CARDIOPULMONARY SUPPORT AND PHYSIOLOGY

DELAYED MYOCARDIAL PRECONDITIONING BY α 1-ADRENOCEPTORS INVOLVES INHIBITION OF APOPTOSIS

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Objective: Previous studies have demonstrated that α 1-adrenoceptor activation increases myocardial resistance to ischemic injury 24 hours later. Here we tested the hypothesis that delayed protection is associated with limited infarction and involves altered expression of pro-apoptotic and/or anti-apoptotic proteins. **Methods:** Rabbits were treated with phenylephrine or an equivalent volume of vehicle (n = 6 per group). Twenty-four hours after pretreatment, isolated hearts were perfused with a bovine erythrocyte suspension in modified Krebs solution, subjected to 45 minutes of global ischemia (37°C), and reperfused for 120 minutes. Infarct size was determined by triphenyltetrazolium chloride staining. Apoptosis was quantified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. Left ventricular tissue from separate groups of animals (n = 5 per group), 24 hours after pretreatment with phenylephrine or vehicle but without ischemia and reperfusion, was analyzed by Western blotting for content of the anti-apoptotic protein, bcl_x, and pro-apoptotic protein, bax. **Results:** Isolated hearts after phenylephrine pretreatment had increased end-reperfusion developed pressures (56.8 ± 4.9 vs 36.2 ± 3.9 mm Hg for vehicle, P = .008) and decreased elevated end-diastolic pressures (26.7 ± 4.5 vs 42.3 ± 5.0 mm Hg for vehicle, P = .04). Phenylephrine pretreatment abrogated infarction (9.9 ± 2.4% vs 42.6 ± 6.3% for vehicle, P = .002) and reduced the number of apoptotic nuclei (24 ± 4.8 vs 51 ± 4.6 for vehicle, P = .038). Analysis by Western blotting showed that the ratio of bcl_x to bax protein increased in phenylephrine-pretreated hearts (2.65 ± 0.5 vs 1.0 ± 0.1 for vehicle, P = .008). **Conclusion:** Delayed myocardial protection to infarction mediated by α 1-adrenoceptor activation involves an increased bcl_x/bax ratio, thereby limiting apoptotic cell death. (J Thorac Cardiovasc Surg 1999;117:980-6)

Previous work suggests that pharmacologic interventions¹⁻⁸ and short periods of ischemia-reperfusion (IR)^{9,10} can limit myocardial infarction 24 hours later. Although restriction of the infarcted area is well docu-

mented as an important therapeutic goal in limiting left ventricular (LV) dysfunction and arrhythmia,^{11,12} the mechanism of this cardioprotective response is not well understood. Data from several in vivo and in vitro models of IR injury suggest that free radicals and disruption of metabolic homeostasis are important contributory factors in myocardial cell death. Thus cytoprotection is thought to involve increased expression of proteins that either suppress free radical accumulation such as superoxide dismutase and catalase^{6,13,14} or maintain metabolic homeostasis.^{1,5,7,10,15} However, several agents can induce delayed ischemic tolerance in intact myocardium^{6,16,17} without increasing the expression of antioxidants and stress-response proteins.^{5,16,17} Whether these diverse agents activate a common protective mechanism

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Fig 1. Treatment protocols used. In protocol 1, isolated hearts undergo global ischemia (time [0]) and reperfusion 24 hours after phenylephrine or vehicle pretreatment. In protocol 2, hearts are harvested 24 hours after pretreatment for molecular studies.

and what factors mediate delayed protection remain unknown.

Myocardial infarction in reperfused ischemic myocardium results from regulated cell death (apoptotic) and unregulated cell death (non-apoptotic).^{18,19} Whereas non-apoptotic cell death is rapid, the initiation and execution of apoptotic cell death is a slower process involving multiple pathways. Common to all of these pathways is the activation of proteases, called *caspases*. In response to oxidative stress, the regulation and initiation of caspase activity is governed by the bcl-2 family of proteins,^{17,18} which either retard (eg, bcl-2, bcl_x) or promote (eg, bax) apoptosis. Thus the question arises as to whether cardioprotective agents inhibit caspase activity and thereby infarction by altering the ratio of anti-apoptotic protein(s) to pro-apoptotic protein(s). If so, then modulation of bcl-2 gene products may provide a general mechanism of delayed cardiac protection.

Here we determined whether α 1-adrenoceptor activation confers delayed ischemic tolerance to the myocyte by modulating the ratio of the anti-apoptotic protein, bcl_x, to the pro-apoptotic protein, bax, thereby limiting apoptosis.

Methods

The animal protocol was reviewed and approved by the Animal Care and Research Committee of the Medical College of Virginia/Virginia Commonwealth University. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 85-23, revised 1985).

Experimental protocols. Two experimental protocols were used (Fig 1). In protocol 1, New Zealand White rabbits (n = 12, 3-4 kg) were randomly assigned to 1 of 2 groups: phenylephrine pretreatment (50 μ g/kg, intravenously) or saline vehicle (n = 6 per group). Successful activation of the

Fig 2. Comparison of diastolic (circles) and systolic (squares) pressures in isolated hearts subjected to ischemia and reperfusion 24 hours after pretreatment with phenylephrine (solid lines) and vehicle (dotted lines). Time (0) denotes onset of ischemia followed by reperfusion 45 minutes later. Data points are pressure $\pm$ SEM. Asterisk (*) denotes significant differences compared with time-matched EDPs after vehicle pretreatment (*t* test, n = 6).

α -adrenoceptors was confirmed in pilot studies where phenylephrine bolus administration caused a near doubling of the mean arterial pressure that gradually returned to baseline over the next 15 minutes. Twenty-four hours later, isolated hearts were perfused as described below. The isolated hearts underwent 60 minutes of equilibration during which the intraventricular balloon was incrementally inflated until a stable diastolic pressure of 5-10 mm Hg was reached. Subsequently, the hearts underwent 45 minutes of normothermic global ischemia followed by 120 minutes of reperfusion. At the end of reperfusion, hearts were rapidly harvested

Fig 3. Left ventricular sections stained with TUNEL and FI-dUTP after ischemia and reperfusion 24 hours after pretreatment. No counterstain was used to allow clear identification of positive staining nuclei for apoptosis (arrows). **a**, Vehicle. **b**, Phenylephrine. **c**, No nuclei are stained in the absence of deoxynucleotidyl transferase (negative control). Bar = 55 μ m.

and LV tissue rapidly processed for triphenyltetrazolium chloride (TTC) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL).

In protocol 2, rabbits were randomly assigned to 1 of 2 groups: phenylephrine pretreatment (50 μ g/kg, intravenously) or saline vehicle ($n = 5$ per group). Twenty-four hours later, each rabbit was anesthetized with pentobarbital (65 mg/kg, intravenously) and given heparin (1000 U/kg, intravenously). The heart was excised. The LV was rapidly divided and frozen in liquid nitrogen for analysis by Western blotting.

Isolated heart model. Experiments using an isolated beating rabbit heart preparation were performed as described previously by our group.²⁰ In brief, rabbits were anesthetized with a ketamine/xylazine mixture (50 mg/kg, 25 mg/kg, intramuscularly, respectively), ventilated with oxygen, and heparinized (1000 U/kg, intravenously). A thoracotomy was performed followed by cardiectomy in rapid sequence. Retrograde coronary perfusion was established via the aortic root (100 cm H₂O) with a total ischemic period of less than 30 seconds. The perfusate consisted of modified Krebs-Henseleit buffer (NaCl 103 mmol/L, KCl 4.0 mmol/L, KH₂PO₄ 1.2 mmol/L, NaHCO₃ 30 mmol/L, MgSO₄ 1.2 mmol/L, ethylenediaminetetraacetic acid 0.4 mmol/L, lactate 1.0 mmol/L, dextrose 11 mmol/L, palmitic acid 0.4 mmol/L, and albumin 4 g/dL) containing bovine erythrocytes at a hematocrit value of 40%. An LV vent was placed in the apex. A water-filled latex balloon connected to a pressure transducer and chart recorder (Gould, Inc, Cleveland, Ohio) was

inserted into the LV through the mitral orifice and secured with a purse-string suture. The pulmonary artery was cannulated for collection of coronary venous flow and the instrumented heart was submerged in saline solution (37°C). Before and periodically during the experiment, blood gas values were measured and maintained within the normal physiologic range. The hearts were paced at 180 beats/min (SDS9 Stimulator, Grass Instrument Co, Quincy, Mass). Physiologic data including heart rate, diastolic pressure, systolic pressure, and coronary flow were measured at the end of equilibration, ischemia, and every 15 minutes during reperfusion.

TTC staining. For evaluation of cellular necrosis, the isolated hearts pretreated with phenylephrine or saline vehicle and subjected to protocol 1 were stained with TTC according to the method of Fishbein and associates.²¹ In brief, the hearts were rinsed with chilled saline solution and the atria and approximately 3 mm of the apex were removed. The remaining LV tissue was divided into 5 transverse slices (5-mm thick), immersed in 1% TTC for 20 minutes (37°C), followed by fixation in 10% formalin (60 minutes) and then photographed. Viable tissue stains red in this assay and infarcted areas are nonstaining (white).

TUNEL. In the same hearts subjected to protocol 1, apoptosis was measured. For this assay, the middle (third) slice of unfixed LV, before processing for TTC staining, was used. LV slices were immersed in 5% paraformaldehyde for 60 minutes (at 23°C) and then impregnated with 30% sucrose in phosphate-buffered saline solution (4°C) over night. Slices

were frozen in liquid nitrogen and cyrosectioned. Next, 10- to 15- μ sections were assayed by TUNEL staining according to the manufacturer's instructions (Apoptosis Detection System, Promega, Madison, Wis) and visualized by either fluorescence microscopy (Zeiss, 25 $\times$ lens, 0.8 NA; Carl Zeiss, Oberkochen, Germany) or confocal microscopy (Zeiss LSM410, 40 $\times$ lens, 1.25 NA). The Promega TUNEL assay has been widely used and validated in detection of apoptosis in a variety of cell lines by many investigators.²² The number of nuclei labeled with fluorescein-dUTP (Fl-dUTP) per unit area of tissue was determined by counting an average of 9 high-power fields (31 $\times$).

Western blotting. LV tissue samples (2 mg) from hearts subjected to protocol 2 were pulverized under liquid nitrogen, resuspended in sodium dodecyl sulfate (SDS) sample buffer (400 μ L, SDS 5%, Tris HCl 0.125 mmol/L, NaOH 6.25 mmol/L, glycerol 10%, β -mercaptoethanol 0.25%, and bromophenol blue 1.25%). Equivalent samples (200 μ g) were subjected to SDS-PAGE (8% polyacrylamide gel electrophoresis).²³ Proteins were transferred over night at 4°C to nitrocellulose membranes. Nonspecific protein binding was blocked with Blotto (Tris HCl 50 mmol/L, NaCl 150 mmol/L, Tween 0.05%, and powdered milk 5%, NaN_3 0.02%). To detect specific proteins, we then incubated the blots with either mouse anti-bcl_x antibody (1:500, Transduction Laboratories, Lexington, Ky), or rabbit anti-bax polyclonal antibodies (N20, 1:500, Santa Cruz Biotechnology, Santa Cruz, Calif). We screened 2 antibodies directed to different primary sequences (Santa Cruz N20 and P19) and found a positive signal with N20. The specificity of the immunologic reaction was controlled by preabsorbing the primary antibody with the corresponding synthetic antigen and by omitting the primary antibody (negative controls). Under these conditions, no specific signal was detected. As a positive control, we used lysates of Nb2FMM lymphoma cells (gift of Dr Witorsch, Medical College of Virginia, Virginia Commonwealth University). Bound antibody was detected with biotinylated secondary antibody (Vector Laboratories, Burlingame, Calif) and enhanced chemiluminescence reagents (Nycomed Amersham, Buckinghamshire, United Kingdom) according to manufacturer's recommendations. Total protein was measured by a commercial Protein Assay kit (Sigma Chemical Co, St Louis, Mo).

Data analysis. Data shown are expressed as the mean $\pm$ SEM. The number of hearts used is indicated. Autoradiographs were analyzed with the Photoshop Image analyzing program (Adobe Systems Incorporated, San Jose, Calif). Integrated densities of autoradiographic bands were determined as a function of pixel intensity per unit area. Developed pressure (DP) was calculated as systolic minus diastolic pressure at each time point. The percent infarcted area was calculated as the ratio of the infarcted to total area at risk. Nested analysis of variance and Bonferroni post hoc test (SAS STATVIEW, SAS Institute, Inc, Cary, NC) determined significant differences within multiple groups. Significant differences between 2 experimental groups were determined by the Student *t* test. The correlation between infarction and

Fig 4. Confocal micrograph of TUNEL-stained end-reperfusion myocardium 24 hours after vehicle pretreatment. TUNEL-positive DNA fragmentation is shown in myocyte (**) and endothelial (*) nuclei. Bar = 100 μ m.

functional parameters was determined by the Pearson product moment test (SigmaStat, Jandel Scientific, SPSS, Inc, Chicago, Ill).

Results

Phenylephrine pretreatment blunts contractile dysfunction by IR. The baseline systolic and diastolic pressures for the phenylephrine- and vehicle-pretreated isolated hearts did not differ significantly at the end of the 60-minute equilibration period. However, phenylephrine-pretreated hearts had significantly less contracture at the end of the ischemic period (38.7 ± 6.4 vs 55.5 ± 2.6 mm Hg for vehicle, $P = .034$). Additionally, they had better DPs at end-reperfusion (56.8 ± 4.9 vs 36.2 ± 3.9 mm Hg for vehicle, $P = .008$). The improved DP was primarily due to better resolution of the postreperfusion elevated end-diastolic pressure (EDP) in the phenylephrine-pretreated hearts (26.7 ± 4.5 vs 42.3 ± 5.0 mm Hg for vehicle, $P = .042$). The systolic pressures for the 2 groups were similar (Fig 2).

Phenylephrine pretreatment limits IR-induced infarction. The fraction of infarcted myocardium in hearts subjected to protocol 1 was determined using TTC-stained LV sections. Infarction was significantly reduced after phenylephrine pretreatment ($9.9\% \pm 2.4\%$ vs $42.6\% \pm 6.3\%$ for vehicle, $P = .002$). The reduction in infarction positively correlated with the reduction in

Fig 5. Autoradiographs depict Western blot analyses of rabbit myocardium 24 hours after pretreatment with phenylephrine or vehicle for bax and bcl_x protein levels. The molecular weight of the specific bands on the gel is noted to the right.

Fig 6. Bar graphs represent the average densitometric scores derived from the Western blot analyses for myocardial bax and bcl_x protein contents. Two pretreatment groups were analyzed: vehicle (black) and phenylephrine (white). Asterisks (**) denote significant difference compared with vehicle, $P = .003$ (t test, $n = 5$).

end-ischemic contracture ($R = +0.68$, $P = .03$) and end-reperfusion EDP ($R = +0.74$, $P = .02$) as determined by the Pearson product moment. Additionally, a significant negative correlation was present between infarction and end-reperfusion DPs ($R = -0.84$, $P = .002$).

Phenylephrine pretreatment decreases apoptosis.

Apoptotic nuclei are represented by bright spots on TUNEL-stained sections (Fig 3). The number of apoptotic nuclei significantly decreased after pretreatment with phenylephrine (24 ± 4.8 vs 51 ± 4.6 per high-power field for vehicle, $P = .038$). Confocal microscopy identified apoptotic endothelial cells in addition to myocytes. It also demonstrated condensed nuclear chromatin with fragmentation (typical apoptotic characteristics) in TUNEL-positive myocyte nuclei (Fig 4). When further analyzed by the Bonferroni post hoc test, each phenylephrine-pretreated heart differed significantly from its matched control. No significant differences were found among the phenylephrine-pretreated hearts; one of the control hearts did differ significantly from the others.

Phenylephrine pretreatment alters levels of bax but not bcl_x proteins.

The protein contents for bax and bcl_x were determined in hearts subjected to protocol 2. Autoradiographs generated using Western blot analyses of LV tissue in hearts 24 hours after phenylephrine and vehicle treatment are shown in Fig 5. The average densitometric scores for bax and bcl_x protein contents are summarized in Fig 6. The phenylephrine- and vehicle-pretreated hearts had similar levels of bcl_x (195.8 ± 8.1 and 173.8 ± 17.7 , respectively, $P = \text{not significant}$). Phenylephrine-pretreated hearts had lower levels of bax protein (86.2 ± 17.0 vs 175.8 ± 12.4 for vehicle, $P = .003$). The averaged ratios of bcl_x to bax protein content more than doubled after phenylephrine pretreatment (2.6 ± 0.5 vs 1.0 ± 0.1 for vehicle, $P = .008$).

Discussion

Previous studies of regional ischemic injury have shown that $\alpha 1$ -adrenoceptor activation can confer both acute²⁴ and delayed⁸ cardioprotection in the rabbit. Here, we evaluated the $\alpha 1$ -adrenoceptor agonist, phenylephrine, in mediating delayed protection to global ischemia. We selected a pharmacologically mediated model of delayed cardioprotection against global IR injury in the rabbit because of its potential clinical applicability in cardiac surgery. In addition, global IR eliminates the inherent variability in measuring regional myocardial function and the inevitable limitations of extrapolated global function from regional data. Additionally, global IR in a larger animal model would require cardiopulmonary bypass that may introduce confounding variables associated with extracorporeal circulation.

Our findings showed improved postreperfusion diastolic recovery (decreased EDP) and contractile function (increased DP) in hearts 24 hours after pretreatment with phenylephrine. This effect was associated with a significant reduction in infarction. This reduction in cell death directly correlated with improved

global functional parameters, suggesting that cell death is an important contributing factor to contractile impairment. Our findings of improved diastolic function in this infarct model are in contrast to improved systolic pressure associated with delayed preconditioning induced by brief IR in rabbits¹⁰ and pigs.²⁵ These findings suggest that the mechanisms limiting infarction in α 1-adrenoceptor-mediated delayed preconditioning may be distinct from that initiated by periods of IR. A possible explanation for this disparity in systolic and diastolic protection may be that different preconditioning methods recruit different endogenous protective mechanisms, therefore influencing the extent and nature of the preconditioning effect, such as differential protection against infarction and stunning. Recent unpublished data from our laboratory confirm α -adrenergic delayed preconditioning against infarction without significant amelioration of stunning.

In the heart, the α -adrenoceptor plays an important role in myocyte growth. α -Adrenoceptor activation triggers a sequel of cellular responses including activation of protein kinase C (PKC) and an increase in intracellular calcium levels. In delayed cardioprotection by repetitive IR periods, activation of PKC has been implicated as a critical event²⁵; however, the proteins that mediate delayed preconditioning by ischemia are unknown. In macrophages the PKC activator, 12-O-tetradecanoylphorbol-13-acetate, blocks nitric oxide induced apoptotic DNA fragmentation and depresses levels of bax protein.²⁶ Because cell growth and cell death are opposing and coupled processes, we asked whether α 1-adrenoceptor activation by phenylephrine affords cellular protection to ischemic injury. In particular, we studied its effects on regulated cell death. Recent studies have demonstrated acute ischemic preconditioning limiting IR-induced apoptosis.²⁷ However, our current study is the first demonstration that pharmacologic pretreatment limits apoptosis and depresses levels of bax in the myocardium 24 hours later. Although an active role for PKC in this phenomenon is suggested, the identity of the intracellular signaling factors involved requires further investigation.

Using the TUNEL assay and confocal microscopy, we have shown that global IR can initiate regulated cell death in the isolated rabbit heart. Our findings support those of others^{18,19} that demonstrate apoptosis within cardiac myocytes in paradigms of ischemic injury. Additionally, we showed apoptosis in endothelial cells in the IR-injured myocardium. We found a significant effect on TUNEL staining by phenylephrine 24 hours after pretreatment, with the number of myocyte and endothelial apoptotic nuclei markedly decreased.

Several hypotheses have been proffered to explain delayed tolerance to IR injury. In general, protection is thought to involve the induction of antioxidant pathways and increased expression of stress response proteins. However, delayed cellular protection is not closely correlated with increased expression of stress response genes such as heat shock proteins and SOD.^{1,5,17} Instead, our data suggest that α 1-adrenoceptor-mediated delayed preconditioning may confer protection by altering the levels of pro-apoptotic proteins and thereby suppress programmed cell death.

Bax protein is found in various tissues including myocytes of human hearts without cardiac disease.^{18,19} It is a member of a family of homologous proteins (including bcl-2, bcl_x) that encompasses 2 groups of genes that govern apoptosis, including caspase-dependent as well as oxidant- and hypoxia-induced cell death. The anti-apoptotic proteins (bcl-2 and bcl_x) prevent cell death by binding the pro-apoptotic protein (bax).^{18,19} Once complexed with bcl-2 or bcl_x, bax is inhibited. Conversely, the pro-apoptotic proteins such as bax, when over expressed, can block the death repressor activity of bcl-2 and bcl_x.²⁸⁻³⁰ Therefore the ratio of these gene products is a critical factor in determining whether the cell survives or dies.^{18,19,25-32} Cell death can be delayed in ventricular myocytes^{31,32} by suppressing the relative content of the pro-apoptotic protein, bax. Consistent with these findings, we have observed that transient α 1-adrenoceptor activation suppressed bax protein levels 24 hours later without significant changes in bcl_x protein levels, thereby markedly altering the bax to bcl_x ratio favoring suppression of apoptosis. The altered ratio was significantly associated with decreased post-IR apoptosis, infarction, and global myocardial dysfunction. Whether the change in bax protein level reflects decreased transcriptional activity and/or increased turnover of the encoding messenger RNA and/or protein is not known. Further studies are required to explore the regulatory mechanisms involved in α 1-adrenoceptor-mediated suppression of bax protein levels, in particular the role of PKC.

In summary, we conclude that increased bcl_x/bax protein ratio is associated with myocardial tolerance to IR injury 24 hours after α 1-adrenoceptor activation. Although an increase in this ratio is sufficient to provide cytoprotection, we cannot exclude other bcl_x homologous proteins (eg, bcl-2, bad, fas, and bak) that may also be involved. Our data implicate apoptosis as a contributory factor in the pathogenesis of IR-induced cell death and suggest that targeted pharmacologic modulation of apoptosis may provide new cardioprotection modalities for use in cardiac surgical procedures.

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