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Original Article

Transient glucose deprivation causes upregulation of heme oxygenase-1 and cyclooxygenase-2 expression in cardiac fibroblasts

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Abstract

Transient glucose deprivation (TGD) has been shown to induce a resistance to a subsequent ischemia and reperfusion injury in the heart. Induction of cyclooxygenase-2 (COX-2) and heme oxygenase-1 (HO-1) is known to mediate the powerful defensive adaptation of the heart against oxidative stress. In this study, we found that a 30-min incubation in the absence of glucose resulted in a rapid increased expression of COX-2 and HO-1 in cardiac fibroblasts as examined by real-time quantitative polymerase chain reaction (PCR) and western blot analysis. Interestingly, TGD increased the generation of reactive oxygen species (ROS) and caused the transient phosphorylation of p38 mitogen-activated protein kinase (MAPK) as well as the translocation of protein kinase C (PKC)-ε from the cytosolic to the membrane fraction. However, no significant change in the distribution of PKC-δ isoform was observed compared with the control. Pretreatment of the cells with an antioxidant, *N*-acetylcysteine (NAC), resulted in the inhibition of p38 MAPK phosphorylation and PKC-ε translocation during TGD. In addition, the induction of COX-2 and HO-1 expression by TGD was prevented by pretreatment with NAC or SB203580, a p38 MAPK inhibitor. Surprisingly, pretreatment with chelerythrine, an inhibitor of PKC, strongly augmented the HO-1 mRNA expression but blocked the COX-2 mRNA induction by TGD. These results demonstrate that briefly removing glucose from cultured cardiac fibroblasts induces COX-2 and HO-1 expression via generation of ROS and p38 MAPK phosphorylation, while the translocation of PKC-ε to the membrane fraction may participate in the induction of COX-2 but not in the HO-1 expression.

Keywords: Glucose; ROS; PKC; MAPK; Metabolic preconditioning

1. Introduction

Heme oxygenase (HO) is the enzyme that degrades heme into biliverdin (which is subsequently converted into the potent antioxidant, bilirubin), carbon monoxide (CO, which may function as a vasodilatory gas), and free iron [1,2]. Till date, three isoforms of HO have been identified: HO-1 is highly inducible whereas HO-2 and HO-3 are constitutively expressed [3,4]. HO-1 is strongly induced by numerous stress stimuli including ultraviolet irradiation, heavy metals, endotoxins, heat shock, and hypoxia [2,5]. Several studies using rat and porcine heart models have shown to induce of HO-1 in response to ischemia and reperfusion both in cardiac myocytes and fibroblasts [6–8]. This induction in the heart following ischemia and reperfusion is considered to be a component of the cellular defense mechanisms against oxidative stress-mediated injury. For example, induction of HO-1 by prior administration of hemin before ischemia could ameliorate cardiac injury following ischemia/reperfusion in rat heart [9,10]. In addition, hearts from transgenic mice overexpressing HO-1 specifically in the cardiac myocytes had reduced infarct size and inflammatory cell infiltration after ischemia and reperfusion compared with that of wild-type mice [11,12].

Cyclooxygenase (COX)-2 is a rate-limiting enzyme in prostaglandin (PG) biosynthesis, which catalyzes the conversion of arachidonic acid to PGH_2. Two distinct COX isoforms have been identified: COX-1 is present in most cells and is constitutively expressed, while COX-2 is induced by

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stresses such as ischemia, pro-inflammatory cytokines, and mitogenic responses [13,14]. Several lines of evidence suggest that COX-2 plays a crucial role in mediating the cardioprotective effects of ischemic preconditioning (IPC), which is the most potent anti-ischemic intervention known to date in terms of the endogenous protection of ischemic myocardium [15,16], because COX-2 metabolites have been shown to exert cardioprotective action such as attenuation of stunning and reduction in infarct size [17–19]. Interestingly, Yoshida et al. [20] reported that IPC failed to adapt the hearts of the heterozygous HO-1 knockout mice compared with the wild-type hearts, suggesting that induction of HO-1 also plays a crucial role in the adaptive modification of the heart mediated by IPC.

Some studies have reported that transient glucose deprivation (TGD) confers a preconditioning (PC)-like protection (metabolic PC) against subsequent ischemic injury in an isolated rat heart model [21] and in cultured rabbit cardiomyocytes [22]. However, the signaling pathways that confer resistance to oxidative stress are not well defined. It remains unclear whether TGD causes transcriptional activation of known cardioprotective genes and increased expression of proteins in the heart. In the present study, we showed that TGD induces both HO-1 and COX-2 expression via the p38 mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways in cardiac fibroblasts, which indicates the possibility that the coordinated upregulation of multiple proteins plays a role in metabolic PC.

2. Materials and methods

2.1. Cell culture

Primary cultures of cardiac fibroblasts were prepared from neonatal rat hearts as described previously [23], according to the Simpson's method. Briefly, hearts from 10 to 15 pups were minced and subjected to serial trypsin digestion to release single cells. After the final digestion, the cells were washed and preplated for 1 h in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) with a glucose concentration of 1000 mg/L, supplemented with 10% fetal calf serum (JRH, Lenexa, KS), penicillin (1000 U/ml), and streptomycin (1 mg/ml) at 37 °C; this medium allows the selective attachment of non-myocardial cells (mostly fibroblasts). The non-myocardial cells that adhered during the preplating step were grown to confluence, trypsinized and subcultured. Subconfluent (~70% confluency) cardiac fibroblasts at first passage were then maintained in serum-free DMEM for 24 h and used for the experiments. To verify the cellular constituents of this culture, immunocytochemical study using rabbit polyclonal antibodies directed against vimentin, sarcomeric muscle actin, and von Willebrand factor (DAKO A/S, Glostrup, Denmark) was performed by dextran polymer method using the DAKO EnVision™ System, Peroxidase (DAKO), which revealed that cultured cells exhibited positive staining for vimentin and negative staining for either sarcomeric muscle actin or von Willebrand factor. From this, we identified the cultured cells as cardiac fibroblasts.

2.2. Experimental protocols

To study the effects of TGD on COX-2 and HO-1 induction, cultured cardiac fibroblasts were incubated in glucose-free DMEM for 30 min in a water-jacketed incubator gassed with 95% air and 5% CO_2 at 37 °C. After incubation, the cells were subjected to post-incubation in complete DMEM for the indicated time periods.

In the control group, cultured cells were incubated in complete DMEM for identical time periods. There were no differences between the levels of lactate dehydrogenase release from cardiac fibroblasts subjected to "a 30-min preincubation" in glucose-free DMEM and from the control cells, suggesting that this treatment does not cause cell injury. Various groups of cardiac fibroblasts were incubated with SB203580 (10 μM; BIOMOL, Plymouth Meeting, PA), *N*-acetylcysteine (NAC, 500 μM; Sigma) or chelerythrine (5 μM; Sigma) for 20 min, prior to the addition of the glucose-free medium.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the cells using the RNeasy Mini kit (Qiagen, Chatsworth, CA) according to the recommended procedures. The RNA concentrations were determined spectrophotometrically at 260 nm. Two micrograms of total RNA was reverse transcribed in a 20-μl volume containing 1 mM of oligo-dT primer, 0.5 mM of dNTP mixture, 10 units of RNase inhibitor (Takara shuzo, Shiga, Japan), and 4 units of Omniscript II reverse transcriptase (Qiagen). The reverse transcription was conducted at 37 °C for 60 min, and the reaction mixture was heated at 95 °C for 5 min to terminate the cDNA synthesis.

2.4. Real-time semiquantitative RT-PCR

Real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using the ABI Prism 7700 Sequence Detection System (PE Biosystems, Foster City, CA) was carried out by following the established protocol [24]. The primers and TaqMan probes (synthesized by Hokkaido System Science, Hokkaido, Japan) for quantitation of COX-2, HO-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, as an internal control) transcripts were designed using the primer design software Primer Express (PE Biosystems) (Table 1). The probes were labeled with 6-carboxyfluorescein. The relative efficiencies of the COX-2 or HO-1 primer/probe sets and the GAPDH primer/probe pair were tested by subjecting serial dilutions of a single

Table 1
Sequence of primer and probe sets for Real-time RT-PCR analysis

Gene	Primer/ probe	Sequence
HO-1	Forward	5′-TTTTTTCACCTTCCCGAGCA-3′
	Reverse	5′-GTGTTCATGCGAGCACGATAG-3′
	Probe	5′-CGACAACCCCACCAAGTTCAAACAGC-3′
COX-2	Forward	5′-CGCTGTACAAGCAGTGGCAAAG-3′
	Reverse	5′-GCGTTTGCGGTACTCATTGAGA-3′
	Probe	5′-CCTCCATTGACCAGAGCAGAGAGATGAAA-3′
GAPDH	Forward	5′-CTTCACCACCTTCTTGATGTCATC-3′
	Reverse	5′-ACCCCCAATGTATCCGTTGTG-3′
	Probe	5′-AGGTTTCTCCAGGCGGGCATGTCAGAT-3′

5′-end reporter dye was FAM (6-carboxylfluorescein). The Quencher fluorescent dye at the 3′-end was TAMRA (6-carboxyltetramethylrhodamine) for all probes.

RNA sample analyzed to real-time PCR analysis. The plot of log input vs. ΔC_t was <0.1, which satisfies the previously established criterion for equivalence of efficiency of amplification [24]. C_t, or threshold cycles, represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected, and ΔC_t refers to the difference between the threshold cycles for the target and the reference. After confirming that the efficiencies of amplification of the COX-2, HO-1 and GAPDH transcripts were approximately equal, the amount of the COX-2 or the HO-1 transcript relative to the GAPDH transcript was determined by using the comparative C_t method described in Perkin Elmer Applied Biosystems User Bulletin #2 (1997). In brief, the reaction mixture containing 4 μl of RT product, 100 nM of TaqMan probe, 400 nM of each forward and reverse primers and 1× TaqMan Universal PCR Master Mix (PE Biosystems) was subjected to PCR analysis using the following cycling parameters: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The data were analyzed using the Sequence Detector software.

2.5. Western blotting

Cells were dissolved in a lysis buffer (CelLytic™-M Mammalian Cell Lysis/Extraction Reagent, Sigma) containing a protease inhibitor (Protease Inhibitor Cocktail, Sigma) and 1.0 mM Na_3VO_4. After centrifugation at 20,000 *g* for 15 min at 4 °C, the supernatants were transferred, and the protein concentrations were determined by Coomassie blue dye binding assay (Bio-Rad Laboratories, Hercules, CA). Lysates (15 μg) were loaded and resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE) followed by transfer to a PVDF membrane (Hybond™-P, Amersham Biosciences, Uppsala, Sweden). The membranes were incubated for 1 h in Tris-buffered saline (TBS) and 0.1% Tween-20 (TBS-T) containing 5% nonfat-powdered milk and were then incubated overnight at 4 °C with specific antibodies against COX-1 and COX-2 (Cayman Chemical, Ann Arbor, MI) at a 1:1000 dilution, HO-1 (Takara Shuzo, Shiga, Japan) at concentration of 10 μg/ml, and actin (Sigma) at a 1:2000 dilution in TBS-T with gentle agitation. For p38 MAPK, membranes were probed overnight at 4 °C with specific antibodies against p38 MAPK or a dual phosphotyrosine–threonine–p38 MAPK, (Cell Signaling Technology, Beverly, MA) at a 1:1000 dilution in 5% bovine serum albumin (BSA) containing TBS-T with gentle agitation. After washing, the blots were incubated with appropriate second antibodies and conjugated to horseradish peroxidase (Amersham Biosciences). Immunoreactive bands were detected using the enhanced chemiluminescence (ECL) kit (Amersham Biosciences) with exposure to X-ray film (Hyperfilm™-MP, Amersham Biosciences). Autoradiograms exposed in the linear range of film density were scanned, and densitometric analysis was performed using the NIH Image software.

2.6. Measurement of prostanoids

After cardiac fibroblasts cultured in glucose-free medium for 30 min, the medium were replaced with glucose-containing medium. After 1 h, concentration of PGD_2 and PGE_2 in the culture medium was assayed using an enzyme-linked immunoassay according to the manufacture's protocol (Cayman Chemical).

2.7. PKC isoform distribution

To study the effect of TGD on PKC translocation, cytosolic and membrane fractions were prepared from cardiac fibroblasts using the Subcellular Proteome Extraction Kit (Calbiochem, San Diego, CA), according to the manufacture's instructions. Equal amounts of cytosolic and particulate proteins (20 μg) were concentrated using PAGEprep™ Protein Clean-Up and Enrichment Kit (Pierce) before SDS-PAGE analysis (10% acrylamide running gel) and used for immunoblot analysis as described in the section on western blotting. The monoclonal anti-PKC-δ and PKC-ε antibodies (BD Transduction Laboratories, Lexington, KY) were used at a 1:1000 dilution in TBS-T containing 5% nonfat-powdered milk, respectively.

2.8. Measurement of intracellular reactive oxygen species generation

Intracellular formation of reactive oxygen species (ROS) during TGD was estimated by an oxidation-sensitive fluorescent dye, hydroxyphenyl fluorescein (HPF) (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) using the method described elsewhere [25]. Cells seeded in 96-well plates were loaded with 10 μM HPF, incubated for 30 min at 37 °C. Dye-loaded cells were then incubated in DMEM with (control, CONT) or without (TGD) glucose for 30 min. To determine the effect of NAC on ROS generation, dye-loaded cells were treated with 500 μM NAC for 20 min prior to "a 30-min

incubation" in a glucose-free medium (TGD + NAC). Fluorescence resulting from intracellular HPF oxidation was measured in a Fluoroskan II plate reader (Labsystems, Helsinki, Finland) using 485-nm excitation and 538-nm emission wavelengths.

2.9. Statistical analysis

The results shown are presented as mean ± S.E. Statistical differences between the groups were analyzed by ANOVA, using Fisher PLSD as the post hoc test. When the Bartlet's test revealed significances between groups, non-parametric comparisons (statistical differences between the groups were analyzed by Kruskal–Wallis test, using Mann–Whitney *U*-test as the post hoc test) were performed on the variables. $P < 0.05$ was considered statistically significant.

3. Results

3.1. TGD rapidly increases both HO-1 and COX-2 mRNA

We first determined the influence of "a 30-min incubation" without glucose on mRNA expression levels of HO-1 and COX-2 in cardiac fibroblasts using real-time semiquantitative PCR. As shown in Fig. 1A, TGD resulted in a rapid increase of HO-1 mRNA. The effects were observed immediately when the cells were incubated in complete DMEM after "a 30-min incubation" in glucose-free DMEM. The time course for the induction of HO-1 gene showed an approximate fivefold increase 1 h after post-incubation in complete DMEM compared to that of control cells. Thereafter, the HO-1 mRNA level was decreased and returned back to the basal levels within 6 h. Similar to the effects on HO-1 mRNA levels, pretreatment with a glucose-free medium for 30 min significantly increased the level of COX-2 mRNA in cardiac fibroblasts (10-fold induction of COX-2/GAPDH relative ratio vs. control). Unlike the induction of HO-1 mRNA, the increased levels of COX-2 mRNA rapidly reduced to the basal level as early as 3 h after post-incubation in complete DMEM. Similar to the induction observed in cardiac fibroblasts, a brief period of glucose deprivation caused upregulation of these genes expression in cultured neonatal cardiac myocytes (purity of 80–85%), but the extent of induction of these gene expression was lower than that of induction in cardiac fibroblasts (COX-2: 4.58 ± 0.43-fold and HO-1: 2.15 ± 0.06-fold increase vs. control, at 1 h after replacement with glucose-containing medium). Using western blotting, we confirmed that the increase in HO-1 mRNA was accompanied by an increase in HO-1 protein expression. The mild increase in HO-1 protein was detected at a maximum level at 1.5 h after post-incubation in complete DMEM in cardiac fibroblasts and it declined gradually within 24 h (Fig. 1B). Furthermore, the COX-2 protein content also increased 0.5 h after post-incubation, whereas the COX-1 protein content did not change. Unlike HO-1 induction, the levels of COX-2 protein returned back to control steady-state levels within 3 h after post-incubation in complete DMEM. In addition, we detected a small, but significant elevation in PGD_2 but not PGE_2 in the medium of cardiac fibroblasts incubated in complete medium for 1 h after "a 30-min preincubation" without glucose (TGD/R: 39.34 ± 4.004 pg/ml; control: non-detectable for below the detection limit; $n = 8$).

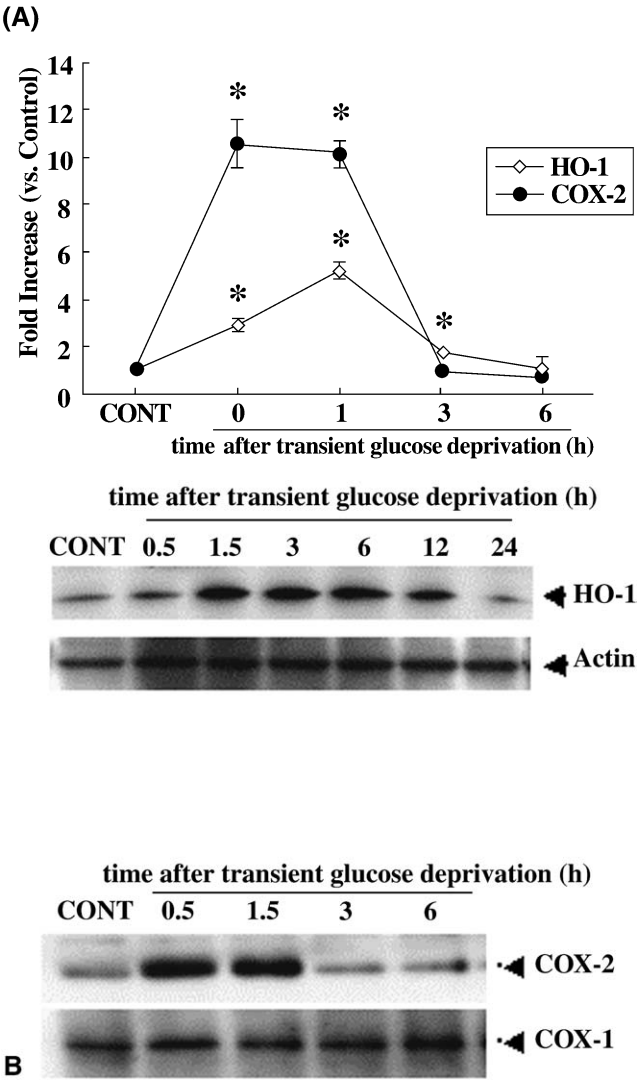

Fig. 1. Time-course of COX-2 and HO-1 expression induced by transient glucose deprivation in cultured cardiac fibroblasts. Cardiac fibroblasts were subjected to incubation in the absence of glucose for 30 min followed by post-incubation in a glucose-containing medium for the indicated time periods. (A) Total RNA was extracted from cardiac fibroblasts at the indicated time periods after TGD. COX-2 and HO-1 mRNA expression was determined by real-time quantitative PCR. Mean ± S.E. ($n = 4$) were normalized to GAPDH levels and graphed as fold induction relative to control value. *$P < 0.05$ compared with control. (B) Western blot analysis of COX-2 and HO-1. Whole cell lysates obtained at the indicated times after TGD were resolved by SDS-PAGE and probed with an antibody against the indicated protein. Immunodetection of actin with an anti-actin antibody, or COX-1 with an anti-COX-1 antibody was performed as an internal control for protein loading and transfer.

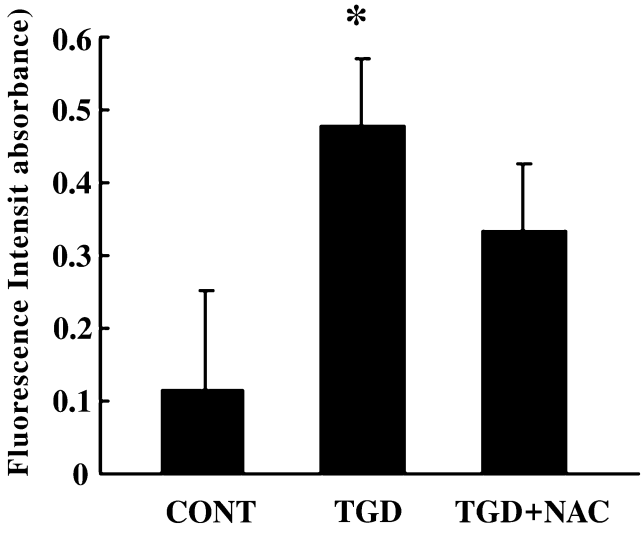

Fig. 2. Detection of ROS generation in cardiac fibroblasts during TGD. Cardiac fibroblasts were loaded with 10 μM HPF, incubated for 30 min at 37 °C. Cells were then incubated in a glucose-containing (CONT) or -free (TGD) medium for 30 min. To determine the effect of NAC on intracellular ROS generation by TGD, HPF-loaded cells were also pretreated with 500 μM NAC for 20 min prior to "a 30-min incubation" in the absence of glucose. The fluorescence intensity was determined at 538 nm with excitation at 485 nm. Data are mean ± S.E. (n = 8). *P < 0.05 compared with control.

3.2. An increase in ROS generation by TGD

Recent studies using human hepatoma cell have reported that the generation of ROS via the mitochondrial electron transport chain increases after prolonged glucose deprivation [26]. We determined whether a brief incubation in a glucose-free medium causes an increase in ROS formation in cardiac fibroblasts. HPF was used as a fluorescence probe to selectively detect highly ROS such as hydroxyl radicals and reactive intermediates of peroxidase (Fig. 2). We observed a very low, but detectable, enhanced fluorescence in the cells after "a 30-min incubation" in a glucose-free medium. In addition, pretreatment of the cells with an antioxidant, NAC, for 30 min prior to TGD suppressed this increase in fluorescence. These results suggest that TGD increases ROS intracellular formation in cardiac fibroblasts.

3.3. MAPK activation and PKC translocation during TGD

Several reports suggest that activation of multiple protein kinases including p38 MAPK and PKC is involved in the signal transduction of HO-1 and COX-2 induction in a variety of tissues [27,28]. We first determined whether p38 MAPK activation was initiated during "a 30-min incubation" in glucose-free DMEM. The protein expression levels of p38 MAPK in cardiac fibroblasts were assessed by western blotting, using the corresponding phosphospecific and non-phosphospecific antibodies. As shown in Fig. 3, transient phosphorylation of p38 MAPK was observed after "a 30-min incubation" without glucose. This phosphorylated kinase

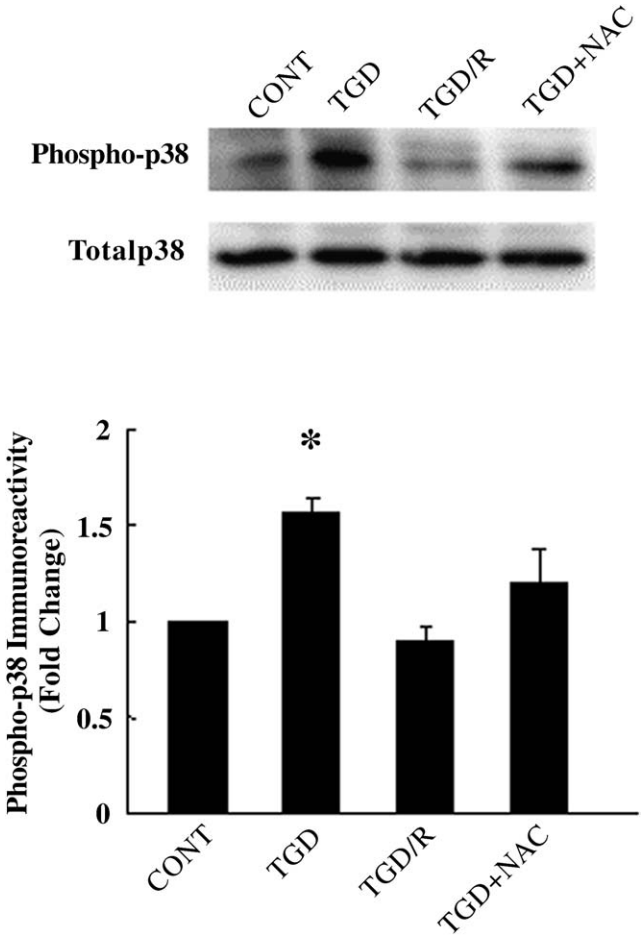

Fig. 3. Transient phosphorylation of p38 MAPK by briefly removing glucose from cultured cardiac fibroblasts. Cardiac fibroblasts were subjected to "a 30-min incubation" in the absence of glucose (TGD) followed by "a 15-min post-incubation" in the presence of glucose (TGD/R). To determine the effect of NAC on p38 MAPK phosphorylation, cardiac fibroblasts were also pretreated with 500 μM NAC for 20 min prior to TGD (TGD + NAC). Whole cell lysates were resolved by SDS-PAGE and probed with an antibody against dual phosphorylated p38 MAPK (top blot) or total p38 MAPK (bottom blot). Densitometric analysis of the phosphorylation of p38 MAPK. The results were normalized by arbitrarily setting the densitometry of the control group. Data are mean ± S.E. (n = 4). *P < 0.05 compared with control.

was decreased 30 min after the cells were subsequently incubated in a glucose-containing medium. In addition, pretreatment with antioxidant NAC partially inhibited the phosphorylation of p38 MAPK during TGD. To determine the activation of PKC isoforms by TGD, the translocation of PKC-δ and -ε isoforms to the membrane fraction during "a 30-min glucose-free incubation" was assessed by western blotting (Fig. 4). TGD resulted in the translocation of PKC-ε isoform to the membrane fraction whereas the immunoreactivity of PKC-δ isoform in the membrane fraction was not different from that of the control level. An increased amount of PKC-ε in the membrane fraction during TGD returned to the control level within 30 min of subsequent incubation with a glucose-containing medium or by pretreatment with NAC before TGD treatment.

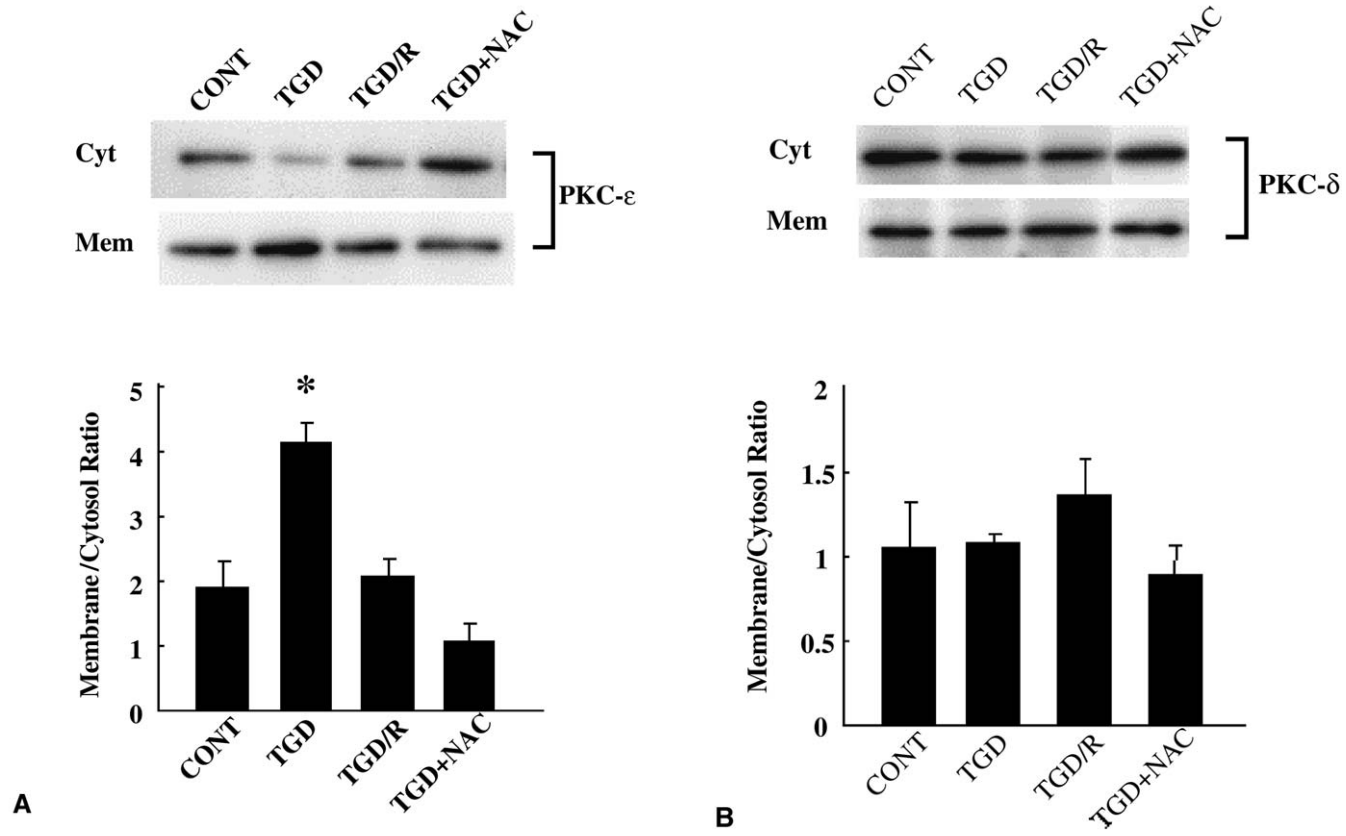

Fig. 4. The subcellular distribution of PKC-ε and -δ isoforms during TGD. Cytosolic and membrane fractions were extracted from cardiac fibroblasts after "a 30-min glucose-free incubation" (TGD) and followed by post-incubation in the presence of glucose for 15 min (TGD/R). Control extracts were obtained from cells before a glucose-free incubation. To determine the effect of NAC on PKC translocation, cardiac fibroblasts were also pretreated with 500 μM NAC for 20 min prior to the TGD (TGD + NAC). Fractions were resolved by SDS-PAGE and analyzed by western blotting with an antibody against the indicated protein. Densitometric analysis of the translocation of PKC-ε (A) and -δ (B) isoforms from cytosolic (Cyt) to membrane (Mem) fractions. Membrane/cytosolic ratios of immunoreactivity are calculated for each isoform as indices of PKC translocation. The data are expressed as mean ± S.E. ($n = 3$). *$P < 0.05$ compared with control.

3.4. Effects of an antioxidant and inhibitors of PKC and p38 MAPK on HO-1 and COX-2 induction mediated by TGD

To address the possibility that ROS generation and the p38 MAPK and PKC signaling pathways mediated the induction of HO-1 and COX-2 expression following TGD in cardiac fibroblasts, we examined the effects of NAC, a selective inhibitor of p38 MAPK (SB203580), and an inhibitor of PKC (chelerythrine) on the induction of HO-1 and COX-2 mRNA. Real-time PCR analysis showed that pretreatment of the cells with either NAC (500 μM) or SB203580 (10 μM) inhibited TGD-mediated upregulation of HO-1 and COX-2 mRNA expression (Fig. 5A,B). Interestingly, pretreatment with chelerythrine (5 μM) strongly enhanced the HO-1 mRNA expression in TGD-treated cells (Fig. 5C), whereas induction of COX-2 mRNA expression by TGD was suppressed on pretreatment with this chemical (Fig. 5A). Furthermore, chelerythrine induced the expression of HO-1 mRNA in cells without the TGD treatment (CONT + chelerythrine) (Fig. 5C).

4. Discussion

Induction of HO-1 and COX-2 in response to ischemia and reperfusion has been shown to be associated with cellular protection against oxidative stress in the heart [10,15]. Brief episodes of ischemia were shown to cause the upregulation of COX-2 mRNA and protein expression, and this increased COX-2 enzymatic activity was considered to mediate the protection afforded by the late phase of IPC [15,16]. Yoshida et al. [20] have reported that IPC failed to adapt the hearts of the heterozygous HO-1 knockout mice compared with wild-type hearts. Moreover, Sharma et al. [8] have examined the expression of HO-1 in the heart during myocardial stunning in anesthetized pigs. They found that HO-1 expression was upregulated at both mRNA and protein levels in the perivascular region (mainly fibroblasts) around blood vessels in the myocardium during the reperfusion phase after a short repetitive ischemia. These reports suggest that the induction of COX-2 and HO-1 plays a crucial role in the adaptive cardioprotection achieved by IPC. TGD of heart has been shown to confer a PC-like protection, termed metabolic PC [21,22]. In

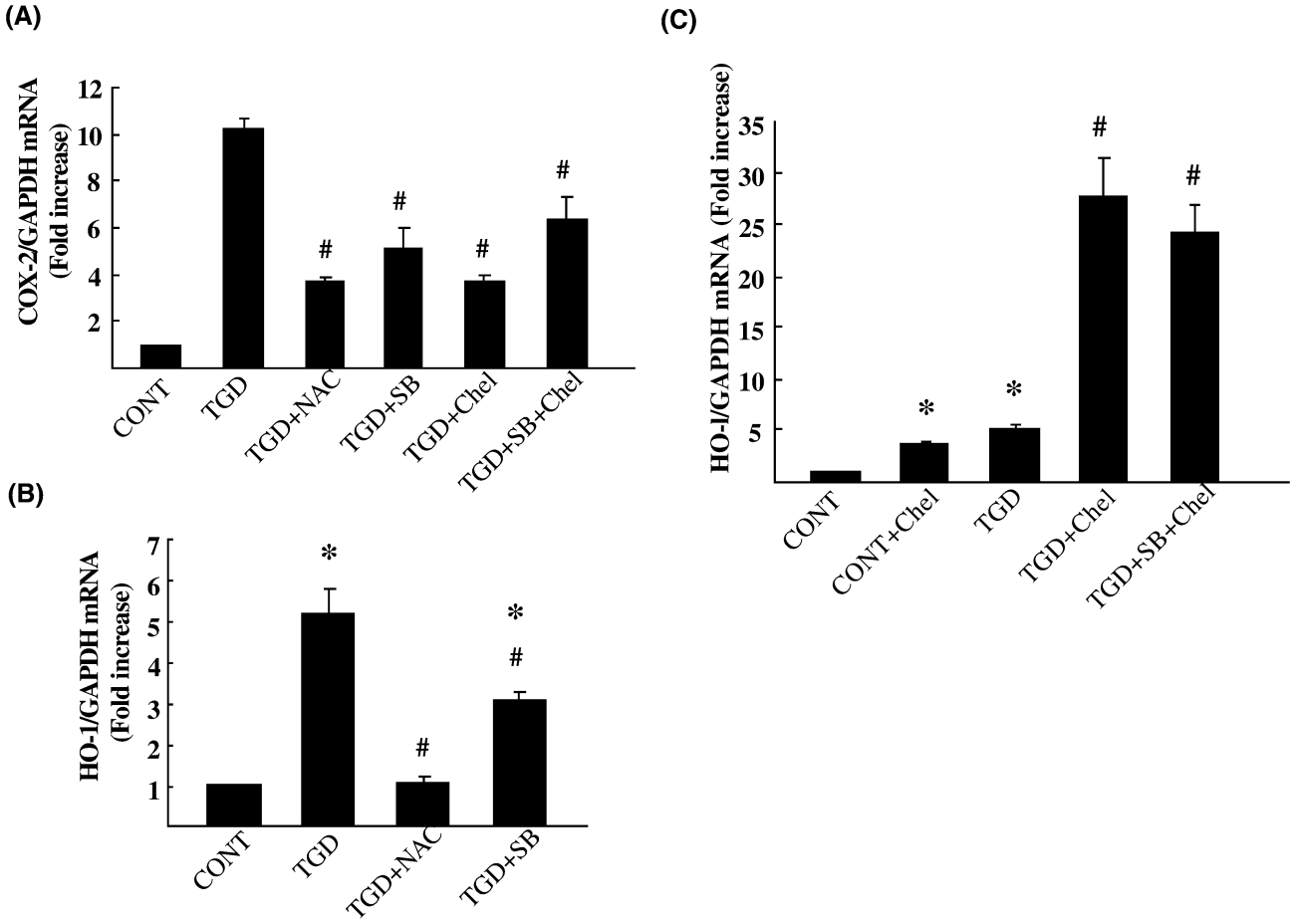

Fig. 5. Effects of an antioxidant and chemical inhibitors of p38 MAPK and PKC on the induction of HO-1 and COX-2 by TGD. Cardiac fibroblasts were pretreated with NAC (500 μM), SB203580 (10 μM), chelerythrine (5 μM), or vehicle for 20 min and then incubated in a glucose-free medium for 30 min. The levels of COX-2 (A) and HO-1 (B,C) mRNA expression were quantified by real-time quantitative PCR, normalized to GAPDH mRNA and graphed as fold inductions of control cells. The results are presented as mean ± S.E. ($n = 4$). *$P < 0.05$ compared with control. #$P < 0.05$ compared with TGD.

this study, we showed that TGD causes upregulation of HO-1 and COX-2 expression in cardiac fibroblasts. Our results suggest the possibility that the induction of HO-1 and COX-2 in cardiac fibroblasts may be an important factor in the cardioprotective signaling pathways of metabolic PC, although little has been reported on whether metabolic PC causes rapid transcriptional upregulation of cardioprotective genes in an intact heart. Given that the upregulation of HO-1 and COX-2 in cardiac fibroblasts is caused during metabolic PC and IPC in an intact heart, the myocardial contents of heme catabolites such as bilirubin and COX-2-derived prostanoids could be elevated. Since exogenous bilirubin added during sustained hypoxia protects cardiac myocytes against the reoxygenation injury [9] and infusions of PGs reduce myocardial infarct size [17,18], it is reasonable to suppose that the expression of cardioprotective enzymes such as HO-1 and COX-2 in cardiac fibroblasts may also play an important role in cellular protection against oxidative stress in the heart. Regarding COX-2-derived prostanoids, we indeed detected a small, but significant elevation in PGD_2 but not PGE_2 in the medium of cardiac fibroblasts incubated in complete medium after "a 30-min preincubation" without glucose. It has been reported that low concentration of PG of J series (PGJs), which synthesized from PGD_2 could enhance cytoprotection against toxic effects of lipid peroxidation product in endothelial cell [29]. Further evidence that PGJs induced HO-1 in porcine aortic endothelial cells [30] suggests that COX-2-derived prostanoids would, in part, contribute to induce several cytoprotective genes in non-myocardial cells.

Several lines of evidence indicate that the mitochondrial generation of ROS such as hydrogen peroxide (H_2O_2) is augmented during glucose deprivation [26]. In a study on human hepatoma (HepG2) cells, Chang et al. [26] reported that prolonged glucose deprivation caused upregulation of HO-1 expression, and the HO-1 induction was blocked by inhibitors of the mitochondrial electron transport chain and a general antioxidant, NAC. Our results using HPF also suggest that TGD is associated with ROS generation, and pretreatment with NAC before TGD inhibits the generation of intracellular oxidants and rapid induction of COX-2 and HO-1 transcripts (Figs. 2 and 5). Furthermore, augmentation of ROS generation from mitochondria and exogenous H_2O_2 treatment has been shown to lead to p38 MAPK phosphory-

lation in cultured cardiac myocytes [31,32]. Consistent with these observations, our results, obtained from cardiac fibroblasts, suggest that phosphorylation of p38 MAPK occurred transiently during "a 30-min incubation" in a glucose-free medium and returned back to basal control level within 15 min of subsequently incubating the cells in a glucose-containing medium (Fig. 3). In addition, the phosphorylation of p38 MAPK was partially inhibited by pretreatment with NAC, suggesting that ROS generated during TGD may be involved in mediating the p38 MAPK activation. Similar transient phosphorylation and activation of p38 MAPK was observed during a multi-cycle IPC in isolated rat and rabbit hearts [33,34]. Marais et al. [34] have reported that the significant activation observed during the first two PC episodes disappears during the third episode. However, the role of the p38 MAPK signaling pathway in the early phase of IPC is still controversial, since several studies reported that these inhibitors could function as cardioprotective compounds [35], and administration of p38 MAPK inhibitor, SB202190, before IPC treatment could not abolish cardioprotection [36]. In addition, our results show that the induction of HO-1 and COX-2 by TGD is prevented by pretreatment with p38 MAPK inhibitor. In agreement with the findings, several lines of evidence have shown that p38 MAPK influences the regulation of HO-1 and COX-2 gene expression in many different cell types [27]. However, other recent studies have also shown that the inhibition of p38 MAPK does not prevent HO-1 induction by prolonged glucose deprivation in HepG2 cells, although antioxidants and inhibitors of the mitochondrial electron transport chain block HO-1 induction [26], suggesting that the intracellular signaling pathways that regulate these gene expressions by glucose deprivation exhibit cell-specific behavior.

In the cellular mechanisms involved in metabolic PC using the isolated rat heart model, Awan et al. [21] suggested that adenosine release, PKC, and activation of the mitochondrial K^+ channel could play a role in the development of the cardioprotective effects of metabolic PC. The present study demonstrates the translocation of PKC-ε but not PKC-δ from the cytosolic to the membrane fractions during brief glucose deprivation in cardiac fibroblasts (Fig. 4). These isoforms have also been observed to translocate due to hypoxic PC in cultured rat cardiac myocytes, and the isoform-selective PKC translocation has been correlated with cardioprotection mediating IPC [37,38]. It has been reported that PKC-ε may play a crucial role in cardioprotection. Activation of PKC-ε seems to be required to mediate IPC to attenuate apoptosis and necrosis, because selective inhibition of PKC-ε using the peptide inhibitor added during simulated ischemia and reperfusion has been shown to abolish the cardioprotective effects of IPC [39]. Further evidence which indicated for the critical role of PKC-ε in cardioprotection can be seen in fact that transgenic mice overexpressing the PKC-ε in the heart are protected from cardiac damage on ischemia and reperfusion [40]. In contrast, several reports suggest that activation of PKC-δ mediates cardiac damage from ischemic insults and inhibition of PKC-δ with the selective inhibitory peptide resulted in cardioprotection from cell injury caused by ischemia and reperfusion [41]. Although whether TGD leads to the translocation of these PKC isoforms to membrane fractions in cardiac myocytes was not examined in our study, the results suggest that the translocation of PKC-ε is a mechanism of the signaling pathways that is common to both metabolic PC and IPC in the rat heart. However, the involvement of PKC in IPC is still controversial since several reports indicate that an increased PKC translocation is not necessarily correlated with the protected state of cardiac myocytes [42]. Further studies are needed to clarify the involvement of the PKC pathway in IPC and metabolic PC.

In the present study, pretreatment of cardiac fibroblasts with a PKC inhibitor, chelerythrine, prevented the increase in COX-2 transcripts induced by TGD, suggesting the involvement of PKC pathway in the COX-2 upregulation. In contrast, pretreatment with this inhibitor resulted in potentiation of HO-1 induction after TGD, suggesting that the PKC pathway may also play a role in the negative regulation of HO-1 induction. However, the mechanisms through which the induction of HO-1 expression is further augmented after TGD by inhibition of the PKC pathway remain to be elucidated. A further important point to be considered is that chelerythrine as well as the other PKC inhibitor has effects independent of PKC. Chelerythrine has been reported to inhibit alanine aminotransferase activity by interaction with the thiol group [43]. Furthermore, the most important problem of PKC-independent effects of this reagent seems to be that the treatment in the commonly used concentration for in vitro experiments (6–10 μM) causes an increase in intracellular ROS and induces rapid apoptosis of neonatal cardiac myocytes [44]. These data suggest that the production of ROS by pretreatment with chelerythrine causes to enhance the induction of HO-1 by TGD via PKC-independent pathways.

In conclusion, the present study provides the first evidence of TGD leading to changes in the subcellular distribution of PKC isoforms and transient activation of p38 MAPK, causing upregulation of COX-2 and HO-1 expression in cardiac fibroblasts. Furthermore, these results might also provide a basis for novel therapeutic strategies aimed at enhancing these cardioprotective genes against ischemic injury.

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