Mechanisms of myocardial ischemic preconditioning are age related: PKC-ε does not play a requisite role in old rabbits

Karin Przyklenk, Guohu Li, Boris Z. Simkhovich, and Robert A. Kloner

1Departments of Emergency Medicine and Anesthesiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655; and 2Heart Institute, Good Samaritan Hospital, and Department of Medicine, Section of Cardiology, University of Southern California, Los Angeles, California 90017

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EXHAUSTIVE EVIDENCE, OBTAINED from numerous models and species, has demonstrated that brief episodes of myocardial ischemia can paradoxically protect or “precondition” the heart and limit necrosis caused by a subsequent, more sustained ischemic insult (21). Moreover, although the mechanisms responsible for preconditioning (PC)-induced cardioprotection remain incompletely resolved, G-protein-coupled signaling and subsequent activation and translocation of protein kinase C (PKC), in particular the ε-isoform, have been proposed to play pivotal roles (16, 20, 21). There are, however, well-described alterations in G-protein-coupled signaling with increasing age (24), and emerging evidence further suggests that both the expression and subcellular redistribution of PKC isoforms in response to receptor stimulation may vary in the aging heart (14, 27, 28). These observations, coupled with the fact that virtually all insight into PC-induced cardioprotection has been derived from juvenile or adult cohorts, may confound the extrapolation of mechanistic data from adults to aging populations.

Presently, there is no consensus as to whether infarct size reduction with PC is maintained in the aging heart. In some models, in particular the isolated buffer-perfused rat heart, there is evidence that PC-induced cardioprotection may wane with increasing age (2, 3, 7, 14, 25). In contrast, in the in vivo rabbit model, infarct size reduction with PC remains comparable, with no loss in efficacy, in ~4-yr-old animals exhibiting definitive hallmarks of cardiovascular aging vs. adult animals (22). There is similar disagreement among clinical studies: both loss of efficacy (1, 10, 15) and continued benefit (11, 13, 18) have been reported in patient subsets ~60–70 yr of age, i.e., the specific population in which the incidence of acute myocardial infarction is greatest and thus cardioprotection is most relevant (4, 9, 22). Most notably, in models in which infarct size reduction with PC is maintained irrespective of increasing cardiovascular age, there is, at present, no information regarding the cellular mechanisms of PC-induced cardioprotection in old vs. adult groups. Accordingly, our present aim was to determine, by 1) pharmacological treatment with PKC and PKC-ε inhibitors and 2) direct assessment of PKC-ε protein by Western immunoblotting, whether the role of PKC-ε in infarct size reduction with PC is altered in aging rabbit heart.

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2563
MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of Good Samaritan Hospital and conforms to the Guide for the Care and Use of Animals (National Academic Press, Washington DC, 1996).

Surgical Preparation

We utilized the well-documented in vivo rabbit model of coronary artery occlusion-reperfusion, which has been previously described in detail (22). Briefly, pathogen-free New Zealand White rabbits of either sex were anesthetized with intramuscular injections of ketamine (200 mg) and xylazine (100 mg) and intubated and ventilated with room air supplemented with 100% oxygen. After the left jugular vein (for administration of fluids) and left carotid artery (for measurement of heart rate and arterial pressure) were cannulated, the heart was exposed through a left thoracotomy and a large marginal branch of the circumflex artery was encircled with a snare for later occlusion-reperfusion. Body temperature was maintained at 38–39°C, and supplemental anesthesia (pentobarbital sodium) was administered intraperitoneally as required (~50 mg/h).

Protocol 1: Effect of PKC Antagonists on Infarct Size Reduction With PC

Protocol IA: adult rabbits. Thirty-four adult rabbits (~6 mo old; 3.1 ± 0.4 kg) were enrolled in the infarct size component of the study. After stabilization and drug treatment (described below), rabbits received either 5 min of PC ischemia followed by 10 min of reperfusion or a matched 15-min control period. This single, 5-min PC stimulus has been shown by our group (unpublished observations) and others to evoke significant cardioprotection in the in vivo rabbit heart, with no further benefit achieved by the addition of multiple PC cycles (5). All animals then underwent 30 min of sustained coronary artery occlusion and 3 h of reflow (Fig. 1). Heart rate and mean arterial pressure were recorded at baseline (before randomization and treatment), immediately before and at 25 min into sustained coronary occlusion, and at 15 min and 3 h postreperfusion. At the end of the protocol, the area at risk of infarction (AR) and area of necrosis (AN) were delineated with the use of routine methods (in vivo injection of blue dye and tetrazolium staining, respectively) and quantified by computerized planimetry (22).

If activation and translocation of PKC-ε contributes to PC-induced cardioprotection in the rabbit, we reasoned that treatment with PKC-ε translocation inhibitor peptide (PKC-ε-TIP; Calbiochem), an octapeptide confirmed to selectively inhibit translocation of the ε-isofrom of PKC (12, 19) and shown to block PC-induced protection in isolated rabbit cardiomyocytes (16), would attenuate the reduction in infarct size achieved with PC. Accordingly, 27 rabbits were randomized to receive PKC-ε-TIP (0.3 mg/kg) or vehicle (1 ml/kg sterile water), administered as a left atrial bolus at 5 min before the onset of the PC or control period (n = 6–8 per group). An additional three animals were randomized to receive scrambled (i.e., presumably ineffective) PKC-ε-TIP (0.3 mg/kg) before PC ischemia, whereas four PC rabbits were pretreated with chelerythrine chloride (Calbiochem), an inhibitor that is selective for PKC but is not isoform specific, at a dose reported to inhibit translocation of PKC-ε and abrogate cardioprotection [5 mg/kg in sterile water (17, 23)]. All agents were administered in a blinded manner, and all measurements of AN and AR were performed without knowledge of the treatment group.

Protocol 1B: old rabbits. We evaluated the consequences of PKC-ε and pan-PKC inhibition on infarct size reduction with PC in ~4-yr-old rabbits shown previously by our group (22) to display definitive hallmarks of cardiovascular aging (i.e., significant myocyte hypertrophy, myocardial fibrosis, and near-total loss of responsiveness to β-adrenergic stimulation). Specifically, 34 animals aged 44–52 mo (mean age of 48 mo; 4.7 ± 0.5 kg) underwent the same 15-min PC or control period and sustained occlusion-reperfusion regimen as described for protocol IA (Fig. 1). Rabbits were randomized to receive blinded treatment with PKC-ε-TIP, chelerythrine, or vehicle at 5 min before PC ischemia or at 5 min before the matched control period (n = 4–8 per group). Among the PC rabbits allocated to peptide treatment, one subset (n = 5) received the dose of 0.3 mg/kg utilized in protocol IA, whereas the remainder (n = 3) received a double dose (0.6 mg/kg) of PKC-ε-TIP. Measurements of AR and AN were performed as described in protocol IA.

Protocol 2: Subcellular Distribution of PKC-ε in Adult and Old Rabbit Hearts

Sixteen rabbits (8 adults and 8 aged ~4 yr) were used to obtain preliminary insight into possible age-associated differences in the subcellular redistribution of PKC-ε in response to brief PC ischemia. After stabilization, animals in each age group were randomized to undergo either 5 min of coronary occlusion or a matched sham control period (n = 4 per group; Fig. 1). In hearts that received PC ischemia, blue dye was injected via the left atrium during the final seconds of occlusion to delineate the AR. At the end of the 5-min ischemic or control period, the hearts were rapidly excised and frozen in liquid nitrogen.

Tissue within the AR (or from the comparable area distal to the suture in sham-subjected controls) was homogenized
in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 0.3% β-mercaptoethanol, 10 mM benzamidine, and 20 µl of protease inhibitor cocktail (Sigma; product P8340) per 1 ml of buffer. Cytosolic and particulate fractions were prepared by using standard methods (26). After initial low-speed centrifugation (1,000 g), the resultant supernatant, containing solubilized cytoplasmic membranes and mitochondrial fragments, was designated as the particulate fraction. PKC-ε in the cytosolic and membrane fractions was assayed by Western immunoblotting. For each heart, equal amounts of protein (35 µg for all particulate fractions; 70 µg for all cytosolic fractions) were electrophoresed onto 10% SDS-polyacrylamide gels and transferred overnight onto polyvinylidene difluoride membranes. Uniform protein loading was documented with Ponceau staining, and gel retention was determined with Coomassie blue staining. Blots were probed with a PKC-ε-specific antibody (Transduction Laboratories), developed with a chemiluminescence system (ECF kit, Amersham), detected with the use of the Molecular Dynamics Storm system (Amersham), and band intensity quantified with the use of ImageQuant software (Amersham). To minimize any confounding influence of variability among gels, each gel was loaded with cytosolic and particulate fractions from four hearts (1 per group), and band intensities were normalized, for each gel and fraction, to the corresponding values recorded for the adult sham samples. In addition, the ratio of PKC-ε in the cytosolic to particulate fractions, considered an index of activation/translocation, was calculated for each group and normalized to adult sham values. All immunoblots were performed in duplicate, and the results were averaged.

**Statistics**

Because protocols 1A and 1B were conducted sequentially, separate statistical analyses were performed for each component of the study. Within each age group, hemodynamics were compared by two-factor ANOVA (for treatment and time) with replication, whereas AR [expressed as a percentage of the left ventricle (LV)] and AN (expressed as a percentage of the AR) were compared by one-factor ANOVA. All post hoc pairwise comparisons were made with the Newman-Keuls test. Infarct sizes in all groups that received interventions were further compared with vehicle controls by analysis of covariance (ANCOVA), incorporating risk region (the major determinant of infarct size in the rabbit) as the covariate. For protocol 2, normalized values of PKC-ε in the cytosolic and particulate fractions were compared among groups by one-factor ANOVA and the Newman-Keuls post hoc test. All data are reported as means ± SE.

**RESULTS**

**Protocol 1A: Adult Rabbits**

**Hemodynamics.** Heart rate was comparable among all groups, both at baseline and throughout the protocol (Table 1). Mean arterial pressure was, by chance, higher at baseline in rabbits later assigned to receive PKC-ε-TIP (Table 1). However, arterial pressure was not significantly altered by PKC-ε-TIP, scrambled peptide, or chelerythrine treatment per se.

**Risk region and infarct size.** Mean values of AR for the six treatment groups ranged from 29 ± 3 to 35 ± 3% of the total LV weight (P = not significant) (Fig. 2).

Infarct size was, as expected, significantly reduced with PC: AN/AR averaged 34 ± 4% vs. 53 ± 4% in the vehicle + PC vs. vehicle + control groups, respectively (P < 0.05). The protective effect of PC was abrogated both by chelerythrine and, most notably, by PKC-ε-TIP. In contrast, administration of scrambled peptide had no effect on the efficacy of PC, and PKC-ε-TIP did not alter infarct size in controls. These results (i.e., significant cardioprotection in the PC and scrambled PKC-ε-TIP + PC groups vs. vehicle controls, but no protection in the chelerythrine + PC and PKC-ε-TIP + PC cohorts) were confirmed by ANCOVA (not shown).

**Protocol 1B: Old Rabbits**

**Hemodynamics.** There were no group differences in heart rate or mean arterial pressure at any time during the protocol (Table 2).

<table>
<thead>
<tr>
<th>Table 1. Hemodynamics of adult rabbits</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Heart rate, beats/min</td>
</tr>
<tr>
<td>Vehicle + control</td>
</tr>
<tr>
<td>Vehicle + PC</td>
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<tr>
<td>PKC-ε-TIP + control</td>
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<tr>
<td>PKC-ε-TIP + PC</td>
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<tr>
<td>Scram-PKC-ε-TIP + PC</td>
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<tr>
<td>Chel + PC</td>
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<tr>
<td>Mean arterial pressure, mmHg</td>
</tr>
<tr>
<td>Vehicle + control</td>
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<tr>
<td>Vehicle + PC</td>
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<tr>
<td>PKC-ε-TIP + control</td>
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<tr>
<td>PKC-ε-TIP + PC</td>
</tr>
<tr>
<td>Scram-PKC-ε-TIP + PC</td>
</tr>
<tr>
<td>Chel + PC</td>
</tr>
</tbody>
</table>

Values are means ± SE. Chel, chelerythrine; PC, preconditioned; PKC-ε-TIP, protein kinase C-ε translocation inhibitor peptide; Scram, scrambled. *P < 0.05 vs. baseline; †P < 0.05 vs. vehicle + control.
Risk region and infarct size. Values of AR/LV were similar to those observed in adults, with no differences among the seven treatment groups (Fig. 3).

Mean AN/AR in vehicle-control rabbits was 51 ± 6%. The efficacy of PC-induced cardioprotection was maintained in ~4-yr-old animals, with infarct size reduced to 34 ± 3% in the vehicle + PC group (P < 0.05). However, in contrast to observations made in the adult cohort, neither chelerythrine, PKC-ε-TIP, nor the double dose of the peptide attenuated the reduction in infarct size achieved with PC. These findings of cardioprotection with PC, and persistent PC-induced limitation of infarct size despite treatment with chelerythrine and PKC-ε-TIP, were corroborated by ANCOVA (not shown).

Table 2. Hemodynamics of 4-yr-old rabbits

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Preocclusion (25 min)</th>
<th>Occlusion (25 min)</th>
<th>Reperfusion (15 min)</th>
<th>Reperfusion (3 h)</th>
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<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle + control</td>
<td>141 ± 6</td>
<td>136 ± 7</td>
<td>143 ± 7</td>
<td>144 ± 7</td>
<td>167 ± 5*</td>
</tr>
<tr>
<td>Vehicle + PC</td>
<td>149 ± 10</td>
<td>150 ± 11</td>
<td>156 ± 9</td>
<td>156 ± 7</td>
<td>167 ± 5</td>
</tr>
<tr>
<td>PKC-ε-TIP + control</td>
<td>134 ± 2</td>
<td>128 ± 2</td>
<td>142 ± 3</td>
<td>141 ± 3</td>
<td>154 ± 11</td>
</tr>
<tr>
<td>PKC-ε-TIP + PC</td>
<td>142 ± 6</td>
<td>153 ± 4</td>
<td>158 ± 7</td>
<td>158 ± 6</td>
<td>158 ± 4</td>
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<tr>
<td>PKC-ε-TIP (&gt;2) + PC</td>
<td>138 ± 10</td>
<td>132 ± 13</td>
<td>138 ± 10</td>
<td>143 ± 10</td>
<td>156 ± 8</td>
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<tr>
<td>Chel + control</td>
<td>133 ± 6</td>
<td>128 ± 11</td>
<td>138 ± 10</td>
<td>143 ± 11</td>
<td>152 ± 8</td>
</tr>
<tr>
<td>Chel + PC</td>
<td>151 ± 5</td>
<td>148 ± 10</td>
<td>154 ± 7</td>
<td>154 ± 6</td>
<td>168 ± 7</td>
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<tr>
<td>Mean arterial pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle + control</td>
<td>94 ± 4</td>
<td>92 ± 5</td>
<td>75 ± 4*</td>
<td>70 ± 4*</td>
<td>50 ± 2*</td>
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<tr>
<td>Vehicle + PC</td>
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<td>71 ± 5*</td>
<td>64 ± 3*</td>
<td>50 ± 3*</td>
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<tr>
<td>PKC-ε-TIP + control</td>
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<td>50 ± 3*</td>
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<td>72 ± 5</td>
<td>65 ± 4*</td>
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<td>45 ± 4*</td>
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<tr>
<td>PKC-ε-TIP (&gt;2) + PC</td>
<td>92 ± 4</td>
<td>86 ± 6</td>
<td>76 ± 7*</td>
<td>72 ± 7*</td>
<td>56 ± 6*</td>
</tr>
<tr>
<td>Chel + control</td>
<td>88 ± 4</td>
<td>84 ± 5</td>
<td>68 ± 5*</td>
<td>60 ± 5*</td>
<td>46 ± 4*</td>
</tr>
<tr>
<td>Chel + PC</td>
<td>85 ± 4</td>
<td>80 ± 8</td>
<td>71 ± 7*</td>
<td>66 ± 5*</td>
<td>45 ± 3*</td>
</tr>
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</table>

Values are means ± SE. ×2, Double dose. *P < 0.05 vs. baseline.
Protocol 2

In adults that received 5 min of PC ischemia, PKC-ε in the particulate fraction was increased to 111% of adult sham values (Fig. 4). In addition, the ratio of PKC-ε in the particulate/cytosolic fractions, normalized to adult sham values, was 1.14, consistent with activation/translocation of the isoform.

Hearts from ~4-yr-old rabbits tended to exhibit lower PKC-ε immunoreactivity in both subcellular fractions, a trend that achieved significance in the old PC group. Moreover, in old animals, the PC stimulus did not elicit an increase in PKC-ε in the particulate fraction (Fig. 4), and the ratio of PKC-ε in the particulate to cytosolic fractions remained unchanged at 0.98 and 1.00 in the old sham and old PC subsets.

DISCUSSION

We report that, in 4-yr-old rabbits, a model shown previously by our group to exhibit de
despite treatment with either PKC-
in the adult population are consistent with this concept. Subsequent investigations, employing quantitative Western immunoblotting, further implicated the specific involvement of PKC-ε: among the 11 known isoforms of the kinase, it was the ε-isoenzyme that exhibited translocation with brief PC ischemia (20). In this regard, our finding that treatment with PKC-ε-TIP (but, importantly, not the scrambled peptide) blocked PC-induced cardioprotection in adult rabbits provides in vivo pharmacological evidence for the specific role of PKC-ε translocation in infarct size reduction with PC. Moreover, our ancillary observation of an increase (albeit modest) in PKC-ε immunoreactivity in the particulate fraction in response to 5 min of PC ischemia in adult rabbits further corroborates this hypothesis.

Preconditioning the Aging Heart

Among the host of studies investigating the phenomena of ischemic PC, the vast majority have employed juvenile or adult models. Indeed, although several protocols have evaluated surrogate indexes of cardioprotection (i.e., recovery of LV function) in hearts harvested from senescent animals (2, 3, 28), only three experimental studies to date have assessed infarct size reduction, the acknowledged gold standard of PC, in aging cohorts. In isolated buffer-perfused rat hearts subjected to either global (7) or regional ischemia (25), infarct size reduction with PC purportedly wanes with increasing age and is ineffective in eliciting protection in 18- to 20-mo-old animals. In contrast, we have found that, in ~4-yr-old rabbits exhibiting conclusive evidence of cardiovascular aging, there was no loss in the in vivo efficacy of PC (22), an observation confirmed in the present study. Clinical studies have yielded similar disagreements. There are reports that preinfarct angina fails to elicit protection in aging patients with acute myocardial infarction (1, 10, 15), whereas others have found continued cardioprotection with brief antecedent ischemia (i.e., preinfarct angina) irrespective of age (11, 13) and, in isolated human atrial tissue subjected to simulated ischemia, persistent PC-induced cardioprotection even in cohorts aged 70–90 yr (18). Thus, presently, there is no consensus as to whether the efficacy of infarct size reduction with PC is maintained in old populations and, perhaps more notably, no mechanistic insight as to why PC-induced cardioprotection continues to be manifest, despite increasing cardiovascular age, in some models, species, or protocols.
Aging, PKC-ε, and Cardioprotection

We found, in our in vivo rabbit model, that infarct size reduction with PC was abrogated by PKC-ε-TIP in adult rabbits, whereas PKC-ε-TIP, even at double the dose administered to adults, failed to attenuate PC-induced cardioprotection in the ~4-yr-old cohort. These data could be interpreted to suggest that 1) PKC-ε activation/translocation is impaired in aging rabbit hearts or, alternatively, 2) PKC-ε translocation is augmented in old hearts such that even high doses of the peptide were insufficient to attenuate the benefits of PC ischemia. In an effort to distinguish between these two possibilities, we utilized Western immunoblotting to obtain insight into potential differences in the expression and subcellular distribution of PKC-ε in adult vs. ~4-yr-old rabbit hearts vs. without brief PC ischemia.

Our results in protocol 2 indicate that, for uniform protein loads, PKC-ε immunoreactivity was ~10–25% lower in subcellular fractions isolated from ~4-yr-old rabbits vs. adults. Although there is a paucity of data on the effects of aging on PKC isoform expression, there is evidence, obtained in isolated buffer-perfused rat hearts, for lower levels of cytosolic PKC-ε in hearts harvested from senescent animals (14). However, it remains unclear whether this reduction in PKC-ε immunoreactivity per unit protein truly reflects a decrease in PKC-ε content within the myocytes (perhaps due to a marked, twofold to eightfold increase in protein phosphatase activity seen by our group (unpublished observations) and others (6) in the aging heart), or, is, at least in part, a secondary consequence of the concurrent fibrosis (i.e., 1.7-fold increase in percent collagen content) documented previously in the characterization of our ~4-yr-old rabbit cohort (22).

We further found that, in contrast to observations made in adults, 5 min of brief PC ischemia did not evoke an increase in PKC-ε in the particulate fraction in hearts from ~4-yr-old rabbits. Although there are few published data on the effect of increasing age on stimulus-mediated activation/translocation of PKC isoforms, our results are consistent with recent evidence showing impaired translocation of PKC-ε in response to α1-receptor stimulation with phenylephrine, administration of 1,2-dioctanoyl-sn-glycerol, and, most notably, brief PC ischemia in hearts harvested from ~1- to 2-yr-old rats (14, 28). However, not all studies have identified PKC-ε as the specific isoform affected by aging: data obtained from isolated rat cardiomyocytes showed an age-associated decrease in the translocation of PKC-δ, but not PKC-ε, after exposure to phorbol myristate acetate (27). It must be acknowledged that, in our ancillary protocol, our primary endpoint was PKC-ε immunoreactivity (rather than phosphotransferase activity), results were obtained at only one time point, and these data do not, in themselves, preclude possible activation/translocation of the isoform at other times during the PC stimulus. Nonetheless, protocols 1 and 2 taken together suggest that the observed inability of PKC-ε-TIP to attenuate PC-induced cardioprotection in old rabbits was due to impaired translocation of PKC-ε, rather than augmented translocation and insufficient dosing, in the old cohort.

Implications and Future Directions

It has been hypothesized, from studies conducted in isolated buffer-perfused rat hearts, that defects in one or more cellular signaling components underlie the purported age-associated loss of PC-induced cardioprotection in this model, with PKC, as well as norepinephrine, the ATP-sensitive potassium channel and others implicated as possible sites of impairment (3, 25, 28). Our present data are consistent with the concept of impaired PKC-ε translocation and signaling with increasing age. However, our results demonstrate that, in the in vivo rabbit model, infarct size reduction with PC was maintained, with no loss in efficacy, despite this age-associated alteration in PKC-ε.

These data raise the question: What kinase(s) or other cellular mediators assume the role of PKC-ε in eliciting infarct size reduction with PC in the aging hearts? One obvious possibility is that other PKC isoforms, in lieu of PKC-ε, contribute to PC-induced cardioprotection in old rabbits. Our finding of persistent limitation of infarct size in PC rabbits, despite treatment with chelerythrine (an inhibitor that is not isoform selective) argues against (but does not disprove) the concept that PC in this aging cohort is primarily due to activation/translocation of one or more “alternative” PKC isoforms. However, multiple signaling mechanisms (i.e., one or more “alternative” isoforms of PKC, together with as-yet unidentified tyrosine kinases and/or mitogen-activated protein kinases) may be recruited and may act in concert to elicit the reduction of infarct size seen with PC in old rabbits. In fact, this concept of synergy among signaling pathways has been explored in other models and species (rat, pig) in which pharmacological inhibition of PKC alone was found to be insufficient to block the benefits of PC (8, 29). Resolution of this important question awaits further comprehensive assessment of the activation/translocation and phosphotransferase activity of multiple PKC isoforms and activity of multiple kinases in adult vs. aging rabbit heart. Nonetheless, our present results demonstrate that, in rabbit, the mechanisms responsible for PC are age related, with no apparent, requisite role of PKC-ε in the aging cohort.

DISCLOSURES

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REFERENCES


