication (solvent, n = 11; ridogrel, 0.31 mg/kg, n = 7; ridogrel, 5 mg/kg, n = 7) was administered 50 min after initiation of thrombosis and 10 min before intravenous administration of streptokinase (1,000 IU/kg bolus injection; 100 IU/kg per min during 90 min). Activated partial thromboplastin time (APTT), plasma fibrinogen and bleeding time are reported as mean values ± SEM. **Open bars** = values before the administration of adjunctive medication (solvent or ridogrel) and streptokinase; **hatched bars** = values at the end of the experiment. *, **$p < 0.05$ versus values *before streptokinase administration or **with solvent.

interaction between thromboxane A_2 synthase inhibition and endoperoxide receptor antagonism in improving coronary reperfusion and maintaining patency with streptokinase. Low dose ridogrel (0.31 mg/kg) strongly reduced serum levels of thromboxane B_2 and increases those of 6-keto-prostaglandin $F_{1\alpha}$ and prostaglandin E_2 throughout the experimental period. In contrast, at peak plasma levels of ridogrel reached by this dose, aggregation of canine platelets, sensitized with l-epinephrine and subsequently stimulated with the thromboxane A_2 mimetic U-46619 (17,20), is

only marginally affected. Although by no means reflecting actual in vivo levels (33,34), the reduction in thromboxane B_2 levels and the reorientation to increased levels of 6-keto-prostaglandin $F_{1\alpha}$ and prostaglandin E_2 as measured by radioimmunoassays in serum from coagulated blood ex vivo demonstrate the capacity for profound and specific thromboxane A_2 synthase inhibition of ridogrel in a low dose, lacking substantial endoperoxide receptor antagonism (17,25). As documented earlier with alternative molecules (5), such thromboxane A_2 synthase inhibition to some extent improves streptokinase-induced thrombolysis. This is reflected by the shorter time to reach reperfusion and the greater extent of maximal reperfusion obtained with such adjunctive medication in comparison with solvent. However, the enhancement of the thrombolytic efficacy of streptokinase obtained by such thromboxane A_2 synthase inhibition is less pronounced than that achieved with high dose ridogrel. This difference is most marked on variables such as time delay to high grade reperfusion, the incidence and maximal extent of coronary recanalization and the reduction in the rate of reocclusion. In contrast to the low dose, the high dose of ridogrel provides additional antagonism of platelet thromboxane A_2–prostaglandin endoperoxide receptors in addition to thromboxane A_2 synthase inhibition. This is evidenced by the reduction in serum thromboxane B_2 and the increase in 6-keto-prostaglandin $F_{1\alpha}$ and prostaglandin E_2 levels ex vivo similar to those obtained with the low dose and, in contrast to the latter, by the attenuation of U-46619–induced canine platelet aggregation in vitro at concentrations of ridogrel equal to peak plasma levels of the compound in vivo after the administration of 5 mg/kg.

Single endoperoxide receptor antagonism to some extent improves coronary thrombolysis by streptokinase in dogs (6). However, in the present experimental conditions of high thrombogenicity due to the presence of a lysing thrombus exposing absorbed thrombin (35), such a pharmacologic effect is not likely to be the sole mechanism by which high dose ridogrel optimizes the efficacy of the fibrinolytic agent for several reasons. 1) The enhancement of thrombolysis versus that in a control group with a similar dose of streptokinase obtained with single endoperoxide receptor antagonism using sulotroban (6) is less pronounced than that we achieved in the present study with high dose ridogrel in a similar canine protocol. 2) Despite a potency of the thromboxane A_2 and prostaglandin endoperoxide receptor antagonist far beyond that of ridogrel at the highest dose we used in the present study (25), single endoperoxide receptor antagonism with sulotroban (6) is inferior to strong thromboxane A_2 synthase inhibition complemented with comparatively modest endoperoxide receptor antagonism (ridogrel, 5 mg/kg) in preventing coronary occlusion in conditions of high grade thrombogenicity created by extensive vessel wall damage in dogs (17). 3) Although single thromboxane A_2 synthase inhibition (20) or endoperoxide receptor antagonism (36–38) to some extent enhances the efficacy of coronary thrombolysis with recombinant tissue-type plasminogen activator (rt-PA) in heparinized dogs, such an upgrading is significantly higher with combined thromboxane A_2 synthase inhibition and endoperoxide receptor antagonism as achieved by compound combinations or by dual-acting ridogrel in comparison with the single interventions (20,38).

Of particular interest is the observation that in contrast to that of rt-PA (39), the enhancement of streptokinase-induced thrombolysis with ridogrel occurs without additional administration of heparin. Similar to conditions during primary prevention of thrombus formation in damaged coronary and carotid arteries of various species (18–20), the present data suggest that thromboxane A_2 synthase inhibition, by reorienting prostaglandin endoperoxide metabolism away from thromboxane A_2 into the inhibitory prostaglandin D_2 and prostacyclin, synergizes with modest endoperoxide receptor antagonism, blocking the shutdown by accumulating prostaglandin endoperoxides on adenylate cyclase by which inhibitory prostanoids increase platelet cyclic adenosine monophosphate (40). By such a mechanism, this dual pharmacologic intervention on arachidonic acid metabolism and receptors significantly attenuates platelet activation during streptokinase-induced coronary thrombolysis. Such an inhibition of platelet reactivity is sufficiently strong to reduce renewed deposition of thrombotic material on the lysing thrombus, thereby accelerating and intensifying coronary recanalization and preventing thrombotic reocclusion after streptokinase. We observed that streptokinase-induced changes in plasma fibrinogen levels and in activated partial thromboplastin time as well as the lysis by streptokinase of diluted canine whole blood are not influenced by ridogrel, whereas template-induced bleeding times are prolonged after the highest ridogrel dose. This observation points to an action of high dose ridogrel on platelet function for enhancing streptokinase-induced thrombolysis rather than on the activity of enzymes involved in the coagulation and fibrinolysis cascades.

At the dose of 5 mg/kg, ridogrel produces combined thromboxane A_2 synthase inhibition and endoperoxide receptor antagonism, and thereby optimal enhancement of the thrombolytic efficacy of streptokinase in restoring and maintaining coronary artery patency. Thus, at this dose, ridogrel significantly reduces the size of the myocardial infarct resulting from a mechanical left anterior descending coronary artery occlusion followed by coronary reperfusion. The absence of effects of combined thromboxane A_2 synthase inhibition and endoperoxide receptor antagonism with ridogrel on heart rate, blood pressure, cardiac performance and regional myocardial blood flow argues against a relevant contribution of major hemodynamic effects of this agent on the preservation of myocardial tissue and points to localized myocardial sites of action during ischemia and reperfusion.

The myocardial salvage we obtained with ridogrel at a dual-acting dose may be due to 1) thromboxane A_2 synthase inhibition, reorienting prostaglandin endoperoxide metabolism away from thromboxane A_2 into increased amounts of protective prostaglandin D_2 and prostacyclin (9,41), or 2)

endoperoxide receptor antagonism, blocking detrimental cellular responses to prostaglandin endoperoxides and thromboxane A_2 (8,10,11,14), separately shown to reduce myocardial infarct size after coronary occlusion and reperfusion, or 3) the combination of thromboxane A_2 synthase inhibition and endoperoxide receptor antagonism (17). The protection against myocardial reperfusion injury by the latter pharmacologic intervention then may be a result of several possible effects, including reductions in microvascular vasospasm (42), platelet activation (42), cytotoxicity and lysosomal enzyme release (8,43) and neutrophil accumulation and activation (44–47).

Conclusions. The present study demonstrates that profound thromboxane A_2 synthase inhibition combined with a comparatively modest degree of endoperoxide receptor antagonism as achieved with ridogrel in doses applicable to humans (24) significantly enhances the efficacy of coronary thrombolysis elicited with streptokinase, even without the concomitant application of heparin as adjunctive medication. Moreover, in contrast to cyclooxygenase inhibition (48,49), such a pharmacologic intervention provides salvage of myocardial tissue at risk of infarction because of coronary occlusion and reperfusion. This observation may have implications for the future treatment of patients with acute myocardial infarction.

We acknowledge the contributions of L. Wouters, F. Verheyen, L. Leyssen, G. Verheyen, I. Hauspie and C. Kooremans to various stages of the manuscript.

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