Mechanisms of Myocardial Ischemic Preconditioning are Age-Related: Protein Kinase C-ε Does Not Play a Requisite Role in Old Rabbits

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ABSTRACT

Data obtained from adult cohorts has implicated activation/translocation of protein kinase C (PKC)-ε as an important cellular mediator of myocardial infarct size reduction with ischemic preconditioning (PC). Age-related alterations in cellular signaling may, however, confound the extrapolation of mechanistic insight derived from adults to the aging population – the specific subset in which cardioprotection is undoubtedly most relevant. Accordingly, our aim was to investigate the role of PKCε as a mediator of infarct size reduction with preconditioning (PC) in old versus adult rabbits. In Protocol 1, we assessed the effect of PKCε translocation inhibitor peptide (PKCε-TIP) and the pan-PKC inhibitor chelerythrine on infarct size reduction with PC in adult and ~4 year old rabbits, a population previously shown to exhibit definitive hallmarks of cardiovascular aging. Rabbits received 5 min of PC ischemia or a matched control period followed by 30 min of coronary artery occlusion and 3 h of reperfusion, with infarct size (delineated by tetrazolium staining) serving as the primary endpoint. In Protocol 2, we obtained insight (by Western immunoblotting) into the subcellular redistribution of PKCε in response to the 5 min PC stimulus in adult and old rabbits. In adults, infarct size reduction with PC was abrogated by both PKCε-TIP and chelerythrine. However, in old rabbits: (i) PC-induced cardioprotection was maintained despite inhibitor treatment; and (ii) brief PC ischemia was not associated with activation/translocation of PKCε. Thus, the mechanisms responsible for PC are age-related in rabbit heart, with no apparent, requisite role of PKCε in aging animals.

Key words: myocardial ischemia; myocardial infarct; signal transduction
Exhaustive evidence, obtained in 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 [22]. Moreover, although the mechanisms responsible for preconditioning (PC)-induced cardioprotection remain incompletely resolved, G-protein-coupled signaling followed by activation and translocation of protein kinase C (PKC) – in particular, the ε-isoform – have been proposed to play a pivotal role [16,20,22]. 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.

There is no current consensus as to whether infarct size reduction with PC is maintained in the aging heart. In some models – in particular, the isolated buffer-perfused rabbit heart – there is evidence that PC-induced cardioprotection may wane with increasing age [2,3,7,14,25] while, in the in vivo rabbit model, infarct size reduction with PC remains comparable, with no loss in efficacy, in ~4 year old animals exhibiting definitive hallmarks of cardiovascular aging when compared with adults [21]. There is similar disagreement among clinical studies, with both loss of efficacy [1,10,15] and continued benefit [11,13,18] having been reported in patient subsets ~60-70 years of age – i.e., the specific population in which the incidence of acute myocardial infarction (MI) greatest and thus cardioprotection is most relevant [4,9,21] 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 versus adult groups. Accordingly, our current aim was to determine, by: (1) pharmacologic treatment with PKC and PKCε inhibitors, and: (2) direct assessment of PKCε protein by Western immunoblotting, whether the role of PKCε in infarct size reduction with PC is altered in aging rabbit heart.
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 Laboratory Animals* (National Academic Press, Washington DC, 1996).

Surgical preparation

We utilized the well-documented, *in vivo* rabbit model of coronary artery occlusion/reperfusion, described in detail previously [21]. Briefly, pathogen-free New Zealand White rabbits of either sex were anesthetized with intramuscular injections of ketamine (200 mg) + xylazine (100 mg), intubated and ventilated with room air supplemented with 100% oxygen. After cannulating the left jugular vein (for administration of fluids) and left carotid artery (for measurement of heart rate and arterial pressure), 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 (sodium pentobarbital: intraperitoneal) was administered as required (~50 mg/h).

**Protocol 1: Effect of PKC antagonists on infarct size reduction with preconditioning** (Figure 1)

**1A: Adult Rabbits**

Thirty-four adult rabbits (~6 months 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 observation] 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 hours of reflow (Figure 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 hours post-reperfusion. At the end of the protocol, the area at risk of infarction (AR) and area of necrosis (AN) were delineated using routine methods (*in vivo* injection of blue dye and tetrazolium staining, respectively) and quantified by computerized planimetry [21].

If activation and translocation of PKCε contributes to PC-induced cardioprotection in the rabbit, we reasoned that treatment with PKCε translocation inhibitor peptide (PKCε -TIP; Calbiochem) – an...
octapeptide confirmed to selectively inhibit translocation the ε isoform 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 PKCε -TIP (0.3 mg/kg) or vehicle (sterile water: 1 mL/kg), administered as a left atrial bolus at 5 min before the onset of the PC/control period (n=6-8 per group). An additional 3 animals were randomized to receive scrambled (i.e., presumably ineffective) PKCε -TIP (0.3 mg/kg) before PC ischemia, while 4 preconditioned rabbits were pretreated with chelerythrine chloride (Calbiochem), an inhibitor that is selective for PKC but not isoform-specific, at a dose reported to inhibit translocation of PKCε 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.

1B: Old Rabbits

We evaluated the consequences of PKCε and pan-PKC inhibition on infarct size reduction with PC in ~4 year old rabbits shown previously by our group to display definitive hallmarks of cardiovascular aging – i.e., significant myocyte hypertrophy, myocardial fibrosis and near-total loss of responsiveness to β-adrenergic stimulation [21]. Specifically, 34 animals aged 44-52 months (mean: 48 months; 4.7±0.5 kg) underwent the same 15 min PC/control period and sustained occlusion/reperfusion regimen as described for adults (Figure 1). Rabbits were randomized to receive blinded treatment with PKCε -TIP, chelerythrine or vehicle at 5 min before PC ischemia or 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 1A, while the remainder (n=3) received a double dose – 0.6 mg/kg – of PKCε -TIP. Measurements of AR and AN were performed as described in Protocol 1A.

Protocol 2: Subcellular distribution of PKCε in adult and old rabbit hearts (Figure 1)

Sixteen rabbits – 8 adults and 8 aged ~4 years – were used to obtain preliminary insight into possible age-associated differences in the subcellular redistribution of PKCε in response to brief PC ischemia. After stabilization, animals in each age group were randomized to undergo 5 min of coronary occlusion or a matched sham-control period (n=4 per group; Figure 1). In hearts that received PC ischemia, blue dye was injected via the left atrium during the final seconds of occlusion in order to delineate
the AR. At the end of the 5 min ischemic/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-controls) was homogenized in ice-cold buffer containing 50 mM Tris-HC pH 7.5, 5 mM EDTA, 10 mM EGTA, 0.3% β-mercaptoethanol, 10 mM benzamidine and 20 µL of protease inhibitor cocktail (Sigma; #P 8340) per 1 mL buffer. Cytosolic and particulate fractions were prepared using standard methods [26]. After initial low-speed centrifugation (1,000xg) to remove nuclei and cellular debris, high-speed centrifugation (45,000xg for 30 min) was applied and the supernatant was removed and used as the cytosolic fraction. The pellet was solubilized in homogenization buffer supplemented with 1% Triton X (30 min at 4ºC) and centrifuged (45,000xg for 15 min); the resultant supernatant, containing solubilized cytoplasmic membranes and mitochondrial fragments, was designated as the particulate fraction.

PKCε in the cytosolic and membrane fractions was assessed 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-polyacrylimide 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 PKCε-specific antibody (Transduction Laboratories), developed using a chemofluorescence system (ECF kit, Amersham), detected using Molecular Dynamics Storm system (Amersham) and band intensity quantified using ImageQuant software (Amersham). To minimize any confounding influence of variability among gels, each gel was loaded with cytosolic and particulate fractions from 4 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 PKCε in the cytosolic/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 averaged.

Statistics

As 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 using 2-factor analysis of variance (for treatment and time) with replication, while AR (expressed as a % of the left
ventricle (LV) and AN (expressed as a % of the AR) were compared by 1-factor analysis of variance. All post-hoc pairwise comparisons were made using the Newman-Keuls test. Infarct sizes in all groups that received interventions were further compared to vehicle controls by analysis of covariance, incorporating risk region – the major determinant of infarct size in the rabbit – as the covariate. For Protocol 2, normalized values of PKCε in the cytosolic and particulate fractions were compared among groups by 1-factor analysis of variance and the Newman-Keuls post-hoc test. All data are reported as mean + SEM.

RESULTS

Protocol 1A: Adult Rabbits

Hemodynamics (Table 1)

Heart rate was comparable among all groups, both at baseline and throughout the protocol. Mean arterial pressure was, by chance, higher at baseline in rabbits later assigned to receive PKCε-TIP. However, arterial pressure was not significantly altered by PKCε-TIP, scrambled peptide or chelerythine treatment per se.

Risk region and infarct size (Figure 2)

Mean values of AR for the 6 treatment groups ranged from 29±3% to 35±3% of the total LV weight (p=ns).

Infarct size was, as expected, significantly reduced with preconditioning: AN/AR averaged 34±4% vs 53±4% in the vehicle + PC versus vehicle + control groups, respectively (p<.05). The protective effect of PC was abrogated both by chelerythine, and, most notably, by PKCε-TIP. In contrast, administration of scrambled peptide had no effect on the efficacy of PC, and PKCε-TIP did not alter infarct size in controls.

These results – i.e., significant cardioprotection in the PC and scrambled PKCε-TIP + PC groups vs vehicle controls, but no protection in the chelerythine + PC and PKCε-TIP + PC cohorts – were confirmed by ANCOVA (not shown).

Protocol 1B: Old Rabbits

Hemodynamics (Table 2)

There were no group differences in heart rate or mean arterial pressure at any time during the protocol.
Risk region and infarct size (Figure 3)

Values of AR/LV were similar to those observed in adults, with no differences among the 7 treatment groups.

Mean AN/AR in vehicle-control rabbits was 51±6%. The efficacy of PC-induced cardioprotection was maintained in ~4 year animals, with infarct size reduced to 34±3% in the vehicle + PC group (p<.05). However, in contrast to observations made in the adult cohort, neither chelerythine, PKCε -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 PKCε -TIP – were corroborated by ANCOVA (not shown).

Protocol 2 (Figure 4)

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

Hearts from ~4 year old rabbits tended to exhibit lower PKCε 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 PKCε in the particulate fraction (Figure 4), and the ratio of PKCε in the particulate/cytosolic fractions remained ~unchanged at 0.98 and 1.00 in the old sham and old PC subsets.

DISCUSSION

We report that, in ~4 year old rabbits – a model shown previously by our group to exhibit definitive morphologic and functional hallmarks of cardiovascular aging [21] – PC-induced cardioprotection was maintained despite treatment with either PKCε translocation inhibitor peptide or chelerythrine. Moreover, preliminary evidence indicates that, in old rabbits, PC ischemia was not associated with a subcellular redistribution of PKCε. Thus, in contrast to observations made in adult animals, our results suggest that activation/translocation of PKCε is not required for infarct size reduction with PC in the aging cohort.

Preconditioning in adult rabbits: role of PKCε

Many previous studies have concluded that, in adult rabbit heart, preconditioning is mediated at least in part by PKC; i.e., pharmacologic agonists mimicked, while chelerythrine and other PKC antagonists
reportedly blocked, the reduction in infarct size achieved with PC (reviewed in [22,26]). Our current results demonstrating abrogation of PC-induced cardioprotection with chelerythrine in the adult population are consistent with this concept. Subsequent investigations, employing quantitative Western immunoblotting, further implicated the specific involvement of the PKCε: 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 PKCε-TIP – but, importantly, not the scrambled peptide – blocked PC-induced cardioprotection in adult rabbits provides in vivo pharmacologic evidence for the specific role of PKCε translocation in infarct size reduction with PC. Moreover, our ancillary observation of an increase (albeit modest) in PKCε 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 phenomenon of ischemic preconditioning, the vast majority have employed juvenile or adult models. Indeed, although several protocols have evaluated surrogate indices of cardioprotection (i.e., recovery of left ventricular function) in hearts harvested from senescent animals [2,3,28], only 3 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 18-20 month old animals. In contrast, we have found that, in ~4 year old rabbits exhibiting conclusive evidence of cardiovascular aging, there was no loss in the in vivo efficacy of PC [21] – an observation confirmed in the present study. Clinical studies have yielded similar disagreement. There are reports that preinfarct angina fails to elicit protection in aging patients with acute MI [1,10,15], while, on the contrary, 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 years [18]. Thus, there is no current 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, PKCε and cardioprotection

We found, in our in vivo rabbit model, that infarct size reduction with PC was abrogated by PKCε translocation inhibitor peptide in adult rabbits while, in contrast, PKCε-TIP, even at double the dose administered to adults, failed to attenuate PC-induced cardioprotection in the ~4 year old cohort. These data could be interpreted to suggest that: (1) PKCε activation/translocation is impaired in aging rabbit hearts; or, alternatively, (2) PKCε 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 2 possibilities, we utilized Western immunoblotting to obtain insight into potential differences in the expression and subcellular distribution of PKCε in adult versus ~4 year old rabbit hearts, with versus without brief PC ischemia.

Our results in Protocol 2 indicate that, for uniform protein loads, PKCε immunoreactivity was ~10-25% lower in subcellular fractions isolated from ~4 year old rabbits versus 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 PKCε in hearts harvested from senescent animals [14]. However, it remains unclear whether this reduction in PKCε immunoreactivity per unit protein truly reflects a decrease in PKCε content within the myocytes – perhaps due to a marked, ~2- to 8-fold increase in protein phosphatase activity seen by our group (unpublished observation) 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 % collagen content) documented previously in the characterization of our ~4 year old rabbit cohort [21].

We further found that, in contrast to observations made in adults, 5 min of brief PC ischemia did not evoke an increase in PKCε in the particulate fraction in hearts from ~4 year old rabbits. Although there is little 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 PKCε 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 ~1-2 year old rats [14,28]. However, not all studies have identified PKCε as the specific isoform affected by aging: data obtained from isolated rat cardiomyocytes showed an age-associated decrease in the translocation of PKCδ, but not PKCε, following exposure to phorbol myristate acetate [27]. It must be acknowledged that, in our ancillary protocol, our
primary endpoint was PKCε immunoreactivity (rather than phosphotransferase activity), results were obtained at only 1 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 PKCε-TIP to attenuate PC-induced cardioprotection in old rabbits was due to impaired translocation of PKCε – rather than augmented translocation and insufficient dosing – in the old cohort.

Implications and future directions

It has been hypothesized, based on 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 current data are consistent with the concept of impaired PKCε transduction 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 PKCε.

These data raise the question: what kinase(s) or other cellular mediators assume the role of PKCε in eliciting infarct size reduction with PC in the aging hearts? One obvious possibility is that other PKC isoforms, in lieu of PKCε, 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 – albeit 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 pharmacologic inhibition of PKC alone was found to be insufficient to block the benefits of preconditioning [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 versus aging rabbit heart. Nonetheless, our current results demonstrate that, in rabbit, the
mechanisms responsible for PC are age-related, with no apparent, requisite role of PKCε in the aging cohort.
REFERENCES


**Table 1.**
**Hemodynamics: Adult Rabbits**

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<th>Occlusion:</th>
<th>Reperfusion:</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Pre-Occlusion</td>
</tr>
<tr>
<td><strong>HEART RATE (beats/min):</strong></td>
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<tr>
<td>Vehicle + Control</td>
<td>159±7</td>
<td>158±7</td>
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<tr>
<td>Vehicle + PC</td>
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<tr>
<td>PKCε-TIP + Control</td>
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<td>PKCε-TIP + PC</td>
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<tr>
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<td>172±10</td>
<td>164±4</td>
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<tr>
<td>Chel + PC</td>
<td>188±13</td>
<td>182±13</td>
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| **MEAN ARTERIAL PRESSURE (mm Hg):** |        |                |        |        |       |
| Vehicle + Control | 84±5   | 79±5           | 68±3 † | 64±3 † | 48±3 †|
| Vehicle + PC     | 91±5   | 84±3           | 73±2 † | 70±2 † | 59±3 †|
| PKCε-TIP + Control | 96±3 * | 90±3 *         | 76±3 † | 70±3 † | 56±3 †|
| PKCε-TIP + PC    | 94±3 * | 84±2           | 75±2 † | 71±2 † | 55±2 †|
| Scram-PKCε-TIP + PC | 74±11  | 69±7           | 66±6   | 62±7 † | 43±6 †|
| Chel + PC        | 77±3   | 74±3           | 62±2 † | 59±2 † | 44±2 †|

Chel = Chelerythrine; PC = Preconditioned; PKCε-TIP = PKCε translocation inhibitor peptide; Scram = scrambled

† p<.05 versus Baseline; * p<.05 versus Vehicle + Control
Table 2.
Hemodynamics: ~4 Year Old Rabbits

<table>
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<td>142±6</td>
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<td>PKCε-TIP (×2) + PC</td>
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<td>Chel + Control</td>
<td>133±6</td>
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<td>Chel + PC</td>
<td>151±5</td>
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<td><strong>MEAN ARTERIAL PRESSURE</strong> (mm Hg):</td>
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<td>Vehicle + Control</td>
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<td>Vehicle + PC</td>
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Chel = Chelerythrine; PC = Preconditioned; PKCε-TIP = PKCε translocation inhibitor peptide; (×2) = double dose

† p<.05 versus Baseline
FIGURE LEGENDS

**Figure 1:** Schematic illustration of experimental protocols. ■ = episodes of coronary artery occlusion.

**Figure 2:** Risk region expressed as a % of the total left ventricular (LV) weight (top) and area of necrosis expressed as a % of the risk region (bottom) for Protocol 1A: adult rabbits. PC = preconditioned; PKC = protein kinase C; PKCε-TIP = PKCε translocation inhibitor peptide.

**Figure 3:** Risk region expressed as a % of the total left ventricular (LV) weight (top) and area of necrosis expressed as a % of the risk region (bottom) for Protocol 1B: ~4 year old rabbits. Abbreviations as in Figure 2.

**Figure 4:** PKCε immunoreactivity in cytosolic and particulate fractions for adult and ~4 year old rabbits subjected to brief preconditioning ischemia or uninterrupted perfusion (sham). **Top:** Representative immunoblot. **Bottom:** Mean PKCε immunoreactivity in the particulate fraction, normalized to adult sham values. *p<.05 versus adult shams; † p<.05 versus adult ischemic values.
Figure 1.

Protocol 1A and 1B:

Sham-Control

Preconditioned

20 min
30 min
3 hours

atrial bolus: vehicle/drug

30 min
3 hours

infarct size

Protocol 2:

Sham-Control

Preconditioned

5 min

subcellular distribution of PKCε
Figure 2.

Area at Risk (% of LV)

Necrosis (% of Risk Region)

* p<.05 vs Control; † p<.05 vs PC
Figure 3.

**Area at Risk (% of LV)**

![Area at Risk Bar Chart](chart1.png)

**Necrosis (% of Risk Region)**

![Necrosis Bar Chart](chart2.png)

* p<.05 vs Control
Figure 4.