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Isoform-specific induction of PKC-ε by high glucose protects heart-derived H9c2 cells against hypoxic injury

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Abstract

We investigated which PKC isoforms are involved in high glucose-induced protection against hypoxic injury. Treatment for 48 h with high glucose (22 mM) markedly increased the expression of PKC-ε in the particulate fraction (213 ± 22.1% of the control) but had no effect on other types of PKC isoforms, suggesting that the high glucose-induced increase in PKC expression is isoform-specific. The mRNA level for PKC-ε was also substantially increased, reaching its peak after 4 h of high glucose treatment. The high glucose increased PKC-ε activity in the particulate fraction up to 183 ± 32.2% of the control. During hypoxia, the amount of PKC-ε in the particulate fraction was remarkably diminished in the low glucose-treated cells, but remained at a higher level in high glucose-treated cells. The treatment with εV1-2 (10 μM), a specific inhibitor of PKC-ε, abolished the protective effect of high glucose against hypoxia. These results suggest that isoform-specific induction of PKC-ε is involved in high glucose-induced protection against hypoxic injury in heart-derived H9c2 cells.

Keywords: High glucose; H9c2; PKC-ε; Hypoxia; Cardioprotection; Isoform-specific

A characteristic of diabetes is the development of progressive cardiomyopathy. This characteristic often manifests contractile dysfunction, which is thought to involve protein kinase C (PKC) activation, because PKC activation and phosphorylation of troponin I in diabetes cause a loss of calcium sensitivity in the intact myofibrils [1]. Previously, we suggested that pathophysiologic alterations such as cardiac dysfunction in diabetes might mimic ischemic preconditioning through PKC activation as a common pathway of cardioprotection [2]. Consistent with this hypothesis, many investigators have demonstrated that hyperglycemia favorably influences the outcome of an ischemic insult in the heart [3,4].

PKC has been increasingly recognized as an important signaling molecule in the cardioprotection mechanism of various models, including dog heart [5], rat heart [6,7], and cardiomyocyte [8]. The PKC family consists of conventional (α, βI, βII, and γ), novel (δ, ε, η, θ, and ι), and atypical (ζ, μ, and λ) types [9]. Although the predominant isozymes expressed in cardiac cells have been reported to be α, βI, δ, ε, and ζ subtypes, the functional significance of these distinct PKC isozymes has not been fully characterized [10].

Our previous study showed that treatment with 22 mM high glucose for 48 h protected H9c2 cells against hypoxic injury and that the PKC inhibitor chelerythrine abolished the high glucose-induced protective effect, suggesting the involvement of PKC in the mechanism of this high glucose effect [11]. Until now, however, the specific role of individual PKC isoforms in high glucose-induced protection has not been examined in H9c2 cells, though substantial evidence exists on the role of PKC isozymes in ischemic preconditioning [12,13]. Accordingly, the goal of the present study is to determine whether high glucose can modulate PKC

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isoforms and which PKC isoforms are involved in high glucose-induced cardioprotection in heart-derived H9c2 cells.

Method

Cell culture and hypoxia system. Heart-derived H9c2 cells were purchased from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose supplemented with 10% fetal bovine serum before experimentation. The cells were maintained for a further 48 h in DMEM containing low glucose (5.5 mM) or high glucose (22 mM). For hypoxic challenges, H9c2 myocytes were transferred into an anaerobic chamber (Forma Scientific, Marietta, OH, USA) maintained at 37 °C with humidified atmosphere of 5% CO_2, 10% H_2, and 85% N_2 as described before [14]. In the anaerobic chamber, the culture medium was replaced with serum-free, glucose-free DMEM that had been saturated with N_2 gas for 1 h. Normoxic incubation of the myocytes in the serum-free DMEM was conducted in a water-jacked incubator gassed with 95% air and 5% CO_2 at 37 °C.

Western blot analysis. To determine whether high glucose can stimulate the expression of PKC isozymes, Western blot analysis was performed. The cells were lysed using 150 μl/well of lysis buffer (20 mM Tris–HCl, 2 mM EDTA, 5 mM EGTA, 5 mM dithiothreitol, 6 mM β-mercaptoethanol, 1 mM PMSF, 20 mM leupeptin, and 50 μg/ml aprotinin, and pH 6.8). The particulate (membrane) and soluble (cytosolic) fractions of the lysates were separated by centrifugation at 100,000*g* for 1 h at 4 °C, and the supernatant was collected for the soluble fraction. Pellets resuspended in the same volume of lysis buffer were centrifuged at 10,000*g* for 10 min at 4 °C and the supernatant was collected for the particulate fraction. A quantity of 100 μg of either the soluble fraction or particulate fraction was separated on 8% SDS–PAGE and transferred onto PVDF membrane (Millipore, Bedford, MD). The membrane was blocked with 5% nonfat dried milk for 90 min and incubated overnight with PKC isotype antibodies (Santa Cruz, Delaware Avenue, CA, USA). The membrane was then incubated with a secondary immunoglobulin antibody conjugated with alkaline phosphatase for 4 h and the PKC band was visualized using the NBT/BCIP method (Sigma–Aldrich, St. Louis, MO, USA).

mRNA expression of PKC-ε. RT-PCR was performed to assess mRNA expression of PKC-ε. Total RNA was extracted from the cells with RNAsol (InTron, Sungnam, Korea) according to the manufacturer's suggested protocol. The total RNA (1 μg) was synthesized into cDNA using random hexamers, avian myeloblastosis virus reverse transcriptase (Promega, Serva, Heidelberg, Germany), and PCR buffer (20 mM Tris–HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, and 100 μg/ml bovine serum albumin). The cDNA (2 μl) was amplified by PCR in a total volume of 20 μl using 0.5 U Taq DNA polymerase (Bioneer, Seoul, Korea), 100 μM dATP, dCTP, and dGTP, 50 μM dTTP (Boehringer–Mannheim, Mannheim, Germany), and 0.5 μM of each primer in PCR buffer. One-minute cycles were performed at 95, 60, and 72 °C in a microprocessor-driven thermal cycler (Perkin–Elmer-Cetus, Emeryville, CA, USA). Primers for PKC-ε were 5′-ACT GCT CCC ACT GCA GAG AT 3′ (sense) and 5′-TAG TTC CTG GTC ACA AGG GG-3′ (antisense), while the primers for GAPDH were 5′-CCA TGG AGA AGG CTG GG-3′ (sense) and 5′-CAA AGT TGT CAT GGA TGA CC-3′ (antisense).

PKC activity assay. The enzyme activity of PKC was measured using a PKC assay kit (Invitrogen, Groningen, The Netherlands), as described previously [15]. The basis of this PKC assay was the phosphorylation of a specific substrate (myelin basic protein) by PKC and its specific inhibition by a PKC inhibitor. Cells were collected in a sample buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 25 μg/ml each aprotinin and leupeptin, and 10 mM β-mercaptoethanol). The collection was homogenized on ice, and centrifuged at 45,000*g* for 30 min, and separated into cytosolic (soluble) and particulate (membrane) fractions. The activities of the PKC in the particulate and the soluble fractions were assayed separately. The mixture of myelin basic protein and [γ-^{32}P]ATP (Amersham–Pharmacia, Piscstaway, NJ, USA) was used as the substrate of PKC for the phosphorylation reaction. The activity of PKC-ε was obtained by using εV1-2, a specific inhibitor for PKC-ε, and subtracting the value of phosphorylation that occurred with εV1-2 from that without εV1-2.

Measurement of cell death. Cell death was microscopically assessed by morphologic observation under phase-contrast optics and a staining assay with 10 μg/ml propidium iodide (PI), which is not normally taken up by living cells [16]. The cell death percentage was calculated from the percentage of cells stained with PI divided by the total number of cells after 8 h of hypoxia.

Statistical analysis. All data were expressed as means ± SD. The numerical data were compared using a Student's *t* test for unpaired observations between the two groups. A *p*-value of <0.05 was considered significant.

Results

Effect of high glucose on PKC isozyme expression

First, we attempted to determine whether the treatment of heart-derived H9c2 cells with 22 mM high glucose could stimulate the expression of PKC isozymes. As shown in Figs. 1A and C, 48 h treatment with 22 mM high glucose produced a 2.1-fold increase in the expression of PKC-ε in the particulate fraction (213 ± 22% of the 5.5 mM glucose), while PKC-ε in the soluble fraction remained unchanged (Figs. 1A and B). High glucose, however, had no effect on other types of PKC (-α, -βI, -δ, and -ζ) in either the particulate or the soluble fractions. We examined the time course effect of the 22 mM high glucose and found that high glucose tended to translocate PKC-ε protein from a soluble to a particulate as early as 2 h after treatment. The level of PKC-ε protein in the particulate markedly increased at 12 and 24 h of high glucose treatment and lasted for 48 h after treatment (Fig. 2A). To clarify whether this stimulatory effect of high glucose on PKC-ε protein is associated with its effect on mRNA, we determined the mRNA level of PKC-ε by RT-PCR. The mRNA levels of PKC-ε began to rise 15 min after the high glucose treatment, reaching their peak after 4 h of treatment. The increased level of mRNA tended to decrease at 8 h of treatment but still maintained at higher level (Fig. 2B).

Effect of high glucose on PKC activity

Since the increased expression of PKC-ε by high glucose does not in itself prove any functional activity for PKC-ε, we used a commercial PKC assay kit to evaluate the ability of high glucose to activate PKC-ε. As illustrated in Fig. 3, PKC-ε activity in the particulate fraction of cells treated with 22 mM glucose significantly increased up to 183 ± 32.2% of the cells treated with 5.5 mM glucose, though the PKC-ε activity in the

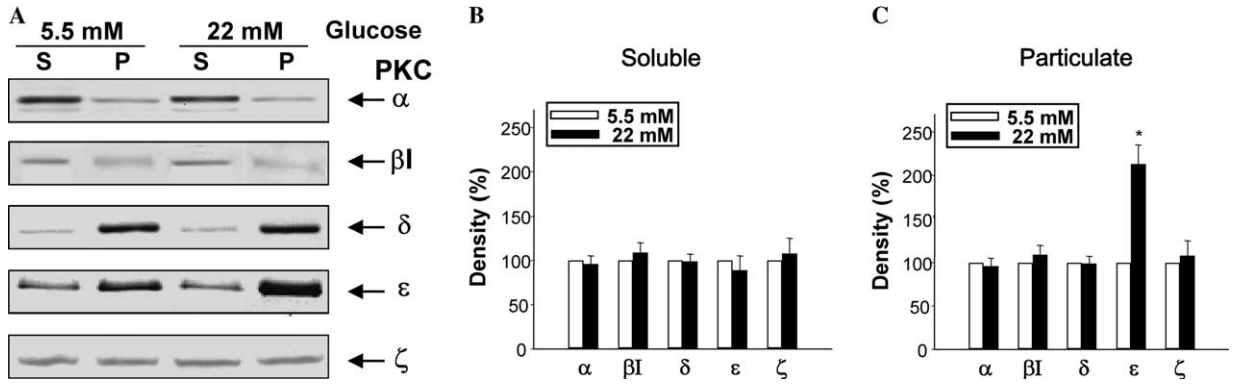

Fig. 1. Western blot analysis and quantitative Western blot analysis of PKC isozymes after the treatment with H9c2 cells with low glucose (5.5 mM) or high glucose (22 mM) for 48 h. (A) Western blots for all PKC isozymes detected in the soluble (S) and the particulate (P) fraction after 48 h of glucose treatment. Shown are representative Western blots of four separate experiments. (B) Quantitative Western blot analysis for PKC isozymes in a soluble fraction. (C) Quantitative Western blot analysis for PKC isozymes in a particulate fraction. Data shown in (B) and (C) as means ± SD ($n = 4$) represent the percentage of cells treated with low glucose (5.5 mM) for each isozyme. *$p < 0.05$ vs. values in cells treated with low glucose.

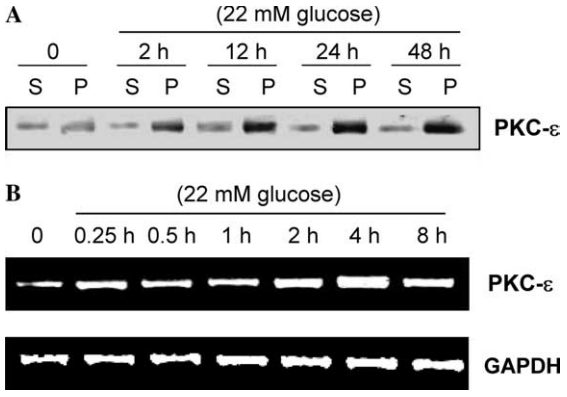

Fig. 2. Western blot analysis (A) and RT-PCR analysis (B) of PKC-ε. Shown are representatives of four separate experiments. (A) Western blots for PKC-ε detected in the soluble (S) and the particulate (P) fraction after various periods of treatment (2, 12, 24, and 48 h) with glucose (22 mM) in H9c2 cells. (B) RT-PCR products after various periods of treatment (15 min, 30 min, 1, 2, 4, and 8 h) with glucose (22 mM). Total RNAs extracted from the H9c2 cells were reverse transcribed with hexanucleotide primers; cDNA was amplified with specific primers for PKC-ε.

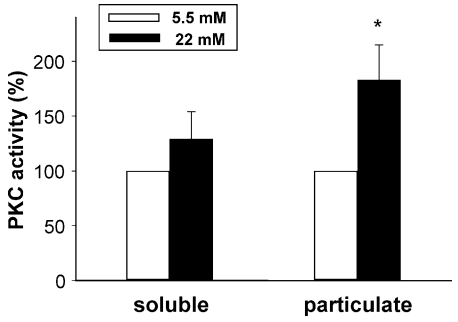

Fig. 3. Activity of PKC-ε at 48 h after the treatment of H9c2 cells with low glucose (5.5 mM) or high glucose (22 mM). PKC activity was determined using the myelin basic protein phosphorylation assay in soluble and particulate fractions. Data shown as means ± SD ($n = 4$) represent the percentage of cells treated with low glucose (5.5 mM). *$p < 0.05$ vs. cells treated with low glucose.

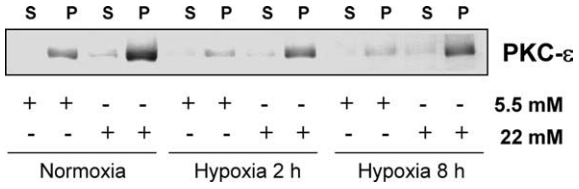

Fig. 4. Effect of high glucose on PKC-ε expression during hypoxia. H9c2 cells were treated with low glucose (5.5 mM) and high glucose (22 mM) for 48 h and then exposed to normoxia or hypoxia (2 and 8 h). Western blots for PKC-ε were detected in the soluble (S) and particulate (P) fraction. Shown are representatives of four experiments.

soluble fraction did not significantly change. This result implies that high glucose can stimulate the PKC activity because the PKC activity in the particulate is sufficient to reflect its in situ activity [17].

Effect of high glucose on PKC-ε expression during hypoxia

We investigated the effect of 22 mM high glucose on PKC-ε for short (2 h) and long (8 h) periods following the onset of hypoxia. In cells treated with 5.5 mM low glucose, particulate PKC-ε was significantly reduced 2 and 8 h after the onset of hypoxia. In cells treated with 22 mM high glucose, particulate PKC-ε decreased slightly during hypoxia but remained at a much higher level than in cells treated with low glucose after 2 h of hypoxia. As shown in Fig. 4, the higher level was maintained for at least 8 h of hypoxia.

The role of PKC-ε in high glucose-induced cardioprotection

To elucidate the role of PKC-ε in high glucose-induced protection, we examined the effect of the pharmacological blockade of PKC-ε on hypoxia-induced

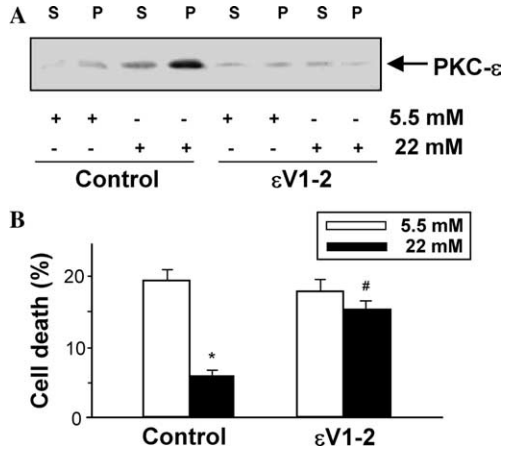

Fig. 5. Effect of PKC-ε inhibitor on PKC expression and cell death after 8 h of hypoxia. H9c2 cells were treated with low glucose (LG, 5.5 mM) or high glucose (HG, 22 mM) for 48 h in the absence (Control) or presence of myristoylated PKC-εV1-2 (εV1-2, 10 μM) before being transferred to an anaerobic chamber, where the culture medium was replaced with serum-free, glucose-free, anoxic DMEM. (A) Representative Western blot that shows PKC-ε protein levels in soluble (S) and particulate (P) fractions after 8 h of hypoxic insult ($n = 4$). (B) Cell death percentages shown as means ± SD ($n = 4$) were calculated from the percentage of cells stained with PI divided by the number of total cells. *$p < 0.05$ vs. cells treated with low glucose, #$p < 0.05$ vs. control.

cells by using εV1-2, a selective cell-permeable inhibitor of PKC-ε [12]. As reported, εV1-2 peptide, which is derived from the first unique region (V1) of PKC-ε (amino acids 14–21), inhibits PKC-ε translocation and activity, and decreases the total amount of PKC-ε in cardiomyocytes [12,18].

First, we examined the ability of εV1-2 (10 μM) to block the high glucose effect on PKC-ε during hypoxia. We observed that during hypoxia the higher level of PKC-ε was remarkably diminished by the εV1-2 in the particulate without increasing the cytosolic fraction (Fig. 5A). These results are consistent with other studies that show a reduced amount of PKC-ε in the soluble and particulate fractions in the presence of εV1-2, and lead us to support the hypothesis that εV1-2-induced inhibition of the translocation of PKC-ε may result in increase in the sensitivity of PKC-ε to degradation [12]. As shown in Fig. 5B, our results demonstrate the ability of εV1-2 to abolish the high glucose-induced resistance to hypoxic injury, by showing that high glucose decreases the number of PI-stained cells 8 h after hypoxia from 19.8 ± 2.13% (cells treated with low glucose) to 5.8 ± 0.79%; furthermore, co-treatment with εV1-2 inhibits the high glucose effect of reducing cell death by 15.1 ± 1.17%.

Discussion

In this study, we provide the first evidence that high glucose produces isoform-specific induction of PKC-ε protein in H9c2 cells, conferring protection against hypoxic injury.

Isoform-specific regulation of PKC-ε by high glucose

PKC has long been known to play a pivotal role in the functional adaptation to myocardial ischemia and the cardioprotection by ischemic preconditioning [12,13]. Previously, we demonstrated with our collaborators that 48 h treatment with 22 mM glucose protected H9c2 cells against hypoxic injury and that PKC inhibitors chelerythrine and staurosporine abolished high glucose-induced protection, suggesting the possible role of PKC in this high glucose effect [11]. On the basis of these results, we hypothesized that 48 h treatment with 22 mM high glucose could affect the activities of PKC isozymes in heart-derived H9c2 cells. Our present findings support this hypothesis by showing the ability of high glucose (22 mM) to increase the activity and expression of PKC-ε. Furthermore, our findings that high glucose had no effect on other PKC isoforms (-α, -β, -δ, and -ζ) suggest that stimulation by high glucose is isoform-specific. Interestingly, however, while the translocation of PKC was not detectable in our results except at the early stage (2 h) of high glucose treatment; the increase in the amount of PKC-ε protein in the particulate was detectable for up to 48 h of treatment. These results are consistent with other studies in which 48 h treatment with 30 mM glucose induces an increase in membrane-associated PKC-δ and PKC-ε [19].

Two distinct regulatory processes of PKC activation have recently been reported. One process translocates PKC from the cytosol to the particulate in an isotype-unselective way. The other process induces PKC in an isotype-selective way at the protein level and the mRNA level [20,21]. Supporting the latter hypothesis of an isotype-selective induction process, our findings indicate that the increased activity of PKC-ε in cells treated with high glucose (Fig. 2) may be associated with the induction at the mRNA transcriptional level and the subsequent protein accumulation of PKC-ε in the particulate fraction (Fig. 3). The results of this study demonstrate four things: the mRNA level of PKC-ε increases as early as 15 min after treatment with glucose; the higher level of PKC-ε lasts for at least 8 h; the protein level of PKC-ε begins to increase after only 2 h of treatment with 22 mM glucose; and the increased level of PKC-ε lasts for up to 48 h of treatment with 22 mM glucose. Although we did not examine the mRNA level of PKC-ε for 48 h of treatment with glucose, our results suggest that the induction of PKC-ε mRNA by high glucose may contribute, at least in part, to the increase in its protein level. Whether the increase of PKC-ε is due to the increase of synthesis or the decrease of degradation or both remains to be investigated.

The role of PKC-ε in high glucose-induced cardioprotection against hypoxic injury

There have been controversial reports about the role of PKC isotypes during an ischemic insult [12,20,22]. Strasser et al. [20] demonstrated that an acute (2.5 min) and prolonged (up to 60 min) hypoxic insult increases both the amount and the translocation of PKC-ε. We previously reported that short-term hypoxia (15 or 30 min) causes a rapid increase in the protein level of PKC-ε in a particulate fraction of H9c2 cells, while a prolonged hypoxic insult (2 and 8 h of hypoxia) significantly diminishes the protein level, suggesting that prolonged hypoxia increases degradation of PKC-ε [23]. Although our previous results on the stimulatory effect of short-term hypoxia on PKC-ε seem to be consistent with other reports [20], a discrepancy still exists between our results and the results of others on the effect of prolonged hypoxia. In contrast with our results, PKC-ε has been shown to translocate from a soluble to a particulate fraction following 30–120 min of hypoxia in primary cultured rat cardiomyocyte [12]. On the other hand, long-term hypoxia has been shown to cause a decrease of PKC-δ rather than PKC-ε in membrane association [22]. Differences between tissues, cell types, and insults might help to explain this discrepancy.

In the present study, we focused on prolonged hypoxia (2 and 8 h) because hypoxia-induced cell death requires more than 6 h of hypoxia. We found that the substantial reduction of PKC-ε shown during hypoxia was inhibited by treatment with 22 mM high glucose. This result encourages us to speculate that maintaining a higher level of PKC-ε in the particulate during hypoxia might play some beneficial roles against hypoxic injury. To investigate the role of maintaining PKC-ε through treatment with high glucose, we examined the influence of a specific blockade of PKC-ε on hypoxia-induced cell death. Co-treatment with εV1-2 and high glucose almost completely abolished not only the maintaining effect of high glucose on PKC-ε in the particulate during hypoxia but also the protective effect of the high glucose against hypoxia-induced cell death. These results support the hypothesis that PKC-ε up-regulated by high glucose plays an essential role in protecting H9c2 cells against hypoxic injury. Although we did not evaluate the ability of high glucose to regulate the expression of PKC-ε at the mRNA level during hypoxia, the possibility of reducing the degradation of PKC-ε protein through high glucose during hypoxia cannot be excluded.

In accordance with our results, others have identified PKC-ε as a mediator of the protection signal; they have demonstrated that hypoxic preconditioning selectively activates PKC-ε, which contributes to the resistance against prolonged hypoxia [12]. In addition, the protective role of PKC-ε has been demonstrated in adenosine-induced neuronal protection against ischemia-reperfusion [24] and in the development of Alzheimer's disease [25]. Thus, PKC-ε appears to be a common mediator of the protection signal in the brain and the heart, though a contrary report asserts that chronic activation of PKC-ε can result in severe conditions such as malignant tumors and vascular disease [26].

In summary, the present results suggest that isoform-specific induction of PKC-ε might be involved in the mechanism of high glucose-induced protection against hypoxic injury in heart-derived H9c2 cells.

Acknowledgments

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