*Japanese Journal of Physiology*, **49**, 409–415, 1999

# Protein Kinase C Inhibitors Abolish the Increased Resistance of Diabetic Rat Heart to Ischemia-Reperfusion Injury

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**Abstract:** Protein kinase C (PKC) has been implicated in ischemic preconditioning, but whether it plays a role in the cardioprotection observed in the diabetic heart is not known. We assessed the possible role of PKC by investigating whether the inhibition of PKC with staurosporine (Stau, 0.01 μM) or chelerythrine (Chel, 1 μM) can abolish the increased resistance to ischemia (25 min)-reperfusion (30 min) injury in Langendorff perfused hearts from streptozotocin-induced 4-week diabetic rats. In the diabetic heart, pre-ischemic left ventricular developed pressure (LVDP), double product (DP: LVDP×heart rate/1,000), ±d*P*/d*t*(max) and coronary flow rate (CFR) were all reduced compared to the control. The pretreatment with Stau or Chel significantly improved these parameters. The post-ischemic contractile function was recovered to a greater extent in the diabetic heart (116.9±20.5% of pre-ischemic DP) than in the control (23.3±2.3% of pre-ischemic DP), indicating the increased resistance of the diabetic heart to ischemia-reperfusion injury. The treatment with Stau or Chel abolished the enhanced recovery in the diabetic heart (36.0±14.6 and 54.1±12.8% of pre-ischemic DP, respectively). The reduction in post-ischemic end diastolic pressure (EDP) and lactate dehydrogenase (LDH) release in diabetes (13.5±2.5 mmHg and 27.2±6.2 U/g heart) compared to the control (55.8±2.9 mmHg and 60.3±5.7 U/g heart) was significantly ($p<0.05$) increased by pretreatment with Stau (39.0±4.9 mmHg and 53.1±7.6 U/g heart) or Chel (36.2±3.0 mmHg and 48.8±4.3 U/g heart). Neither Stau nor Chel had any influence on the post-ischemic values of LVDP, DP, ±d*P*/d*t*(max), EDP and LDH release in the control heart. In the conclusion, the present results suggest that PKC activation may, at least in part, contribute to the increased resistance of the diabetic heart to ischemia-reperfusion injury. [Japanese Journal of Physiology, 49, 409–415, 1999]

*Key words*: diabetic heart, PKC, ischemia-reperfusion, cardioprotection.

Although controversy still exists as to whether the diabetic heart is more or less susceptible to ischemic injury, a number of studies, including our previous report [1], have convincingly demonstrated that the diabetic heart is more resistant to ischemic injury [2]. However, the mechanism of this resistibility of the diabetic heart is not yet clearly understood.

Protein kinase C (PKC) is a serine-threonine protein kinase that is relatively abundant in cardiovascular tissues and plays an important role in the regulation of cell growth and contractile function. There is much current interest in the potential role of PKC in ischemic preconditioning and various evidence supports this hypothesis; PKC activators such as phorbol esters can mimic the protective effect of ischemic preconditioning, and PKC inhibitors including chelerythrine block ischemic preconditioning [3–6]. Among PKC isoforms, PKC-α, δ, ε isoforms are suggested to be involved in conferring the cardioprotection of ischemic preconditioning in normal rats [7].

PKC can be upregulated by hyperglycemia in the diabetic model [8, 9]. Increased PKC activities have

Received on June 9, 1999; accepted on August 6, 1999
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been demonstrated in the membrane of heart [10], aorta [11] and retina of the diabetic rat [12], and in heart from human type-I insulin-dependent diabetes mellitus (IDM) [13]. In addition, it has been reported that PKC activity in the diabetic rat heart and aorta increased in parallel with the increase in diacylglycerol (DAG) level in the same tissue [14]. It has been thus suggested that the increased activity of membranous PKC is probably due to elevated DAG levels since DAG is a physiological activator of PKC [14]. Despite a number of previous reports supporting the increased activity of PKC in the diabetic heart, there is no information about the role of PKC in the cardioprotective mechanism in the diabetic rat.

In the present study, we tested the possible role of PKC by assessing whether the inhibition of PKC with staurosporine (Stau) and chelerythrine (Chel) can block the cardioprotective effect of the Langendorff hearts from streptozotocin (STZ)-induced diabetic rats.

## METHODS

**Induction of diabetes.** Male Sprague-Dawley rats weighing 200–230 g were fasted for 24 h. Then animals were made diabetic by injecting streptozotocin (STZ, 50 mg/kg, I.P.) dissolved in 0.1 M citrate buffer (pH 4.5, 4°C). Non-diabetic control animals (age-matched controls) were injected with an equivalent volume of the citrate buffer only. Blood glucose levels were measured by using a glucometer (Johnson & Johnson), and diabetic rats (blood glucose >300 mg/dl) were used 4 weeks after the induction of diabetes.

**Isolated Langendorff heart preparation.** An isolated heart perfusion experiment was performed by a method previously described [15]. Rats were anesthetized with sodium pentobarbital (50 mg/kg, I.P.) and then injected with heparin (1,000 IU/kg) intravenously through the tail vein. The trachea was intubated and rats were mechanically ventilated with a rodent ventilator (Model 7025, Ugobasile, Italy). After thoracotomy, the heart was perfused *in situ* with modified Krebs-Henseleit bicarbonate buffer (KH buffer, mM: NaCl 116, $NaHCO_3$ 24.9, KCl 4.7, $MgSO_4$ 1.1, $KH_2PO_4$ 1.17, $CaCl_2$ 2.52, glucose 8.32 and pyruvate 2.0) at pH 7.4 and 37°C by retrograde aortic cannulation. The hearts were then excised and moved to a Langendorff apparatus (H.S.E., Germany) where they were perfused with KH buffer, which was oxygenated with carbogen (95% $O_2$/5% $CO_2$) at a constant perfusion pressure (65 mmHg). A water-filled latex balloon attached to a metal cannula was placed in the left ventricle through the pulmonary vein and connected to an Isotec pressure transducer (H.S.E.) for the measurement of left ventricular-developed pressure (LVDP). The hearts were allowed to equilibrate for 15 min, at which time left ventricular end-diastolic pressure (EDP) was adjusted to 10 mmHg, and this balloon volume was maintained throughout the experiment.

**Experimental conditions.** After equilibration, Stau or Chel was added to the perfusate to give final concentrations of 0.01 and 1.0 μM, respectively, and perfused in a retrograde fashion for 5 min prior to ischemia. The concentrations and exposure time of PKC inhibitors were chosen on the basis of preliminary studies (data not shown). Isolated hearts were then subjected to global ischemia by completely shutting off the perfusate. After 25-min ischemic time, reperfusion with KH buffer (without Stau and Chel) was initiated by opening the perfusion-flow line to the heart and flow continued for 30 min. To prevent the myocardium from drying out during global ischemia following reperfusion, and to maintain the cardiac temperature at 37°C throughout the experiment, hearts were submerged in a chamber filled and circulated with 37°C KH buffer.

**Determinants for cardiac function and injury before and after ischemia-reperfusion.** LVDP, as an indicator of cardiac contractile function, was calculated by subtracting EDP from left ventricular peak systolic pressure. Heart rate (HR) was recorded by using tachometer amplifiers (Grass Instrument Co.). Double product (DP), an important parameter for assessing cardiac performance, was calculated by multiplying LVDP by HR. Maximum $+dP/dt$ ($+dP/dt$(max)) and maximum $-dP/dt$ ($-dP/dt$(max)), indicators of the rate of contractile and relaxant response, respectively, were determined by differentiating LVDP (Differentiator, Grass 7P20C). Coronary flow rate (CFR) was determined by the collection of coronary effluent for 1 min. Samples of coronary effluent during 30 min-reperfusion were collected to determine lactate dehydrogenase (LDH) release, as a sensitive index for cellular injury, by an optimized spectrophotometric assay kit (340-LD, Sigma Chemical Co., St. Louis, MO, USA). EDP measured at 30-min after reperfusion was used as an index for myocardial contracture.

**Chemicals.** Streptozotocin (STZ, Sigma) was used for the induction of diabetes. Staurosporine (Stau) and chelerythrine (Chel), purchased from Sigma Chemical Co., were dissolved in dimethyl sulfoxide (DMSO) and phosphate-buffered saline, respectively, and diluted with Modified Krebs-Henseleit

**Table 1. Effect of staurosporine and chelerythrine on cardiac function and coronary flow rate before and after ischemia-reperfusion.**

| | Control | | | Diabetes | | |
|---|---|---|---|---|---|---|
| | Pre-ischemia | | Post-ischemia | Pre-ischemia | | Post-ischemia |
| | Pre-drug | Post-drug | R30 | Pre-drug | Post-drug | R30 |
| LVDP (mmHg) | | | | | | |
| Veh | 71.5±5.1 | 72.3±5.1 | 20.0±2.2[a] | 48.2±4.7[c] | 48.8±4.2[c] | 53.2±2.0[c] |
| Stau | 68.0±5.3 | 73.8±4.3 | 14.5±1.5[a] | 51.7±4.1[c] | 72.3±6.0[a,b] | 16.7±4.7[a,b] |
| Chel | 68.1±3.3 | 65.0±3.7 | 15.5±3.8[a] | 49.8±4.7[c] | 70.8±5.0[a,b] | 27.4±3.1[a,b] |
| HR (beats/min) | | | | | | |
| Veh | 337±5.7 | 332±5.5 | 290±5.4[a] | 258±9.6[c] | 254±7.2[c] | 249±4.9[c] |
| Stau | 335±6.0 | 348±9.4 | 295±15 | 250±4.6[c] | 247±3.6[c] | 241±4.2[c] |
| Chel | 331±6.0 | 323±6.0 | 266±12[a] | 260±6.3[c] | 262±4.8[c] | 239±8.7 |
| CFR (ml/min) | | | | | | |
| Veh | 17.3±1.1 | 17.4±1.0 | 9.4±0.9[a] | 9.0±1.4[c] | 9.0±1.4[c] | 13.0±1.8[a,c] |
| Stau | 16.0±0.4 | 19.9±1.0[a] | 13.2±1.0[b] | 9.4±0.8[c] | 19.7±1.2[a,b] | 14.2±2.2[a] |
| Chel | 17.3±0.9 | 18.5±1.4 | 12.9±1.0[b] | 9.8±0.6[c] | 12.8±0.8[a,c] | 11.9±0.8 |

LVDP, left ventricular developed pressure; HR, heart rate; CFR, coronary flow rate; R30, post 30min-reperfusion; Veh, vehicle; Stau, staurosporine 0.01 μM; Chel, chelerythrine 1.0 μM. [a] $p<0.05$ vs. pre-drug; [b] $p<0.05$ vs. Veh (vehicle); [c] $p<0.05$ vs. control.

bicarbonate buffer (KH buffer) to give final concentrations of 0.01 and 1.0 μM, respectively. The final concentration of DMSO (0.01%) was found to have no effect on developed pressure or recovery from ischemia-reperfusion, thus it was used for the vehicle (Veh). Drugs (Veh, Stau, Chel) were perfused into the heart through the aortic cannula (not circulated) by using a Gilson peristaltic pump.

**Data analysis.** All values are expressed as mean ±SEM. Data were analyzed by unpaired Student's *t*-test between two groups. All statistical differences were determined at $p<0.05$ level.

## RESULTS

### Effect of PKC inhibitors on HR, LVDP and CFR

In this study, we used 4-week diabetic rats (270±17.9 g body weight, 438±39 g/dl blood glucose) and age-matched control rats (355±20 g body weight, 88±7.5 g/dl blood glucose). Table 1 shows the absolute values for pre-ischemic and post-ischemic cardiac functions. In the diabetic vehicle group, all the pre-ischemic values of LVDP, HR and CFR were significantly ($p<0.05$) reduced from their control vehicle values of 74.5±5.1 mmHg, 337±5.7 beats/min and 17.3±1.1 ml/min to 48.2±4.7 mmHg, 258±9.6 beats/min and 9.0±1.4 ml/min, respectively, indicating the development of diabetes-induced cardiac dysfunction. The reduced values of pre-ischemic LVDP and CFR in the diabetic heart were increased by treatment with Stau (72.3±6.0 mmHg, $p<0.05$, and 19.7±1.2 ml/min, $p<0.05$) or Chel (70.8±5.0 mmHg, $p<0.05$, and 12.8±0.8 ml/min, respectively). In the control heart, pre-ischemic CFR but not LVDP increased by treatment with Stau (24.4±2.4%) or Chel (6.9±2.0%). Our results also showed that post-ischemic recovery of LVDP, HR and CFR after 25 min-ischemia followed by 30 min-reperfusion was greater in the vehicle-treated diabetic heart (118.3±16.3, 96.9±3.6 and 146.9±12.4% of pre-ischemic values, respectively) than that in the vehicle-control heart (27.9±2.8, 86.2±2.2 and 52.6±6.2% of pre-ischemic values, respectively), confirming the increased resistance to ischemia-reperfusion injury in the diabetic heart. The greater recovery of post-ischemic LVDP in the diabetic heart was significantly ($p<0.05$) diminished by pretreatment with Stau (36.9±14.4% of pre-ischemic LVDP) or Chel (54.1±12.8% of pre-ischemic LVDP), while the improved recovery of post-ischemic CFR in the diabetic heart was not altered by Stau nor Chel. In the control heart, neither Stau nor Chel had any influence on post-ischemic decrease in LVDP or HR, while post-ischemic recovery of CFR was improved by Stau (83.0±8.0% of pre-ischemia) or Chel (74.6±6.4% of pre-ischemia) compared with that in the vehicle-control heart (52.6±6.2% of pre-ischemia).

### Effect of PKC inhibitors on cardiac performance

To assess the effect of Stau and Chel, we used another parameter for cardiac performance, double product (DP), calculated by multiplying LVDP by HR. As shown in Fig. 1, pre-ischemic DP in vehicle-diabetes (12.4±1.4) was reduced more than that of the vehicle-control (24.0±1.7), and this dysfunction in the diabetic heart was significantly ($p<0.05$) improved by treatment with Stau or Chel (17.9±1.6 and 18.6±1.5, respectively). In the control heart, the pre-ischemic value of DP (24.0±1.7) remained unaltered after treatment with Stau or Chel (25.7±1.8 and 21.0±3.8, respectively). The resistance of the diabetic heart to post-ischemic dysfunction, as shown by greater post-ischemic DP in vehicle-diabetes (13.3±0.7) than vehicle-control (5.8±0.7), was almost completely abolished by pretreatment with Stau or Chel (4.0±1.1 and 6.4±0.7, respectively), while the post-ischemic DP of vehicle-control (5.8±0.7) remained unaltered after pretreatment with Stau or Chel (4.2±0.3 and 4.0±0.9, respectively). The abolishing effect of Stau or Chel on the resistance of the diabetic heart to post-ischemic dysfunction was further demonstrated by other parameters, $+dP/dt$(max) (rate of contractile function) and $-dP/dt$(max) (rate of relaxant function) (Fig. 2).

### Effect of PKC inhibitors on myocardial contracture following ischemia-reperfusion

Pre-ischemic EDP was adjusted to 10 mmHg in all groups. Post-ischemic EDP in the vehicle-control heart increased up to the value of 55.8±2.9 mmHg due to myocardial contracture following ischemic in-

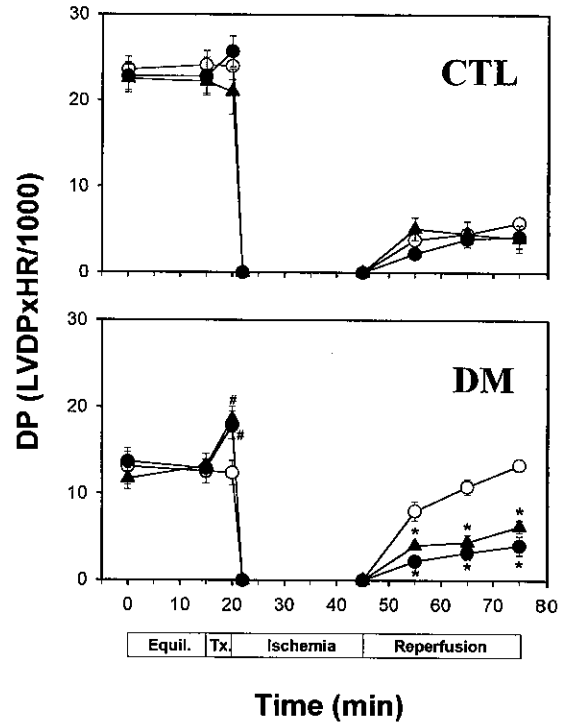


**Fig. 1. Effect of staurosporine (Stau) and chelerythrine (Chel) on double product (DP) before and after 25-min ischemia followed by 30-min reperfusion in isolated heart from normal control (CTL) and diabetic (DM) rats ($n$=7–8).** DP was calculated by multiplying left ventricular developed pressure (LVDP) by heart rate (HR). After 15-min equilibration (Equil.), the treatment (Tx.) with vehicle (Veh, 0.01% DMSO), Stau (0.01 μM) or Chel (1.0 μM) was performed by the addition of drugs to the perfusate 5 min prior to ischemia. ○, vehicle; ●, Stau; ▲, Chel. # $p<0.05$ vs. pre-drug, * $p<0.05$ vs. vehicle-treated DM.

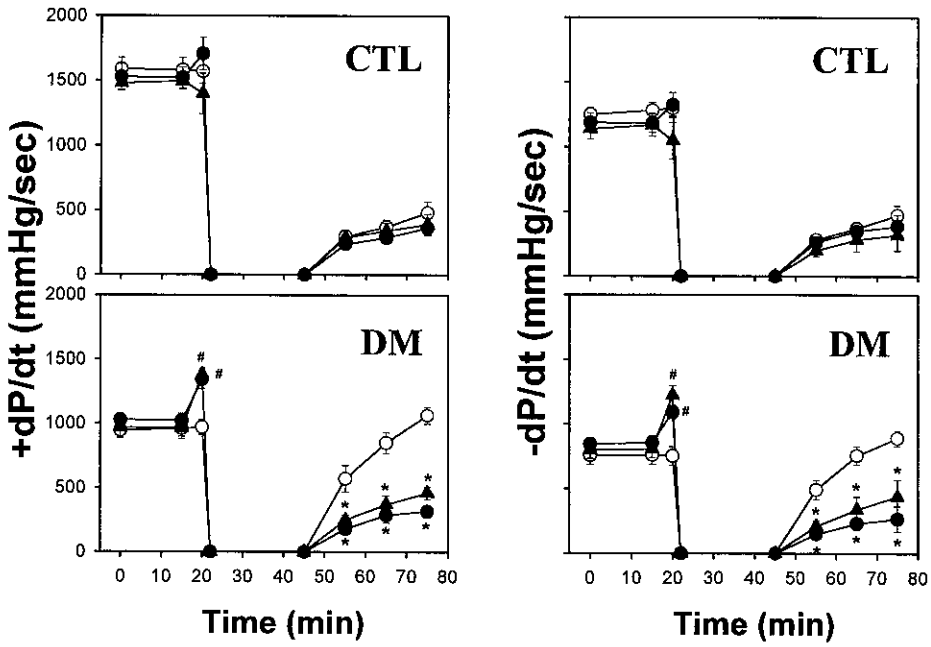


**Fig. 2. Effect of staurosporine (Stau) and chelerythrine (Chel) on $+dP/dt$(max) and $-dP/dt$(max) before and after 25-min ischemia followed by 30-min reperfusion in isolated heart from normal control (CTL) and diabetic (DM) rats ($n$=7–8).** Time schedule for equilibration (15 min) and treatment (5 min) was the same as shown in Fig. 1. Vehicle (Veh, 0.01% DMSO), Stau (0.01 μM) or Chel (1.0 μM) was added to perfusate 5 min prior to ischemia. ○, vehicle; ●, Stau; ▲, Chel. # $p<0.05$ vs. pre-drug, * $p<0.05$ vs. vehicle-treated DM.

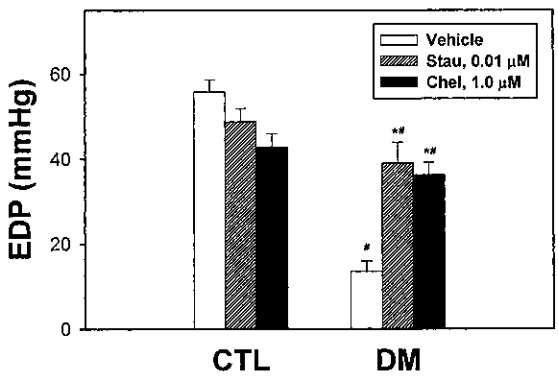


**Fig. 3. Effect of staurosporine (Stau) and chelerythrine (Chel) on end diastolic pressure (EDP) after 30-min reperfusion in heart from control (CTL) and diabetic (DM) rats ($n$=7–8).** Pre-ischemic EDP was adjusted to 10 mmHg in all groups. Vehicle (Veh, 0.01% DMSO), Stau (0.01 μM) or Chel (1.0 μM) was added to perfusate 5 min prior to ischemia. #$p<0.05$ vs. vehicle-treated CTL, *$p<0.05$ vs. vehicle-treated DM.

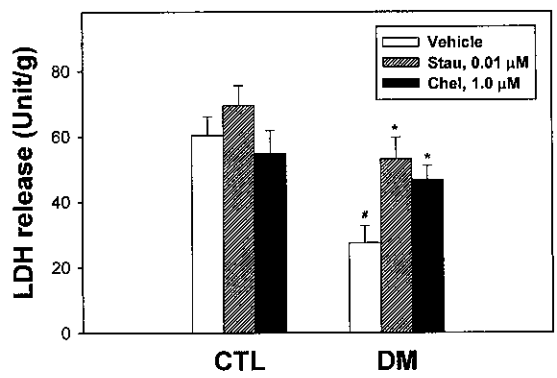


**Fig. 4. Effect of staurosporine (Stau) and chelerythrine (Chel) on lactate dehydrogenase release (LDH) during 30-min reperfusion in isolated heart from control (CTL) and diabetic (DM) rats ($n$=7–8).** Vehicle (Veh, 0.01% DMSO), Stau (0.01 μM) or Chel (1.0 μM) was added to perfusate 5 min before ischemia. #$p<0.05$ vs. vehicle-treated CTL, *$p<0.05$ vs. vehicle-treated DM.

sult, while the increase in post-ischemic EDP of the vehicle-diabetic heart was far less (13.5±2.5 mmHg), indicating greater recovery of the post-ischemic diastolic function in diabetic myocardium. This improvement in the diabetic heart was aggravated by Stau or Chel, as shown in Fig. 3, where post-ischemic EDP in the diabetic heart (13.5±2.5 mmHg) was increased by treatment with Stau or Chel (39.0±4.9 and 36.2±3.0 mmHg, respectively).

### Effect of PKC inhibitors on tissue injury following ischemia-reperfusion

As shown in Fig. 4, there was a significant ($p<0.05$) reduction in post-ischemic LDH release in the diabetic heart (27.5±6.2 U/g heart) compared to that in the control (60.3±5.7 U/g heart), indicating less injury following ischemia-reperfusion in the diabetic heart. This cardioprotective effect observed in the diabetic heart was completely abolished when Stau or Chel was treated for 5 min before ischemic insult, so that LDH release in the Stau- and Chel-treated diabetic groups (53.1±7.6 and 48.8±4.3 U/g heart, respectively) were not significantly different from that in the vehicle-treated control group (60.3±5.7 U/g heart).

## DISCUSSION

The present study demonstrates that staurosporine and chelerythrine (nonselective and highly selective PKC inhibitors, respectively) can abolish the increased resistance of the diabetic heart to ischemia-reperfusion injury.

In this study, we hypothesized that PKC might play a role in the preconditioning effect of the diabetic rat heart. This hypothesis was based on two facts: 1) PKC activation is an essential component in the protective effect of ischemic preconditioning [4], and 2) PKC is activated in diabetic rat heart [16]. Although we did not measure the activity of PKC nor determine which PKC isoforms are involved in diabetic heart, as an alternative way to test this hypothesis, we investigated the effect of PKC inhibitors on the resistibility of a diabetic heart.

Our results showed that the magnitude of the pre-ischemic basal contractile function (LVDP, DP) was significantly reduced in the diabetic heart as compared with that in a control heart. These results are in agreement with the report that the increased activity of PKC in the diabetic heart stimulates the phosphorylation of myocardial proteins, troponin and the troponin-tropomyosin complex, which may decrease cardiac muscle contractility [17]. Hug and Sarre [18] suggested that the persistent upregulation of PKC activity in the diabetic heart might lead to many changes in cardiac function, including impairment of myocardial contractility. Our observation that this depressed contractile function (LVDP, DP) was significantly improved up to the control level by staurosporine and chelerythrine suggests that the inhibition of PKC can restore the impaired cardiac function in diabetics. This effect of PKC inhibition in diabetes may be explained by the decreased phosphorylation of myocardial proteins. Our finding that the reduced $+dP/dt(\max)$ (the rate of contraction) in diabetes was enhanced by PKC inhibitors (Fig. 2) seems to be associated with the increased $Ca^{2+}$ handling through PKC-mediated modulation of sarcolemmal $Ca^{2+}$ channels

[19]. On the other hand, the enhancing effect of PKC inhibitors on $-dP/dt(max)$ (the relaxation rate) may be explained by the improved $Ca^{2+}$ uptake through the sarcoplasmic reticulum (SR) since the $Ca^{2+}$ uptake to SR is impaired by PKC activation [20].

We observed that the basal coronary flow rate was reduced in the diabetic heart (Table 1). This could also be related to increased PKC activity in the vessels of the diabetic heart since the increase in PKC activity in vascular smooth muscle is thought to induce sustained contraction, possibly by phosphorylation of the contractile proteins calponin and caldesmon [21]. Therefore, the greater improvement of reduced coronary flow in the diabetic heart than in the control heart, as induced by staurosporine and chelerythrine, may result from the greater extent of inhibition on PKC-mediated contraction in diabetic vessels.

As for the post-ischemic function, this study confirms our previous finding [1] by showing that the contractile function of the diabetic heart following ischemia-reperfusion recovered to a greater extent than that of the control heart. This is consistent with other reports that the diabetic heart can actually be more resistant than non-diabetic heart to ischemic injury *in vitro* and *in vivo* an animal models [22–25]. In addition, our study demonstrated that both the enhanced recovery of post-ischemic contractile function (DP, $\pm dP/dt(max)$, EDP) and protection against post-ischemic tissue injury (LDH release) in the diabetic heart were almost completely abolished by pretreatment with staurosporine and chelerythrine. From these results, it is suggested that alterations in PKC activity in the diabetic myocardium can actually be beneficial to the heart during prolonged ischemia followed by reperfusion. Consistent with our findings, Liu *et al.* [24] suggested that diabetic rat hearts are more readily preconditioned than control hearts. The previous studies by others have shown that the administration of PKC activator such as phorbol ester and diacylglycerol can produce a similar degree of protection against ischemia-reperfusion injury as that seen after ischemic preconditioning [26]. Ytrehus *et al.* [4] suggested that activated PKC during ischemic preconditioning might phosphorylate a secondary effector, which may be able to induce protective effects of preconditioning. Thus, it is appealing to speculate that pathophysiologic alterations such as cardiac dysfunction in diabetes might mimic ischemic preconditioning in a non-diabetic heart through PKC activation as a common pathway, with a resultant triggering of cardioprotective responses against injury from a prolonged period of ischemia-reperfusion. Our findings that the disappearance of cardioprotective effect in the diabetic heart by treatment with PKC inhibitors might be explained by the PKC inhibition–associated increase in myocardial work and oxygen demand prior to ischemia-reperfusion, and the resultant abolition of such preconditioning effect.

We observed that pretreatment with PKC inhibitors failed to decrease post-ischemic coronary function in the diabetic heart (Table 1) despite their ability to reduce post-ischemic contractile function. In addition, post-ischemic coronary function in control hearts was rather enhanced by pretreatment with saurosporine or chelerythrine as compared to the vehicle-control. These results could be consistent with other reports that endothelial dysfunction induced by ischemia-reperfusion is prevented by staurosporine [27]. Our findings, therefore, suggest that the abolition of cardioprotective effect can be caused without being accompanied by coronary dysfunction following ischemia-reperfusion in isolated rat heart.

In conclusion, the present results suggest that PKC activation may be, at least in part, involved in the increased resistance to ischemia-reperfusion injury in hearts from STZ-induced diabetic rats, although this study does not provide explanations for the mechanisms by which PKC activation plays a role in the diabetes-induced resistibility. We further suggest that the diabetic rat heart can be a useful model in studies about the mechanism for cardioprotection against ischemia-reperfusion injury.

This research was supported by a grant from the Graduate School of Medicine, Ajou University.

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