Endogenous Adenosine Inhibits Platelet Aggregation During Myocardial Ischemia in Dogs

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The goal of this study was to clarify that blockade of adenosine receptors during myocardial ischemia causes further reductions in coronary blood flow due to platelet aggregation. Coronary perfusion pressure in 47 open-chest dogs was reduced such that coronary blood flow decreased to one fifth of the control value; thereafter, coronary perfusion pressure was maintained at the low levels. During hypoperfusion, coronary flow was kept low but constant with a massive release of adenosine. When 8-phenyltheophylline, an adenosine receptor antagonist, was infused during coronary hypoperfusion, coronary blood flow (18±2 ml/100 g/min) gradually decreased at 5–10 minutes of ischemia and reached almost zero at 20 minutes. Three minutes after the onset of ischemia, before further reduction of coronary flow, the microscopic examination revealed the existence of thromboembolization in the small coronary arteries, and the number of platelets in the regional coronary venous blood were significantly decreased, indicating that a further reduction of coronary flow due to treatment with 8-phenyltheophylline is attributed to thromboembolism caused by platelet aggregations. This reduction of coronary flow and formation of thromboembolism were inhibited by the treatments with dibutyryl cAMP, forskolin, and yohimbine, indicating that this thromboembolization during a lack of adenosine activity is due to platelet aggregation and that platelet aggregation caused by 8-phenyltheophylline is triggered by stimulation of \(\alpha\)-adrenoceptors by released norepinephrine during ischemia. We demonstrate that adenosine, generated endogenously in response to ischemia, inhibits platelet aggregation. The finding that adenosine is not merely a vasodilator but that it also regulates thrombosis has major implications for designing new strategies of myocardial salvage. (Circulation Research 1991;69:1402–1408)

Although platelet aggregation is known to be capable of plugging coronary arteries and thereby worsening myocardial ischemia,\(^1\) the factors that trigger and inhibit such aggregation have not been defined. During ischemia, a massive amount of adenosine is released and is thought to relax coronary smooth muscle and increase coronary blood flow.\(^2,3\) Besides this view, in previous in vitro experiments adenosine has been introduced as a potent inhibitor of platelet aggregation through \(\mathrm{A}_2\)-adenosine receptors of the platelets.\(^4–8\) However, until now, there has been no report that endogenous adenosine during myocardial ischemia benefits inhibition of thromboembolization due to platelet aggregation in coronary small arteries. We report here evidence that, during ischemia, released adenosine potentially inhibits thromboembolism that is primarily due to platelet aggregation. This novel and fundamental phenomenon preserves normal functional capacities of coronary circulation during and after ischemia.

Materials and Methods

Instrumentation

Forty-seven mongrel dogs weighing 16–22 kg were anesthetized with pentobarbital sodium (30 mg/kg). The trachea was intubated, and the dog was ventilated with room air with oxygen. The chest was opened through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. We cannulated and perfused the left anterior descending coronary artery with blood via the carotid artery.
through an extracorporeal bypass tube after heparinization (500 units/kg). Coronary blood flow (CBF) was measured with an electromagnetic flow probe attached at the bypass tube, and coronary perfusion pressure (CPP) was monitored at the tip of the coronary arterial cannula. A small, short collecting tube was cannulated into a small coronary vein near the center of the perfused area to sample coronary venous blood. The drained venous blood was collected in a reservoir placed at the level of the left atrium. A high fidelity of left ventricular pressure and its first derivative were measured by a micromanometer (model P-5, Konigsberg Instruments, Inc., Pasadena, Calif.) placed in the left ventricular cavity through the apex. A pair of ultrasonic crystals was placed in the center of the perfused area ~1 cm apart to measure myocardial segment length with an ultrasonic dimension gauge (5 MHz, Schuessler, Cardiff by the Sea, Calif.). End-diastolic length (EDL) was determined at the R wave of the electrocardiogram, and end-systolic length (ESL) was determined at the minimal dP/dt.\(^{9,10}\) Fractional shortening was calculated by (EDL – ESL)/EDL as an index of myocardial contractility of the perfused area.\(^{9,10}\)

**Experimental Protocols**

After stabilization, CPP was decreased with partial occlusion of the bypass tube to the left anterior descending coronary artery such that CBF decreased to one fifth of the control flow. The low CPP was maintained for 20 minutes. The same procedures were performed under treatments with 8-phenyltheophylline (30 \(\mu g/kg/min\) i.c., \(n=5\)). To test whether coronary flow reduction due to 8-phenyltheophylline treatment is attributed to platelet aggregation, we further tested whether the effects of 8-phenyltheophylline in the ischemic hearts were attenuated by infusions with dibutyryl cAMP (db-cAMP, 40 \(\mu g/kg/min\) i.c., \(n=5\)) and forskolin (0.3 \(\mu g/kg/min\) i.c., \(n=5\)). This is because both db-cAMP and forskolin are reported to inhibit platelet aggregation.\(^{4-8}\) Moreover, to investigate the triggering mechanisms of the platelet aggregation due to adenosine receptor antagonization, propranolol (10 \(\mu g/kg/min\), \(n=5\)), prazosin (4 \(\mu g/kg/min\) i.c., \(n=5\)), and yohimbine (4 \(\mu g/kg/min\) i.c., \(n=5\)) were administered in the ischemic hearts under the treatment with 8-phenyltheophylline.

**Chemical Analysis**

**Lactate measurements.** Lactate was assessed by enzymatic assay.\(^{9,10}\) and the lactate extraction ratio was obtained by coronary arteriovenous difference in lactate concentration multiplied by 100 and divided by arterial lactate concentration.

**Adenosine measurements.** The method of adenosine measurements has been reported previously.\(^{9-13}\) One milliliter of blood was drawn into a syringe containing 0.5 ml of 0.03% dipyridamole and 0.1 ml of 160 mM MnCl\(_2\) to make the final concentration of each chemical 0.01% and 10 mM, respectively. Dipyridamole blocked the uptake of adenosine by both red blood cells and platelets, and MnCl\(_2\) blocked the degradation of adenosine. Sampled blood was quickly put into iced water to prevent release of adenosine from red blood cells. After centrifugation, the supernatant was obtained, and radioimmunoassay methods were used for analyzing the adenosine content.

Briefly, adenosine in the plasma (100 \(\mu l\)) was succinylated by 100 \(\mu l\) dioxane containing succinic acid anhydride and triethylamine. After a 10-minute incubation, the mixture was diluted with 800 \(\mu l\) of 0.3 M imidazole buffer (pH 6.5). The assay mixture contained 100 \(\mu l\) sample, 100 \(\mu l\) succinyl [\(^3\)H]adenosine (25,000 counts/min in an amount of 1 pmol), and 100 \(\mu l\) diluted anti-adenosine serum. After the mixture was kept in an ice-cold water bath for 24 hours, a cool suspension of dextran-coated charcoal (500 \(\mu l\)) was added. The charcoal was spun down, and 0.5 ml supernatant was counted for radioactivity in a liquid scintillation counter. The amount of adenosine degradation during the sampling procedure and degradation rate of adenosine were negligible.\(^{9,11}\)

**Noradrenaline measurements.** The method of noradrenaline measurements has been described previously.\(^{14}\) Five milliliters of coronary arterial and venous blood taken into a tube containing EDTA was immediately placed in iced water and centrifuged for 20 minutes. Plasma noradrenaline was adsorbed on alumina and separated by high-performance liquid chromatography (LC-3A pump, Zpax-SCX column, Shimazu Seisakuso Co.). Plasma noradrenaline was determined spectrofluorometrically by the trihydroxyindole method (RF-500LCA spectrofluorophotometer, Shimazu).

**Morphological Studies**

After completion of measurements of hemodynamic parameters and blood sampling, heart tissues were prepared for the light microscopic analysis to examine the changes in small coronary arteries as well as in the myocardium.

**Statistical Analysis**

Statistical analysis was performed with unpaired \(t\) test. Multiple analysis of variance was also used to assess the differences of time–response curve between each group. All values were expressed as mean±SEM, and \(p<0.05\) was considered significant.

**Results**

In the nonischemic control condition, CPP and CBF were 113±8 mm Hg and 89±2 ml/100 g/min, respectively. Intracoronary administrations of 8-phenyltheophylline, forskolin, db-cAMP, propranolol, prazosin, and yohimbine changed neither CBF, lactate extraction ratio, nor fractional shortening in the nonischemic condition.

Figure 1 shows the changes in CPP and CBF after the reduction of CPP. Without any treatment (open circles in Figure 1, \(n=5\)), both CPP and CBF were maintained unchanged in the low levels. Coronary arteriovenous differences of adenosine were signifi-
enhanced to 876±32 pmol/ml in the 8-phenyltheophylline–infused condition (\(p<0.05\) versus the untreated condition), suggesting that endogenous adenosine may attenuate release of norepinephrine in the ischemic myocardium. After 20 minutes of ischemia, there was hardly any remaining coronary flow in the perfused area.

When CPP was returned to the control level 20 minutes after the onset of ischemia in the untreated condition, CBF was also returned to normal (93±4 ml/100 g/min) after reactive hyperemia (CBF, 339±13 ml/100 g/min). However, in the 8-phenyltheophylline–treated condition, although the occluder was released and administration of 8-phenyltheophylline was discontinued, reactive hyperemia was not observed (85±34 ml/100 g/min) and the basal coronary flow (7±2 ml/100 g/min) did not return to control levels, suggesting that gradual decreases in CBF during ischemia in the 8-phenyltheophylline–treated condition are not due to functional changes in coronary smooth muscle (e.g., vasoconstriction) but due to organic changes or obstruction of coronary arteries. Therefore, we checked for histological evidence of organic obstruction in the small coronary vessels. Figure 2 shows typical sections of myocardium at 3 minutes after ischemia in the untreated condition (top panel) and in the 8-phenyltheophylline–treated condition (bottom panel). There were no abnormalities in the myocytes and small coronary arteries in the untreated condition. In striking contrast, the 8-phenyltheophylline–treated hearts exhibited widespread plugging of the small coronary vessels, implying that treatment with 8-phenyltheophylline during ischemia causes thromboembolism in small coronary vessels.

To test the idea that the reduction of CBF caused by 8-phenyltheophylline during ischemia is related to the activation of platelets, we examined the effects of db-cAMP and forskolin on this flow reduction. Administrations of both forskolin and db-cAMP attenuated (\(p<0.01\)) the effects of 8-phenyltheophylline during ischemia (Figure 1), indicating that the gradual flow reduction under the treatment with 8-phenyltheophylline is mainly attributed to the platelet activation during ischemia. Furthermore, the number of platelets in the coronary venous blood (the untreated condition, 15.1±0.5×10³/mm³; 8-phenyltheophylline–treated condition, 9.9±1.1×10³/mm³; \(p<0.01\)) was significantly decreased 3 minutes after the reduction of CPP at the time when CBF was about to decrease (Figure 1), although numbers of erythrocytes (4.37±0.23×10¹⁰/mm³ versus 4.34±0.10×10¹⁰/mm³, \(p=NS\)) and leukocytes (7.4±0.2×10⁵/mm³ versus 7.4±0.2×10⁵/mm³) in the coronary venous blood were not decreased significantly. This selective reduction of the number of platelets before the decreases in CBF strongly argues against the hypothesis that the stagnant bloodstream due to the treatment with 8-phenyltheophylline would cause platelet aggregations.

We investigated the triggering mechanisms of the platelet aggregation during ischemia without the

\[\text{FIGURE 1. Graphs showing serial changes in coronary perfusion pressure (panel A) and coronary blood flow (panel B) after reduction of coronary perfusion pressure. Coronary perfusion pressure was not changed throughout. Because adenosine receptors of the coronary arteries are blocked by 8-phenyltheophylline (8PT), coronary perfusion pressure, which maintained one fifth of the coronary flow, was higher than that in the untreated condition. Although coronary perfusion pressure was kept constant in each group, coronary blood flow gradually decreased toward zero at 20 minutes after ischemia in the 8PT-treated condition. In the untreated condition, coronary blood flow did not decrease during 20 minutes of ischemia. Exposures of dibutyl cAMP (db-cAMP) and forskolin prevented this decrease in coronary blood flow in the 8PT-treated condition, although neither db-cAMP nor forskolin in this protocol altered values in the nonischemic condition (db-cAMP: 94.6±4.4 vs. 92.3±1.1 ml/100 g/min for control, \(p=NS\); forskolin: 92.1±2.1 vs. 90.5±1.9 ml/100 g/min for control, \(p=NS\)).}\]
effects of adenosine. The most likely possibility is that catecholamine, released during ischemia, may affect platelets. However, \( \beta \)-adrenoceptor blockade did not prevent this gradual decrease in CBF (Figure 3). Administration of prazosin could not inhibit this decrease in CBF under the treatment with 8-phenyltheophylline (CBF, 1.5±0.5 ml/100 g/min at 20 minutes of ischemia). In contrast, an infusion of yohimbine significantly \( (p<0.01) \) attenuated the reduction of coronary flow by 8-phenyltheophylline \( (n=5) \), indicating that a gradual decrease in CBF under an infusion of 8-phenyltheophylline is attributed to \( \alpha_2 \)-
adrenoceptor stimulation by released norepinephrine during ischemia (Figure 3). Histological examination also confirmed this result: yohimbine inhibited this plugging in the small coronary vessels, but neither prazosin nor propranolol was effective in this regard.

The functional implications of our findings are striking. Figure 4 shows the changes in regional myocardial contractility (fractional shortening) and lactate extraction ratio. Treatment with 8-phenylethylpholine significantly \( p<0.01 \) decreased both fractional shortening and lactate extraction ratio during myocardial ischemia compared with the untreated condition, indicating that the reduction of CBF due to the plugged small coronary vessels during ischemia directly causes myocardial contractile and metabolic dysfunction.

Taken together, our data suggest that inhibition of adenosine receptors activated primarily platelets during ischemia and triggered thromboembolization in the coronary small arteries that was mediated through stimulation of \( \alpha_2 \)-adrenoceptors.

**Discussion**

In the present study, to test the hypothesis that endogenous adenosine plays a key role in the inhibition of thromboembolism due to platelet aggregation in the ischemic condition, we used in vivo hearts, because the ischemic condition is only made in the blood-perfused experimental model. Ischemic myocardium per se can be mimicked in the myocardial cells or myocardial strips perfused with the crystalloid perfusate; however, the realistic changes in environment due to ischemia are only accomplished in the in vivo beating hearts. Indeed, our novel phenomenon is profoundly related to the adenosine released from the myocardium, the norepinephrine released from the presynaptic nerve ending, and platelets in the coronary flow stream. Our conclusion is that an unbalance of the combination of these factors (e.g., when the adenosine receptor blockade promotes the cascade of the platelet aggregation and exerts the deleterious results to the small coronary arteries) can only be obtained in the in vivo experimental model with the set of these factors.

We should be careful when we consider the effects of db-cAMP and forskolin in the present study, because these chemicals are reported to increase myocardial contractility in high doses. However, the doses of db-cAMP and forskolin that were used in the present study did not exert these effects on myocardial and coronary smooth muscle cells, gauged by fractional shortening and coronary flow, respectively. Nevertheless, these doses of db-cAMP and forskolin can inhibit platelet aggregation, because these chemicals affect platelets in the coronary blood stream directly compared with their effects on myocardial and coronary smooth muscle cells. The relative potencies of these chemicals in myocardial and coronary smooth muscle cells may be weakened by the barrier of endothelium.

Stimulations of \( \alpha_2 \)-adenosine receptors in platelets are responsible for increases in cAMP content and inhibition of platelet aggregation.\(^{15}\) On the other hand, \( \alpha_2 \)-adrenoceptor stimulation by norepinephrine is reported to cause platelet aggregation.\(^{16}\) In ischemic hearts, both adenosine and norepinephrine are massively released from myocytes and nerve endings, respectively; however, there are no reports concerning the interaction of these two factors. When moderate ischemia is caused by reduction of the coronary flow to one third of control flow, adenosine is massively released, although norepinephrine is not,\(^{13,17}\) suggesting that adenosine release may affect release of norepinephrine.\(^{18}\) In such a situation, we did not observe the flow reduction and platelet aggregation that were due to treatment with 8-phenylethylpholine.\(^{13}\) During severe ischemia that enhances release of norepinephrine,\(^{17}\) platelet aggregation was not observed while adenosine was released; platelet aggregation progressed only when adenosine receptors were blocked and \( \alpha_2 \)-adrenoceptors were potently stimulated in platelets. These lines of evidence support the idea that, in ischemic hearts, endogenous adenosine potentially inhibits platelet aggregation.
It is intriguing to consider the role of adenosine in the neurotransmitter functions of the presynaptic nerve endings. The released adenosine inhibits norepinephrine release and attenuates both catecholamine and ischemic injuries. The earlier and present studies agree well. However, once this negative feedback mechanism is disorder by blockade of adenosine receptor activities, norepinephrine is continuously released and \( \alpha_2 \)-adrenergic receptors are further stimulated. Indeed, the present study provides supportive evidence that adenosine receptor blockade by 8-phenylethophylline enhances the release of norepinephrine during ischemia, which further enhances platelet aggregation in the small coronary vessels and worsens myocardial ischemia. Adenosine is revealed to be an important element in this crucial feedback mechanism.

In the clinical settings, thromboembolism in the small coronary arteries can be observed in acute myocardial infarction. When myocardial ischemia is prolonged, the capability of adenosine production may be weakened, and adenosine receptors may be desensitized, both attenuating the stimulatory effects of adenosine receptor in platelets. In contrast, release of norepinephrine is closely related to the duration and extent of ischemia: prolonged ischemia facilitates release of norepinephrine, which further stimulates \( \alpha_2 \)-adrenoceptors in platelets. This unbalance that decreases cAMP concentrations in platelets may cause thromboembolism in coronary small vessels. To prevent the activation of this deleterious vicious cycle, adenosine infusion or potentiation of adenosine production may be promising for the treatment of ischemic heart diseases.

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References


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