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Protective Effect of Defibrotide on Splanchnic Injury following Ischemia and Reperfusion in Rats

Soo Ran Choi, Ji Hoon Jeong, Jin Ho Song, and Yong Kyoo Shin

Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, Korea

A splanchic artery occlusion for 90 min followed by reperfusion of the mesenteric circulation resulted in a severe form of circulatory shock, characterized by endothelial dysfunction, severe hypotension, marked intestinal tissue injury, and a high mortality rate. The effect of defibrotide, a complex of single-stranded polydeoxyribonucleotides having antithrombotic effect, was investigated in a model of splanchnic artery occlusion (SAO) shock in urethane anesthetized rats. Occlusion of the superior mesenteric artery for 90 min produced a severe shock state, resulting in a fatal outcome within 120 min of reperfusion in many rats. Defibrotide (10 mg/kg body weight) 10 min prior to reperfusion significantly improved mean arterial blood pressure in comparison to vehicle treated rats ($p<0.05$). Defibrotide treatment also significantly attenuated in the increase of plasma amino nitrogen concentration, intestinal myeloperoxidase activity, intestinal lipid peroxidation, infiltration of neutrophils in intestine and thrombin induced adherence of neutrophils to superior mesentric artery segments. Superoxide anion and hydrogen peroxide production in 1 μM formylmethionylleucylphenylalanine (fMLP)-activated PMNs was inhibited by defibrotide in a dose-dependent fashion. Defibrotide effectively scavenged hydrogen peroxide, but not hydroxyl radical. Treatment of SAO rats with defibrotide inhibited tumor necrosis factor-α, and interleukin-1β productions in blood in comparison with untreated rats. These results suggest that defibrotide partly provides beneficial effects by preserving endothelial function, attenuating neutrophil accumulation, and antioxidant in the ischemic reperfused splanchnic circulation

Key Words: Defibrotide, Ischemia, Reperfusion

INTRODUCTION

Splanchnic artery occlusion shock (SAO) is an experimental type of circulatory shock, which is the consequence of a prolonged ischemia of the splanchnic region. Occlusion of the splanchnic circulation followed by reperfusion results in a severe form of circulatory shock, characterized by severe hypotension, hemoconcentration, intestinal injury and a high mortality rate (Lefer & Lefer, 1993; Zimmermann et al, 1993). An important component of SAO shock is endothelial dysfunction (Carey et al, 1992; Lefer & Lefer, 1993) which was originally attributed to oxygen-derived free radicals released from both the reperfused endothelium (Ratych et al, 1987; Lefer & Lefer, 1993) from activated adherent polymorphonuclear leukocytes (PMNs) (Bittermann et al, 1988; Mullane et al, 1988). Endothelial dysfunction predisposes to vasospasm, platelet deposition, and increased neutrophil adherence which exacerbates the shock state. Ischemia-reperfusion is a stimulus for leukocyte-endothelial interaction (Granger, 1977). Leukocyte-endothelial interaction involves a complex system of adhesion molecules, including selectins, β_2 integrins and immunoglobulin superfamily (Butcher, 1993). Leukocyte interaction with the endothelium begins with leukocyte rolling, followed by adherence and transendothelial migration. P-selectin, a member of the selectin family of adhesion molecules, is believed to play a major role in the initial phase of leukocyte emigration, and characterized by the rolling of leukocytes along the vascular endothelial surface. Although P-selectin is necessary for early neutrophil contact with the endothelium, P-selectin-mediated leukocyte-endothelial interaction is not sufficient to allow neutrophil emigration from the vessel. A firmer adherence of the neutrophil to the endothelial surface is required for transendothelial migration (Butcher, 1992). This firm adherence involves the interaction of β_2 integrins (i.e. CD11/CD18) on the polymorphonuclear leukocytes (PMNs) surface, and intercellular adhesion molecule 1 (ICAM-1) on the endothelial cell surface (Lawrence & Springer, 1991; Butcher, 1992). Experimental studies have also shown that *in vivo* administration of antibodies raised against ICAM-1 reduced neutrophil infiltration into the inflamed lungs and protects the development of SAO-induced injury in rabbit. Neutrophil activa-

Corresponding to: Yong Kyoo Shin, Department of Pharmacology, College of Medicine, Chung-Ang University, 221 Heukseok-dong, Dongjak-gu, Seoul 156-756, Korea. (Tel) 82-2-820-5680, (Fax) 82-2-817-7115, (E-mail) SYK@cau.ac.kr

ABBREVIATIONS: SAO, splanchnic artery occlusion; fMLP, formylmethionylleucylleucylphenylalanine; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

tion at sites of injury results in a large production of superoxide anions which in turn contributes to tissue damage seen post reperfusion in several ischemic organs including kidney (Morpurgo et al, 1996); stomach (Yoshikawa et al, 1990), intestine (Zimmermann et al, 1993), skin (Goossens et al, 1990) and heart (Ambrosio & Flaherty, 1992; Grill et al, 1992). Some important tissue damaging and pro-inflammatory roles attributed to superoxide anion include: endothelial cell and increased microvascular permeability (Haglind et al, 1994; Xia et al, 1995), formation of chemotactic factors such as leukotrienes B_4 (Deitch et al, 1990), recruitment of neutrophils at sites of inflammtion (Salvemini et al, 1999a,b), lipid peroxidation and DNA single-strand damage (Dix et al, 1996). In addition, superoxide anion by interacting with nitric oxide (NO) destroys the biological activity of this mediator, attenuating important anti-inflammatory and tissue protective properties of NO; namely maintenance of blood vessel tone, platelet reactivity, cytoprotective effect on numerous organs (including heart, intestine and kidney), and release of anti-inflammatory and cytoprotective prostacyclin (via activation of constitutive cyclo-oxygenase enzyme) (Salvemini et al, 1999a, b). The product formed as a result of superoxide anion interaction with NO is peroxynitrite ($ONOO^-$), a well described, potent cytotoxic and proinflammatory molecule (Crow & Beckman 1995; Misko et al, 1998; Salvemini et al, 1999a, b). Therefore, removal of superoxide protects NO and reduces formation of the cytotoxic peroxynitrite.

Defibrotide is a complex single-stranded polydeoxyribonucleotides isolated by controlled depolymerization of porcine intestinal mucosa DNA and comprises a cluster of chains of different length and base sequences (Ma et al, 1991; Bianchi et al, 1993; Lanzarotti et al, 1993) with a molecular size of 20~30 kDa. This substance either preserves or enhances the release of antiaggregatory eicosanoids (Niada et al, 1986), and exerts fibrinolytic activity by liberating tissue plasminogen activator and decreasing its inhibitor (Klcking,1992). Recently, defibrotide has been found to exert cytoprotective action in acute inflammation disorders, probably by preserving nitric oxide (NO) release by the endothelium (Palmer & Goa, 1993). However, the exact biological function of defibrotide in SAO has not been clarified.

Therefore, the objectives of this investigation were to determine if defibrotide provided beneficial effects in a well established rat model of splanchnic ischemia and reperfusion, and to elucidate mechanisms involved.

METHODS

Experimental protocol

Sprague-Dawley male rats, body weight 250~300 g, were anesthetized with intraperitoneal injection of urethane (1.5 g/kg). The trachea was cannulated to maintain a patent airway throughout the experiment. A polyethylene catheter was inserted in one carotid artery to monitor mean arterial blood pressure (MABP), and a second catheter was placed in the contralateral external jugular vein for infusion of rutin or its vehicle. MABP was recorded on a Grass model 7 oscillographic recorder using Statham P23 AC pressure transducers (Gould, Cleveland, OH). The abdominal cavity was opened via a midline laparotomy, and the superior mesenteric arteries (SMA) were isolated near their aortic origin.

After stabilization, SMA was completely occluded for 90 minutes using nontraumatic arterial clamps. At the end of the ischemic period, the clamp was removed, and the splanchnic circulation was reperfused. Rats were observed for 2 hours after reperfusion or until their MABP declined to 50 mmHg, at which time the experiment was terminated. Rats experiencing massive acute circulatory collapse (i.e., MABP$<$50 mmHg) within the first 30 minutes post- reperfusion were excluded from the study, since this is usually associated with a significant degree of hemorrhage. Rats were randomly assigned to one of four groups: a) sham-operated control rats, b) sham-operated control plus defibrotide rat (10 mg/kg bolus i.v.) in which all surgical procedures were performed except that the mesenteric arteries were not occluded, c) SAO plus vehicle (saline 0.3 ml bolus i.v.), or d) SAO plus defibrotide (10 mg/kg bolus). Bolus administration of either vehicle or defibrotide was given 10 minutes before reperfusion. At the time of circulatory collapse or at the end of 2 hour reperfusion period, the SMA was removed and studied using isolated vascular ring experiments, and a section of ileal tissue was removed and used for analysis of myeloperoxidase (MPO) activity and lipid peroxidation.

Determination of tissue myeloperoxidase (MPO)

Ileal MPO activity, an enzyme occurring virtually exclusively in polymorphonuclear leukocytes (PMN), was determined using the method of Bradley et al. (1982) as modified by Mullane et al (1985). A hemorrhage-free area of the ileum, approximately 8 to 10 cm in length, and at least 30 cm distal to the stomach, was dissected and carefully rinsed in 0.9% NaCl. The sample was then homogenized in 0.5% HTAB (hexadecyltrimethyl ammonium bromide, Sigma Chemical Co., St. Louis, MO, which was dissolved in 50mM potassium phosphate buffer at pH 6.0) using a Polytron (PCU-2) homogenizer (Kinematica GMBH, Luzern, Switzerland). Homogenates were centrifuged at 12,500 g and 4°C for 30 minutes. The supernatants were then collected and reacted with 0.167 mg/ml of *o*-dianisidine dihydrochloride (Sigma Chemical Co.) and 0.0005% H_2O_2 in 50 mM phosphate buffer at pH 6.0. The resultant change in absorbance was determined spectrophotometrically at 460 nm. One unit of MPO is defined as the quantity of enzyme to hydrolyse 1 mmole of peroxide/min at 25°C.

Rat neutrophil isolation

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

Rat neutrophil labeling

Isolated rat neutrophils were then labeled with Zynaxis PKH-2 cell linker (Zynaxis Cell Science Inc., prepared for Sigma Immunochemical, Malvern, PA) according to the procedure of Yuan and Fleming (1990). One ml of diluents was added to a loose cell pellet containing 20 million cells. After mixing the cell suspension with 20 μl of PKH-2GL dye for 5 min by inversion, two ml of PBS containing 10% rat plasma was added to stop the reaction, and another 8 ml of PBS was used to underlay the suspension. Cells were then centrifuged at 350 g for 10 min, and the supernatant was removed. The cells were resuspended in PBS, recounted and employed for adherence studies. This labeling procedure yields >95% of the cells possessing normal morphology and function (Yuan & Fleming, 1990).

In vitro adherence of PMN to thrombin stimulated superior mesenteric artery endothelium

Thrombin stimulation leads to rapid up-regulation of P-selectin (Lorant et al, 1991). In order to gain additional insight into the effects of defibrotide on PMN adherence, superior mesenteric artery segments obtained from additional anesthetized control rats were removed and placed into warmed Krebs-Henseleit (K-H) buffer consisting of (in mmol/L): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 12.5, and glucose 10. The arteries were cut into 2~3 mm rings, opened, and placed into cell culture dishes filled with 3 ml of K-H buffer. These opened segments were incubated with 2 U/ml thrombin (Sigma immunochemical) for 10 min in order to stimulate the endothelial cells. After 10 min of incubation, the opened segments were removed and placed in fresh K-H solution. Labeled inactivated rat PMNs (4×10^5 PMNs/ml) were added to thrombin-stimulated endothelium alone or in combination with increasing concentrations of defibrotide (0.1~0.5 mg/ml). After 20 min of incubation in a metabolic shaker bath at 37°C, the segments were washed in K-H buffer and placed endothelial side up on microscope slides, and adherent PMNs were counted using an epifluorescent microscope (Laborlux 12, Leitz, Germany). Five different fields each of endothelial surface were counted, and the results were expressed as adherent PMNs/mm^2 of endothelial surface.

Ex vivo PMN adherence to superior mesenteric artery endothelium

PMNs were isolated and fluorescently labeled as previously described. Segments from superior mesenteric artery were isolated from each rat and placed into warm K-H buffer. Arteries were cut into rings of 2 to 3 mm length. The rings were then opened, and placed with the endothelial surface up into a cell culture dish filled with 3 ml of oxygenated K-H solution and incubated in culture dishes with autologous labeled PMNs (1.2×10^6 cells) for 20 min at 37°C in a shaker bath which stimulates shear forces. Following 20 min of incubation, segments were washed with K-H buffer and placed on slide glass. PMNs adhering to the endothelium were counted by using epifluorescence microscope (Laborlux 12, Leitz, Germany). Five different fields of each endothelial surface were counted, and the results were expressed as adherent PMNs/mm^2 of endothelial surface.

Plasma free amino-nitrogen

Blood samples were kept on ice, centrifuged at 2,500 g for 20 min at 4°C, and the supernatant was employed for biochemical assay. Plasma free amino-nitrogen concentration was determined by using plasma samples deproteinized with 5% trichloroacetic acid. The free amino-nitrogen concentration was used as an index of total plasma proteolysis and expressed in units per milliliter (U/ml). One unit is equivalent to 10 nmol of serine.

Measurement of lipid peroxidation

A hemorrhage-free area of the ileum, 8 to 10 cm in length, and at least 30 cm distal to the stomach, was dissected and carefully rinsed in 0.9% NaCl. The sample was then homogenized in 10 volumes K-H buffer by using a Polytron (PCU-2) homogenizer (Kinematica GMBH, Luzern, Switzerland). Homogenates were centrifuged at 12,500 g at 4°C for 30 min. The supernatants were then collected. Lipid peroxidation of the ileum was estimated from malondialdehyde concentration measured by thiobarbituric acid method. The supernatant of ileum (1 mg protein/ml) was added to the reaction mixture consisting of 150 mM KCl and 50 mM NaH_2PO_4, pH 7.4. The reaction was started by adding ileal tissue to the mixture, final volume 1.0 ml. After 30 min of incubation, the reaction was stopped by adding 1.0 ml of 1% 2-thiobarbituric acid (TBA) in 50 mM NaOH and 1.0 ml of 2.8% trichloroacetic acid. The chromophore was developed by boiling in a water bath for 10 min. After cooling to room temperature, the absorbance was measured at 532 nm (Gutteridge, 1981). The concentration of malondialdehyde was expressed as nmol/mg protein, using molar extinction coefficient of 1.52×10^6/M/cm (Placer et al, 1966).

Measurement of superoxide anion production

The superoxide anion produced was assayed by superoxide dismutase-inhibitable reduction of ferricytochrome c. The reaction mixtures (200 μl) in 96 well microplate contained 3×10^5 PMNs, 75 μM ferricytochrome c, stimulating agent and DMEM, pH 7.4 and were placed at 5% CO_2 incubator, for 4 h at 37°C. The absorbance was measured in a microplate reader (Molecular Devices, Spectra MAX 340, Molecular Devices, Co., Sunnyvale, CA, U.S.A.). The amount of reduced ferricytochrome c was represented as nmole using the extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} at 550 nm (Pick & Mizell, 1981; Markert et al, 1984).

Measurement of hydrogen peroxide production

PMNs (3×10^5 cells/well) were incubated in 200 μl of DMEM, containing 0.1 mg/ml phenol red and 0.2 mg/ml horseradish peroxidase, for 4 h at 37°C. The reaction was terminated by adding 20 μl of 1 N NaOH, and absorbance was measured at 610 nm (Pick and Mizell, 1981). The concentration of hydrogen peroxide was calculated using hydrogen peroxide solution as the standard.

Measurement of 2-α deoxyribose degradation

Effect of defibrotide to decompose hydroxyl radicals was determined by assaying malondialdehyde chromogen formation due to 2-α deoxyribose degradation (Aruoma, 1994;

Halliwell & Gutteridge, 1989). The reaction mixtures contained, in a final volume of 1.0 ml, 2 mM 2-α deoxyribose, 50 μM $FeCl_3$, 50 μM EDTA, 500 μM H_2O_2, 100 μM ascorbate, 150 mM KCl, and 50 mM NaH_2PO_4 buffer, pH 7.4 and other compounds (10 μg/ml defibrotide). After 30 min of incubation, 1 ml of 1% thiobarbituric acid in 50 mM NaOH and 1 ml of 2.8% trichloroacetic acid were added to stop the reaction. Absorbance was measured at 532 nm.

Measurement of H_2O_2 decomposition

The concentration of hydrogen peroxide was measured by the method of Allen et al (1952). The reaction mixtures contained, in a final volume of 1 ml, 120 mM KCl, 0.1 mM H_2O_2, 10 μM sodium azide, 50 mM Tris-HCl, pH 7.4 and other compounds (10 μg/ml defibrotide). After a reaction stopping solution (25 mg/ml of potassium biphthalate, 2.5 mg/ml NaOH, 82.5 mg/ml potassium iodide and 0.25 mg/ml ammonium molybdate) was added to the above mixture, change of absorbance was read spectrophotometrically at 350 nm.

Measurement of cytokine production

The amounts of cytokines produced in blood were measured by enzyme-linked immunosorbent assay (ELISA) with commercial kit for rat TNF-α and IL-1β (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Their concentrations were calculated by interpolation of the regression curve of known amounts of recombinant cytokines (rIL-1β and rTNF-α purchased from Amersham Pharmacia Biotech), and expressed as pg/ml.

Statistical analysis

All values in the text and figures are presented as mean± standard errors of the mean (SEM) of n independent experiments. Statistical analysis was performed using Student's *t*-test for paired data. Probabilities of 0.05 or less were considered to be significant in all cases.

RESULTS

Effects of defibrotide on mean arterial blood pressure (MABP)

Occlusion of the superior mesenteric artery resulted in an increase of MABP (50~70 mmHg) with mean arterial blood pressures reaching 160~168 mmHg for all SAO groups (Fig. 1). During 90 min of occlusion, there was a slow decline in MABP to levels comparable to pre-occlusion values. However, upon reperfusion, all SAO rats exhibited a precipitous decline of MABP to approximately 60~70 mmHg. The administration of defibrotide (10 mg/kg) had no significant effect on MABP over 2 hour observation period in sham trauma rats. This finding indicates that neither the surgical procedures nor the administration of defibrotide contributed to the alterations in MABP in rats subjected to SAO shock. SAO rats given only with vehicle demonstrated slight increase (p=NS) of MABP following the initial decline of MABP which was followed by a steady secondary decline in MABP during the remainder of the reperfusion period. In contrast, those SAO rats treated with defibrotide (10 mg/kg) showed an immediate and sustained increase of MABP following the initial decline which persisted over the entire 120 min reperfusion. In fact, at the end of the observation, no significant differences in MABP were observed between sham-operated control and defibrotide receiving SAO rats.

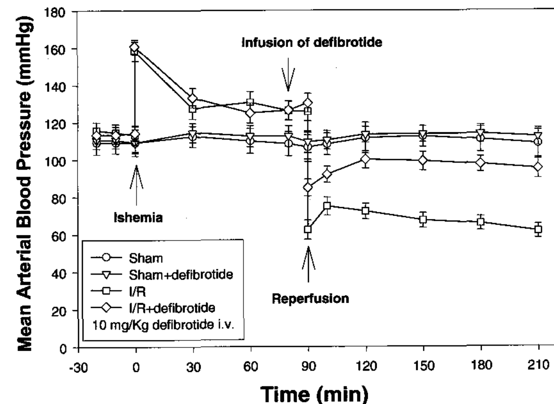

Fig. 1. Time course of mean arterial blood pressure in rats subjected to sham, sham+defibrotide, I/R, and I/R+defibrotide. All values are means±standard error of surviving animals at each time point, n=5-6.

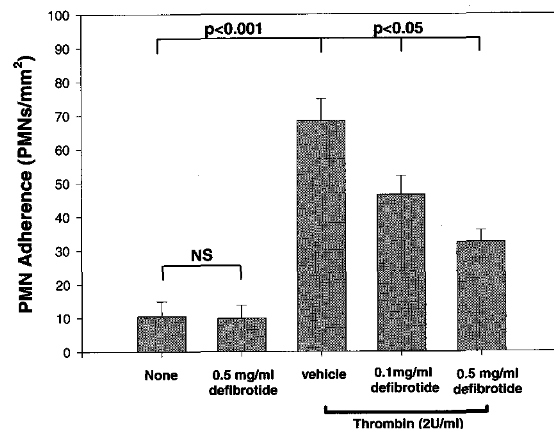

Fig. 2. In vitro effect of defibrotide on PMN adherence to thrombin-stimulated (2 U/ml) rat superior mesenteric artery. Data are expressed as number of PMNs/mm^2. Bar heights represent means and brackets indicate±SEM, n=5.

Effects of defibrotide on the PMNs adherence to the SMA vascular endothelium

One of the early events in PMNs-mediated reperfusion injury is an increase in the adherence of PMNs to the reperfused endothelium. In order to determine whether defibrotide inhibits the adherence of rat PMNs to SMA endothelial cells, the effects of defibrotide on unactivated PMN adherence to stimulated SMA endothelium in vitro were determined (Fig. 2). Few unactivated PMN adhered to unstimulated SMA endothelium. However, stimulation of SMA endothelium with thrombin (2 U/ml) for 10 min resulted in a significant ($p<0.001$) 7-fold increase of adherent PMNs to the endothelium, in comparison to unstimulated endothelium. Addition of defibrotide inhibited PMN adherence

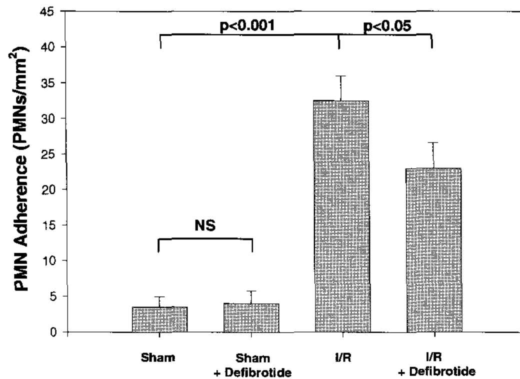

Fig. 3. Effects of in vivo administration of defibrotide on the in vitro adherence of unstimulated PMN to non ischemic-reperfused superior mesenteric artery endothelium and ischemic-reperfused superior mesenteric artery endothelium. Data are expressed as number of PMNs/mm^2. Bar heights represent means and brackets indicate ± SEM.

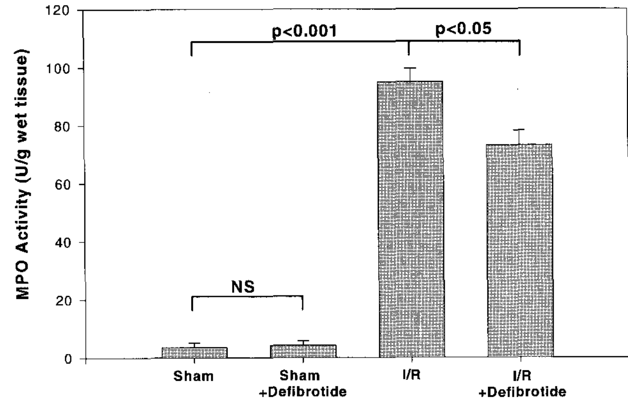

Fig. 4. Effects of defibrotide on ileal myeloperoxidase (MPO) activity in sham, sham+defibrotide, I/R, and I/R+defibrotide. Data are expressed as Unit/g wet tissue of ileum. Bar heights represent means and brackets indicate ±SEM, n=5.

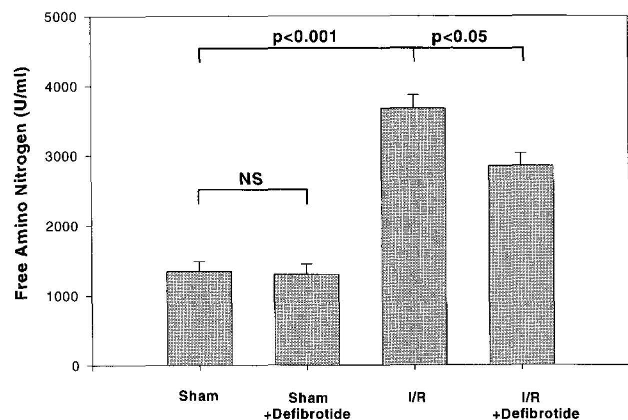

Fig. 5. Effects of defibrotide on plasma free-amino nitrogen (FAN) in sham, sham+defibrotide, I/R, and I/R+defibrotide. Data are expressed as Unit/ml blood. Bar heights represent means and brackets indicate ± SEM, n=5.

to the endothelium in a concentration-dependent manner. Incubation of SMA segments with 0.5 mg/ml defibrotide resulted in 55.8% inhibition of PMN adherence. We measured the adherence of PMNs, following in vivo administration of defibrotide (10 mg/kg) (Fig. 3). When the unstimulated autologous PMNs were incubated with noniischemic SMA segments for 20 min, relatively few PMN adhered to the endothelium regardless of the group of origin. In SAO rats receiving defibrotide, unstimulated PMNs incubated with the ischemic SMA segments resulted in a significant decrease of PMN adherence ($p < 0.05$). These results suggest that a protective effect of defibrotide is contributed in part by inhibiting of the interaction between neutrophils and mesenteric vascular endothelium.

Effects of defibrotide on ileal myeloperoxidase activity

Accumulation of PMNs in the ischemic-reperfused intestinal tissue is considered to be one of the primary contributory mechanisms to reperfusion injury. Therefore, as a marker for PMN accumulation, the MPO activity in the intestinal tissue was measured (Fig. 4). The MPO activity was low in all sham-operated control rats (3.5±1.6 U/g wet tissue). However, ischemia of the mesenteric circulation followed by reperfusion in untreated rats resulted in high increase of ileal MPO activity (95.0±4.5 U/g wet tissue). Treatment of SAO rats with 10 mg/kg defibrotide significantly ($p < 0.05$) attenuated ileal MPO activity, in comparison to untreated rats, indicating that defibrotide retarded the accumulation of PMNs in the post-ischemic mesentery.

Effects of defibrotide on plasma free amino-nitrogen

We also measured free plasma amino-nitrogen level as a index of plasma proteolysis, which results from the liberation of proteolytic enzymes into the circulation following circulatory shock. Only moderate levels of proteolysis occurred in sham operated control rats (Fig. 5), whereas, ischemia of the mesenteric circulation in ntreated rats followed by reperfusion resulted in significant ($p < 0.001$) increases in plasma free amino-nitrogen. Treatment of SAO rats with defibrotde significantly ($p < 0.05$) inhibited the level, in compared to untreated rats.

Effects of defibrotide on lipid peroxidation of intestine

We measured lipid peroxidation in intestine as an index of oxidant-induced injury tissue, resulting from the production of reactive oxygen species (i.e. superoxide radical, hydroxyl radical, hydrogen peroxide). The lipid peroxidation was low in all sham operated control rats. However, ischemia of the mesenteric circulation in untreated rats followed by reperfusion resulted in a 4-fold increase in lipid peroxidation. Treatment of SAO rats with defibrotide significantly inhibited ($p < 0.05$) lipid peroxidation of intestine in comparison to untreated rats (Fig. 6).

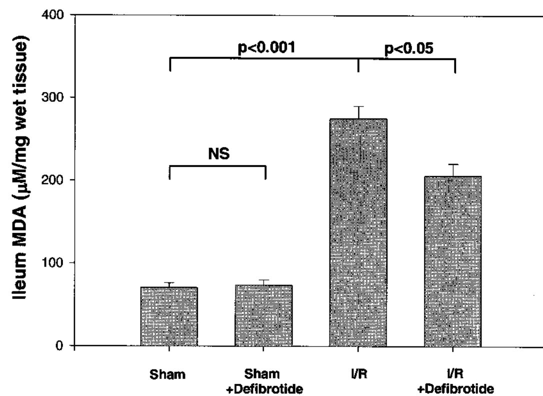

Fig. 6. Effects of defibrotide on ileal lipid peroxidation in sham, sham+defibrotide, I/R, and I/R+defibrotide. Data are expressed as malondialdehyde nmol/mg protein, using the molar extinction coefficient of 1.52×10^6/M/cm. Bar heights represent means and brackets indicate±SEM, n=5.

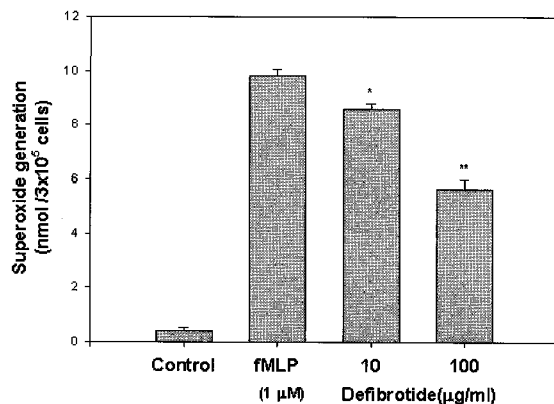

Fig. 7. Effect of defibrotide on fMLP stimulated superoxide generation in PMNs. PMNs was stimulated with 1 μM fMLP in the presence of defibrotide. Defibrotide significantly decreased the superoxide production in dose-dependent manners. Data are expressed as nmol/3×10^5 cells. Data are mean±standard error of mean (SEM). *$p<0.05$, **$p<0.01$ vs. fMLP treatment alone.

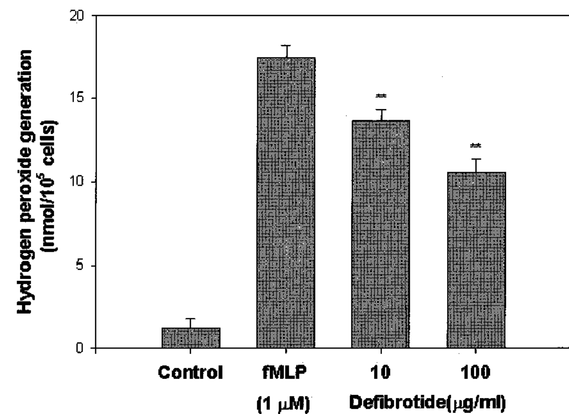

Fig. 8. Effect of defibrotide on fMLP-stimulated hydrogen peroxide generation in PMNs. PMNs was stimulated with 1 μM fMLP in the presence of defibrotide. Defibrotide significantly decreased the hydrogen peroxide production in dose-dependent manners. Data are expressed as nmol/3×10^5 cells. Data are mean standard error of mean (SEM). **$p<0.01$ vs. fMLP treatment alone.

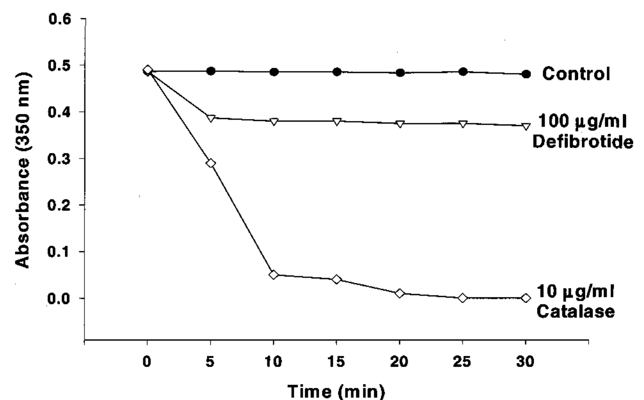

Fig. 9. Effect of defibrotide on hydrogen peroxide decomposition. Scavenging actions of defibrotide (100 μg/ml) and catalase (10 μg/ml) on 100 μM H_2O_2 were measured. Data are expressed as change in absorbance.

The effects of defibrotide on superoxide and hydrogen peroxide generation in PMNs

Our data obtained above indicate significantly beneficial ileal protective effects of defibrotide in SAO rat. Therefore, we investigated whether defibrotide had a direct effect on superoxide and hydrogen peroxide generation in PMNs. PMNs produce superoxide and hydrogen peroxide in response to fMLP.

Since FMLP have been shown to significantly stimulate superoxide and hydrogen peroxide production (Han et al, 1997), the effects of defibrotide on superoxide and hydrogen peroxide production in fMLP-stimulated PMNs were examined. One μM fMLP-stimulated PMNs produced 9.84±0.26 (n=5) nmol of superoxide anion/3×10^5 cells, and the production in was inhibited by defibrotide in a dose dependent fashion (Fig. 7). One μM fMLP-stimulated PMNs produced 17.43±0.52 (n=5) nmol of hydrogen peroxide/3×10^5 cells, the production was also inhibited by defibrotide in a dose dependent fashion (Fig. 8).

Scavenging action of defibrotide on hydroxyl radical and hydrogen peroxide

In biological systems, hydroxyl radical and hydrogen peroxide have been implicated as a precursor for more reactive oxygen species, and can also form complexes with metal ions.

Autooxidation of iron liberates reactive oxygen species, and iron causes formation of hydroxyl radical and iron-oxygen complexes. Therefore, hydroxyl radical produced was measured with TBA reactivity of 2-α deoxyribose. Defibrotide did not inhibit the increased TBA reactivity of 2-α deoxyribose in the presence of defibrotide (data not shown). Fig. 9 shows that 100 μM H_2O_2 was significantly decomposed by 10 μg/ml of catalase and was also decomposed by 100 μg/ml defibrotide.

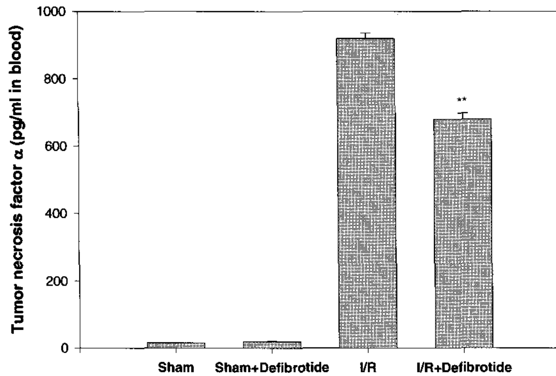

Fig. 10. Effect of defibrotide on tumor necrosis factor α (TNF-α) production. Reperfusion of the ischemic splanchnic circulation leads to profound increase in plasm TNF-α. The dose of defibrotide was 10 mg/kg rat body weight. Data are expressed as pg/ml blood. Data are mean±standard error of mean (SEM). $^{**}p<0.01$ vs. I/R (ischemia/reperfusion).

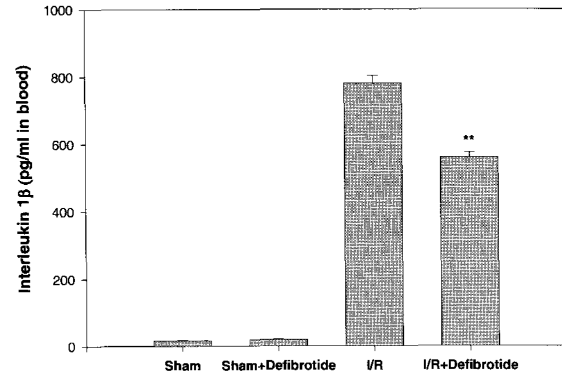

Fig. 11. Effect of defibrotide on interleukin-1β (IL-1β) production. Reperfusion of the ischemic splanchnic circulation leads to profound increase in plasm IL-1β. The dose of defibrotide 10 mg/kg rat body weights. Data are expressed as pg/ml blood. Data are mean± standard error of mean (SEM). $^{**}p<0.01$ vs. I/R (ischemia/reperfusion).

Effects of defibrotide on cytokine production in SAO rat

To examine the effect of defibrotide in SAO rat, we investigated whether defibrotide had a direct effect on TNF-a and IL-1b production. Therefore, the amounts of cytokines produced in blood were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions with commercial kit for rat TNF-α and IL-1β (Amersham Pharmacia Biotech).

The amounts of TNF-α and IL-1β were 15.0±1.0 pg/ml, and 16.0±1.0 pg/ml in sham rats, respectively (Fig. 10, 11), however, they were markedly increased in I/R rats (920.0± 16.0 pg/ml, 780.0±23.0 pg/ml, respectively). Treatment of I/R rats with defibrotide significantly inhibited ($p<0.01$) the TNF-α and IL-1β production in blood, compared with I/R rats (Fig. 10, 11).

DISCUSSION

The celiac, superior mesenteric, and inferior mesenteric arteries supply blood to the liver, pancreas, and intestine with up to 90% of the blood flow. An occlusion of superior mesenteric artery results in an abrupt increase in systemic arterial blood pressure by approximately 25 to 50 mmHg, which is due primarily to a decrease in the baroreceptor input to the central medullary vasomotor center. The large increase of MABP diminishes during ischemia and returns to near pre-occlusion levels by the end of ischemic period. Upon reperfusion of splanchnic circulation, a precipitous fall in MABP ensues with values ranging from 50 to 70 mmHg. This is followed by a moderate increase in pressure within the first 30 minutes of reperfusion and a steady decline throughout the remainder of the reperfusion period to values incompatible with life (i.e., approximately 45 mmHg). In this investigation, we observed a response similar to the above indicating that the rats in the study were subjected to severe circulatory shock due to SAO/ reperfusion. This type of circulatory shock is characterized by the formation and accumulation of toxic factors produced during the ischemic period that are released into the systemic circulation upon reperfusion of splanchnic organ (Lefer and Barenholz, 1972). These toxic mediators induce proteolysis, severe hypotension, hemoconcentration, cardiac depression, and changes in vasoactivity. Occlusion alone of major splanchnic arteries results in disturbances in parenchymal and microvascular permeability, and the production of cytotoxic substances in pancreas and platelets. Upon reperfusion, these potentially toxic metabolites, including a myocardial depressant factor and thromboxane A2, are introduced into the systemic circulation where they exacerbate the shock state. In addition, the restoration of blood flow in the previously ischemic region also allows for the interaction of leukocytes, particularly PMNs, with the endothelium which has been identified as one of the early phases of the inflammatory process (Butcher, 1991) and these PMNs also release leukotrienes, cytokines, reactive oxygen species and proteases (Grisham et al, 1986; Buerke et al, 1994; Rubin et al, 1994).

During the process of reperfusion injury, PMNs are involved in the injury to splanchnic organs as well as in the development and progression of endothelial dysfunction (Lefer et al, 1991; Rubin et al, 1994). Endothelial dysfunction associated with ischemia and subsequent reperfusion is an early event following reperfusion (Lefer et al, 1991). This dysfunction is due in large part to an increased superoxide and reduced NO generation by the endothelium (Lefer & Lefer, 1993), evidenced by a decreased relaxation of the mesenteric arteries to endothelium dependent vasodilators (Karasawa et al, 1991; Carey et al, 1992). Additionally, the dysfunctional endothelium becomes activated, and interactions between leukocytes and the endothelium significantly increase 30 min after reperfusion (Weyrich et al, 1993). By 2 hours post-reperfusion, numerous PMNs have extravasated through the endothelium and migrated into the interstitium (Weyrich et al, 1993). We observed about fifty fold increase in ileal MPO activity following SAO and reperfusion, indicating a large number of PMN residing in the interstitium.

Once PMNs become adherent to the vascular endothelium or migrate to the interstitium, they release proteases along with oxygen-derived free radicals which propagate localized tissue injury. Oxygen radicals released by PMNs

are capable of inactivating nitric oxide as well as endogenous protease inhibitors, enabling serine proteases, such as elastase and cathepsin G, to exacerbate tissue injury (Karasawa et al, 1991). This is followed by the release of chemoattractants, such as platelet activating factor, leukotriene B_4, interleukin-1 , and tumor necrosis factor-α, by activated PMNs and endothelial cells. The release of these substances results in the recruitment of additional PMNs to the site of injury, thereby exacerbating existing endothelial dysfunction and tissue injury.

Inflammation is a process involved in several states, and limitation of the inflammatory process has been the target of numerous therapeutic strategies for these disorders. Administration of monoclonal antibodies directed against adhesion molecules (Weyrich et al, 1993), blockers of adhesion molecule ligands (Buerke et al, 1994; Takada et al, 1997), and substances which inhibit adhesion molecule expression (Lefer et al, 1993) have all proven to be successful in limiting the severity of the inflammatory process. In addition to these approaches, recent studies demonstrated a beneficial effect of defibrotide as an anti-inflammatory agent (Kim et al, 2002). Due to highly integrated cascade of events surrounding the inflammatory response, several mechanisms have been suggested to be involved in the anti-inflammatory properties of defibrotide. Data from this study show that defibrotide, which lacks direct antithrombin activity, is capable of attenuating unstimulated PMN adherence to thrombin stimulated endothelium, thus, indicating that defibrotide was capable of attenuating the adherence of PMNs to endothelial cells when either endothelium or PMNs are stimulated.

The production of a large amount of superoxide and hydrogen peroxide increases the number of rolling or adherent PMNs (Suzuki et al, 1989; Johnston et al, 1996), primarily by overwhelming endogenous NO. NO is able to inactivate both superoxide and hydrogen peroxide (Rubanyi et al, 1991; Huie & Padmaja, 1993; Johnston et al, 1996), thereby protecting surrounding cells from free radical mediated damage. In addition, nuclear factor-κB (NF-κB), a transcription factor which up-regulates P-selectin, E- selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 gene expression have been shown to be inhibited by NO (De Caaterina et al, 1995). NO has also been shown to prevent the expression of key adhesion molecules on the cell surface (Armstead et al, 1997). Oxygen-derived free radicals (Huie & Padmaja, 1993) have also been shown to directly activate NF-κB, enhancing the synthesis of key adhesion molecules. Administration of defibrotide might inhibit superoxide, hydroxyl radical, and hydrogen peroxide release from activated PMNs and ischemic vascular endothelium. Defibrotide also inhibited cytokine (i.e. TNF-α, IL-1β) production in blood. Therefore, defibrotide may provide significant protective effects by inhibiting the action of potentially injurious oxygen radicals, thereby attenuating endothelial dysfunction and preventing the stimulation of adhesion molecule transcription factors. These results are consistent with previous studies which demonstrated beneficial effect of defibrotide on liver ischemia-reperfusion (Kim et al, 2002).

In this study, we have shown that the administration of defibrotide attenuates hypotension in rats subjected to 90 min of splanchnic ischemia followed by reperfusion. The beneficial effects of defibrotide are due at least in part to an attenuation of endothelial dysfunction which is an early consequence of ischemia and reperfusion of the splanchnic vasculature. This was evidenced by the fact that defibrotide treatment significantly preserved endothelial function and inhibited both the *in vitro* PMN adherence to vascular endothelium as well as *in vivo* PMN accumulation within the splanchnic organs. It appears that the primary mechanism of protection involves the inhibition of toxic mediators released by activated neutrophils during reperfusion of the splanchnic circulation. This serves to protect the endothelium, and preserve endothelial function and inhibit the interaction between leukocytes and endothelial cell. The present study, together with previous investigations may demonstrate potential therapeutic benefits of defibrotide during inflammatory states such as splanchnic artery occlusion and reperfusion.

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