Amelioration of ischemia-reperfusion injury with cyclic peptide blockade of ICAM-1

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Merchant, Shakil H., Debbie M. Gurule, and Richard S. Larson. Amelioration of ischemia-reperfusion injury with cyclic peptide blockade of ICAM-1. Am J Physiol Heart Circ Physiol 284: H1260–H1268, 2003. First published December 19, 2002; 10.1152/ajpheart.00840.2002.—Neutrophils are pivotal in the pathogenesis of ischemia-reperfusion (I/R) injury leading to muscle damage. Firm adhesion of neutrophils to the endothelium is initiated by an interaction between intercellular adhesion molecular-1 (ICAM-1) on the endothelium and β2-integrins on neutrophils. Inhibition of ICAM-1-dependent binding using monoclonal antibodies has been shown to be efficacious in ameliorating I/R injury by preventing the influx of neutrophils into the ischemic tissue. We recently described a cyclic peptide that is a potent and selective inhibitor of ICAM-1 (IP25) in vitro. In this study, we tested the hypothesis that IP25-mediated blockade of ICAM-1 would inhibit neutrophil influx during reperfusion of ischemic tissue and consequently attenuate muscle injury in a tourniquet hindlimb murine model of I/R injury. Varying amounts of peptide drug were injected at the beginning of the reperfusion period. The neutrophil influx and size of infarction at the end of 2 h of reperfusion were compared with those in untreated control mice and contralateral nonischemic limbs. Mice receiving IP25 immediately before reperfusion showed a 56% reduction in neutrophil infiltration in the ischemic muscle, accompanied by a 40% reduction in the infarct size. No effect on I/R injury was seen if IP25 administration was delayed for 60 min after reperfusion. We conclude that IP25 effectively inhibits ICAM-1-mediated adhesion of neutrophils to the endothelium in mice leading to a protective effect and suggests that synthetic peptide antagonists have a potential role as therapeutic tools.

Infarction; peptide antagonist; cell adhesion

PROLONGED INTERRUPTION of the blood supply to the heart results in cell death and irreversible tissue damage (15, 16, 37, 47). In myocardial ischemia, there is a direct correlation between the amount of tissue necrosis and the prognosis of the patient (16). Salvage of the ischemic tissue depends on early reperfusion. However, there is evidence that reperfusion leads to a series of events that significantly worsens the tissue injury (15, 47). Reperfusion injury after ischemia [ischemia-reperfusion (I/R) injury] determines the outcome in several clinical scenarios including myocardial infarction, stroke, organ transplant, and skeletal muscle ischemia during abdominal aortic aneurysm surgery (15, 20, 22, 38, 43, 47). A sizable number of observations indicate that neutrophils [polymorphonuclear cells (PMNs)] play a pathogenetic role in tissue damage after ischemia and reperfusion, including the observations that treatment with monoclonal antibodies (MAb) that inhibit neutrophil (PMNs) activation or influx into the myocardium result in a significant decrease in the size of infarction in experimental animals (8–10, 21, 31, 33, 40–42).

Increased PMN adhesiveness to the endothelium appears to be a critical step early in the sequence of events leading to I/R injury. PMN extravasation is dependent on increased adhesive interactions between PMNs and the vascular endothelial cell surface, together with the decreased shear force produced by vascular leakiness at sites of inflammation. Coordinated expression and usage of adhesion molecules results in physiological events that lead to PMN localization to a tissue or organ (3, 23, 26, 33). Members of the selectin family present on PMNs (L-selectin) and endothelial cells (P-selectin and E-selectin) initially mediate the transition from rapid flow to rolling (1, 3, 23, 25, 55). The loosely interacting (rolling) PMNs are then activated by chemokines to a more adhesive state (arrest), making the cell more resistant to being sheared off the endothelial lining by local microhemodynamic shear forces. Initial in vitro observations have suggested that the arrest and transmigration of PMNs was mediated by two members of the β2-integrin family [LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18)] binding to intercellular adhesion molecule-1 (ICAM-1) on endothelial cells (33). However, more recent evidence suggests that LFA-1 binding to ICAM-1 alone is insufficient for PMN emigration, because MAb directed at the LFA-1 α-subunit completely block hypoxia-induced adhesion of neutrophils to endothelial cells (49, 50). Furthermore, PMN from mice genetically deficient in ICAM-1 or LFA-1, but not Mac-1, will not extravasate efficiently during inflammatory processes, and I/R injury is ameliorated in these animals (13, 33, 49).

Currently, effective intervention strategies for modulating clinically relevant I/R injury do not exist. Yet, pharmacological modulation of the reperfusion injury may be beneficial in several scenarios, including reduc-

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ing myocardial infarct size. Several investigators have shown that MAb directed against ICAM-1 or β₂-integrin reduce PMN infiltration and consequently infarct size in experimental animals (26, 28, 29, 49, 50, 54). However, anaphylactic reactions and secondary physiological effects have hampered the clinical use of these antibodies in humans. Thus we have recently developed a small peptide antagonist of ICAM-1 that inhibits ICAM-1/LFA-1-dependent cell adhesion. An initial weaker nonapeptide ICAM-1 antagonist (IP04) was identified using phase display (44). IP04 was shown to specifically block ICAM-1/LFA-1 binding, ICAM-1-dependent homotypic aggregation of human and mouse cells, and neutrophil binding to the activated endothelium under flow conditions in a parallel plate flow chamber. With the use of alanine and homologous amino acid substitutions, IP04 was further mutagenized to increase its potency for inhibition of homotypic aggregation as well as PMN binding to the endothelium in vitro under flow conditions in a parallel plate flow chamber (IP25) (Refs. 4 and 44 and E. J. Burks, L. O. Sillerud, M. J. Wester, D. C. Brown, and R. S. Larson, unpublished observations). Recently, we have solved the tertiary structures of IP04 and IP25 and proposed a model for how these antagonists bind to ICAM-1 (Ref. 4; E. J. Burks, L. O. Sillerud, M. J. Wester, D. C. Brown, and R. S. Larson, unpublished observations). In this model, IP25 binds to ICAM-1 proximal to the binding site of the native ligand LFA-1 as defined by mutagenesis studies (6, 48), thus competing with LFA-1 to bind ICAM-1. Because of the small size and ability to block cell-cell adhesion, we postulated that IP25 may be a useful tool in the pharmacological modulation of reperfusion injury by blocking emigration of PMNs to the ischemic segments. The purpose of the study was to evaluate the usefulness of the peptide antagonist of ICAM-1 in reducing I/R injury. With the use of a mouse model of tourniquet hindlimb skeletal muscle I/R injury, we evaluated the hypotheses that 1) the peptide antagonist of ICAM-1 reduces PMN infiltration in the areas of infarction, and 2) the attenuation of PMN infiltration into muscle is associated with a decrease in infarct size.

METHODS

Reagents

Blocking MAb against murine ICAM-1 (clone 3E2B) was purchased from Endogen (Woburn, MA) and used in the in vivo blocking studies. MAb directed against murine ICAM-1 (MEM111) was purchased from Caltag (Uden, The Netherlands) and used in immunoperoxidase studies. The disulfide-linked cyclic nonapeptide IP25 (CLLRMKSAC) was synthesized by Biopeptide (San Diego, CA). The purity (95%) was verified by HPLC, and the structure was verified by mass spectroscopy and two-dimensional NMR as part of concurrent structural studies (Refs. 4 and 44 and E. J. Burks, L. O. Sillerud, M. J. Wester, D. C. Brown, and R. S. Larson, unpublished observations).

Animals

Female BALB/c mice (Harlan Sprague Dawley) weighing between 20 and 25 g were used for all experiments. The mice were maintained at the animal care facilities of the University of New Mexico (Albuquerque, NM).

Experimental Protocol

The model for tourniquet hindlimb ischemia and reperfusion was performed as described previously (3). In brief, mice were anesthetized using an inhalant anesthetic (1.5% Halothane, Halocarbon Laboratories; River Edge, NJ) and placed on a circulating hot water blanket (Baxter; Deerfield, IL) to maintain a constant body and muscle temperature. Latex O-rings (Miltex Instruments; Bethpage, NY) were fixed above the greater trochanter using a McGivney hemorrhoidal ligator (Miltex Instrument; Bethpage, NY) to interrupt the arterial blood supply to the hindlimbs. After 2 h of hindlimb ischemia, the O-rings were removed, initiating hindlimb reperfusion. Reperfusion of the previously occluded artery was confirmed by visual inspection.

Exclusions

All animals surviving the full I/R protocol were included in the data analysis. There were no deaths during the experimental protocol in the IP25-treated mice.

Animal Groups

In all, 53 mice were used in this study. The experimental protocol was divided into two different sets of experiments.

Assessment of the time course of I/R injury. The animals were randomized in two groups: group A (n = 5) mice remained anesthetized for the entire duration of the study (4 h) but did not go through the I/R protocol, and group B (n = 20) mice underwent 2 h of ischemia and varying periods of reperfusion (ranging from 1 to 4 h). Each experiment was performed with five mice: one mouse from group A and four mice from group B (group B mice underwent 1, 2, 3, and 4 h of reperfusion, respectively).

Peptide blockade of ICAM-1 on I/R injury. Animals were randomized into five groups: group A (n = 11) animals underwent 2 h of ischemia, followed by 2 h of reperfusion (untreated mice); group B (n = 15) animals underwent 2 h of ischemia followed by 2 h of reperfusion and additionally received varying amounts of the peptide drug IP25 (0.025–0.114 mg/g body wt in three divided doses every 30 min, beginning at the start of the reperfusion period); group C (n = 4) animals underwent 2 h of ischemia, followed by 2 h of reperfusion and additionally received a peptide drug with a scrambled IP25 sequence (identical amino acid composition as IP25 but with a fixed randomized sequence); and group D (n = 4) animals underwent 2 h of ischemia, followed by 2 h of reperfusion and additionally received blocking MAb directed against ICAM-1 (2 mg/kg body wt) at the start of the reperfusion period. Group E (n = 3) animals were administered 0.114 mg/g IP25 in three doses at 60, 75, and 90 min after reperfusion. The contralateral nonischemic hindlimb of each animal served as a paired control; 0.114 mg/g was the maximum dose that could be given due to peptide solubility. Because the serum half-life of peptide antagonists is typically short, three injections were given to maintain the serum concentration of the peptide antagonist. Each experiment in this set was performed using at least one mouse from groups A, C, and D and multiple mice from group B (receiving different amounts of the peptide drug IP25).
**Tissue Collection**

At the end of the reperfusion period, the animals were euthanized and blood samples were collected by heart puncture. Serum was used to measure lactate dehydrogenase (LDH) levels. Skeletal muscles (quadriceps and hamstrings) were harvested from both hindlimbs.

**Histological and Immunohistochemical Evaluation of Area of Infarction, PMN Infiltration, and Myocyte Damage**

The quadriceps were fixed in 10% neutral buffered formalin, processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin (H-E) for routine histological studies. Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue sections using the avidin-biotin-peroxidase complex (ABC) method in a Dako Autostainer (Carpinteria, CA). Immunohistochemical staining for the infarcted area was performed using rabbit anti-human myoglobin polyclonal antibody (Dako) as a primary antibody and anti-rabbit IgG biotinylated MAb as a secondary antibody (Vectastain, ABC Kit Elite Rabbit IgG PK-6101, Vector Laboratories; Burlingame, CA). To determine the expression of ICAM-1, immunohistochemical staining using MAb against ICAM-1 (clone MEM111, dilution 1:100, Monosan; Uden, The Netherlands) was performed. The pathologists (R. S. Larson and S. H. Merchant) were blinded to the treatment of the animals until all the data were analyzed.

Immunoperoxidase staining was done using the LSAB2 peroxidase kit (Dako). Briefly, deparaffinized sections were placed in a thermostresistant container filled with citrate buffer solution (pH 6.0), steamed for 45 min, and then cooled for 20 min before being stained. The antigen-antibody reaction was visualized using 3,3′-diaminobenzidine as a chromogen.

We evaluated four aspects of histological changes related to reperfusion injury: 1) total area of infarction, 2) PMN infiltration, 3) degree of myocyte damage, and 4) ICAM-1 expression. The histological aspects were evaluated as follows. The total surface area of the skeletal muscle in each section was measured, and the area of infarction was measured from the H-E-stained histological sections and confirmed by immunohistochemical stains for myoglobin (infarcted area seen as loss of myoglobin staining in the infarcted area). The infarcted areas were measured using a measuring grid and expressed as the percentage of the total surface area of the skeletal muscle.

**Statistical Analysis**

We tested the differences in myocyte damage, LDH levels, infarct size, and neutrophil influx using ANOVA and Student's *t*-tests. One-way ANOVA was used first to show statistical differences among the groups, and Student's *t*-test showed differences between specific groups. A *P* value of <0.05 was considered statistically significant. Statistical calculations were performed using GraphPad statistical software (San Diego, CA).

**RESULTS**

**Time Course of I/R Injury**

To examine I/R injury and determine the earliest time period at which measurable alterations were evident, animals were evaluated at the end of varying periods of reperfusion ranging from 1 to 4 h.

**Skeletal Muscle Histology**

Morphometric estimation of the infarct zone size showed an increase in the size of infarct as a function of time of reperfusion (Fig. 1). The infarct was evident at 1 h and increased over the 4-h reperfusion period. The skeletal muscle also showed a dramatic increase in PMNs beginning at 1 h and increasing over the 4 h of reperfusion (*P* < 0.05 compared with control at all time periods) (Figs. 1 and 2). The PMN infiltration correlated with the presence of myocyte injury and was seen as preferentially localized in the border zones surrounding the area of myocyte damage. PMNs were observed in the interstitium and infiltrating the skeletal muscle fibers (Fig. 2).

Morphological examination to assess qualitative myocyte damage (using morphological criteria, as listed in Table 1) revealed evidence of myocyte damage within 1 h of reperfusion (Figs. 1 and 2). Myocyte damage scores only assessed the presence of myocyte injury, not the size of infarction. Because the highest degree of myocyte damage was evident within 1 h of reperfusion, with increasing time of reperfusion, the myocyte damage scores remained constant, although these changes were seen spreading to larger zones of the skeletal muscle over the period of reperfusion. Necrosis or extravasated PMNs were not observed in any muscle from the control mice or the contralateral legs.

**Table 1. Histopathology scoring system to assess myocyte damage**

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
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<tbody>
<tr>
<td>0</td>
<td>No abnormal findings</td>
</tr>
<tr>
<td>1</td>
<td>Isolated focus of early ischemic changes (edema, waviness of fibers, or loss of cross striation)</td>
</tr>
<tr>
<td>2</td>
<td>Multifocal early ischemic changes</td>
</tr>
<tr>
<td>3</td>
<td>Single focus of moderate coagulative necrosis (shrunken eosinophilic cytoplasm, pyknosis, or loss of nuclei)</td>
</tr>
<tr>
<td>4</td>
<td>Multifocal moderate coagulative necrosis</td>
</tr>
<tr>
<td>5</td>
<td>Multifocal severe necrosis with total loss of tissue architecture (total disintegration of myocytes)</td>
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**Serum LDH**

Blood was collected by heart puncture at the end of the reperfusion period. Serum LDH levels were measured using a spectrophotometric assay on an Ektachem 250 analyzer (Ortho-Clinical Diagnostics; Rochester, NY), and results are expressed in units per liter.

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Serum LDH Studies

Serum LDH enzyme levels were used as an indicator of skeletal muscle damage and to confirm the histological observations. The serum LDH levels at the end of the I/R protocol were markedly increased compared with control mice (Fig. 1). Statistically significant elevation in the serum LDH levels was observed within 2 h of reperfusion (17,984 ± 2,814 U/dl, P = 0.004) compared with control mice (3,912 ± 1,101 U/dl).

ICAM-1 Expression

The expression of ICAM-1 in vascular endothelium and myocytes was observed in the same experimental animals described above using the MAb against ICAM-1 for immunostaining. Expression of ICAM-1 was observed in capillary, venous, and arterial endothelium of all skeletal muscle sections of all experimental animals, including control mice not exposed to I/R injury. No ICAM expression was identified in the myocytes from control mice (Fig. 3A). At each time point after injury, there was a qualitative increase in myocyte expression of ICAM-1 (Fig. 3B), but ICAM-1 expression in endothelium did not appreciably increase. Myocyte staining was limited to the small foci of myocytes, which were often concentrated adjacent to the necrotic areas (Fig. 3B). Additionally, infiltrating neutrophils were often concentrated in the ischemic regions in the vicinity of vessels and myocytes staining for ICAM-1, indicating neutrophil emigration occurs in ischemic muscle concurrent with the induction of ICAM-1 expression.

Effect of IP25 on I/R Injury

To evaluate the in vivo effect of IP25 on I/R injury, varying amounts of drug were injected in the tail vein beginning immediately before the reperfusion period, and the results were compared with those in untreated animals.

PMN Influx, Infarct Area, LDH Levels, and Histology

The area of infarction and PMN influx were significantly decreased in mice receiving IP25 (16 ± 2% of muscle and 3.2 ± 0.6 PMNs/mm², respectively, using the 0.114 mg/g dose) compared with untreated mice at the end of the 2-h period of reperfusion (27 ± 3% of muscle and 7.2 ± 1.3 PMNs/mm², P = 0.0034 and P = 0.037, respectively, n = 4 in each group) (Figs. 4A and 5). In addition, there was a linear dose response from 0 to 0.102 mg/g IP25, in that with increasing concentrations of IP25, smaller infarct areas, lower LDH levels, and lower PMN influx were observed (Fig. 4, B and C). Increasing the dose from 0.102 to 0.114 mg/g IP25 did not alter infarct size or PMN influx. The results obtained with IP25 doses >0.102 mg/g were comparable with those obtained by MAb blockage of ICAM-1 (Fig. 4A). No decrease in the size of infarction or PMN infiltration was obtained when a peptide with a scrambled IP25 sequence or, alternatively, no peptide was included in the I/R protocol.

Fig. 1. Relationship of area of infarct, neutrophil influx, and myocyte damage at increasing times of reperfusion (R). A: area of infarction (percentage of total muscle) and PMNs per millimeter squared (means ± SE) were measured at various intervals of reperfusion as described in METHODS (n = 4 for each time point; statistically significant differences were seen between the control group and each of the other groups by Student’s t-test, P < 0.05). Area of infarction and myocyte damage were measured as described in METHODS. C: serum lactate dehydrogenase (LDH) levels (means ± SE) at various intervals of reperfusion (n = 4 for each time point). Control mice underwent 4 h of anesthesia without the ischemia-reperfusion (I/R) protocol. There was a significant increase in LDH levels after 2 h of reperfusion (*P = 0.004) compared with controls. δ represents both control mice that underwent 4 h of anesthesia without the I/R protocol as well as the contralateral limb of mice undergoing I/R (n = 16). ANOVA analysis indicated that differences among groups in each of A–C were statistically significant. No significant difference was seen among the two control groups.
used (Fig. 4A). In contrast to the reduction in PMN and area of infarction, myocyte damage scores were not reduced in mice receiving IP25 (data not shown). This reflects the presence of some muscle damage (i.e., wavy fibers) still present in foci (Fig. 5). We also administered the peptide blockade after 1 h of reperfusion (Fig. 4A). Statistically significant reductions in neutrophil influx or the size of infarct were not observed.

ICAM-1 Expression in IP25-Treated Mice

Similar to the untreated mice described above (Fig. 4), ICAM-1 expression was not identified in the myocytes but was present in endothelium from the control group. Regardless of treatment, ICAM-1 expression in myocytes was induced during reperfusion, whereas the expression of ICAM-1 as detected with immunoperoxidase did not qualitatively change. No significant differences in ICAM-1 expression in myocytes or endothelium were seen at any time points among the different treatment groups (data not shown).

DISCUSSION

Experimental studies have shown that inflammatory tissue damage due to I/R is caused primarily by PMNs (41, 42). ICAM-1 is an adhesive glycoprotein receptor expressed on endothelial cells and required for PMN emigration from blood into tissue (3, 23, 26, 33, 54). MAbs directed against ICAM-1 or its ligand, \textbeta_2-integrin, as well as studies using animals genetically deficient in ICAM-1 or integrin receptors have shown that the extent of reperfusion injury is attenuated through ICAM-1 blockade or absence as assessed by 1) decreased myocardial necrosis, 2) a reduction in neutrophil accumulation in I/R myocardium, 3) recovery of endothelium-dependent vasorelaxation in regional ischemia models, and 4) improvement of cardiac function and myocardial energy status in global ischemia models (3, 17, 23, 26, 28, 29, 34, 50, 54). We previously described a novel cyclic peptide that is a potent, selective inhibitor of ICAM-1-dependent cell adhesion in vitro (Refs. 4 and 44 and E. J. Burks, L. O. Sillerud, M. J. Wester, D. C. Brown, and R. S. Larson, unpublished observations). The main aim of the present study was to demonstrate the in vivo efficacy of IP25 by employing a mouse model of tourniquet hindlimb I/R injury. This study provides direct evidence for the in vivo protective effect of IP25 by demonstrating an attenuation of infarct size (40% reduction) and PMN

Fig. 2. Representative histological sections (hematoxylin and eosin stain, \( \times 400 \)) of skeletal muscle. A: control limbs; B: 2-h ischemia and 1-h reperfusion; C: 2-h ischemia and 4-h reperfusion. No polymorphonuclear neutrophil (PMN) infiltration is seen in control limbs. At 1 h of reperfusion, early PMN influx with tagging of muscle sarcolemmal membranes is seen. At 4 h of reperfusion, there is dense accumulation of PMNs in the interstitium.

Fig. 3. Representative immunoperoxidase staining for ICAM-1 (\( \times 400 \)) as described in METHODS. A: normal skeletal muscle in control mice. Inset, ICAM-1 staining in the endothelium of the vessel. ICAM-1 expression is not seen in myocytes. B: ischemic muscle in mice undergoing the I/R protocol. Inset, area indicated by arrow. Dense membrane staining of damaged myocytes is seen. Immunoreaction is restricted to the endothelium in nonischemic muscles, whereas intense staining is evident in the ischemic myocytes.
influx (56% reduction) after experimental ischemia and reperfusion. Thus inhibition of ICAM-1-dependent binding after reperfusion of the ischemic myocytes has been investigated extensively, but this is the first study to show the efficacy of a peptide antagonist in amelioration of I/R injury. Our results were comparable with those obtained by MAb blockade of ICAM-1 in parallel experiments, indicating that the use of this cyclic peptide in humans may be therapeutically beneficial.

In vitro and in vivo studies have clearly demonstrated that the adhesion and transmigration of PMNs through the endothelium into tissue is dependent on β₂-integrin binding to ICAM-1 during I/R injury (3, 23, 26, 33). In addition, after emigration into tissue, it is well appreciated that PMN-mediated myocardial cell injury may also involve β₂-integrin-ICAM-1 interaction (3, 26, 28, 29). In agreement with these previous observations, ICAM-1 expression was detected in the endothelium and myocytes in our study as well (3, 24, 53). ICAM-1 expression in endothelial cells was easily detected by immunoperoxidase staining in normal and infarcted tissue. However, the increased expression of ICAM-1 in the endothelium in the areas of infarction was not easily appreciated. With the use of immunoperoxidase staining, other investigators have also seen similar results, and this likely relates to the fact that this staining technique is not sensitive to increasing expression of antigens (24). In contrast, the induced expression of ICAM-1 is easily appreciated in myocytes, because ICAM-1 is not detected before ischemia. ICAM-1 expression in myocytes is most intense within the areas of infarction and in viable myocytes at the border zones of infarction. Immunostaining was evident within 1 h of reperfusion in the form of bright membranous staining of the myocytes (Fig. 4B). Our findings and those of others (3) of ICAM-1 expression in skeletal muscle I/R injury are similar to those observed in cardiac myocytes during I/R injury. In these previous cardiac I/R studies, ICAM-1 mRNA expression was induced as early as 1 h after reperfusion and increased over 24 h, although ICAM-1 protein expression on myocytes was not seen until 24 h (24, 53). ICAM-1 mRNA expression was most intense in the cardiac myocytes in the ischemic viable “border zone” within the first few hours of reperfusion. From these current observations on ICAM-1 expression and neutrophil influx inhibition, as well as our previous studies (Refs. 4 and 44 and E. J. Burks, L. O. Sillerud, M. J. Wester, D. C. Brown, and R. S. Larson, unpublished observations), it is clear that IP25 inhibits neutrophil binding to and transmigration through the endothelium. However, whether IP25 may directly antagonize PMN-myocyte interaction, although expected, is not demonstrated. Inhibition of leukocyte-myocyte binding after I/R injury has been shown to improve rat cardiac myocyte contractility (46).

Previous studies also have indicated that PMN influx is most intense and increases during the first 4 h of ischemia, in agreement with our findings (8, 41, 42). We were able to detect PMN at 1 h, but the most intense PMN influx occurred at 3–4 h. The PMN influx

Fig. 4. A: comparison of the area of infarction and PMN influx at the end of 2 h of ischemia and 2 h of reperfusion in mice receiving drug (0.114 mg/g IP25) with mice receiving sham PBS injection (no peptide), control peptide with scrambled sequence (0.114 mg/g), and MAb against ICAM-1 (2 mg/kg 3E2B). Bars indicate SE; n = 4 in each group. ANOVA shows statistically significant differences among groups (P < 0.05). **Student t-tests compared with the control group indicated by asterisks and the control group (P < 0.05). B: dose-response graph showing the area of infarction and PMN influx at the end of 2 h of ischemia and 2 h of reperfusion in mice receiving the amounts of IP25 indicated. Equivalent volumes of PBS were injected in control mice not receiving any drug (n = 4 for each group, see METHODS for details). Bars indicate SE. ANOVA analysis showed statistically significant differences among groups (P < 0.05). C: serum LDH values in control mice (sham PBS injection) and indicated doses of IP25. Bars indicate SE. ANOVA analysis of groups indicated statistically significant differences (P < 0.05).
is most intense in the border zones of infarction and is of greater magnitude in larger areas of infarct. Myocyte damage was also detectable within 1 h of I/R injury. However, focal myocyte damage was present even when neutrophil influx was inhibited and could not be detected, suggesting that some myocyte damage may be independent of ICAM-1-dependent mechanisms, and includes complement and endothelial cell-derived factors as suggested by others (2, 12, 55). Although there was a reduction in the area of infarct and LDH levels with IP25 treatment, LDH levels were elevated in IP25-treated mice compared with control mice, consistent with the histological observation of some persistent muscle damage and infarct in the absence of neutrophil influx.

Our studies show the efficacy of IP25 in inhibiting PMN influx and reducing infarct size after 2-h ischemia and 2-h reperfusion. Previous studies with dogs and rodents have indicated that there may be only a “window of time” in which it is possible to reduce I/R injury by neutrophil influx inhibition (3, 7, 21). This may be due to the ischemia injury being so severe that the tissue is necrotic before reperfusion and therefore not amenable to reperfusion injury. We chose to study the efficacy of IP25 after 2 h of ischemia, because significant PMN and myocyte changes were readily evident to examine the efficacy of IP25, and we were concerned about the potential short half-life of peptide drugs. However, our studies do not fully address the effects of ischemia duration and how it related to neutrophil influx inhibition. Furthermore, it is unclear whether it will be beneficial to inhibit for 24 h or more, because the suppression of PMN influx at later times after reperfusion may prevent myocardial healing, as has been observed with glucocorticoids (45). In contrast, others have shown that ICAM-1 inhibition has no protective role in myocardial remodeling at later stages (30, 36). In addition, the effectiveness of IP25 may also be enhanced by constant infusion or repetitive bolus administration.

There does appear to be a shift in the selectin used for the initial PMN rolling in that P-selectin is utilized during the first 60 min of reperfusion, whereas at later times (>4 h) E-selectin is preferentially utilized (1, 3, 25, 55). Thus alteration in selectin utilization and the value of selectin blockade appears to relate to the change in expression of the various selectins in the endothelium at different time points after reperfusion. In contrast, ICAM-1 is expressed before ischemia, and its expression increases with reperfusion. In our studies, injection of IP25 after 1 h of reperfusion did not alter I/R injury. This later finding is consistent with the concept that neutrophil influx begins within minutes of reperfusion and the effects of early infiltration cannot be reversed with later ICAM-1 blockade or that later infiltration is not ICAM-1 dependent.

Ischemia-reperfusion injury is of significant clinical interest. Although there is overwhelming evidence that MAbs directed against β2-integrins or ICAM-1 reduce PMN infiltration and consequently infarct size in experimental animals (17, 36, 50, 54), several clinical trials directed at blocking adhesion molecule binding in humans have not shown efficacy (11, 39, 51). There are a number of potential explanations. First, the design of the trials may not have been adequate, because two of these studies had mortality rates in all groups lower than the reported rates in similar populations, making the studies too small to draw any statistically significant information. Second, current animal models may not adequately reflect I/R in humans. This may relate to the presence of non-adhesion-related pathways playing a more dominant role in humans or that the dominant pathway is dramatically influenced by the length of ischemia. In support of this notion, a recent study (19) demonstrated that myocyte injury in longer ischemia times became increasingly dominated by a caspase-dependent apoptotic pathway. Finally, the clinical trials used MAb therapy. The clinical application of MAbs is hampered by potential therapeutic hazards, including life-threatening anaphylactic reactions. In addition, some anti-ICAM-1 or integrin MAb that block function in vitro have actually been found to activate neutrophils in vivo and are therefore inappropriate to use in clinical trials (53). Small peptide inhibitors such as IP25 would be less immunogenic and circumvent the secondary physiological effects of antibodies (14, 19, 27, 32). In addition, the short half-life of peptide inhibitors is likely to be beneficial if ICAM-1-
dependent events at later time points of myocardial healing are adverse to healing (30, 36). One of the chief difficulties in treating I/R injury is its multifactorial etiology, although a significant component is mediated by PMN infiltration. Endothelial cell-derived mediators (including arachidonic acid metabolites, endothelin, and endothelium-derived relaxing factor), complement, and apoptosis inhibitors are among the other more well-characterized factors that have shown to be intimately involved with reperfusion injury (2, 12, 15, 18, 35). Modulations of these factors have also been shown to reduce infarct size in experimental animals (2, 12, 15, 35). Because the mechanism of action of these mediators are different at different time points after reperfusion, future studies will evaluate whether modulation of one of these factors in combination with peptide-mediated blockade of ICAM-1 has a synergistic effect in preventing I/R injury. In all, the results of the present study indicate the potential of synthetic chemically modified peptides as an alternative therapeutic approach to I/R injury.

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ICAM-1 PEPTIDE BLOCKS I/R INJURY


