Short Communication

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Protein Kinase C Inhibitors Attenuate Protective Effect of High Glucose against Hypoxic Injury in H9c2 Cardiac Cells

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Abstract: In this study, we demonstrated that PKC inhibitors significantly attenuated the cardioprotective effect produced by high-glucose (22 mM) treatment for 48 h against hypoxic injury in H9c2 cardiac cells. PKC activators mimicked the cardioprotective effect of high glucose. These results suggest a possible role of PKC activation in high-glucose–induced protection. [Japanese Journal of Physiology, 50, 645–649, 2000]

Key words: high glucose, cardioprotection, PKC.

Several previous studies including ours [1] have demonstrated the cardioprotective effect of diabetic heart against ischemic injury, suggesting a contribution of a high level of plasma glucose to the cardioprotection. In our preliminary report [2], we provided a demonstration about the cardioprotective effect of high glucose *in vitro* on hypoxic injury by using a cultured cardiac cell system.

Protein kinase C (PKC) has been considered to mediate ischemic preconditioning; PKC activators such as phorbol esters or synthetic diacylglycerol analogues mimic the cardioprotection afforded by ischemic preconditioning, and various PKC inhibitors attenuate the effect of preconditioning on infarct size, contractile dysfunction, and arrhythmias during reperfusion [3]. It has been reported that the activity of PKC is increased in hyperglycemic rat heart and various cardiovascular cells cultured in high glucose condition [4, 5]. We previously suggested that the activation of PKC associated with hyperglycemia may play a role in the cardioprotection from the results that PKC inhibitors abolished the cardioprotective effect of diabetic heart against ischemic injury [1].

In the present study, we investigated the role of PKC in high-glucose–induced cardioprotection *in vitro* by examining whether the pharmacological inhibition of PKC can block the cardioprotective effect. We also tested whether exogenous activation of PKC can mimic the cardioprotection.

H9c2 cardiac myocytes were obtained from the American Type Culture Collection (Rockville, MD). H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose supplemented with 10% fetal bovine serum before experiment; cells were then further maintained for 48 h in DMEM containing low glucose (5.5 mM), high glucose (22 mM), or mannitol (16.5 mM) with low glucose. For PKC activation, myocytes were stimulated with a PKC activator, 4β-phorbol 12-myristate 13-acetate (PMA, 10 nM) or 1,2-dioctanoyl-*sn*-glycerol (DOG, 100 μM), in 5.5 mM glucose–containing DMEM for 30 min and 8 h, respectively, before hypoxia. A PKC inhibitor, chelerythrine (Chel, 3 μM) or staurosporine (Stau, 10 nM), was added to the medium 1 h before hypoxia. The concentrations and durations of drug treatment were chosen on the basis of preliminary studies (data not shown). For hypoxic challenges, H9c2 myocytes were transferred into an anaerobic chamber (Forma Scientific) maintained at 37°C with a humidified atmosphere of 5% CO_2, 10% H_2, and 85% N_2, as described earlier [6]. In the anaerobic chamber, a culture medium was replaced with serum-free, glucose-free DMEM saturated with N_2 gas for 1 h before use. Normoxic incubation of myocytes in serum-free DMEM was conducted in a water-jacked incubator gassed with 95% air and 5% CO_2 at 37°C. Overall cell injury was assessed by measuring the activity of lactate dehydrogenase (LDH) released into the medium

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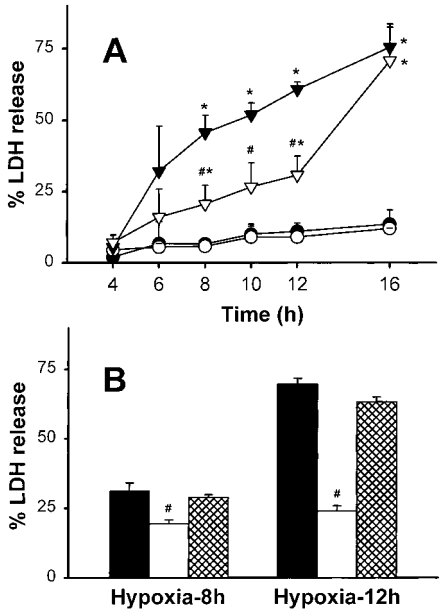

Fig. 1. Effects of high glucose (A) and mannitol (B) on lactate dehydrogenase (LDH) release after hypoxic insult. H9c2 cells were maintained for 48 h in low glucose (5.5 mM), high glucose (22 mM), or mannitol (16.5 mM) with low glucose (5.5 mM) before being transferred to an anaerobic chamber, where the culture medium was replaced with serum-free, glucose-free, anoxic DMEM. A serum-free normoxic medium was used for the normoxic group. Data represent mean±SEM. (n=5–6). Percent of LDH was calculated from the maximum LDH release (100%) induced by 0.1% triton X-100. A: Closed circle, 5.5 mM glucose normoxia; open circle, 22 mM glucose normoxia; closed triangle, 5.5 mM glucose hypoxia; open triangle 22 mM glucose-hypoxia. B (8 or 12 h after hypoxic insult): closed, 5.5 mM glucose; open, 22 mM glucose; hatched, 16.5 mM mannitol + 5.5 mM glucose. * $p<0.05$ vs. 5.5 mM glucose normoxia; # $p<0.05$ vs. 5.5 mM glucose hypoxia.

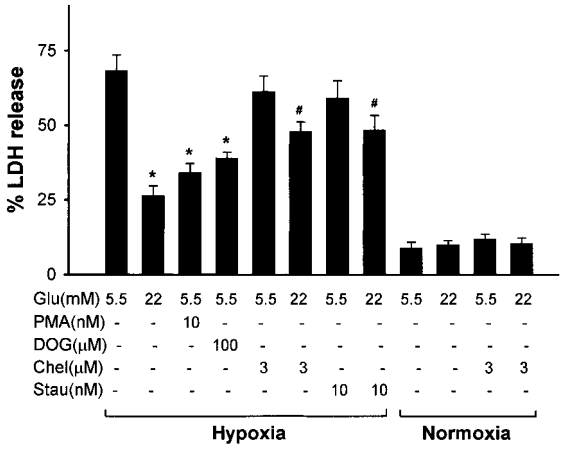

Fig. 2. The effects of PKC activators and inhibitors on LDH release 10 h after hypoxic insult. H9c2 cells were maintained for 48 h in low glucose (5.5 mM) or high glucose (22 mM) before being transferred to an anaerobic chamber, where the culture medium was replaced with serum-free, glucose-free, anoxic DMEM. 4β-Phorbol 12-myristate 13-acetate (PMA, 10 nM) and 1,2-dioctanoyl-*sn*-glycerol (DOG, 100 μM) were treated for 30 min and 8 h, respectively, before hypoxia. Staurosporine (Stau, 10 nM) and chelerythrine (Chel, 3 μM) were treated for 1 h before hypoxia. Data represent mean±SEM (n=5–6). Percent of LDH release was calculated from the maximum LDH release (100%) induced by 0.1% triton X-100. * $p<0.05$ vs. 5.5 mM glucose hypoxia, # $p<0.05$ vs. 22 mM glucose hypoxia. Glu: glucose.

10 h after hypoxic insult as previously described [7]. The percent of LDH was calculated from the maximum LDH release (100%) induced by lysing with 0.1% triton X-100. To examine the extent of cell death, we performed a morphologic observation microscopically under phase-contrast optics and staining assay with propidium iodide (PI, 10 μg/ml), which is normally not taken up by living cells [8]. All data were expressed as mean±SEM. Numerical data were compared by using a Student's *t*-test for unpaired observations between two groups. The p value of <0.05 was considered significant.

Under normoxic conditions, LDH release in 5.5 and 22 mM glucose–treated cells after 10 h were 11.0±2.8 and 9.1±3.3%, respectively (Fig. 1A). As shown in Fig. 1A, when cells cultured in 5.5 mM glucose media were incubated under hypoxic conditions, LDH release after 10-h hypoxia was increased markedly (60.7±2.6%), but far less in 22 mM glucose-treated cells (30.7±6.7%). The duration of 22 mM glucose-treatment, 48 h, was chosen from the result that the maximum protective effect of 22 mM glucose was shown at the treatment duration of 48 h instead of 24 or 36 h (data not shown). To examine whether the effect of 22 mM glucose is associated with its high osmolarity, we observed the effect of 16.5 mM mannitol added to 5.5 mM glucose media, to give the same osmolarity as 22 mM glucose. The LDH release in mannitol-treated cells was not different from that in 5.5 mM glucose (Fig. 1B). When H9c2 cells were treated with PMA or DOG, LDH release after 10-h hypoxia was significantly decreased (34.2±3.1 and 39.1±2.0%, respectively) compared with that in 5.5 mM glucose-media (68.3±5.2%) (Fig. 2). Treatment with Chel (3 μM) or Stau (10 nM) 1 h before hypoxia significantly ($p<0.01$) inhibited 22 mM glucose–induced cardioprotection, increasing LDH release (48.0±3.3 and 48.5±4.9%, respectively, Fig. 2) compared with that in 22 mM glucose–treated control (26.5±3.3%). As shown in Fig. 3, H9c2 cells under normoxic conditions remained quite intact (1.0% cell death). After 8 h of hypoxia, 30.3±1.6% of low-glucose–treated cells were dead, as indicated by their permeability to PI, and retracted from adhesion sites and became round, but only a few cells (4.3±1.8%) were

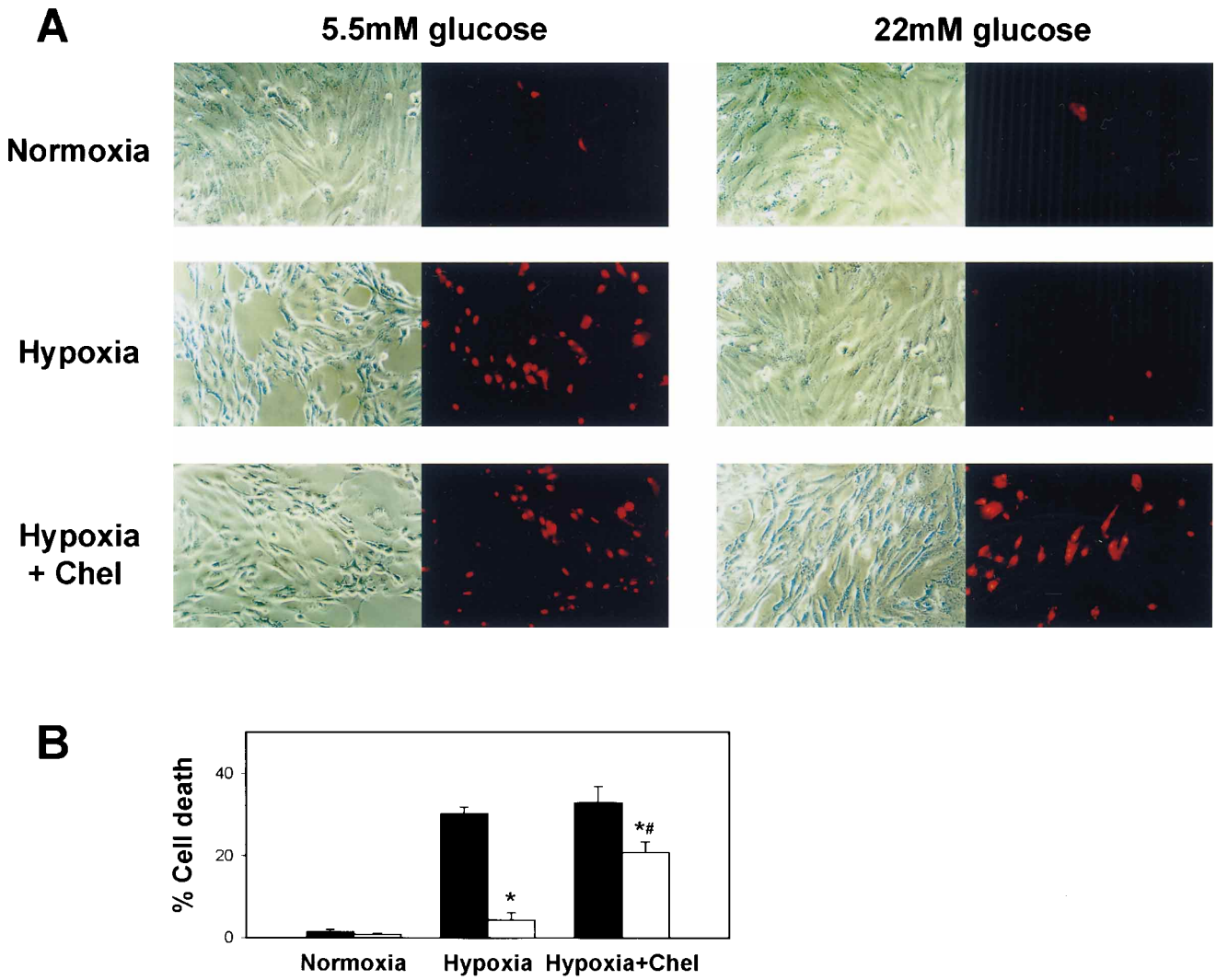

Fig. 3. Effect of chelerythrine on cell death 8 h after hypoxic insult. H9c2 cells were maintained for 48 h in low glucose (5.5 mM) and high glucose (22 mM) before being transferred to an anaerobic chamber where the culture medium was replaced with serum-free, glucose-free, anoxic DMEM. Chelerythrine (Chel, 3 μM) was applied for 1 h before hypoxia. **A**: Phase contrast and propidium iodide (PI)–stained photographs. Data shown are representative of 4 experiments. **B**: Percent of cells stained with PI, mean±SEM (n=5). Closed bar: 5.5 mM glucose; open bar: 22 mM glucose. * p<0.05 vs. 5.5 mM glucose hypoxia, # p<0.05 vs. 22 mM glucose hypoxia.

stained with PI when treated with high glucose. The treatment with Chel (3 μM) 1 h before hypoxia significantly (p<0.01) increased (20.7±2.6%) the uptake of PI after 8-h hypoxia in high-glucose–treated cells with no influence on low-glucose–treated cells.

In this study we investigated the possibility that high glucose can induce protection from hypoxic injury through PKC activation. This hypothesis was because high glucose increases PKC activity [4, 5], and PKC activation is thought to be essential in the mechanism of ischemic preconditioning [3].

Our results demonstrated that high glucose can produce a >50% protection against hypoxic injury in an H9c2 cardiac cell culture model, as determined by LDH release and PI-staining after hypoxic (serum-, glucose-, and oxygen-free) insult. In agreement with this high-glucose effect, we previously reported the protective effect of high glucose against hypoxic injury in smooth muscle cells [9]. A recent report is very consistent with the present result, which shows the protective effect of 25 mM high glucose against chemical hypoxia in neonate cardiomyocytes [10]. High-glucose–induced protective effect has also been reported by other studies demonstrating that administration of high glucose reduces brain damage in vivo [11], and that high glucose protects neuron cells against excitotoxins [12]. Controversial results remain, however, about the effect of high glucose, suggesting that high glucose exaggerates ischemic injury in endothelial cells [13]. This discrepancy may be due

to cell type specificity.

Twenty-two millimolar glucose used in this study has higher osmolarity than 5.5 mM glucose. Because high osmotic stress can trigger a stress-regulated protein kinase cascade with a resultant alteration of apoptotic cell death [14], we tested the possibility that the effect of high glucose is associated with its high osmolarity. The possibility, however, can be excluded from the result that the same osmolarity of mannitol (mannitol 16.5 mM+glucose 5.5 mM) as 22 mM glucose did not alter hypoxic injury (Fig. 1B). This suggests that high glucose per se may trigger some intracellular signals leading protection against hypoxic insult in cardiac cells.

PKC has been known to play a critical role in cardioprotection by ischemic preconditioning in several animals [15], humans [16], and cultured cardiomyocytes [17]. During ischemic preconditioning, PKC is activated through a cellular signaling pathway involving adenosine A1 receptors and G protein–stimulated phospholipase C activity with a resultant increase in diacylglycerol (DAG) level, which elevates the translocation of PKC from cytosol to membrane [3]. In this context, hyperglycemia-, and/or high-glucose–induced PKC activation has been demonstrated in diabetic heart and various cardiovascular cells cultured in a high glucose condition [4]. The mechanism of this PKC activation seems different from that in preconditioning, since it includes an increase in *de novo* synthesis of DAG and phosphatidic acid, a precursor for DAG. The enhanced *de novo* synthesis of phosphatidic acid from the elevated glycolytic intermediates results in increased *de novo* synthesis of DAG [4]. Regarding the role of this PKC activation, we previously suggested that the PKC activation might play a role in the hyperglycemia-induced mimicking of preconditioning in isolated rat heart model [1]. In the present *in vitro* study using cultured cardiac cells, we provide evidence supporting this PKC hypothesis. Our results demonstrated that the treatment with Chel or Stau inhibited quite significantly ($p<0.01$) the high-glucose–induced cardioprotection with no effect on low-glucose–treated cells (Figs. 2 and 3), although the inhibition was partial but not complete, suggesting that some mechanism besides PKC activation may also be included in this cardioprotection mechanism or that PKC inhibition by Chel or Stau is incomplete. Our results also showed that the activation of PKC by PMA and DOG produced protective effect against hypoxic injury. These results suggest that cardioprotection afforded by high glucose, at least in part, correlates with PKC activation, and they further support our previous suggestion [1] that the PKC activation may be a common pathway for cardioprotection afforded by either hyperglycemia (high glucose) or preconditioning, although its upstream mechanism for activation by high glucose appears different from that by preconditioning.

There are multiple isozymes of PKC, and at least six different ones have been identified in rat cardiac myocytes [18], where individual isozymes translocate to characteristic intracellular sites following activation with each isozyme playing a different role in myocardial function. Although PKC-δ [19] and PKC-ε [18, 19] are suggested to mediate the protective effect of hypoxic preconditioning, the role of each PKC isozyme in preconditioning is not yet thoroughly understood. Moreover, further study is needed to determine which PKC isotype is activated by high glucose and which is essential for the high-glucose–induced cardioprotection shown in this study.

In conclusion, the present results suggest that PKC activation may be at least partially involved in the high-glucose–induced protection against hypoxic injury in H9c2 cardiac cells.

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