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This information is current as
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J Immunol 2001; 167:5375-5380; ;
doi: 10.4049/jimmunol.167.9.5375
<http://www.jimmunol.org/content/167/9/5375>

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Novel Small Molecule Inhibitor of C1s Exerts Cardioprotective Effects in Ischemia-Reperfusion Injury in Rabbits¹

Michael Buerke,^{2*} Hansjörg Schwertz,* Werner Seitz,[†] Jürgen Meyer,* and Harald Darius*

Myocardial ischemia-reperfusion injury can be related to complement activation with generation of chemotactic agents, adhesion molecule expression, release of cytokines and oxygen-derived free radicals, and subsequent neutrophil accumulation. In the present study the cardioprotective effects of a novel highly selective small molecule C1s inhibitor (C1s-INH-248, Knoll) were examined in a rabbit model of myocardial ischemia (I) and reperfusion (R; i.e., 60 min I + 180 min R). In *in vitro* tests (enzyme activity and SRBC lysis) C1s-INH-248 demonstrated profound inhibitory potency. In *in vivo* C1s-INH-248 (1 mg/kg body weight) administered 5 min before reperfusion significantly attenuated myocardial injury (31.9 ± 2.5 vs $8.9 \pm 1.6\%$ necrosis/area at risk; $p < 0.01$). The cardioprotective effect was dose dependent. The reduction of myocardial injury was also observed as diminished plasma creatine kinase activity in C1s-INH-248-treated animals (70.7 ± 6.8 vs 45.1 ± 3.9 U/g protein after 3 h of reperfusion, $p < 0.05$). Further, cardiac myeloperoxidase activity (i.e., a marker of PMN accumulation) in the ischemic and necrotic area was significantly reduced following C1s-INH-248 treatment (1.31 ± 0.23 vs 0.4 ± 0.05 U/100 mg tissue in necrotic area, $p < 0.01$). Thus, blocking the classical complement pathway with a highly specific and potent synthetic inhibitor of the activated C1 complex appears to be an effective mean to preserve ischemic myocardium from injury following reperfusion. *The Journal of Immunology*, 2001, 167: 5375–5380.

Reperfusion of ischemic myocardium results in additional myocardial injury (i.e., reperfusion injury) (1), generation of radicals, complement activation leading to endothelial dysfunction, and enhanced neutrophil accumulation, which results in tissue injury. The complement system is thought to play a major role in initiating some of the inflammatory events occurring in ischemia and reperfusion (2–5). The classical complement pathway can be activated by certain sensitizing Abs, cardiac mitochondrial particles, cardiolipin, or the fibrinolytic system. Subsequent generation of the terminal membrane attack complex (MAC³; C_{5b-9}) can induce direct cytotoxic effects. In this regard, blocking of the classical complement pathway with exogenous C1 esterase inhibitor isolated from plasma exerted cardioprotective effects following ischemia and reperfusion (6, 7). In addition, C1 esterase inhibitor caused a reduction of P-selectin and ICAM-1 expression following myocardial ischemia and reperfusion (8). Further, soluble complement receptor 1 (sCR1) and Ab directed against C5 exerted protective effects in different *in vitro* (9) and *in vivo* (10, 11) models of myocardial ischemia and reperfusion. sCR1 has been shown to function as inhibitor of the C3 conver-

tases. C5 Abs have been shown to prevent cleavage of C5 and thus inhibit generation of C5a and the membrane attack complex.

C1 esterase inhibitor is an endogenous inhibitor of the classical complement pathway which might be insufficiently active in situations like ischemia and reperfusion, since C1 esterase inhibitor can be inactivated by neutrophil released proteases such as elastase (12). Further, beside its inhibitory effect on the activated C1 complex (C1q, C1r, C1s), C1 esterase inhibitor has blocking potency on the mannose-binding lectin (MBL) pathway, the kallikrein system, the coagulation system, and the fibrinolytic system. Until now there was no specific inhibitor of the classical complement system available.

Therefore, the major purposes of this study were to determine the effects of a novel synthetic small molecule inhibitor of C1s on 1) complement activation, 2) myocardial tissue injury, and 3) neutrophil accumulation in a well-established rabbit model of myocardial ischemia and reperfusion.

Materials and Methods

Determination of C1s-INH-246 inhibitory activity

Determination of biochemical C1s and C1r activity. Complement C1s and C1r proteases were purified from human and rabbit plasma according to a procedure described by Lane et al. (13). Enzyme activity was measured spectrophotometrically using the artificial substrate Cbz-Gly-Arg-S-bzl (custom synthesis from Polypeptide, Wolfenbuettel, Germany). Release of Bzl-SH was quantified by adding an excess of 5,5'-dinitro-bis(2-nitrobenzoic acid) (no. 43760, Fluka, Buchs, Switzerland) and detection of yellow color ($\lambda = 405$ nm).

Determination of mannan binding protein-associated serine protease 1 (MASP1) inhibition. MASP1 protease was purified from human plasma by the method of Tan et al. (14). Enzyme activity was detected spectrophotometrically using the artificial substrate Cbz-Gly-Arg-S-bzl. Bzl-SH release was quantified by adding an excess of 5,5'-dinitro-bis(2-nitrobenzoic acid) and by a colorimetric readout at $\lambda = 405$ nm.

Determination of thrombin inhibition. Thrombin was purified from human plasma (T8885, Sigma, Deisenhofen, Germany). Potency of inhibitors was measured spectrophotometrically using a standard chromogenic assay with the artificial substrate S2238 (Chromogenix, Molndal, Sweden) and spectrophotometric measurement of yellow reaction product at $\lambda = 405$ nm. IC₅₀ curves of inhibitors C1s-INH-248 (peptidomimetic inhibitor,

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Received for publication May 1, 2001. Accepted for publication August 31, 2001.

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¹This work supported in part by Grant Bu 819/3-1 from the Deutsche Forschungsgemeinschaft.

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³Abbreviations used in this paper: C_{5b-9}, MAC, membrane attack complex; C1s-INH-248, synthetic small molecule inhibitor of C1s; C1-INH, C1 esterase inhibitor; CK, creatine kinase; LAD, left anterior descending; MASP1, mannan binding protein-associated serine protease 1; MBL, mannose-binding lectin; MI, myocardial ischemia; MPO, myeloperoxidase; PMN, polymorphonuclear leukocytes; R, reperfusion; sCR1, soluble complement receptor 1; SRBC, sheep red blood cells.

originally derived from thrombin inhibitor D-Phe-Pro-Arg, BASF patent application EP00/027710, m.w. 520.5; Knoll, Ludwigshafen, Germany) and C1-INH in concentrations of 0.1 nM to 100 μ M were determined routinely for C1s, C1r, MASP-1 and thrombin after 10-min preincubation.

Complement-mediated SRBC lysis.

To determine the ability of C1s-INH-248 to block the classical complement pathway, we used an erythrocyte hemolytic assay as described previously (8). SRBC (Nobis, Endingen, Germany) were incubated with 0.1–20 μ l rabbit serum. Absorbance in the presence of 20 μ l rabbit serum was considered 100% of hemolytic activity. The complement activity of the other tubes was calculated by dividing the absorbance of each tube by the absorbance of 20 μ l serum \times 100 and expressed as the percent RBC hemolysis.

To compare the effect of the C1s-INH-248 or the C1 esterase inhibitor (C1-INH, Berinert, Aventis-Behring, Marburg, Germany) we incubated sensitized sheep erythrocytes with 10 μ l rabbit serum in the presence of different concentrations of C1s-INH-248 (50 ng/ml to 0.1 mg/ml) or C1 INH (0.1–5 U/ml).

Experimental protocol and determination of myocardial necrosis

Adult male rabbits (2.3–3.6 kg) were anesthetized with sodium pentobarbital (30–60 mg/kg i.v.). An intratracheal cannula was inserted through a midline incision, and the rabbits were placed on intermittent positive pressure ventilation (TSE ventilator, TSE Bad Homburg, Germany). A polyethylene catheter was inserted into the right external jugular vein for additional pentobarbital infusion or for administration of C1s-INH-248, C1-INH, or its vehicle. A polyethylene catheter was inserted through the right femoral artery and positioned in the abdominal aorta for the measurement of mean arterial blood pressure. After a midsternal thoracotomy, the anterior pericardium was incised and a 3-0 silk suture was placed around the left anterior descending (LAD) coronary artery 5–8 mm from its origin. The electrocardiogram (ST segment elevation, heart rate) and mean arterial blood pressure were continuously monitored and recorded on a chart recorder every 20 min. The pressure rate index, an approximation of myocardial oxygen demand, was calculated as product of mean arterial blood pressure and heart rate divided by 1000.

Myocardial ischemia (MI) was induced by tightening the initially placed reversible ligature around the LAD so that the vessel was completely occluded. This was designated time zero. C1s-INH-248 (i.e., 0.1, 0.5, and 1 mg/kg body weight), C1-INH (100 and 200 U/kg body weight), or vehicle (saline) was given i.v. as a bolus 55 min after the coronary occlusion (i.e., 5 min before reperfusion (R)). Five minutes later (i.e., after a total of 60 min ischemia) the LAD ligature was untied, and the ischemic myocardium was reperused for 3 h.

The rabbits were randomly divided into seven major groups. Following administration of C1s-INH-248 blood samples were drawn to determine C1s-INH-248 elimination with HPLC analysis following bolus administration. Sham MI+R rabbits were subjected to the same surgical procedures as MI+R rabbits, except that the LAD coronary artery was not occluded.

Determination of myocardial necrosis

At the end of the 180-min reperfusion period, the ligature around the LAD was again tightened. Fifteen milliliters of 0.5% Evans blue was rapidly injected into the left ventricle to stain the area of myocardium that was perfused by the patent coronary arteries. Immediately following this injection, the heart was rapidly excised and placed in cold saline. The right ventricle, great vessels, and fat tissue were carefully removed, and the left ventricle was sliced parallel to the atrioventricular groove in 3-mm-thick sections. The unstained portion of the myocardium (i.e., the total area at risk) was separated from the Evans blue-stained portion of the myocardium (i.e., the area not at risk). The area at risk was sectioned into small cubes (2 \times 2 \times 2 mm) and incubated in 0.1% nitro blue tetrazolium in phosphate buffer at pH 7.4 and 37°C for 10 min. The irreversibly injured or necrotic portion of the myocardium at risk that did not stain was separated from the stained portion of the myocardium (i.e., the ischemic, but nonnecrotic, area). The three portions of the myocardium (i.e., nonischemic, ischemic nonnecrotic, and ischemic necrotic tissues) were subsequently weighed and indexed. In additional animals myocardial tissue was taken to perform histologic analysis of tissue injury and neutrophil infiltration and immunohistochemical analysis for determination of C_{5b-9} deposition.

Plasma creatine kinase (CK) analysis

Arterial blood samples (2 ml) were drawn immediately before ligation and every 60 min thereafter. The blood was collected in polyethylene tubes

containing 50 IU heparin sodium. Samples were centrifuged at 2000 \times g at 4°C for 20 min, and the plasma was decanted for biochemical analysis. Plasma protein concentration was assayed using the biuret method of Gornall et al. (15). Plasma CK activity was measured using the method of Rosalki (16) and expressed as international units per gram protein.

Determination of myocardial myeloperoxidase (MPO) activity

The myocardial activity of MPO, an enzyme occurring virtually exclusively in neutrophils, was determined using the method of Bradley et al. (17) and modified by Mullane et al. (18) and described previously (6). One unit of MPO is defined as that quantity of enzyme hydrolyzing 1 mmol peroxide/min at 25°C.

Measurement of superoxide radical release from rabbit polymorphonuclear leukocytes (PMN)

The rate of superoxide anion production by PMN was measured spectrophotometrically by the reduction of ferricytochrome *c* (19). Isolation of rabbit PMN was performed using a Percoll density gradient. The final PMN pellet was resuspended in HBSS (Sigma). Five hundred-microliter samples of the rabbit PMN suspension containing 5×10^6 cells were preincubated with ferricytochrome *c* (100 μ M; Sigma) in a total volume of 900 μ l for 15 min at 37°C in 1.5 ml spectrophotometric cells in a spectrophotometer model. The PMNs were stimulated with 100 nM leukotriene B₄ or PMA (100 nM) in a final reaction volume of 1.0 ml with either C1s-INH-248 (50 ng/ml to 0.1 mg/ml) or vehicle. The absorbance at 550 nm was measured every 30 s, and superoxide anion production (nanomoles per 5×10^6 PMNs) was calculated by dividing the absorbance of the samples by the extinction coefficient for the reduction of ferricytochrome *c* (21.1 mM⁻¹ cm⁻¹).

Analysis of PMN-endothelium interaction

Isolation of rabbit PMN was performed using 6% dextran for sedimentation and a Percoll density gradient. PMNs were collected from 62 to 80%. Isolated autologous PMNs were then labeled with a fluorescent dye (Sigma) according to the method of Yuan and Fleming (20).

Rabbit aortas were isolated and placed in warm, oxygenated Krebs Henseleit solution. Fat and connective tissue were removed, and the aortas were cut into rings 2–3 mm in length. The aortas were then opened and placed with the endothelial surface up into a cell culture dish filled with 3 ml Krebs Henseleit solution. To stimulate endothelial cells to increase their adhesiveness for neutrophils, aortic rings were activated with thrombin and subsequently coincubated with fluorescence-labeled neutrophils with or without C1s-INH-248. In addition, neutrophil were activated with leukotriene B₄ to augment PMN adherence. Adhered PMNs were counted using epifluorescence microscopy (Zeiss, Gottingen, Germany) and expressed as PMNs per square millimeter.

Statistical analysis

All values in the text and figures are presented as the mean \pm SEM of *n* independent experiments. All data were subjected to ANOVA followed by Fisher's protected least significant difference test. *p* \leq 0.05 was considered statistically significant.

Results

Biochemical analysis of inhibitory potency of C1s-INH-248 and C1-INH on protease activity of C1r, C1s, MASP1, and thrombin

The inhibitory activity of C1s-INH-248 and C1-INH was analyzed on a panel of related serine proteases (i.e., human C1r, C1s, MASP-1, thrombin, and rabbits C1s). From the analysis of biochemical activity it was evident that C1s-INH-248 had superior activity on human C1s compared with C1-INH (i.e., 10 times more

Table I. Determination of IC₅₀ (micromoles) of C1s-INH-248 and C1-INH on enzyme activity

	Human				Rabbit
	C1r	C1s	MASP-1	Thrombin	C1s
C1s-INH-248	9	0.002	2	2.08	0.0007
C1-INH	>0.2	0.02	0.007	>2.0	0.004

Table II. Effect of preincubation of C1s-INH-248 and C1-INH on inhibitory activity on human C1s and C1r

	Preincubation 10 min IC ₅₀ (μM)		Preincubation 60 min IC ₅₀ (μM)	
	C1r	C1s	C1r	C1s
C1s-INH-248	9	0.002	9	0.002
C1-INH	>0.2	0.3	>0.2	0.02

potent; Table I). However, C1r was only affected by both compounds in the micromolar range. In contrast to C1-INH, C1s-INH-248 did not inhibit MASP-1. Neither inhibitor affected thrombin activity. In addition, related serine proteases such as plasma kallikrein, XIa, and XIIa were tested for inhibition by C1s-INH-248. However, we did not observe inhibition to a significant extent (IC₅₀ = >10 μM; data not shown). C1s-INH-248 had superior activity on rabbit C1s compared with C1-INH (i.e., 7 times more potent; Table I).

Since C1s-INH-248 (m.w. 520) is a rather small molecule compared with C1-INH (i.e., 150 kDa) we determined the maximal inhibitory effect 10 and 60 min following incubation with C1s-INH-248 and C1-INH. Interestingly, C1s-INH-248 resulted in a rapid and potent inhibition of C1s. C1-INH reached similar IC₅₀ activity when incubated 60 min before activation, indicating a lower *k_{on}* compared with C1s-INH-248 (Table II).

Effects of C1 inhibition on complement-mediated red cell hemolysis

Incubation of sensitized SRBC with rabbit serum resulted in a concentration-dependent serum-induced hemolysis of the red cells (Fig. 1A). Ten microliters of rabbit serum exerted 70–80% hemolytic activity. Coincubation of 10 μl rabbit serum with C1s-INH-248 (50 ng/ml to 100 μg/ml) resulted in a concentration-dependent inhibition of the hemolytic activity to almost a complete inhibition at 5 μg/ml (Fig. 1B). These results clearly demonstrate the efficacy of the C1s-INH-248 to inhibit complement activation. Compared on equimolar basis C1s-INH-248 demonstrated superior potency (0.19 nM vs 1.6 μM for IC₅₀ of SRBC hemolysis).

Cardiac electrocardiographic and hemodynamic changes

In the sham MI rabbits, we observed that an i.v. bolus administration of 1 mg/kg body weight C1s-INH-248 had no detectable effect on any of the measured hemodynamic, electrocardiographic, or biochemical variables. In the two groups of MI/R rabbits, there

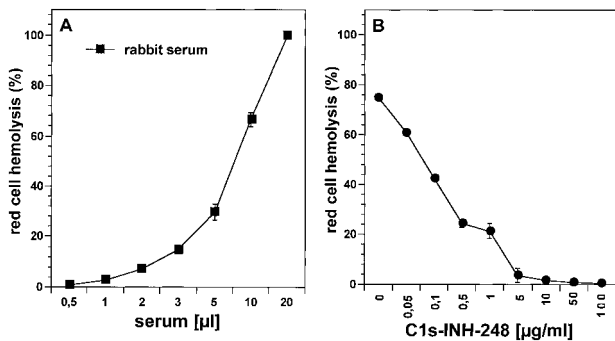


FIGURE 1. Inhibition of rabbit serum induced hemolysis of sensitized SRBC by C1s-INH-248 (expressed as percent hemolysis). A, Dose-dependent rabbit serum induced hemolysis. The inhibition of hemolysis by C1s-INH-248 was dose dependent over the range from 0.05 to 1 mg/ml (B). Values are the mean ± SEM for seven experiments.

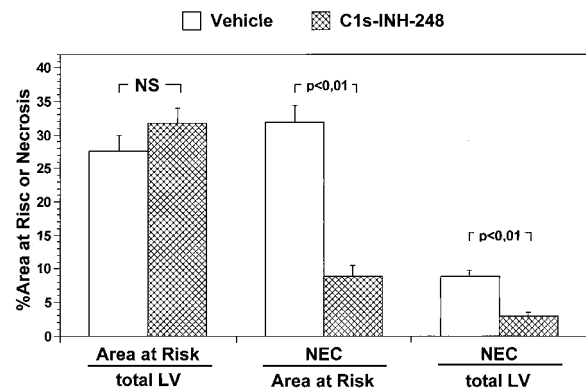


FIGURE 2. Tissue wet weight of area at risk as a percentage of the total left ventricular wet weight, and necrotic tissue as a percentage of area at risk and of the total left ventricle for the two MI+R groups. The heights of the bars are means; brackets represent ±SEM for seven rabbits.

were no significant differences in any of the variables observed before coronary occlusion. After reperfusion, the ST segment decreased to nearly control values, since coronary reperfusion had been effective. In both MI/R groups were no significant differences of the pressure rate index readings, suggesting that C1s-INH-248 did not appear to alter myocardial oxygen demand (data not shown).

Effect of C1s-INH-248 on myocardial injury following reperfusion

To ascertain the effects of C1 inhibition on the degree of actual myocardial salvage of ischemic tissue following reperfusion, we measured the amount of necrotic cardiac tissue expressed as a percentage of either the area at risk or the total left ventricular mass. There was no significant difference in the wet weights of the areas at risk between the two ischemic groups (Fig. 2). About 30% of the ischemic myocardium became necrotic in the vehicle group when indexed to the area at risk or 9% when indexed to the total left ventricle. However, in the C1s-INH-248-treated ischemic-reperfused group, the amount of necrotic tissue was <9 or 3%, respectively (p < 0.01). Therefore, C1s-INH-248 (1 mg/kg body weight) significantly protected against necrotic injury in ischemic-reperfused rabbits (Fig. 2).

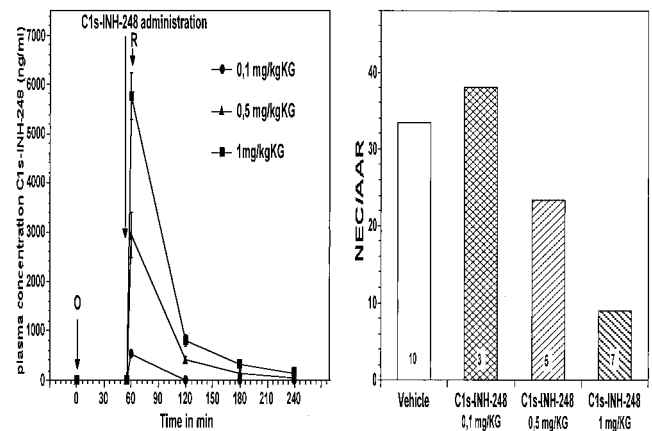


FIGURE 3. Left, Time course of the plasma concentration of C1s-INH-248 following bolus administration of 0.1, 0.5, and 1 mg/kg body weight at different time points. Right, Reperfusion injury for the different MI+R groups treated with vehicle and 0.1, 0.5, and 1 mg/kg body weight C1s-INH-248. The heights of bars are the means for three to seven rabbits.

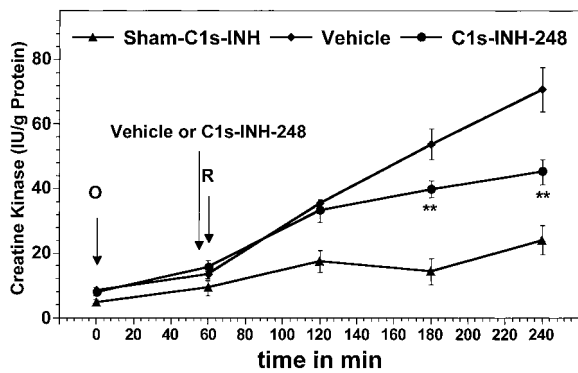


FIGURE 4. Plasma CK activity expressed as international units per micrograms of protein measured hourly throughout the experiment for all three groups. All values are the mean \pm SEM for five to seven rabbits. **, $p < 0.01$ compared with MI+C1s-INH-248. O, occlusion.

In additional animals we used a dose of 0.5 and 0.1 mg/kg body weight following 60-min ischemia. When we compared the effects on necrosis with the plasma concentration of C1s-INH-248, we saw a dose-dependent decrease following bolus administration (Fig. 3). After 3 h of reperfusion we detected in the 0.1 mg/kg body weight C1s-INH-248-treated animals a similar degree of necrosis compared with that in vehicle-treated animals (33 vs 38% necrotic area/area at risk). Animals treated with 0.5 mg/kg body weight C1s-INH-248 showed a partial protective effect (23% necrotic area/area at risk; Fig. 3, right).

When we compared the effects of C1s-INH-248 with administration of C1-INH we observed with even high doses treatment (i.e., 200 U/kg C1-INH) less cardiac protection (15% necrotic area/area at risk; i.e., 50% reduction) as with the C1s-INH-248 treatment (1 mg/kg; 9% necrotic area/area at risk, i.e., 70% reduction).

To confirm the preservation of ischemic tissue, we determined the effect of C1s-INH-248 on CK activity, a biochemical marker of myocardial tissue injury. In sham MI/R rabbits receiving C1s-INH-248, the plasma CK activity increased slightly. In the two ischemic groups, plasma CK activity increased slightly during the period of myocardial ischemia. However, a marked washout of CK into the circulating blood occurred in rabbits receiving vehicle. In contrast, MI/R rabbits treated with C1s-INH-248 had significantly lower plasma CK activities compared with vehicle-treated rabbits ($p < 0.01$; Fig. 4).

Neutrophil accumulation in the ischemic-reperfused area

Since accumulation of neutrophils in the ischemic region during reperfusion has been thought to be one of the major mechanisms responsible for reperfusion injury, we measured MPO activity as a marker for neutrophil accumulation in the myocardium (Fig. 5). MPO activity was very low in nonischemic myocardium of both MI groups (no significant difference). However, MI rabbits receiving only the vehicle exhibited a slight increase in MPO activity in the ischemic region. MPO activity in the ischemic region of C1s-INH-248-treated animals tend to be lower compared with that in vehicle-treated animals ($p < 0.05$). MPO activity increased in the necrotic portion to 1.31 ± 0.23 IU/100 mg tissue. In contrast, C1s-INH-248-treated MI/R rabbits exhibited significantly lower MPO activity in the necrotic myocardial tissue (0.40 ± 0.05 IU/100 mg tissue; $p < 0.001$).

Similarly, in histologic sections of myocardial tissue we were able to detect reduced neutrophil accumulation following C1s-INH-248 treatment (data not shown). In addition, immunohistochemical analysis of MAC (C_{5b-9}) showed that reduced neutrophil

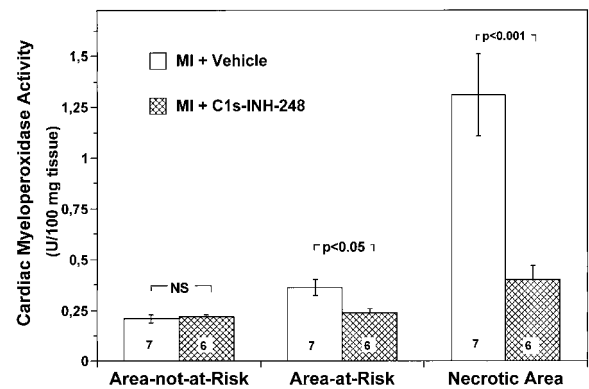


FIGURE 5. Cardiac MPO activity in area at risk, necrotic area, and area not at risk expressed as units per 100 mg tissue wet weight for the two MI+R groups. The heights of bars are the means; brackets represent \pm SEM for seven animals in each group.

accumulation was accompanied by less immunostaining for MAC, indicating inhibition of complement activation in C1s-INH-248-treated animals compared with vehicle-treated animals (data not shown).

Effects of C1s-INH-248 on number of circulating white blood cells

To determine whether C1s-INH-248 exerted any neutropenic effects that could contribute to its cardioprotection, we counted circulating white blood cells at the beginning and during the experimental period. White blood cells counts did not change significantly over the course of the experiment, and there were no significant differences between these groups (Table III).

Effects of C1s-INH-248 on superoxide radical release from PMNs

To determine whether C1s-INH-248 exerted any inhibitory effects on PMN radical generation following activation with leukotriene B₄ or PMA, we performed spectrophotometric analysis. The rate of superoxide anion production by PMN following leukotriene B₄ or PMA activation did not change significantly when PMN were coincubated with C1s-INH-248 (50 ng/ml to 0.1 mg/ml). Similar results were observed in human PMN.

Effects of C1s-INH-248 on PMN-endothelium interaction

To determine whether C1s-INH-248 exerted any effect on PMN adhesion to the vascular endothelium, we performed an in vitro adhesion assay. Thrombin activation resulted in increased neutrophil adherence to the aortic vascular endothelium. However, PMN adherence was not affected by C1s-INH-248 (50 ng/ml to 0.1 mg/ml).

Discussion

Our data clearly demonstrate significant cardioprotective activities of the novel small molecule C1 inhibitor (C1s-INH-248) in myocardial ischemia and reperfusion. The cardioprotection exerted by C1s-INH-248 was characterized by a reduction of necrosis and decreased serum CK activity compared with rabbits given the vehicle only. The cardioprotective effect of C1s-INH-248 was dose dependent when administered as a 0.1–1 mg/kg body weight bolus injection. Even compared with the treatment with the C1 esterase inhibitor (C1-INH) C1s-INH-248 demonstrated superior potency. The protection of C1s-INH-248 also resulted in inhibition of PMN accumulation in the reperfused myocardium. Further, the protective effect could be attributed to decreased deposition of C_{5b-9} on

Table III. White blood cell count at different time points (cells per microliter)

	0 min	60 min	120 min	180 min	240 min
Sham + C1s-INH-248	3900 ± 841	2900 ± 604	3750 ± 534	4850 ± 1571	4916 ± 1301
MI/R + Vehicle	4221 ± 591	3385 ± 459	3385 ± 428	4628 ± 790	4400 ± 408
MI/R + C1s-INH-248	5216 ± 877	5314 ± 1090	5542 ± 813	6071 ± 778	6092 ± 978

ischemic reperfused myocardium or vascular endothelial cells. To our knowledge, this is the first study demonstrating cardioprotection with a highly specific synthetic C1 inhibitor following myocardial ischemia and reperfusion.

To date, cardioprotective effects of complement inhibition were demonstrated for the C1 esterase inhibitor (C1-INH), sCR1, and Abs directed against C5 or C5a (6, 10, 11, 21, 22). C1 esterase inhibitor blocks the classical complement pathway by binding to the activated C1 complex (C1q, C1s, C1r), which results in dissociation of the complex (23). Further, C1-INH can be inactivated by elastase released from activated neutrophils, human proteinase (3), plasmin, or thrombin (12, 24, 25). Thus, inactivation of C1-INH can occur locally in inflamed tissues such as ischemic reperfused myocardium. Therefore, administration of exogenous C1-INH was able to reduce myocardial reperfusion injury (6–8).

C1s-INH-248 is a small molecule (m.w. 520) nonpeptide inhibitor that is highly specific for C1s. Inhibition of C1s can occur for the activated and nonactivated complexes. C1s-INH-248 acts as reversible and competitive inhibitor of activated C1s. Since C1s-INH-248 is a really small molecule, it is able to inactivate C1 quickly, whereas C1-INH inhibitory activity can be augmented 10 times when preincubated for 60 min. In contrast to C1s-INH-248, C1-INH not only inhibits activated serine proteinases C1s and C1r, it is also a major inhibitor of activated factor FXII, as well as an inhibitor of kallikrein and activated factor XI (12, 23). Therefore, the previous observed cardioprotective effects cannot be solely attributed to complement inhibition. However, with the present study we were able to demonstrate with the highly specific C1s inhibitor the important role of the classical complement pathway following myocardial ischemia and reperfusion.

In this regard, we were able to demonstrate inhibition of complement-mediated red cell hemolysis by C1s-INH-248 and C1-INH following rabbit serum administration. However, C1s-INH-248 had on equimolar basis a superior potency ($IC_{50} = 0.19$ nM vs 1.6 μ M). From the *in vitro* testing it was evident that C1-INH blocks also the MBL pathway (i.e., MASP1, MASP2, lectin complement pathway). Administration of C1s-INH-248 did not inhibit MASP1, nor did it affect the MBL pathway (data not shown). Therefore, it is unlikely that activation of the MBL pathway plays a dominant role in our model.

However, accumulation of the first component of the classical complement pathway (i.e., C1q) has been demonstrated in the ischemic-reperfused myocardium and has been related to increased neutrophil accumulation in this area (8, 26). C1q binds to membrane particles, mitochondrial fragments, or other subcellular components of the ischemic tissue and is able to activate the complement cascade with subsequent generation of C3a, C5a, and C_{5b-9} (4). C_{5b-9} deposits were observed in myocardial tissue from patients with myocardial infarction (27) and in animal hearts following ischemia and reperfusion (10, 28). We observed in the present study, in accordance with other studies, significant deposition of C_{5b-9} following myocardial ischemia and reperfusion. However, C1s-INH-248 treatment retarded C_{5b-9} deposition and tissue injury. In a similar approach of myocardial ischemia and reperfusion

in C6-deficient rabbits, reduction of myocardial necrosis was related to a reduction in C_{5b-9} deposition (29).

Our results clearly show that C1s-INH-248, when administered 5 min before reperfusion as bolus injection (0.1, 0.5, and 1 mg/kg body weight), markedly retards, dose-dependently, postreperfusion cardiac injury. The reduction in tissue injury exerted by C1s-INH-248 cannot be attributed to any hemodynamic effects, since the bolus injection did not alter the hemodynamic parameters. Similarly, C1s-INH-248 did not exert any anti-thrombin activity, since the bolus injection did not alter the activated partial thromboplastin time compared with vehicle (data not shown).

Treatment with C1s-INH-248 resulted, aside from its complement inhibitory effect, in reduction of 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 C1s-INH-248, however, cannot be attributed to changes in circulating white blood cell counts, since bolus injection of C1s-INH-248 did not result in a significant drop of white blood cell counts. These data eliminate the possibility that C1s-INH-248 exerts cardioprotection due to neutropenia, a phenomenon known to reduce reperfusion injury (30). Further, C1s-INH-248 did not affect neutrophil radical generation following leukotriene B_4 or PMA activation and did not directly inhibit PMN-endothelium interaction. Therefore, the direct anti-neutrophil potency of C1s-INH-248 cannot be attributed to the cardioprotective effects. Similar, in other experiments complement depletion with cobra venom factor resulted in significant inhibition of myocardial injury and reduced PMN infiltration (2).

Neutrophil adherence to the vascular endothelium is an early and important event following reperfusion of ischemic myocardium that is mediated by various adhesion molecules (31, 32). The complement system stimulates neutrophil-endothelium interaction, since C_{5b-9} and C5a induce rapid translocation of P-selectin from Weibel-Palade bodies to the endothelial surface (33, 34). In this regard, blocking of selectins with either a mAb or a soluble sialyl Lewis^x-containing oligosaccharide reduced myocardial reperfusion injury in cats (35, 36). Further, C5a induces the synthesis and release of cytokines, including IL-1, IL-6, and TNF- α , which can induce the expression of ICAM-1 or E-selectin (37). In a previous study we could demonstrate that blocking of complement activation results in reduced expression of P-selectin and ICAM-1 on the vascular endothelium (8). Therefore, it is most likely that the reduced neutrophil accumulation following C1s-INH-248 treatment can be attributed to indirect reduction of PMN-endothelium interaction.

In conclusion, we have demonstrated that *in vivo* administration of the small molecule C1 inhibitor C1s-INH-248 attenuates myocardial injury following ischemia and reperfusion. These protective effects could be in part attributed to reduced PMN accumulation as well as decreased C_{5b-9} deposition following C1s-INH-248 administration in the reperfused myocardium. Furthermore, these

in vivo results demonstrate the important role of classical complement pathway activation for tissue injury in inflammatory states such as myocardial ischemia and reperfusion.

Acknowledgments

We thank Prof. S. Bhakdi (Department of Microbiology, Johannes Gutenberg University, Mainz, Germany) for his critical comments. We thank Dr. Martin Schmidt and Dr. Heinz Hillen (Knoll AG, BASF Pharma, Ludwigshafen, Germany) for their tremendous support and critical discussion during the course of the study.

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