Blocking of Classical Complement Pathway Inhibits Endothelial Adhesion Molecule Expression and Preserves Ischemic Myocardium from Reperfusion Injury

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ABSTRACT

Myocardial injury after ischemia (I) and reperfusion (R) is related to leukocyte activation with subsequent release of cytokines and oxygen-derived free radicals as well as complement activation. In our study, the cardioprotective effects of exogenous C1 esterase inhibitor (C1 INH) were examined in a rat model of myocardial I + R (i.e., 20 min + 24 hr or 48 hr). The C1 INH (10, 50 and 100 U/kg) administered 2 min before reperfusion significantly attenuated myocardial injury after 24 hr of R compared to vehicle treated rats (P < .001). Further, cardiac myeloperoxidase activity (i.e., a marker of PMN [polymorphonuclear leukocyte] accumulation) in the ischemic area was significantly reduced after C1 INH treatment compared to vehicle treated animals (0.81 ± 0.1, 0.34 ± 0.13, 0.13 ± 0.1 vs. 1.44 ± 0.3 U/100 mg tissue, P < .001). In addition, C1 INH (100 U/kg) significantly attenuated myocardial injury and neutrophil infiltration even after 48 hr of reperfusion compared to vehicle treatment. Immunohistochemical analysis of ischemic-reperfused myocardial tissue demonstrated activation of classical complement pathway by deposition of C1q on cardiac myocytes and cardiac vessels. In addition, expression of the endothelial adhesion molecules P-selectin and intercellular adhesion molecule 1 (ICAM-1) was observed after reperfusion of the ischemic myocardium. In this regard, C1 INH administration abolished expression of P-selectin and ICAM-1 on the cardiac vasculature after myocardial ischemia and reperfusion. Blocking the classical complement pathway by exogenous C1 INH appears to be an effective mean to preserve ischemic myocardium from injury after 24 and 48 hr of reperfusion. The mechanisms of this cardioprotective effect appears to be due to blocking of complement activation and reduced endothelial adhesion molecule expression with subsequent reduced PMN-endothelium interaction, resulting in diminished cardiac necrosis.

Although early reperfusion of the ischemic myocardium is a desired goal, evidence indicates that reperfusion itself contributes to additional myocardial injury (i.e., reperfusion injury) (Farb et al., 1993). This injury is preceded by endothelial dysfunction (Tsao et al., 1990) with decreased release of basal NO resulting in enhanced neutrophil adhesion (Ma et al., 1993) to the coronary endothelial cell surface (i.e., within 20 min after the onset of reperfusion) and neutrophil accumulation within the reperfused myocardium. Among other factors which are thought to mediate the reperfusion injury are cytokines (i.e., IL-1 and TNF), generation of radicals and complement activation (Entman et al., 1991).

The complement system is thought to play a major role in initiating some of the inflammatory events occurring in ischemia and reperfusion (Maroko et al., 1978). The classical complement pathway can be activated by certain sensitizing antibodies, cardiac mitochondrial particles, cardiolipin or the fibrinolytic system. C3a and C5a, anaphylatoxins of the complement cascade are potent leukocyte chemotactic agents, and C5a induces synthesis and release of cytokines such as IL-1, IL-6 and TNF-α in macrophages. The terminal MAC (C5b-9) stimulates the synthesis of reactive oxygen metabolites and LTB4 in neutrophils (Seeger et al., 1986). Further, the complement system activates the adhesion of neutrophils to the endothelium, since the MAC and C5a induces rapid translocation of P-selectin from Weibel-Palade bodies to the endothelial surface (Hattori et al., 1989; Foreman et al., 1994). The adhesion process after reperfusion of the ischemic myocardium starts with neutrophil rolling largely mediated

ABBREVIATIONS: Ab, antibody; C1 INH, C1 esterase inhibitor; CK, creatine kinase; MPO, myeloperoxidase; ICAM-1, intercellular adhesion molecule 1, C5b-9, MAC membrane attack complex; NO, nitric oxide; TNF, tumor necrosis factor; LWF, left ventricular free wall; IL, interleukin; PAF, platelet-activating factor; LAD, left anterior descending coronary artery; LVFW, left ventricular free wall; MAb, monoclonal antibody; PMN, polymorphonuclear leukocyte; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene difluoride.
by endothelial P-selectin expressed on the endothelial surface, and by constitutively expressed L-selectin on the neutrophil surface (Bevilacqua and Nelson, 1993). Some of the ligands for selectins are presumably glycolipids or glycoproteins containing carbohydrates like sialyl Lewis\(^a\) or Lewis\(^a\). The rolling process tethers the neutrophils to the endothelial cell surface leading to PAF-mediated PMN activation (Butcher, 1991). This leads to tight adhesion mediated by the interaction of CD11/CD18 with ICAM-1 which can result in transmigration of the neutrophils into the extravascular space. In this regard, monoclonal antibodies directed against either P-selectin, L-selectin or a sialyl Lewis\(^a\) containing oligosaccharide have been shown to prevent neutrophils from adhering to the coronary endothelium, preserve coronary endothelial function, and attenuate myocardial necrosis after myocardial ischemia and reperfusion (Buerke et al., 1994a and b).

However, in different in vitro (Shandelya et al., 1993) or in vivo (Weisman et al., 1991; Buerke et al., 1995; Horstick et al., 1997) models of myocardial ischemia and reperfusion inhibition of the complement cascade has been shown to be cardioprotective. However, little data are available on the precise mechanism of complement inhibition with its effects on adhesion molecule expression and reduction of reperfusion injury after long-term reperfusion in vivo.

Therefore, the major purposes of this study were to determine the effects of exogenous C1 esterase inhibitor on 1) complement activation, 2) myocardial tissue injury, 3) neutrophil accumulation and 4) adhesion molecule expression in a well-established rat model of myocardial ischemia and long-term reperfusion.

### Materials and Methods

**Determination of C1 esterase inhibitor activity.** To determine the ability of C1 INH to block the classical complement pathway, we used an erythrocyte hemolytic assay. One ml of sensitized sheep erythrocytes (Nobis, Endingen, Germany) were incubated with 0.01 to 2 l human or rat serum. The tubes were stored at room temperature for 60 min. The unlysed cells were removed by centrifugation with 1000 rpm at room temperature for 10 min. The absorbance of the supernatant was determined spectrophotometrically at 405 nm. Absorbance in the presence of different concentrations of the C1 INH (0.1–2 U/ml), in the presence of different concentrations of the C1 INH (0.1–2 U/ml), and determined hemolytic activity as described above.

**Experimental protocol.** Male Sprague-Dawley rats (225–250 g) were anesthetized with ether before surgery. A skin incision was made over the left thorax and the pectoral muscles were retracted to expose the ribs. A purse string suture was placed loosely through the skin and underlying musculature to facilitate rapid closure of the chest wall. A thoracotomy was performed at the level of the fifth intercostal space. Myocardial ischemia was produced by briefly exteriorizing the heart, and placing a 4–0 silk suture around the left anterior descending coronary artery, approximately 2 to 3 mm from its origin effectively occluding the vessel. After the slip knot was tied, the heart was replaced in the thoracic cavity, air was evacuated from the thorax and the chest wall, muscles and skin were rapidly closed by means of the previously placed purse-string suture. Ischemia was maintained for 20 min, at which time the slip knot was released, initiating the 24 or 48 hr period of reperfusion. Sham-operated control rats (Sham MI) underwent the same surgical procedures except that the suture which was passed under the LAD was not tied. After 24 or 48 hr of reperfusion, rats were anaesthetized with pentobarbital (35 mg/kg, i.p.) and their hearts were excised and placed in ice cold 0.9% NaCl. The left ventricular free wall and septum were dissected free and homogenized in cold 0.25 M sucrose (1:10, v/v) containing 1 mM EDTA and 1 mM mercaptoethanol with an Ultra-Turrax homogenizer (Jahnke + Kunkel, Staufen, Germany). Homogenates were centrifuged with 36,000 \(x\) g at 4°C for 30 min. The supernatants were decanted and analyzed spectrophotometrically for creatine kinase and myeloperoxidase activities.

The rats were randomly divided into three major groups. These are: 1) sham MI, 2) MI + R rats receiving vehicle and 3) MI + R rats receiving C1 INH (10, 50, 100 U/kg) dissolved in 0.5 ml saline. The C1 esterase inhibitor (Berinert, Behring Company, Marburg, Germany, isolated from human plasma, 1U \(\pm\) 0.15 mg) or its vehicle was administered by single i.v. bolus injection 2 min before reperfusion. We chose two different reperfusion periods (i.e., 24 and 48 hr) to determine the effect of the C1 esterase inhibitor on the cardiac integrity after long-term reperfusion.

In four additional sham rats we determined the hemodynamic parameters and white blood cell counts after C1 esterase inhibitor administration. There were no significant changes in any of the cardiovascular variables (heart rate, blood pressure) or any drop in white blood cell count measured in sham operated rats treated with 100 U/kg C1 esterase inhibitor i.v.

In 18 additional rats we determined the histological analysis of neutrophil infiltration and tissue injury as well as immunohistochemical analysis of cardiac P-selectin and ICAM-1 expression after myocardial ischemia and reperfusion.

**Creatine kinase analysis.** Protein concentration was assayed using the Biuret method of Cornell (1949). CK activity of left ventricular free wall and septum was measured using the method of Rosalki (1967). The supernatants of the homogenized myocardium (i.e., LWFW and septum) were incubated with ADP and phosphocholine (Sigma Chemical Co., Deisenhofen, Germany) and the change in absorbance was measured spectrophotometrically at 340 nm. The difference of CK in the LVFW was calculated by subtracting CK_{LWFW} from CK_{septum} and expressed as CK difference in International Units (IU/g) protein; since the CK washout from the LVFW is a useful index of tissue injury after ischemia and reperfusion (Kjekshus and Sobel, 1970). All assays were measured without previous knowledge as to the group of treatment of each individual rat.

**Determination of myocardial myeloperoxidase activity.** The myocardial activity of myeloperoxidase, an enzyme occurring virtually exclusively in neutrophils, was determined using the method of Bradley et al. (1982) and modified by Mullane et al. (1985). The supernatants of the homogenized myocardium (i.e., left ventricular free wall and septum) were reacted with 0.167 mg/ml of O-dianisidine dihydrochloride (Sigma) and 0.0005% \(\text{H}_2\text{O}_2\) in 50 mM phosphate buffer at pH 6.0. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO is defined as that quantity of enzyme hydrolyzing 1 mmol of peroxide/min at 25°C. The MPO increase in the LVFW was calculated by subtracting MPO_{septum} from MPO_{LVFW} and expressed as MPO difference (U/100 mg wet weight tissue). All assays were measured without prior knowledge as to the group of origin of each rat.

**Histology and immunohistochemistry.** For histologic and immunohistochemical analysis, 18 additional rats were subjected to no ischemia, 20 min I + 0 min R, 20 min I + 20 min R, 20 min I + 8 hr R and 20 min I + 24 hr R. At the end of the reperfusion period, the hearts were removed and immediately cannulated via the aorta. The hearts were perfused at 50 mmHg with K-H buffer for 2 min. After the hearts were cleared of blood, perfusion was switched to 4% paraformaldehyde in phosphate-buffered saline (pH 7.4; 4°C) for 5 min to perfusion fix the hearts. Full thickness slices of the ischemic...
and nonischemic left ventricular wall (1 mm in thickness and 5 mm in width) were fixed for 1.5 hr at 4°C in 4% paraformaldehyde. After 1.5 hr, the ventricular slices were dehydrated in a graded series of acetone solutions (i.e., 50, 70, 90 and 100%) at 4°C. After dehydration, the sections were infiltrated with methacrylate (ImmunoBed; Polyscience, Eppelheim, Germany) at room temperature for 24 hr, and subsequently embedded in methacrylate at 4°C for 12 hr. Glass knives were used to cut 2-μm thick tissue sections. The tissue sections were placed on coated slides.

To perform histologic analysis the slides were stained with hematoxylin/eosin (Sigma) and examined using an Olympus light microscope (Olympus Optical Co., Hamburg, Germany).

Immunohistochemical procedures on plastic sections were performed using methods described previously by Beckstead et al. (1986) using the avidin-biotin immunoperoxidase technique (Vectastain ABC Reagent; Vector Laboratories, Burlingame, CA). Immunohistochemical analysis was performed with antibodies directed against C1q (Calbiochem, Bad Soden, Germany), P-selectin (Pharmingen, Hamburg, Germany), and ICAM-1 (Pharmigen, Hamburg, Germany). Procedure for the immunohistochemical staining is described extensively for C1q and the same protocol was used for the other antibodies. Incubation of the primary anti-C1q monoclonal antibody were carried out overnight at room temperature at dilutions of 1:10, 1:50, 1:100 of the C1q MAb. Of these dilutions, 1:50 dilution gave the highest degree of immunolocalization combined with the least amount of nonspecific background staining. The sections were lightly counterstained with Gill's hematoxylin 3 (Sigma), and examined using an Olympus light microscope (Olympus Optical Co., Hamburg, Germany). Tissue sections from each rat heart were analyzed in 10 separate fields for each tissue section. The number of stained vessels was assessed in random tissue sections as an index of the occurrence of adhesion molecule expression. The total number of vessels was assessed with ECL detection reagents (Amersham Corp., Arlington Heights, IL).

**Statistical analysis.** All values in the text and figures are presented as means ± S.E.M. of n independent experiments. All data were subjected to analysis of variance followed by Fisher’s t test. P ≤ .05 or less were considered to be statistically significant.

**Results**

**Inhibitory Effects of C1 Esterase Inhibitor on Complement-Mediated Hemolysis**

Incubation of sensitized sheep red cells with human and rat serum resulted in a concentration-dependent serum induced hemolysis of the red cells (i.e., activation of the complement pathway) (fig. 1A). One μl of human or rat serum exerted a 80% hemolytic activity. Coincubation of 1 μl rat or human serum with C1 INH (0.1–2 U/ml) resulted in a concentration-dependent inhibition of the hemolytic activity to almost a complete inhibition at 2 U/ml. These results clearly demonstrate the efficacy of the C1 INH to inhibit activation of the classical complement pathway after administration of rat or human serum (fig. 1B).

**Immunohistochemical localization of C1q after myocardial ischemia and reperfusion**

The presence of C1q in the ischemic-reperfused myocardium was detected by an anti-C1q antibody using an avidin-biotin immunoperoxidase procedure. Nonischemic sections of heart tissue (i.e., those taken from myocardium perfused by the right coronary artery (i.e., septum) or from myocardium without ischemia did not demonstrate any immunostaining.

Similarly, immunohistological preparations, in which either the primary antibody (anti-C1q Ab) or the biotinylated secondary antibody (mouse IgG) was replaced with non-immune serum, did not exhibit any observed labeling of myocardial or endothelial cells (fig. 2A).

In contrast to these controls, C1q was evident in sections from all ischemic groups exposed to reperfusion periods of 0 to 24 hr. Intense immunolocalization of the antibody directed against C1q was evident at 20 min and 8 hr after reperfusion (fig. 2B (20 min I and 20 min R) and 2C (20 min I and 8 hr R)). Significant immunolocalization of anti C1q MAb was also seen at 20 min ischemia and 0 min of reperfusion, although the staining reaction tended to be patchier. Immunolocaliza-

![Fig. 1. Hemolytic activity of human and rat serum and its inhibition of sensitized sheep red cell hemolysis by C1 INH expressed as % hemolysis. A total of 0.01 to 2 μl of human or rat serum induced dose-dependent red cell hemolysis (A). One μl of human or rat serum resulted in 80% red cell hemolysis. Inhibition of hemolysis by C1 INH was dose dependent over the range from 0.1 to 2 U/ml (B). Points are means ± S.E.M. for five experiments.](https://example.com/fig1.png)
tion of C1q was prevalent on cardiac myocytes and in the coronary vasculature (i.e., particularly the endothelium). However, C1 INH-treated animals demonstrated the same amount of C1q deposition. These results indicate that reperfusion of the ischemic myocardium results in deposition of C1q in cardiac tissue in our experimental model which might lead to further activation of the complement cascade.

To demonstrate cross-reactivity of the C1q antibody, human C1q, human serum and rat serum containing 5 to 10 μg of protein were separated by electrophoresis on 10% acrylamide SDS gels and transferred to PVDF membranes. After blotting with the primary and secondary antibody the subunit A-B dimer (MW 52,750) of C1q could be observed in human and rat serum which indicates cross-reactivity of the C1q antibody in rats (fig. 2D).

Effect of C1 INH on Myocardial Injury after 24 Hr of Reperfusion

To evaluate the extent of ischemic injury, we measured creatine kinase activity in homogenates of nonischemic (i.e., septum) or ischemic (i.e. LVFW) myocardial tissue after 24 hr of reperfusion. Sham MI/R rats receiving 100 U/kg C1 INH demonstrated almost no myocardial injury (fig. 3). Sham-operated rats receiving vehicle showed similar CK differences compared with sham MI/R rats treated with C1 INH (data not shown). However, coronary occlusion and reperfu-
sion in rats given only the vehicle resulted in significant depletion of creatine kinase from the left ventricular wall (P < .001, compared with sham MI rats) (fig. 3). In contrast, the bolus injection of C1 INH (100 U/kg) 2 min before reperfusion significantly attenuated the CK loss from ischemic-reperfused myocardium. Further 50 U/kg C1 INH exerted a partial degree of cardioprotection whereas 10 U/kg C1 INH did not result in a significant cardioprotection. These results clearly indicate that bolus administration of C1 INH prevents myocardial injury after reperfusion of ischemic myocardium in a dose-dependent manner.

Neutrophil accumulation in the ischemic myocardium after 24 hr of reperfusion. Accumulation of neutrophils in the ischemic region during reperfusion has been thought to be a major mechanism responsible for reperfusion injury. Therefore, we measured MPO activity in the nonischemic (i.e., septum), and ischemic (i.e., LVFW) portions of the myocardium and calculated the difference as an indicator of neutrophil accumulation. Sham-operated control rats receiving C1 INH showed low levels of MPO-differences which were similar to those obtained from sham MI/R rats treated with vehicle (data not shown). MI rats receiving vehicle exhibited a marked increase in MPO activity in the left ventricular free wall indicating increased neutrophil accumulation in the reperfused myocardium after an ischemic episode. In contrast, administration of 100 U/kg C1 INH i.v. 2 min before reperfusion significantly abolished the MPO increase in the ischemic-reperfused myocardium. Bolus injection of 50 and 10 U/kg C1 INH exerted a partial inhibition of PMN accumulation. Figure 4 summarizes these data. Further, histological analysis of ischemic reperfused myocardium confirmed the results of the biochemical assay (table 1). These results indicate that the cardioprotective effect of C1 INH may be partially related to inhibition of neutrophil accumulation in the ischemic-reperfused myocardium.

Effect of C1 INH on Myocardial Injury and Neutrophil Accumulation in the Ischemic Myocardium after 48 Hr of Reperfusion

To evaluate the effect of C1 INH on long-term reperfusion we measured the loss of creatine kinase in homogenates of the ischemic-reperfused left ventricular free wall in comparison to the nonischemic septum as a marker of myocardial tissue damage after 48 hr of reperfusion. Sham MI/R rats receiving 100 U/kg C1 INH demonstrated only a small difference in CK activity between septum and left ventricular free wall. However, coronary occlusion and reperfusion in rats given only the vehicle resulted in a significant depletion of creatine kinase from the left ventricular wall (P < .001), compared with sham MI rats. In contrast, the bolus injection of C1 INH (100 U/kg) attenuated the CK loss from ischemic-reperfused myocardium even after 48 hr of reperfusion (fig. 5a). There was a significant reduction of reperfusion injury in the C1 INH-treated group when compared with vehicle treated rats (P < .001).

We measured MPO activity in the nonischemic (i.e., septum), and ischemic (i.e., LVFW) portions of the myocardium after 48 hr of reperfusion and calculated the difference as an indicator of neutrophil accumulation. Sham-operated control rats receiving C1 INH showed low levels of MPO-differences. However, MI rats receiving vehicle exhibited a marked increase in MPO activity in the LVFW indicating increased neutrophil accumulation in the reperfused myocardium after an ischemic episode. In contrast, administration of 100 U/kg C1 INH significantly reduced MPO activity in the ischemic-reperfused myocardium. Figure 5b summarizes these data. These results indicate that the inhibition of neutrophil accumulation persists even after 48 hr of reperfusion.

**TABLE 1**

<table>
<thead>
<tr>
<th>Number of infiltrating neutrophils into the left ventricular free wall after 20-min ischemia and 24 hr of reperfusion</th>
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<tr>
<td>Sham MI + C1 INH (100 U/kg)</td>
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<tr>
<td>PNM/mm²</td>
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*P < .05 compared to vehicle-treated animals.

**Fig. 5.** Effect of single administration of C1 INH on myocardial injury (left) or neutrophil infiltration (right) after 48 hr reperfusion of ischemic myocardium. Differences in creatine phosphokinase (CK) activity in the LVFW and septum are expressed in IU/g protein. Difference of cardiac myeloperoxidase (MPO) activity in the LVFW and septum expressed as U/100 mg tissue wet weight. Rats were treated with either 100 U/kg body weight C1 INH or vehicle. All values are means ± S.E.M. for five or six rats in each group. ***P < .001 compared to MI/R + vehicle.
Effect of C1 INH on Adhesion Molecule Expression (P-selectin and ICAM-1) after Myocardial Ischemia and Reperfusion

Immunohistochemical localization of P-selectin. Coronary endothelial P-selectin was detected by using the primary antibody in combination with a sensitive avidin-biotin immunoperoxidase procedure applied to 2-μm thick plastic embedded sections of heart tissue. Nonischemic sections of heart tissue (i.e., those taken from myocardium perfused by the right coronary artery) occasionally demonstrated faint patchy cytoplasmic immunostaining primarily located on the endothelium in less than 5% of coronary venules and arterioles. Similarly, the ischemic nonreperfusion group did not develop any significant immunostaining (fig. 6a). Immunohistological preparations, in which either the primary antibody or the biotinylated secondary antibody (mouse IgG) was replaced with nonimmune serum, did not exhibit labeled endothelial cells. In contrast to nonreperfused controls, intense immunolocalization of endothelial P-selectin was evident 20 min and 8 hr after myocardial reperfusion in vehicle-treated animals (figs. 6b and c) with the peroxidase reaction product covering the full cell height. However, endothelial P-selectin expression returned to initial values after 24 hr of reperfusion. Administration of the C1 INH 2 min prior to reperfusion resulted in a significant inhibition of P-selectin expression on the ischemic reperfused vasculature (fig. 6d). Immunohistochemical localization of P-selectin was observed in coronary arterioles as well as venules, although the number of arterioles and degree of staining were less than that observed in coronary venules. Immunolocalization of P-selectin was most prevalent (P < .01) in coronary venules after 20 min of reperfusion where 35 ± 4% of the coronary venules demonstrated positive immunostaining (fig. 7). This labeling index decreased at longer reperfusion time points (8 hr of reperfusion, 28 ± 3% positive vessels). Ischemic-reperfused myocardium of rats treated with C1 INH demonstrated remarkably diminished immunostaining (i.e., 14 ± 4%, P < .05 compared to vehicle-treated rats), indicating a reduced expression after C1 INH treatment (fig. 7). These results indicate that reperfusion of the ischemic myocardium results in expression of P-selectin on cardiovascular endothelium and that C1 INH treatment appears to be an effective inhibitor of endothelial P-selectin expression after ischemia and reperfusion.

Immunohistochemical localization of ICAM-1. Tissue sections obtained from nonischemic and ischemic nonreperfused rats exhibited small amounts of MAb immunolocalized on vascular endothelium (i.e., between 2–5% stained vessels).

Fig. 6. Photomicrographs of plastic embedded heart tissue incubated with MAb directed against P-selectin and labeled with peroxidase substrate solution. Brown reaction product is present at sites of antigen localization. A, Coronary venule exposed to 20 min of ischemia followed by 0 min of reperfusion. B, Coronary venule exposed to 20 min of ischemia followed by 20 min of reperfusion. C, Coronary venule exposed to 20 min of ischemia followed by 8 hr of reperfusion. D, Coronary venule exposed to 20 min of ischemia followed by 20 min of reperfusion of an animal treated with C1 INH. All figures are lightly counterstained with Gill’s hematoxylin. Magnification ×400.
Immunolocalization of MAb 1A29 in nonischemic coronary venules was presumably due to constitutive ICAM-1 present on the endothelial surface of the coronary vasculature. Immunohistochemical preparations in which the primary antibody or the biotinylated secondary antibody were replaced by nonimmune serum exhibited no immunostaining. Little immunostaining was seen in coronary venules from the control nonreperfused group or in ischemic-reperfused myocardium (20 min I or 20 min R). However, after 8 hr of reperfusion, significant immunolocalization was observed on endothelial cells lining coronary venules (fig. 8) from vehicle-treated rats. After 24 hr of reperfusion expression of ICAM-1 returned to basal levels. Bolus administration of the C1 INH resulted in reduced expression of ICAM-1 on the ischemic reperfused vasculature after 8 hr of reperfusion. ICAM-1 expression was observed in coronary arterioles as well as venules. Immunolocalization of ICAM-1 was most prevalent in coronary venules after 8 hr of reperfusion where 39 ± 6% of the coronary venules demonstrated positive immunostaining (fig. 9). This labeling index decreased at longer reperfusion time points (24 hr of reperfusion, 5 ± 3% positive vessels). Ischemic-reperfused myocardium of rats treated with C1 INH demonstrated remarkably diminished immunostaining for ICAM-1 (i.e., 19 ± 4% after 8 hr, P < .05 compared to vehicle-treated rats), indicating reduced expression after C1 INH treatment (fig. 8b). These results indicate that reperfusion of the ischemic myocardium results in expression of ICAM-1 on cardiovascular endothelium and that C1 INH treatment appears to be an effective inhibitor of endothelial ICAM-1 expression after ischemia and reperfusion.

**Discussion**

Our data clearly demonstrate significant cardioprotective activities of the C1 INH in myocardial ischemia and long-term reperfusion. The cardioprotection exerted by C1 INH in this study was characterized by a reduction in CK loss from the ischemic-reperfused myocardium compared to rats given only the vehicle for C1 INH. The protection of C1 INH also extended to inhibition of PMN accumulation in the reperfused myocardium. The decreased neutrophil infiltration could be explained by significant reduction of adhesion molecules expression (i.e., P-selectin and ICAM1) in the vascu-

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**Fig. 7.** Number of P-selectin positive stained venules expressed as percent of the total number of venules [(stained venules/total venules) × 100]. The ratio of stained venules to the total number of vessels was calculated for each rat heart exposed to 20 min of ischemia and 0 min, 20 min, 8 hr and 24 hr of reperfusion. The data are presented as means ± S.E.M. for five rats in each group. *P < .05 compared to vehicle-treated animals.

**Fig. 8.** Photomicrographs of plastic embedded rat myocardium incubated with MAb directed against ICAM-1 and labeled with peroxidase substrate solution. Brown reaction product is present at sites of antigen localization (arrows). A, Coronary venule exposed to 20 min of ischemia followed by 8 hr of reperfusion. B, Coronary venule exposed to 20 min of ischemia followed by 8 hr of reperfusion of a C1 INH-treated animal. Magnification ×400.

**Fig. 9.** Number of ICAM-1 positive stained venules expressed as percent of the total number of venules [(stained venules/total venules) × 100]. The ratio of stained venules to the total number of vessels was calculated for each rat heart exposed to 20 min of ischemia and 0 min, 20 min, 8 hr and 24 hr of reperfusion. The data are presented as means ± S.E.M. for five rats in each group. *P < .05 compared to vehicle-treated animals.
lature of the reperfused myocardium after C1 INH administration.

C1 INH has been studied thus far in several models of inflammation-like pulmonary dysfunction after LPS and vascular leakage (Guerrero et al., 1993). The common findings in these studies are that blocking of the classical complement pathway by C1 INH exerted significant protective effects resulting in a smaller degree of tissue injury. In this regard, we were able to demonstrate inhibition of complement-mediated red cell hemolysis by C1 INH after human or rat serum administration. However, C1 INH blocks further MASP1, MASP2 (lectin complement pathway), the kininogen pathway and several enzymes in the coagulation pathway. However, accumulation of the first component of the classical pathway (i.e., C1q) has been demonstrated in the ischemic reperfused myocardium and has been related to increased neutrophil accumulation in this area. Our immunohistochemical findings of C1q deposition in ischemic myocardium (i.e., 20 min ischemia) and in ischemic-reperfused myocardium (i.e., 20 min I + 20 min R and 20 min I + 8h R) support the results of Rossen et al. (1985). In addition, we were able to demonstrate cross-reactivity of the C1q Ab in rats. In immunohistochemical sections C1q was detected predominately on the coronary vascular endothelium although C1q could be observed on cardiac myocytes. C1q binds to membrane particles, mitochondrial fragments or other subcellular components of the ischemic myocardium and is able to activate the complement cascade. Further, C1q and C5a were found in cardiac lymph within the first 4 hr of reperfusion (Dreyer et al., 1989). The C1 activation results in generation of the bimolecular complex C4b, C2a which is referred to as C3 convertase and forms C3a and C3b. Further splitting of C5 into C5a and C5b with subsequent creation of the membrane attack complex (i.e., MAC) C5b-9. In this regard, immunohistochemical analysis of autopsy material obtained from patients with myocardial infarction identified C5b-9 deposits in myocardial tissue (Schäfer et al., 1986) and C5b-9 deposition has been observed on capillaries and venules after myocardial ischemia and reperfusion (Mathey et al., 1994; Weisman et al., 1991).

Our results clearly show that C1 INH, when administered 2 min before reperfusion at a dose of 10 to 100 U/kg body weight markedly retards postreperfusion cardiac injury (i.e., loss of creatine kinase) (P < .001). The reduction in tissue injury exerted by C1 INH cannot be attributed to any hemodynamic effects, because the bolus injection did not alter the hemodynamic parameters. Further, variations in collateral flow in the ischemic myocardium (i.e., alterations in oxygen supply) as a possible mechanism for the cardioprotective effect are highly unlikely, because rat hearts have almost no coronary collateral blood flow. Blocking of the classical complement pathway by C1 INH in vivo has been shown to reduce reperfusion injury and preserve myocardial contractility in a feline model of ischemia and reperfusion (Buerke et al., 1995). In vitro, Shandleya et al. (1993) used a soluble complement receptor 1 (sCR1) and observed improved cardiac contractile function and coronary flow in postischemic rat hearts.

One important component of the myocardial salvage afforded by C1 INH is very likely caused by its ability to diminish neutrophil infiltration. Clearly, neutrophils are involved in myocardial ischemia-reperfusion damage in our model, since we observed significant increases in MPO activities in vehicle-treated ischemic myocardial tissue. The effects of C1 INH, however, cannot be attributed to changes in circulating white blood cell counts because bolus injection of C1 INH did not result in a significant drop of white blood cell counts. These data eliminate the possibility that C1 INH administration in vivo exerted leukopenic effects, a phenomenon known to be cardioprotective in myocardial ischemia reperfusion injury (Engler et al., 1986). The reduced neutrophil accumulation after C1 INH observed in our study coincides with other ischemia-reperfusion experiments, where complement depletion with cobra venom factor resulted in significant inhibition of myocardial injury and reduced PMN infiltration into the ischemic myocardium (Maroko et al., 1978).

Neutrophil adherence to the vascular endothelium is an early and important event after reperfusion of ischemic myocardium which is mediated by various adhesion molecules (Lefer et al., 1994). The adhesion sequence starts with neutrophil rolling, commence with tight adhesion and might result in transmigration of the neutrophils into the extravascular tissue (Butcher, 1991). In pathophysiological processes, these endothelial adhesion molecules P-selectin, ICAM-1 and E-selectin are up-regulated on the endothelial cell surface after exposure to inflammatory mediators such as oxygen-derived free radicals, cytokines, PAF and thrombin (Lorant et al., 1991; Patel et al., 1992). In vitro studies have demonstrated that stimulated expression of endothelial P-selectin is rapid (i.e., within 10 min), whereas induction of endothelial ICAM-1 is dependent on protein synthesis and therefore requires several hours (Lorant et al., 1991; Bevilacqua et al., 1987). Our in vivo results indicate that maximal expression of endothelial P-selectin occurs 20 min after reperfusion of the ischemic coronary vasculature. Moreover, P-selectin expression is sustained at lower levels for up to 8 hr after reperfusion. Maximal expression of ICAM-1 was observed after eight hr of reperfusion when 40% of coronary venules expressed ICAM-1. The time course of endothelial cell expressed P-selectin and ICAM-1 after ischemia and reperfusion directly coincides with the time course of neutrophil infiltration into ischemic-reperfused myocardium.

Oxygen-derived free radicals may stimulate translocation of P-selectin from Weibel-Palade bodies to the endothelial cell surface. Patel et al. (1992) found that peroxides also induce rapid translocation of P-selectin, allowing P-selectin to tether circulating neutrophils to the endothelium. Similarly, Palluy et al. (1992) found that endothelial cells exposed to anoxia and reoxygenation exhibit significantly increased neutrophil adherence, an effect that could be blocked by a monoclonal antibody to P-selectin as well as by superoxide dismutase or catalase. If free radical generation is continuous, P-selectin expression can be sustained on the endothelial surface for hours (Patel et al., 1992). In addition, the complement system stimulates neutrophil-endothelial adhesion (Vercellotti et al., 1991), because the MAC (i.e., C5b-9) and C5a induces rapid translocation of P-selectin from Weibel-Palade bodies to the endothelial surface (Hattori et al., 1989; Foreman et al., 1994). Furthermore, complement induced generation of oxygen-free radicals might be an important stimulus for endothelial P-selectin expression (Patel et al., 1991). In this regard, blocking P-selectin either with a MAb or a
soluble sialyl Lewisα containing oligosaccharide reduced myocardial reperfusion injury in cats (Weirich et al., 1993; Buerke et al., 1994a). Our in vivo results support these in vitro studies and suggest that complement as well as oxygen-derived free radicals released at the onset of reperfusion induce P-selectin expression by endothelial cells within minutes and this expression is sustained at lower levels with increasing periods of reperfusion up to 8 hr.

In contrast to endothelial cell expressed P-selectin that was rapidly induced within 10 to 20 min of reperfusion and gradually dissipated, expression of endothelial cell ICAM-1 was relatively low during the early stages of reperfusion and was significantly increased at 8 hr of reperfusion, probably due to stimulation with cytokines (Smith, 1990). Previous studies have demonstrated that TNF-α is significantly increased after myocardial ischemia and reperfusion (Kukielka et al., 1993). In vitro studies have clearly demonstrated that endothelial cells exposed to TNF-α undergo marked up-regulation of ICAM-1 on their cell surface (Smith, 1990). Different complement factors exert a variety of inflammatory effects. C3a and C5a are potent leukocyte chemotactic agents. C5a induces synthesis and release of cytokines including IL-1, IL-6 and TNF-α in macrophages. These cytokines can induce the expression of ICAM-1. Interestingly, the MAC (i.e., C5b-9) augments TNF-α induced synthesis of ICAM-1 and E-selectin on endothelial cells (Kilgore et al., 1995). Blocking of either ICAM-1 or CD18 significantly reduced myocardial injury after myocardial ischemia and reperfusion (Ma et al., 1992).

In our study we have demonstrated that blocking of the classical complement pathway with C1 INH results in reduced expression of P-selectin and ICAM-1 with subsequently reduced PMN infiltration. The complement-mediated myocardial injury after ischemia and reperfusion can be attributed to direct cytotoxic actions of complement (i.e., MAC, C5b-9) and can be augmented by infiltrating neutrophils. After administration of exogeneous C1 INH we are blocking the classical complement cascade in its first step and prevent thereby further complement activation with subsequent release of chemotactic agents, PMN accumulation and tissue injury.

In conclusion, we have demonstrated that in vivo administration of C1 INH attenuates myocardial injury resulting from myocardial ischemia and long-term reperfusion. These protective effects could be largely attributed to reduced PMN accumulation after C1 INH administration in the reperfused myocardium. Furthermore, these in vivo results demonstrate the important role of complement inhibition for the alleviation of tissue injury, adhesion molecule expression and neutrophil infiltration in inflammatory states like myocardial ischemia and reperfusion.

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References


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