Preconditioning reduces tissue complement gene expression in the rabbit isolated heart

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Tanhehco, Elaine J., Koji Yasojima, Patrick L. McGee, Ruth A. Washington, Kenneth S. Kilgore, Jonathon W. Homeister, and Benedict R. Lucchesi. Preconditioning reduces tissue complement gene expression in the rabbit isolated heart. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H2373–H2380, 1999.—Both preconditioning and inhibition of complement activation have been shown to ameliorate myocardial ischemia-reperfusion injury. The recent demonstration that myocardial tissue expresses complement components led us to investigate whether preconditioning affects complement expression in the isolated heart. Hearts from New Zealand White rabbits were exposed to either two rounds of 5 min global ischemia followed by 10 min reperfusion (ischemic preconditioning) or 10 µM of the ATP-dependent K+ (KATP) channel opener pinacidil for 30 min (chemical preconditioning) before induction of 30 min global ischemia followed by 60 min of reperfusion. Both ischemic and chemical preconditioning significantly (P < 0.05) reduced myocardial C1q, C1r, C3, C8, and C9 mRNA levels. Western blot and immunohistochemistry demonstrated a similar reduction in C3 and membrane attack complex protein expression. The KATP channel blocker glyburide (10 µM) reversed the depression of C1q, C1r, C3, C8, and C9 mRNA expression observed in the pinacidil-treated hearts. The results suggest that reduction of local tissue complement production may be one means by which preconditioning protects the ischemic myocardium.

ischemia-reperfusion injury: adenosine 5′-triphosphate-dependent potassium channels; membrane attack complex

The phenomenon of ischemic preconditioning, whereby brief episodes of ischemia protect the myocardium from a prolonged ensuing ischemic insult, has been recognized for over 10 years (29). Preconditioned hearts exhibit smaller infarct zones than naive hearts subjected to a similar ischemic event (29). Efforts to delineate the primary mechanisms of ischemic preconditioning have uncovered a number of potential mediators, including adenosine (25, 26, 41), protein kinase C (7, 47), and activation of ATP-dependent K+ (KATP) channels (14, 15). However, relatively little is known about preconditioning-induced modulation of the immune response to ischemia-reperfusion, namely its effects on complement activation.

Chemical preconditioning refers to the ability of pharmacological agents to reduce infarct size in the reperfused myocardium when administered before ischemia. Of the compounds found effective in mimicking ischemic preconditioning, KATP channel openers have been observed to be the most universal in terms of attenuating reperfusion injury in a variety of experimental models (2, 4, 27, 38). Successful employment of KATP channel openers in decreasing infarct size has led to the theory that the KATP channel represents an endogenous effector that confers cardioprotection when activated. KATP channels normally open under conditions where intracellular ATP concentrations decrease, as during tissue ischemia. The exact mechanism by which KATP channel openers ameliorate reperfusion injury remains to be elucidated. As with ischemic preconditioning, studies concerning chemical preconditioning have overlooked the effects on immunological factors that determine tissue viability.

Complement-mediated tissue damage contributes to the myocardial injury associated with ischemia-reperfusion (18). Augmented membrane attack complex (MAC) formation and assembly has been noted on irreversibly injured myocytes during reperfusion (20, 36). Several studies have demonstrated that inhibitors of complement activation attenuate myocardial reperfusion injury in vivo (1, 24, 28). It has been assumed that complement components are deposited from the plasma on reperfused tissue, resulting in MAC assembly and ultimately cell lysis. We recently reported that isolated, perfused hearts express C3 and C9 mRNAs as well as their protein products (45). This endogenous complement expression significantly increases during ischemia-reperfusion (45). The modulation of tissue complement protein synthesis during ischemia-reperfusion suggests that local generation of complement may participate in the phenomenon of reperfusion injury.

Our study sought to investigate whether ischemic or pharmacological preconditioning affects endogenous complement expression in myocardium subjected to ischemia-reperfusion. We used the rabbit isolated heart model to examine tissue complement apart from plasma-derived complement proteins. Unlike previous work concerning preconditioning, we did not measure infarct size as an end point since the rabbit hearts were made globally ischemic during preconditioning and during the prolonged ischemic insult. Our results indicate that preconditioning alters local complement activation in the reperfused myocardium.

METHODS

Guidelines for Animal Research. The procedures used in this study were in accordance with the guidelines of the...
University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association of Accreditation of Laboratory Animal Health Care, and the animal care use program conforms to the standards in The Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 86-23].

Langendorff perfused heart. Male New Zealand White rabbits (1.8-2.2 kg) were rendered unconscious by cervical dislocation. Hearts were removed, mounted, and perfused on a Langendorff apparatus with modified, oxygenated Krebs-Rabbits (1.8–2.2 kg) were rendered unconscious by cervical dislocation. Hearts were removed, mounted, and perfused on a Langendorff apparatus with modified, oxygenated Krebs-Rabbits (1.8–2.2 kg) were rendered unconscious by cervical dislocation. Hearts were removed, mounted, and perfused on a Langendorff apparatus with modified, oxygenated Krebs-Rabbits (1.8–2.2 kg) were rendered unconscious by cervical dislocation. Hearts were removed, mounted, and perfused on a Langendorff apparatus with modified, oxygenated Krebs-Rabbits (1.8–2.2 kg) were rendered unconscious by cervical dislocation. Hearts were removed, mounted, and perfused on a Langendorff apparatus with modified, oxygenated Krebs-Rabbits (1.8–2.2 kg) were rendered unconscious by cervical dislocation. 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digestion reaction was carried out for 2 h at 37°C. The digested PCR products were analyzed by electrophoresis on a 6% nondenaturing polyacrylamide gel.

Western blot analysis. Western blots were performed on the cytosolic fraction of homogenates of rabbit heart and liver.

C3 analysis. Tissue samples were homogenized in 1% SDS in Tris-buffered saline containing a protease inhibitor cocktail (Complete, Mini; Boehringer Mannheim). Homogenates were centrifuged at 7,200 g at 4°C for 15 min. The protein content of the supernatants was determined using a commercially available kit (BCA Protein Assay Kit; Pierce, Rockford, IL). Protein (40 µg) was boiled with 2 µl lithium dodecyl sulfate sample buffer (NOVEX, San Diego, CA) for 10 min. Samples were loaded on a 10% NuPAGE Bis-Tris gel. Life Technologies precasted molecular mass markers were used (GI-BCO-BRL, Gaithersburg, MD). After 50 min of electrophoresis (200 volts), the proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) at 25 volts for 90 min. Membranes were blocked in 5% nonfat milk for 1 h before incubation with anti-rabbit C3 antibody (1:500; Cappel, Costa Mesa, CA). Immunoblots were then washed twice with Tris-buffered saline 0.1% Tween 20 before incubation with anti-goat IgG (1:5,000; Sigma) labeled with horseradish peroxidase. Immunoreactivity was visualized with the Immunopure Metal Enhanced DAB Substrate Kit (Pierce Chemical) for 3 min.

MAC analysis. Heart samples were homogenized in 5-fold volume per protein extraction buffer (0.02 M Tris·HCl, pH 7.5) containing the protease inhibitors phenylmethylsulfonyl fluoride (10 µg/ml), aprotinin (10 µg/ml), and EDTA (1 mM). Homogenates were centrifuged at 18,000 g at 4°C for 30 min. The protein content of the supernatants was determined, and the samples were diluted in SDS sample buffer (60 mM Tris, pH 6.8, 2.5% SDS, 5% β-mercaptoethanol) to a final protein content of 1 mg/ml and were boiled for 3 min. Because of the high molecular mass of the MAC, modifications of the electrophoresis and protein transfer steps were required. Samples containing 10 µg of protein were loaded on a 3% polyacrylamide gel, and separation was carried out for 2.5 h at 100 V in a cold room with the apparatus surrounded by ice. The transfer to the membranes was then carried out at 100 V for 2 h in the cold. Membranes were blocked in 5% low-fat milk for 2 h. The immunoblots were then treated for 4 h at room temperature with a chicken anti-rabbit MAC antibody (1:500 dilution). The membranes were washed and treated for 3 h with a goat anti-chicken IgG (1:8,000; Sera Laboratories). Immunoreactivity was visualized by incubation with SuperSignal CL-HRO chemiluminescent substrate (Pierce Chemical). After being dried, the membranes were covered in clear plastic wrapping and were exposed to X-ray film (Hyperfilm ECL; Amersham Life Science) for 20 s.

MAC immunofluorescence. At the completion of the experiments, hearts were removed from the Langendorff apparatus, cut into transverse sections, and frozen in liquid N2. The apex and atrial tissue were discarded. Sections were embedded in optimum cutting temperature compound embedding medium (Miles, Elkhart, IN), cut at 3 µm, and placed on polylysine-coated slides. After being rinsed with PBS, sections were incubated with 4% paraformaldehyde in PBS at room temperature. Heart sections were rinsed with PBS and incubated with 1% BSA for 15 min to minimize nonspecific staining. After being rinsed with PBS, sections were incubated with a polyclonal chicken anti-rabbit MAC antibody at a 1:50 dilution at room temperature for 1 h. Sections were then rinsed with PBS and incubated at room temperature for 1 h with a donkey anti-chicken fluorescein isothiocyanate-conjugated antibody (Accurate Antibodies, Westbury, NY) at a 1:50 dilution. After a final rinse with PBS, sections were mounted with Fluoromount-G (Electron Microscopy Sciences) and protected with a coverslip. Controls included sections in which the primary antibody was omitted.

Statistical analysis. Data are expressed as means ± SE. Differences between control and experimental groups were checked for statistical significance (P < 0.05) by ANOVA followed by Student’s t-test for unpaired observations. Dunnett’s t-tests with Holm’s (17) correction for multiple comparisons was used for determining significant differences.

RESULTS

RT-PCR amplification from total RNA extracts was used to establish the presence and relative levels of the mRNAs for C1q, C1r, C3, C8, and C9 in all heart samples. Identification of PCR products from typical RT-PCR experiments is illustrated in Fig. 1. The primers chosen to amplify each cDNA yielded a single product corresponding to the anticipated size based on the known sequences. The C1q primers generated a product of 361 bp, which gave the expected fragments of 214 and 147 bp when treated with the restriction enzyme SacI. The C1r primers generated a product of 218 bp, which was cleaved by Sau3A1 to yield the predicted digestion fragments of 65 and 153 bp. The C3 primer generated a product of 298 bp, and treatment with HinclI yielded fragments of 253 and 45 bp. The C8 primers yielded a product of 441 bp, and treatment with Msel gave fragments of 160 and 281 bp. The C9 primers generated a product of 202 bp, and treatment with BamHI resulted in fragments of 125 and 67 bp. The cyclophilin primers yielded a product corresponding to the calculated size of 206 bp (data not shown). These results establish that unique reaction products were being amplified that correspond to each complement mRNA being analyzed.

The relative intensities of all gel bands were determined as described in METHODS, and the quantitative values were expressed as relative OD units. Figure 2 illustrates the significant increases (P < 0.001) of C1q, C1r, C3, C8, and C9 mRNAs in hearts exposed to reperfusion injury. Baseline mRNA values were obtained from hearts that were perfused for 5 min before removal from the apparatus (untreated group). Hearts subjected to 30 min global ischemia followed by 60 min of reperfusion exhibited significantly higher (P < 0.001) mRNA levels.
C1q, C1r, C3, C8, and C9 mRNA levels than untreated hearts that were not made ischemic. Ischemic preconditioning significantly (P < 0.001) attenuated this increase compared with nonpreconditioned hearts subjected to an identical (30 min) ischemic insult (Fig. 2). Chemical preconditioning with the K$_{\text{ATP}}$ channel opener pinacidil (10 µM) also significantly attenuated (P < 0.001) myocardial C1q, C1r, C3, C8, and C9 mRNA upregulation after ischemia-reperfusion (Fig. 2). Although the preconditioned hearts demonstrated slightly higher values for C1q, C1r, C3, C8, and C9 mRNA versus the untreated hearts, no significant difference was seen between the groups (P > 0.05) compared with group 2 hearts subjected to ischemia-reperfusion.

The K$_{\text{ATP}}$ channel blocker glyburide reversed the effects of pinacidil (Fig. 2). C1q, C1r, C8 mRNA (P < 0.001), and C3 mRNA (P < 0.02) levels were significantly greater in glyburide-pinacidil hearts compared with pinacidil-treated hearts, whereas C9 mRNA did not significantly differ between the groups (P > 0.05). Similarly, C1q, C1r, C3, and C8 mRNA levels of glyburide- and pinacidil-treated hearts were significantly greater (P < 0.001) than the ischemically preconditioned hearts. Again, C9 mRNA did not significantly differ between the groups (P > 0.05). Hearts treated with glyburide alone did not significantly differ from hearts subjected to ischemia-reperfusion for any of the complement mRNAs (Fig. 2) but demonstrated significantly higher levels for each mRNA than the ischemic preconditioned or pinacidil-treated samples.
with the nonpreconditioned ischemia-reperfusion heart. These results support the mRNA data, which indicate that both ischemic and chemical preconditioning attenuate ischemia- and reperfusion-induced complement expression in the myocardium. The MAC immunofluorescence also suggests that the rabbit myocardium is capable of synthesizing all of the complement components needed to form the MAC.

It cannot be excluded that resident leukocytes are the cells exhibiting MAC immunofluorescence. However, hematoxylin and eosin staining of isolated heart samples from other experiments performed in our laboratory demonstrate an almost complete absence of leukocytes in the preparation, diminishing the possibility that leukocytes are the source of complement proteins. Tissue samples from buffer-perfused hearts that had undergone ischemia-reperfusion were fixed in Formalin and paraffin embedded. Sections were prepared and stained with hematoxylin and eosin. Representative random sections were examined for the presence of accumulated leukocytes by a board-certified pathologist (J. W. Homeister) using light microscopy. No focalization of diffuse intravascular or interstitial accumulations of leukocytes or erythrocytes were identified in any sections, as expected in isolated buffer-perfused hearts harvested from untreated animals (unpublished observations). It is evident from the data that perfusion of isolated hearts with buffer removes blood-borne elements from the organ.

**DISCUSSION**

The results of this study demonstrate that ischemic and chemical preconditioning substantially attenuate the upregulation of C1q, C1r, C3, C8, and C9 mRNA expression and C3 and MAC protein in rabbit myocardium subjected to ischemia-reperfusion. Because complement activation advances the pathogenesis of reperfusion injury, we propose that reduction of local tissue complement expression may be one mechanism by which both ischemic and chemical preconditioning exert their cardioprotective effects. Traditionally, the success of preconditioning has been measured according to alteration of infarct size. Due to the use of global ischemia in our model, infarct size was not a suitable end point for assessment of myocardial damage. However, several studies have shown that preconditioning induced by global ischemia in isolated hearts decreases infarct size after a prolonged ischemic insult followed by reperfusion (22, 25). We modified our protocol based on previous work which demonstrated that 5 min of regional ischemia, followed by 10 min of reperfusion, reduces infarct size in the isolated heart (22, 25). The concentration of pinacidil employed in our experiments (10 µM) has also been shown to exert a cardioprotective effect (8, 16).

We previously determined that myocardial tissue locally expresses augmented C3 and C9 mRNA and the corresponding proteins after reperfusion, causing us to
The use of the rabbit isolated heart allows a clearer evaluation of the effects of preconditioning on myocardial tissue by removing the heart from the influence of plasma complement and blood cellular components. We acknowledge the difficulty of determining the importance of endogenous tissue complement in reperfusion injury in vivo. However, what is already known about complement-mediated tissue damage enables us to postulate potential roles for tissue complement in this setting. Because the MAC consists of independent subunits, tissue-expressed complement components may interact with plasma-derived complement and facilitate MAC formation. Myocardial tissue may also serve as an alternate source of complement. Last, cytokines, which are upregulated during ischemia-reperfusion, may increase local complement production. Tumor necrosis factor-α, interleukin (IL)-1, IL-6, and interferon-γ are known to stimulate complement synthesis by hepatocytes (43). Cytokines have also been shown to upregulate complement components in neural, kidney, and human umbilical cord vein endothelial cells in vitro (9, 11, 23).

Other organs, such as the brain and kidney, also appear to sustain a basal level of complement production, which is augmented in disease states (11, 35, 39, 40, 45). Preconditioning via similar mechanisms as myocardial preconditioning has been described in the brain, lungs, liver, and skeletal muscle (13, 30, 31, 33). Complement-mediated tissue damage has been implicated in reperfusion injury concerning these organs (32, 34, 43). The similarities between the mechanisms of preconditioning in these tissues and myocardial preconditioning indicate that the results of this study may provide insight regarding the mechanism of preconditioning in various organs.

The results of this study confirm and extend our previous data showing upregulation of myocardial complement C3 and C9 mRNAs in reperfusion injury. Additional data were obtained for C1q, C1r, and C8, exhibiting similar increases. Ischemic and chemical preconditioning significantly inhibits ischemia- and reperfusion-induced C1q, C1r, C3, C8, and C9 mRNA expression. Complement mRNA levels in preconditioned hearts were not significantly different from complement mRNA levels in untreated hearts. Glyburide alone also did not affect myocardial complement mRNA expression in the rabbit isolated hearts, suggesting that pinacidil may be acting to reduce complement expression via modulation of the K ATP channel. The presence of the MAC in the ischemia-reperfusion heart and its complete absence in the untreated heart indicate that ischemia-reperfusion provokes full activation of the complement cascade, including assembly of the autodestructive MAC. In addition to attenuating complement mRNA expression, ischemic preconditioning and chemical preconditioning with pinacidil reduce MAC formation.

In conclusion, we demonstrate that ischemic and chemical preconditioning inhibit the upregulation of C1q, C1r, C3, C8, and C9 mRNA expression and C3 and MAC protein expression caused by ischemia-reperfusion injury in the rabbit isolated heart model. This may represent a mechanism by which preconditioning exerts its documented cardioprotective effects. The results presented in this study suggest that tissue complement may have an important role in advancing tissue necrosis during reperfusion and that preconditioning inhibits this process.

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