A Study on Dilazep: I. Mechanism of Anti-ischemic Action of Dilazep Is Not Coronary Vasodilation but Decreased Cardiac Mechanical Function in the Isolated, Working Rat Heart†

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ABSTRACT—In the isolated, perfused working rat heart, ischemia (15 min) decreased the mechanical function and the tissue levels of adenosine triphosphate and creatine phosphate and increased the levels of lactate and free fatty acids. Reperfusion (20 min) did not restore the mechanical function, but restored incompletely the levels of metabolites, with the exception of free fatty acids, which increased further during reperfusion. Dilazep was given 5 min before starting ischemia until the end of ischemia. Dilazep at 5 or 10 pM decreased the cardiac mechanical function, but did not affect coronary flow in the pre-ischemic heart. Dilazep at 5 or 10 pM accelerated the recovery of mechanical function and coronary flow during reperfusion, and it attenuated metabolic changes induced by ischemia and reperfusion. Dilazep at 1 pM neither decreased the pre-ischemic mechanical function nor restored the mechanical function during reperfusion, although it attenuated the accumulation of free fatty acids during reperfusion. These results suggest that dilazep attenuates both ischemia- and reperfusion-induced myocardial damage and that the anti-ischemic action of dilazep is not due to coronary vasodilation but probably due to an energy-sparing effect and other effects that remain to be studied.

Keywords: Dilazep, Working heart, Ischemia, Adenosine triphosphate, Free fatty acid

Dilazep inhibits uptake of adenosine by cardiac cells and hence potentiates the action of adenosine to dilate the coronary artery (1), suggesting that it has an anti-ischemic action. In fact, the cardioprotective (or anti-ischemic) effect of dilazep was reported in the dog heart (2–4). In clinical studies, dilazep has been used successfully as a drug for treatment of patients with ischemic heart disease (5, 6). However, the coronary vasodilating action of dilazep is not very marked, suggesting that there is another mechanism for the anti-ischemic action of dilazep.

In the present study, therefore we examined whether dilazep has anti-ischemic action in concentrations that do not increase coronary flow. To exclude extracardiac factors, we employed an isolated working rat heart preparation. Ischemia-induced damage was evaluated by both mechanical and metabolic functions of the heart; ischemia reduces cardiac mechanical function, decreases the tissue levels of high-energy phosphates such as adenosine triphosphate (ATP) and creatine phosphate (CrP), and increases the tissue levels of lactate and free fatty acids (FFA) (7, 8). Post-ischemic reperfusion (simply expressed as reperfusion in the present study) returns the mechanical and metabolic levels toward their pre-ischemic levels, except for the levels of FFA, which further increase during reperfusion (8, 9). Therefore, reperfusion-induced damage was evaluated by the tissue levels of FFA, particularly the ratio of the level of arachidonic acid in the ischemic heart to that in the non-ischemic heart, and the ratio of the arachidonic acid level in the reperfused heart to that in the ischemic heart, because arachidonic acid increases markedly during both ischemia and reperfusion.

MATERIALS AND METHODS

Heart perfusion
Male Sprague-Dawley rats (280–340 g; Sankyo Labo Service Corporation, Sapporo) were anesthetized with so-
dium pentobarbital (50 mg/kg, i.p.). After thoracotomy, the hearts were quickly removed and then perfused according to the Langendorff method followed by the working heart method. The solution for perfusion was a modified Krebs-Henseleit bicarbonate (KHB) buffer (119.4 mM NaCl, 4.7 mM KCl, 2.9 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25.0 mM NaHCO3, 11.0 mM glucose and 0.5 mM EDTA-2 Na) equilibrated with a gas mixture of 95% O2 + 5% CO2 and maintained at 37°C. Hearts were initially perfused using the Langendorff method at a constant pressure of 90 cmH2O for 10 min and then perfused using the working heart method at a left atrial filling pressure of 12.5 cmH2O and an afterload pressure of 90 cmH2O for 15 min (8). Ischemia was induced by lowering the afterload pressure from 90 cmH2O to 0 cmH2O (i.e., no-afterload ischemia) (10). Reperfusion was induced by returning the afterload pressure to its initial level (90 cmH2O). Aortic pressure and heart rate were monitored with a pressure transducer placed in the aortic cannula. Cardiac mechanical function was defined as RPP (peak aortic pressure multiplied by heart rate). Coronary flow (defined as the flow coming from the cannula inserted into the pulmonary artery) was measured with a graded glass cylinder.

Experimental protocol and heart groups

The hearts were divided into 4 groups: control (no drug), 1 μM dilazep, 5 μM dilazep and 10 μM dilazep groups. In each group, the hearts were divided further into 3 subgroups: non-ischemia or pre-ischemia (designated as NI), ischemia (designated as I), and reperfusion (designated as R) subgroups. In our previous study, we found that 15 min of ischemia is sufficient to produce irreversible damage to the heart in terms of mechanical and metabolic damage. Therefore in the present study, ischemia was performed for 15 min, and reperfusion was started immediately after ischemia and continued for 20 min. In NI, I and R groups, the hearts were freeze-clamped immediately before starting ischemia, immediately after the end of ischemia, and immediately after the end of reperfusion, respectively. In the drug-treated groups, dilazep (1 μM, 5 μM or 10 μM) was administered to the perfusion solution 5 min before starting ischemia until the end of ischemia, and then reperfusion was performed with normal KHB buffer that did not contain dilazep (Fig. 1). Therefore, there were the following 12 groups: control: NI (n=10), control: I (n=10), control: R (n=12); 1 μM dilazep:NI (n=6), 1 μM dilazep: I (n=7), 1 μM dilazep: R (n=8), 5 μM dilazep:NI (n=7), 5 μM dilazep: I (n=7), 5 μM dilazep: R (n=7), 10 μM dilazep:NI (n=6), 10 μM dilazep: I (n=7), 10 μM dilazep: R (n=7).

Preparation of frozen cardiac tissue samples

The heart was frozen with a Wollenberger’s clamp previously cooled with liquid nitrogen (−173°C). The freeze-clamped cardiac tissue samples were stored in liquid nitrogen until the biochemical analysis was performed.

Assay of the tissue high-energy phosphates and lactate

A part of the frozen cardiac tissue sample (about 0.8 – 1.0 g) was pulverized in a mortar cooled with liquid nitrogen, and tissue FFA were extracted from the pulverized tissue with perchloric acid, and then neutralized with KOH solution. These metabolites of energy metabolism were assayed by standard enzymatic methods (11 – 13) using a spectrophotometer (Gilford System 2600; Gilford Instrument Laboratories, Inc., Oberlin, OH, USA). Energy charge potential (ECP) was calculated according to the following formula:

\[ \text{ECP} = \frac{(\text{ATP} + 0.5 \text{ ADP})}{(\text{ATP} + \text{ADP} + \text{AMP})} \]

Assay of the tissue FFA

The levels of tissue FFA were measured according to the method described in our previous report (8). Briefly, the frozen cardiac tissue (about 150 mg) was pulverized in a mortar cooled with liquid nitrogen, and tissue FFA were extracted from the pulverized tissue with chloroform/methanol (2:1) containing 0.05% butylated hydroxytoluene (an anti-oxidant), and then the FFA in the extract were converted to their fluorescent derivatives with 9-anthryldiazomethane in methanol. After incubation at room temperature for 1 hr, the fluorescent-derivatives of FFA were filtered with a Millipore™ filter (FH 0.5 μm; Nihon Millipore Kogyo K.K., Yonezawa) and injected into a reverse-phase high-performance liquid chromatography system with a Zorbax-ODS column (0.46 x 25 cm; DuPont, Philadelphia, PA, USA). Methanol/distilled water (100:80) was used as the mobile phase. The level of individual FFA was determined by comparing the peak height of the FFA with that of a known amount of heptadecanoic acid (an internal standard).

Drugs

Dilazep hydrochloride was kindly supplied by Kowa Company (Tokyo). The drug was dissolved in the normal KHB buffer used in the present study. Biochemicals, reagents, and enzymes were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Statistical analyses

All data are expressed as means±S.E.M. The significance of difference between means was analyzed by the
analysis of variance, followed by Duncan's multiple-range test for unpaired observations and by Student's t-test for paired observations. A P value of 0.05 or less was considered significant.

RESULTS

Coronary flow and RPP

Changes in coronary flow and those in RPP during non-ischemia, ischemia and reperfusion in the presence or absence of dilazep are illustrated in Fig. 1. A change of perfusion from the Langendorff to the working heart method increased coronary flow and RPP. In the control experiments, ischemia decreased coronary flow to 0 ml/min (upper panel), decreased the heart rate to 0/min and also decreased aortic pressure to 0 mmHg; therefore, RPP became 0 mmHg/min after ischemia (lower panel). After reperfusion following ischemia, coronary flow recovered, although incompletely, but RPP that had been decreased by ischemia did not recover, indicating that ischemia and reperfusion produced mechanical dysfunction of the heart.

In the pre-ischemic (i.e., non-ischemic) heart, dilazep at 1 μM, 5 μM or 10 μM did not affect coronary flow significantly (upper panel, from 10 to 15 min), but it decreased RPP in a concentration-dependent manner (lower panel, from 10 to 15 min). The decrease in RPP of the pre-ischemic heart induced by dilazep at 5 μM or 10 μM was significant statistically. Even in the presence of dilazep, ischemia decreased both coronary flow and RPP. The dilazep-treated heart was then reperfused. After 20 min of reperfusion of the ischemic heart treated with dilazep at 5 μM or 10 μM, there was a significant degree of recovery in both coronary flow and RPP, indicating that dilazep at 5 μM or 10 μM restored coronary flow and mechanical function during reperfusion following ischemia toward the pre-ischemic level. Dilazep at 1 μM, which did not significantly change RPP in the pre-ischemic heart, however, did not accelerate the recovery of either coronary flow or RPP during reperfusion following ischemia.

Because dilazep in the concentrations used in the present study did not have a coronary dilating effect in the non-ischemic heart, the dilazep-induced increase in coronary flow during reperfusion is regarded as a phenomenon secondary to an increase in the cardiac mechanical function induced by the drug, which increases coronary venous drainage, hence coronary flow.

High-energy phosphates

In Fig. 2, changes in the levels of both ATP (upper panel) and CrP (lower panel) during non-ischemia, ischemia and reperfusion in the presence or absence of dilazep at 1 μM, 5 μM or 10 μM are shown. In the control group, the tissue level of ATP markedly decreased after ischemia, but it recovered after reperfusion, although incompletely; the level of ATP in the control:I group was significantly lower than that in the control:N1 group and also significantly lower than that in the control:R group. Dilazep at 1 μM, 5 μM or 10 μM did not affect the basal level of ATP in the non-ischemic heart. The levels of ATP in the 10 μM dilazep:I and 5 μM dilazep:R groups, however, were significantly higher than that in the control:I group. The levels of ATP in the 10 μM dilazep:I and 5 μM dilazep:R groups, however, were significantly higher than that in the control:R group.

The level of CrP in the control group decreased marked-
ly after ischemia and recovered completely after reperfusion. Dilazep at 10 μM increased the basal level of CrP in the non-ischemic heart. Dilazep at 10 μM or 5 μM attenuated the decrease in CrP caused by ischemia significantly, and it increased the level of CrP after reperfusion significantly. Higher levels of CrP induced by dilazep before ischemia are probably due to a decrease in cardiac mechanical function induced by the drug. The CrP levels in the 5 μM dilazep:I or 10 μM dilazep:I group were significantly higher than that in the control:I group, and the CrP levels in the 5 μM dilazep:R and 10 μM dilazep:R groups were also significantly higher than that in the control:R group. Changes in ECP are shown in the upper panel of Fig. 3. The results with ECP were essentially the same as those with ATP.

**Lactate**

Changes in the level of lactate during ischemia and reperfusion are shown in the lower panel of Fig. 3. In the control group, the level of lactate increased significantly after ischemia compared to the non-ischemic group and

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**Fig. 2.** The levels of ATP (upper panel) and CrP (lower panel) in the rat heart. In the NI (non-ischemia), I (ischemia) and R (reperfusion) groups, the heart was freeze-clamped immediately before ischemia, at the end of ischemia and at the end of reperfusion, respectively. See the text for names of the groups (Materials and Methods). Drugs were given from 5 min before ischemia to the end of reperfusion. Each group consists of more than 6 hearts. All data are expressed as means±S.E.M. *P<0.05, between two subgroups in the control, 1 μM dilazep, 5 μM dilazep or 10 μM dilazep group. **P<0.05, compared with the value in the corresponding subgroup (NI, I or R) in the control group.

**Fig. 3.** The tissue levels of ECP (upper panel) and lactate (lower panel) in the rat heart in Fig. 2. Symbols are the same as those in Fig. 2.
decreased markedly after reperfusion compared to the ischemic group. The lactate level in the 10 μM dilazep:I group was significantly lower than that in the control:I group. The level of lactate in the 10 μM dilazep:R or 5 μM dilazep:R group was significantly lower than that of the control:R group.

Arachidonic acid and other FFA

In Fig. 4, changes in the levels of arachidonic (upper panel) and palmitoleic (lower panel) acids during ischemia and reperfusion are illustrated. In the control group, the levels of arachidonic and palmitoleic acids increased after ischemia, and further increased markedly after reperfusion. The level of arachidonic acid in the 10 μM dilazep:I group was significantly lower than that in the control:I group, and the arachidonic acid levels in the 1 μM dilazep:R, 5 μM dilazep:R and 10 μM dilazep:R groups were significantly lower than that in the control:R group. A similar result was obtained with palmitoleic acid. It should be noted that accumulation of arachidonic and palmitoleic acids was more markedly attenuated by dilazep during reperfusion than that during ischemia. In Fig. 5, changes in the levels of linoleic (upper panel) and stearic (lower panel) acids are illustrated. In the control group, these FFA increased after ischemia and further increased markedly after reperfusion. The effect of dilazep on the tissue levels of linoleic and stearic acids was essentially the same as that on the levels of arachidonic and palmitoleic acids; dilazep at 10 μM significantly attenuated the accumulation of stearic acid during ischemia, and dilazep at 1 μM, 5 μM or 10 μM significantly attenuated the accumulation of both linoleic and stearic acids during reperfusion.

**Fig. 4.** The tissue levels of arachidonic acid (upper panel) and palmitoleic acid (lower panel) in the rat heart in Fig. 2. Symbols are the same as those in Fig. 2.

**Fig. 5.** The levels of linoleic acid (upper panel) and stearic acid (lower panel) in the rat heart in Fig. 2. Symbols are the same as those in Fig. 2.
DISCUSSION

The results of the present study clearly demonstrate that dilazep has an anti-ischemic action in concentrations that do not increase coronary flow of the pre-ischemic heart. Both ischemia-induced and reperfusion-induced damages were attenuated by dilazep. The anti-ischemic effect of dilazep is possibly due to a decrease in cardiac mechanical function, because dilazep decreased pre-ischemic cardiac mechanical function concentration-dependently and hence conserved the ATP level after ischemia. The degree of recovery of post-ischemic mechanical function was shown to depend on the level of ATP after ischemia (Fig. 6). Therefore, the anti-ischemic effect of dilazep on the ischemic heart can be interpreted as an energy-sparing effect (14). Recovery of coronary flow during reperfusion induced by dilazep at 5 μM or 10 μM is probably due to recovery of mechanical function as mentioned before.

What is the mechanism of the decrease in cardiac mechanical function induced by dilazep? According to Tamura et al. (15), dilazep has a local anesthetic action, the extent of which is comparable to that of lidocaine. The fact that dilazep inhibits myocardial hypercontracture induced by veratridine (16), which slows the inactivation process of the Na⁺ channel, supports the view that the protective effect of dilazep is due to its Na⁺ channel blocking effect, leading to a decrease in mechanical function of the heart. There are some interesting reports showing that dilazep produces a Ca²⁺ antagonizing effect in the guinea pig taenia coli (17) and in dog coronary artery (18). Dilazep also inhibits the histamine-stimulated cytosolic Ca²⁺ increase in cultured endothelial cells (19).

In the heart, both ischemia and reperfusion produce release of arachidonic acid and other FFA in the myocardium (8, 20, 21). This is because ischemia and reperfusion increase the intracellular calcium concentration, leading to increased activity of the phospholipases and hence to increased release of FFA from phospholipids of the cell membrane (22-24). Anti-ischemic drugs such as β-adrenoceptor antagonists and calcium channel blockers have been reported to attenuate the ischemia-reperfusion-induced FFA accumulation during both ischemia and reperfusion (8, 25). In addition, d-propranolol, which has practically no β-adrenoceptor antagonistic action but has a local anesthetic action, and lidocaine attenuate FFA accumulation during ischemia and reperfusion (26, 27).

By what mechanism did dilazep attenuate the ischemia- and reperfusion-induced FFA accumulation? FFA accumulation is thought to be a result of increased deacylation and/or decreased reacylation of membrane phospholipids (28). During ischemia, there was a decline in the level of ATP that regulates the activity of acyl-CoA synthetase, which is a rate limiting factor of phospholipid synthesis (29). This suggests that one of the major mechanisms of FFA accumulation during ischemia is a decrease in reacylation to phospholipids. Because dilazep attenuates the decrease in the ATP level during ischemia, it inhibits FFA accumulation during ischemia. Deacylation of

![Graph](image)

Fig. 6. The relationship between the tissue levels of ATP and the RPP values. Upper panel: Relationship between the decrease in RPP before ischemia and the decrease in the ATP level induced by ischemia, showing that the more RPP decreases before ischemia, the smaller the ischemia-induced decrease in the ATP level is. Lower panel: Relationship between the value of RPP at the end of reperfusion and the ATP level immediately after ischemia, showing that the higher the ATP level after ischemia is, the higher the RPP level after reperfusion is. In both the upper and lower panels, the data are those in Fig. 1 (lower panel) and Fig. 2 (upper panel), and only the mean values in the control, dilazep at 1 μM, 5 μM and 10 μM groups are plotted. Taking both data into account, a decrease in RPP (mechanical function) would minimize the decrease in the ATP level after ischemia, leading to a better recovery of mechanical function.
phospholipids should be accelerated by ischemia, because there is an increased activity of phospholipase A₂ during ischemia (30), which results from an increase in intracellular levels of Ca²⁺.

The mechanism of FFA accumulation during reperfusion is not the same as that during ischemia, because further accumulation of FFA occurs during reperfusion even when the ATP level increases. There is circumstantial evidence that myocardial phospholipases A₂ and C mainly contribute to the post-ischemic reperfusion injury (31, 32). Because the optimum pH range for the myocardial phospholipase A₂ is neutral to alkaline (33), the enzyme would be activated during reperfusion to a greater extent than during ischemia. If dilazep inhibits reperfusion-induced intracellular Ca²⁺ increase, it would attenuate reperfusion-induced damage. In vitro experiments have demonstrated that dilazep inhibits release of myristic acid from L-α-dimyristoyl phosphatidylcholine by the phospholipases A₂ or C (34). Dilazep also inhibits the thrombin-induced release of arachidonic acid in platelets (35), probably by directly inhibiting release of arachidonic acid from phospholipids in the platelet cell membrane. Therefore, it is possible to assume that dilazep inhibits release of FFA during ischemia and reperfusion by inhibiting the cardiac mechanical function and by attenuating the ischemia-reperfusion-induced increase in the activity of phospholipases directly or indirectly. It is of interest that dilazep at 1 μM did not restore mechanical function during reperfusion, but it attenuated FFA accumulation during reperfusion. This indicates that dilazep at 1 μM has an anti-ischemic action although it is not great enough to restore the mechanical function, and that FFA accumulation is a sensitive indicator of ischemia-induced damage, particularly reperfusion-induced damage.

There are some other possible mechanisms for the anti-ischemic action of dilazep. Dilazep is known to protect the shape change of erythrocytes induced by storage in the cold for a period of more than one month (36), and therefore it is likely that dilazep has a non-specific membrane-protecting effect. Nevertheless, the main mechanism of the non-specific membrane-protecting effect of dilazep is unclear. There is also another possibility: dilazep attenuates ischemia-reperfusion-induced changes because it may attenuate the effect of lysophosphoglycerides. Our recent studies have revealed that lysophosphatidylcholine produces ischemia-reperfusion-like changes (including decreases in the ATP and CrP levels and increases in the lactate and FFA levels) in the isolated rat heart and that dilazep attenuates the lysophosphatidylcholine-induced changes (37). Because there is accumulation of lysophosphoglycerides in the myocardium during ischemia (38, 39), the above assumption would be possible.

In conclusion, dilazep attenuates the ischemia-reperfusion-induced damage of the heart probably because of its energy-sparing effect and some other effects that remain to be studied.

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