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Effects of LEX032, a novel recombinant serine protease inhibitor, on N^G-nitro-L-arginine methyl ester induced leukocyte–endothelial cell interactions

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Abstract

We studied the effects of LEX032, a novel serine protease inhibitor, on N^G-nitro-L-arginine methyl ester (L-NAME) induced leukocyte–endothelium interactions in vivo, utilizing intravital microscopy of the rat mesentery. Superfusion of the rat mesentery with 50 μM L-NAME, a nitric oxide (NO) inhibitor, for 90 min resulted in a significant and time-dependent increase in leukocyte rolling, leukocyte adherence, and transmigration of leukocytes, compared to control rats superfused with Krebs–Henseleit (K–H) solution. However, systemic administration of LEX032 (15 mg/kg bolus injection followed by a 15 mg/kg per hour infusion) to L-NAME superfused rats significantly attenuated leukocyte rolling and adherence along the venular endothelium of the rat mesentery, and also inhibited transmigration of leukocytes through the microvascular endothelial wall. Moreover, no significant changes were observed in mean arterial blood pressure or local venular shear rates following systemic administration of LEX032. Our data demonstrate that systemic inhibition of serine proteases by LEX032 reduces enhanced leukocyte–endothelium interactions provoked by inhibition of NO synthesis. These results also explain some of the beneficial effects exerted by serine protease inhibitors in ischemia-reperfusion and other inflammatory states.

Keywords: Intravital microscopy; Nitric oxide (NO) synthase inhibitor; Shear rate; Leukocyte adherence; Transendothelial migration

1. Introduction

Polymorphonuclear leukocytes, also known as neutrophils, play an important role in protecting the host organism by destroying invading pathogens. These neutrophils, once activated, release oxygen radicals (e.g., superoxide and peroxide) as well as proteolytic enzymes, including serine proteases (e.g., neutrophil elastase and cathepsin G), in an effort to eliminate invading microorganisms (Weiss, 1989). Although these processes are crucial during bacterial infection, they can also result in injury to viable host tissue and thus contribute to severe inflammatory states. Three early and key steps in this inflammatory response are leukocyte rolling, leukocyte adherence, and transmigration of leukocytes through the endothelium to the underlying inflamed area.

Several recent studies have examined the role of serine proteases and their inhibitors (i.e., serpins) in limiting neutrophil-mediated host injury. In this regard, inhibition of elastase and cathepsin G reduces neutrophil-mediated lung injury (Mulligan et al., 1993). Zimmerman and Granger (1990) have demonstrated the key role of neutrophil elastase during reperfusion of the ischemic bowel. Furthermore, in vitro studies have implicated neutrophil-release of serine proteases in damaging cultured endothelial cells as well as in degrading the extracellular matrix (Inauen et al., 1990).

In lieu of these data, it is plausible that serpins may be able to significantly reduce neutrophil-mediated tissue injury. Two important naturally occurring serpins found in humans are α-1 protease inhibitor, and α-1 antichymotrypsin. The former is an inhibitor of neutrophil elastase and cathepsin G, whereas the latter attenuates the effects of chymotrypsin, pancreatic elastase, and cathepsin G. α-1 antichymotrypsin is also thought to play a role in limiting the inflammatory response. LEX032 is a serpin which, via recombinant technology, possesses the inhibitory and inflammatory control properties of α-1 antichymotrypsin

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along with the neutrophil elastase inhibiting ability of α-1 protease inhibitor (Sands and Hook, 1997).

Delyani et al. (1996) have previously shown that LEX032 attenuates myocardial reperfusion damage in cats through its serpin activity, and Scalia et al. (1995) have reported that LEX032 increases survival time in murine traumatic shock. It has also been proposed that LEX032 maintains endogenous levels of nitric oxide (NO) by preserving the vascular endothelium (Scalia et al., 1995). NO is involved in many physiological functions, including inhibition of platelet aggregation, attenuation of neutrophil adherence, reduction of microvascular leakiness, as well as vasodilation of blood vessels (Lefer and Lefer, 1993, 1996).

In light of these findings, the goal of the current study was to determine if LEX032, a novel recombinant serine protease inhibitor, could affect N^G-nitro-L-arginine-methyl ester (L-NAME) induced leukocyte–endothelial cell interactions. We elected to evaluate the role of LEX032 on the three main steps of leukocyte–endothelium interaction, namely leukocyte rolling, leukocyte adherence to the endothelium, and the transmigration of leukocytes across the endothelium.

2. Materials and methods

Sprague–Dawley rats weighing 250–275 g were anesthetized with sodium pentobarbital (40 mg/kg) injected intraperitoneally. A tracheotomy was performed to maintain a patent airway throughout the experiment. A polyethylene catheter was inserted into the left carotid artery to monitor mean arterial blood pressure. Mean arterial blood pressure was recorded on a Grass Model 7 oscillographic recorder using a Statham P23AC pressure transducer (Gould, Cleveland, OH, USA). A midline laparotomy was performed in order to exteriorize a loop of ileal mesentery which was then placed in a temperature controlled, fluid filled Plexiglas chamber for observation of the mesenteric microcirculation via intravital microscopy as earlier described (Davenpeck et al., 1994). A jugular vein was cannulated for administration of supplementary sodium pentobarbital, as needed to maintain a surgical plane of anesthesia throughout the observation period. The mesentery was placed over a Plexiglas pedestal in the superfusion chamber, and the ileum was secured for stabilization of the viewing field. A modified Krebs–Henseleit solution (containing, in mM: 118 NaCl, 4.74 KCl, 2.45 $CaCl_2$, 1.19 KH_2PO_4, 1.19 $MgSO_4$, 12.5 $NaHCO_3$) warmed to 37°C and bubbled with 95% N_2 and 5% CO_2, was used to superfuse the ileum and mesentery throughout the 90 min observation period. A Microphot microscope (Nikon, Tokyo, Japan) with a 40× objective lens and a 10× ocular lens was used to visualize the mesenteric microcirculation. The image was projected by a video camera (Hamamatsu, Hamamatsu, Japan) onto a color Sony high resolution computer monitor and recorded with a videocassette recorder. Red blood cell velocity was determined on-line using an optical Doppler velocimeter (Borders and Granger, 1984), obtained from the Microcirculation Research Institute (College Station, TX, USA).

The rats were allowed to stabilize for 20–30 min following surgery. Following stabilization, a 30–50 μm diameter postcapillary venule was chosen for observation. A baseline recording was made to establish basal values for leukocyte rolling, adherence, and transmigration. The mesentery was then superfused with N^G-nitro-L-arginine-methyl ester (L-NAME, 50 μM) in a modified Krebs–

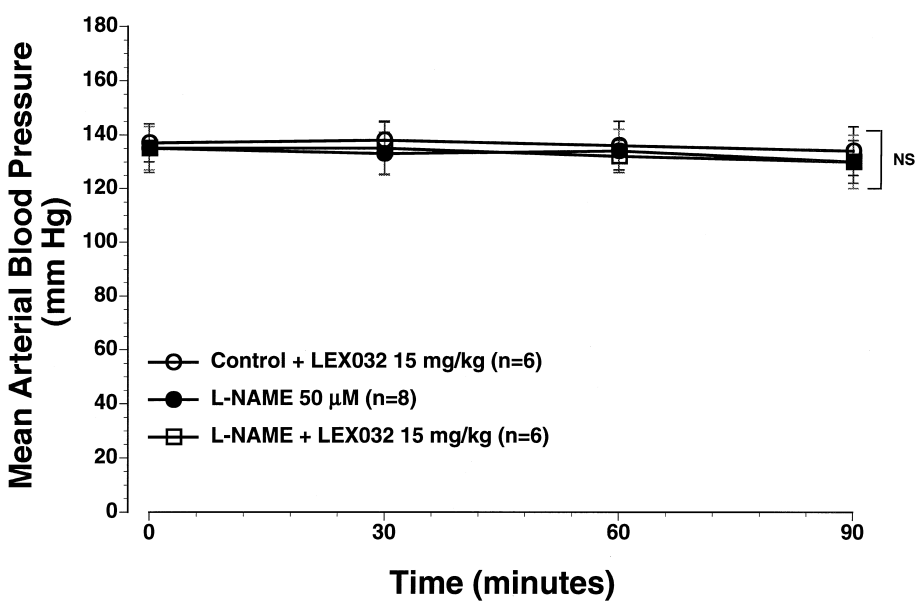

Fig. 1. Mean arterial blood pressures of the three experimental groups of rats. All values are means ± S.E.M. for 6–8 rats in each group.

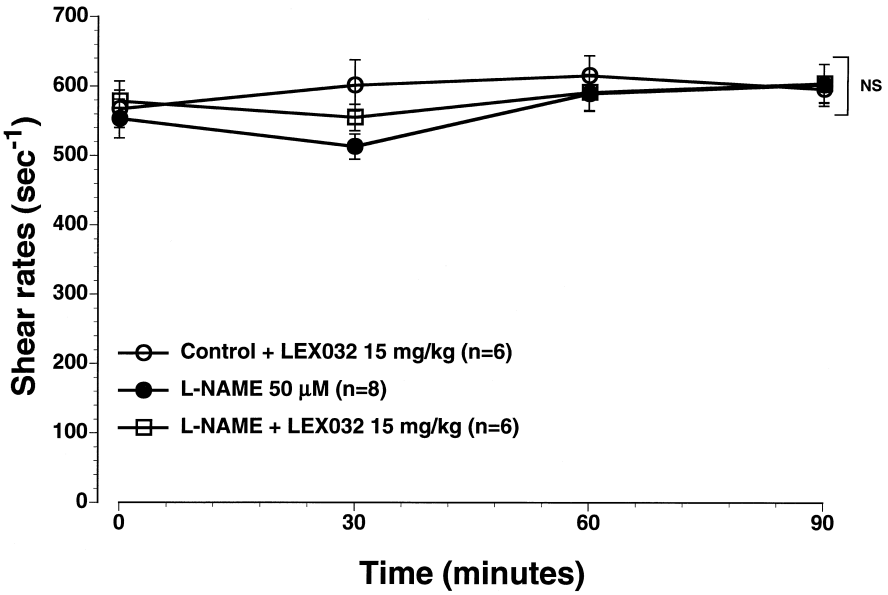

Fig. 2. Mean venular shear rate values of the three experimental groups of rats. All values are means ± S.E.M. for 6–8 rats in each group.

Henseleit solution for 90 min. Video recordings were made at 30, 60, and 90 min after initiation of L-NAME superfusion for quantification of leukocyte rolling, adherence, and transmigration. LEX032 was administered as a bolus (15 mg/kg i.v.) and as an infusion (15 mg/kg per hour i.v.). Immediately thereafter, L-NAME superfusion was started. Rats were randomly divided into three groups: (a) control rats which received LEX032, (b) L-NAME superfused rats, and (c) L-NAME superfused rats treated with LEX032. Preliminary studies indicated that control rats receiving 0.9% NaCl responded identically to control rats receiving LEX032, so there was no further need to study additional untreated control rats.

The number of rolling, adherent, and transmigrated leukocytes was determined off-line by playback of the videotape. Leukocytes were considered to be rolling if they were moving at a velocity significantly slower than that of red blood cells. Leukocyte rolling is expressed as the

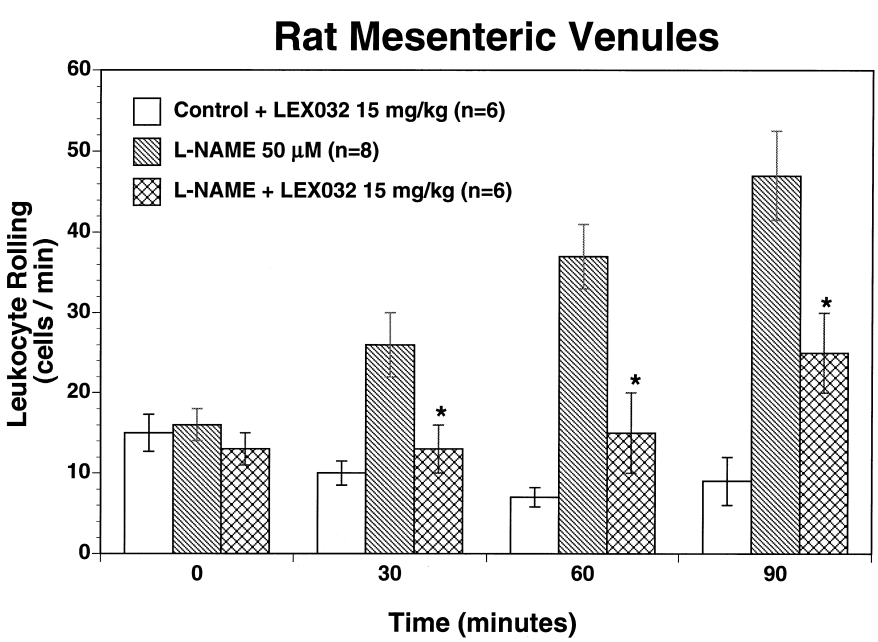

Fig. 3. Leukocyte rolling in rat mesenteric venules observed in control rats, rats subjected to 50 μM N^G-Nitro-L-Arginine Methyl Ester (L-NAME), or to L-NAME + LEX032 (15 mg/kg bolus + 15 mg/kg per hour i.v.). All values are means ± S.E.M. for 6–8 rats in each group. L-NAME clearly increased WBC rolling over the time-course of 30 to 90 min. This was significantly attenuated by LEX032 from 30 to 90 min. * $P < 0.05$ vs. L-NAME.

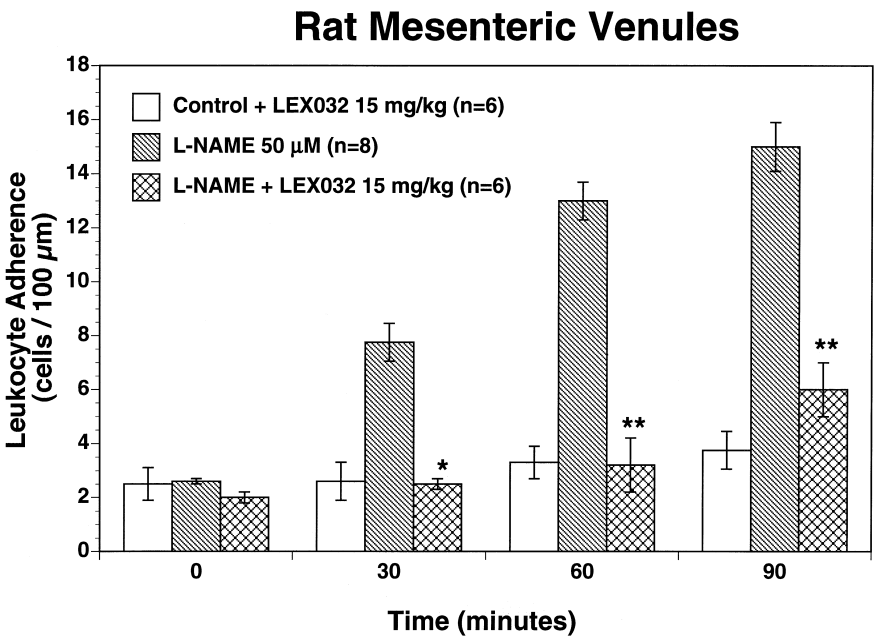

Fig. 4. Leukocyte adherence in rat mesenteric venules from either control rats, rats subjected to 50 μM N^G-Nitro-L-Arginine Methyl Ester (L-NAME), or to L-NAME + LEX032 (15 mg/kg bolus +15 mg/kg per hour i.v.). All values are means ± S.E.M. for 6–8 rats in each group. L-NAME significantly increased WBC adherence from 30 to 90 min. This was significantly attenuated by LEX032 from 30 to 90 min. $* P < 0.05$ and $** P < 0.01$ vs. L-NAME.

number of cells moving past a designated point per minute (i.e., leukocyte flux). A leukocyte was judged to be adherent if it remained stationary for > 30 s (Granger et al., 1989). Adherence is expressed as the number of adherent leukocytes/100 μm of vessel length. In order to quantify the number of transmigrated leukocytes, the tissue area adjacent to the 100 μm length of postcapillary venule over a distance of 20 μm from the vessel wall was used. The

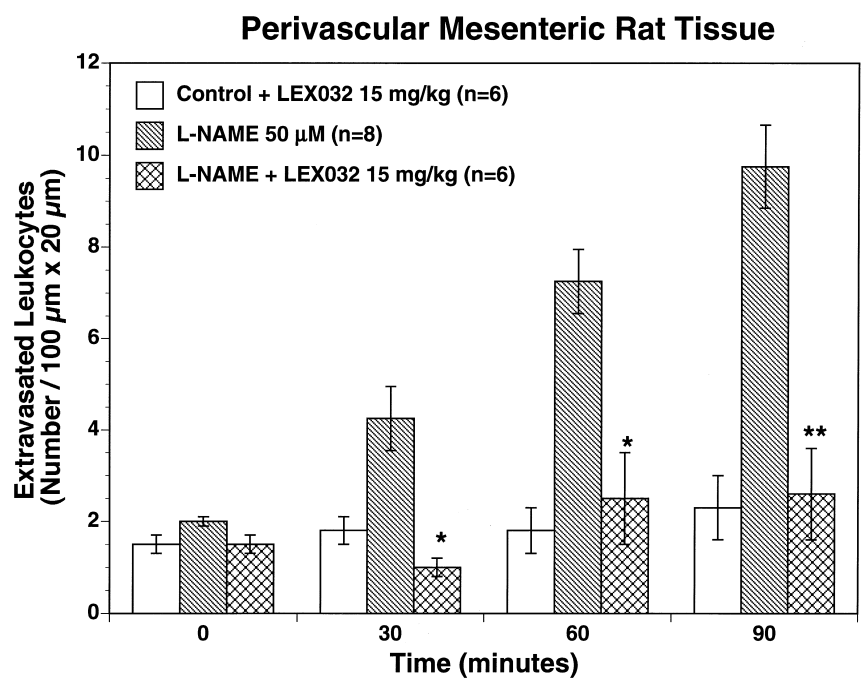

Fig. 5. Leukocyte extravasation within a 20 μm distance from the vessel wall in the rat mesenteric microvasculature. Bar heights show the number of transmigrated leukocytes for all experimental groups of rats. All values are means ± S.E.M. observed at 0, 30, 60, and 90 min for each group. Numbers in parentheses indicate numbers of rats studied. $* P < 0.05$ and $** P < 0.01$ vs. L-NAME.

number of extravasated leukocytes was counted and normalized with respect to area (20×100 μm). Red blood cell velocity and vessel diameter were used to calculate venular wall shear rate (g) employing the formula $g = 8 (V_{mean}/D)$ ($V_{mean} = V_{rbc}/1.6$) where V = velocity and D = diameter (Granger et al., 1989).

All values in the text and figures are presented as means ± S.E.M. of n independent experiments. All data were subjected to analysis of variance (ANOVA) followed by post hoc analyses using Fisher's t-test. $P < 0.05$ was considered statistically significant.

3. Results

Neither superfusion of the rat mesentery with 50 μM L-NAME nor the bolus and infusion administrations of LEX032 caused any significant effect on either systemic or regional hemodynamics, since there were no consistent changes in mean arterial blood pressure (Fig. 1) or in venular shear rates (Fig. 2) over the 90 min experimental period. Thus, these hemodynamic results indicate that alterations in leukocyte–endothelial cell interactions were not due to changes in physical hydrodynamic forces brought about by the infusion of the LEX032, or to spontaneous hemodynamic alterations.

The local application of L-NAME resulted in a time-dependent increase in leukocyte rolling (Fig. 3), adherence (Fig. 4), and transmigration (Fig. 5). The increase in rolling was statistically significant as early as 30 min after the onset of superfusion ($P < 0.05$ vs. control rats). The same pattern was noticed with leukocyte adherence ($P < 0.05$) and transmigration 20 μm from the venule wall ($P < 0.05$). LEX032 (15 mg/kg bolus i.v., 15 mg/kg per hour infusion i.v.) consistently reduced the L-NAME induced leukocyte rolling (Fig. 3), adherence (Fig. 4), and transmigration (Fig. 5).

Since rolling, adherence, and transmigration occur sequentially, these data indicate that the effects of LEX032 were progressive, rather than being transient. Moreover, maintained inhibition of leukocyte rolling by LEX032 led to fewer adherent neutrophils and subsequently lower numbers of leukocytes transmigrated across the vascular endothelium.

4. Discussion

The present data indicate that LEX032, a recombinant serine protease inhibitor (i.e., serpin), significantly attenuated L-NAME induced leukocyte rolling, adherence, and transmigration. Furthermore, administration of LEX032 consistently reduced leukocyte–endothelial cell interactions brought about by L-NAME in rats. All this occurred without any significant systemic hemodynamic effect (i.e., changes in mean arterial blood pressure or venular shear rates). The initial blood concentration of LEX032 following the bolus should equilibrate at about 150 μg/ml. Since LEX032 does not cross the blood–brain barrier, and is a large protein molecule, its circulating levels were probably 100–150 μg/ml in this study (Sands and Hook, 1997)

Leukocyte rolling, adherence, and subsequent transmigration through the endothelial wall of the mesenteric microcirculation are key steps in the inflammatory response brought about by several pathophysiological states (e.g., trauma, ischemia-reperfusion, and shock). These activated neutrophils produce pro-inflammatory modulators including oxygen derived free radicals and elastase, which serve to activate additional neutrophils (Sands and Hook, 1997) and thus further propagate tissue injury (Inauen et al., 1990). These relationships have been confirmed by the fact that neutrophil depletion or administration of antibodies directed against specific cell adhesion molecules exert a beneficial effect during ischemia-reperfusion, trauma, and shock (Vedder et al., 1989; Mileski et al., 1990; Lefer et al., 1994). A key early event during these conditions is the occurrence of endothelial dysfunction. This is characterized by reduced endogenous release of NO (Lefer and Lefer, 1993), a substance involved in the inhibition of platelet aggregation, attenuation of neutrophil adherence, and reduction in microvascular leakiness (Scalia et al., 1995). Davenpeck et al. (1994) also showed that reduced endogenous NO results in the upregulation of P-selectin on the endothelial surface of mesenteric vessels. Loss of NO would thus result in enhanced leukocyte–endothelium interaction (Kubes et al., 1991), and an increase in the permeability of the endothelium (Kurose et al., 1995).

Upon migration through the endothelial wall, activated leukocytes may release superoxide radicals which can further aggravate tissue injury. These superoxide radicals bind NO (Rubanyi and Vanhoutte, 1986), and the reduced levels of endogenous NO cause an upregulation of cell adhesion molecules, thereby increasing leukocyte–endothelial cell interactions (Lefer and Lefer, 1996). LEX032 has been shown to directly inhibit superoxide production by rat and human neutrophils (Kilpatrick et al., 1991; Murohara et al., 1995). This serpin could thus maintain endogenous NO levels, resulting in fewer leukocyte–endothelium interactions and thereby slowing this sequence of events.

Elastase is thought to play a role in the cleavage of CD43, a long, rigid molecule expressed at the surface of neutrophils. This molecule is negatively charged, and prevents leukocyte–endothelial interactions through charge repulsion, so the cleavage of CD43 by elastase-like proteins stimulates such interactions (Sands and Hook, 1997). The same study has shown that LEX032, which inhibits neutrophil elastase, prevents the cleavage of CD43. Thus, it follows that this serpin (i.e., LEX032) should reduce leukocyte–endothelial cell interactions. Furthermore, cathepsin G, another serine protease, upregulates the re-

lease of LTB_4 and platelet-activating factor (Camussi et al., 1988, 1989) thereby enhancing leukocyte–endothelium interactions and neutrophil chemotaxis (Lorant et al., 1991). LEX032 is known to inhibit elastase and cathepsin G activity, and the results of the current study are thus consistent with other proposed mechanisms of the actions of LEX032.

In conclusion, it has been demonstrated that LEX032, a novel recombinant serine protease inhibitor, significantly limits the microvascular effects of L-NAME, a known NO inhibitor which causes an increase in leukocyte–endothelium interactions (i.e., leukocyte rolling, adherence, and transmigration). Although we only examined the effects of a 90 min L-NAME mesenteric superfusion, the striking effects of LEX032 are highly significant over this time. The increased leukocyte–endothelium interactions brought about by L-NAME are comparable to those which occur during trauma or ischemia-reperfusion, and thus provide key mechanistic information on the protective effects of LEX032 in these important pathophysiological states.

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