Effects of Diazoxide on Norepinephrine-Induced Vasocontraction and Ischemic Myocardium in Rats

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Effects of diazoxide on norepinephrine-induced vasocontraction in vitro and global ischemia-induced lactate accumulation in the myocardial tissue in vivo were studied in rats. Diazoxide produced relaxation of the isolated rat aorta contracted by norepinephrine in a dose dependent manner. The relaxation of the aorta was associated with reduction of intracellular Ca²⁺ concentration. This reduction may be due either to activation of K⁺ channels or Na⁺-K⁺ ATPase, or to both. Global ischemia induced by aorta constriction for 30 min in vivo increased the myocardial tissue level of lactate. Pretreatment with diazoxide (10mg·kg⁻¹, i.v.) significantly attenuated the accumulation of lactate due to global ischemia. The present study suggests that diazoxide reduces ischemic influence on the myocardium partly through its vasodilatory action.

Keywords diazoxide; norepinephrine; fura-2; myocardial ischemia; lactate

Diazoxide, a derivative of benzo thiadiazine, has a vasodilatory effect, but no diuretic effects.1,2 We demonstrated previously,3 that the vasodilatory action of diazoxide is due to opening of the ATP-sensitive K⁺ channels and inhibiting of receptor-operated Ca²⁺ movements from either the intracellular store sites or extracellular space to the cytoplasm. In addition, diazoxide improves Ca²⁺ perturbation in the vascular smooth muscle, leading the deoxycorticosterone acetate (DOCA)-induced hypertensive rats to normotensive.4 Myocardial ischemia increases the release of norepinephrine in the myocardial tissue, and enhances ischemic damage of the heart.5 The released norepinephrine may contract the vasculature in the myocardium and worsen the ischemic deterioration. It is possible that diazoxide has some beneficial effects on the ischemic myocardium via its inhibition of norepinephrine-induced vasocontraction. The present study, therefore, was undertaken to examine the effect of diazoxide on norepinephrine-induced contraction of vascular smooth muscle in vitro and on ischemic myocardium in vivo.

MATERIALS AND METHODS

In Vitro Experiments In the first series of experiments, the thoracic aorta was carefully removed from male Wistar rats weighing 250—350 g which had been anesthetized with pentobarbital sodium (30 mg·kg⁻¹, i.p.), and dissected free from the adjacent tissues in a Krebs-bicarbonate solution (118 mm NaCl, 4.7 mm KCl, 2.5 mm CaCl₂, 1.2 mm MgSO₄, 1.2 mm KH₂PO₄, 12.5 mm NaHCO₃, 11.1 mm dextrose, and 0.01 mm EDTA-2Na) gassed with 95% O₂-5% CO₂. The endothelium was removed by gently rubbing the intimal surface with a cotton swab moistened with a Krebs-bicarbonate solution and then cut into helical strips 2 mm in width and 10 mm in length. The tissue strips were suspended under 1 g of preload tension with steel hooks in an organ bath containing 10 ml of a Krebs-bicarbonate solution aerated with 95% O₂-5% CO₂ at 37°C, and kept in it for 60—90 min for equilibration. The isometric contraction of the aortic strips was measured with a force-displacement transducer (Toyo Baldwin T-7-8-240, Tokyo, Japan) and recorded on a polygraph (Nihon Kohden, Tokyo, Japan). The high K⁺ solution was made by replacing NaCl in the normal solution with equimolar KCl.

When the intracellular concentration of Ca²⁺ was measured together with the contraction, the aorta strip was loaded with 10 μM acetoxyethyl ester of fura-2 for 2—3 h in the presence of 0.2% Cremophor EL (a non- cytotoxic (detergent) at 37°C. The strip was then washed once and held horizontally in a bath (10 ml in capacity) at 37°C connected to a fluorimeter (CAF-100, Jasco Corporation, Tokyo, Japan). One end of the aorta strip was connected to a strain gauge transducer (Toyo Boldwin, Tokyo, Japan) and the other end was fixed. Again, the strip was washed several times with Krebs-bicarbonate solution and equilibrated over a period of 1 h. The aortic strip was illuminated alternately (50 Hz) with two excitation wavelengths (340 and 380 nm) and the 500 nm fluorescence emitted from the strip was collected into a photomultiplier. The amount of 500 nm fluorescence induced by 340 nm excitation (F₃₄₀) and that induced by 380 nm excitation (F₃₈₀) was measured and the ratio of these two fluorescent (R₃₄₀/₃₈₀) was calculated.6

Norepinephrine-induced contraction was obtained by exposing the tissue strip to norepinephrine at a final concentration of 1×10⁻⁶ M. Twenty minutes after the plateau contraction was observed, the tissue strip was washed with Krebs-bicarbonate solution 5—6 times over a 60-min period, and allowed to return to its resting basal line. Again, the strip was exposed to 1×10⁻⁶ M norepinephrine. After observing the maximal response to norepinephrine, diazoxide was added to the bath at final concentrations of 1×10⁻⁵, 5×10⁻⁵, and 1×10⁻⁴ M. Because the drug was dissolved in saline—NaOH solution, one of a pair of the tissue strips always served as a vehicle control treated with saline—NaOH solution. The aorta strip loaded with fura-2 was exposed to norepinephrine using the procedure described above. Diazoxide (1×10⁻⁴ M) was added to the bath 10 min after the exposure of norepinephrine. Ten minutes after the addition of...
diazoxide, glibenclamide at $1 \times 10^{-6}$ M was also added. In the experiments with ouabain, ouabain ($1 \times 10^{-3}$ M) was loaded for 1 h prior to the final addition of $1 \times 10^{-6}$ M norepinephrine.

**In Vivo Experiments** In the second series of experiments, rats were anesthetized with pentobarbital sodium (30 mg·kg⁻¹, i.p.), tracheally intubated, and ventilated with room air. A left side thoracotomy was performed to expose the heart. The aorta proximal to the heart was encircled with a silk ligature. Ischemia was initiated by ligating the aorta 10 min after diazoxide (10 mg·kg⁻¹) or vehicle was injected into the right femoral vein. Thirty minutes after the onset of ischemia, the heart was removed and immediately frozen with freezing clamps prechilled in liquid nitrogen. Heart samples were also obtained from a rat whose aorta was not ligated. The frozen myocardial samples were pulverized in liquid nitrogen with a pestle and mortar, and extracted with 6% perchloric acid. The level of lactate in neutralized perchloric acid extract was determined according to standard enzymatic procedures.⁷

**Statistics** Data are expressed as mean ± S.E. Results were analyzed by one-way analysis of variance followed by Dunnnett’s $t$ test. $p$ value less than 0.05 was considered statistically significant.

**RESULTS**

**Effects of Diazoxide on Norepinephrine-Induced Contraction** The effects of diazoxide on norepinephrine-induced aortic contraction are illustrated in Fig. 1. Vehicle containing NaOH did not change aortic contraction induced by $1 \times 10^{-6}$ M norepinephrine (control). Diazoxide at either dose significantly decreased the norepinephrine-induced aortic contraction, and the decrease was in a dose dependent manner.

**Intracellular Ca²⁺ Concentration** Figure 2 shows a representative trace of effects of diazoxide and glibenclamide on changes in intracellular Ca²⁺ concentration and tension induced by norepinephrine. The results are summarized in Fig. 3. Addition of norepinephrine to the bath produced an immediate and transient increase in $R_{340/380}$, indicating increase in the intracellular Ca²⁺ concentration. Tension was slowly increased by norepinephrine, and reached maximum 10 min after the addition. At this point, diazoxide ($1 \times 10^{-4}$ M) was added. Diazoxide decreased the tension associated with the decrease in intracellular Ca²⁺ concentration. Slope of the decrease in intracellular Ca²⁺ in diazoxide-treated aorta was steeper than that in vehicle-treated aorta. Addition of glibenclamide ($1 \times 10^{-6}$ M) tended to increase the tension and to slow the decrease in the intracellular Ca²⁺.

We also studied the effect of diazoxide on 30 mM KCl induced contraction of the aorta. The results are shown in Fig. 4. KCl immediately but transiently increased intracellular Ca²⁺ concentration with a gradual increase in tension. Diazoxide facilitated the decrease in $R_{340/380}$ and produced relaxation of the aorta. Glibenclamide appeared to restore the aortic contraction with slowing of the decrease in $R_{340/380}$.

**Effects of Diazoxide on Norepinephrine-Induced Contraction in Aorta Preloaded with Ouabain** To examine the effect of diazoxide on the Na⁺-K⁺ ATPase, the aorta was preloaded with $1 \times 10^{-3}$ M ouabain (Fig. 5). Diazoxide produced a significant vasodilation in the presence of ouabain.

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**Fig. 1. Effects of Diazoxide on NE-Induced Aorta Contraction**

Data are expressed as % relaxation of the aorta contracted with NE of $1 \times 10^{-6}$ M. Note that the bottom of the ordinate shows 100% relaxation. Result of diazoxide at 0 M was obtained in the aorta treated with vehicle (NaOH–saline solution). Values are means S.E. of 8–17 observations in each group.

**Fig. 2. Representative Tracing of Intracellular Ca²⁺ Concentration and Contraction**

Either diazoxide of $1 \times 10^{-4}$ M or vehicle was added to the bath 10 min after treatment with $1 \times 10^{-4}$ M norepinephrine (NE), and glibenclamide of $1 \times 10^{-8}$ M was added 10 min after the addition of vehicle or diazoxide.
of ouabain. However, diazoxide-induced relaxation of the aorta in the presence of ouabain was significantly less than that in the absence of ouabain.

**In Vivo Experiment** The effect of diazoxide on accumulation of lactate in the ischemic myocardium is shown in Fig. 6. Myocardial ischemia significantly increased the tissue level of lactate. Injection of diazoxide at a dose of 10 mg·kg⁻¹ significantly attenuated the accumulation of myocardial lactate caused by ischemia.

**DISCUSSION**

Diazoxide produces vasodilation in the vascular smooth muscle precontracted with norepinephrine,⁸⁻¹¹ serotonin,¹² or angiotensin II.¹¹,¹² Diazoxide also shows a significant relaxation in the aortic tissues contracted with 30 and 40 mM KCl, but not in those with 80 mM KCl.³

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**Fig. 3.** Effects of Diazoxide and Glibenclamide on Intracellular Ca²⁺ Concentration (Upper Panel) and Contraction (Lower Panel) after Treatment with Norepinephrine (NE)

Experimental protocol is the same as that in Fig. 2. ● represents diazoxide (1 x 10⁻⁴ M) treatment and ○ represents vehicle treatment. The intracellular Ca²⁺ concentration and contraction are expressed as percentages of maximum changes in R₂₀₀₀ø and contraction, respectively, induced by norepinephrine. Values are means ± S.E. of 4—7 observations. *p<0.05; **p<0.01, compared with the corresponding values in vehicle-treated control group.

**Fig. 4.** Effects of Diazoxide and Glibenclamide on Intracellular Ca²⁺ Concentration (Upper Panel) and Contraction (Lower Panel) after Treatment with 30 mM KCl

Experimental protocol and symbols are the same as those in Fig. 3. Values are means ± S.E. of 4—7 observations. *p<0.05, compared with vehicle-treated control group.

**Fig. 5.** Effects of Ouabain on Diazoxide-Induced Aorta Relaxation

In all groups, the aorta was contracted with 1 x 10⁻⁴ M norepinephrine, and then vehicle (control) or diazoxide at 1 x 10⁻⁴ M (diazoxide) was added. Ouabain (1 x 10⁻⁵ M) was loaded for 1 h prior to the addition of norepinephrine (ouabain + diazoxide). Values are means ± S.E. of 5—17 observations. **p<0.01, compound with "control". "p<0.01, compared with "diazoxide".
Because glibenclamide attenuates the vasodilatory effects of diazoxide induced by 50 mm KCl, ATP-sensitive K⁺ channels may be involved in the mechanism of vasorelaxation with diazoxide. However, we have also demonstrated that diazoxide reduces norepinephrine induced contraction through inhibition of receptor-operated Ca²⁺ movement. In the present study, vasodilation with diazoxide was associated with reduction of intracellular Ca²⁺ concentration in the aorta contracted with either norepinephrine or KCl (Figs. 3 and 4). Glibenclamide almost completely blocked diazoxide induced vasodilation in aorta contracted with KCl, whereas it incompletely blocked the dilation in the aorta contracted with norepinephrine. This suggests that the mechanism of diazoxide-induced relaxation in the aorta contracted with norepinephrine involves other factors in addition to the opening of ATP-sensitive K⁺ channels. Activation of Na⁺-K⁺ ATPase in the cell membrane may decrease the intracellular Ca²⁺ concentration and dilate the vascular smooth muscle. Increase in Na⁺ efflux decreases the intracellular Na⁺ ion concentration, and then stimulates the Na⁺-Ca²⁺ exchange system. Finally, increase in Na⁺ influx via Na⁺-Ca²⁺ exchange decreases intracellular Ca²⁺ concentration. If diazoxide were to dilate the vascular smooth muscle via Na⁺-K⁺ ATPase activation, inhibition of Na⁺-K⁺ ATPase with ouabain could attenuate diazoxide induced vasorelaxation. Ouabain significantly inhibited the diazoxide-induced relaxation of the aorta contracted with norepinephrine (Fig. 5). It is possible that one of the mechanisms of diazoxide-induced vasodilation is activation of Na⁺-K⁺ ATPase. Because diazoxide has multiple-mechanisms of vasodilation, it dilates the aorta contracted with KCl more strongly than other KATP channel openers.

In the present study, ischemia was induced by cessation of the systemic circulation. In the preliminary experiments, we attempted to make regionally ischemic hearts by suturing the coronary artery of anesthetized open chest rats. However, after 30 min of ischemia, it was hard to take the tissue samples rapidly from only the ischemic region of the myocardium. The lactate level in the ischemic myocardium showed wide variation. Because the method of aorta clamping used is not unusual for making the isolated perfused rat heart globally ischemic, we used this method in the in vivo experiment. Ischemia in the heart augments release of norepinephrine in the myocardium. Norepinephrine increases myocardial energy requirement and decreases energy supply, because it increases cardiac work and contracts the coronary vasculature. Because diazoxide relaxes the vascular smooth muscle contracted with norepinephrine, this drug may exert a beneficial effect on the ischemic myocardium. Ischemia accelerates anaerobic carbohydrate metabolism and causes accumulation of some deteriorative end products in the myocardium, such as lactate. Accumulation of tissue lactate caused by global ischemia was significantly attenuated by diazoxide (Fig. 6), indicating a protective effect of the drug on the ischemic myocardium. The blood pressure is decreased by diazoxide injection under the same experimental conditions. Because the vascular tone is maintained by norepinephrine in vivo, diazoxide decreases the blood pressure by its vasodilatory action. This may be one of the mechanisms operating in the cardioprotective effect of diazoxide.

In conclusion, diazoxide relaxes the vascular smooth muscle contracted with norepinephrine through reduction of the intracellular Ca²⁺ concentration, and may have some protective effect on the ischemic myocardium.

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REFERENCES