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Hypoxic preconditioning promotes the translocation of protein kinase C ε binding with caveolin-3 at cell membrane not mitochondrial in rat heart

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Protein kinase C has been shown to play a central role in the cardioprotection of ischemic preconditioning. However, the mechanism underlying PKC-mediated cardioprotection is not completely understood. Given that caveolae are critical for PKC signaling, we sought to determine whether hypoxic preconditioning promotes translocation and association of PKC isoforms with caveolin-3. A cellular model of hypoxic preconditioning from adult rat cardiac myocytes (ARCM) or H9c2 cells was employed to examine PKC isoforms by molecular, biochemical and cellular imaging analysis. Hypoxia was induced by incubating the cells in an airtight chamber in which O_2 was replaced by N_2 with glucose-free Tyrode's solution. Cells were subjected to hypoxic preconditioning with 10 minutes of hypoxia followed by 30 minutes of reoxygenation. Western blot data indicated that the band intensity for PKCε, PKCδ or PKCα, but not PKCβ and PKCζ was enhanced significantly by hypoxic preconditioning from the caveolin-enriched plasma membrane interactions. Immunoprecipitation experiments from the caveolin-enriched membrane fractions of ARCM showed that the level of PKCε, PKCδ and PKCα in the anti-caveolin-3 immunoprecipitates was also increased by hypoxic preconditioning. Further, our FRET analysis in H9c2 cells suggested that there is a minimum FRET signal for caveolin-3 and PKCε along cell peripherals, but hypoxic preconditioning enhanced the FRET signal, indicating a potential interaction between caveolin-3 and PKCε. And also treatment of the cells with hypoxic preconditioning led to a smaller amount of translocation of PKCε to the mitochondria than that to the membrane. We demonstrate that hypoxic preconditioning promotes rapid association of PKCε, PKCδ and PKCα with the caveolin-enriched plasma membrane microdomain of cardiac myocytes, and PKCε via direct molecular interaction with caveolin-3. This regulatory mechanism may play an important role in cardioprotection.

Introduction

Protein kinase C (PKC) exists as a family of at least 12 isoforms. They play important roles in the cellular protection of ischemic preconditioning (IPC).[1,2] Upon activation, PKC isoforms can translocate to multiple subcellular localizations.[3-5] Such as plasma membrane, mitochondria or nucleus. Different subcellular location relations with the substrate specificity of activated PKC isoforms. Although the translocation of specific PKC isoforms to the plasma membrane is believed to be a critical step in the signal transduction of IPC, the precise mechanism underlying PKC-mediated cardioprotection remains largely unknown.

In the heart, a variety of signaling molecules are localized to caveolae.[6,7] Caveolae are specialized plasma membrane microdomains associated with numerous signaling transduction events and entry pathways of biological molecules.[8-12] These microdomains provide subsarcolemmal signaling compartments by recruiting interacting signaling molecules. Caveolins are the main structural components of caveolae. They comprise a family of 3 distinct isoforms: caveolin-1 and -2 are almost ubiquitously expressed, whereas caveolin-3 is a muscle-specific isoform.[13,14] Interestingly, cardiac myocyte caveolae have been shown to be the focal points for activated PKC isoforms and their downstream signaling molecules.[9] Different PKC isoforms may mediate distinct cellular functions by phosphorylating specific subsets of target proteins. Substrate specificity is most likely associated with differential targeting of activated PKC isozymes to distinct subcellular locations.

Although it has been reported that caveolae are down-regulated during ischemia but preserved by IPC,[15] the link

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between PKC and caveolae in the cardioprotection of ischemic preconditioning is still unknown. In the present study, we tested the hypothesis that preconditioning promotes selective translocation and association of PKC isoforms with caveolin-3, the primary structural component of caveolae in cardiac myocytes. We used a cellular simulated IPC model from isolated adult rat cardiomyocytes (ARCM) and the rat heart-derived H9c2 cells to examine whether hypoxic preconditioning induces PKC translocation to the caveolin-rich plasma membrane and whether translocated PKC isoforms interact with caveolin-3. We chose to examine 5 major isoforms of PKC that are known to be expressed in the adult rat cardiomyocytes. We demonstrate that hypoxic preconditioning promoted rapid association of PKCα, ε and δ, but not PKCβ1 and PKCζ with caveolin-3 on the plasma membrane.

Materials and Methods

Materials and cDNA constructs

Mouse or goat IgG directed against caveolin-3 or caveolin-1, and rabbit IgG directed against PKCε were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit IgG purchased from Cell Signaling Technology (Danvers, MA) were used to against GAPDH, PKCα, β1, δ or ζ. The selective PKC inhibitor chelerythrine was supplied from Sigma Aldrich (St. Louis, MO). Trypan blue was purchased from Fisher Scientific (Pittsburgh, PA). Caveolin-3-CFP was provided by Dr. Jefferey R Martens (University of Michigan, Ann Arbor), and PKCε-YFP was obtained from Dr. Ralf Kubitz (Heinrich-Heine University of Düsseldorf, Düsseldorf, Germany). The full length caveolin-3 was ligated into pECFP-C1 and PKCε was ligated into pEYFP-N1.

Cell culture and transfection

Rat heart-derived H9c2 cells were cultured in DMEM/F12 (Dulbecco's modified Eagle's medium) supplemented with 10% FBS, 2mM glutamine, and penicillin streptomycin.[16] H9c2 cells were transfected with cDNAs using Fugene6 according to the manufacturer's instructions. Experiments were carried out 48 to72 hours after transfection.

Sprague Dawley rats (250 to 300 g) purchased from Animal Co., Ltd. (Beijing Laboratory Animal Research Center) were kept in isolator plastic cages cages under specific pathogen free conditions of light and dark (12 h: 12 h) with an ambient temperature of 24°C ± 2°C. They were fed adlibitum with the standard laboratory chow and tap water for 1 week before the experiments commenced. Animals were observed twice daily. Then the rat was anesthetized with chloral hydrate and adult rat ventricular myocytes were isolated from rat heart by enzymatic dissociation. The experiments were approved by and carried out in accordance with the regulation of Animal Experimentation Committee of Jilin University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Isolation of cardiomyocytes

Adult rat ventricular myocytes were isolated from Sprague Dawley rats (250 to 300 g) by enzymatic dissociation.[17] In brief, hearts were excised and retrogradely perfused via the aorta with gas (5% CO_2–95% O_2) Tyrode's solution containing (mM) NaCl 126, KCl 5.4, NaH_2PO_4 0.33, HEPES 10, $CaCl_2$ 1.0, $MgCl_2$ 1.0, and glucose 10. The steps of heart perfusion and subsequent operation were performed at 37°C. The perfusate was then changed to a Tyrode's solution that is nominally Ca^{2+} free but otherwise has the same composition. Therefore, perfusion with the same solution was added 0.06% (w/v) crude collagenase for 20 minutes. Softened ventricular tissues were removed, cut into small pieces, and mechanically dissociated by trituration.

Hypoxic preconditioning

Hypoxia was induced by incubating the cells in an airtight chamber in which O_2 of the gas was replaced by N_2 (5 % CO_2–95% N_2). Moreover, the cell culture medium was glucose-free Tyrode's solution containing (mmol/L) 139 NaCl, 4.7 KCl, 0.5 $MgCl_2$, 1.0 $CaCl_2$, and 5 HEPES, pH 7.4, at 37°C. Hypoxic preconditioning (HP) was achieved by subjecting cells to 10 min of hypoxia and glucose-free Tyrode's solution, then 30 min of reoxygenation and glucose-contained Tyrode's solution. As we described previously,[16] The sustained hypoxia/reoxygenation (H/R) was provided with 90 min of hypoxia followed by 120 min of reoxygenation.

Cell viability assessment

Trypan blue reagent could penetrate the degenerated cell membrane of dead cells and combine with the dissolved DNA. Therefore, we can use it to distinguish viable cells from dead cells by haemocytometer cell counts under the microscope. Cells that are able to exclude the blue stain were considered viable, and the percentages of non-blue cells over total cells were used as an index of cell viability. Cells were counted <15 min to minimize variability associated with changes in the ratio of stained/unstained cells over time.

Small interfering RNA and transfection

The small interfering RNA (siRNA) oligonucleotide targeting caveolin-3 was purchased from Ambion Inc.. (Austin, TX, USA). Three pairs of pre-validated Cav-3 specific siRNAs were used for caveolin-3. (sense, GGUUCCUCUCAAUUCCACCtt, and antisense GGUGGAAUUGAGAGGAACCtc; sense, CGUUCACCGUCUCCAAGUtt, and antisense, UACUUGGAGACGGUGAACGtg; sense, GCUUCGACGGUGUAUGGAAtt, and antisense, UUCCAUACACCGUCGAAGCtg). A negative control siRNA (scrambled:5-GGGATTCCGACCTTACGAT-3) was included to monitor nonspecific effects. H9c2 cells were transfected with siRNA using Amaxa kit (Amaxa, Gaithersburg, MD) immediately after pre-plating step. Forty-eight hours after transfection, westerblot was carried out to examine the knockdown of targeted proteins.

Purification of caveolin-enriched membrane fractions

Caveolin-rich fractions from adult rat cardiomyocytes or H9c2 cells were prepared by using a previously described detergent-free method with some modification.[20] Briefly, cells were pretreated with or without hypoxic preconditioning and then were spin down and resuspended in 0.5 M sodium carbonate (pH 11.0; 2 ml). Subsequently the cell homogenates were subjected to centrifugation at 4°C, and the supernatant was received. The supernatant was adjusted to 45% sucrose by addition of an equal volume of 90% sucrose in MBS (25 mM Mes, pH 6.5/0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube to be centrifuged. A 5–35% discontinuous sucrose gradient (in MBS containing 250 mM sodium carbonate) was formed above, by overlaying with 4 ml of 35% sucrose (prepared in MBS with 250 mM sodium carbonate) and then 4 ml of 5% sucrose (again prepared in MBS with 250 mM sodium carbonate). After adding continuous layers to the tubes, the tubes were centrifuged at 39,000 rpm for 18–20 hr in an SW41 rotor. Twelve 1-ml distinct membranous fractions were seen from the top to the bottom of the gradient for subsequent analysis by Western blot. Caveolin-rich fractions (fractions 4–6) contain caveolin but exclude most other cellular proteins. In order to concentrate the caveolar to a pellet, we centrifuged the tubes at 40,000 *g* for 2 hr. Caveolin-rich fractions (fractions 4–6) were then suspended in lysis buffer and sonicated. To immunoisolate the caveolar, samples were electrophoresed by SDS-PAGE, transferred onto a nitrocellulose membrane, and analyzed by probing with various antibodies. The relative purity of caveolar or cellular fractions was evaluated by antibody against the marker proteins caveolin-3 or GAPDH, respectively.

Western blot and Co-immunoprecipitation

Immunoblot analysis was carried out as described in details previously.[18] Briefly, the cellular and caveolar fractions were lysed and denatured in a sample buffer. Equal amounts of proteins were seperated by 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4), immunoblotted with primary antibodies in TBS, 0.1% Tween 20 for 2 hr at room temperature or overnight at 4°C. After washing, the blots were reacted with peroxidase-conjugated secondary antibodies for 45 minutes and the protein concentrations were determined by the ECL detection system.[19]

Immunoprecipitation experiments were performed according to the previous report.[20,21] The cardiac myocytes were isolated and then pretreated with or without hypoxic preconditioning prior to homogenization. The cells were lysed and centrifuged to get supernatant. Following the 2 hr at 4°C incubation supernatant and antibody against caveolin-3 complex were captured with r-protein-G agarose. Agarose beads slurry were washed 4-times with solubilization buffer before removal of bound proteins by boiling at 100°C for 5min in SDS sample buffer. Samples were loaded in duplicate and separated by SDS-polyacrylamide gels. Resolved proteins were transferred onto nitrocellulose membranes, blocked, incubated with primary and secondary antibodies, then analyzed by the ECL detection system.

Analysis of fluorescence resonance energy transfer (FRET)

H9c2 cells were transfected with PKCε-YFP/caveolin-3-CFP and PKCε-YFP/ Mitochondria-CFP. Images were acquired sequentially through CFP, YFP and FRET filter channels as we described previously. Filter sets used were the donor CFP, the acceptor YFP, and FRET. A background value was determined from a region in each image without any cells. The background value was subtracted from the raw images before carrying out FRET calculations. Corrected FRET ($FRET^C$) was calculated for entire images or selected regions of images, such as cell peripheral regions, by using the equation: $FRET^C = FRET - (0.5 \times CFP) - (0.5 \times YFP)$, where FRET, CFP and YFP correspond to background-subtracted images of cells co-expressing CFP and YFP acquired through the FRET, CFP and YFP channels, respectively. The 0.5 value is the fractions of bleed through of CFP or YFP fluorescence, estimated from cells expressing either CFP- or YFP-fusion proteins. Mean $FRET^C$ values were calculated from mean fluorescence intensities for each selected sub-region. All calculations were performed using MetaMorph. $FRET^C$ images are presented as a quantitative pseudocolor image.

Data analysis

Group data were presented as mean ± SE. Unpaired t-test was used to compare between groups. Multiple group means were compared by ANOVA followed by LSD post hoc test. Differences with a 2 tailed $P < 0.05$ were considered statistically significant.

Results

Effect of hypoxic preconditioning on translocation of PKC isoforms to the caveolin-rich plasma membrane

We have previously shown that using a detergent-free sucrose gradient extraction-procedure the muscle-specific caveolin-3 isoform is enriched in the fraction 4–6 from adult rat cardiomyocytes.[20] Thus, in the present study caveolin-rich fractions 4–6 were collected to further determine whether hypoxic preconditioning increases the expression of PKC isoforms within caveolae by translocating PKC to the caveolin-rich plasma membrane. The blots were probed with anti-caveolin-3 antibody to confirm the caveolin-rich fractions. Freshly isolated adult cardiomyocytes were treated with or without hypoxic preconditioning with 10 minutes of hypoxia followed by 30 minutes of reoxygenation.[22] As shown in **Figure 1A**, hypoxic preconditioning did not cause significant alterations in the expression of total cellular PKC isoforms including PKCε, δ, α, β1 and ζ. However, the band intensity for PKCε, PKCδ and PKCα in the caveolin-rich fractions increased significantly in response to hypoxic preconditioning. Small fractions of immunoreactivity for all 5 PKC isoforms were detected in the caveolin-rich fractions under control condition. The antibody against caveolin-3 detected abundant caveolin-3, a

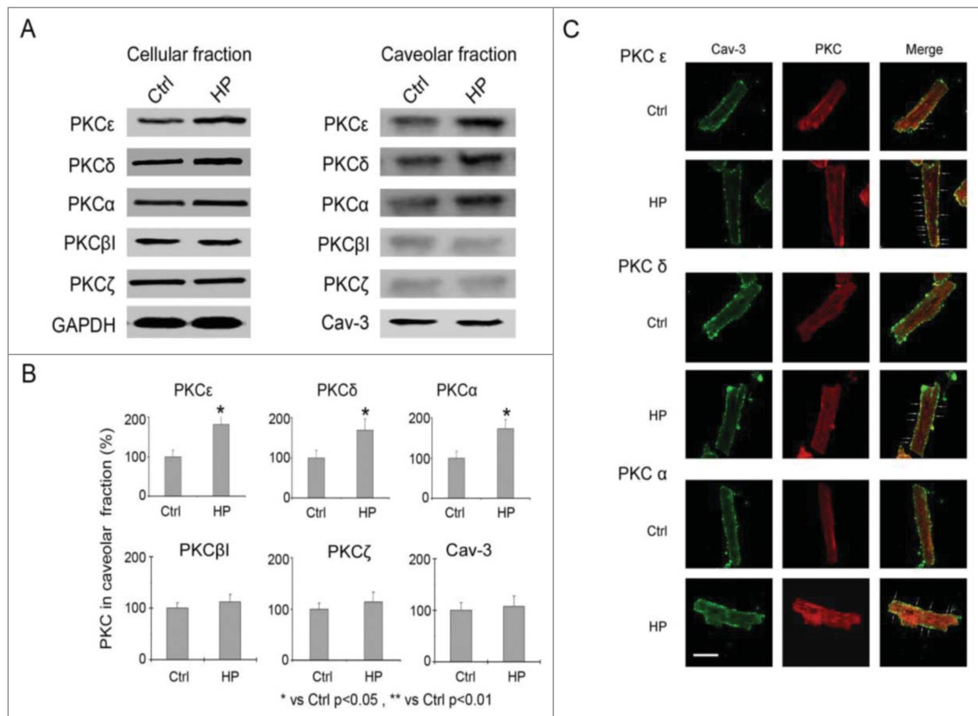

Figure 1. Effect of hypoxic preconditioning on PKC isoforms translocation to the caveolin-rich plasma membrane and colocalization of PKC and caveolin-3 in adult rat cardiac myocytes. Cells were treated with or without hypoxic preconditioning (HP) prior to fractionation. (**A**) Representative Western blots of total cellular or caveolar fraction from 4 independent experiments. (**B**) Expression of PKC isoforms in the caveolin-rich fractions calculated by relative densitometry and normalized to 100% of control. Hypoxic preconditioning caused significant increase in the expression of PKCε, PKCδ and PKCα in the caveolin-rich fractions, but not in total cellular lysates. *$p < 0.05$ vs. control, $n = 4$. (**C**) Double labeling confocal images of adult rat ventricular myocytes with anti-PKCε, PKCδ and PKCα and anti-Cav-3 antibodies. Punctate areas of colocalization (represented by yellow) are apparent along the plasma membrane, Scale bar: 10 μM. $n = 40$.

signature protein for the caveolae, indicating that isolated fractions were purified caveolin-enriched fractions. Densitometric analysis revealed that hypoxic preconditioning treatment significantly enhanced the expression of PKCε, δ and α in the caveolin-rich fractions (**Fig. 1B**; PKCε: 181.29 ± 36 .41%, PKCδ: 168.53 ± 27 .53%, PKCα: 172.67 ± 23 .64%, $p < 0.05$ vs. control, $n = 4$). In contrast, hypoxic preconditioning did not increase the recruitment of PKCβ1 and ζ to the caveolin-rich domains. These results confirm that hypoxic preconditioning promotes rapid translocation of PKCε, δ and α to the caveolin-rich plasma membrane microdomains of cardiac myocytes.

Colocalization of PKC isoforms and caveolin-3

To investigate whether PKC isoforms colocalizes with caveolin-3, we employed immunofluorescence confocal microscopy.[23] **Figure1C** shows fluorescence images from adult rat ventricular myocytes. The antibody against caveolin-3 (green) or PKC ε, δ and α (red) demonstrated a prominent surface sarcolemmal punctate staining area. Merged images showed significant punctuate areas of colocalization for PKC ε, δ and α and caveolin-3 along the plasma membrane. These data suggests that hypoxic preconditioning induce PKC ε, δ and α translocate to cell membrane and localized in caveolin-3-associated membrane domains.

Effect of hypoxic preconditioning on the association of caveolin-3 with PKC isoforms

Caveolin-3 has been shown to interact with various signaling proteins localized in caveolae. PKC isoforms have been shown to localize to caveolae and interact with caveolin in a caveolin-subtype and PKC isoform-dependent manner.[17,24] To determine whether caveolin-3 associates with some of PKC isoforms in H9c2 cells and whether this association is up-regulated by hypoxic preconditioning, we performed co-immunoprecipitation for PKC isoforms and caveolin-3 in the caveolin-3-enriched fractions from H9c2 cells pretreated with or without hypoxic preconditioning. Caveolin-rich fractions containing equal amounts of total proteins were immunoprecipitated with anti-caveolin-3 antibody. The immune complex was collected with protein G beads and analyzed by immunoblotting. As shown in **Figure 2C**, there was a minimum association of caveolin-3 with PKCε, δ or α under control condition. Treatment with hypoxic preconditioning did not alter the recovery of caveolin-3, but significantly increased the association of caveolin-3 with PKCα (169.23 ± 19.56 vs. control, $p < 0.05$), PKCε (175.16 ± 18.47 vs. control, $p < 0.05$) and PKCδ (163.57 ± 11.36 vs. control, $p < 0.05$). The same experiments were repeated 3 times. Other PKC isoforms such as PKCβ1 and ζ were not detected in the caveolin-3 immunoprecipitates under either control or hypoxic preconditioning condition. The proteins detected in the caveolin-3 immunoprecipitates were not detected in the lysates that were immunoprecipitated with control IgG. These results demonstrate that hypoxic preconditioning promotes the rapid association of PKCα, ε and δ with caveolin-3 in H9c2 cells.

Role of PKC in the protective effect of hypoxic preconditioning

We have previously shown that hypoxic preconditioning with 10 minutes of hypoxia followed by 30 minutes of reoxygenation provides the protection against hypoxia-induced cell death.[16] To confirm the protection and the role of PKC activity under our

cellular model of hypoxic preconditioning, we studied the effect of the PKC activity inhibitor chelerythrine on the cytoprotection of hypoxic preconditioning from adult rat cardiac myocytes. The cell viability assay with trypan blue staining revealed that the percentage of viable cells after prolonged hypoxia and reoxygenation (120 minutes of hypoxia and 160 minutes of reoxygenation) was significantly higher than that in the group treated with hypoxic preconditioning prior to the prolonged hypoxia/reoxygenation (**Fig. 3**, 82.2% ± 7.4% vs. 43.6% ± 5.0%, $p < 0.01$). As expected, the preconditioning effect was largely eliminated in cells pretreated with chelerythrine (10 μM, 49.4% ± 3.5%, $p < 0.01$), indicating the role of PKC in the protection of hypoxic preconditioning. The data were obtained from 4 independent experiments. These results indicate that PKC activity is involved in the cellular protection of hypoxic preconditioning and our hypoxic preconditioning protocol provides a valid tool for studying relevant molecular mechanisms.

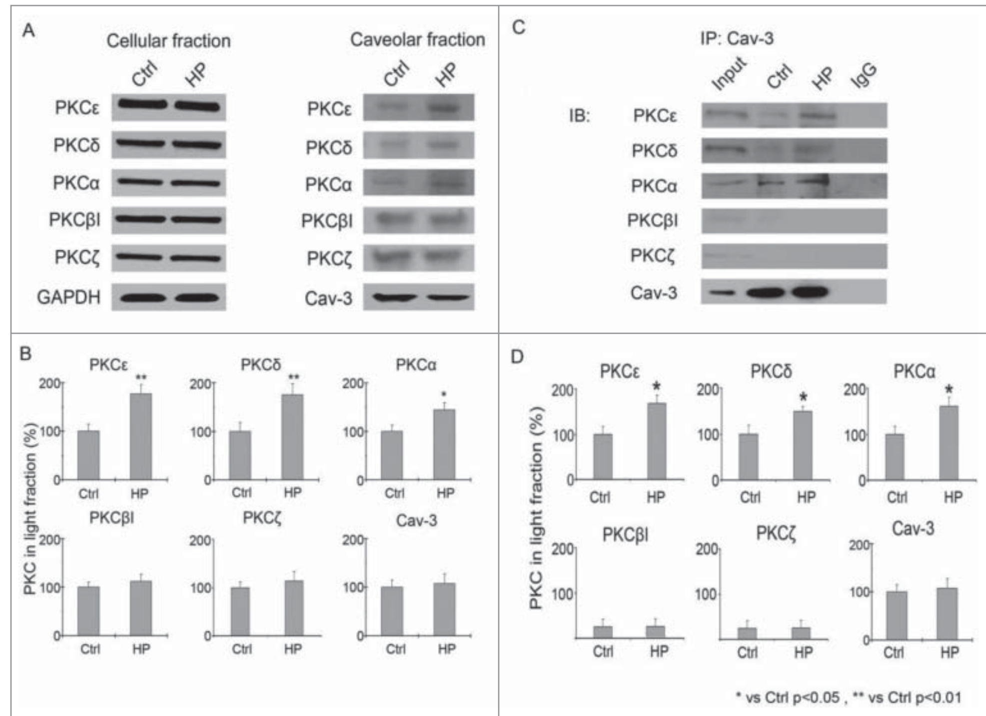

Figure 2. Effect of hypoxic preconditioning on PKC translocation to caveolin-rich plasma membrane and co-precipitation of caveolin-3 and PKC isoforms in H9c2 cells. Cells were treated with (HP) or without hypoxic preconditioning (Ctrl) prior to fractionation. (**A**) Representative Western blots of total cellular or caveolar fraction from 3 independent experiments. (**B**) Expression of PKC isoforms in the caveolin-rich fractions calculated by relative densitometry and normalized to 100% of control. Hypoxic preconditioning caused significant increase in the expression of PKCε, PKCδ and PKCα in the caveolin-rich fractions, but not in total cellular lysates. (**C**) The caveolin-rich fractions form cells treated with or without HP were immunoprecitated with anti-caveolin-3 antibody, followed by immunoblotting with antibodies against various PKC isoforms. IgG denotes immunoprecipitation with control IgG from the protein lysates of HP-treated cells. (**D**) Expression of PKC isoforms in the caveolin-3 immunoprecipitates from the caveolin-rich fractions. Percent expression of PKC isoforms were calculated by relative densitometry and normalized to 100% of control. Hypoxic preconditioning promoted association of caveolin-3 with PKCε, α and δ, but not PKCβ1 and PKCζ. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control, $n = 3$.

Effect of hypoxic preconditioning on translocation of PKC isoforms to the caveolin-rich plasma membrane

We also examined the effect of hypoxic preconditioning on translocation of endogenous PKC isoforms in H9c2 cells. We found that PKCα, β1, ε, δ and ζ were all expressed in H9c2 cells. The level of PKC ε, δ and α but not β1 and ζ increased in the caveolin-rich membrane fractions in response to hypoxic preconditioning (**Fig. 3B**, PKCε: 173.04 ± 18.16%, PKCδ: 189.73 ± 27.21%, $p < 0.01$ vs. control; PKCα: 143.01 ± 10.73%, $p < 0.05$ vs. control, $n = 4$).

To determine whether destroying caveolae by siRNA knockdown of caveolin-3 expression alters the regulation of PKC translocation by hypoxic preconditioning, we investigated the effect of caveolin-3 siRNA on PKC translocation. We first examined whether a pre-designed siRNA oligonucleotide for caveolin-3 can reduce endogenous caveolin-3 protein level H9C2 cells. We found that caveolin-3 siRNA significantly suppressed caveolin-3 expression 48 h after transfection with siRNA. In contrast, control siRNA had no effect (**Fig. 3A**). As shown in **Figure 3B**, transfection of H9c2 cells with caveolin-3 siRNA largely eliminated the stimulatory effect of hypoxic preconditioning on PKC ε, δ and α translocation (**Fig. 3B**, siRNA-Cav-3: PKCε: 53.72 ± 5.53%, $p < 0.01$ vs. HP PKCδ: 62.26 ± 6.21%, $p < 0.01$ vs. HP; PKCα: 76.62 ± 8.58%, $p < 0.05$ vs. HP, $n = 4$). Taken together, these findings suggest that hypoxic preconditioning-mediated translocation of PKC ε, δ and α in the H9c2 cell requires intact caveolae where PKC ε, δ and α and caveolin-3 may associate.

FRET analysis on the interaction between PKC and caveolin-3

Our immunoprecipitation experiments indicate that caveolin-3 associates with several PKC isoforms. This association could be via specific protein-protein interaction or both proteins reside in a lipid environment that remains intact throughout the immunoprecipitation process. To further define whether hypoxic preconditioning promotes direct molecular interaction between caveolin-3 and PKC isoforms, we used PKCε as an example to test in the rat heart-derived H9c2 cells whether YFP fused to the

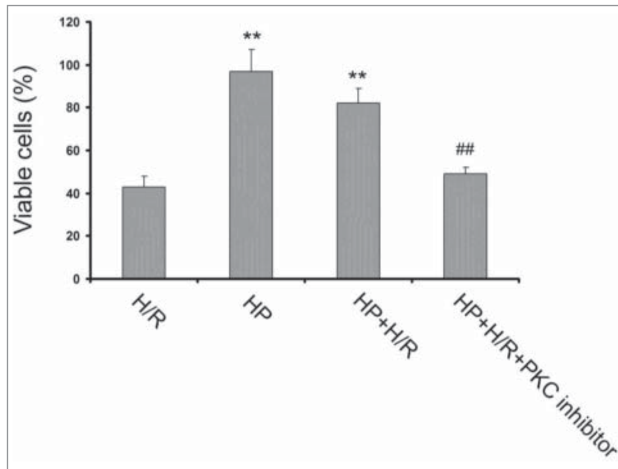

Figure 3. Role of PKC on hypoxic preconditioning (HP)-mediated protective effect against hypoxia/reoxygenation (H/R)-induced cell damage. Adult rat cardiomyocytes were treated with or without hypoxic preconditioning, in the presence or absence of the PKC inhibitor chelerythrine (Che). Cell viability was assessed by trypan blue staining. Five $\times$ 10^5 cells were in each group. **$p < 0.01$ vs. H/R, ##$p < 0.01$ vs. HP+H/R, $n = 4$.

C-terminus of PKCε is sufficiently close to CFP fused to the N-terminus of caveolin-3 to yield FRET. We used H9c2 cells because they are easily transfected and have been used for many studies in hypoxia and protein kinases.[25,26] Cells were transfected with caveolin-3-CFP, PKCε-YFP or both. As shown in **Fig. 5**, $FRET^C$ was minimal in all regions of cells that were not subjected with hypoxic preconditioning (**Fig. 5E**), indicating that there was no significant amount of PKCε localized in caveolae. PKCε appeared to be mostly in the cytosol. In particular, FRET signals were not observed in the plasma membrane enriched with caveolin-3-CFP. This validates the accuracy of our method of correction for non-FRET component of the FRET images. Treatment of the same cell with hypoxic preconditioning led to rapid translocation of PKCε to the caveolin-enriched plasma membrane microdomains and exhibited FRET signals due to energy transfer from CFP at the N-terminus of caveolin-3 to YFP at the C-terminus of PKCε. And fluorescence intensity measured along the line. To compare FRET efficiencies, the $FRET^C$ was normalized to the intensity of PKCε-YFP after background subtraction. As shown in panel G, hypoxic preconditioning significantly increased FRET efficiency when compared to control (0.49 ± 0.01 vs. control, $p < 0.01$). The data were analyzed from a total of 40 cells. Our results suggest that hypoxic preconditioning promoted the direct interaction between Cav-3-CFP and PKCε-YFP.

FRET analysis on the interaction between PKC and mitochondrial

We also used PKCε to test in H9c2 cells whether YFP fused to the C-terminus of PKCε is sufficiently close to CFP fused to mitochondria to yield FRET. H9c2 cells were transfected with Mito-CFP, PKCε-YFP or both. As shown in **Fig. 5F**, $FRET^C$ was minimal in all regions of cells that were not subjected with hypoxic preconditioning (**Fig. 3F**), indicating that there was no significant amount of PKCε localized in mitochondria. Treatment of the cells with hypoxic preconditioning led to a small amount of translocation of PKCε to the mitochondria microdomains and exhibited FRET signals due to energy transfer from Mito-CFP to YFP at the C-terminus of PKCε and fluorescence intensity measured along the line. To compare FRET efficiencies, the $FRET^C$ was normalized to the intensity of PKCε-YFP after background subtraction. As shown in panel H, hypoxic preconditioning significantly increased FRET efficiency when compared to control (0.24 ± 0.01 vs. control, $p < 0.05$). The data were analyzed from a total of 40 cells. Our results suggest that hypoxic preconditioning promoted a little interaction between Mito-CFP and PKCε-YFP.

Discussion

The present study demonstrated that hypoxic preconditioning promotes targeting of PKC isoforms, PKCα, ε and δ, to the caveolin-rich plasma membrane microdomains. We found that hypoxic preconditioning with 10 minutes of hypoxia followed by 30 minutes of reoxygenation induced a rapid association of PKCα, ε and δ with caveolae or caveolin-3 in both adult rat cardiac myocytes and heart-derived H9c2 cells. Specifically, we demonstrated that hypoxic preconditioning promoted the targeting of PKCε, δ and α to the caveolin-rich plasma membrane microdomains. Importantly, we showed that PKCε directly interacted with caveolin-3 by FRET. This observation is consistent with our hypothesis that PKC isoforms are selectively recruited to the caveolae of cardiac myocytes by hypoxic preconditioning, possibly by interacting with caveolin-3.

Whereas many endogenous neurotransmitters, peptides, and hormones have been proposed to play a role in the signal transduction pathways mediating the cardioprotective effect of IPC, nearly universal evidence indicates the involvement of PKC.[27-29] However, apart from this general consensus, the molecular mechanism by which PKC isoform contribute to IPC is not largely unknown. Importantly, the mechanism by which PKC is translocated to plasma membrane is not known. It has been reported that caveolae are downregulated during ischemia but preserved by IPC16, indicating importance of caveolae or caveolin-3 in cardioprotection. In the present study, we employed a simulated ischemic preconditioning model and examined the effect of hypoxic preconditioning on the selective translocation and caveolar targeting of PKC isoforms. We found that under basal condition the level of immunoreactivity for PKCε, α and δ in cardiomyocyte caveolae was minimal, but enhanced significantly following hypoxic preconditioning stimulation. In contrast, the level of other PKC isoforms PKCβ1 and PKCζ was not altered by hypoxic preconditioning. Our data also show that caveolin-3 associates with PKCε, α and δ, but not PKCβ1 and ζ. These observations provide the novel evidence that hypoxic preconditioning induces the selective translocation and targeting of PKCε, δ and α to the caveolin-rich plasma membrane. To address further the molecular interaction that may confer PKC

translocation to the caveolar plasma membrane, we used PKCε as an example and studied the effect of hypoxic preconditioning on direct molecular interaction between PKCε and caveolin-3. Our observation with FRET analysis revealed that there was no significant interaction between PKCε and caveolin-3 as evidenced by minimum FRET signal. However, hypoxic precondition with 10 minutes of hypoxia and 30 minutes of reoxygenation induced substantial increase in FRET signal between PKCε and caveolin-3, indicating that hypoxic preconditioning increased translocation of PKCε to the caveolar membrane, possibly by direct interaction of PKCε to caveolin-3.

While our data demonstrate that hypoxic preconditioning promotes selective translocation of PKCε, δ and α, there is no evidence that all 3 PKC isoforms are involved in the cardioprotective effect of hypoxic preconditioning. Depending on experimental conditions and preconditioning protocols, more than one PKC isoforms could be activated, each serving a different role under specific physiological or pathophysiological conditions. In addition to translocation of PKC isoforms, our finding further indicates the importance of caveolae microdomains in PKC-mediated cardioprotection. Even though different PKC isoforms may be targeted to caveolae upon activation, activated PKC isoforms within caveolae may associate with distinct cellular functions due to differential targeting of separate populations of caveolae which may contain different sets of signaling proteins.[30]

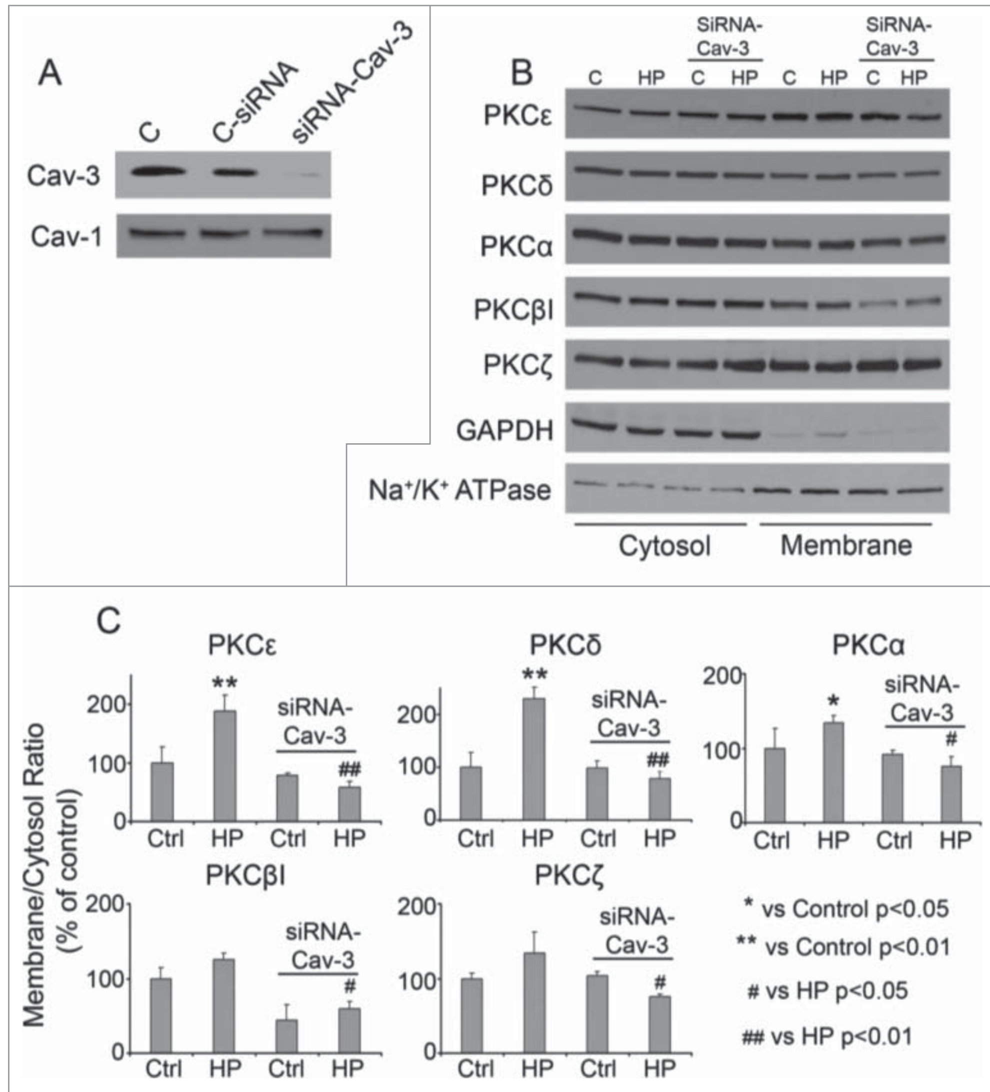

Figure 4. Knockdown of caveolin-3 expression with siRNA prevents HP mediated translocation of PKC isoforms. (**A**) Western blot shows significant reduction of endogenous caveolin-3 (Cav-3) by siRNA against caveolin-3 (Cav-3 siRNA) and control-siRNA (scramble siRNA). (**B**) siRNA prevents HP mediated translocation of PKC ε,δ,αbut not PKC β1,ζ. (**C**) Percent expression of PKC isoforms were calculated by relative densitometry and normalized to 100% of control. HP promoted translocation of PKCε, δ, α but not PKC β1,ζ. * vs control $p < 0.05$,** vs control $p < 0.01$, # vs HP $p < 0.05$,##vs. HP $p < 0.01$. $n = 3$.

The intact caveolae structure in cardiac myocytes has been shown to be required for the cardioprotection of ischemic preconditioning.[15,31] Our observation that hypoxic preconditioning promotes the selective translocation of PKCε, δ and α to the caveolar plasma membrane microdomain further implies that caveolae may serve as a focal point for efficient PKC signaling transduction in cardioprotection. Nevertheless, our results do not exclude the possibility that hypoxic preconditioning may promote translocation of PKC isoform to other subcellular sites such as mitochondria. As mitochondria plays an essential role in regulation of apoptosis, mitochondria closely relates with cell survival. In fact, mitochondrial PKCε has been linked to cardioprotection against ischemia.[28,32, 33] While PKC isoforms do not have consensus mitochondrial N-terminal targeting sequence, they have been consistently shown to translocate to mitochondria by ischemic preconditioning. Studies have reported that HSP90 and some mitochondrial import machineries are crucial for targeting PKC to mitochondria.[4,34] However, how PKC translocation to mitochondria is initiated and whether caveolar PKC signaling is positively linked to mitochondrial translocation of PKC in cardioprotection are interesting questions and remain to be elucidated.

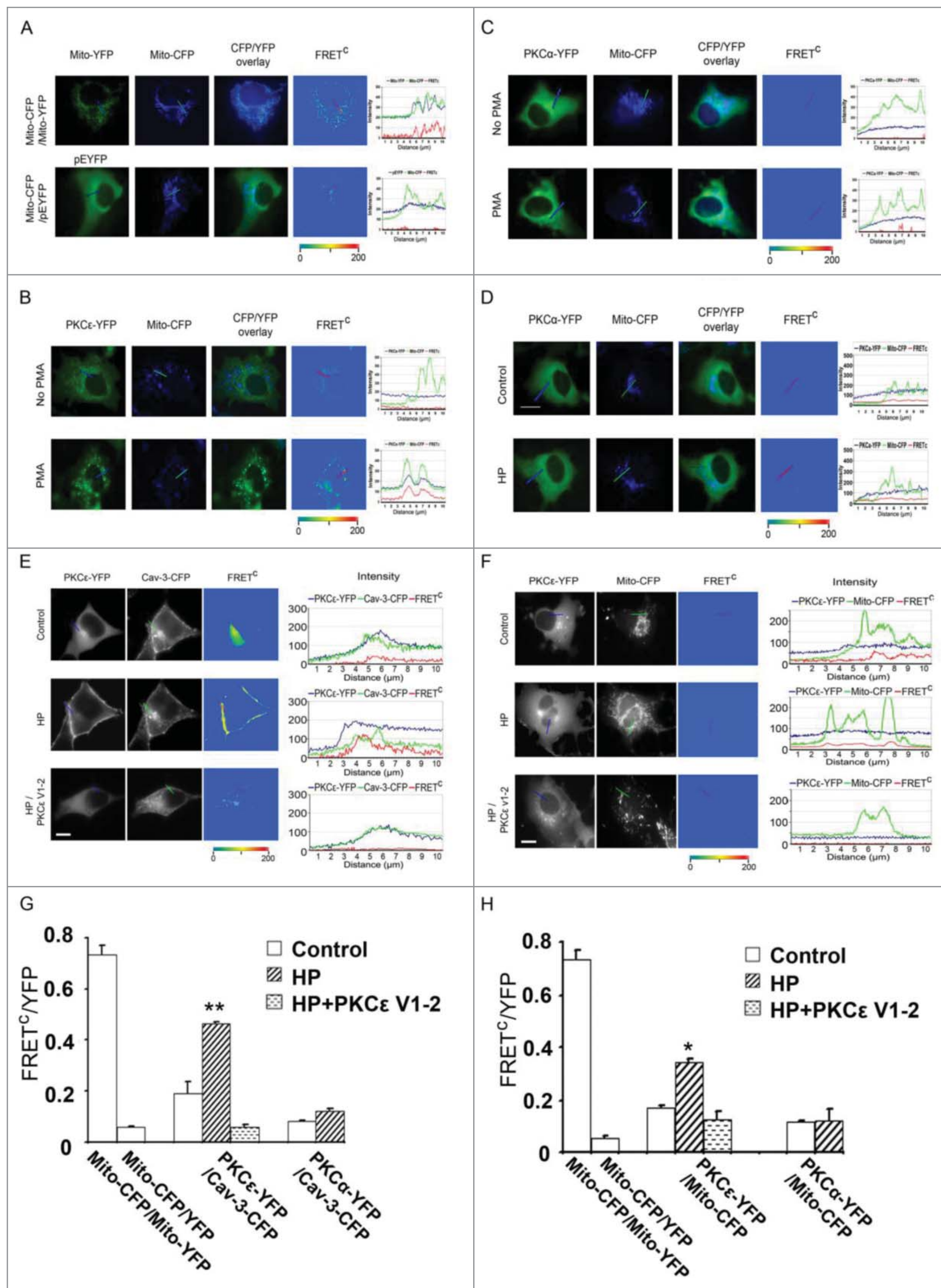

Figure 5. Effect of hypoxic preconditioning on the interaction of PKC with caveolin-3 and PKC in mitochondrial. H9c2 cells were co-transfected with PKCε-YFP/Cav-3-CFP and PKCε-YFP/Mito-CFP cDNAs. The images were captured from the same cell before and after treatment with hypoxic preconditioning. (**A**) Representative images of Mito-YFP, Mito-CFP and FRET^C showing the background-subtracted and corrected FRET in quantitative pseudocolor. Scale bar: 10 μM. 400 × ; n = 20. (**B**) Representative images of PKCε-YFP, Mito-CFP and FRET^C. PMA led to FRET signals due to energy transfer from CFP of mitochondrial to YFP of PKCε. n = 20. (**C and D**) Representative images of PKCα-YFP, Mito-CFP and FRET^C. HP&PMA led to a little FRET signals due to energy transfer from CFP of mitochondrial to YFP of PKCε. (ABCD) as the positive control. 400 × ;n = 40. (**E**) Representative images of PKCε-YFP, Cav-3-CFP and FRET^C. Hypoxic preconditioning led to FRET signals due to energy transfer from CFP of caveolin-3 to YFP of PKCε. Fluorescence intensity measured along the line. 400 × ;n = 20. (**F**) Representative images of PKCε-YFP, Mito-CFP and FRET^C. Hypoxic preconditioning led to FRET signals due to energy transfer from CFP of Mito to YFP of PKCε. Fluorescence intensity measured along the line. 400 × ;n = 20. (**G and H**) FRET efficiency (FRET^C/YFP) was expressed as values that were normalized by dividing corrected FRET (FRET^C) by the mean intensity of YFP (PKCε-YFP). 400 × ;n=20. *$p < 0.05$ vs. control, **$p < 0.01$ vs. control.

In the present study, we show that hypoxic preconditioning induces selective translocation of PKC isoforms to the caveolar membrane, possible by binding of PKC to caveolin-3. However, the signaling cascade that leads to the activation of PKC isoforms in response to preconditioning stimulus is still not completely understood. It is generally believed that IPC are coupled to several G protein-coupled receptors, resulting in signal cascade involving activation of PKC.[29] The present study suggests that the recruitment of activated PKC isoforms to caveolae could lead to activation of downstream signaling molecules. A key signaling cascade that could be activated by PKC isoforms within caveolae is the ERK subfamily of mitogen activated protein kinases.[9] Another important substrate for PKC activation could be ATP sensitive K^+ channels (K_{ATP}) since cardiac K_{ATP} channels are mostly localized in caveolae and functionally regulated by adenosine receptors and PKC.[35-38]

In summary, our data demonstrate that hypoxic preconditioning promotes selective translocation and caveolar targeting of PKCε, δ and α but not PKCβ1 and ζ. This finding provides new mechanistic insight into our understanding the role of caveolae in PKC-mediated cardioprotection.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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