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Protein kinase C signaling pathway involvement in cardioprotection during isoflurane pretreatment

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Abstract. The well-known cardioprotective effect of isoflurane, a type of volatile anesthetic, against myocardial ischemia/reperfusion (I/R) injury has become an important focus in cardiovascular research. During reperfusion numerous oxidants, such as H_2O_2, are produced. Aldehyde dehydrogenase 2 (ALDH2) is a protective factor in myocardial I/R, and once phosphorylated and activated ALDH2 may confer cardioprotection. The present study investigated whether cardioprotection by isoflurane depends on the activation of ALDH2 and aimed to determine how protein kinase C (PKC)δ is involved in isoflurane-induced cardioprotection. Anaesthetized rats were used to produce I/R injury models by imposing 40 min of coronary artery occlusion followed by 120 min of reperfusion. The animals were assigned randomly to the following groups: Untreated controls, and isoflurane preconditioning with and without the PKCδ inhibitor. I/R injury was estimated by the activity of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB). Isoflurane pretreatment was observed to attenuate the release of LDH and CK-MB, and enhance the phosphorylation of ALDH2. Activation of ALDH2 and cardioprotection induced by isoflurane preconditioning were enhanced by a PKCδ inhibitor. The results suggest that the activation of ALDH2 by the inhibition of the mitochondrial translocation of PKCδ is important in the protection of the myocardium from I/R injury, and that the effect of PKCδ on isoflurane preconditioning is directly opposed to that of PKCε. PKCε activation was involved in isoflurane pretreatment, which consequently activated downstream signaling pathways and aided cardioprotection. Isoflurane pretreatment also led to attenuated mitochondrial translocation of PKCδ.

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Introduction

Millions of people succumb to acute myocardial infarction (AMI) each year (1), thus AMI makes a significant contribution to the global burden of disease. In the past few decades, it was identified that experiencing short periods of myocardial ischemia and reperfusion (I/R), prior to restoration of full coronary reperfusion, has a protective effect on cardiomyocytes against subsequent prolonged I/R injury; a phenomenon termed 'ischemic preconditioning' (IPC) (2). Further studies have shown that volatile anesthetics, such as isoflurane, can simulate the effect of IPC. When administered prior to a period of myocardial I/R, volatile anesthetics induce cardioprotective effects, which are referred to as 'anesthetic preconditioning' (APC) (3,4). APC can lead to increased resistance of cardiomyocytes against (I/R) injury by eliciting endogenous protective mechanisms. This was observed in various animal models, as well as in humans (3-8). In contrast to IPC, APC may not cause a reduction in blood flow, thus it exhibits greater ethical acceptability and clinical safety.

Protein kinase C (PKC)ε activation is required to protect the heart from (I/R) injury (9,10). Recent evidence suggests that PKCε is targeted to the mitochondria and interacts with numerous mitochondrial proteins, including mitochondrial aldehyde dehydrogenase 2 (ALDH2) (11). The mitochondrial isoform of ALDH2 is key in the metabolism of acetaldehyde and other toxic aldehydes, and phosphorylation and activation by PKCε are required to confer cardioprotection (10,11). Overexpression of ALDH2 alleviates I/R injury, post-I/R injury and ischemic ventricular dysfunction (12,13). Consistent with this, ALDH2 expression was downregulated during cardiomyocyte hypoxia (14), and ALDH2 knockout exacerbated the I/R injury (15). These data support the essential role of ALDH2 in the protection against I/R injury in the heart. The mechanisms underlying ALDH2-induced protection against I/R injury are likely to be various and diverse, involving bioactivation of nitroglycerin; decreasing the production of free radicals (16) and the formation of 4-hydroxy-2-nonenal (HNE)-protein adducts (17); the activation of c-Jun N-terminal kinases 1/2 and extracellular signal-regulated kinases 1/2 (18); and mitochondrial dysfunction (19-24), which are all hallmarks of I/R injury. It is suggested that during I/R injury, the overall levels of PKCε and PKCδ are regulated by the proteasome, a multi-subunit

complex found predominantly in the cytosol of mammalian cells, which can result in the degradation of PKCδ (22,25). The proteasome regulates the ratio of pro-apoptotic PKCδ to pro-survival PKCε in the mitochondria, and thus determines the ultimate fate of the cell and may be viewed as an indicator of cellular viability (22). Uecker *et al* (11) reported that PKCδ was translocated exclusively to the mitochondria in response to isoflurane treatment, rather than the cell membrane, suggesting the importance of this isoform in mitochondrial adenosine triphosphate (ATP)-dependent potassium channel-mediated cardiac protection by isoflurane. However, a recent report by Xu *et al* (39) has shown that APC increased the levels of PKCε and PKCδ in the cell membrane, and decreased the levels in the cytosol. The role of PKCδ in APC and the mechanism conferring cardioprotection have not yet been elucidated. Therefore, the present study aimed to investigate the role of PKCδ in APC and its underlying mechanism of action.

Materials and methods

Animals. The present study was approved by the Ethics Committee of Shanxi Medical University (Taiyuan, China). Male Sprague-Dawley (SD) rats, weighing 200-220 g, were used in this study. The animals were provided by The Experimental Animal Center of Tsinghua University (Beijing, China). The rats were placed in a quiet, temperature- (23±3˚C) and humidity- (60±5%) controlled room, with a 12/12 h light-dark cycle (light beginning at 0800). Rats had free access to a standard diet and drinking water. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the China National Institutes of Health.

In vivo I/R injury experimental protocol. The acute myocardial I/R injury model was performed by left anterior descending (LAD) coronary artery ligation. Male SD rats were anesthetized by intraperitoneal administration of 30 mg/kg pentobarbital sodium. After a tracheotomy had been performed, rat lungs were ventilated mechanically with positive pressure ventilation using a 30-40% air/oxygen mixture to maintain arterial blood gas pH within a physiological range by adjusting the respiratory rate and tidal volume throughout the experiment. Myocardial infarction (MI) was caused by ligation of the LAD coronary artery. Briefly, the thorax was opened at the fourth or fifth left intercostal space. After left thoracotomy and pericardiotomy, MI was induced by LAD ligation 2-3 mm from the origin with a 6-0 silk suture (Hairmer Co., Xi'an, China). All animals (except for the rats in the sham groups) were subjected to 40 min of regional myocardial ischemia followed by 120 min of reperfusion (26). To confirm isoflurane-induced APC, a minimal alveolar concentration of isoflurane of 1.0 (2.1%) was administered at the end of the stabilization period for 30 min, followed by 30 min of washout with oxygen prior to coronary occlusion.

Rats were randomly assigned to the following groups (n=8 per group): Sham group, a non-ischemic control group of sham-operated rats without isoflurane pretreatment (chest walls were opened without ligating the LAD coronary artery for 160 min); non-ischemic control group comprising sham-operated rats pretreated with isoflurane; I/R group (40 min of myocardial ischemia and 120 min of reperfusion) without isoflurane pretreatment; and I/R group with isoflurane pretreatment. To evaluate the role of PKCδ in phosphorylation of ALDH2 and in isoflurane-induced APC, a direct inhibitor of PKCδ, rottlerin (1 μM), was administered 5 min prior to ischemia with and without isoflurane to subgroups of the rats.

Analyzing the activity of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) in plasma. Serum CK-MB analysis is a widely used biomarker to detect cardiac injury. Proportionally greater serum CK-MB relative to the total CK activity can evaluate acute myocardial injury. At the end of the reperfusion period, 5 ml blood samples were taken. Serum was separated by centrifugation at 5,000 x g for 5 min on a tabletop centrifuge and the supernatant was stored in liquid nitrogen. The samples were thawed for analysis. LDH and CK-MB were assayed using LDH and CK-MB commercially available kits (Roche, Manheim, Germany), respectively, by an automatic analyzer 7600 (Hitachi, Tokyo, Japan).

Preparation of whole cell extracts from myocardium and western blot analysis. To identify the effect of isoflurane preconditioning on PKCδ activation and translocation, mito-PKCδ and total-PKCδ expression in all groups was measured (Sham, I/R, Sham+isoflurane and I/R+isoflurane groups) by western blot analysis. The effect of rottlerin on signal pathway proteins (phos-ALDH2 and total-ALDH2) was also assayed by western blot analysis.

Upon completion of the experimental period, the myocardium and cardiomyocytes were lysed in ice-cold radioimmunoprecipitation assay lysis buffer containing 1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin at 4˚C for 15 min. The homogenate was incubated and centrifuged at 5,000 x g for 5 min at 4˚C. The supernatant was collected and the protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA) according to the manufacturer's instructions. The detergent soluble supernatant was frozen with liquid N_2 and stored at -70˚C.

The supernatant was mixed with 5X loading buffer and heated for 5 min at 100˚C. Soluble extracts (50 μg) were loaded in each lane and separated by SDS-polyacrylamide gel electrophoresis. Following electrophoresis, proteins were electrophoretically transferred to a polyvinylidene difluoride filter membrane (0.45 μm, GE Healthcare, Beijing, China). The membrane was blocked in Tris-buffered saline with Tween-20 (TBST) with 5% non-fat milk and incubated overnight with the corresponding primary antibodies at 4˚C. The following primary antibodies were used: Rabbit monoclonal anti-PKCδ and rabbit monoclonal PKCε (both Abcam, Cambridge, UK). The membrane was then incubated for 1 h with secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (Beyotime Institute of Biotechnology, Haimen, China) diluted with TBST (1:2,000). The signals of detected proteins were visualized by an enhanced chemiluminescence reaction system (Millipore, Billerica, MA, USA). The staining was quantified by scanning the films and the band density was

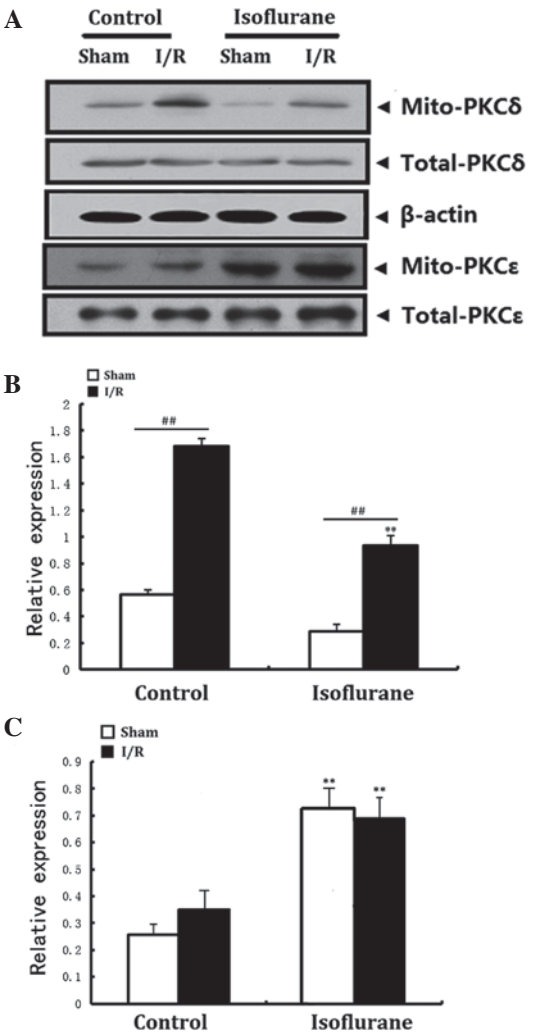

Figure 1. Increase in PKCε translocation and inhibition of PKCδ translocation to mitochondria triggered by isoflurane preconditioning. Values are presented as the mean ± standard error of the mean, n=8 per group. ##P<0.01 vs. the sham control group and **P<0.01 vs. the corresponding group without isoflurane pretreatment. (A) Representative western blot of mitochondrial PKCε (mito-PKCε)/mitochondrial PKCδ and total-PKCε/total-PKCδ in the sham group and the I/R group with or without isoflurane preconditioning. Isoflurane preconditioning enhanced translocation of PKCε and attenuated translocation of PKCδ in the sham and I/R groups. β-actin was used to demonstrate equal protein loading. (B) Quantification of the mitochondrial PKCδ level, normalized to the total PKCδ. (C) Quantification of the mitochondrial PKCε level, normalized to the total PKCε. PKC, protein kinase C; I/R, ischemia/reperfusion.

determined with Image-Pro software (Media Cybernetics, Inc., Rockville, MA, USA).

Statistical analysis. Continuous values are expressed as the mean ± standard error of the mean. Comparisons between multiple-group means were performed using one-way analysis of variance and comparisons between groups were performed using the least significant difference test and Student-Newman-Keuls test. The number of animals per group and statistical significance for all data are listed in the figures and figure legends. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Effect of isoflurane pretreatment on PKCδ activity and mitochondrial PKCδ levels. Regional myocardial ischemia for 40 min by LAD coronary artery ligation followed by 120 min of reperfusion led to a significant increase in PKCδ levels in the mitochondria of cardiomyocytes compared with the sham control group (P<0.01). Isoflurane markedly inhibited the I/R-induced mitochondrial translocation of PKCδ in cardiomyocytes, and significantly decreased the mitochondrial PKCδ concentration in cardiomyocytes (P<0.01 ; Fig. 1A and B). Data suggested that pretreatment with isoflurane decreased the dynamic mitochondrial translocation of PKCδ in response to I/R.

As shown in Fig. 1A, the decrease in the mitochondrial concentration of PKCδ in cardiomyocytes indicated the translocation of PKCδ from the mitochondria to the cytosol, and the corresponding increase in cytosolic PKCδ levels during isoflurane pretreatment, as the total cellular PKCδ levels remained constant.

Role of PKCδ in isoflurane-induced ALDH2 phosphorylation and cardioprotection. PKCδ is involved in isoflurane-induced ALDH2 phosphorylation and cardioprotection. PKCδ activation at the beginning of reperfusion mediates cardiomyocyte apoptosis and necrosis via mitochondrial regulation, whilst PKCδ inhibition alleviates the myocardial I/R injury (27). Decreased levels of PKCδ and elevated levels of phosphorylation of ALDH2 are required to protect the heart from I/R injury. Isoflurane-induced ALDH2 phosphorylation level decrease (P<0.01, Fig. 2A and B). LDH (P<0.05) and CK-MB (P<0.01) release (Fig. 2C and D) induced by I/R was mimicked by the PKCδ inhibitor, rottlerin. Western blot analysis showed that inhibition of PKCδ activity by rottlerin after I/R injury significantly enhanced the phosphorylation level of ALDH2 regardless of isoflurane preconditioning. Consequently, the inhibition of PKCδ was associated with ALDH2 activation and was observed to induce cardioprotection, demonstrated by decreased serum CK-MB and LDH activity *in vivo* (Fig. 2B-D). Similarly, isoflurane preconditioning inhibited PKCδ activity, thus inhibiting its mitochondrial translocation and reducing I/R-induced myocardial injury. A significant difference was observed between the group treated with rottlerin alone and the group that received co-treatment with rottlerin and isoflurane in terms of LDH and CK-MB release (P<0.01), which indicates a synergistic effect on decreasing the two biochemical indicators (Fig. 2C and D). However, no such effect on ALDH2 phosphorylation was observed (Fig. 2A and B).

Discussion

Clinically, volatile anesthetics have been in use for a considerable period of time. A number of studies, in agreement with the current study, have demonstrated the cardioprotective effects of volatile anesthetics applied before a deleterious ischemic event and at the beginning of reperfusion, which share common characteristics with IPC.

However, the rapid induction of pro-survival pathways sufficient to prevent damage immediately after the reperfusion

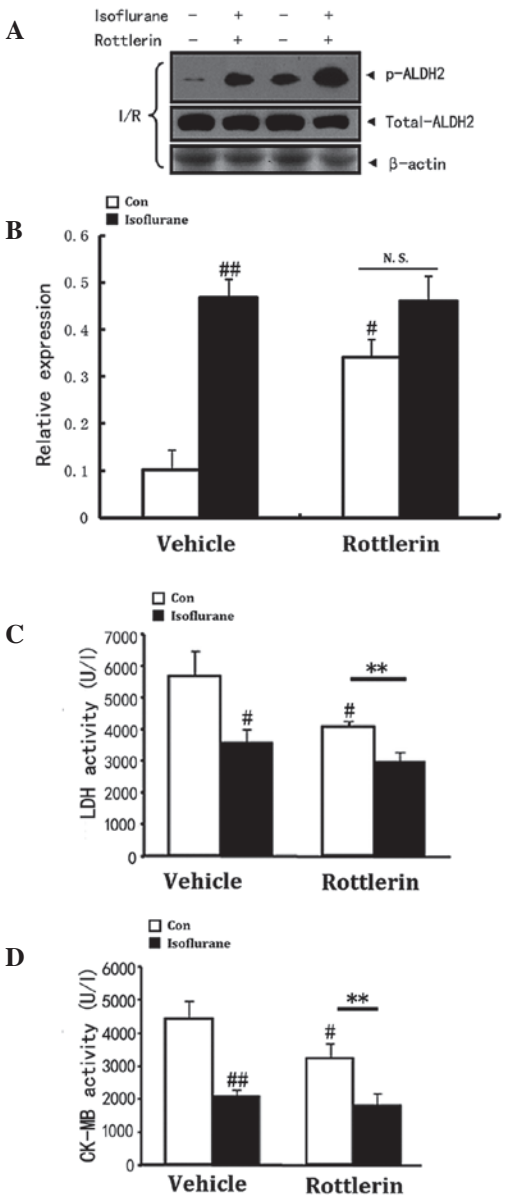

Figure 2. Decrease in PKCδ translocation to the mitochondria involves ALDH2 phosphorylation and cardioprotection induced by isoflurane. Values are presented as the mean ± standard error of the mean, n=8 per group. #P<0.05, ##P<0.01 vs. the vehicle control group and **P<0.01 vs. the corresponding control. (A and B) The roles of PKCδ in isoflurane preconditioning induced ALDH2 activation. Representative western blot of phospho-ALDH2, total ALDH2 after I/R injury. Inhibition of PKCδ significantly increased the phosphorylation of ALDH2 regardless of isoflurane preconditioning. β-actin was used to demonstrate equal protein loading. (C and D) Serum LDH and CK-MB concentrations were analyzed. Isoflurane inhibited LDH and CK-MB release induced by I/R, which was mimicked by rottlerin (a PKCδ inhibitor). Serum LDH and CK-MB concentrations were analyzed. There was no significant difference between the single rottlerin treatment group, and the rottlerin and isoflurane co-treatment group in ALDH2 relative expression, which suggested the effects of isoflurane and rottlerin were not synergistic. In terms of the LDH and CK-MB levels, there was a significant difference between the single rottlerin treatment group, and the rottlerin and isoflurane co-treatment group, indicating a synergistic effect of isoflurane and rottlerin. ALDH2, aldehyde dehydrogenase 2; PKC, protein kinase C; I/R, ischemia/reperfusion; LDH, lactate dehydrogenase; CK-MB, creatine kinase-MB.

event occurs, is likely to be difficult to achieve in practice. Recently, attention has paid to mitochondria as a target of volatile anesthetics when inducing cardioprotection (11,28,29).

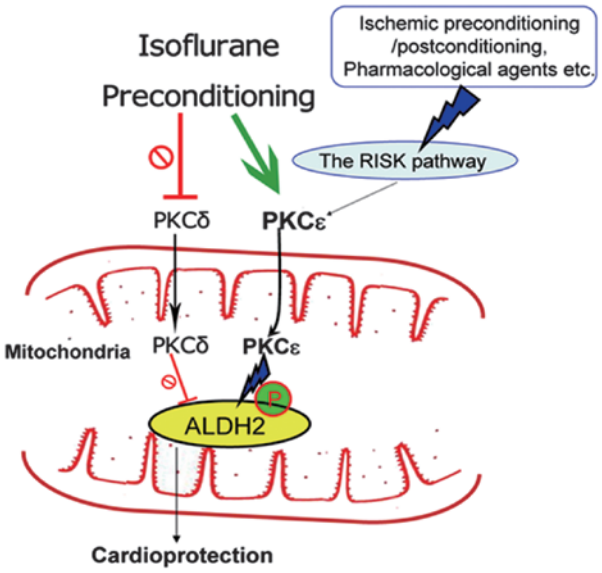

Figure 3. Hypothetical scheme demonstrating that phosphorylation of ALDH2, through increase in PKCε mitochondrial translocation and inhibition of PKCδ mitochondrial translocation, is important in the cardioprotection induced by isoflurane preconditioning in myocardial I/R injury. Ischemic preconditioning/postconditioning and pharmacological agents result in the activation of the RISK pathway, which leads to the phosphorylation and mitochondrial translocation of PKCε. By contrast, isoflurane treatment can suppress the translocation of PKCδ, which may be an inhibitor of ALDH2. These effects are responsible for cardioprotection. RISK, reperfusion injury signaling kinase; PKC, protein kinase C; I/R, ischaemia/reperfusion.

Mitochondria are fully involved in the pathways induced by volatile anesthetics, leading to the cardioprotective effects via production of ATP and regulation of cell death (30).

The mechanisms by which isoflurane ultimately limits infarct size are not known. Apoptosis (31-33) and inflammation (31,34) have been implicated in cardiac I/R injury. In agreement with our previous results (35), isoflurane-treated mice subjected to ischemia and 2 weeks of reperfusion showed lower expression of proapoptotic genes, significantly decreased expression of cleaved caspase-3 and significantly decreased TUNEL staining, as compared with the control group (5).

ALDH2 is best known for its role in metabolizing the ethanol intermediate acetaldehyde, which is a toxic aldehyde with such high activity that it can react with protein, forming aldehydic adducts and leading to protein dysfunction and tissue injury. It has been reported that overexpression of ALDH2 may alleviate I/R injury, post-I/R injury and ischemic ventricular dysfunction (36). Consistent with this, I/R injury may be exacerbated by ALDH2 knockout (37). These data support the results of the present study, which demonstrate that ALDH2 is essential in isoflurane-induced cardioprotection against I/R injury. It has been shown that overexpression of ALDH2 significantly attenuated acetaldehyde- and ethanol-induced oxidative stress (ROS generation), activation of stress signal molecules and apoptosis in fetal human cardiomyocytes (36).

There are various perspectives regarding the role of PKCδ in cardioprotection. The present results showed that during I/R injury, PKCδ translocates from the cytosol to the mitochondria, which suggests that PKCδ may mediate I/R injury by interacting with the mitochondria, consistent with previous studies

(38). However, no significant difference was identified in the total-PKCδ levels between the control and isoflurane groups. This seems to contradict the proteasome regulation mechanism suggested by Churchill *et al* (22). Furthermore, it was identified that following the inhibition of PKCδ, the level of ALDH2 phosphorylation is upregulated, which indicates that PKCδ is involved in the cardioprotective effect induced by isoflurane via the ALDH2 pathway. Thus in the PKC signaling pathway, the roles of PKCε and PKCδ are opposing. The former enhances the phosphorylation and activation of ALDH2, causing cardiac protection, while the latter attenuates the effect. This conclusion is in accordance with several previous studies (39).

In our previous studies it was observed that PKCε activation was involved in isoflurane pretreatment, which consequently activated downstream signaling pathways and aided cardioprotection (35). Our previous studies also identified an anti-apoptotic effect mediated by PKCε in isoflurane preconditioning, demonstrated by decreased caspase-3 activity and apoptotic cell number *in vitro*. This suggested that PKCε may exhibit a key role in apoptosis inhibition during cardioprotection (35). By contrast, the association between PKCδ and apoptosis has also been reported. PKCδ was shown to be activated by various apoptotic stimulants and further translocated to the mitochondria, the Golgi apparatus and the nucleus, causing multiple biological effects (40-42). Emoto *et al* (43,44) observed the cleavage of PKCδ during apoptosis into catalytic products by caspase-3 (45). Leverrier *et al* (46) reported that overexpression of PKCδ catalytic segments induced PARP cleavage, which activated caspase-3. It was further suggested that a positive feedback cycle between PKCδ and caspase-3 was involved in apoptosis, although the mechanisms remained unclear (46,47). The findings regarding the roles of PKCδ phosphorylation and translocation in I/R injury are in accordance with previous studies, thus it is plausible to suggest that the two subtypes of PKC (PKCε and PKCδ) have different roles in myocardial I/R following isoflurane pretreatment, effecting ALDH2 phosphorylation and mitochondrial translocation. This eventually leads to inhibition of the apoptotic signaling pathway, in which caspase-3 is involved, and thereby contributes to cardioprotection.

PKCε has been demonstrated to be a critical protein kinase in ALDH2 activation, whereas few studies have elucidated the role of PKCδ in regulating ALDH2. To the best of our knowledge, our previous study (35) showed for the first time that ALDH2 phosphorylation is the crucial step in the anti-apoptotic effect mediated by isoflurane pretreatment protecting against I/R injury in rat cardiomyocytes *in vivo*. ALDH2 was the key factor in the protective effect and ALDH2 inhibition eliminated the effect.

In conclusion, to the best of our knowledge the present study showed for the first time that isoflurane pretreatment resulted in significantly elevated mitochondrial levels of PKCε accompanied by phosphorylation of ALDH2, as well as attenuated mitochondrial translocation of PKCδ. The bi-directional regulation of the activation of the two PKC subtypes during I/R may further activate ALDH2 and strengthen resistance to I/R injury (Fig. 3). Thus, isoflurane preconditioning may activate the PKC (PKCε and PKCδ)-ALDH2 signaling pathway to induce protective effects against myocardial I/R injury. This study may facilitate the application of APC to induce cardioprotection in the clinical setting.

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