Inhibition of Neutrophil Proteinases by Recombinant Serpin Lex032 Reduces Capillary No-Reflow in Ischemia/Reperfusion-Induced Acute Pancreatitis¹

E. VON DOBSCHUETZ, T. HOFFMANN,² and K. MESSMER

Institute for Surgical Research, Klinikum Grosshadern, Ludwig-Maximilians-University, Munich, Germany

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ABSTRACT

Because neutrophil proteinases such as elastase and cathepsin G are considered to play a major role in inflammatory tissue damage, the microcirculatory effect of the serine proteinase inhibitor (serpin) Lex032 after ischemia/reperfusion (I/R)-induced pancreatitis was investigated. Lex032 inhibits these proteinases by recombinant combination of α1-antitrypsin and α1-antichymotrypsin. Twenty-eight anesthetized rats received either Lex032 or NaCl 0.9% as a control solution during baseline conditions or after 1 h of complete reversible ischemia induced by microclip occlusion of the pancreatic arteries. The number of erythrocyte-perfused capillaries (functional capillary density) and the leukocyte adherence in postcapillary venules were assessed by intravital microscopy 45, 90, and 120 min after administration. In the baseline group, Lex032 increased leukocyte adherence compared with the NaCl 0.9% baseline group, without changing any other parameter. I/R without Lex032 treatment resulted in a 50% reduction in functional capillary density, a 2-fold increase in leukocyte adherence, an increase in interleukin-6 serum concentration, and a significant fall in blood pressure during reperfusion time compared with baseline animals. Treatment with Lex032 in I/R resulted in significant preservation of capillary perfusion, an absence of interleukin-6 increase, and preservation of mean arterial pressure during reperfusion time, without changing the leukocyte adherence, compared with the NaCl 0.9% I/R group. Because of its considerable amelioration of microcirculatory perfusion, Lex032 might be useful in the treatment of pancreatic I/R tissue damage (e.g., cardiac bypass surgery, pancreas transplantation, and hemorrhagic shock) by prevention of capillary perfusion failure.

Ischemia/reperfusion (I/R) plays a causative role in acute pancreatitis. Cases of ischemia followed by acute pancreatitis are described after vascular occlusion due to embolism of pancreas-supplying arteries, pancreas transplantation, cardiac bypass surgery accompanied by hypoperfusion of the pancreas, hemorrhagic shock, and operations of the abdominal aorta clipping the blood supply to the pancreas (Gullo et al., 1996; Hoffmann et al., 1997). Capillary perfusion failure as it occurs in experimental acute pancreatitis causes ischemic damage of the pancreatic tissue. It is thought to be an aggravating factor in the pathophysiology of acute pancreatitis. I/R in the pancreas is characterized by microcirculatory perfusion failure (no reflow), intensified leukocyte accumulation and adhesion in postcapillary venules (reflow paradox), and impairment of endothelial barrier function, indicating the onset of cellular and organ dysfunctions.

Activated leukocytes play a major role in tissue damage in such an inflammatory organ reaction by the following mechanisms: 1) the release of superoxide radicals (oxidative burst) and proteolytic proteases (elastase, cathepsin G, and protease III) by activated leukocytes is known to disturb endothelial cell function and destroy cells and tissue of the inflamed organ, 2) plugging of capillaries by neutrophils contributing to the no-reflow phenomenon, and 3) stimulation of platelets by leukocytes and endothelial cells to release cytokines and vasoactive compounds (Lehr and Arfors, 1994; Nolte and Messmer, 1995; Massberg et al., 1998). Before circulating leukocytes can enter inflamed tissue, they have to roll along endothelium, adhere to it, and transmigrate through the endothelial barrier (Granger and Kubes, 1994). Adhesion molecules such as selectins and integrins are responsible for that accumulation and activation of leukocytes during reperfusion.

Therapeutic approaches undertaken to deal with these harmful actions of leukocytes have been variable, including inducing neutropenia after ischemia by radiation, introduc-

ABBREVIATIONS: I/R, ischemia/reperfusion; ACT, α1-antichymotrypsin; α1-PI, α1-protease inhibitor; FCD, functional capillary density; HNE, human neutrophil elastase; ICAM-1, intercellular adhesion molecule 1; IL-6, interleukin-6; MAP, mean arterial pressure; PMN, polymorphonuclear neutrophil.

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² Present address: Maria-Theresia-Klinik, Munich, Germany.

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ing monoclonal antibodies against leukocytes or filtering leukocytes out of the circulation, inhibiting leukocytes during the activation and accumulation process by antibodies directed against adhesion- and rolling-mediating molecules, drugs such as superoxide scavengers, antioxidants, and proteinase inhibitors, to combat the cytotoxic release products of activated leukocytes (Schmid-Schönbein, 1993; Nolte and Messmer, 1995). Serine proteinase inhibitors (serpins) make up 10% of blood plasma and are involved in the regulation of proteolytic enzymes to sustain homeostasis. Lex032 is a recombinant serpin in which the properties of two very similarly structured protease inhibitors, $\alpha_1$-protease inhibitor ($\alpha_1$-PI) and $\alpha_1$-antichymotrypsin (ACT), were combined by replacing six equivalent amino acids of ACT with the critical amino acids, which gives $\alpha_1$-PI its human neutrophil elastase (HNE)-inhibiting property (Rubin et al., 1994). The resulting novel human protein retains enzyme inhibition and secondary anti-inflammatory actions of ACT and gains the ability to inhibit HNE (Sands and Hook, 1997). In addition to their direct tissue destruction, HNE and cathepsin G are involved in many inflammatory actions such as detachment of adhering leukocytes from the endothelium (Cai and Wright, 1996), regulation of the neutrophil oxidase that generates superoxide radicals (Kusner and King, 1989), activation of platelets, and transmigration of leukocytes through the endothelial barrier. Lex032 has proven its beneficial anti-inflammatory properties in several in vivo studies of myocardial I/R (Murohara et al., 1995; Delyani et al., 1996), ischemic stroke (Homma et al., 1997), and trauma-induced hemorrhagic shock (Scalia et al., 1995). In ex vivo studies, Lex032 showed an antiadhesive effect of neutrophils on mesentery artery endothelium, coronary artery vessel rings (Murohara et al., 1995; Delyani et al., 1996), and fibronectin-coated tissue plates (Carney et al., 1998), suggesting adhesion as a key mechanism in the anti-inflammatory action of Lex032. The in vivo proof of such mechanisms in I/R damage by intravital fluorescence microscopy has been missing until now. The aim of our study was to investigate the microcirculatory effects of Lex032 on pancreas after I/R-induced pancreatitis and during baseline conditions. Assessment of capillary perfusion allows investigation of Lex032’s effects on the no-reflow phenomenon. Actions on leukocyte adherence in postcapillary venules are of special interest because of the findings of former studies indicating an anti-inflammatory effect of Lex032 by reduction of adhesion.

Materials and Methods

Anesthesia and Monitoring. Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 180 to 260 g were anesthetized by ether and pentobarbital sodium (50 mg/kg b.wt. i.p.) after an overnight fast with free access to tap water. After tracheotomy, a tube was inserted into the trachea, and the rat was ventilated (frequency, 57–65 breaths/min; tidal volume, 2–2.5 ml; inspiratory oxygen fraction (FIO2), 0.25–0.40; Harvard Rodent Ventilator 683; Harvard Apparatus, South Natick, MA). The right carotid artery and the right jugular vein were cannulated with a polyethylene catheter (PE-50, 0.58 mm i.d.; Portex, Hythe, Kent, UK) for continuous monitoring of mean arterial pressure (MAP), heart rate, and continuous volume replacement (3–5 ml/h NaCl 0.9%) by a syringe pump. Rectal temperature was kept between 36.5 and 37.5°C by means of a heating pad (Fa. Effenberger, Pfaffing, Germany). Adequate anesthesia was maintained by injection of pentobarbital (6 mg · kg$^{-1}$ · h$^{-1}$ i.v.) and $N_2O$ (0.65–0.75). Arterial blood gases were measured intermittently (ABL 300 Radiometer; Copenhagen, Denmark) and were adjusted to the following values for baseline conditions by means of ventilator adjustment and sodium bicarbonate 8.4% injection: pO2 100 to 120 mm Hg, pCO2 30 to 40 mm Hg, pH 7.39 ± 0.02, and base excess 0 ± 2. Hematocrit values were measured by Coulter counter T540 (Coulter Electronics, Hialeah, FL). The experiments were performed in accordance with German legislation on protection of animals.

Animal Model and Experimental Protocol. After transverse laparotomy, complete ischemia of the pancreas was induced by clipping the four arteries (left gastric artery, gastroduodenal artery, splenic artery, and caudal pancreaticoduodenal artery) to the pancreas by means of microvascular clips (clamping force 3 g; Aesculap, Tuttingen, Germany). The complete microsurgical technique was described elsewhere (Hoffmann et al., 1995). Sham-operated animals received only preparation without induction of ischemia. After a stabilization period of 15 min, animals were randomly assigned to four groups: 1) sham-operated group without ischemia receiving 2.2 ml NaCl 0.9%/kg b.wt. after operation procedures (sham control, n = 7); 2) sham-operated group without ischemia receiving 50 mg/kg (2.2 ml/kg b.wt.) of Lex032 (sham Lex032, n = 7); 3) 1 h-I/R receiving 2.2 ml/kg NaCl 0.9%/kg b.wt. during the onset of reperfusion (ischecmia control, n = 7); 4) 1 h-I/R receiving 50 mg/kg (2.2 ml/kg b.wt.) of Lex032 during the onset of reperfusion (ischemia Lex032, n = 7). One milliliter of arterial blood samples was taken before administration of the solutions, 15 min after administration and at the end of the experiment. Fifteen minutes after administration of the solutions, the pancreas and spleen were exteriorized for intravital microscopy on an adjustable stage and covered by a thin Teflon membrane to prevent drying. MAP was continuously determined and registered on a recorder (Siemens XT Kompensograph; Siemens, Munich, Germany). Heart rate was counted from the pressure amplitude. Heart rate, arterial blood gases from the carotid artery, and hematocrit were measured at baseline during ischemia and 15, 60, and 120 min after administration of the solutions. The experiments were finished by administration of an overdose of pentobarbital to the rats.

Drugs. The genetically engineered serpin Lex032 was generated as described elsewhere (Murohara et al., 1995). With the Escherichia coli fermentation technique in human ACT, six amino acids in the active loop were replaced by the amino acids that provide $\alpha_1$-PI its HNE-inhibiting property. Lex032 was provided on dry ice by Sparta Pharmaceuticals (Horsham, PA) in 25-ml plastic containers (lot no. CPP-156–001). Containers with the solved drug were stored at −20°C. Before use, the drug was thawed on ice and aliquotted to sterile syringes that were stored at 4°C until use. The characteristics of the product described by the manufacturer were as follows: pH 7.4, endotoxin/limulus amebocyte lysate <0.013 equivalent units/ml. One hundred percent purity of the product was ensured by HPLC and polyacrylamide gel electrophoresis by the manufacturer. NaCl 0.9% solution was purchased from Braun AG (Melsungen, Germany).

Intravital Microscopy and Quantification of Microcirculation. Before intravital microscopic measurements, 0.15 ml of 0.75% FITC-HAES [hydroxyethyl starch (M, 200,000) labeled with fluorescein isothiocyanate; Laevosan, Linz, Austria] and 0.1 ml of 0.2% rhodamine 6G (M, 497; Sigma, St. Louis, MO) was injected i.v. for contrast enhancement of microvessels and for in vivo staining of cytochrome c-containing cells (leukocytes), respectively. Intravital microscopy of the pancreas was performed with a modified Leitz-Orthoplan microscope (Leitz, Wetzlar, Germany) with a mercury lamp (100 W, HBO) attached to a Plamox-Pak illuminator with I2/3 excitation 450–490 nm emission >515 nm) and N2 (excitation 530–560 nm, emission >580 nm) filter blocks (Leitz) for epi-illumination. Use of saltwater immersion objective (SW 25 ×/0.6; Leitz) allowed magnification of ×800. The observations were recorded by means of a charge-coupled device (CCD) video camera (PK 6980; Cohu, Prospective Measurements, San Diego, CA) on a videotape (video...
Results

Effects of Lex032 on Capillary Perfusion of Pancreas. After exteriorization of the pancreas, FCD decreased significantly during 2 h of observation in all experimental groups except the Lex032-treated sham compared with the 45-min values in each individual group (Fig. 1). One hour of ischemia in the pancreas resulted in a rapid decrease in FCD from 265 ± 14.9 cm⁻¹ (45 min reperfusion) to 187 ± 11.7 cm⁻¹ (2-h reperfusion). These FCD values of ischemia control were significantly (p < .05) lower than all other groups at each time point. Lex032 treatment improved capillary perfusion significantly (p < .05) after 2 h of reperfusion (267 ± 13.8 cm⁻¹) compared with the NaCl 0.9%-treated I/R (187 ± 11.7 cm⁻¹). In the two sham groups, there was no significant difference between the Lex032 (2-h value, 341 ± 8.6 cm⁻¹) and the NaCl 0.9% control treatment (314 ± 8.6 cm⁻¹).

Effect of Lex032 on Leukocyte Endothelium Interaction. A significant (p < .05) increase of the number of adherent leukocytes during the observation time was registered in the NaCl 0.9% and the Lex032-treated I/R compared with the 45 min values of each individual group (Fig. 2). At 90 min and 120 min the Lex032-treated sham group, as well as the NaCl 0.9% and the Lex032-treated I/R showed a significantly (p <
A higher number of adherent leukocytes than the NaCl 0.9% treated sham group. Between these three groups there was no statistical difference at any time point.

**Effect of Lex032 on Concentration of α-Amylase and IL-6 Serum Level.** Amylase activity was measured as an indicator for acute pancreatitis (Fig. 3). To evaluate the degree of inflammation, IL-6 serum concentration was measured as a prognostic parameter (Fig. 4). Sham and I/R control groups showed a significant (p < .05) increase in serum activity of amylase. In contrast, Lex032 treatment showed no increase in amylase activity during the experiment. The amylase activity in the Lex032-treated I/R group was significantly (p < .05) lower than in the NaCl 0.9%-treated I/R group at the end of the experiment.

Serum IL-6 concentrations were significantly (38%) higher in the I/R control group compared with the sham control group at the end of the experiment. In contrast to the other groups, IL-6 serum concentration was elevated in the I/R group not receiving Lex032 compared with its baseline value. Lex032 treatment resulted in a significantly (49%) lower IL-6 concentration in the I/R compared with the NaCl 0.9%-treated I/R at the end of the experiment.

**Effect of Lex032 on Hemodynamics, Acid-Base Balance, and Hematocrit Values.** The baseline values of MAP (Fig. 5), heart rate, hematocrit (Table 1), pH, and base excess (Table 2) were not significantly different among the four groups. To investigate the systemic hemodynamic influence of inflammatory mediators at onset of I/R and the possible impact of Lex032 on this effect, MAP was registered more frequently after the opening of microclips (Fig. 6). There was a slight increase in MAP after the injection of NaCl 0.9% at 2 and 5 min, respectively, with return to baseline values after 15 min in the sham group. Lex032 treatment of the sham-operated animal resulted in constant MAP and heart rate values during the whole experiment. In the I/R groups, there were no changes in MAP and heart rate during ischemia time. I/R induced a rapid decrease in MAP during the onset of reperfusion, and MAP remained significantly lower than baseline values for the whole reperfusion time in the NaCl 0.9%-treated I/R group. There was no significant change in heart rate in either I/R. Lex032 treatment in the I/R group resulted in restoration of MAP after injection and stabilized MAP at values that were significantly higher than in the NaCl 0.9%-treated control group after 1 and 2 h of reperfusion.

**Effects of Lex032 on Granulocyte Infiltration in Pancreatic Tissue.** Transmigrated leukocytes were seen only in close proximity to the endothelium of the vessels. There were no significant differences comparing the numbers of extravasated leukocytes between the experimental groups (sham control, 1.0 ± 0.6/mm²; sham Lex032, 1.5 ± 1.0/
mm²; ischemia control, 35.4 ± 23.3; ischemia Lex032, 10.0 + 8.5/mm²).

### Discussion

This is the first study investigating the effect of the recombinant serpin Lex032 on leukocyte adherence and microcirculatory perfusion in an I/R model in vivo. Lex032 injected during baseline conditions resulted in a significant increase in leukocyte adherence without elevation in IL-6 serum concentration or amylase activity and without alteration of capillary perfusion in pancreas microcirculation. After 1-h induction of ischemia, Lex032 almost completely abolished the microcirculatory perfusion failure of the pancreas during reperfusion, as evidenced by the I/R control group. Because there was a significantly smaller increase in IL-6 serum concentration and amylase activity as well as preservation of MAP during the reperfusion in the Lex032-treated I/R group, we were able to demonstrate a beneficial effect of Lex032 on I/R-induced pancreatitis. The adherence of leukocytes in postcapillary pancreatic venules was not different in the Lex032-treated I/R compared with the ischemia control group, indicating that the anti-inflammatory effect of Lex032 is not the result of an antiadhesive action of the drug.

Because IL-6 serum concentration is used as an endpoint parameter in clinical trials (Kingsnorth et al., 1995) testing a therapy for severe acute pancreatitis, its diagnostic value for prognosis of pancreatitis is accepted (Inagaki et al., 1997). So far, it is unclear whether IL-6 plays a pro- or anti-inflammatory role in inflammatory diseases (Xing et al., 1998). It is up-regulated in the early, acute-phase response of acute pancreatitis, and its serum concentration correlates well with the severity and prognosis of patients suffering from acute pancreatitis (Norman, 1998). The lower concentration of IL-6 in serum in the Lex032-treated I/R and sham group indicates that Lex032 does not cause further inflammatory damage despite the increased number of adherent leukocytes in pancreatic venules.

In addition to the decrease in capillary perfusion, activation of neutrophil granulocytes with consecutive release of serine proteinases and NADPH oxidase-dependent generation of superoxide radicals from these granulocytes are the hallmarks of inflammatory tissue damage during I/R (Lehr and Arfors, 1994; Hoffmann et al., 1997). Lex032 treatment has proven to reduce the tissue damage and infarct size after experimental induction of I/R in heart (Murohara et al., 1995; Delyani et al., 1996) and brain (Sands and Hook, 1997). It also improved the outcome from experimental uncontrolled hemorrhagic shock (Scalia et al., 1995). Lex032 was further shown to reduce the release of superoxide radicals by PMNs in an in vitro study (Murohara et al., 1995).

There is one major question arising from these studies: Which mechanisms influenced by Lex032 lead to the preservation of microcirculatory pancreatic perfusion after I/R in our study? Lex032 is a recombinant construct in which six amino acid residues of ACT are replaced around its active loop by six amino acids of α₁-PI. Thus the neutrophil elastase-inhibiting property of α₁-PI was added to the cathepsin G-inhibiting property of ACT (Rubin et al., 1994; Sands and Hook, 1997).
In addition to their direct tissue-degrading properties (Crockett Torabi and Ward, 1996), elastase and cathepsin G have other secondary inflammatory effects that could explain the results of our study. After incubation of PMNs with Lex032, the ex vivo adhesion of leukocytes to fibronectin-coated plates (Carney et al., 1998), coronary endothelial rings (Delyani et al., 1996), and endothelium of the superior mesenteric artery (Murohara et al., 1995) was markedly reduced. Lex032 did not influence the expression of the adhesion molecules 1-selectin, CD18, and CD29 on the surface of activated PMNs in an in vitro experiment (Carney et al., 1998), suggesting that these adhesion-related molecules are also not influenced in vivo.

Recent works discussing the major role of HNE and cathepsin G on the adhesion and transmigration process of activated granulocytes are controversial. Inhibition of neutrophil elastase alone has been shown to reduce leukocyte infiltration after I/R in skeletal muscle (Carden and Korthuis, 1996) and small bowel (Zimmerman and Granger, 1990). The latter study showed a more pronounced reduction in leukocyte extravasation rather than a decline in leukocyte adherence. Neutrophil elastase is considered to play a major role in the process of leukocyte extravasation either by degradation of the basement membrane itself or by conversion of gelatinase and collagenase from its proforms to active proteinases (Borregaard and Cowland, 1997), which are key mediators in the extravasation process. Elastase is also considered to enhance adhesion of neutrophils to endothelium by extension of CD11b/CD18 adhesion molecules expressed on neutrophils counteracting the intercellular adhesion molecule 1 (ICAM-1) molecule expressed on endothelial cells (Woodman et al., 1993). In an experimental study of post-transplantation pancreatitis (Yamaguchi et al., 1998), elevated ICAM-1 mRNA expression after reperfusion was prevented by a specific HNE inhibitor. Additionally, this study showed elevated ICAM-1 mRNA after stimulation of human umbilical vein endothelial cells by HNE. In contrast, studies (Cai and Wright, 1996) with an antibody against HNE prevented detachment of PMNs from fibrogenic-coated surfaces, indicating that HNE has an antiadhesive property. In a study in leukotriene B4-activated hamster cheek-pouch microcirculation (Rosengren and Arfors, 1990), HNE inhibitors had no effect on the leukocyte adherence and diapedesis in postcapillary venules. α, integrin (Si Tahar et al., 1997). Thus, the inhibition of both cathepsin G and neutrophil elastase by Lex032 would suggest that Lex032, by its comprehensive combination of two serpins, is a potent drug to inhibit platelet activation by neutrophils and may prevent capillary perfusion failure, as seen in our study by such a mechanism.

Another effect of cathepsin G that could have affected our results involves platelets. Experiments from our laboratory quantified for the first time accumulation and adhesion of activated platelets in precapillary arterioles and postcapillary venules of mouse small intestine after I/R (Massberg et al., 1998). These results let us assume an important role of occlusive platelet aggregates in causing capillary perfusion failure after I/R. Cathepsin G has proved, in several in vitro studies, to be the major neutrophil-mediated activator of platelets (Renesto and Chignard, 1993). HNE is not able to directly activate platelets on its own but can promote the cathepsin G activation step by cleavage of one subunit of the αIIb-β3 integrin (Si Tahar et al., 1997). Thus, the inhibition of both cathepsin G and neutrophil elastase by Lex032 would suggest that Lex032, by its comprehensive combination of two serpins, is a potent drug to inhibit platelet activation by neutrophils and may prevent capillary perfusion failure, as seen in our study by such a mechanism.

Reduced shear rate after I/R is a key factor in the enhancement of leukocyte adherence in postcapillary venules, because the adhesion of adherent leukocytes is greatly decreased when enhancing the shear forces in I/R-predamaged mesenterium (Kubes, 1997). Because there was no sign of altered pancreatic microperfusion or systemic macrohemodynamic values in the Lex032-treated sham group compared with the sham control group, it is unlikely that Lex032 influences the adhesion process in the sham group by such a mechanism. The preserved macrohemodynamics and pancreatic FCD in the Lex032-treated I/R group suggests an elevated shear rate that should result in reduced leukocyte adherence. A rise in pancreas perfusion also increases the number of collisions of circulating neutrophils with the vessel wall by elevation of the effective “diffusion” coefficient of the leukocyte (McIntire and Eskin, 1984). This could be a mechanism explaining the absence of reduced leukocyte adherence in postcapillary venules in the Lex032-treated I/R group.

In conclusion, Lex032 has the potential to act against I/R tissue damage by blocking several serine proteinases. Significant improvement in postischemic capillary flow, reduction in IL-6 and amylase serum concentration, and preservation of hypotension during reperfusion in the I/R-treated group indicate an anti-inflammatory potential of Lex032 in I/R.
induced pancreatitis independent of a reduction in leukocyte adherence. Lex032 might be useful in the treatment of pancreatic I/R damage that occurs during pancreas transplantation, hemorrhagic shock, and cardiac bypass and aorta surgery. The probable usefulness of Lex032 in the treatment of microcirculatory failure after acute pancreatitis could be the subject of further experiments.

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References


Send reprint requests to: Ernst von Dobschuetz, Institute for Surgical Research, Klinikum Grosshadern, Marchioninistrasse 15, 81366 Munich, Germany. E-mail: edobschu@ifc.med.uni-muenchen.de