Differences in the Expression of Protein Kinase C Isoforms and Its Translocation After Stimulation With Phorbol Ester Between Young-Adult and Middle-Aged Ventricular Cardiomyocytes Isolated From Fischer 344 Rats

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It is known that the tolerance against ischemia–reperfusion and the effects of preconditioning decrease in aged hearts, but the mechanisms responsible for this diminished ischemic tolerance and reduced efficacy of preconditioning remain unknown. To determine the age-related changes in these mechanisms, protein kinase C (PKC) isoform expression and its translocation by phorbol ester were analyzed because PKC is believed to be involved in preconditioning. Immunoblotting and immunostaining analysis were performed with isoform-specific PKC antibodies using cardiomyocytes isolated from young-adult (12-week-old: 12W) and middle-aged (50-week-old: 50W) Fischer 344 rats. There was significantly greater PKC- expression in both the cytosolic and membrane fractions of 12W cardiomyocytes than in 50W ones. Exposure of cardiomyocytes to 100 nmol/L 4- α -phorbol 12-myristate 13-acetate (PMA) caused translocation of PKC- from the cytosol to the membrane in the 12W group, whereas in the 50W group, the translocation was attenuated. Immunostaining confirmed the PKC- translocation in the 12W cardiomyocytes. Oil pellet examination showed that the translocation of PKC- induced by preconditioning was associated with cell protection from ischemic injury in the 12W group only. Age-related changes in PKC isoform expression and activation in cardiomyocytes might be responsible for the reduced ischemic tolerance and less efficient preconditioning that accompanies aging. (*Jpn Circ J* 2001; 65: 1071–1076)

Key Words: Aging; Myocytes; Phorbol ester; Preconditioning; Protein kinase C

The increasing size of the elderly population is having an impact on geriatric medicine, resulting in a greater number of elderly patients suffering from ischemic heart disease (IHD). Clinical studies reveal that the prognosis for elderly patients with IHD is poorer because of the morphologic and functional changes in the cardiovascular system that occur with aging.^{1–4} Specifically, an increase in the myocardial collagen content and its cross-linking, as well as amyloid deposition, leads to increased myocardial stiffness, and ‘drop-out’ of myocytes, tubular dilation, lipid deposition, and the appearance of lipofuscin granules account for the degenerative changes in the myocardium.³ These changes may explain why the senescent heart is less able to respond to acute hemodynamic challenges. Other comorbid conditions, including cerebrovascular and peripheral vascular diseases and renal insufficiency, are also more common in older patients and may limit therapeutic procedures, resulting in a poorer prognosis. However, at the cellular level, neither the mechanisms of ischemic tolerance nor their age-related changes, which might regulate the ability of cardiomyocytes to tolerate ischemia–reperfusion, have been determined. In the clinical field, people who suffer from IHD are usually

middle aged or older and therefore if the mechanisms of ischemic tolerance change with age, we need a new strategy against ischemia–reperfusion injury for the elderly.

Classically, a series of brief coronary occlusions, each separated by a brief period of reperfusion (ischemic preconditioning; PC), makes the heart better able to withstand a subsequent longer ischemic insult.⁵ Previous studies have confirmed that certain drugs or exposure to hypoxia also have a protective effect similar to PC.^{6,7} Furthermore, PC can be blocked by protein kinase C (PKC) inhibitors and/or simulated by direct activation of PKC using diacylglycerol or phorbol ester analogues.^{8–12} Based on these results, PKC is believed to play an important role in the PC signaling cascade in cardiomyocytes.

The effects of aging on the ability to tolerate ischemia or PC have been studied in aged rat hearts: the ischemic tolerance of the myocardium decreases and the cardioprotective effects of PC not only diminish with age, but PC actually worsens the myocardial injury.^{13–15} In the present study, we hypothesized that the activity of PKC decreased with age in rat cardiomyocytes, causing diminished PC effects in old rat hearts. To test this hypothesis, we determined the age-related changes in PKC isoform expression and the translocation of PKC using phorbol ester, one of the potent PKC activators, in isolated rat ventricular cardiomyocytes.

Methods

Animals

The experiments were carried out using hearts from male Fischer 344 rats, a strain that has been used extensively as a

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model of aging. The animals were maintained in the Animal Care Facility of Keio University School of Medicine and this investigation conformed to the principles outlined in the *Guide for the Care and Use of Laboratory Animals* published by the Keio animal care committee (Keio University School of Medicine, Tokyo, Japan).

Heart Perfusions and Myocyte Isolations

The preparation used in this study has been described previously.¹⁶ In brief, following the intraperitoneal injection of sodium pentobarbital (40 mg/kg), hearts were removed from young-adult (12-week-old: 12W, n=12) and middle-aged (50-week-old: 50W, n=8) rats. The excised heart was perfused for 5 min using a modified non-recirculating Langendorff technique with Ca²⁺-free buffer (Buffer A) containing (in mmol/L) 134 NaCl, 5.4 KCl, 10 HEPES, 10 glucose, 1 MgCl₂, 0.33 NaH₂PO₄ (pH 7.4). The coronary perfusion flow rate was set at 10 ml/min. The buffer was gassed with 100% oxygen and the entire perfusion system was maintained at 37°C.

To isolate ventricular myocytes, the perfusion buffer was changed to Buffer A containing 300 U/ml collagenase (type 2, Worthington Biochemical, Lakewood, CA, USA), 0.1 mg/ml protease (type 1, Sigma Chemical, St Louis, MO, USA), and 0.04 mmol/L CaCl₂, and the hearts were perfused for 20 min in a recirculating manner. The heart was removed from the perfusion cannula and the atrial tissue was excluded. Ventricular tissue was minced with scissors in 15 ml of Buffer A containing 0.2 mmol/L CaCl₂, incubated at 37°C for 15 min in a shaking bath (30 Hz), and then filtered through a 210-mesh nylon screen. The supernatant buffer was aspirated off and the cells were resuspended in 20 ml of Buffer A containing 0.5 mmol/L CaCl₂ for 10 min. The final cell pellet was suspended in Buffer A containing 1.0 mmol/L CaCl₂. Myocytes were used only when there were more than 80% of rod-shaped cells.

Preparation of Cytosolic and Membrane Fractions

Myocytes from each age heart were divided into 2 groups. One group (n=6 for 12W, n=4 for 50W) was incubated with 100 nmol/L 4- α -phorbol 12-myristate 13-acetate (PMA) for 10 min at room temperature, and the other group (n=6 for 12W, n=4 for 50W) was incubated without PMA and served as a control group.

Cell pellets were resuspended in ice-cold separation buffer containing (in mmol/L) 20 HEPES, 250 sucrose, 2 EDTA, 2 EGTA, and 10 β -mercaptoethanol (pH 7.5). Cells were homogenized with leupeptin (~1 mmol/L) and phenylmethanesulfonyl fluoride (Pmsf, ~1 mmol/L) using a sonicator (Ultrasonic Processor GE50, EYELA, Tokyo, Japan). The homogenate was centrifuged at 100,000 G for 40 min, and the supernatant was designated as the cytosolic fraction (c). The pellet was resuspended in separation buffer containing 0.5% (vol/vol) Triton-X, leupeptin, and Pmsf, followed by centrifugation at 100,000 G for 40 min and the resultant supernatant represented the membrane fraction (m).

Western Blot Analysis of PKC Isoforms

Twenty micrograms each of the cytosolic and membrane proteins were analyzed by one-dimensional electrophoresis on 10% (wt/vol) SDS-polyacrylamide gels. The gels were transferred to nitrocellulose membranes at 400 mA for 3.5 h. Blots were blocked overnight with 5% (wt/vol) skim milk at room temperature before they were incubated for 2.5 h with one of the primary antibodies (anti-PKC-

- α , - β , or - γ ; dilution, 1:1,000; rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the blotting buffer containing (in mmol/L) 137 NaCl, 20 Tris base, and 0.2% (vol/vol) Tween20 (pH 7.6). After incubation with a primary antibody, blots were exposed for 1 h at room temperature to a peroxidase-conjugated secondary antibody (dilution, 1:5,000; anti-rabbit IgG, Amersham International, Buckinghamshire, UK). Membranes were rinsed 3 times with phosphate buffered saline (PBS)-Tween20 and finally developed with the enhanced chemiluminescence Western blotting system (ECL, Amersham). AttoPhos (Amersham) was used to identify each PKC isoform on the blot. Cytosolic- and membrane-associated PKC bands were quantified using an optical densitometer (ImageQuANT FluorImager 595, Molecular Dynamics, Sunnyvale, CA, USA).

To assess the PKC translocation stimulated by PMA, the membrane-cytosol ratio (m/c) was calculated as the band density for the membrane fraction divided by the band density for the cytosolic fraction.

Immunostaining

Ventricular cells were isolated as described before. The PMA stimulation protocol for myocytes was similar to that used for the immunoblot analysis. Immediately after incubation with/without PMA, the cells were sedimented onto 12-mm glass coverslips coated with laminin. Excess buffer was removed and replaced by a solution containing 4% formaldehyde dissolved in PBS containing (in mmol/L) 145 NaCl, 8 Na₂HPO₄·12H₂O, and 2 NaH₂PO₄·2H₂O (pH 7.4), and incubated overnight at 37°C to fix the cells. Cells were subsequently washed 3 times with PBS. Nonspecific binding was blocked for 1 h incubation at room temperature with PBS containing 5% (wt/vol) normal blocking serum (Santa Cruz Biotechnology). Coverslips were then incubated with one of the anti-PKC- α , - β , - γ , or - δ primary antibodies dissolved in PBS-blocking serum (1 μ g/ml) overnight at 4°C. Cells were then incubated with biotinylated secondary antibody solution for 30 min, followed by incubation with Avidin Biotin (AB) Enzyme Reagent for 30 min at room temperature. Cells were washed with 1% (vol/vol) Triton X-100 in PBS, rinsed with distilled water, and dried. DAB staining solution (Santa Cruz Immunoperoxidase Staining Kit, Santa Cruz Biotechnology) was added to cells for 5–10 min, and rinsed with distilled water. After staining, mounting medium and coverslips were placed on the cells, and the stained cells were analyzed by light microscopy. To assess translocation of the PKC isoforms to the membrane, myocytes were scanned along their short axis and density profiles were obtained.

Oil Pellet Examination

Myocytes isolated from each age group were subjected to simulated ischemia under oil shield buffer for 120 min (Oil+) in a microtube at room temperature. After 120 min of simulated ischemia or oxygenated incubation (Oil-), cells were stained on a slideglass with trypan blue and the percentage of the stained cells in a total of 300 cells were counted as an index of ischemic injury. Before the 120 min-simulated ischemia, PC was induced by 10 min of oil pelleting incubation followed by oxygenated incubation for 15 min (Oil-PC). To determine which PKC-isoform was associated with PC, Western blot analysis was done as described before using Oil-PC myocytes just before undergoing 120-min simulated ischemia.

Fig 1. Expression of protein kinase C (PKC) isoforms in the cytosolic and membrane fractions of ventricular cardiomyocytes determined by immunoblotting. PKC- bands significantly decreased in 50-week-old (50W) myocytes compared with 12-week-old (12W) ones in both the cytosolic and membrane fractions. Values are reported as mean $\pm$ SE (n=6 for 12W, n=4 for 50W). 12c: cytosolic fraction of the 12W group; 12m: membrane fraction of the 12W group; 50c: cytosolic fraction of the 50W group; 50m: membrane fraction of the 50W group. * p <0.05 vs 12W.

Fig 2. Translocation of protein kinase C (PKC) isoforms by stimulation with 4- β -phorbol 12-myristate 13-acetate (PMA). The relative degree of each isoform translocation was assessed by the 'm/c ratio' (the ratio of density of the membrane fraction to that of the cytosolic fraction). In 12-week-old myocytes, only the m/c ratio of PKC- increased significantly in the presence of PMA compared with controls (C). In 50-week-old myocytes, there were no significant changes in the m/c ratio of each isoform, implying that none of the PKC isoforms translocated after stimulation with PMA (A–D). Values are reported as mean $\pm$ SE (n=6 for 12W, n=4 for 50W). () control group; () PMA group. * p <0.05 vs control.

Fig 3. The density profiles of immunocytochemical staining for protein kinase C (PKC) - in 12-week-old ventricular cardiomyocytes. The surface-to-cytosol staining ratio dramatically increased in PMA-stimulated cells. The lower panels show the density profiles of staining (arbitrary units) along the short axis of the myocytes shown in the upper panels. In the PMA-stimulated cell, the density was higher on both sides of the myocyte, indicating that PKC- translocated to the cell membrane following PMA stimulation. Values are reported as mean $\pm$ SE (n=6) in the lower panels.

Fig 4. The density profiles of immunocytochemical staining for protein kinase C (PKC) - in 50-week-old ventricular cardiomyocytes. There were no significant differences in the localization of immunoreactive PKC- between control myocytes and PMA-stimulated cells. The density profiles in the lower panels show that there is no characteristic staining pattern in myocytes, irrespective of their exposure to PMA. Values are reported as mean $\pm$ SE (n=4) in the lower panels.

Chemicals

Chemicals were obtained from Wako Chemicals (Osaka, Japan), Nakarai Chemicals (Kyoto, Japan), Sigma Chemical Co (St Louis, MO, USA), or Pharmacia Biotechnology (Uppsala, Sweden).

Statistical Analysis

Data are represented as mean $\pm$ SE of 4–6 experiments. Comparisons between 2 groups were analyzed by unpaired Student's *t*-test. Differences between groups were determined by one-way ANOVA followed by Fisher's protected

least significant difference test. A *p* value of less than 0.05 was considered statistically significant.

Results

PKC Isoform Expression in 12W or 50W Control Cardiomyocytes

As shown in Fig 1A, B and D, the density (arbitrary units) of the PKC- α , - β , and - δ bands for both the cytosolic and membrane fractions was similar in the 12W and 50W control cardiomyocytes, although the PKC- α of both fractions appeared to be greater in the older rat cardiomyocytes (Fig 1A). However, the PKC- α was significantly lower in the older control myocytes for both the cytosolic and membrane fractions (Fig 1C).

Translocation of PKC Isoforms in Rat Cardiomyocytes Stimulated With PMA

After exposure to PMA (100 nmol/L) the PKC- α , - β , - δ , or - ϵ content was the same for the 12W and 50W cardiomyocytes. In contrast, the expression of PKC- α was lower in the 50W group compared with the 12W group (data not shown). In the 12W myocytes, only the m/c ratio for PKC- α increased significantly in the presence of PMA compared with control incubations (Fig 2C). This suggests that only PKC- α translocated from the cytosolic fraction to the membrane fraction during PMA stimulation. Although the m/c ratio for PKC- α in the 12W myocytes tended to increase after PMA treatment, the change was not significant ($p=0.16$) (Fig 2A). There were no significant changes in the m/c ratio for any of the PKC isoforms in the 50W cells, suggesting that the PKC isoforms did not translocate after stimulation with PMA in the older rats (Fig 2A–D).

Immunostaining Studies

Immunocytochemical analysis was performed to confirm PKC- α translocation from the cytosolic fraction to the membrane fraction in PMA-stimulated young (12W) myocytes (Fig 3, upper panel). In contrast, immunocytochemical staining showed no significant changes in the cellular staining pattern in the older (50W) myocytes after PMA stimulation (Fig 4, upper panel). The density profiles demonstrated that staining was diffuse in control cardiomyocytes of both ages, but that staining became gradually more intense from the center to the periphery of 12W cardiomyocytes stimulated with PMA (Figs 3, 4, lower panels).

Oil Pellet Examination

In younger myocytes, the percentage of stained cells was significantly less in Oil-PC than Oil+ cells ($33.3 \pm 7.2\%$ vs $65.5 \pm 14.7\%$, respectively; $p < 0.05$) (Fig 5A), and the beneficial effect of Oil-PC was lost in the older ones (Fig 5B). Western blot analysis showed that only PKC- α translocated from the cytosol to the membrane after Oil-PC in the younger myocytes (Fig 6A); in the older myocytes, none of the PKC-isoforms translocated (Fig 6B).

Discussion

In the present study, we demonstrated that PKC-expression decreases in both the cytosolic and membrane fractions of ventricular cardiomyocytes isolated from older rats. We also showed that the PKC- α translocation from the cytosol to membrane caused by a PKC stimulator dimin-

Fig 5. The percentage of stained myocytes with trypan blue, as an index of ischemic injury, in oil pellet examination. In 12-week-old myocytes, the percentage of stained cells was significantly less in the Oil-PC group than in the Oil+ group (A). In 50-week-old myocytes, there were no significant changes between the Oil-PC and Oil+ groups (B). Values are reported as mean $\pm$ SE ($n=4$ for each age). () Oil- group; () Oil+ group; (■) Oil-PC group. * $p < 0.05$ vs Oil+.

Fig 6. Western blot analysis of protein kinase C (PKC) isoforms in oil pellet examination. In 12-week-old (12W) myocytes, the band density of PKC- α of the membrane fraction was stronger than that of the cytosolic fraction in the Oil-PC group compared with the Oil-group, but the other PKC-isoform bands showed no change, indicating that only PKC- α translocated to the membrane fraction after oil pellet preconditioning (A). In 50-week-old (50W) myocytes, the bands of each PKC-isoform expressed similar density (B). 12c: cytosolic fraction of the 12W group; 12m: membrane fraction of the 12W group; 50c: cytosolic fraction of the 50W group; 50m: membrane fraction of the 50W group.

ishes with aging, and that the relationship between PKC-translocation and PC seen in the young rat ventricular cardiomyocytes is lost in the older ones.

Changes in PKC Isoform Expression With Aging

Changes in the expression of PKC isoforms in the heart during development have been demonstrated in a number of studies using chromatographic separation, immunoblotting, or immunocytochemistry, but different conclusions have been reached,¹⁷ most likely because of different preparations of hearts (whole hearts, dispersed myocytes, or cultured myocytes), different strains of animals, different stages of development (fetus, neonate, or adult), and technical differences. However, based on those results, it is widely accepted that PKC- α , PKC- β , PKC- γ , and PKC- δ are present in adult rat isolated cardiomyocytes.^{18–22} We demonstrated in preliminary experiments that PKC- α , PKC- β , and PKC- γ are the dominant isoforms in isolated cardiomyocytes from both young-adult and middle-aged rats, although a small amount of PKC- δ is also present. Therefore, we used these 4 isoforms to investigate the age-related changes in PKC isoform expression in the present study, which demonstrated that only PKC- δ decreased in older cardiomyocytes.

Rybin et al investigated age-dependent differences in PKC isoform expression in extracts from the fetal (14-day-old), neonatal (2–15-day-old), and adult rat whole heart, as well as in cultured neonatal and isolated adult rat ventricular myocytes, and reported that PKC- δ was present in both fetal and neonatal myocytes, but not in adult myocytes.²³ PKC- δ was not detected in the adult cardiomyocytes probably because of inappropriate immunologic technique (ie, the lack of sensitivity and specificity of the antisera²⁴). PKC- δ was present in a greater amount in the fetal and neonatal myocytes than in adult myocardial extracts. PKC- δ was present in all cell preparations, but in greatest abundance in the extracts from neonatal myocytes. Additionally, PKC- α , PKC- β , and PKC- γ translocated from a soluble fraction to a particulate fraction in response to stimulation with phorbol ester. They hypothesized that sympathetic innervation might regulate PKC isoform expression during development. Although they did not examine middle-aged myocytes, the age-dependent decline in PKC- δ content is consistent with our results.

Our studies also demonstrated an additional feature that PKC- δ translocation in response to PMA was impaired in older rats. Another report demonstrated that PKC- δ immunoreactivity in liver activated by heat shock diminished in aged rats (96-week-old) compared with young (8-week-old) rats.²⁵ Pucat et al studied the differences in the expression and regulation of PKC isoforms during development using immunoblotting in neonatal (1–2-day-old) and adult (200–250 g, age not described) isolated rat cardiomyocytes; they detected PKC- α , PKC- β , and PKC- γ in both neonatal and adult cardiomyocytes, and PKC- δ was detected in greater abundance in neonatal cells. Furthermore, the responses to several agonists (phenylephrine, ATP, PMA, endothelin, and carbachol) were different for each PKC isoform.¹⁹ These results suggest that the regulatory systems change during development.

PKC and PC

In spite of numerous investigations, the cellular mechanisms responsible for PC have not been fully determined. A number of neuroendocrine and paracrine triggers have been identified, including catecholamines, bradykinin,

adenosine, opioids, endothelin, and angiotensin II.²⁶ These triggers are coupled through G-proteins to phospholipase C, and the diacylglycerol formed by phospholipase C activates PKC. Kitakaze et al hypothesized that PC directly activates PKC and the activated PKC phosphorylates ecto-5'-nucleotidase, leading to adenosine production and thereby causing cardioprotection.²⁷ Furthermore, PKC activates mitochondrial KATP channels, which mediate some of the beneficial effects of PC.²⁸ Therefore, PKC is thought to be an important mediator in the signal cascade of PC in several animal species including rats.^{8,10–12} PC is blocked by specific PKC inhibitors and also simulated by direct activation of PKC with a diacylglycerol analogue or PMA.^{8–12} and recent studies have attempted to determine the specific PKC isoforms that may be responsible for PC.

Yoshida et al demonstrated translocation of the PKC- α , PKC- β , and PKC- γ isoforms to the membrane fraction during PC in adult rat whole heart, and also showed that a highly specific PKC inhibitor, chelerythrine, inhibits both the improvement of contractile dysfunction during reperfusion and the translocation of PKC- α , PKC- β , and PKC- γ by PC.²⁹ They suggested that any of these 3 PKC isoforms can mediate the PC effect. Additionally, they demonstrated that 1 μ mol/L PMA treatment can induce translocation of the PKC- α , PKC- β , and PKC- γ isoforms. Although the experimental preparation and PC procedure in their study were different from ours, the result that only PKC- δ translocated after 100 nmol/L PMA treatment shown in the present study seems to be inconsistent and may result from the lesser concentration and shorter duration of PMA treatment used in our study.

In the setting of hypoxia or pharmacological preconditioning by phorbol esters, PKC- α , PKC- β , and PKC- γ translocated in both intact rat hearts and isolated cardiomyocytes, suggesting that these 3 isoforms might participate in PC.^{20,21} In particular, PKC- δ is reported to act through a different pathway than the other isoforms,^{11,19,21,29} supporting the hypothesis that activated PKC- δ plays a pivotal role in PC.^{11,30} Our study also showed an association between the beneficial effects of PC against ischemic injury and PKC- δ in young-adult rat ventricular cardiomyocytes. Therefore, the decreased expression and translocation of PKC- δ in older rats seen in the present study might explain the impaired tolerance to an ischemic insult and less efficacious PC in aged rats.^{13–15}

Study Limitations and Future Directions

PC is a complicated, multifactorial phenomenon and the effect of aging on PC may not be solely explained by differences in PKC isoform expression and translocation. Studies are necessary to elucidate the effect of aging on PC, for instance, in transgenic animals or using an isoform-specific activator of PKC, or on the downstream pathways of PKC (especially PKC- δ) activation. In vivo, interactions with other cell types, including fibroblasts and endothelial cells, and the effects of the autonomic nervous system and hormones cannot be ignored and remain unknown.

In conclusion, we found age-related changes in PKC isoform expression and activation in ventricular cardiomyocytes, which may play a role in the impairment of ischemic tolerance and less efficacious preconditioning that accompanies aging.

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