Role of PKC-δ during Hypoxia in Heart-Derived H9c2 Cells

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Abstract: In the present study, we investigated the role of protein kinase C (PKC) isoforms during hypoxia in heart-derived H9c2 cells. Hypoxia caused a rapid translocation of PKC-δ from soluble to particulate fraction and a down-regulation of PKC-ε and PKC-ζ, whereas PKC-α and PKC-βI remained unaltered. When H9c2 cells were pretreated with PKC-δ inhibitor rottlerin (3 µM), hypoxia-induced apoptotic and necrotic cell death were significantly increased. Hypoxic insult also caused an activation of extracellular signal-regulated protein kinase (ERK) and p38 MAPK with no change in c-Jun NH2-terminal protein kinase (JNK) phosphorylation. Hypoxia-induced cell death was increased by treatment with ERK1/2 inhibitor U0126 (10 µM), but attenuated by p38 MAPK inhibitor SB202190 (10 µM). Treatment with rottlerin completely blocked the hypoxia-induced ERK phosphorylation, whereas it significantly increased p38 MAPK phosphorylation. The hypoxia-induced translocation of PKC-δ was not altered by U0126 and/or SB202190. From these results, it is suggested that hypoxia causes a rapid translocation of PKC-δ and subsequently ERK activation and p38 inactivation, rendering H9c2 cells resistant to hypoxia-induced cell death. [The Japanese Journal of Physiology 54: 405–414, 2004]

Key words: PKC-δ, ERK1/2, p38, hypoxia, H9c2.

Ischemic/hypoxic stress causes a series of signal pathways in various cell types and tissues that lead to cell death and organ dysfunction [1]. In heart, prolonged ischemia causes cell death and contractile dysfunction, whereas brief episodes of ischemia can render the heart resistant to a subsequently prolonged ischemia [2]. As an important mediator of ischemia-induced changes, protein kinase C (PKC) family members are recognized in various cell types [3, 4]. In heart, PKCs have been demonstrated to play roles in the protection against ischemic injury [5]. Our previous studies support this concept by demonstrating the protective role of PKC against ischemic injury in isolated rat heart model [6] and in heart-derived H9c2 cells [7]. Besides this beneficial role in hearts and cardiomyocytes, PKCs have been suggested to play roles in the pathophysiological consequences of myocardial ischemia [8]. They are classified into three subfamilies, which include the conventional (α, βI, βII, γ), the novel (δ, ε, η, θ, ι), and the atypical (ζ, µ, λ) isoforms based on Ca2+ and phospholipid sensitivity [9]. Although the predominant isoforms in rat heart have been reported to be α, β, δ, ε, and ζ subtypes [10], the identification of the PKC isoforms translocated during hypoxia and their roles in cardiomyocytes have not been fully characterized.-

Previously, we have demonstrated that metabolic inhibition causes PKC-ε translocation, resulting in H9c2 cell death [11]. In this study using H9c2 cells exposed to anaerobic hypoxia, we found that PKC-δ was specifically translocated to particulate fraction during hypoxia, whereas PKC-ε and PKC-ζ were downregulated. To assess the role of PKC-δ translocation, we examined the effect of its inhibitor rottlerin on hypoxia-induced H9c2 cell death. We also investigated its possible downstream signaling molecules, mitogen-activated protein kinases (MAPKs), during hypoxia.


METHODS

Cell culture and hypoxia system. Heart-derived H9c2 cells were purchased from American Type Culture Collection (Rockville, MD) and cultured before experimentation in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose supplemented with 10% fetal bovine serum. For hypoxic challenges, H9c2 cells were transferred into an anaerobic chamber (Forma Scientific, Marietta, OH, USA) maintained at 37°C with a humidified atmosphere of 5% CO₂, 10% H₂, and 85% N₂, as described before [7]. In the anaerobic chamber, the culture medium was replaced with serum-free, glucose-free DMEM that had been saturated with N₂ gas for 1 h. Normoxic incubation of the cells in the serum-free DMEM was conducted in a water-jacked incubator gassed with 95% air and 5% CO₂ at 37°C.

Chemicals and treatment. PKC inhibitors, such as Go 6976 (inhibitor for PKC-α and -βI, 10 nM) [12], rottleriin (inhibitor for PKC-δ, 3 µM) [13, 14], myristoylated PKC εV1-2 (inhibitor for PKC-ε, 10 µM) [15], and myristoylated PKC ζ (inhibitor for PKC-ζ, 10 µM) [16], were purchased from Biomol Research Lab. (Plymouth Meeting, PA, USA). MAPKs inhibitors, such as U0126 (inhibitor for ERK, 10 µM) [17, 18], SB202190 (inhibitor for p38 MAPK, 10 µM) [19], and SP600125 (inhibitor for JNK, 5 µM) [20], were purchased from Calbiochem (San Diego, CA, USA). Several inhibitors were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1%, and this concentration of DMSO was found to have no effect on H9c2 cell viability. H9c2 cells were treated with inhibitors 30 min before and during hypoxia.

Lactate dehydrogenase (LDH) assay. To measure overall cell injury, we assayed the activity of LDH released into the medium 10 h after hypoxic insult as described previously [7], i.e., by spectrophotometric analysis at 340 nm. The percent of LDH was calculated from the maximum LDH release of DMSO was 0.1%, and this concentration of DMSO was used as a control.

Flow cytometric analysis for Propidium iodide (PI)-, Annexin V staining. Entry into apoptosis was shown to lead to a loss of phospholipid asymmetry with an exposure of phosphatidylserine in the outer leaflet [21]. In some assays, annexin V-positive/propidium iodide (PI)-negative cells were considered apoptotic [22]. We have used this binding of annexin V to detect phosphatidylserine exposure on the membranes of apoptotic cells, which were assessed with an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Oncogene Research Products, Boston, MA). The cells were detached by 0.25 g/l trypsin-0.1 g/l EDTA treatment, gently resuspended in cold binding buffer to 5 × 10⁵ cells, and incubated with annexin V-FITC and PI at room temperature in the dark. Living cells were defined as those negatively stained for both annexin V and PI (annexin V-negative/PI-negative). Early apoptotic cells were defined as those stained only with annexin V (annexin V-positive/PI-negative), late apoptotic cells were defined as those stained with both annexin V and PI (annexin V-positive/PI-positive). Necrotic cells were defined as those stained only with PI (annexin V-negative/PI-positive). The fluorescence intensity of the cell surface was then measured by flow cytometry (BD Biosciences, Lexington, KY, USA). The values are expressed as the percentage of annexin V-positive cells to total cells counted.

Western blot analysis for PKC. The cells were lysed with 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, 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 supernant was collected for the soluble fraction. The pellets were resuspended in the same volume of lysis buffer containing 1% Triton X-100 were centrifuged at 10,000 × g for 10 min at 4C, and the supernatant was collected for the particulate fraction. A quantity of 40 µg protein of either the soluble fraction or the particulate fraction was separated on 8% SDS-PAGE and transferred onto PVDF membrane (Millipore, Bedford, MD). The membrane was blocked with 5% nonfat dry milk for 90 min and incubated overnight with PKC isoform antibodies for PKC-α, -βI, -δ, -ε, TdT and digoxigenin-conjugated dUTP for 1 h at 37°C. We visualized the labeled DNA with peroxidase-conjugated antidigoxigenin antibody and 3',3'-diaminobenzidine (DAB) as a chromogen. The nuclei was counterstained with hematoxyline.

In situ terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL). The in situ labeling of fragmented DNA was performed by TUNEL assay with the commercially available ApopTag Plus kit (Oncor, Gaithersburg, MD, USA). Monolayers of H9c2 cells were grown on 24 well plates and fixed with 4% paraformaldehyde (PFA). The cells fixed with PFA were rinsed in PBS and immersed in equilibration buffer (5 min, 37°C), and then incubated with the reaction mixture containing TdT and digoxigenin-conjugated dUTP for 1 h at 37°C. We visualized the labeled DNA with peroxidase-conjugated antidigoxigenin antibody and 3',3'-diaminobenzidine (DAB) as a chromogen. The nuclei was counterstained with hematoxyline.
The membrane was then incubated with a secondary immunoglobulin antibody conjugated with alkaline phosphate for 4 h, and the PKC band was visualized by the NBT/BCIP method (Sigma, St. Louis, MO, USA). To normalize the differences in protein loading, the blots were reprobed with a monoclonal antibody to actin (Sigma, St. Louis, MO, USA) and cytochrome C oxidase subunit IV (Cox IV, Molecular Probes, Eugene, OR, USA).

Western blot analysis for MAPKs. The cells were lysed in cell lysis buffer (62.5 mM Tris-HCl, pH 6.8, 5% β-mercapto-ethanol, 2 mM phenylmethylsulfonyl fluoride, 2% sodium dodecyl sulfate, and 10 mM EDTA) and heated at 100°C for 10 min. A quantity of 10 µg protein was loaded to each lane of a 10% gel for separation. The blots were incubated 90 min with MAPKs antibodies for ERK, p38, and JNK (Cell Signaling Technology, Beverly, MA, USA) after incubation in the blocking solution. This was followed by incubation with secondary alkaline phosphate-conjugated antirabbit goat globular protein antibodies (Bio-Rad, Hercules, CA, USA). Bound antibodies were detected by the use of NBT/BCIP premixed solution. We used a GS-700 imaging densitometer (Bio-Rad, Hercules, CA, USA) to analyze the results.

**Statistical analysis.** All data were expressed as mean ± S.D. The numerical data were compared by a Student’s t-test for unpaired observations between the two groups. A P value of <0.05 was considered significant.

**RESULTS**

**Protein expression of PKC isoforms during hypoxia.** We investigated which PKC isoforms are activated during anaerobic hypoxia (oxygen-, and glucose-free) in heart-derived H9c2 cells by Western blot analysis. As shown in Fig. 1A, hypoxia leads to a rapid translocation of PKC-δ from soluble to particulate fraction, whereas the protein levels of PKC-ε and PKC-ζ are decreased during hypoxia. PKC-α and -βI did not change in particulate fraction. The amount of PKC-δ protein in the particulate fraction began to increase (1.5-fold) at 5 min after hypoxia and further increased to remain

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**Fig. 1.** The expression of PKC isoforms during hypoxia. **Panel A:** Western blots for PKC isoforms detected in soluble (S) and particulate (P) fractions after various periods of hypoxic time in H9c2 cells. An equal loading of proteins was confirmed by probing with monoclonal antibody to actin and cytochrome C oxidase subunit IV (Cox IV), markers for soluble and particulate, respectively. The data shown are representative of 5 separate experiments. **Panel B:** Quantitative analysis of Western blots for PKC-δ in soluble and particulate fraction. The data shown as mean ± SD (n = 5) represent the percentages (%) of the time 0 (control). *P < 0.01 vs. time 0 (control) in particulate fraction.
Roles of PKC isoforms in hypoxia-induced cell death. To investigate the roles of PKC isoforms during hypoxia in terms of hypoxia-induced cell death, we measured LDH activity released during hypoxia after treatment with PKC isoform inhibitors. Hypoxia-induced LDH release after 10 h of hypoxia (43 ± 1.9% of max.) was significantly increased (79.5 ± 1.4%) by treatment with 3 µM rottlerin (PKC-δ inhibitor), but by no other inhibitors, although PKC-ζ inhibitor appeared to have its own toxicity in normoxic condition (Fig. 2).

Role of PKC-δ in hypoxia-induced cell death. Figure 3A shows the time course of LDH release during hypoxia in the absence or presence of 3 µM rottlerin. In its presence, LDH release was remarkably increased above 50% as early as after 6 h of hypoxia (59.3 ± 2.1%) and reached a submaximum level after 8 h of hypoxia (79.9 ± 1.9%), compared to the vehicle group (16.2 ± 1.2% and 31.3 ± 4.8%, respectively), suggesting an accelerating role of rottlerin in hypoxia-induced cell death. TUNEL staining was performed for an evaluation of apoptotic cell death. The amount of TUNEL-positive cells was 13.6 ± 2.1% in vehicle-treated cells exposed to hypoxia for 4 h, and this amount was significantly increased by treatment with rottlerin (37.4 ± 3.2%). The enhancement of hypoxia-induced apoptotic cell death by rottlerin in H9c2 cells was also demonstrated by labeling with annexin V. The amount of apoptotic cells (annexin V-positive) was increased after 4 h of hypoxia (21.29 ± 1.9%), and this amount was significantly increased by treatment with rottlerin (48.51 ± 3.2%). On the other hand, the amount of necrotic cells (annexin V-negative/PI-positive) was not increased after 4 h of hypoxia (16.2 ± 4.9%), and this amount was increased by treatment with rottlerin (32.02 ± 2.6%).

Phosphorylation of MAPKs during hypoxia. As shown in Fig. 4, ERK1/2, phosphorylation was increased approximately threefold after 15 min of hypoxia, and this increased level was maintained up to 4 h of hypoxia, after which there was a subsequent decrease (Fig. 4A). The phosphorylation of p38 began to increase at 5 min after hypoxia, and it further increased about twofold after 1 h of hypoxia. It was remarkably decreased after 4 h of hypoxia (Fig. 4B). On the other hand, JNK phosphorylation remained unaltered during hypoxia after up to 6 h of hypoxia (Fig. 4C).

Roles of MAPKs inhibitors in hypoxia-induced cell death. To investigate the roles of MAPKs during hypoxia, we used a specific inhibitor for MEK1/2 (U0126) [17, 18] and for p38 MAPK (SB202190) [19]. As shown in Fig. 5A, when H9c2 cells were treated with 10 µM U0126, hypoxia-induced LDH release after 10 h of hypoxia (41.1 ± 2.4%) was further increased to 64.9 ± 2.2%. The inhibition of ERK phosphorylation by U0126, shown in Fig. 5A was analyzed by Western blot. On the other hand, p38 inhibition by 10 µM SB202190 decreased the hypoxia-induced LDH release from 43.6 ± 6.7% to 26.9 ± 3.2% after 10 h of hypoxia (Fig. 5B). TUNEL staining was performed for an evaluation of apoptotic cell death during hypoxia. The amount of TUNEL-positive cells was 13.6 ± 2.1% in vehicle-treated cells exposed to hypoxia for 4 h, and this amount was not different from U0126-treated cells (Fig. 5C). On the other hand, hypoxia-induced TUNEL-positive cells were decreased by treatment with SB202190. The effect of U0126 and SB202190 during hypoxia was also demonstrated by annexin V and PI staining analyzed by flow cytometry (Fig. 5D). Although hypoxia-induced apoptotic cells were very slightly increased by treatment with U0126, this difference was not statistically significant. On the other hand, the amount of necrotic cells was increased by U0126 (22.05 ± 2.2%). SB202190 decreased pri-
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Panel A: Inhibition of PKC-δ translocation by rottlerin (upper) and the time course effect of 3 µM rottlerin on hypoxia-induced LDH release (bottom). The data are expressed as mean ± SD (n = 4). *P < 0.05 vs. hypoxia alone. Nm, normoxia; Hx, hypoxia; Veh, vehicle; rott, rottlerin. Panel B: TUNEL-stained photographs. Arrows indicate TUNEL-positive cells. The data shown are representative of 3 separate experiments. Panel C: Quantitative assay of apoptotic and necrotic cells. The intensities of fluorescence were analyzed by flow cytometry. The apoptotic cells (annexin V-positive) were evaluated as a percentage of total cells. The necrotic cells (annexin V-negative/PI-positive) were evaluated as a percentage of total cells. The data are expressed as mean ± SD (n = 3). *P < 0.05 vs. Veh-hypoxia. #P < 0.05 vs. Veh-normoxia.

Fig. 3. The role of PKC-δ during hypoxia. Panel A: Inhibition of PKC-δ translocation by rottlerin (upper) and the time course effect of 3 µM rottlerin on hypoxia-induced LDH release (bottom). The data are expressed as mean ± SD (n = 4). *P < 0.05 vs. hypoxia alone. Nm, normoxia; Hx, hypoxia; Veh, vehicle; rott, rottlerin. Panel B: TUNEL-stained photographs. Arrows indicate TUNEL-positive cells. The data shown are representative of 3 separate experiments. Panel C: Quantitative assay of apoptotic and necrotic cells. The intensities of fluorescence were analyzed by flow cytometry. The apoptotic cells (annexin V-positive) were evaluated as a percentage of total cells. The necrotic cells (annexin V-negative/PI-positive) were evaluated as a percentage of total cells. The data are expressed as mean ± SD (n = 3). *P < 0.05 vs. Veh-hypoxia. #P < 0.05 vs. Veh-normoxia.

Fig. 4. Western blot analysis for phosphorylation of ERK1/2 (p44/42), p38, and JNK in H9c2 cells during hypoxia. H9c2 cells were exposed to hypoxia (serum-/glucose-free DMEM) for the indicated periods. The quantification of MAPKs phosphorylation during hypoxia is also shown. The data are expressed as mean ± SD (n = 5). *P < 0.05 vs. time 0 (control).
marily apoptotic cell deaths. JNK inhibition by SP600125 showed no effect on hypoxia-induced cell death (data not shown).

Link between PKC-δ and MAPK activation.

To investigate the relationship between PKC-δ and MAPK activation, we examined the effect of U0126 and SB202190 on PKC-δ translocation and the effect of PKC-δ inhibitor on ERK1/2 and p38 phosphorylation. As shown in Fig. 6A, the hypoxia-induced translocation of PKC-δ was not changed by U0126 or SB202190. Whereas hypoxia-induced ERK1/2 phosphorylation was completely blocked by treatment with rottlerin, hypoxia-induced p38 phosphorylation was further increased by rottlerin (Fig. 6B), suggesting PKC-δ as an upstream signaling molecule of both ERK1/2 and p38. We also examined the effect of ERK1/2 inhibitor and p38 inhibitor on p38 phosphorylation and ERK phosphorylation, respectively, to investigate cross talk between ERK1/2 and p38. As shown in Fig. 7, ERK1/2 phosphorylation was not affected by SB202190, and p38 phosphorylation was not affected by U0126.

Fig. 5. The effects of MAPKs inhibitors on hypoxia-induced cell death. H9c2 cells were incubated with MEK1/2 specific inhibitor (U0126, 10 μM) and p38 inhibitor (SB202190, 10 μM) for 30 min before and during hypoxia. Panel A: The effect of U0126 on hypoxia (Hx)- or normoxia (Nm)-induced cell death was examined by LDH release. The inhibition of ERK phosphorylation by U0126 was analyzed by Western blot analysis. The data are expressed as mean ± SD (n = 5). *P < 0.05 vs. hypoxia alone. Panel B: The effect of SB202190 on hypoxia (Hx)- or normoxia (Nm)-induced cell death was examined by LDH release. The inhibition of p38 phosphorylation by SB202190 was analyzed by Western blot analysis. The data are expressed as mean ± SD (n = 5). *P < 0.05 vs. hypoxia alone. Panel C: TUNEL-stained photographs. Arrows indicate TUNEL-positive cells. The data shown are representative of 3 separate experiments. Panel D: Quantitative assay of apoptotic and necrotic cells. The intensities of fluorescence were analyzed by flow cytometry. Veh, vehicle; SB, SB202190 (10 μM). The data are expressed as mean ± SD (n = 3). *P < 0.05 vs. Veh-hypoxia. #P < 0.05 vs. Veh-normoxia.
DISCUSSION

In this study, we demonstrate that PKC-δ and ERK are activated at an early stage of hypoxia in H9c2 cells, rendering cells resistant to prolonged hypoxia-induced cell death.

There have been controversial reports about the activation of PKC isoforms during ischemic insult [23–25]. Strasser et al. [24] demonstrated that acute (2.5 min) and prolonged (up to 60 min) hypoxic insult induced the translocation of PKC-ε from cytosolic to particulate fraction, a marker for PKC activation, in primary cultured rat cardiomyocyte. On the other hand, we previously reported that in heart-derived H9c2 cells, the protein level of PKC-ε in particulate fraction markedly diminished by after prolonged hypoxic insult (2 h, 8 h of hypoxia), despite a very slight increase in PKC-ε after short-term hypoxia (15, 30 min). We therefore have suggested that the differential mode of PKC-ε activation depends on cell types and severity of injuries [15]. In this study, we obtained similar results for PKC-ε showing a hypoxia-induced decrease in the protein level of PKC-ε.

With PKC-δ, however, our results showed a significant translocation from soluble to particulate fraction at an early time of hypoxia (from 5 min to 2 h, Fig. 1). These results are consistent with others that PKC-δ is translocated from soluble to particulate fraction after 5 min of ischemia/reperfusion (3 times) [26]. Moreover, PKC-δ activation is induced by a variety of apoptotic stimuli in different cellular systems, including neutrophils [27] and fibroblasts [28]. However, there are still reports in contrast with ours, showing that PKC-δ was inactivated by hypoxia in human endothelial cells [29] and that PKC-δ remained unaltered after ischemia in rat heart [8]. As with PKC-ε this discrepancy in PKC-δ activation may be explained by differences between tissues, cells, and injuries.

In various cell types, PKC family members have been found to be important mediators of hypoxia-induced apoptosis and of adaptation to cytotoxic stimuli [29]. However, the roles for different PKC isoforms are still controversial [30]. Maher has reported that the downregulation of PKC-δ blocks oxidative stress-induced cell death in neuronal cells [19]. Despite numerous studies indicating a proapoptotic role of PKC-δ, there are a few studies pointing out...
its antiapoptotic role [31]. PKC-δ protects RAW 264.7 macrophages from nitric oxide induced apoptosis, and this effect was attributed to the inhibitory effect of PKC-δ on the JNK and p38 MAPK pathways [32]. The beneficial effect of PKC-δ against ischemic injury was also suggested in cardiomyocytes expressed with constitutively active isoforms of PKC-δ [33]. Our results in this study support this concept by demonstrating that the inhibition of PKC-δ by rottlerin [13, 14] increases hypoxia-induced apoptotic cell death as well as necrotic cell death. As for PKC-ε, we have previously reported its detrimental and beneficial roles by showing that PKC-ε plays a detrimental role for metabolic inhibition-induced cell death [11] and that the maintenance of PKC-ε by high glucose during hypoxia gives protective effects [15]. Other studies also demonstrated the antiapoptotic and proapoptotic roles of PKC-ε in various cell types, including cancer cells [30, 34, 35]. It is interesting that the present study showed that the inhibition of PKC-ε had no effect on cell death during prolonged hypoxia (Fig. 2). Taken together, the differential roles of PKC isoforms appear to depend on types of cells and injuries.

Several studies in whole hearts and isolated cardiomyocytes have described the activation of the MAPK family during ischemia [36]. Whereas ERK1/2 is predominantly activated by growth factors, JNK and p38 MAPK are generally activated by stresses such as cytokines and ischemic injury. p38 MAPK and JNK, as well as ERK1/2, have been demonstrated to be activated during hypoxia in cardiomyocytes [37, 38] or after ischemia/reperfusion [34, 37, 39]. Our previous studies are consistent with these results by showing the metabolic inhibition induced activation of p38 MAPK and JNK in H9c2 cells [11]. In this study, we found that the phosphorylation of ERK1/2 and p38 MAPK in H9c2 cells is increased rapidly during hypoxia, but not the phosphorylation of JNK (Fig. 4). Although the time courses of ERK1/2 activation and p38 activation are similar, there was no cross talk between ERK1/2 and p38 signaling (Fig. 7). From the present results, which reveal that an inhibition of ERK aggravates hypoxia-induced cell death whereas p38 inhibitor attenuates it, we suggest that ERK plays the beneficial role and p38 MAPK the detrimental role during hypoxia-induced H9c2 cell death. At an early time, 4 h of hypoxia, rottlerin remarkably increased both apoptotic and necrotic cell death (Fig. 3), whereas U0126 to some extent increased only necrotic cell death, with no effect on apoptotic cell death (Fig. 5). Furthermore, SB202190 significantly blocked apoptotic cell death at this time. From these results, it is suggested that there may be a PKC-δ- and p38-dependent, but ERK-independent, antiapoptotic protective mechanism in the early phase of hypoxia (4–6 h). Our results further suggest that PKC-δ- and ERK-dependent protective mechanisms against necrotic cell death may be involved in the late phase of hypoxia (longer than 10 h).

On the basis of recent evidence, including our previous report [11] implicating the possible link between PKC and MAPKs [34], we investigated the presence of a putative link between PKC and MAPKs during hypoxia-induced H9c2 cell death. Since the beneficial role against hypoxia-induced H9c2 cell death appeared to be related with PKC-δ and ERK1/2, we investigated the link between PKC-δ and ERK1/2 and found that ERK1/2 activation appeared to be downstream of PKC-δ because rottlerin blocks the phosphorylation of ERK1/2 during hypoxia, and because U0126 has no effect on the hypoxia-induced modulation of PKC-δ (Fig. 6). As for p38, inactivation appears to be a downstream signaling of PKC-δ based on the results that rottlerin increases the phosphorylation of p38 during hypoxia and that SB202190 has no effect on hypoxia-induced modulation of PKC-δ (Fig. 6).

In summary, our results suggest that hypoxia (oxygen- and glucose-free) induces the isofrom specific translocation of PKC-δ and subsequently the phosphorylation of ERK1/2, which appears to enhance H9c2 cell survival during hypoxia by decreasing apoptotic and necrotic cell death. Our results further suggest that hypoxia induces p38 phosphorylation as a proapoptotic signaling pathway, which appears to be inactivated as a downstream molecule for the PKC-δ isofrom.

This work was supported by a Korea Research Foundation Grant (KRF-2001-003-F00232).

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