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Molecular mechanism underlying adenosine receptor-mediated mitochondrial targeting of protein kinase C

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ABSTRACT

Activation of protein kinase C (PKC) via adenosine receptors is known to be involved in the cardioprotection of ischemic preconditioning (IPC). Specifically, activation of PKCε is critical for cardioprotection. There is ample evidence that PKCε resides in cardiac mitochondria. However, the signals that promote translocation of PKCε are largely unknown. The present study was designed to determine whether and how adenosine receptor activation induces translocation of PKCε to mitochondria. Freshly isolated adult rat cardiac myocytes and rat heart-derived H9c2 were used in the study. Immunofluorescence imaging of isolated mitochondria showed that PKCε but not PKCδ was localized in mitochondria and this mitochondrial localization of PKCε was significantly increased by adenosine treatment. The adenosine-induced increase in PKCε-positive mitochondria was largely prevented not only by PKC inhibitor chelerythrine, but also by the HSP90 inhibitor geldanamycin and by siRNA targeting HSP90. Immunoblot analysis from percoll-purified mitochondria further demonstrated that adenosine mediated a significant increase in mitochondrial PKCε but not PKCδ. This effect was blocked by inhibiting PKC activity with chelerythrine and bisindolylmaleimide. Furthermore, co-immunoprecipitation data showed that PKCε but not PKCδ was associated with TOM70 and HSP90, and this association was enhanced by adenosine treatment. Moreover, adenosine-induced association of PKCε with TOM70 was reduced by suppressing HSP90 expression with siRNA. In conclusion, we demonstrate that adenosine induces HSP90-dependent translocation of PKCε to mitochondria, possibly through mitochondrial import machinery TOM70. These results point out a novel mechanism in regulating PKC in mitochondria and suggest an important implication in ischemic preconditioning or postconditioning.

1. Introduction

Activation of protein kinase C (PKC) has been shown to be involved in the protective effect of ischemic preconditioning (IPC) and postconditioning [1–3]. Specifically, activation of PKC isoform PKCε is critical for cardioprotection [4–6]. Numerous reports have shown that selective activation of PKCε confers cardioprotection whereas inhibition of PKCε prevents protection.

It has been known that upon activation, PKC translocates to multiple subcellular localizations including mitochondria, nucleus and plasma membrane [4,7,8]. There is ample evidence that PKC substrates reside in cardiac mitochondria [4,8–11]. While some studies demonstrate that PKCε is present constitutively within cardiac mitochondria, others suggest that mitochondrial translocation of PKCε is required for protective effect [4,6,9]. Given that most mitochondrial proteins are nuclear-encoded, synthesized in the cytosol, and transported to mitochondria through the protein-conducting pores formed by translocases of the outer (TOM) and inner (TIM) mitochondrial membrane [12–14], PKCε may be translocated to mitochondria via import mechanism in order to interact with its substrates within mitochondria.

Adenosine receptors are G-protein-coupled receptors and consist of 4 subtypes — A1, A2A, A2B and A3. It is generally believed that A1 and A3 receptors are coupled to Gi/Go proteins whereas A2A and A2B are linked to Gs proteins. While different receptor subtypes may be associated with distinct signaling pathways, all of these receptor subtypes are shown to be cardioprotective against ischemia/reperfusion-induced injury [15]. Importantly, the adenosine-receptor-mediated cardioprotection has been shown to be associated with the activation and translocation of PKC isoforms from cytosolic to membrane fractions [16–19]. However, whether adenosine receptors induce translocation of PKC isoforms to mitochondria is not known. In recent years, studies have shown that molecular chaperone heat shock protein (HSP)90 and HSP70, which are linked to IPC, can facilitate mitochondrial protein transport by association with import machinery TOM70 in mammalian cells [20,21]. Further, a recent

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report indicates that some components of the mitochondrial import machinery are down-regulated during ischemia but preserved by IPC [22]. In the present study, we tested the hypothesis that adenosine induces mitochondrial targeting of PKCε, and this process is regulated by HSP90-dependent mitochondrial import mechanism. We examined the effect of adenosine on the dynamic movement of PKCε from cytoplasm to mitochondria and its regulation by TOM70 and HSP in the freshly isolated adult cardiac myocytes and rat heart-derived H9c2 cells. We demonstrated that adenosine promoted PKCε translocation to mitochondria. This regulatory mechanism largely depends on cellular HSP90, possibly via TOM70. Our data indicate a novel mechanism underlying adenosine-induced PKC targeting to mitochondria.

2. Materials and methods

2.1. Materials

Rabbit IgG directed against PKCε or PKCδ was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Adenosine, adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), chelerythrine, bisindolylmaleimide hydrochloride, HSP90 inhibitor geldanamycin were supplied from Sigma-Aldrich (St. Louis, MO). Adenosine was dissolved in deionized water. All other drugs were dissolved in dimethyl sulfoxide, which did not exceed a final concentration of 0.1%.

2.2. Isolation of cardiomyocytes

Adult rat ventricular myocytes were isolated from adult Sprague Dawley rats (250 to 300 g) by enzymatic dissociation [23,24]. In brief, hearts were excised and retrogradely perfused via the aorta with oxygenated (100% O_2) Tyrode's solution containing (mM) NaCl 126, KCl 5.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, NaH_2PO_4 0.33, HEPES 10, and glucose 10 at 37 °C. The perfusate was then changed to a Tyrode's solution that is nominally Ca^{2+} free but otherwise had the same composition. The hearts were perfused with the same solution containing collagenase for 20–30 min. Softened ventricular tissues were removed, cut into small pieces, and mechanically dissociated by trituration. The digested cells were initially stored in a solution containing (mM) KCl 20, KH_2PO_4 10, glucose 10, potassium glutamate 70, ß-hydroxybutyric acid 10, taurine 10, mannitol 5, and EGTA 5, along with 1% albumin, and then transferred to normal Tyrode solution. Cells were incubated in Tyrode solution with various agents for 5 to 10 min at 37 °C prior to subsequent biochemical and immunofluorescence experiments.

2.3. Small interfering RNA

The small interfering RNA (siRNA) oligonucleotide targeting rat HSP90 α and β was purchased from Ambion Inc. (Austin, TX, USA). A negative control siRNA (scrambled) was included to monitor non-specific effects.

2.4. Cell culture and transfection

Rat heart-derived H9c2 cells were cultured in Dulbecco's modified Eagle's medium DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin–streptomycin [25]. H9c2 cells were transfected with siRNA using Hi-perfect siRNA transfection reagent [25]. Experiments were carried out 48 to 72 h after transfection.

2.5. Preparation of the cytosolic or mitochondrial fraction or intact mitochondria

Intact mitochondria or mitochondrial or cytosolic fractions were prepared from H9c2 cells and adult rat cardiomyocytes by differential centrifugation [19,25,26]. For Western blot experiments, mitochondrial fraction was further purified by 30% percoll ultracentrifugation [25,26]. For mitochondrial colocalization experiments, cells were stained with a mitochondrial marker MitoTracker (200 nM) for 15 min before fractionation. In brief, cells after treatment with various agents at 37 °C were collected in an ice-cold homogenizing buffer containing (in mM) 250 mM sucrose, 5 mM HEPES, 5 mM EDTA, and protease inhibitor cocktail. Two 15 s homogenization cycles were performed on ice. The homogenate was centrifuged at 1000 *g* for 10 min at 4 °C to remove nuclei and debris. The mitochondrial and cytosolic fractions were prepared by centrifugation at 8500 × *g* for 20 min at 4 °C. The supernatants were then centrifuged at 27,000 *g* for 1 h at 4 °C, and the resulting supernatant was used as the soluble cytosolic fraction. The pellet containing the mitochondrial fraction was resuspended in the homogenizing buffer and further centrifuged at 8500 *g* for 20 min at 4 °C. The washed mitochondria were then resuspended. For percoll purification, the crude mitochondrial suspension (0.5 ml) was laid on the top of 10 ml of a solution containing 30% percoll, 0.25 M sucrose, 1 mM EDTA and 10 mM HEPES (pH 7.4). Self-generating percoll gradient was developed by centrifugation at 95,000 *g* for 30 min at 4 °C. Mitochondrial band was collected by a Pasteur pipette and washed in the homogenizing buffer.

2.6. Western blotting

Immunoblot analysis was carried out from the cytosolic and mitochondrial fractions [19,26]. The purity of mitochondrial or cytosol fractions was evaluated using antibody against the mitochondrial marker protein prohibitin, the cytosol marker GAPDH and the plasma membrane marker Na^+/K^+ ATPase to ensure there is no significant contamination in the cytosolic or mitochondrial fraction. Briefly, the percoll-purified mitochondrial and cytosolic fractions were denatured in a sample buffer. Equal amounts of proteins were loaded and electrophoresed on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The transferred blots were blocked with 5% nonfat milk in Tris-buffered saline (TBS, 150 mM NaCl, 20 mM Tris–HCl, pH 7.4) and incubated for 2 h at room temperature or overnight at 4 °C with primary antibodies in TBS, 0.05% Tween 20. After washing, the blots were reacted with peroxidase-conjugated secondary antibodies for 45 min and developed using the ECL detection system.

2.7. Co-immunoprecipitation

Immunoprecipitation experiments were performed as reported previously [19,27,28]. Cells were pretreated with or without adenosine prior to homogenization. The mitochondrial fraction was incubated with primary antibody for 2 h at 4 °C. Antigen-antibody complexes were captured with r-protein-G agarose (4 °C, 2 h). Agarose beads were washed 4-times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane, and analyzed by probing with various antibodies.

2.8. Immunofluorescence microscopy

As described previously [27], intact mitochondria were isolated from cells pretreated with MitoTracker and/or other agents. Mitochondria were fixed with 4% formaldehyde in PBS for 30 min, blocked, permeabilized in 5% goat serum in PBS with 0.1% Triton X-100 (30 min), and labeled with primary antibody for 2 h. Mitochondria

were then washed three times and labeled with fluorescence-conjugated secondary antibody for 1 h. Immunofluorescence was visualized with Nikon fluorescence microscope. All images were analyzed using a background subtraction method offline.

2.9. Data analysis

Group data were presented as means ± SE. Multiple group means were compared by ANOVA followed by LSD *post hoc* test. Differences with a two-tailed $P<0.05$ were considered statistically significant.

3. Results

3.1. Effect of adenosine on mitochondrial localization of PKCε

To investigate role of adenosine on mitochondrial localization of PKCε, we examined whether adenosine promotes mitochondrial translocation of PKCε and whether this translocation is dependent on PKC activation. Given that determining whether proteins are localized in mitochondria is difficult in intact adult cardiomyocytes due to tightly packed myofibrils and sub-organelles, we isolated mitochondria from adult rat cardiac myocytes and analyzed mitochondria for the presence of protein of interest. This method has been demonstrated to be an effective way to localize proteins in mitochondria [20,26]. The freshly isolated adult rat cardiac myocytes were pretreated without or with adenosine (10 μM), adenosine plus chelerythrine (10 μM), or adenosine plus DPCPX (100 nM) for 5–10 min. The concentration of the PKC inhibitor chelerythrine was chosen based on our previous studies [26,29,30]. Cells were also stained with a mitochondria-selective probe MitoTracker-green before mitochondrial fractionation. Intact mitochondria were obtained from cardiac myocytes by differential centrifugation. As shown in Fig. 1A, a small portion of PKCε was localized in mitochondria that were identified by MitoTracker-green. However, adenosine pretreatment significantly increased mitochondrial localization of PKCε, as evidenced by increased yellow punctate staining. The adenosine-induced increase in mitochondrial PKCε was largely blocked by PKC inhibitor chelerythrine. The quantification analysis (Fig. 1B) revealed that the percent of PKCε-positive mitochondria, which was normalized to total mitochondria, was significantly increased by adenosine treatment (72.3 ± 7.6% vs. 21.1 ± 4.5%, adenosine vs. control, $p<0.01$) and this effect was largely eliminated by DPCPX (31.5 ± 6.4% vs. 72.3 ± 7.6%, adenosine plus DPCPX vs. adenosine, $p<0.01$). The adenosine effect on PKCε was also prevented by chelerythrine (39.8 ± 6.69% vs. 72.3 ± 7.6%, adenosine plus chelerythrine vs. adenosine, $p<0.01$). The data were collected from 4 to 6 independent experiments. Fig. 1B (right panel) shows that PKCδ was not detected in mitochondria from cells with or without adenosine treatment.

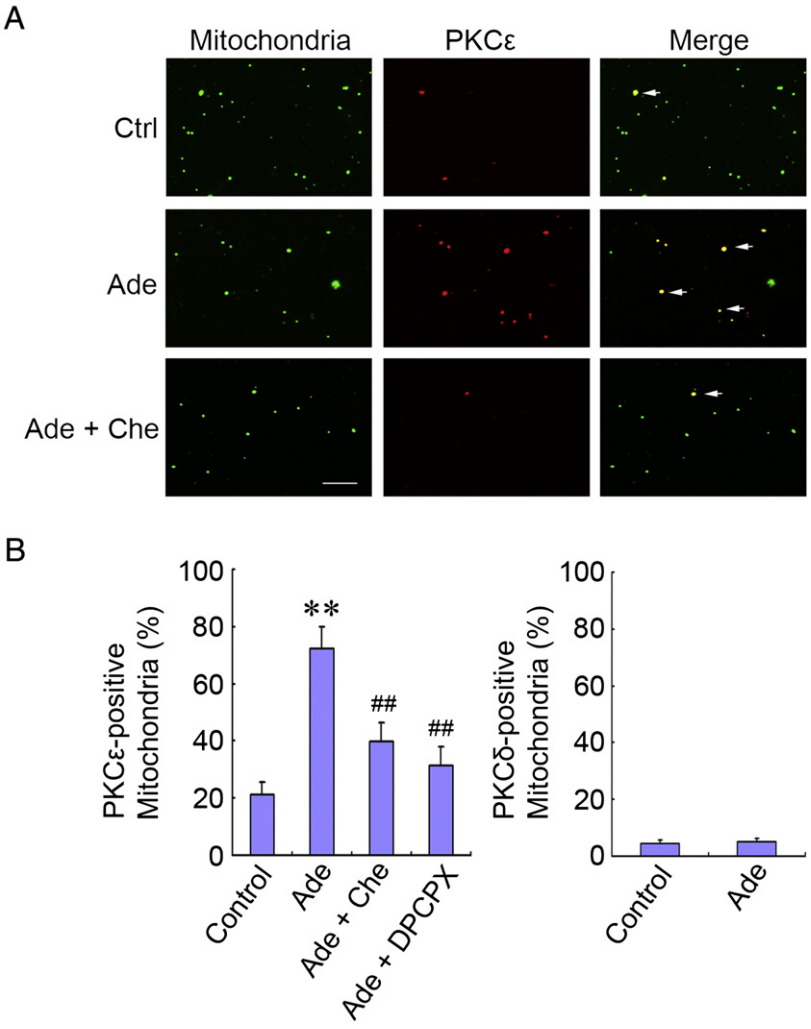

Fig. 1. Effect of adenosine on mitochondrial localization of PKCε. Isolated mitochondria were prepared from adult rat cardiac myocytes treated without or with adenosine (Ade), Ade + chelerythrine (Che) or Ade + 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX). (A) Representative immunofluorescence images of isolated mitochondria double labeled with anti-PKCε antibody and MitoTracker Green. Arrows indicate PKCε-positive mitochondria. Scale bar: 10 μm. (B) Percentage of mitochondria with PKCε-positive or PKCδ-positive fluorescence. Results are data from 4 to 6 independent experiments. $^{**}p<0.01$ vs. control, $^{\#\#}p<0.01$ vs. adenosine.

We also examined other major PKC isoforms including PKCα, β1, and β2 in the rat cardiac myocytes. We found that none of them was significantly present in mitochondria in response to adenosine stimulation (data not shown). These results suggest that PKCε is present in mitochondria at a relatively low level under control condition, but can be increased by adenosine-induced PKC activation.

3.2. Effect of adenosine on translocation of PKCε from the cytosol to mitochondrial fraction

To further confirm the effect of adenosine on mitochondrial translocation of PKCε, immunoblot analysis was carried out for percoll-purified mitochondrial fraction prepared from adult rat cardiomyocytes pretreated without or with adenosine, adenosine plus chelerythrine, or adenosine plus bisindolylmaleimide (30 nM) for 5–10 min. Enrichment of mitochondria was identified by antibody against prohibitin, a molecular marker of mitochondria. Immunoblots with antibody against Na^+/K^+-ATPase, a plasma membrane protein marker revealed no significant contamination of mitochondrial or cytosolic fraction with plasma membrane. GAPDH and prohibitin also serve as loading control for cytosol and mitochondrial fractions, respectively. Western blot with antibody against PKCε revealed a prominent protein band in mitochondrial fraction whereas antibody against PKCδ detected a faint band in mitochondria (Fig. 2A). Adenosine treatment induced a significant redistribution of PKCε but not PKCδ from the cytosol to mitochondria. The mitochondria to cytosol ratios of PKCε increased about 2.5 fold in adenosine-treated cells when compared to control group (Fig. 2B, 274.4 ± 92.1% vs. control, $p<0.05$, $n=3$). The PKC inhibitor chelerythrine blocked the effect of adenosine-induced translocation of PKCε to mitochondria (166.7 ± 16.48% vs. 274.4 ± 92.1%, adenosine plus chelerythrine vs. adenosine, $p<0.05$, $n=3$). Similar results were also obtained with a highly selective PKC inhibitor bisindolylmaleimide (115.7 ± 8.1% vs. 202.5 ± 37.4%, adenosine plus bisindolylmaleimide vs. adenosine, $p<0.05$, $n=3$). In contrast, no significant amount of PKCδ was redistributed to mitochondria in response to adenosine treatment. These results indicate that adenosine induces translocation of PKCε to mitochondria from cytosol via activation of PKC.

3.3. Effect of inhibiting HSP90 activity on adenosine-mediated mitochondrial localization of PKCε

HSP90 has been linked to cardioprotection of ischemic preconditioning and mitochondrial import [31]. We sought to determine

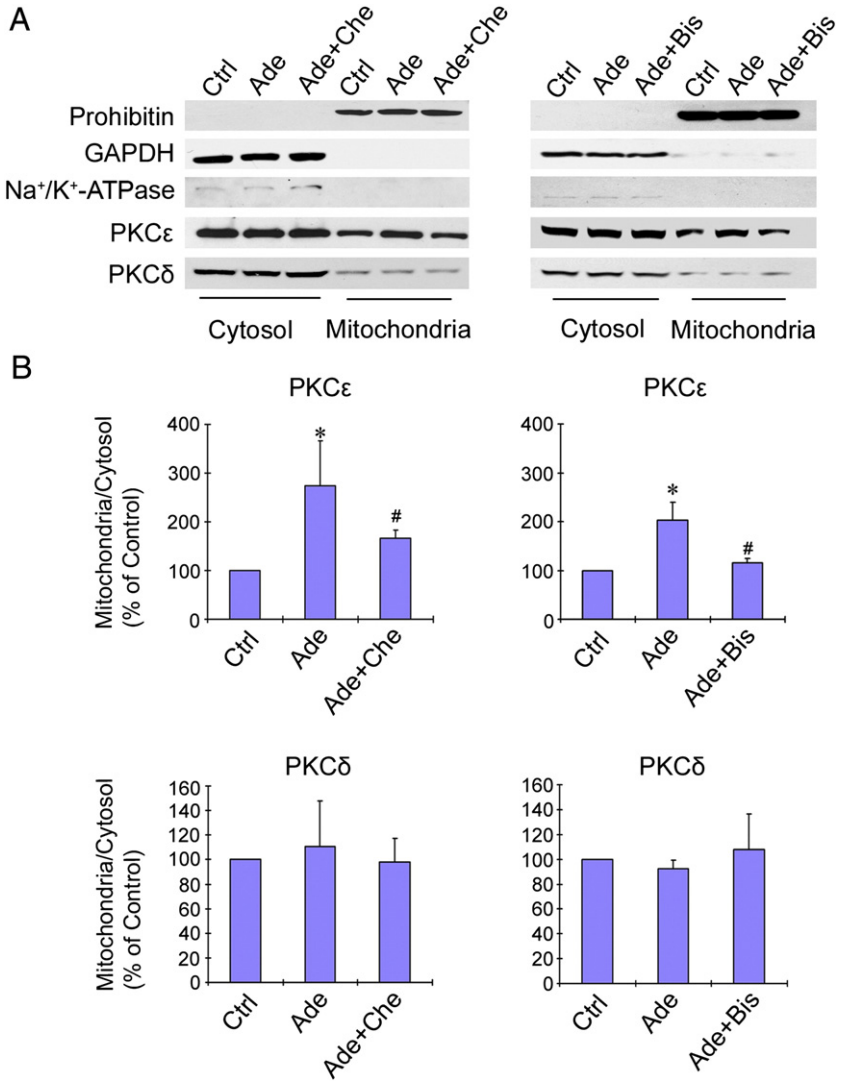

Fig. 2. Effect of adenosine on translocation of PKCε to mitochondria from cytosol. Subcellular fractionation was performed from adult rat cardiac myocytes which were treated without or with adenosine (Ade), or Ade + chelerythrine (Che) or bisindolylmaleimide (Bis). (A) Representative Western blots with antibody against PKCε or PKCδ. (B) Mitochondria to cytosol ratio as indexes of PKCε or PKCδ translocation to mitochondrial from cytosol. They were calculated by relative densitometry and normalized to control. Results are data from 3 independent experiments. *$p<0.05$ vs. control, #$p<0.05$ vs. adenosine.

whether HSP90 is involved in adenosine-induced mitochondrial translocation of PKCε. Cardiomyocytes freshly isolated from adult rat hearts were pretreated with or without adenosine or adenosine plus a selective HSP90 inhibitor geldanamycin (1 μM) for 5–10 min before mitochondrial fractionation. As shown in Fig. 3, geldanamycin significantly inhibited adenosine-induced increase in mitochondrial PKCε, as indicated by reduction in PKCε-positive mitochondria. Quantitative data showed that adenosine induced about 3-fold increase in PKCε-positive mitochondria which was significantly eliminated by geldanamycin (38.2 ± 7.4% vs. 72.3 ± 7.6%, adenosine plus geldanamycin vs. adenosine, $p<0.01$). These data indicate that adenosine increases translocation of PKCε to mitochondria, possibly through enhancing HSP90 activity.

3.4. Effect of suppression of HSP90 with siRNA on adenosine-mediated mitochondrial localization of PKCε

In order to confirm the specific role of HSP90 on adenosine-induced mitochondrial translocation of PKCε, we studied the effect of suppressing endogenous HSP90 with siRNA targeting HSP90 (α and β isoforms) in H9c2 cells. While H9c2 cells are myoblast cell line derived from embryonic rat heart, these cells have been used extensively for studies involving cardioprotection, heat shock proteins and protein kinases. We employed H9c2 cells because they display many properties similar to those in adult cardiomyocytes and are easily transfected [25,32]. As shown in Fig. 4, transfection of H9c2 cells with siRNA targeting HSP90 significantly reduced the expression of endogenous HSP90. Similar to that in cardiac myocytes, adenosine pretreatment in H9c2 cells increased the mitochondrial targeting of PKCε (53.6 ± 4.7% vs. 14.5 ± 2.1%, adenosine vs. control, $p<0.01$), and the effect was largely prevented by pretreatment with chelerythrine (21.6 ± 5.9% vs. 53.6 ± 4.7%, adenosine plus chelerythrine vs. adenosine, $p<0.01$). Interestingly, suppression of HSP90 with siRNA significantly prevented adenosine-induced translocation of PKCε to mitochondria (23.60 ± 7.21% vs. 53.6 ± 4.7%, adenosine plus HSP90-siRNA vs. adenosine, $p<0.01$), while the negative scrambled siRNA did not have any effect on adenosine-induced increase in PKCε-positive mitochondria (Fig. 4A and C,). The adenosine-induced increase in mitochondrial PKCε was also inhibited by DPCPX (Fig. 4C, 20.8 ± 4.2% vs. 53.6 ± 4.7%, adenosine plus DPCPX vs. adenosine, $p<0.01$). Fig. 4B showed significant suppression of HSP90 expression with siRNA targeting HSP90 (α and β). The data were analyzed from 4 to 6

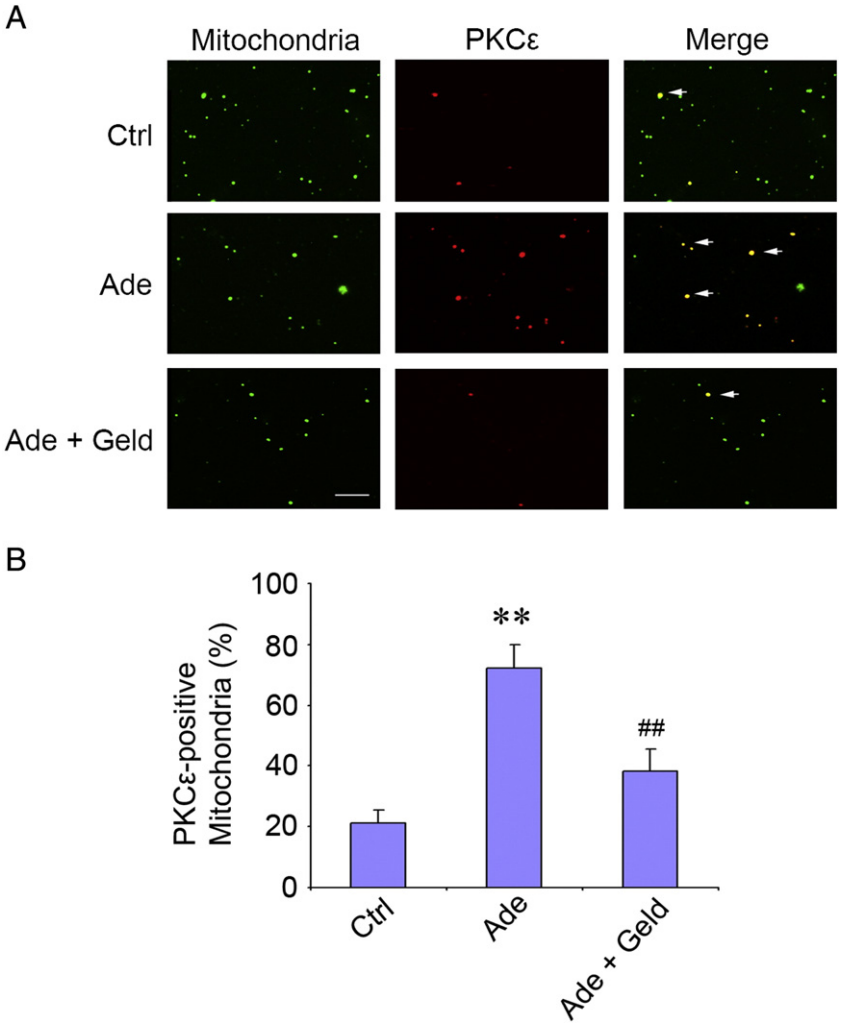

Fig. 3. Effect of inhibiting HSP90 activity on adenosine-induced increase in mitochondrial PKCε. Isolated mitochondria were prepared from adult rat cardiac myocytes treated without or with adenosine (Ade), or Ade + geldanamycin (Geld). (A) Representative immunofluorescence images of isolated mitochondria double labeled with anti-PKCε antibody and MitoTracker Green. Arrows indicate PKCε-positive mitochondria. Scale bar: 10 μm. (B) Percentage of mitochondria with PKCε-positive fluorescence. Results are data from 4 to 6 independent experiments. $^{**}P<0.01$ vs. control, $^{\#\#}p<0.01$ vs. adenosine.

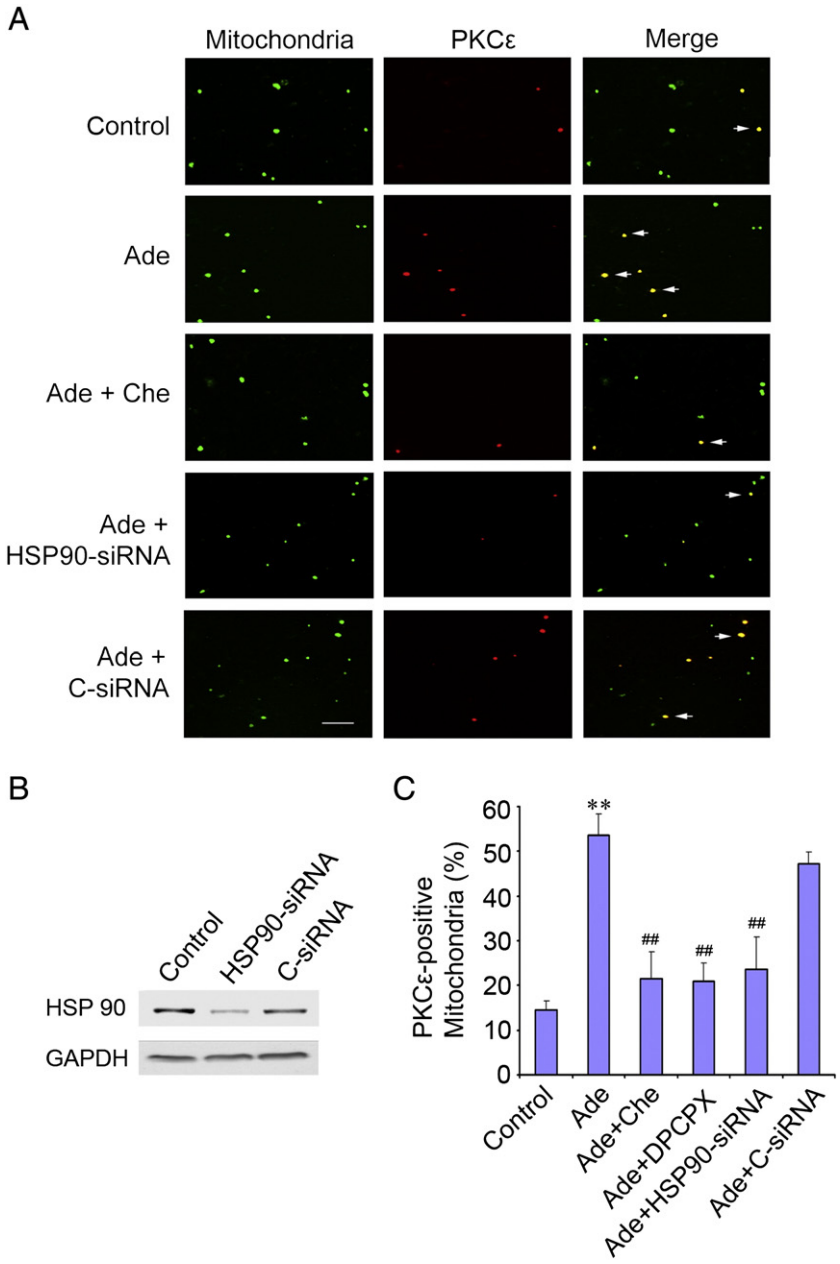

Fig. 4. Effect of suppressing HSP90 protein expression with HSP90 siRNA on adenosine-induced increase in mitochondrial PKCε. Mitochondria were prepared from rat heart-derived H9c2 cells without or with the treatment of adenosine (Ade), Ade + chelerythrine (Che), Ade + HSP90-siRNA, or Ade + control siRNA (C-siRNA). (A) Representative immunofluorescence images of isolated mitochondria double labeled with anti-PKCε antibody and MitoTracker Green. Arrows indicate PKCε-positive mitochondria. Scale bar: 10 μm. (B) Western blot of total cellular fraction from H9c2 cells transfected with HSP90-siRNA or C-siRNA. (C) Percentage of mitochondria with PKCε-positive fluorescence. Results are data from 4 to 6 independent experiments. $^{**}P<0.01$ vs. control, $^{\#\#}p<0.01$ vs. adenosine.

independent experiments. These results further demonstrated that HSP90 is involved in adenosine-induced translocation of PKCε to mitochondria.

3.5. Effect of adenosine on co-immunoprecipitation of PKCε, HSP90 and TOM70

To determine whether PKCε associates with HSP90, HSP70 or/and TOM70 and whether this association can be up-regulated by adenosine, we performed co-immunoprecipitation in the mitochondrial fraction prepared from cardiomyocytes pretreated with or without adenosine (10 μM). The total cellular HSP90 or HSP70 was not altered by adenosine treatment (data not shown). When the mitochondrial homogenates were immunoprecipitated with anti-HSP90, anti-PKCε or anti-TOM70 antibody, the immunoblotting revealed that HSP90, PKCε and TOM70 were associated with each other. In contrast, no significant amount of PKCδ or HSP70 was detected in the HSP90, TOM70 or PKCε precipitates (Fig. 5A). Pretreatment with adenosine significantly increased PKCε co-precipitated by anti-HSP90 antibody (214.3 ± 56.3% vs. control, $p<0.05$) or anti-TOM70 antibody (228.3 ± 30.8% vs. control, $p<0.05$). Similarly, adenosine also increased TOM70 level co-precipitated by anti-HSP90 (195.9 ± 17.8% vs. control, $p<0.05$) or anti-PKCε (215.8 ± 36.7% vs. control, $p<0.05$), and increased HSP90 co-precipitated by anti-PKCε (183.3 ± 4.3% vs. control, $p<0.05$) or anti-TOM70 antibody (187.9 ± 17.6% vs. control, $p<0.05$). However, HSP70 level co-precipitated by anti-PKCε, anti-HSP90 or anti-TOM70 antibody was not increased significantly by adenosine. Adenosine did not significantly alter the recovery of HSP90, PKCε or TOM70 in the HSP90, PKCε and TOM70 precipitates, respectively. As a control, HSP90, PKCε or TOM70 was not detected in the homogenates treated with control

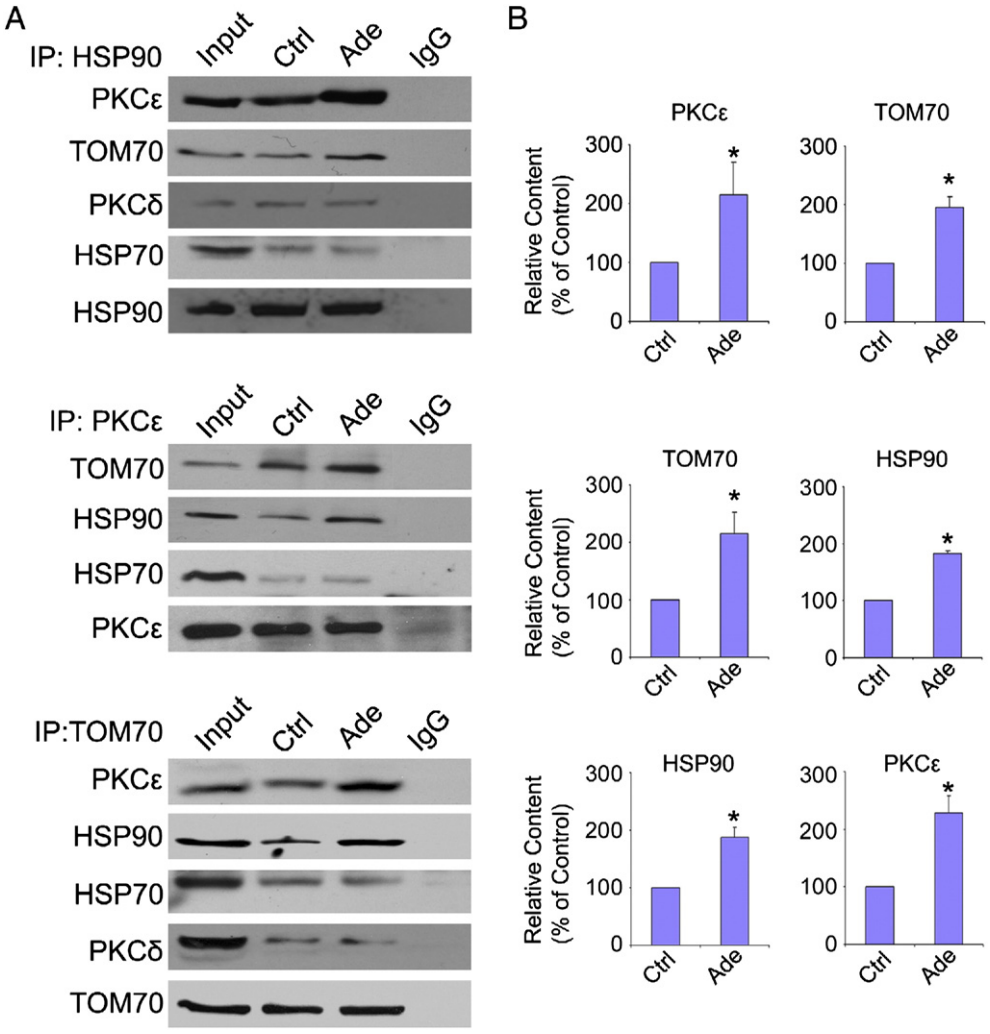

Fig. 5. Co-immunoprecipitation of HSP90, PKCε and TOM70 from adult cardiac myocytes. (A) Immunoprecipitation was performed with anti-HSP90, anti-PKCε or anti-TOM70 antibody from mitochondrial fraction or protein lysate (Input) of cardiac myocytes treated without or with adenosine (Ade). (B) Quantification of co-precipitated HSP90, PKCε and TOM70. Results are data from 3 independent experiments. *P<0.05 vs. control.

antibody IgG. These results indicate that pretreatment with adenosine for 5–10 min did not significantly increase HSP90 expression level but promoted association of HSP90, TOM70 and PKCε.

3.6. Effect of suppressing HSP90 expression on association of PKCε and TOM70

To determine whether adenosine-mediated association of PKCε with TOM70 is dependent on HSP90, we performed co-immunoprecipitation using the mitochondrial fraction prepared from H9c2 cells pretreated with or without adenosine (10 μM). Our data showed that TOM70 was co-precipitated by PKCε under the basal condition and this association was enhanced by adenosine treatment (Fig. 6, 186.6 ± 16.7% vs. control, $p<0.05$). However, suppression of HSP90 with siRNA significantly inhibited adenosine-induced association of PKCε with TOM70 (97.6 ± 16.2% vs. 186.6 ± 16.7%, adenosine plus HSP90-siRNA vs. adenosine, $p<0.05$). The basal level of association of PKCε and TOM70 was not altered by HSP90-siRNA, indicating the importance of adenosine-induced increase in HSP90 activity for association of PKCε with TOM70. As a control, TOM70 was not detected in the homogenates treated with control antibody IgG. These results indicate that pretreatment with adenosine for 5–10 min significantly enhanced association of PKCε with TOM70; the effect was prevented by suppression of HSP90 expression.

4. Discussion

Our study has demonstrated that adenosine promotes translocation of a novel PKC isoform, PKCε, to mitochondria. We found that adenosine receptor activation induced a rapid association of PKCε with TOM70, which is dependent on HSP90. Specifically, we showed that activation of adenosine receptors induced a selective translocation of PKCε (but not PKCδ) from the cytosol to mitochondria. We also showed that adenosine-mediated PKCε translocation to mitochondria was significantly reduced by inhibiting HSP90 function or suppressing HSP90 expression. Further, our data indicate that HSP90 is critical for association of PKCε and TOM70. We demonstrate for the first time that adenosine receptor activation induces selective translocation of PKCε to mitochondria. This translocation process is associated with the mitochondrial import machinery TOM70 and is dependent on HSP90 function.

It is well known that upon stimulation, PKC isoforms are translocated to their distinct subcellular membrane regions where the activated isoforms are anchored close to their particular substrates [33]. Studies have shown that PKCε is present in the mitochondrial inner membrane and interacts with mitochondrial components, such as the pore component of cardiac mitochondrial permeability transition pore [4,10]. Phosphorylation by PKCε enhances the activity of cytochrome c oxidase subunit IV [11]. Although numerous evidence suggests that adenosine receptors are linked to PKC activation [16,34],

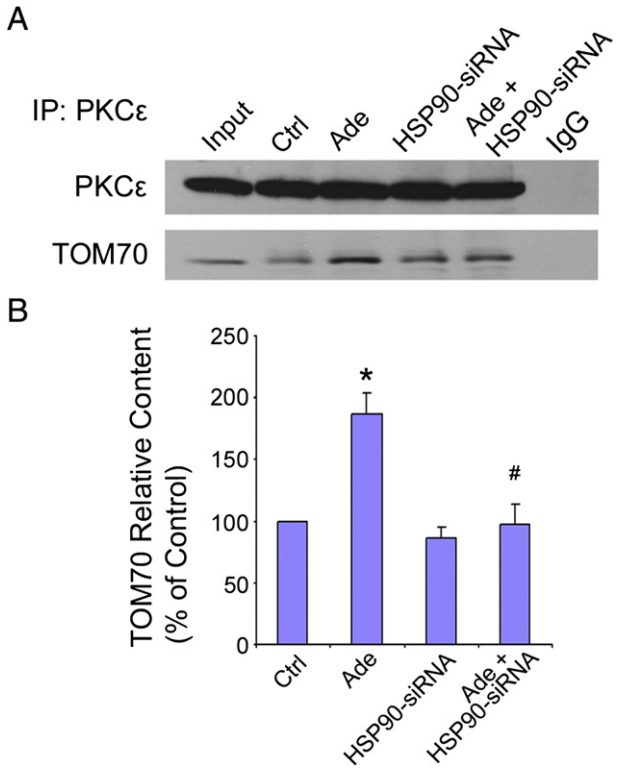

Fig. 6. Effect of HSP90 siRNA on co-precipitation of PKCε and TOM70. (A) Immunoprecipitations of mitochondrial fractions from H9c2 cells treated without or with adenosine (Ade), HSP90-siRNA, or Ade + HSP90-siRNA. (B) Quantification of TOM70 co-precipitated by PKCε. Results are data from 3 independent experiments. *$P<0.05$ vs. control, #$p<0.05$ vs. adenosine.

no study has directly addressed translocation of PKC isoforms to mitochondria after activation of adenosine receptors. In the present study, experiments were designed to determine whether PKCε is indeed localized to mitochondria under basal condition and whether the mitochondrial localization of PKCε is regulated by adenosine. Specifically, we examined whether adenosine induced translocation of PKCε from cytosol to mitochondria. We found that under basal conditions, the level of PKCε in mitochondria was relatively low but enhanced significantly following adenosine stimulation. In contrast, no significant amount of PKCδ was detected in mitochondria with or without adenosine. These observations provide the novel evidence that adenosine induces selective translocation of PKCε to mitochondria.

Although many studies have reported PKCε localized to mitochondria [4,10], the existence of PKCε in mitochondria is still puzzling, in particular on the mitochondrial inner membrane. It is well known that many proteins translocated into mitochondria possess N-terminal mitochondrial targeting sequence. Translocases in mitochondria recognize the signal sequence and then mediate the import and sorting of proteins. However, no mitochondrial targeting sequence in PKCε and other PKC isoforms has been found. It is therefore not clear how PKCε gets into mitochondria. It is generally believed that mitochondrial import machinery TOM20 recognizes classical N-terminal mitochondrial targeting sequences whereas TOM70 interacts with internal targeting sequences such as those in the multitransmembrane carrier proteins of the inner mitochondrial membrane [35–37]. It is noted that some proteins lack the N-terminal mitochondrial targeting sequence but are targeted to mitochondrial inner membrane [20]. The present study shows that adenosine promotes association of PKCε with the mitochondrial import machinery TOM70, indicating the importance of mitochondrial import in PKCε translocation to mitochondria. Furthermore, the translocation process appears to be dependent on HSP90, a protein that has been shown to assist with mitochondrial import [31]. Our data demonstrate that suppressing HSP90 expression with specific siRNA prevented adenosine-induced association of PKCε and TOM70, indicating that both HSP90 and TOM70 are important for mitochondrial translocation of PKCε. It has been shown that some components of the mitochondrial import machinery are down-regulated during ischemia but preserved by IPC [22], suggesting that mitochondrial import could be a novel mechanism by which PKCε confers cardioprotection [31]. Indeed, a recent study by Budas and colleagues indicates that HSP90-mediated mitochondrial import of PKCε plays an important role in the protection of the myocardium from ischemia and reperfusion injury [38].

In addition to translocation to mitochondria, PKC has been shown to translocate to the plasma membrane, nucleus and other subcellular organelles upon activation. We have recently discovered that adenosine A1 receptor activation induces PKCε and PKCδ translocation to the caveolar plasma membrane [19]. Here, we showed that adenosine receptor activation also caused PKCε translocation of mitochondria. Although different receptor subtypes and their signaling may be associated with differential translocation of PKC isoforms, our observations suggest that adenosine receptor signaling can potentially influence various subcellular localizations of different PKC isoforms and may serve distinct roles in a variety of cellular events. There is growing evidence that adenosine contributes significant cardioprotection via adenosine receptor subtypes and their distinct signal transduction pathways, such as PKC isoforms and their down-stream signaling including mitogen-activated protein kinase and PI3 kinase/protein kinase B [39]. While it is well-known that adenosine A1 receptors are associated with PKC activation and translocation to the plasma membrane via PLC/PLD, recent reports indicate that adenosine A1 receptors-induced PKC activation may further facilitate adenosine A2b receptor signaling which then regulates survival kinases including PI3 kinase and ERK [39–41]. Because PI3 kinase has also been shown to activate PKC either via direct activation by lipid products or via phosphorylation by PDK1 [42], it is likely that adenosine receptors may cause PKC activation and targeting to distinct subcellular localizations such as mitochondria via adenosine A2b receptor-dependent signaling pathways. A recent study indicates that A2b receptors are present in or near mitochondria rather than on the sarcolemma, suggesting the possible involvement of mitochondria in adenosine A2b receptors-mediated signaling [43].

Our finding indicates a novel mechanism involved in the adenosine-mediated PKC signaling that is dependent on mitochondrial import machineries. Adenosine receptor signaling has been associated with cardioprotection against ischemic/reperfusion injury [15]. Given that most of mitochondrial proteins are imported from cytosol via translocons on the mitochondrial membranes, the dynamic regulation of protein import to mitochondria by adenosine signaling could be a novel mechanism mediating cardioprotection. The number of PKC substrates in mitochondria has been proposed, such as aldehyde dehydrogenase 2, cytochrome c oxidase subunit IV, mitochondrial permeability transition pore, and mitochondrial ATP-sensitive K+ channels [4]. The present study provides a detailed understanding of the molecular mechanism underlying adenosine-mediated mitochondrial targeting of PKC isoforms, which increase our knowledge about adenosine-mediated cardioprotection against ischemic injury, and may lead to identification of potential therapeutic targets.

In summary, we demonstrate that adenosine receptor activation induces translocation of PKCε to mitochondria, and this process is associated with TOM70 and dependent on HSP90. Our observations may have important implication in cardioprotection associated with adenosine receptor signaling.

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