Preconditioning reduces tissue complement gene expression in the rabbit isolated heart

ELAINE J. TANHEHCO, KOJI YASOJIMA, PATRICK L. MCGEER, RUTH A. WASHINGTON, KENNETH S. KILGORE, JONATHON W. HOMEISTER, AND BENEDICT R. LUCCHESI

University of Michigan Medical School, Department of Pharmacology, Ann Arbor, Michigan 48109; and Kinsmen Laboratory of Neurological Research, University of British Columbia, Vancouver, British Columbia, Canada L6T 1Z3

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–Henseleit buffer (pH 7.44, 37°C) through the aorta at a constant flow (20–24 ml/min). The perfusion medium was composed of (in mM): 118.5 NaCl, 4.7 KCl, 1.2 MgSO3, 1.2 KH2PO4, 24.8 NaHCO3, 2.5 CaCl2·2H2O, and 10.0 glucose. The buffer was passed through a membranous “lung” composed of Silastic Medical Grade Tubing (Dow Corning, Midland, MI). The membrane lung was gassed continuously with a mixture of 95% O2-5% CO2. The hearts were paced through the right atrium with electrodes attached to a laboratory stimulator (180 impulses/min, 2-ms duration, 4 V; Grass SD-5, Quincy, MA). A left ventricular drain, thermistor probe, and a latex balloon were placed via the left atrium and secured with a purse string suture at the atrial appendage. The latex balloon was expanded with water to achieve a left ventricular end-diastolic pressure of 5 mmHg. The preparation has been described in detail previously (45).

Experimental protocol. Isolated hearts were stabilized under normoxic conditions for a 10- to 15-min equilibration period before beginning the protocol. Eight experimental groups were studied. Group 1 was composed of hearts that were perfused for 5 min only; after 5 min, the hearts were removed from the apparatus and sectioned, and the tissue was frozen immediately in liquid N2 until analyzed. Group 2 (no preconditioning) consisted of hearts that had been perfused for 30 min before induction of 30 min of global ischemia, followed by 60 min of reperfusion. Group 3 (ischemically preconditioned) consisted of hearts subjected to two rounds of 5 min of global ischemia followed by a 10-min reperfusion period before induction of 30 min of global ischemia and 60 min of reperfusion. Group 4 (chemically preconditioned) consisted of hearts treated with 10 μM of the KATP channel opener pinacidil for 30 min before induction of 30 min of global ischemia followed by 60 min of reperfusion. The drug was present for the duration of the protocol. Group 5 consisted of hearts perfused with 10 μM of the KATP channel blocker glyburide for 10 min before the addition of 10 μM pinacidil, and the remainder of the protocol was identical to that of group 4. Group 6 consisted of hearts that were treated with 10 μM glyburide for 40 min before the onset of 30 min global ischemia followed by 60 min reperfusion. Glyburide was present for the duration of the protocol in groups 5 and 6. Group 7 underwent the identical protocol as group 4 except that the hearts were perfused in the presence of the pinacidil vehicle (0.02% ethanol). Group 8 was subjected to a protocol identical to group 5 except glyburide was replaced by glyburide vehicle (0.07% DMSO), and the pinacidil was replaced by pinacidil vehicle. Induction of total global ischemia was accomplished by stopping the flow of perfusate to the heart. Reperfusion of the heart was established by turning on the pump to the original flow rate. Groups 1–6 consisted of six hearts per group, and groups 7 and 8 consisted of three hearts each.

Choice of specific primers. The DNA sequences of rabbit C3, C8, C9, and cyclrophilin were obtained from the GenBank database (accession numbers M32434, U20055, L26980, and YO0052, respectively). Cyclrophilin mRNA was chosen as the internal standard because it is expressed at a relatively constant level in virtually all tissues. We previously reported on the primer sequences used to detect mRNAs for C3, C9, and cyclorphilin (45). The primer sequences chosen for amplifying C8 were as follows: forward 5'-TAAAAGACCGCA-CCAAAGGGAACAC-3' and reverse 5'-ATGAAGACCAGCGA GACCCAGAAC-3'. The DNA sequences for C1q and C1r have not been reported. We therefore used our primers for human C1q and C1r (46) to determine whether rabbit cDNA products could be obtained. Total RNA from rabbit heart was reverse transcribed, and the cDNAs were amplified with the human C1q and C1r primers by the methods previously described in detail (45, 46). Single products close to the expected sizes were obtained on polyacrylamide gels. These products were then subcloned into TEGM-T plasmid vector (Promega) for sequencing. The sequences were determined by the cycle-sequencing method using T7 and SP6 sequencing primers on an autosequencer (NAPS Unit; UBC). The sequenced rabbit clone for C1q was 361 base pairs (bp) long compared with 358 for the comparable human cDNA product. There was 82.6% homology overall, with 100% homology in the primer region. Primers for rabbit C1r were as follows: forward 5'-CCACGGGATAAAAGGAGAGAAGG-3' and reverse 5'-GCCGTGTTAGGTGAATGATTAG-3'. The assigned GenBank accession number is AF089083. The sequenced rabbit clone for C1r was 218 bp long compared with 216 for the human product. There was 85.3% homology overall with only 1 bp difference in the primer region. The assigned GenBank accession number is AF108768. New primers for rabbit C1r were designed as follows: forward 5'-GGCTCCTGTGACACGATCTTCTA-3' and reverse 5'-TGGCCTGGTTAGTAGGTGC-3'.

RNA preparation and RT-PCR. Total RNA from ~500 mg of each tissue sample was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (6). The extracted RNA was quantified by scanning spectrophotometry. The 260- to 280-nm absorbance ratio of all preparations was >1.8. The RNA was then reverse transcribed and specific cDNAs amplified by the PCR technique as previously described in detail (45, 46).

In preliminary studies, we found that the amount of PCR product increased exponentially from 20 cycles to 29 cycles for cyclorphilin and from 25 cycles to 37 cycles for the complement cDNAs. A plateau phase was reached after 29 and 37 cycles, respectively, due to the plateau effect (21). Accordingly, each cDNA sample was treated by the PCR procedure with the cyclorphilin product being amplified for 27 cycles and the complement products for 35 cycles. Each PCR reaction product was electrophoresed through a 6% polyacrylamide gel, and the product was visualized by incubation for 10 min in a solution containing 10 ng/ml ethidium bromide. Resulting gel bands were imaged using a GDS 6700 image analyzer (Ultra Violet Products, Uplands, CA). The relative intensities of the bands, expressed as optical density (OD) units, were quantitatively analyzed using NIH Image software 1.61. Each complement mRNA amplification was run in parallel with a cyclorphilin mRNA amplification to provide an internal standard. Direct OD values were analyzed, as well as values relative to cyclorphilin. Polaroid photographs of the gels were taken.

Restriction digest analysis. The PCR products were purified by the ethanol precipitation procedure (46). Unique restriction sites and restriction enzymes were selected using the DNA Strider computer program. The restriction enzymes chosen were as follows: SacI for C1q; Sau3AI for C1r; HincII for C3; MseI for C8; and BamHI for C9. The restriction
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 prestained 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 two times 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 Immunobond Metal Enhanced DAB Substrate Kit (Pierce Chemical) for C3.

MAC analysis. Heart samples were homogenized in 5× 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 9 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 poly-L-lysine-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 Fluormount-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 314 and 147 bp when treated with the restriction enzyme SacI. The C1r primers generated a product of 218 bp, which was cleaved by Sau3AI to yield the predicted digestion fragments of 65 and 153 bp. The C3 primer generated a product of 298 bp, and treatment with HincII yielded fragments of 253 and 45 bp. The C8 primers yielded a product of 441 bp, and treatment with MseI 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)
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$_{ATP}$ 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, in no case did the difference reach the P < 0.05 level of significance (Fig. 2).

The K$_{ATP}$ 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 and pinacidil preconditioned hearts (P = 0.001 to 0.01; Fig. 2).

Western blot analysis (Fig. 3) demonstrated bands for C3 in the soluble extract of hearts that were subjected to ischemia-reperfusion, but not the ischemically preconditioned or pinacidil-treated hearts, suggesting that preconditioning attenuated C3 protein expression. The C3 product displayed bands at 120 and 80 kDa, as reported previously (45). Blots for MAC also indicated the strong presence of the complex in the soluble extract of the ischemia-reperfusion heart (Fig. 4, lane 1). There was no apparent signal in the untreated heart (Fig. 4, lane 4). A detectable but weaker MAC signal was observed in the pinacidil-treated heart, and an even weaker signal was seen in the ischemically preconditioned heart compared with ischemia-reperfusion hearts (Fig. 4, lanes 1–3). Western blot analysis was not done for the other proteins, because antibodies against rabbit C1r, C1q, C8, and C9 are not available.

MAC immunofluorescence staining suggests that ischemic and chemical preconditioning also attenuate MAC formation in the ischemic myocardium (Fig. 5). An intense red fluorescence was detected in the ischemia-reperfusion heart. The ischemically preconditioned and pinacidil-treated hearts did not demonstrate any areas of intense red fluorescence compared to the untreated heart.
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 localization 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...
H2378  PRECONDITIONING REDUCES COMPLEMENT GENE EXPRESSION

reevaluate the current assumption that myocardial tissue injury due to complement activation is dependent solely on plasma-derived complement (45). Complement injures tissue directly by lysing cells through formation of the MAC and/or by augmenting the in vivo inflammatory response in the reperfused area at risk (18). Murohara et al. (28) demonstrated that both the classical and alternative pathways of complement activation participate in ischemia-reperfusion injury. The upregulation of C1q and C1r in our study implies that activation of the classic pathway may occur during reperfusion of the isolated heart. C1 esterase inhibitor, which blocks the classic pathway of complement, has been shown to ameliorate myocardial reperfusion injury (5, 28). It is also conceivable that activation of the alternative complement pathway occurs in this setting, as free radicals are produced during reperfusion and have been shown to activate the alternative pathway (3).

Chemical preconditioning accomplished with pinacidil attenuated C1q, C1r, C3, C8, and C9 mRNA upregulation. This attenuation was prevented and reversed by glyburide. The data suggest that the decrease in C1q, C1r, C3, C8, and C9 mRNA results from modulation of the KATP channel. Preconditioning may be decreasing C1q, C1r, C3, C8, and C9 mRNA upregulation primarily through an intracellular event elicited by opening of the KATP channel or secondarily by preservation of the myocardial tissue after ischemia-reperfusion. It has been demonstrated that activation of KATP channels attenuates hydrogen peroxide-mediated cytotoxicity, which contributes to reperfusion injury by perpetuating the production of free radicals (10). Free radicals regulate transcription of several genes and activate the ubiquitous transcriptional regulator nuclear factor-\(\kappa\)B (37). Our laboratory has also found that free radicals upregulate complement transcription in the isolated heart (unpublished observations). If reduction of complement expression is a by-product of the cytoprotective effects of KATP channel opening, then other anti-ischemic agents may suppress the pathways that increase complement generation by the reperfused myocardium.

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-\(\alpha\), interleukin (IL)-1, IL-6, and interferon-\(\gamma\) 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 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 KATP 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.

We are grateful to Dr. S. Bhakdi for the kind donation of the rabbit C5b-9 antigen, which made the MAC immunofluorescence staining and Western blot analysis possible.

This study was supported by the Cardiovascular Research Fund, University of Michigan, and donations from individual British Columbians.

Address for reprint requests and other correspondence: B. R. Lucchesi, Dept. of Pharmacology, Univ. of Michigan, 1301 MSRB III, Ann Arbor, MI 48109-0632 (E-mail: benluc@umich.edu).

Received 9 April 1999; accepted in final form 13 July 1999.
REFERENCES


