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Effect of mitochondrial ATP-sensitive potassium channel opening on the translocation of protein kinase C epsilon in adult rat ventricular myocytes

H. Li, T. Yang, Z. Long and J. Cheng

Department of Anesthesiology, Xinqiao Hospital,
The Third Military Medical University, Chongqing, China

Corresponding author: H. Li
E-mail: lhdoccn@yeah.net

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ABSTRACT. This study aimed to investigate the effects of mitochondrial ATP-sensitive potassium ($MitoK_{ATP}$) channel opening on the translocation of protein kinase C epsilon (PKCε). In addition, we aimed to determine the relationship between PKCε translocation and the production of reactive oxygen species (ROS). PKCε protein expression in cultured adult rat ventricular myocytes was investigated by immunofluorescence and Western blotting. Diazoxide (DZ), a selective $MitoK_{ATP}$ channel activator, caused a significant translocation to myofibrillar-like structures in cultured adult rat ventricular myocytes. *N*-2-Mercaptopropionylglycine, a free radical scavenger, could partially inhibit the translocation of PKCε induced by DZ. By contrast, chelerythrine, a selective PKC inhibitor, could completely block the translocation of PKCε induced by DZ. The opening of $MitoK_{ATP}$ channels might activate and cause PKCε to translocate into myofibrillar-like structures. PKCε activation occurred downstream of the $MitoK_{ATP}$ channel, possibly as a result of ROS production that

occurred after the MitoK$_{ATP}$ channels opened.

Key words: Mitochondrial ATP-sensitive potassium channel; Cardiomyocyte; Protein kinase C epsilon

INTRODUCTION

Ischemic preconditioning (IP) offers a strong endogenous protection against ischemia-reperfusion injury. Many studies have demonstrated that the opening of mitochondrial ATP-sensitive potassium (MitoK$_{ATP}$) channels functions as an end effector in cardioprotection by IP and is key a signaling trigger in the signal transduction pathways involved in IP (Obal et al., 2005; Kaneda et al., 2008; Maack et al., 2009). Protein kinase C epsilon (PKCε) is another important signaling molecule in IP. PKCε deficiency results in the loss of cardioprotection induced by IP or an α1 adrenergic receptor agonist (Gray et al., 2004; Yun et al., 2012). However, the function and mechanisms of PKCε in the opening of MitoK$_{ATP}$ channels remain unclear. In this study, we investigated the effects of diazoxide (DZ) pretreatment on PKCε translocation and the relationship between the translocation of PKCε and the production of reactive oxygen species (ROS). This investigation was performed with cultured adult rat ventricular myocytes utilizing various methods, including immunofluorescence, Western blotting, and other methods.

MATERIAL AND METHODS

Animals

Twenty healthy male Sprague-Dawley (SD) rats (weight = 180 to 250 g) were supplied by the Animal Center of Daping Hospital of the Third Military Medical University, China. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Third Military Medical University.

Isolation and identification of adult rat ventricular myocytes

Thoracotomy was performed after SD rats were anesthetized and disinfected as described previously (Gupta et al., 2005). The aorta was exposed and intubated from the distal end to the root in order to connect with the Langendorff apparatus. The heart was removed, rapidly mounted on the Langendorff apparatus, and perfused in a non-recirculating mode with cold Krebs-Henseleit buffer (perfusion pressure, 70 to 100 cm H_2O) that contained all of the components (except $CaCl_2$) for 5 min and bubbled with 95% O_2/5% CO_2. After the heart was perfused for 5 min, a mixture of collagenase (0.1%) and hyaluronidase (0.1%; Sigma-Aldrich Corp., St. Louis, MO, USA) was added to the buffer. The heart was perfused again in a recirculating mode for 30 min at 37°C. All of the heart components were then removed except for the ventricles. These ventricles were placed in a beaker that contained a calcium-free buffer and agitated with 0.25% trypsin solution in a shaking bath (37°C) for 5 min. The released cells

were purified by allowing them to settle naturally three times, and one-gradient centrifugation was performed in 6% bovine serum albumin. The cells were then resuspended in DMEM supplemented with 10% fetal bovine serum and cultured at 37°C in a humidified 5% CO_2-95% air atmosphere. The purified cells (85%) were rod shaped and identified as ventricular myocytes by α-actin antibody staining (98% purity; Santa Cruz Biotechnology Inc., CA, USA).

Experimental protocol

At 1 h after the cells were cultured, and randomly divided into 4 groups: Group A, control group; Group B, DZ (Sigma) preconditioning; Group C, DZ + *N*-2-mercaptopropionylglycine (MPG; Sigma) preconditioning; and Group D, DZ + chelerythrine (CH; Sigma) preconditioning. Each preconditioning group was pretreated for 10 min and cultured for 20 min after the drugs were removed. The final concentrations of DZ, MPG, and CH were 200, 400, and 2 μM, respectively, as previously reported (McPherson and Yao, 2001; Seymour et al., 2003; González et al., 2010; Law et al., 2010).

Immunofluorescence

Using conventional methods (Stawowy et al., 2005; Poulin et al., 2009), the cell suspension was placed on glass slides that were pretreated with poly-L-lysine and fixed in cold acetone at 4°C for 5 min after drying. The slides were then incubated with normal goat serum at room temperature for 30 min and with 1% mouse anti-rat PKCε monoclonal antibody (Santa Cruz Biotechnology Inc.) in Tris-buffered saline at 4°C overnight. The negative control group was incubated without a primary antibody. The other groups were incubated with 1% fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody in a dark chamber at 37°C for 2 h. The cover slips were mounted with a mixture of equal amounts of glycerin and phosphate buffer (0.5 M, pH 9.5), examined, and photographed under a fluorescence microscope.

Western blot analysis

Myocardial cytoplasmic and membranous proteins were extracted according to the method of Miyamae et al. (1998). Proteins were quantified by the bicinchoninic acid method (Beyotime Institute of Biotechnology, Ningbo, China). The proteins were then loaded and electrophoresed on sodium dodecyl sulfate-polyacrylamide gel, transfected to a polyvinylidenedifluoride membrane for western blot analysis of PKCε, visualized by the chemiluminescence method, developed, and fixed on an x-ray film. The results were analyzed by a gel imaging analysis system (ProteinSimple, USA). A value x area (A x mm^2) of each band was calculated as the protein content. The percentage of PKCε in total PKCε indicated the amount of translocated PKCε.

Statistical analysis

Data are reported as means ± SE. Homogeneity of variance analysis was performed using SPSS 10.0. Statistical significance was determined using one-way ANOVA and Tamhane analysis. $P < 0.01$ was considered to represent a statistically significant difference.

RESULTS

Immunofluorescence detection

All of the groups were observed under an inverted fluorescence microscope after immunofluorescence staining. A few scattered punctate fluorescence specs were observed in group A (Figure 1A). Strips of fluorescence appeared in group B and were approximately arranged in the same direction as the myofilaments (Figure 1B). A very weak strip of fluorescence and some scattered punctate fluorescence was observed in groups C (Figure 1C) and D (Figure 1D). No specific fluorescence was observed in the negative control group (data not shown).

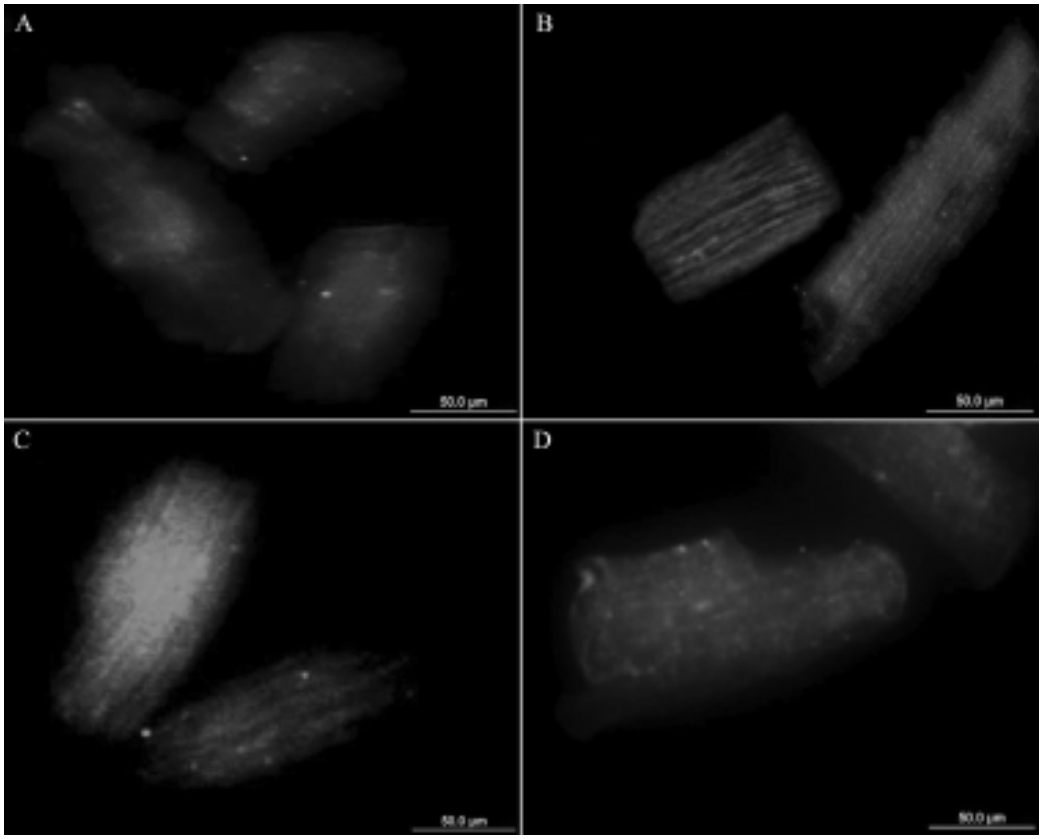

Figure 1. Binding of cardiomyocytes with FITC-anti PKC epsilon under a fluorescence microscope (400X). **A.** control group; **B.** DZ preconditioning group; **C.** DZ + MPG preconditioning group; **D.** DZ + CH preconditioning group.

Expression of cytoplasmic and membranous PKCε

The abundance of cytoplasmic and membranous PKCε were detected by Western blot analysis (Figure 2), and the percentage of the membranous PKCε in total PKCε corresponded to the amount of PKCε that translocated to the membrane (Figure 3). The percentages of mem-

branous PKCε in groups B and C were 65.22 ± 3.25% and 44.06 ± 2.60%, respectively, which were much higher than those in group A (27.42 ± 3.44%). The amount of PKCε in group C was lower than that in group B. The membranous PKCε in group D was 31.90 ± 4.66%, and was not significantly different from that in group A.

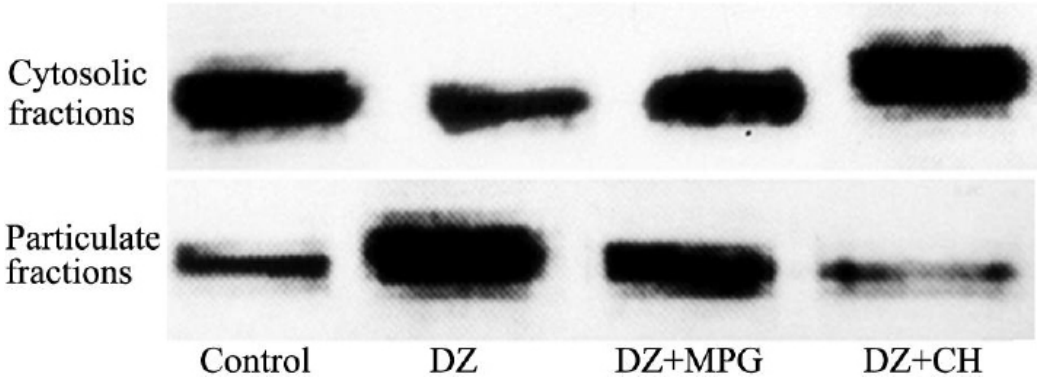

Figure 2. Expression of PKCε on cytosolic fractions and particulate fractions in cultured adult rat ventricular myocytes measured by Western blot.

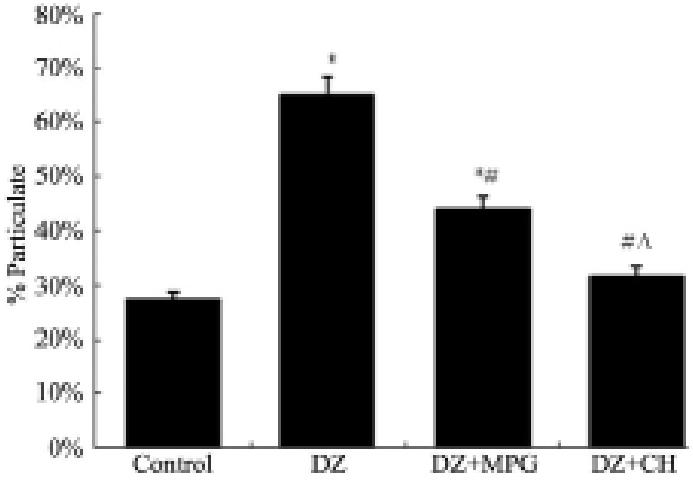

Figure 3. Effect of different preconditioning factors on translocation of PKCε in cultured adult rat ventricular myocytes (means ± SE, N = 5). $^{*}P < 0.01$ *vs* control; $^{\#}P < 0.01$ *vs* DZ preconditioning; $^{\Delta}P < 0.01$ *vs* DZ + MPG preconditioning.

DISCUSSION

This study demonstrated that the opening of MitoK_{ATP} channels may activate and cause PKCε to translocate to the cell membrane, particularly to myofibrillar-like structures. This opening could be prevented by either scavenging ROS produced after the MitoK_{ATP} channel opening or PKC activity inhibition.

Ohnuma et al. (2002) discovered that activated PKCε causes MitoK$_{ATP}$ channels to open during the signal transduction of IP, indicating that PKCε may be an upstream signal of MitoK$_{ATP}$ channels. However, whether or not the opening of MitoK$_{ATP}$ channels in turn activates PKCε remains unclear and, if this opening could activate PKCε, its potential mechanisms are yet to be determined. In this study, we found that the opening of MitoK$_{ATP}$ channels could activate and cause PKCε translocation, suggesting that PKCε may be a downstream signal of MitoK$_{ATP}$ channels. Therefore, a positive feedback loop could be present between the activated PKCε and the opening of MitoK$_{ATP}$ channels in the signal transduction of IP. IP activates PKCε, which causes MitoK$_{ATP}$ channels to open. Once MitoK$_{ATP}$ channels are opened, PKCε becomes activated; thus, the cardioprotective signal is amplified to elicit a cardioprotective effect.

The mechanisms by which MitoK$_{ATP}$ channels are opened and PKCε is activated remain unclear. ROS are very important intracellular signaling molecules, which are associated with PKC activation (Otani, 2004; Cosentino-Gomes et al., 2012) in IP, whereas the opening of MitoK$_{ATP}$ channels can cause the release of ROS (Eaton et al., 2005; Costa and Garlid, 2008; Gordon et al., 2009; Hirata et al., 2011; Jin et al., 2012). Our findings also show that scavenging the ROS produced after the MitoK$_{ATP}$ channels were opened could inhibit the translocation and activation of PKCε. Therefore, these ROS may be involved in PKCε activation and function as a vital signal that links PKCε with the opening of MitoK$_{ATP}$ channels.

PKCε may have an important function in the signal transduction of IP via MitoK$_{ATP}$ channel opening. ROS produced after the MitoK$_{ATP}$ channels were opened may have a significant function in activating PKCε. Activated PKCε could then translocate to myofibrillar-like structures to protect the cardiomyocytes.

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REFERENCES

Cosentino-Gomes D, Rocco-Machado N and Meyer-Fernandes JR (2012). Cell signaling through protein kinase C oxidation and activation. *Int. J. Mol. Sci.* 13: 10697-10721.

Costa AD and Garlid KD (2008). Intramitochondrial signaling: interactions among mitoK$_{ATP}$, PKCε, ROS, and MPT. *Am. J. Physiol. Heart Circ. Physiol.* 295: H874-H882.

Eaton M, Hernandez LA and Schaefer S (2005). Ischemic preconditioning and diazoxide limit mitochondrial Ca overload during ischemia/reperfusion: role of reactive oxygen species. *Exp. Clin. Cardiol.* 10: 96-103.

González G, Zaldívar D, Carrillo E, Hernández A, et al. (2010). Pharmacological preconditioning by diazoxide downregulates cardiac L-type Ca^{2+} channels. *Br. J. Pharmacol.* 161: 1172-1185.

Gordon LI, Burke MA, Singh AT, Prachand S, et al. (2009). Blockade of the erbB2 receptor induces cardiomyocyte death through mitochondrial and reactive oxygen species-dependent pathways. *J. Biol. Chem.* 284: 2080-2087.

Gray MO, Zhou HZ, Schafhalter-Zoppoth I, Zhu P, et al. (2004). Preservation of base-line hemodynamic function and loss of inducible cardioprotection in adult mice lacking protein kinase C ε. *J. Biol. Chem.* 279: 3596-3604.

Gupta A, Aberle NS, Ren J and Sharma AC (2005). Endothelin-converting enzyme-1-mediated signaling in adult rat ventricular myocyte contractility and apoptosis during sepsis. *J. Mol. Cell Cardiol.* 38: 527-537.

Hirata N, Shim YH, Pravdic D, Lohr NL, et al. (2011). Isoflurane differentially modulates mitochondrial reactive oxygen species production via forward versus reverse electron transport flow: implications for preconditioning. *Anesthesiology* 115: 531-540.

Jin C, Wu J, Watanabe M, Okada T, et al. (2012). Mitochondrial K^+ channels are involved in ischemic postconditioning in rat hearts. *J. Physiol. Sci.* 62: 325-332.

Kaneda K, Miyamae M, Sugioka S, Okusa C, et al. (2008). Sevoflurane enhances ethanol-induced cardiac preconditioning through modulation of protein kinase C, mitochondrial K_{ATP} channels, and nitric oxide synthase, in guinea pig hearts. *Anesth. Analg.* 106: 9-16.

Law JK, Yeung CK, Yiu KL, Rudd JA, et al. (2010). A study of the relationship between pharmacologic preconditioning and adenosine triphosphate-sensitive potassium (K_{ATP}) channels on cultured cardiomyocytes using the microelectrode array. *J. Cardiovasc. Pharmacol.* 56: 60-68.

Maack C, Dabew ER, Hohl M, Schafers HJ, et al. (2009). Endogenous activation of mitochondrial K_{ATP} channels protects human failing myocardium from hydroxyl radical-induced stunning. *Circ. Res.* 105: 811-817.

McPherson BC and Yao Z (2001). Morphine mimics preconditioning via free radical signals and mitochondrial K(ATP) channels in myocytes. *Circulation* 103: 290-295.

Miyamae M, Rodriguez MM, Camacho SA, Diamond I, et al. (1998). Activation of ε protein kinase C correlates with a cardioprotective effect of regular ethanol consumption. *Proc. Natl. Acad. Sci. U. S. A.* 95: 8262-8267.

Obal D, Dettwiler S, Favoccia C, Scharbatke H, et al. (2005). The influence of mitochondrial K_{ATP}-channels in the cardioprotection of preconditioning and postconditioning by sevoflurane in the rat *in vivo*. *Anesth. Analg.* 101: 1252-1260.

Ohnuma Y, Miura T, Miki T, Tanno M, et al. (2002). Opening of mitochondrial K_{ATP} channel occurs downstream of PKC-ε activation in the mechanism of preconditioning. *Am. J. Physiol. Heart Circ. Physiol.* 283: H440-H447.

Otani H (2004). Reactive oxygen species as mediators of signal transduction in ischemic preconditioning. *Antioxid. Redox. Signal.* 6: 449-469.

Poulin B, Maccario H, Thirion S, Junoy B, et al. (2009). Ubiquitination as a priming process of PKC α and PKC ε degradation in the αT3-1 gonadotrope cell line. *Neuroendocrinology* 89: 252-266.

Seymour EM, Wu SY, Kovach MA, Romano MA, et al. (2003). HL-1 myocytes exhibit PKC and K_{ATP} channel-dependent delta opioid preconditioning. *J. Surg. Res.* 114: 187-194.

Stawowy P, Margeta C, Blaschke F, Lindschau C, et al. (2005). Protein kinase C ε mediates angiotensin II-induced activation of β1-integrins in cardiac fibroblasts. *Cardiovasc. Res.* 67: 50-59.

Yun N, Kim SH and Lee SM (2012). Differential consequences of protein kinase C activation during early and late hepatic ischemic preconditioning. *J. Physiol. Sci.* 62: 199-209.