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Effect of C1 Esterase Inhibitor on the Cardiac Dysfunction Following Ischemia and Reperfusion in the Isolated Perfused Rat Heart

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Complement-mediated neutrophil activation has been hypothesized to be an important mechanism of reperfusion injury. It has been proposed that C1 esterase inhibitor (C1 INH) may prevent the complement-dependent activation of polymorphonuclear leukocytes (PMNs) that occurs within postischemic myocardium. Therefore, The effect of C1 INH was examined in neutrophil dependent isolated perfused rat heart model of ischemia (I) (20 min) and reperfusion (R) (45 min). Administration of C1 INH (5 mg/Kg) to I/R hearts in the presence of PMNs (100×10^6) and homologous plasma improved coronary flow and preserved cardiac contractile function ($p < 0.001$) in comparison to those I/R hearts receiving only vehicle. In addition, C1 INH significantly ($p < 0.001$) reduced PMN accumulation in the ischemic myocardium as evidenced by an attenuation in myeloperoxidase activity. These findings demonstrate the C1 INH is a potent and effective cardioprotective agent inhibits leukocyte-endothelial interaction and preserves cardiac contractile function and coronary perfusion following myocardial ischemia and reperfusion.

Key Words: Ischemia/Reperfusion, Rat heart, PMNs, Complement esterase inhibitor (C1 INH)

INTRODUCTION

The first suggestion that complement activation is involved in myocardial tissue injury was made in 1971 by Hill & Ward (1971), who showed that ischemic myocardial tissue released a protease responsible for the formation of a chemotactic molecule by cleavage of C3. Rossen et al (1988) proposed that myocardial ischemia results in the release of subcellular constituents that bind C1q and activate complement, thereby generating the anaphylatoxin (i.e., C3a, C4a and C5a) and stimulating infiltration of PMNs, which may exacerbate tissue injury. In addition to the description above, studies of the complement system indicated that activation of the cascade can occur as a result of interactions with oxygen metabolites. Shingu & Nobunaga (1984) demonstrated that hydrogen peroxide can cause hydrolysis of C5, leading to the generation of a C5a-like molecule that has chemotactic activity. Similarly, hydrogen peroxide and related oxygen species produced by PMNs mediated complement activation (Shingu et al, 1992).

The complement system is thought to play an important role in initiating some of the inflammatory events occurring in ischemia and reperfusion (Maroko et al, 1978; Crawford et al, 1988). The classic complement pathway can be activated by certain sensitizing antibodies, cardiac mitochondrial particle, cardiolipin, or the fibrinolytic system (Crawford et al, 1988; Rosen, 1993). C3a and C5a are potent leukocyte chemotactic agents, and C5a induces the synthesis and release of cytokines such as interleukin-1, interleukin-6, and tumor necrosis factor in macrophages. Infusion of C5a into the coronary arteries of pigs also results in reduced cardiac contractile function (Ito et al, 1990). Additional components of the complement cascade C5b-9, known as the terminal membrane attack complex (MAC), stimulate the synthesis of reactive oxygen metabolites and leukotriene B4 in neutrophils (Campbell & Morgan, 1985; Seeger

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et al, 1986). The complement system also activates the adhesion of neutrophils to the endothelium, because the MAC induces rapid translocation of P-selectin from Weibel Plade bodies to the endothelial surface (Hattori et al, 1989). Similary, CD11b/CD18, the 2 integrin leukocyte adhesion complex, functions as complement receptor 3 (Vercellotti et al, 1991). Deposition of complement including the MCA in myocardial infarction, particularly in the marginal zone, suggests that complement could contribute to the ischemic tissue damage (Hugo et al, 1990). The MCA may lyse myocytes and thereby directly contribute to cell death, whereas sub-lytic attack may induce various functional disturbances of the myocardium (Homeister et al, 1992; Berger et al, 1993).

The process of reperfusion injury is characterized by an inflammatory response in which polymorphonuclear leukocytes (PMNs) are believed to play an important role (Entman et al, 1991). Upon reperfusion, many activated PMNs accumulate in the microvasculature resulting in microvascular plugging, and an impairment in coronary perfusion (Engler et al, 1983; Lefer et al, 1991). The activated PMNs induce tissue injury by the release of a variety of cytotoxic substances including oxygen derived free radicals, inflammatory cytokines, and proteolytic enzymes (Weiss, 1991). Many of these substances may mediate vascular endothelial dysfunction as well as contribute to myocardial injury (Buerke et al, 1994). This is also consistent with evidence that either decreasing the number of circulating PMNs or administration of monoclonal antibodies directed against cell adhesion molecules can lead to a significant cardioprotection against reperfusion injury (Ma et al, 1992; Lefer et al, 1994).

Inhibition of the complement cascade at the receptor level has been shown to be cardioprotective in different in vitro (Shandelya et al, 1993) and in vivo (Weisman et al, 1991) models of myocardial ischemia and reperfusion. However, few data are available on the effect on reperfusion injury of complement system blockade at an early step in the complement cascade. Therefore, The major purpose of this study was to investigate whether the administration of C1 esterase inhibitor (C1 INH) is able to protect against cardiac contractile dysfunction and PMN accumulation associated with ischemia reperfusion injury in a carefully controlled model of rat myocardial ischemia/reperfusion which is dependent upon PMNs to mediate the cardiac contractile dysfunction.

METHODS

Determination of C1 INH activity

To determine the ability of C1 INH to block the classic complement pathway, we used an erythrocyte hemolytic assay. Two hundred microliters of sensitized sheep erythrocytes (1.5×10^8 cells/ml) were incubated with the first component of the classic complement pathway, C1q (0.01 to 0.25 g/ml) and either 10 μl C1q-depleted human serum or normal human serum. The volume was then adjusted to 550 μl with gelatin veronal bufferr and the tubes were placed in a shaker bath at 37°C for 15 minutes; then 1 ml ice cold gelatin veronal buffer was added to each tube to stop the reaction. The unlysed cells were removed by centrifugation at 800 g at 4°C for 10 minutes. The absorbance of the supernatant was determined spectrophotometrically at 412 nm. Absorbance in the presence of normal serum was considered 100% of complement activity. The complement activity of the other tubes was calculated by dividing the absorbance of each tube by the absorbance of the normal serum.

To determine the effect of C1 INH on C1q-induced hemolysis, we incubated sensitized sheep erythrocytes with C1q-depleted human serum in the presence of 0.1 μg/ml C1q with and without different concentrations of C1 INH and determined hemolytic activity as described above.

Isolated rat heart experiment

Male Sprague-Dawley rats (250~300 g) were anesthetized with 40 mg/kg sodium pentobarbital and administered 1,000 U sodium heparin i.p. (Abbott Laboratories Diagnostic Division). Following a midline thoracotomy, the hearts were rapidly excised, the ascending aorta was cannulated, and retrograde perfusion of the heart was initiated on a Langendorff apparatus at a constant pressure of 80 mm Hg. These isolated hearts were perfused with a Krebs bicarbonate buffer of the following composition (in mmol/liter): glucose, 17; NaCl, 120; $NaHCO_3$, 25; $CaCl_2$, 2.5; EDTA, 5.9; and $MgCl_2$, 1.2 maintained at 37°C. The perfusate was oxygenated with 95% O_2+5% CO_2 which equilibrated at a pH of 7.3 to 7.4. Two sidearms in the perfusion line located just proximal to the heart inflow cannula allowed infusion of PMN and plasma directly into the coronary inflow line. To asses cardiac contractile function, a 2.5 Fr microtip

catheter transducer (Millar Instruments, Inc.) was inserted directly into the left ventricular cavity as previously reported (Pabla et al, 1996; Lefer et al, 1997). Left ventricular pressures, maximal rate of development of left ventricular pressure (+dP/dt min), coronary flow, and heart rate were all recorded using a MacLab data acquisition system (ADI Diagnostics Inc.) in conjunction with a Power Macintosh 7600 computer (Apple Computers). All data were stored and analyzed at the end of each experiment.

Rat neutrophil isolation

Neutrophil donor rats (300~350 g) received a 10 ml i.p. injection of 0.5% glycogen. Eighteen hours later, the rats were anesthetized with ethyl ether and the PMNs were harvested by peritoneal lavage in PBS. The peritoneal lavage was centrifuged at 3,000 rpm and 4°C for 10 minutes as previously described (Lefer et al, 1997). Finally, the PMNs were washed in Krebs buffer and counted using a microscope and hemocytometer. These neutrophil preparations were > 95% pure, and >95% viable using exclusion of 0.3% trypan blue as the criterion for viability. Furthermore, PMNs obtained by this method have been found to respond normally in cell adhesion tests (Lefer et al, 1997).

Additional PMNs were isolated from blood according to the method of Williams et al (Williams et al, 1987) using the hetastarch exchange transfusion technique in 400 gram rats anesthetized with pentobarbital sodium 40 mg/kg administered i.p.. Yields of 120×10^6 PMNs per rat were obtained which were > 95% pure and 95% viable using the trypan blue exclusion test. These blood PMNs were washed 5~6 times with PBS to remove the hetastarch prior to use.

Rat plasma preparation

Whole blood was obtained by performing an intracardiac puncture in anesthetized rats with a 20 ml plastic syringe with a 20 gauge needle (Becton Dickinson Co.) containing 2.0 ml of sodium citrate-phosphate-dextrose solution. The whole blood was immediately spun in a refrigerated centrifuge (GSGR; Beckman Instruments, Inc. Palo Alto, CA) at 3,000 rpm for 10 min, and the plasma was decanted.

Perfused heart experimental protocol

After 15 minute stabilization period, baseline left ventricular developed pressure (LVDP), +dP/dt max., and coronary flow were measured every 5 minutes for 15 minutes to ensure complete equilibration of the hearts. Flow of Kreb's buffer was then reduced to zero, creating global, total ischemia. This ischemia was maintained for 20 minutes. The flow was then restored to that of pre-ischemic level and reperfusion of the heart was initiated. At reperfusion, 100×10^6 PMNs and 5 ml of plasma was infused directly into the coronary circulation over a period of 5 minutes via a set of side ports situated just proximally to the heart in the perfusion line. The PMNs were suspended in 5 ml of Kreb's buffer in a 5 ml syringe. The plasma was also placed in a 5 ml syringe located just proximal to the inflow port to the coronary circulation. The hearts were allowed to reperfuse for a total of 45 minutes during which time data were collected every 5 minutes for the first 30 minutes and at the 45 minute time point. The C1 INH was dissolved in Krebs buffer at a concentration of 5 mg/Kg (rat body weight) and infused with the over the first 5 minutes of reperfusion. The dose was determined as an effective cardioprotective dose.

Determination of cardiac tissue myeloperoxidase

Myocardial tissue myeloperoxidase (MPO), an enzyme occurring virtually exclusively in PMNs (Mullane et al, 1985), and therefore, increased cardiac MPO activity indicates a significant accumulation of PMNs in the myocardium. One unit of MPO is defined as that quantity of enzyme hydrolyzing 1 mmol of peroxide per minute at 25°C. MPO was determined spectrophotometrically by the method of Bradley et al (Bradley et al, 1985) as modified by Mullane et al (Mullane et al, 1985). The assays were performed without knowledge of the group to which each sample originated.

Chemicals

Sensitized sheep erythrocyte, C1q-depleted human serum, gelatin veronal buffer, sodium heparin, type II oyster glycogen, citrate phosphate dextrose, hexadecyltrimethyl ammonium bromide, and o-dianisidine were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of analytical grade. C1 inhibitor was generously donated by Dr. B. Eisele (Behringwerke AG, Marburg, Germany).

Statistical analysis

All data are presented as mean±SEM. Data were compared by ANOVA using post-hoc analysis with fisher's corrected test. The data on coronary flow and left ventricular function were analyzed by ANOVA incorporating repeated measures. Probability values of 0.05 or less were considered to be statistically significant.

RESULTS

Inhibitory effects of C1 INH on complement-mediated hemolysis

Incubation of sensitized sheep erythrocytes with C1q in C1q-depleted serum resulted in a concentration dependent C1q-induced hemolysis of the cells (i.e., activation of the classic complement pathway). Ten microliters of C1q hemolyzed 92±7% of the erythrocytes, whereas C1q-depleted serum by itself hemolyzed only 28±3% (Fig. 1). Coincubation of 10 μl C1q (0.1 μg/ml) with C1 INH (0.05 to 1 mg/ml) resulted in a concentration-dependent inhibition of the hemolytic activity that was almost complete at 1 mg/ml. These results clearly demonstrate the efficacy of C1 INH in inhibiting activation of the classic complement pathway.

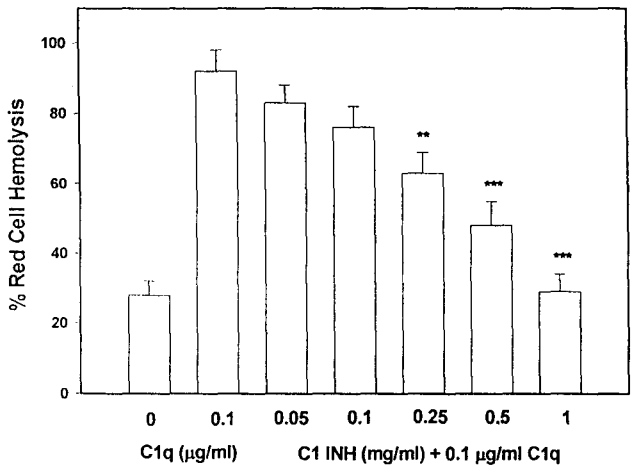

Fig. 1. Hemolytic activity of C1q, the first component of the classic component pathway, and the inhibition of sensitized sheep red cell hemolysis by C1 esterase inhibitor (C1 INH). Data were expressed as percent hemolysis. C1q (0.1 μg/ml) hemolyzed 92% of the cells. The inhibition of hemolysis by C1 INH was concentration dependent over the range of 0.05 to 1 mg/ml. Values are mean±SEM for five individual experiments. $^{**}P<0.01$, $^{***}P<0.001$.

Protective effects of C1 INH on coronary flow and cardiac contractility

To determine whether physiologically relevant concentration of C1 INH, in a well characterized model of myocardial ischemia reperfusion, can attenuate leukocyte-endothelial interactions and improve cardiac contractile function, we perfused rat hearts at control flow for 80 minutes, or for 15 minutes of control flow followed by 20 minutes of total global ischemia and 45 minutes of reperfusion at control flows either with or without PMNs and plasma. Perfusion of rat hearts with C1 INH at control flow for 80 minutes during sham ischemia or during ischemia/reperfusion without PMNs resulted in no change in coronary flow (CF), left ventricular developed pressure (LVDP), or first derivative of LVDP (+dP/dt max) at the end of the observation period, indicating that C1 INH did not exert any direct effects on cardiodynamics. Also, perfusion of sham ischemic hearts with PMNs did not alter any of the cardiac functions measured, indicating that PMNs do not induce cardiac dysfunction in normal non-ischemic hearts. Only in ischemic reperfused rat hearts perfused with PMNs was there a marked reduction

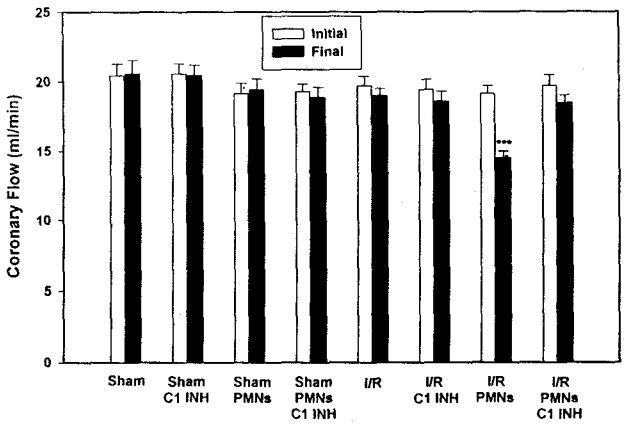

Fig. 2. Initial and final coronary flow. Data were expressed in ml/minutes in the isolated perfused rat hearts subjects to global total ischemia for 20 minutes and 45 minutes of reperfusion. Ischemic hearts were perfused in the presence or absence of PMNs (100 million). All values are expressed as mean±SEM for seven individual experiments. $^{***}P<0.001$.

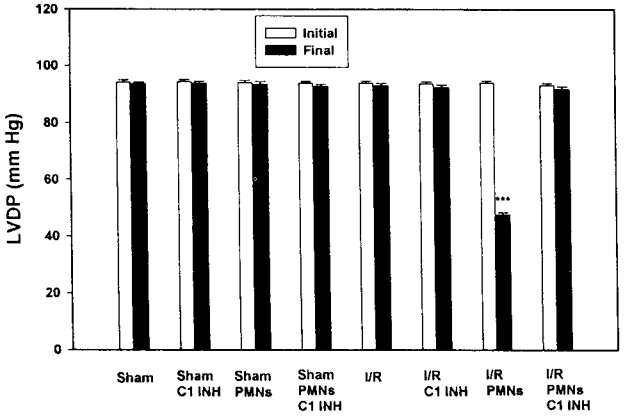

Fig. 3. Initial and final left ventricular developed pressure (LVDP) in the isolated perfused rat hearts prior to ischemia and following reperfusion. Ischemic hearts were perfused in the presence or absence of PMNs. PMNs caused a marked contractile dysfunction which was attenuated by C1 INH. All values are expressed as mean ± SEM for seven individual experiments. ***$P <$ 0.001.

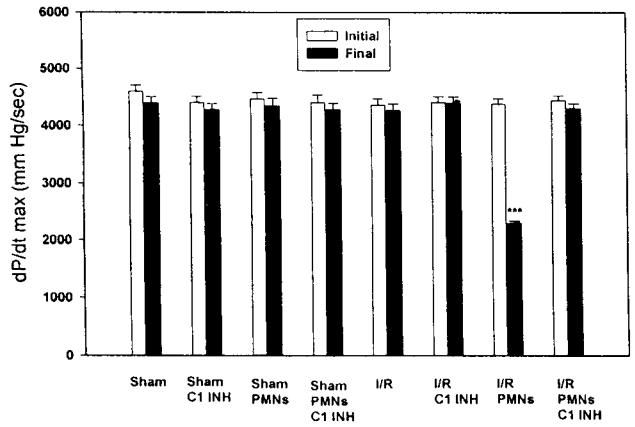

Fig. 4. Initial and final derivative LVDP (+dP/dt max) in rat hearts subject to ischemia and reperfusion. Ischemic hearts were perfused in the presence of PMNs. PMNs caused a significant impairment which was eliminated by the C1 INH. All values are expressed as mean ± SEM for seven individual experiments. ***$P < 0.001$.

in cardiac contractile function and coronary flow.

Ischemic-reperfused hearts perfused with PMNs and plasma exhibited significant cardiac dysfunction effects in these hearts ($P < 0.001$). Coronary flow maintained at zero during ischemia showed a recovery of 76 ± 2% of control in the presence of PMNs (Fig. 2). However, with the same number of PMNs under the same conditions in the presence of C1 INH

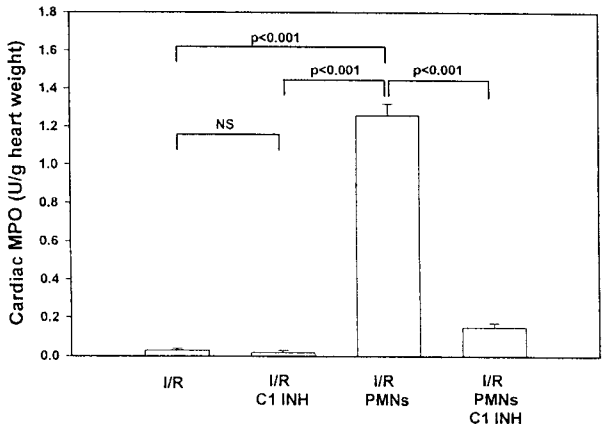

Fig. 5. Cardiac myeloperoxidase (MPO) activity in cardiac sample obtained from ischemic-reperfused rat hearts either in the presence or absence of PMNs and C1 INH. C1 INH significantly attenuated the increase of MPO in ischemic perfused hearts with PMNs. MPO activity is expressed in units per gram of wet tissue weight. All values are expressed as mean ± SEM for seven individual experiments.

coronary flow recovered almost completely (Fig. 2).

Changes in left ventricular function (i.e., LVDP) were similar to that of coronary flow. In ischemic reperfused rat hearts perfused with PMNs, LVDP recovered only partially following reperfusion stabilizing at a deficit in LVDP of 50.6 ± 4% ($p < 0.001$) (Fig. 3). However, in hearts perfused with the same number of PMNs under the same I/R conditions, C1 INH showed a significant protective effect in LVDP to 98 ± 2% of initial values.

These same relationships were obtained with the first derivative of LVDP (+dP/dt max). There was a 47.8 ± 3% reduction in the final +dP/dt max in untreated PMN perfused hearts subject to ischemia/reperfusion ($P < 0.001$) (Fig. 4). However, ischemic/reperfused hearts given C1 INH showed a significantly marked recovery of cardiac contractility, comparable to that of control values (i.e., 96.9 ± 3% of control).

At the end of the reperfusion period of each experiment, left ventricular tissue samples were obtained, frozen at −70°C and subsequently analyzed for PMN accumulation using myeloperoxidase (MPO) activity as a marker for PMNs. In all of the control heart and non-PMN perfused ischemia/reperfusion hearts, no cardiac MPO activity could be detected, therefore indicating that in non-ischemic hearts, or in I/R hearts without addition of PMNs there are very

few resident PMNs. However, I/R hearts perfused in the presence of PMNs showed a highly significant MPO activity signifying PMN accumulation ($P < 0.001$). Furthermore, when these ischemic/reperfused hearts were perfused with PMNs and given C1 INH after reperfusion, there was a significant attenuation of the MPO activity ($P < 0.001$) (Fig. 5) indicating an anti-neutrophil effect of C1 INH.

DISCUSSION

The present study clearly demonstrates a significant cardioprotective action of C1 esterase inhibitor (C1 INH) in a Langendorff perfused heart model of myocardial ischemia/reperfusion. This cardioprotection was characterized by a significant maintenance of post-reperfusion coronary flow, left ventricular developed pressure, and the first derivatives of left ventricular pressure (i.e., dP/dt max) indicating a significant attenuation of cardiac dysfunction by C1 INH. C1 INH did not exert a cardioprotective effect by directly influencing hemodynamics (i.e., inducing coronary flow or increased cardiac contractility) because C1 INH did not alter the functional cardiac index of reperfusion of non-ischemic heart.

Since myocardial reperfusion injury has been shown to be related to PMNs infiltrating into ischemic cardial tissue (Tsao et al, 1990; Weyrich et al, 1993), one very important component of the protection affored by C1 INH is due to its inhibition of PMN accumulation in the ischemic myocardium. Approximately a 88% attenuation of cardiac MPO activity was oberved in C1 INH treated ischemic-reperfused hearts as compared to those I/R hearts given only the vehicle. This appears to be the key cardioprotective effects of C1 INH. Without C1 INH, PMNs adhere to the endothelium of the vasculature and release cytotoxic substances such as proteases, eicosanoids, cytokines, and oxygen-derived free radicals (Weiss, 1989), each of which can mediate tissue injury and exacerbate endothelial dysfunction. These humoral mediators have been found to lead to coronary endothelial injury, disruption of the endothelial basement membranes, PMN extravasation, and myocardial necrosis (Weiss, 1989; Lefer et al, 1991; Entman & Smith, 1994). Our finding of reduced MPO accumulation supports the concept that C1 INH attenuates PMN-endothelial cell interactions.

There is considerable evidence that myocardial ischemia is associated with the activation of the complement system and that this process promotes further cardiac injury with the enhancement of a series of inflammatory events including PMNs chemotaxis and activation (Entrain et al, 1991). This cellular inflammatory response with PMNs activation has been hypothesized to result in the increased generation of reactive oxygen free radicals (Von Andrian et al, 1992) and coronary dysfunction (Tsao et al, 1990), which in turn cause further myocardial injury (Lucchesi, 1990). In the presence of the non-specific complement inhibitor cobra venom factor, it has been demonstrated that myocardial injury can be significantly reduced. With the administration of cobra venom factor in primates before ligation of the coronary artery, a reduction in infarct size was observed 24 hours later (Crawford et al, 1988).

Different complement factors exert a variety of inflammatory effects. C3a and C5a are potent leukocyte chemotactic agents. Blocking C3b by sCR1 results in cardioprotective effects both in vitro (Shandelya et al, 1993) and in vivo (Weisman et al, 1991). C5a induces synthesis and release of cytokines (interleukin-1, interleukin-6, and tumor necrosis factor-α) in macrophages. These cytokines induce the expression of immunoglobulin superfamily adhesion molecules such as ICAM-1, which serves as a major counterreceptor for CD11b/CD18 on neutrophils. Blocking of either ICAM-1 or CD18 significantly reduces myocardial injury in ischemic-reperfused cats (Ma et al, 1991; Ma et al, 1992). Further infusion of C5a into coronary arteries of pigs results in reduced contractile function (Ito et al, 1990). The terminal membrane attack complex (MAC), C5b-9 has been shown to stimulate the reactive oxygen metabolites (Campbell & Morgan, 1985) and leukotriene B_4 PMNs (Seeger et al, 1986). These mediators lead to the accumulation and activation of PMNs and promote the conversion of reversibly injured myocytes to irreversibly injured myocytes (i.e., they promote reperfusion injury). In addition, the complement system neutrophil-endothelium adhesion (Vercellotti et al, 1991), because the MAC C5b-9 induces rapid translocation of P-selectin from Weibel-Plade bodies to the endothelial surface (Hattori et al, 1989). Furthermore, complement-induced generation of oxygen free radicals might be an important stimulus for endothelial P-selectin expression (Patel et al, 1991). Rapid expression of P-selectin is an important trigger for neutrophil rolling, which precedes activation and

tight adherence of the PMNs (Patel et al, 1991; Zimmerman et al, 1992; Bevilacqua & Nelson, 1993). Blocking P-selectin either with MAb or a soluble sialyl Lewis^x-containing oligosaccharide reduces myocardial reperfusion injury in cats (Weyrich et al, 1993; Buerke et al, 1994).

In conclusion, the complement-mediated myocardial injury after ischemia and reperfusion can be attributed to direct pathophysiological actions of complement (Homeister et al, 1992) and can be indirectly augmented by complement-activated PMNs (Shandelya et al, 1993). The cardioprotective effects of the C1 INH observed in the present study are quite dramatic, because we blocked the complement cascade in its first step and thereby prevented all the subsequent steps of the cascade (particularly C3). Blocking the classic complement pathway by administration of C1 INH significantly attenuates many key events, including PMNs accumulation, endothelial activation, and PMN-endothelium interaction in this model of myocardial ischemia and reperfusion. These studies show that in addition to previously shown attenuation of myocardial cell necrosis by C1 INH in ischemia/reperfusion, C1 INH preserves cardiac mechanical function by an anti-neutrophil mechanism.

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