Effect of aging on the ability of preconditioning to protect rat hearts from ischemia-reperfusion injury

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Schulman, Daniel, David S. Latchman, and Derek M. Yellon. Effect of aging on the ability of preconditioning to protect rat hearts from ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 281: H1630–H1636, 2001.—Ischemic preconditioning (IP) reduces infarct size in young animals; however, its impact on aging is underinvestigated. The effect of variations in IP stimuli was studied in young, middle-aged, and aged rat hearts. Isolated hearts underwent 35 min of regional ischemia and 120 min of reperfusion. Hearts with IP were subjected to either one ischemia-reperfusion cycle (5 min of ischemia and 5 min of reperfusion per cycle) or three successive cycles before 35 min of regional ischemia. Additional studies investigated the effects of pharmacological preconditioning in aged hearts using the adenosine A1 receptor agonist 2-chloro-N6-cyclopentyladenosine, the protein kinase C analog 1,2-dioctanoyl-sn-glycerol, and the mitochondrial ATP-sensitive potassium (KATP)-channel opener diazoxide. Infarct sizes indicated that the aged rat heart could not be preconditioned via ischemic or pharmacological means. The middle-aged rat heart had a blunted IP response compared with the young adult (only an increased IP stimulus caused a significant reduction in infarct size). These results suggest that there are defects within the IP signaling cascade of the aged heart. Clinical relevance is important if we are to use any IP-like mimetics to the benefit of an aging population.

infarct size; adenosine; protein kinase C; ATP-sensitive potassium channels

THE HEARTS OF AGED ANIMALS are less tolerant than those of young-adult animals to ischemia-reperfusion injury (27, 40). Anatomical, mechanical, ultrastructural, and biochemical alterations may compromise the adaptive responses of the aged heart (24, 37). cardioprotection through ischemic preconditioning (IP) (34, 44), where transient myocardial ischemia results in acute and delayed protection against a sustained myocardial ischemic insult, is one area of research that is of increasing interest in the aging animal model. It is of particular relevance if we are able to understand the pathological processes that occur in the aging heart and are then able to devise the means to overcome the defects of the age-related process. Although acute IP has been shown not to protect against stunning even in young healthy models (7, 35), research (using function as an endpoint) has also demonstrated a failure of IP to benefit left ventricular (LV) contractile recovery in the aged rat heart (2, 42). With respect to the primary endpoint of IP (namely, infarction), a recent publication (13) has shown the failed ability of ischemic preconditioning to reduce infarct size in the aged heart.

A large number of signal transduction pathways have been extensively studied in physiologically healthy young rat hearts; however, a developing body of research already demonstrates abnormalities within various signaling cascades in the aged rat heart. Age-related changes in local adenosine release, membrane-receptor sensitivity, and cardiac responses to adenosine (33) may account for the increase in susceptibility to ischemia-reperfusion injury (15, 22). Alterations in G proteins do occur in different physiological and pathological states [e.g., hypertension, heart failure, and aging (25, 31, 38)] and probably contribute to altered responses associated with those conditions.

Our hypothesis was to determine whether IP would be attenuated in the aged rat heart, and if so, whether there was a variable change in the preconditioning response with age. If changes were noted, would increasing the preconditioning stimulus affect a change in infarct size? Furthermore, we wanted to investigate the effects of pharmacological preconditioning in the reduction of infarct size in the aged rat hearts compared with young-adult hearts. The drugs used included the selective adenosine A1 receptor (A1R) agonist 2-chloro-N6-cyclopentyladenosine (CCPA), the protein kinase C (PKC) analog 1,2-dioctanoyl-sn-glycerol (DOG), and the mitochondrial ATP-sensitive K (KATP)-channel opener diazoxide. These drugs, which have been demonstrated to reduce infarct size in young-adult rat hearts, were used in this study to target the actions of a key trigger, mediator, and effector, respectively, of the classic ischemic preconditioning response.
METHODS

The animals utilized for experiments performed in this study were cared for and used in accordance with the UK home-office guidelines set out in the Animals (Scientific Procedures) Act in 1986.

In vivo blood pressure measurement. Before the Langendorff-based experiments could be performed, accurate in vivo blood pressure measurement was needed to ascertain whether the aged hearts would need to be buffer perfused at differing perfusion pressures. Three age categories of male Sprague-Dawley rats were studied: young adults (age 3 mo, weight 300–350 g, n = 8), middle-aged adults (age 12 mo, weight 700–840 g, n = 8), and aged adults (age 18–20 mo, 670–850 g, n = 8). All rats were fed a standard diet and were anesthetized with pentobarbital sodium (40–50 mg/kg ip). Once surgical anesthesia was obtained, a tracheostomy was rapidly performed and the rat was mechanically ventilated (Harvard Apparatus; Edenbridge, UK). The right carotid artery was cannulated for measurement of hemodynamic, blood-chemistry and pH parameters. Body-core temperature was monitored using a rectal thermometer and was maintained at 37.0 ± 0.5°C. Constant blood pressure monitoring was achieved via a three-way connection between the carotid cannula and a hydrostatic pressure transducer (P23 XL; Gould Instrument Systems; London, UK). After a 15-min stabilization period, pulse rate and systolic, diastolic, and mean blood pressures were recorded.

Isolated rat-heart preparation. The hearts were excised, placed in ice-cold Krebs-Henseleit buffer, and rapidly mounted onto the aortic cannula of a Langendorff perfusion system. Perfusion was established within 30 s after thoracotomy. The Krebs-Henseleit buffer (pH 7.4, 95% O2-5% CO2) contained (in mM): 118 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.8 CaCl2·2H2O, 25.2 NaHCO3, and 11 glucose. The perfusion pressure was maintained at 80 mmHg, and the myocardial temperature was kept at 37.0 ± 0.5°C. A water-filled latex balloon that was connected to a hydrostatic pressure transducer (P23 XL; Viggo-Spectramed) and coupled to a recorder (Multitrace 2; Lectromed) was inserted into the left ventricle through an incision in the left atrial appendage and inflated to set an end diastolic pressure of 5–10 mmHg. Coronary flow was measured by timed collection of effluent measured over 1 min at each sampling point. A 3-0 silk suture was passed around the main branch of the left coronary artery, and the ends were threaded through a small vinyl tube to form a snare. Regional ischemia was achieved by pulling the snare and confirmed by a substantial decrease in both LV developed pressure (LVDP) and coronary flow (CF). All hearts underwent 15 min of stabilization before being subjected to 35 min of regional ischemia and 2 h of reperfusion (control group). Baseline values for functional parameters were obtained after 5 and 15 min. Heart rate (HR), CF, and LVDP were measured at regular intervals throughout the experiments.

Ischemic preconditioning protocol. Nine groups were included in this study (three from each age category: young adult, middle-aged, and elderly; see Fig. 1A). Within each age category, one group (the controls) underwent 35 min of regional ischemia and 2 h of reperfusion, whereas the second group had a single 5-min preconditioning stimulus before 35 min of regional ischemia and 2 h of reperfusion (1PC), and the third group had three successive preconditioning stimuli before 35 min of regional ischemia and 2 h of reperfusion (3PC). Each preconditioning stimulus involved 5 min of global ischemia before 5 min of reperfusion.

Pharmacological preconditioning protocol. In a subsequent study, young-adult and aged rat hearts were pretreated with either 200 nM CCPA, 30 μM DOG, or 30 μM diazoxide (see Fig. 1B). Each drug was used alone within each age group, and was perfused for 10 min with a subsequent 5-min wash-out period before the standard 35-min of regional ischemia and the 2-h reperfusion protocol. The hearts treated with CCPA, and their respective control groups were paced either at 300 beats/min (in the young-adult hearts) or 240 beats/min (in the aged hearts). DOG was dissolved in a final solution of 0.02% DMSO. This vehicle was also used in the respective control groups. All drugs were supplied by Sigma Chemical (Dorset, England).

Determination of infarct size. At the end of each experiment (after 2 h of reperfusion), the silk suture was reocccluded and a 5% solution of Evan’s blue dye was infused into the perfusate to mark the risk zone as unstained (not blue) tissue. The hearts were then frozen and cut into 2-mm-thick slices parallel to the atrioventricular groove. The slices were thawed and incubated in a 1% tetrozolium chloride (TTC) phosphate-buffered solution (pH 7.4) at 37°C for 15 min and fixed in 10% formalin to enhance the contrast of the Evan’s blue and TTC staining. Slices were then compressed to a uniform 2-mm thickness by placing them between two glass plates separated by a 2-mm spacer. The areas of the left ventricle (stained blue), the risk zone (stained red by TTC), and the infarcted region (unstained) were traced onto acetate...
transparencies. With the use of a computerized planimetry program (Summa Sketch II; Summa Graphics; Seymour, CT), the respective volumes were calculated. Infarct size was expressed as a percentage of risk zone. All measurements were performed in a blinded fashion.

**Statistical analysis.** Data from the experiments are expressed as means ± SE. Differences between the means were compared using Student's t-test. The LV, risk, and infarct volumes were tested for group differences by one-way ANOVA. Comparisons of CF (normalized to the heart weight and expressed as milliliters per minute per gram of tissue) and rate-pressure product (RPP; calculated from HR × LVDP) were performed by repeated-measures ANOVA. *P < 0.05 was considered significant.

**RESULTS**

A total of 92 young-adult, 38 middle-aged, and 60 aged male Sprague-Dawley rats were used in the described experiments. Results from the in vivo measurement of blood pressure (see Table 1) showed there to be no significant difference in mean arterial pressure between young, middle-aged, and aged male Sprague-Dawley rats. All of the isolated hearts were then perfused at the same pressure (80 mmHg) as is accepted in this well-characterized model. Hypertension was not documented in any of the rats.

**A single 5-min preconditioning stimulus significantly reduced infarct size in young-adult but not middle-aged or aged rat hearts.** There was no significant difference in infarct size in the control experiments between rats of the three different age groups (35 min of regional ischemia and 2 h of reperfusion; refer to Fig. 2). Young-adult rats had a control infarct size of 51.8 ± 2.2% of the volume at risk (n = 24). Middle-aged rats had a control infarct size of 51.8 ± 2.2% of the volume at risk (n = 10). Aged rats had a control infarct size of 51.5 ± 5.7% of the volume at risk (n = 10).

After a single cycle of preconditioning (1PC), the infarct size of the young-adult rat hearts significantly reduced to 20.5 ± 3.2% of the volume at risk (n = 12; P < 0.01). In contrast, no reduction in infarct size was achieved in the middle-aged or aged rat hearts after 1PC (45.8 ± 5.5% of the volume at risk, n = 8; and 45.1 ± 4.0% of the volume at risk, n = 8, respectively).

**Three 5-min preconditioning cycles significantly reduced infarct size in young-adult and middle-aged but not aged rat hearts.** The control experiments remained as for the 1PC study (refer to Fig. 2). In the 3PC study, the young-adult rats had an infarct size of 16.9 ± 3.4% of the volume at risk (n = 8). This result was not significantly different from the 1PC group, although it was significantly smaller than the control (51.8 ± 2.2%; P < 0.01). Most notable was that in the middle-aged rats, three preconditioning cycles were able to significantly reduce infarct size compared with the control [29.6 ± 2.1% (n = 12) vs. 51.8 ± 2.2% (control) of the volume at risk; P < 0.01]. There was again no significant reduction in infarct size in the aged rat hearts [40.5 ± 4.5% (n = 8) vs. 51.5 ± 5.7% (control) of the volume at risk].

**CCPA treatment significantly reduced infarct size in young-adult but not aged rat hearts.** In the young-adult rats, the selective A1R agonist CCPA (200 nM) had a significant infarct-lowering effect (refer to Fig. 3). The control infarct size of 50.8 ± 2.2% of the volume at risk (n = 18) was reduced to 27.2 ± 5.2% of the volume at risk (n = 8) after treatment with CCPA (P < 0.01). However, in the aged rats, no reduction in infarct size was seen after treatment with CCPA [52.2 ± 5.7% (control, n = 7) vs. 57.4 ± 3.6% (CCPA, n = 6) of the volume at risk].

**DOG treatment significantly reduced infarct size in young-adult but not aged rat hearts.** In the young-adult rats, the PKC analog DOG (30 μM) had a significant effect on lowering infarct size to 26.4 ± 3.0% of the volume at risk (n = 6) compared with the control.

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**Table 1. In vivo blood pressures of young adult (3 mo), middle age (12 mo), and aged (18–20 mo) rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean BP, mmHg</th>
<th>Systolic BP, mmHg</th>
<th>Diastolic BP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young adult</td>
<td>126 ± 5</td>
<td>134 ± 6</td>
<td>117 ± 6</td>
</tr>
<tr>
<td>Middle age</td>
<td>130 ± 8</td>
<td>136 ± 8</td>
<td>114 ± 6</td>
</tr>
<tr>
<td>Aged</td>
<td>131 ± 9</td>
<td>137 ± 8</td>
<td>112 ± 8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 rats/group. BP, blood pressure.
infarcts at 50.8 ± 2.2% of the volume at risk (n = 18; P < 0.01; refer to Fig. 3). In the aged rats, no reduction in infarct size was seen after treatment with DOG [52.2 ± 5.7% (control, n = 7) vs. 60.8 ± 2.6% (DOG, n = 6) of the volume at risk].

Diazoxide treatment significantly reduced infarct size in young-adult but not aged rat hearts. In the young-adult rats, the mitochondrial K_{ATP}-channel opener diazoxide (30 μM) had a significant effect on lowering infarct size to 22.2 ± 3.8% of the volume at risk (n = 8) compared with the control infarcts at 50.8 ± 2.2% of the volume at risk (n = 18; P < 0.01; refer to Fig. 3). Once more there was no reduction in infarct size in the aged hearts after treatment with diazoxide [52.2 ± 5.7% (control, n = 7) vs. 60.2 ± 7.2% (diazoxide, n = 7) of the volume at risk].

RPP, coronary flow rate, body weight-to-heart weight ratio, and ischemic risk zone analysis. The RPP (refer to Table 2) and coronary flow rate (CFR; refer to Table 3) analyses showed there to be no significant difference between the groups at baseline (5-min stabilization). The RPP decreased significantly into ischemia, and there was only partial recovery during reperfusion. The CFR (expressed in milliliters per minute per gram of tissue) values were similar at baseline and decreased to a similar extent during regional ischemia. Mean body weight and heart weight measurements are listed in Table 3. The mean heart weight-to-body weight ratio remained similar for all three age groups (see Table 3). The ischemic risk volumes (zones) were also similar within each age group. There was no difference in the risk zone-to-LV zone ratio between the three age groups (see Table 3).

DISCUSSION

This study has highlighted a number of important issues that may well reflect on our understanding and future study of the aged population as a whole. A number of novel findings can be summarized as follows. We have demonstrated that aging is associated with a progressive loss in the preconditioning response. With a more powerful IP stimulus, the middle-aged rat heart could be preconditioned to a significant extent, whereas the aged rat heart still failed to respond to an increased preconditioning stimulus. With the use of CCPA (the selective adenosine A1 agonist), DOG (the protein kinase C activator), and diazoxide (the mitochondrial K_{ATP}-channel opener), we have found an inability to precondition the aged heart compared with the young-adult rat heart.

The possibility of death due to coronary artery disease increases progressively with age (3, 30, 43), and though a significant part of this may be the result of the reduction in the use of thrombolytic therapy, Maggioni and colleagues (30) demonstrated that age is an independent predictor of both in-hospital and postdischarge mortality rates in patients presenting with their first myocardial infarction who received thrombolytic therapy (they excluded a correlation between age-related higher mortality for myocardial infarction and more extensive coronary artery disease).

With advancing age, vital constitutive changes take place within the myocardial cell that have a substantial impact on cardiovascular function and reduce the capacity of the heart to tolerate and adapt to ischemia-reperfusion injury. Aged mitochondria have been shown to have reduced phospholipid cardiolipin and mitochondrial membrane-permeability transition pore function as well as impaired efficiency of oxidative phosphorylation and antioxidant systems (8, 11, 14, 20). This coupled with cytosolic and mitochondrial calcium overload (21, 32), opening of the mitochondrial membrane-permeability transition pore (20), increased release of reactive oxygen species and toxic lipid peroxidation products, and mitochondrial dysfunction at 50.8 ± 2.2% of the volume at risk (n = 18; P < 0.01; refer to Fig. 3). In the aged rats, no reduction in infarct size was seen after treatment with DOG [52.2 ± 5.7% (control, n = 7) vs. 60.8 ± 2.6% (DOG, n = 6) of the volume at risk].

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membrane dysfunction, results in a cascade of events leading to cell death via apoptotic and/or necrotic pathways (20, 26).

Lesněfsky and colleagues (27) showed in 1994 that LV contractile recovery after global ischemia was decreased in aged versus adult rat hearts using an isolated heart-buffer-perfused Langendorff model. In our own study, the RPP of the control aged heart versus the young heart was decreased at the end of reperfusion (though not significantly). Our aged animals were 18–20 mo old compared with 24 mo in that previous study. In addition, it must be emphasized that the interpretation of functional recovery as an endpoint of muscle injury must be viewed with caution in our model, which is not representative of a physiological “working” heart.

Interestingly, in our experiments there was no significant rise in infarct size between the young-adult and aging cohorts in the control groups studied. There is only one previous study where infarct size was used as an endpoint in aged animals (13), and there again no difference in infarct sizes between the control groups was noted. Our study is unique in investigating the effects of variable ischemic and pharmacological preconditioning stimuli in the aged rat. It is conceivable that the control infarct size in the aged animals may have been increased within an in vivo study where blood-borne and neurohumoral factors would have influence. This has yet to be tested. Indeed, using even older rats and extending the reperfusion period beyond 2 h could also have resulted in larger infarct sizes.

Although the vast majority of research into IP has focused mostly on physiologically healthy models, there is now an increasing interest in aging as a basis for investigating the IP phenomenon, probably as investigators realize that it is this segment of our population that is at greatest risk in a setting of ischemia and reperfusion (3, 30, 43). Abete and colleagues (1) suggested that in adult patients (age <65 yr), previous angina 48 h before acute myocardial infarction was responsible for a lower incidence of in-hospital deaths and congestive heart failure compared with patients without previous angina. The protective effect of previous angina was not related to use of thrombolytic therapy, antianginal drugs, or demographic variables such as previous myocardial infarction. More interestingly, however, in elderly patients (age >65 yr), previous angina was seen to have no protective effect on myocardial infarction within 48 h of the event.

These clinical findings are indirectly supported in a number of recently published experimental studies. In a small study using myocardial infarction as an endpoint after 40 min of global ischemia and 30 min of reperfusion, it was shown that 9-mo-old rat hearts could be preconditioned with ischemia; however, the protective effect was greatly attenuated compared with 3-mo-old rat hearts (4). In the study by Fenton and colleagues (13), aged rat hearts (age 22 mo) showed no reduction in infarct size in an isolated heart model of global ischemia-reperfusion after two 5-min preconditioning stimuli. In our study, we have further demonstrated that in the middle-aged rat heart, three IP cycles (as opposed to one) must be used to obtain a significantly beneficial effect in the reduction of infarct size. It is likely that with a more powerful preconditioning stimulus, greater amounts of endogenous triggers are released from the preconditioned myocardium with the importance of each preconditioning trigger being dependent on the relative concentrations of the other triggers present (39). It seems that although there was a trend toward an infarct-lowering effect with increased IP in the aged heart, no significant reduction of infarct size was achieved. After in vivo blood pressure measurements, there was no suggestion that the aged hearts were underperfused during the experiments, and the maintenance of CFR between the

Table 3. Coronary flow rate at baseline, body weight, heart weight, heart weight/body weight ratio, risk zone, and risk zone/left ventricular zone ratio analyses for all groups

<table>
<thead>
<tr>
<th>Group</th>
<th>CFR, ml/min ( \cdot )g tissue(^{-1} )</th>
<th>BW, g</th>
<th>HW, g</th>
<th>HW/BW</th>
<th>Risk Zone, cm(^2)</th>
<th>Risk Zone LV Zone</th>
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<tr>
<td>3-Mo</td>
<td>Control</td>
<td>12.0±1.0</td>
<td>316±16</td>
<td>1.2±0.0</td>
<td>3.8±0.3</td>
<td>0.60±0.02</td>
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<td></td>
<td>1PC</td>
<td>12.5±0.5</td>
<td>312±16</td>
<td>1.2±0.0</td>
<td>3.8±0.2</td>
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<td>0.56±0.02</td>
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<td></td>
<td>CCPA</td>
<td>12.0±0.9</td>
<td>305±7</td>
<td>1.2±0.0</td>
<td>3.9±0.1</td>
<td>0.49±0.06</td>
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<td></td>
<td>DOG</td>
<td>13.2±0.9</td>
<td>300±21</td>
<td>1.2±0.0</td>
<td>4.0±0.4</td>
<td>0.58±0.04</td>
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<tr>
<td></td>
<td>Diazoxide</td>
<td>14.1±0.6</td>
<td>319±8</td>
<td>1.2±0.0</td>
<td>3.8±0.3</td>
<td>0.52±0.03</td>
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<td>12-Mo</td>
<td>Control</td>
<td>12.5±1.5</td>
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<td></td>
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<td>2.3±0.1</td>
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<td>0.81±0.01</td>
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<td></td>
<td>3PC</td>
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<td>813±24</td>
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<td>0.86±0.03</td>
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<td>18-Mo</td>
<td>Control</td>
<td>11.7±0.8</td>
<td>820±20</td>
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<td></td>
<td>1PC</td>
<td>10.7±0.3</td>
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<td>2.9±0.3</td>
<td>0.83±0.04</td>
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<tr>
<td></td>
<td>3PC</td>
<td>11.9±0.6</td>
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<td>0.85±0.04</td>
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<td>0.87±0.05</td>
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<td>0.72±0.05</td>
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<td></td>
<td>Diazoxide</td>
<td>12.0±0.8</td>
<td>784±22</td>
<td>2.4±0.3</td>
<td>3.1±0.4</td>
<td>0.88±0.05</td>
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</table>

All values are means ± SE. CFR, coronary flow rate; BW, body weight; HW, heart weight; LV, left ventricular.
age groups confirmed the adequate perfusion and delivery of perfusate-borne substrates. It seems likely therefore that the preconditioning signaling machinery is not functional in the aged heart.

In all of the studies to date in which IP has been examined in the aged heart, very few investigations of the signaling mechanisms have yet been studied. In a “nonworking” isolated heart model, using contractile function as an endpoint in 50-wk-old rats (middle-aged rats), both diazoxide and DOG significantly improved function at the end of a short reperfusion period compared with control hearts (41). To our knowledge, we are the first to test the efficacy of the adenosine A1 agonist CCPA, the direct PKC activator DOG, and the mitochondrial KATP-channel opener diazoxide to reduce infarct size in an aged model. Our rationale for using CCPA, DOG, and diazoxide in the study was based on the accepted understanding of the known signaling mechanisms. All doses of drugs used were in the upper range of those used in previous studies to rule out the possibility of “underdosing” the aged hearts.

Studies using rat ventricular tissue have demonstrated that adenosine A1R-mediated inhibition of adenylyl cyclase activity is attenuated during aging (16). This decrease in function is related to a loss in high-affinity A1R binding sites, which results in diminished coupling of A1R to associated Gα and Gβ proteins (19). Interestingly, several reports have shown that adenosine production in the heart is higher in the aged than in the young (9, 12). Thus the reduction in A1R function, which may be mediated by the uncoupling of the receptor from the G protein, may be an adaptive response to A1R overstimulation (5, 36). Conversely, the increased production of adenosine in the ventricles of old rats may be a compensatory response that develops during aging in an attempt to maintain the protective effects of adenosine as A1R function decreases with aging. Either way, there is an age-related decline in ventricular A1R function that may contribute to impaired cardioprotection by adenosine in hearts of old rats.

The study of PKC within the framework of aging and ischemia-reperfusion injury has resulted in some interesting data (23). In aged rats, after an isolated buffer-ischemia-reperfusion injury has resulted in some inter-

In conclusion, it would seem that the aged heart is accompanied by an impaired ability to translate both endogenous stress signals and pharmacological activators into biochemical steps necessary for induction of the cardioprotective response. Further investigation is needed to fully understand the mechanisms of this altered signaling cascade if we hope to transfer some clinical benefits of preconditioning to our aging patients.

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REFERENCES


