Inhibitory Effects of Glibenclamide and Pertussis Toxin on the Attenuation of Ischemia-Induced Myocardial Acidosis Following Ischemic Preconditioning in Dogs

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Ischemic preconditioning is known to be mediated by several humoral factors, such as adenosine, norepinephrine, and bradykinin. We examined intracellular signal transduction of ischemic preconditioning following receptor stimulation. Alterations in the pH of the ischemic bed were monitored to assess the response of control and ischemic-preconditioned myocardium to glibenclamide and pertussis toxin. Pentobarbital-anesthetized open-chest dogs were subjected to 40 min of ligation of the left anterior descending coronary artery. Ischemic preconditioning was elicited by 25-min periods of coronary ligation followed by 5 min of reperfusion before a 40-min period of ligation. Glibenclamide (0.3 mg/kg) was given iv 20 min before the onset of ischemic preconditioning. Pertussis toxin (6–10 μg/kg) was given iv 3 days before the experiment. Tissue myocardial pH was measured by a glass micro-pH electrode. Ischemia for 5 min decreased myocardial pH and reperfusion returned it to the preischemic levels. Ischemia for 40 min decreased the myocardial pH from 7.43 ± 0.06 to 6.43 ± 0.08. Ischemic preconditioning significantly attenuated the decrease in myocardial pH (6.57 ± 0.06) induced by 40 min of ischemia. Pretreatment with either glibenclamide or pertussis toxin completely abolished the effect of ischemic preconditioning on ischemic myocardial acidosis. Ischemic preconditioning can attenuate ischemia-induced myocardial acidosis in dogs, and this effect is mediated by activation of adenosine triphosphate-sensitive potassium channels and pertussis toxin-sensitive guanosine triphosphate-binding protein.

Key Words: Ischemic preconditioning; Myocardial pH; Adenosine triphosphate-sensitive potassium (K_{ATP}) channel; Gi protein

The phenomenon whereby single or multiple short episodes of ischemia renders the heart more tolerant to a subsequent more prolonged ischemia is termed ischemic preconditioning. Mediation by the following mechanisms has been proposed to explain this phenomenon: the adenosine A_{1} receptor, the adrenergic α_{1} receptor, protein kinase C, adenosine A_{3} receptors of mast cells, antioxidants, lipoxygenase metabolites, stress proteins, and the bradykinin B_{2} receptor. Certainly adenosine A_{1} receptors play an important role in ischemic preconditioning, and adenosine triphosphate-sensitive potassium (K_{ATP}) channels are also involved, as demonstrated by Auchampach and Gross. It is postulated that, in the dog, activation of adenosine A_{1} receptors leading to the opening of K_{ATP} channels results in myocardial ischemic preconditioning. Glibenclamide, a selective K_{ATP} channel antagonist, completely abolishes the protective effect of ischemic preconditioning, and the effect of RP 52891, a selective K_{ATP} channel opener, on the ischemic myocardium mimics that of ischemic preconditioning. Kirsch et al have demonstrated in rat ventricular myocytes that opening of K_{ATP} channels is coupled to adenosine A_{1} receptors by

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guanosine triphosphate-binding protein (G proteins), and Lasley et al\textsuperscript{14} have reported the role of inhibitory G (Gi) proteins in attenuating myocardial ischemic damage by A\textsubscript{1} agonists. Thornton et al\textsuperscript{15} have also reported that the effect of ischemic preconditioning on myocardial infarct size in rabbits is partly dependent on Gi proteins. However, pertussis toxin does not modify the antiarrhythmic effect of ischemic preconditioning in rats\textsuperscript{16}.

Asimakis et al\textsuperscript{17} and Kida et al\textsuperscript{18} have reported that ischemic preconditioning attenuates myocardial acidosis in isolated perfused rat hearts and porcine hearts, respectively. Myocardial pH is a useful indicator for assessing the degree of ischemic myocardial damage\textsuperscript{18–20} Thus, we examined the effects of glibenclamide and pertussis toxin on ischemic preconditioning in terms of alterations in myocardial pH during ischemia and reperfusion.

**Materials and Methods**

**Animal Preparation**

Healthy mongrel dogs of either sex weighing 7.2–19.5 kg were anesthetized with pentobarbital (30 mg/kg iv) and ventilated with room air. Left thoracotomy was performed between the fourth and fifth ribs, and the left ventricle was exposed. After the heart was suspended in a pericardial cradle, the left anterior descending coronary artery (LAD) was dissected free, distal to the first diagonal branch, and was loosely encircled with a silk thread ligature. The LAD flow was measured by a magnetic flow probe positioned at the LAD proximal to the ligature. A micro-glass pH electrode (Fuji Keisoku, Mitaka) was inserted in the area perfused by the LAD at a depth of 6–8 mm, and changes in myocardial pH were recorded continuously on a pen recorder via a pH-meter (F-7II, Horiba, Kyoto). The pH electrode was calibrated with standard pH solution (pH 6.84 and 7.38) before each experiment. Heart rate was counted from the ECG (standard limb lead II). Arterial blood pressure was measured via a cannula introduced into the left femoral artery.

All animal experiments conformed to the guidelines of the American Physiological Society.

**Experimental Protocol**

After about 60 min of stabilization, the LAD in the ischemic preconditioning group was ligated with a silk thread ligature for 5 min, and then released for 5 min. This procedure was repeated twice. Finally, the LAD was ligated for a longer period of 40 min. In the control group, the LAD was not ligated for the first 20 min, and then ligated for 40 min. In the glibenclamide-pertreated group, 0.3 mg/kg glibenclamide was injected over a period of 30 sec from the left femoral vein 40 min before the onset of 40 min of ischemia. The volume of dimethylsulfoxide given was less than 1 ml per animal. In the preliminary experiment, this volume did not modify the myocardial pH even during ischemia. In the pertussis toxin-pretreated group, pertussis toxin (7.5 μg/kg) dissolved in saline solution was given iv 3 days before each experiment. The effect of pertussis toxin was checked by blockade of acetylcholine-induced hypotension before the start of experiments. In each group, the LAD was ligated for 40 min with ischemic preconditioning or without ischemic preconditioning (control). Thus, the present study consisted of the following 6 groups: the control group (n=7), the ischemic preconditioning group (n=7), the glibenclamide+control group (n=5), the glibenclamide+ischemic preconditioning group (n=7), the pertussis toxin+control group (n=5), and the pertussis toxin+ischemic preconditioning group (n=7).

**Calculation and Statistics**

All values are expressed as means±SE. Because a pH value is defined as a negative logarithm of the hydrogen ion concentration, we cannot simply calculate the mean value and SE from the pH value itself. Each value of pH measured by the pH electrode should be converted to the hydrogen ion concentration [H\textsuperscript{+}] according to the equation [H\textsuperscript{+}]=10\textsuperscript{-pH}. The mean±SE value was calculated and the statistical analysis performed in terms of [H\textsuperscript{+}] instead of pH values. Mean values of [H\textsuperscript{+}] were then converted back to pH values again. The significance of differences in myocardial pH during 40 min of ischemia between groups was evaluated by 2-way analysis of variance followed by the Tukey-Kramer test. Differences in hemodynamics within groups were compared by the paired Student t test. A value of p<0.05 was considered statistically significant.

**Results**

**Mortality and Exclusion**

Initially, 56 dogs were used in the study, but 18 were excluded because of ventricular fibrillation (VF) during reperfusion (control group, 2; ischemic preconditioning group, 0; glibenclamide+control group, 1; glibenclamide+ischemic preconditioning group, 3; pertussis toxin+control group, 2; and pertussis toxin+ischemic preconditioning group, 10). Thus, 38 dogs were included in data analysis.
Hemodynamic Changes

In the control group, systolic and diastolic blood pressures significantly decreased during the first 5 min of the 40-min ischemic period. Heart rate was not changed by ischemia. In the ischemic preconditioning group, 5 min of ischemia before 40 min of ischemia significantly decreased systolic blood pressure. Diastolic pressure and heart rate did not change during the first and second 5-min periods of ischemia. Reperfusion following 5 min of ischemia restored the systolic pressure to normal and significantly increased the coronary flow beyond the preischemic level, indicating reactive hyperemia. Ischemia for 40 min did not significantly change systolic and diastolic blood pressures. Glibenclamide increased systolic and diastolic blood pressures slightly but significantly. Reactive hyperemia in the glibenclamide + ischemic preconditioning group was significantly lower than in the ischemic preconditioning group. The other hemodynamic responses to 5 min ischemia and reperfusion and 40 min ischemia were similar to those in the control and ischemic preconditioning groups. Pretreatment with pertussis toxin significantly decreased diastolic blood pressure and coronary flow. Reactive hyperemia in the pertussis toxin + ischemic preconditioning group was also significantly reduced compared with the ischemic preconditioning group. The changes in the other parameters are the same as those in the corresponding groups without pertussis toxin.

Myocardial pH

The effects of ischemic preconditioning on ischemia-induced myocardial pH decrease with or without glibenclamide are illustrated in Fig 1 and those with or without pertussis toxin in Fig 2. In the control group, the myocardial pH significantly decreased from 7.43 to 6.43 during 40 min of ischemia. In the ischemic preconditioning group, the myocardial pH was decreased by the first and second 5-min periods of ischemia, and was returned toward its preischemic level by each reperfusion. The decrease in myocardial pH as a result of the first 5-min period of ischemia was greater than the decrease in the second period; the pH during the first 5-min period of ischemia (6.94 ± 0.06) was significantly lower than during the second (7.09 ± 0.04). The 40-min period of ischemia after ischemic preconditioning decreased myocardial pH from 7.36 to 6.57, but more slowly than in the control group. There was a significant difference in myocardial pH changes during 40 min of ischemia between the control and ischemic preconditioning groups. Glibenclamide injection did not change the myocardial pH before the onset of ischemia. In the glibenclamide + control group, the decrease in myocardial pH during 40-min ischemia appeared to be more potent than that in the control group, although the difference was not significant. In the glibenclamide + ischemic preconditioning group, the changes in myocardial pH during the first and second 5-min ischemic periods and each reperfusion were similar to those in the ischemic preconditioning.
group. During 40 min of ischemia, the myocardial pH decreased to the same extent in the glibenclamide+control group. The effect of ischemic preconditioning was completely abolished by glibenclamide.

Pretreatment with pertussis toxin 3 days before the experiment did not alter the myocardial pH. In the pertussis toxin+control group, 40 min of ischemia decreased the myocardial pH, and there was no significant difference from the control group. In the pertussis toxin+ischemic preconditioning group, the myocardial pH during the first and second 5-min periods of ischemia decreased to the same extent. The decrease in myocardial pH during the second 5 min of ischemia was more potent than that in the ischemic preconditioning group. The subsequent 40 min of ischemia decreased the myocardial pH, and a significant difference was no longer observed between the pertussis toxin+control and pertussis toxin+ischemic preconditioning groups.

Blood Glucose Level and Acetylcholine-Induced Hypotension

In the glibenclamide-pretreated group, blood glucose levels were determined before and 20 and 80 min after glibenclamide injection. Glibenclamide decreased the blood glucose level from $137 \pm 7$ to $123 \pm 8$ mg/100 ml blood at 20 min after the injection, and to $110 \pm 11$ mg/100 ml blood at 80 min after the injection, but the differences were not significant. In the pertussis toxin-pretreated group, acetylcholine-induced hypotension was determined before starting each experiment. Acetylcholine at a dose of 4 $\mu$g/kg iv decreased systolic and diastolic blood pressures in the pertussis toxin-treated animals from 118.0±6.1 and 66.8±3.2 mmHg to 104.7±6.7 (11% reduction) and 51.5±3.0 mmHg (23% reduction), respectively, whereas in the normal animals these values decreased from 171.9±7.1 and 129.4±7.7 to 121.3±10.6 (30% reduction) and 78.8±12.0 mmHg (40% reduction), respectively. Pretreatment with pertussis toxin significantly attenuated the decreases in systolic and diastolic blood pressures induced by acetylcholine.

Discussion

The protocol of a study on ischemic preconditioning usually includes reperfusion after a sustained period of ischemia following repeated brief ischemia-reperfusion. However, in the present study we examined the effect of ischemic preconditioning on myocardium only after 40 min of ischemia. As reported in isolated perfused rat heart in vitro,17,18 myocardial pH was reduced less by sustained ischemia (to only 6.57 rather than 6.43) following ischemic preconditioning in dog hearts in vivo (Fig 1). Although the effect of ischemic preconditioning appears small, it is similar to that obtained with $\beta$-adrenergic blockers such as propranolol, nifedipin, and amosuralol.21

One of the important mechanisms involved in ischemic preconditioning is the opening of $K_{ATP}$ channels via activation of adenosine A1 receptors. Auchampach and Gross3 have demonstrated that activation of adenosine A1 receptors produces myocardial preconditioning in the canine heart by opening
K\textsubscript{ATP} channels. Ischemic preconditioning increases adenosine release and 5'-nucleotidase activity during ischemia and reperfusion\textsuperscript{22} and glibenclamide, a K\textsubscript{ATP} channel blocker, prevents myocardial preconditioning in dogs\textsuperscript{23}. In the present study, glibenclamide completely abolished the attenuation of 40-min ischemia-induced myocardial acidosis by ischemic preconditioning. Thornton et al\textsuperscript{23} have reported that glibenclamide does not modify the effect of ischemic preconditioning on myocardial infarct size in rabbits. However, Toombs et al\textsuperscript{24} have found that glibenclamide can reverse the infarct size reduction in rabbits via ischemic preconditioning. They have explained that the difference between the results is due to the anesthesia used: Thornton et al used pentobarbital, whereas Toombs et al\textsuperscript{24} used ketamine and xylazine.

The hypoglycemic action of glibenclamide may make some contribution to abolishing the effect of preconditioning or worsening the ischemia-induced myocardial acidosis. Omar et al\textsuperscript{25} have reported that in isolated perfused rabbit hearts the protective effect of preconditioning occurs only in the presence of high glucose concentrations in the perfusate during reperfusion. Thus, a reduction in glucose level in the present study may be one of the mechanisms by which glibenclamide blocked preconditioning on the myocardial pH. However, glibenclamide at a relatively low dose (0.3 mg/kg) did not cause a significant decrease in blood glucose level in the present study, and abolition of hypoglycemia by glucose infusion does not ameliorate the damaging effects of ischemia caused by glibenclamide\textsuperscript{26}. It seems unlikely that the hypoglycemic effect of glibenclamide is responsible for loss of ischemic preconditioning.

Kirsch et al\textsuperscript{13} have demonstrated in rat ventricular myocytes that opening of K\textsubscript{ATP} channels is coupled to adenosine A\textsubscript{1} receptors by Gi proteins. Gi proteins are coupled to various cardiac ion channels, including K\textsubscript{ATP} channels\textsuperscript{27}. Lasley et al\textsuperscript{14} have reported that Gi proteins play a role in attenuating myocardial ischemic damage by adenosine A\textsubscript{1} receptor agonists in isolated perfused rat hearts, because muscarinic agonists, which act via similar Gi proteins, also improve postischemic myocardial dysfunction. More direct evidence has been provided by Thornton et al\textsuperscript{15}. They found that pretreatment with pertussis toxin blocks the protective effects of ischemic preconditioning in the rabbit heart. On the other hand, Piacentini et al\textsuperscript{16} failed to find a blocking action of pertussis toxin on the antiarrhythmic action of ischemic preconditioning in rats. The present study clearly shows that ischemic preconditioning completely abolishes the ischemic acidosis induced by pertussis toxin pretreatment in dogs.

In the ischemic preconditioning group, the reduction in myocardial pH occurring during the second period of ischemia was smaller than that occurring during the first period. The myocardium may have been preconditioned by the first 5-min ischemia, although the reactive hyperemia observed after each ischemic period was almost identical. Reactive hyperemia after each 5-min ischemic period was significantly reduced by either glibenclamide or pertussis toxin in the ischemic preconditioning groups. Because adenosine may be responsible for this phenomenon\textsuperscript{26} both glibenclamide and pertussis toxin may block the coronary vasodilation induced by adenosine after brief ischemia.

To test the efficacy of pertussis toxin, Thornton et al\textsuperscript{15} and Piacentini et al\textsuperscript{16} observed the blockade of acetylcholine-induced negative chronotropic action. In contrast, we tested its efficacy using acetylcholine-induced hypotension because acetylcholine produces reflex tachycardia in spite of negative chronotropism in vivo. Although pertussis toxin partially blocked acetylcholine-induced hypotension, it completely prevented the effect of ischemic preconditioning effect on myocardial acidosis during sustained ischemia. The sensitivity of Gi proteins to pertussis toxin may differ according to the organ examined or agonist used.

In conclusion, ischemic preconditioning attenuates myocardial acidosis induced by subsequent prolonged ischemia. At least in dog hearts in vivo, ischemic preconditioning may involve opening of K\textsubscript{ATP} channels via pertussis toxin-sensitive Gi proteins.

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