Application of C1-Esterase Inhibitor During Reperfusion of Ischemic Myocardium

Dose-Related Beneficial Versus Detrimental Effects

Georg Horstick, MD; Oliver Berg, MD; Axel Heimann, MVD; Otto Götze, MD; Michael Loos, MD; Gerd Hafner, MD; Benjamin Bierbach; Steffen Petersen, MD; Sucharit Bhakdi, MD; Harald Darius, MD; Marina Horstick, MD; Jürgen Meyer, MD; Oliver Kempski, MD

Background—Complement activation during reperfusion of ischemic myocardium augments myocardial injury, and complement inhibition with C1-esterase inhibitor (C1-INH) at the time of reperfusion exerts marked cardioprotective effects in experimental studies. Application of C1-INH in newborns, however, was recently reported to have dangerous and even lethal side effects. This study addresses the essential role of dosage in studies using C1-INH.

Methods and Results—Cardioprotection by C1-INH was examined in a pig model with 60 minutes of coronary occlusion followed by 120 minutes of reperfusion. C1-INH was administered intravenously 5 to 10 minutes before coronary reperfusion without heparin at a dose of 40, 100, and 200 IU/kg body wt. Compared with the NaCl controls, C1-INH 40 IU/kg reduced myocardial injury (44.1 ± 13.8% versus 76.7 ± 4.6% necrosis of area at risk, \( P<0.05 \)) and significantly suppressed local C3a and C5a generation. Myocardial protection was accompanied by reduced plasma concentrations of creatine kinase and troponin T. In contrast, no beneficial effects were observed when 100 IU/kg C1-INH was used. Furthermore, application of 200 IU/kg C1-INH provoked severe side effects and coagulation disorders.

Conclusions—When applied at the correct dose, C1-INH significantly protects ischemic tissue from reperfusion damage. However, overly high doses (>100 IU/kg) of C1-INH will provoke detrimental side effects, probably via its procoagulatory action. (Circulation. 2001;104:3125-3131.)

Key Words: inhibitors ■ reperfusion ■ ischemia ■ myocardium ■ myocardial infarction

Coronary angioplasty and thrombolytic therapy are the standard measures that limit the extent of infarct size after coronary occlusion.1 Their beneficial effects can be counteracted by reperfusion injury, which appears to ensue from a number of events, including rapid metabolic reenergization with contraction band necrosis and calcium overload2-3 and normalization of tissue osmolality4 and pH5 that leads to posts ischemic intracellular edema, cell swelling and rupture,6 and apoptosis.7 Local inflammatory reactions and hemorrhage8 worsen the microcirculatory situation and lead to increased microvascular obstruction9 that prevents functional recovery.10-12

Complement activation occurring in reperfused tissues may contribute significantly to reperfusion damage. Administration of C1-esterase inhibitor (C1-INH) proved to be potentially cardioprotective in experimental myocardial infarction (MI).13-14 A dose of 20 IU/kg infused into the coronary artery directly after reopening of the vessel without heparin administration was found to be cardioprotective in pigs,14 and a higher dose (75 IU/kg) given intravenously showed a tremendous reduction of MI in a heparinized cat model.15 In one clinical study, C1-INH infusion led to improved myocardial function after emergency CABG for failed PTCA.16

This promising background has recently been shattered by a tragic report. Thirteen newborns and babies received up to 500 IU/kg C1-INH in an attempt to prevent capillary leakage syndrome after cardiopulmonary bypass operation for congenital heart disease. Great-vein thrombosis developed in all patients, and 9 patients died of embolic events,16 which had been observed once before.17

Here, we decided to examine the role of C1-INH dosage in myocardial protection studies. We confirmed its cardioprotective effects at a dose of 40 IU/kg IV but observed serious side effects when the inhibitor was applied at dosages >100 IU/kg.

Methods

Experimental System

Twenty-seven German Landrace pigs of either sex weighing 27±2 kg were randomized to intravenous treatment with either 40, 100, or...
200 IU purified human C1-INH (Aventis-Behring Co), 0.9% saline infusion (vehicle), or sham operation. Experimenters were blinded to the treatment regimen. After anesthesia with intravenous α-chloralose and intubation, animals were mechanically ventilated with a Dräger respirator AV-1 (O₂ in room air, FIO₂ 0.25 to 0.3, PCO₂ controlled), and venous and arterial lines were introduced. A snare around the coronary artery was established by tunneling the left anterior descending coronary artery (LAD) with a monofilament suture between the proximal and medial thirds, and at the same level, the vena cordis magna was cannulated for blood analysis. A myocardial PO₂ probe was implanted into the expected center of the area at risk (AR) as previously published.14

Group 1 (n=6) received 0.9% saline intravenously before reperfusion. C1-INH at a concentration of 40 IU/kg was infused in group 2 (n=6), in which 1 animal had to be omitted because of technical problems from the microsphere analysis. Group 3 (n=6) received 100 IU/kg, and 1 animal was omitted because of coagulation problems after drug administration. Animals of group 4 (n=3) received C1-INH 200 IU/kg. Group 5 (n=6) underwent the surgical procedure without coronary occlusion (sham animals). Human C1-INH was provided as a solution containing 50 IU/mL. Activity of C1-INH was determined as previously described.14 C1-INH was applied intravenously over 5 minutes starting 10 minutes before reperfusion.

Experimental Protocol
Baseline values were acquired during a preischemic period of 1 hour. Coronary occlusion was achieved for 60 minutes by tightening the snare around the LAD. The snare was then loosened, and a reperfusion period of 120 minutes followed. Ten minutes before reperfusion, either 40, 100, or 200 IU/kg body wt of C1-INH or vehicle was infused intravenously in a blinded fashion. Hemodynamic and PO₂ measurements and blood samples were obtained before coronary occlusion, 50 minutes after coronary occlusion (before drug or vehicle application), and after 10, 30, 60, 90, and 120 minutes of coronary reperfusion. At the end of reperfusion, the animals were killed, and the hearts were recovered for further analysis.

Hemodynamic Parameters
The ECG and right atrial, pulmonary artery, left atrial, and arterial pressures were recorded on a Siemens Sirecust 404-1 at different time points (see design of experiment). Cardiac output was determined by thermodilution (5 mL NaCl 0.9%, room temperature) and calculated for each area. For digestion and dye extraction, we used a modification that the purified anti-human monoclonal antibody 13E1 (Dako) together with an anti-rabbit horseradish peroxidase conjugate (Bio-Rad) and ABTS as a detection system.

Cardiac Enzyme Analysis
Venous blood samples (3 mL) were drawn to determine troponin T (TnT) and total creatine kinase (CK) activity. Blood was collected in polypropylene tubes containing citrate to inhibit blood coagulation. The samples were centrifuged at 2000g for 15 minutes at 4°C, and plasma was decanted. Plasma CK activity was determined by use of a biochemical assay and expressed as international units per milliliter (IU/mL).

Determination of Infarct Size
After 120 minutes of reperfusion, the LAD was reoccluded. Evans blue dye (40 mL) (2% wt/vol solution) was injected into the pulmonary artery to stain the perfused myocardium. Unstained myocardium was defined as the AR and was separated from the Evans blue–stained portion and immersed in triphenyltetrazolium chloride (TTC, Sigma Chemical Co Inc) to stain the vital myocardium according to the published protocol.14 The ischemic but nonnecrotic, red-stained tissue was separated from the unstained infarcted tissue. The 3 tissue sections, nonischemic (area not at risk), ischemic nonnecrotic (vital), and ischemic necrotic tissue (MI), were weighed.

Data were expressed as total left ventricular muscle mass (LVMM), AR, AR as percent of LVMM, and MI as percent of LVMM and AR.

Electron Microscopic Examinations
Before termination of the experiment, 1 transmural myocardial biopsy was obtained from the center of the AR, immersed and fixed in 2% glutaraldehyde, and prepared for transmission electron microscopy (Philips).

Myocardial Perfusion Measurements
Myocardial blood flow (MBF) was examined at baseline, during ischemia before drug treatment (CO 50 minutes), at early reperfusion (R 10 minutes), and at the end of the experiment by use of fluorescence-labeled microspheres (Triton Technology Inc). Two million to 2.5 million spheres per color were injected in a randomized manner into the left atrium over 30 seconds via the left atrial pressure catheter. A reference blood sample of 10 mL was drawn from the abdominal aorta over a period of 3 minutes starting 30 seconds before microsphere injection. At the end of the experiment, Evans blue and TTC staining were performed to differentiate between nonischemic, infarcted, and vital tissue. Mean MBF was calculated for each area. For digestion and dye extraction, we used a previously published protocol modified for the TTC red-stained tissue samples.

Statistics
Data are presented as mean±SEM. Statistical analysis was performed with Sigma Stat (Jandel Corp). The statistical significance of changes from baseline values within each group was tested with ANOVA for repeated measures. Differences between groups were statistically analyzed by 1-way ANOVA comparing several groups. If values did not show a normal distribution, ANOVA for nonparametric values (Kruskal-Wallis test) with the multiple-comparison method (Student-Newman-Keuls test) was used. Statistical significance was accepted at an error probability of P≤0.05 after pairwise testing. Data of group 4 (n=3) are presented in the text and figures.
but were not tested statistically, because group 4 was omitted from the study because of severe adverse events.

Results

In the course of the blinded investigations, severe coagulation disorders were encountered in 4 animals. Blood aspiration from the intravenous catheter was impossible because clots had formed. The study coordinator revealed to us that 3 of the 4 animals had received 200 IU/kg and 1 animal had received 100 IU/kg C1-INH. At this point, therefore, experiments with 200 IU/kg C1-INH (group 4) were terminated.

Myocardial Necrosis

Total LVMM and total wet weight of the AR were determined at the end of the experiment. Wet weight of the LVMM was not significantly different between groups (Figure 1A; group 1, NaCl, 69.0±2.3 g; group 2, C1-40, 68.3±1.4 g; group 3, C1-100, 72.3±2.0 g; group 4, C1-200, 66, 55, and 71 g; and group 5, sham animals, 63.2±2.1 g). Neither AR (Figure 1B; group 1, 24.4±2.2 g; group 2, 22.3±1.1 g; group 3, 25.4±2.6 g; group 4, 22, 18, and 43 g; and group 5, 14.8±1.7 g) nor wet weight of the AR expressed as percentage of LVMM (Figure 1C; group 1, 35.1±2.1%; group 2, 32.8±1.8%; group 3, 34.9±2.9%; group 4, 32%, 32%, and 60%; and group 5, 23.1±1.9%) showed a significant difference between groups 1, 2, and 3. It followed that the region of myocardial ischemia was comparable in size in all groups.

In the NaCl group, 76.7±4.6% of the ischemic AR became necrotic (Figure 1D). Treatment with C1-INH 40 IU/kg markedly reduced the area of necrosis (Figure 1D; 44.1±13.8%; P≤0.05). In contrast, 100 IU/kg and 200 IU/kg C1-INH had no beneficial effect (Figure 1D; 72.7±3.8% and 85.7±4.1%). Calculations of MI/LVMM yielded analogous results (group 1, 27.1±2.6%; group 2, 15.0±4.9%; group 3, 25.0±1.5%; and group 4, 36.5±8.9%).

Transmission electron microscopic observations underlined the findings of “viable islets” inside the AR that will profit from reperfusion (Figure 7).

Hemodynamic Parameters

Mean arterial pressure, mean pulmonary artery pressure, left atrial pressure, central venous pressure, and heart rate displayed no significant differences between groups. Cardiac output was not significantly different between the vehicle and C1-INH groups at any time during the experiment and decreased in all groups with myocardial ischemia versus sham animals at the end of the experiment (Figure 2A).

Myocardial Oxygen Pressure

Intramyocardial Po2 served as a sensitive and rapid indicator of myocardial ischemia and successful reperfusion (Figure 2B). Absolute values of myocardial Po2 depend on the position of the sensor and varied between groups (group 1, 25.3±2.3 mm Hg; group 2, 19.1±4.7 mm Hg; group 3, 34.2±2.4 mm Hg; group 4, 29.9, 26.0, and 24.9 mm Hg; and group 5, 34.2±2.4 mm Hg). Therefore, data in Figure 2 are expressed as percentages of baseline values. During the first 5 minutes of coronary occlusion, myocardial Po2 dropped dramatically in all groups. ThePo2 increased within the first minutes of reperfusion in all groups, with group 2 presenting significantly higher values than groups 1 and 3.

Blood Gas and Lactate Analysis

Values of arteriovenous O2 difference did not differ at baseline (group 1, 9.5±0.5 mm Hg; group 2, 9.8±0.8 mm Hg; group 3,
9.8±0.6 mm Hg; and group 5, 10.2±0.2 mm Hg). There was a significant drop of arteriovenous O\textsubscript{2} difference in groups 1, 2, and 3 at 10 and 30 minutes after reperfusion (P<0.05). Values of the ischemic groups were lower than those of sham animals after reperfusion. At the end of reperfusion, the decrease of arteriovenous O\textsubscript{2} difference was still significant in groups 1 and 3 but not in group 2, but without any difference between groups (group 1, 5.5±0.9 mm Hg; group 2, 6.3±3.7 mm Hg; group 3, 4.7±1.5 mm Hg; group 4, 3.4, 0.8, and 0.4 mm Hg; and group 5, 9.5±0.2 mm Hg).

Arteriovenous lactate difference was equal in all groups at baseline (group 1, 0.9±0.1 mmol/L; group 2, 0.9±0.4 mmol/L; group 3, 0.9±0.2 mmol/L; group 5, 0.8±0.1 mmol/L) and decreased at 10 minutes of reperfusion in groups 1 to 3 (group 1, -2.0±0.7 mmol/L; group 2, -0.8±0.3 mmol/L; group 3, -1.1±0.6 mmol/L; group 5, 0.6±0.1 mmol/L).

Myocardial necrosis was accompanied by increases in potassium levels in plasma drawn from the vena cordis magna. Potassium was elevated at early reperfusion versus baseline in all groups with ischemia and reperfusion (baseline: group 1, 5.9, 3.7, 3.6 mmol/L; group 2, 5.5, 3.7, 3.6 mmol/L; group 3, 5.9, 3.7, 3.6 mmol/L; group 5, 6.1±1.1 mmol/L; group 5, 3.7±0.1 mmol/L). Potassium returned to normal values at the end of the experiment in all groups.

Arterial Po\textsubscript{2} did not differ between all groups at baseline (group 1, 134±13 mm Hg; group 2, 134±9 mm Hg; group 3, 136±12 mm Hg; group 5, 139±8 mm Hg) and remained unaltered throughout the experiments (group 1, 108±12 mm Hg; group 2, 131±3 mm Hg; group 3, 114±8 mm Hg; group 5, 129±9 mm Hg).

Hematocrit was equal among groups (group 1, 30±1.0%; group 2, 29±1.7%; group 3, 30±2.4%; group 5, 29±0.8%), without significant changes during the experiment.

Arterial Pco\textsubscript{2} and sodium did not differ between groups.

Cardiac CK and TnT
Washout of CK into the circulation occurred during the first minutes of reperfusion (Figure 3A). Values were significantly higher in groups 1 and 3 than in group 2 (Figure 3A, P<0.05). CK maximum values in animals of group 4 were 10 160, 12 600, and 13 020 U/mL.

Analogous results were obtained with TnT measurements. Values were significantly lower in animals receiving 40 IU/kg C1-INH than with normal saline and C1-INH 100 IU/kg (Figure 3B). The 3 animals in group 4 had TnT levels of 100, 137, and 101 U/mL.

Plasma Anaphylatoxin Levels
Measured values of the anaphylatoxins were expressed in percentage of baseline values (Figure 4, A and B). On reperfusion, increases in C3a and C5a concentrations occurred that were significantly attenuated by application of C1-INH 40 IU/kg but not of higher doses of C1-INH. The 3 animals of group 4 (not shown in the figure) presented with very high C3a and C5a levels (group 4, C1-200: C3a, 577%, 366%, and 329%; C5a, 588%, 423%, and 326%).

**Figure 3.** CK (A) and TnT (B): CK and TnT release of group 2 (C1-40) was lower than groups 1 and 3 (P<0.05).

C1-INH Plasma Levels
The concentrations of C1-INH were determined, and results are shown in Figure 5A. The ELISA was specific for human C1-INH, and so the starting values were zero. Ten minutes after reperfusion, C1-INH plasma levels were significantly different between groups 2 and 3 (group 2, C1-40, 121±15 μg/mL; group 3, C1-100, 215±16 μg/mL). Values of group 4 were clearly higher than plasma levels of groups 2 and 3 (C1-200, 922, 711, and 557 μg/mL). C1-INH plasma levels in all treated animals decreased during the experiment. Values of group 3 were significantly higher than those of group 2 until the end of the observation period (group 2, C1-40, 97±11 μg/mL; group 3, C1-100, 174±16 μg/mL; group 4, C1-200, 660, 550, and 527 μg/mL).

Thrombin-Antithrombin Measurements
Thrombin-antithrombin (TAT) levels increased in animals of group 1 (NaCl) and group 3 (C1-100, Figure 5B). Animals treated with C1-INH 40 IU/kg presented no changes during the experiments. The maximum values of TAT during reperfusion were higher in group 3 (C1-100, 400±107 μg/L) than group 2 (C1-40, 118±14 μg/L, P<0.05) (group 1, NaCl, 274±73 μg/L; group 5, sham, 101±11 μg/L). TAT values in all 3 animals of group 4 showed a wide range of elevated values, to peak values of >660 μg/L observed in all animals.

Myocardial Perfusion Measurements
MBF of the nonischemic, Evans blue–positive myocardium was normal throughout the experiment (Figure 6, A and B). During ischemia, MBF decreased significantly in the AR (Evans blue–negative myocardium; Figure 6, C through F).
MBF reduction in the ischemic, TTC-positive vital area of the AR did not decrease completely to zero because of contamination from the nonischemic border zone. After reperfusion, hyperemia occurred and returned to baseline values by the end of the experiments (Figure 6, C and D). In contrast, MBF of the ischemic TTC-negative tissue remained below baseline values in groups 1 and 3 during early reperfusion and was significantly higher after 2 hours of reperfusion in group 2 versus group 3 (P<0.05, Figure 6, E and F). The effect was more pronounced in the endocardium than the epicardium. Sham animals presented no changes.

**Discussion**

Administration of 40 IU/kg of C1-INH IV had a marked cardioprotective effect. Infarct size was significantly reduced, and this was paralleled by inhibition of complement activation and reduction of CK and TnT release. These results were comparable to those obtained with intracoronary application of 20 IU/kg.14 In marked contrast, we observed serious side effects when higher concentrations of C1-INH were used. Under blinded study conditions, aspiration of myocardial coronary venous blood was thwarted by coagulation disturbances, which were

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**Figure 4.** Anaphylatoxins C3a (A) and C5a (B): Values are presented as percent changes from baseline values. Area under curve (AUC) of relative C3a changes in group 2 (C1-40) was significantly lower than group 3 (C1-100, *P*<0.05). AUC of C5a changes in group 2 (C1-40) was significantly lower than groups 1 (NaCl) and 3 (C1-100, *P*<0.05).

**Figure 5.** C1-INH plasma levels (A) and TAT (B): Increase of TAT values was significant vs baseline in groups 1 (NaCl) and 3 (C1-100) (*P*<0.05).

**Figure 6.** MBF of Evans blue–positive normal perfused myocardium (A, B), TTC-positive (C, D), and TTC-negative ischemic myocardium (E, F) (absolute values in A, C, and E; percent changes of baseline values in B, D, and F). MBF of ischemic TTC-negative myocardium at end of reperfusion was higher in group 2 (C1-40) than group 3 (C1-100, *P*<0.05).
observed in group 4 animals receiving 200 IU/kg C1-INH. When the inhibitor was administered at 100 IU/kg, no cardioprotective effects could be observed, as reflected by infarct size reduction and CK and TnT plasma concentrations. TAT plasma levels mirrored the coagulation problems in the high-dose C1-INH groups 3 and 4. In contrast, infusion of 40 IU/kg resulted in TAT values comparable to those seen in sham animals. Because heparin can potentiate C1-INH activity, we omitted heparin in our experiments.22

High-dose C1-INH treatment was recently suspected to be the reason for severe side effects in 13 newborns and babies that received up to 500 IU/kg body wt C1-INH IV to prevent capillary leakage syndrome after cardiopulmonary bypass operation. All patients developed severe great-vein thrombosis, and 9 children died of emboli.16 Treatment with 300 IU/kg C1-INH and heparin led to superior vena cava thrombosis in 2 children after anatomic repair of transposition of the great arteries, and an extended renal vein thrombosis occurred in 1 neonate.17 The clinical data16,17 and procoagulatory effects observable at overly high doses highlight the hazards of C1-INH.

Two major activators of the fibrinolytic system are factor XIIa and kallikrein. Both are inactivated by C1-INH, and this alone would explain why high-dose C1-INH will have dangerous procoagulatory side effects.23–30 Furthermore, C1-INH inhibits bradykinin release. Bradykinin has net antiplatelet function and activates release of tissue plasminogen activator as well as formation of nitric oxide.31–34 In addition, complement binding to the cell surface has procoagulatory effects.35

Apparent conflicting data have been presented in a rat model of myocardial ischemia in which a cardioprotective effect of 100 IU/kg C1-INH was reported.36 The method used in that study was associated with high mortality in sham-operated animals, however (>40%), and postinterventional complications might therefore have been obscured.18 Moreover, because of species differences of coronary collateral circulation, CK washout of transmural myocardial biopsies, which was used as a marker of myocardial protection in that study, is not comparable to the detection of ischemic tissue with Evans blue that we used.37–39

Although TTC-negative areas are acceptable estimations of MI, there are local viable islets within the ischemic area, as seen in Figure 7D, that could profit from increased blood flow in the reperfused myocardium.40 The increase of MBF in the ischemic TTC-negative area of group 2 is mirrored by increases in intramyocardial Po2 values during reperfusion. Despite higher plasma levels of C1-INH in groups 3 and 4, complement activation was not attenuated dose-dependently. This might be a result of the diminished MBF after reperfusion because of microvascular obstruction and coagulation disturbances in animals receiving 100 and 200 IU/kg.

Heparin potentiates the inhibitory effect of C1-INH, and the anticoagulant was therefore omitted in this study.22 Administration of 75 IU/kg body wt C1-INH IV in combination with heparin led to a marked reduction of MI in cats.13,38 Results of first clinical experiences with heparin treatment and application of C1-INH as an intravenous bolus for emergency CABG indicated a cardioprotective effect at a lower dose of C1-INH.15

In conclusion, a correct dose of C1-INH (40 IU/kg body wt IV) is as cardioprotective as 20 IU/kg body wt administered intracoronarily. Benefit is lost and replaced by detrimental side effects at higher doses of inhibitor (100 and 200 IU/kg). The effects of heparin coapplication require a separate study.

Figure 7. Evans blue staining (A) of left ventricle and TTC staining (B) of Evans blue–negative ischemic AR of 1 animal treated with C1-INH 40 IU/kg. B, Pattern of vital myocardium (V) in animals receiving 40 IU/kg C1-INH. C and D, Transmission electron microscopy from center of ischemic region. C, Panoramic view of irreversible myocardial damage with contraction band necrosis in C (×10 500). D, Irreversibly injured myocardium and vital myocardium without enlarged I band and saved myofibers as an example of viable islets in TTC-negative areas (×22 000).
Acknowledgments

The authors thank G. Sonntag, H. Erbes, E. Dörr, M. Malzahn, and L. Kopacz for their excellent technical assistance. We gratefully acknowledge Dr. Diekneite and Dr. Jürs from Aventis-Behring Co for their critical comments and supply of C1 esterase inhibitor. This article contains data from the doctoral theses of Oliver Berg, Thomas Gast, and David Goldberg.

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Circulation. 2001;104:3125-3131
doi: 10.1161/hc5001.100835

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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