Effect of FR128998, a novel PAF receptor antagonist, on endotoxin-induced disseminated intravascular coagulation

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Abstract

This study aimed to evaluate the effect of FR128998, (1s,6s)-1-benzyl-10-(3-pyridyl-methyl)-7-thia-10-azaspiro [5,6]-dodecan-11-one 7,7-oxide hydrochloride, a novel platelet activating factor (PAF) receptor antagonist, on endotoxin lipopolysaccharide-induced disseminated intravascular coagulation in rats. Experimental disseminated intravascular coagulation was induced by an infusion of lipopolysaccharide at 0.25 mg/kg/h for 4 h. Simultaneous infusion of FR128998 (0.25 and 1.0 mg/kg/h) with lipopolysaccharide dose dependently inhibited thrombocytopenia, but not leukopenia. The changes in coagulation parameters of disseminated intravascular coagulation, i.e., prolongation of activated partial thromboplastin time and elevated levels of fibrinogen/fibrin degradation products, were also prevented by the treatment with FR128998. In addition, FR128998 attenuated the increase in serum tumor necrosis factor (TNF) which appeared during the initial stage of disseminated intravascular coagulation. FR128998 (10 μM) also inhibited the TNF production by peripheral blood leukocytes or alveolar macrophages stimulated by lipopolysaccharide in vitro. Furthermore, TNF production induced by PAF itself in vitro was also inhibited in the presence of FR128998. These data indicate that PAF plays a pivotal role in the development of disseminated intravascular coagulation via TNF production.

Key words FR128998, PAF (platelet activating factor), Disseminated intravascular coagulation, Lipopolysaccharide, TNF (tumor necrosis factor)

1. Introduction

The alkyl-ether phospholipid platelet-activating factor (PAF-acether, PAF), identified as 1-O-alkyl-2-acyl-sn-glyceryl-3-phosphorylcholine (Demopoulos et al., 1979, Benveniste et al., 1972), is one of the most potent inducers of platelet aggregation and has a variety of biological actions such as increasing vascular permeability, bronchoconstriction and hypotension (Vargaftig et al., 1981). Moreover, PAF is believed to be involved in septic shock and disseminated intravascular coagulation induced by bacterial lipopolysaccharide endotoxins (Chang et al., 1987; Doebber et al., 1985). Using a variety of PAF receptor antagonists, the role of PAF in endotoxemia was studied (Saunders and Handley, 1987). Although effects of PAF receptor antagonists on septic shock induced by endotoxin have been reported (Terashita et al., 1985, Casals-Stenzel, 1987; Rabmovici et al., 1990; Yue et al., 1990, Fernandez-Gallardo et al., 1990), only very few studies examined their effect on the development of disseminated intravascular coagulation induced by lipopolysaccharide, except for the study of CV-3988 (Imura et al., 1986).

Like PAF, tumor necrosis factor (TNF) is implicated as a major mediator of the pathophysiological syndrome induced by endotoxin (Ziegler, 1988). Because it seems that lipopolysaccharide exerts its biological effects in part through the production of PAF and TNF (Engelberts et al., 1991), it is important to study the relationship between these two mediators. However, the relationship between these two mediators in endotoxemia is not fully understood. Previous evidence indi-
cated that the in vitro TNF production by human monocytes is enhanced by PAF (Ruis et al., 1991; Bonavida and Braquet, 1988). A reciprocal relationship has also been reported from the in vitro studies of TNF-induced PAF synthesis and release from macrophages (Camussi et al., 1987, Valone and Epstein, 1988). However, the relationship is still debated because Camussi et al. (1987) and Dubos et al. (1989) indicated that no change in TNF production was observed when macrophages were cultured with PAF alone. In addition, the relationship between these two mediators in the septic shock models has not been established in vivo. BN 50739, a PAF receptor antagonist, attenuated the lipopolysaccharide-induced elevation of plasma TNF in rats (Rabuovici et al., 1990), but not in rabbits (Yue et al., 1990). Unfortunately, only few studies attempted to examine the possible link between PAF and TNF in disseminated intravascular coagulation induced by lipopolysaccharide.

FR128998, (1s,6s)-1-benzyl-10-(3-pyridyl-methyl)-7-thia-10-azaspiro[5,6]-dodecan-11-one 7,7-dioxide hydrochloride, is a novel PAF receptor antagonist, belonging to the derivatives of spiro-thiazepine (Fig 1). We investigated the effect of FR128998 on the development of lipopolysaccharide-induced disseminated intravascular coagulation in rats and examined whether or not the effect of FR128998 on the experimental disseminated intravascular coagulation model is related to inhibition of TNF.

2. Materials and methods

2.1 Animals

Male Wistar rats (6–7 weeks old) were purchased from Japan SLC (Shizuoka, Japan), and male Japanese white rabbits (3–3.5 kg) purchased from Kitayama Labes Co. (Nagano, Japan) were maintained on a standard pellet chow and distilled water ad libitum. In the experiment with the disseminated intravascular coagulation model, the rats were deprived of food but not water for 14 h prior to the experiment.

2.2 Drugs

FR128998 was synthesized in our laboratories. Actinomycin D, PAF (1-O-octadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lipopolysaccharide (from *Escherichia coli* No. 0111 B4) were purchased from Sigma Chemicals Co (St Louis, MO, USA). Bovine serum albumin (F-V) was purchased from Nacalai Tesque (Kyoto, Japan). PAF was dissolved in ethanol and stored at −20°C. FR128998 and lipopolysaccharide were dissolved in 0.9% saline.

2.3 Platelet aggregation in vitro

Platelet aggregation was performed according to the method of Terasita et al. (1983). Blood was collected in 3.8% citrate anticoagulant (1/10 volume) from healthy adult human volunteers or Japanese white rabbits, respectively. Platelet-rich plasma was obtained by centrifuging the blood at 1000 rpm for 10 min at room temperature and warmed at 37°C in siliconized cuvettes with continuous stirring. FR128998 was dissolved and diluted in dimethyl sulfoxide (DMSO). FR128998 or DMSO was added to platelet-rich plasma before the stimulation. Human and rabbit platelets were stimulated by PAF at 0.35 μM or 35 nM, respectively. The maximal aggregation was measured and the inhibition percentage was calculated by comparison with the maximal amplitude obtained in the control aggregation performed in the presence of DMSO.

2.4 PAF-induced mortality

PAF was dissolved at a concentration of 10 μg/ml with 0.9% saline containing 0.25% bovine serum albumin and was injected intravenously into male Wistar rats at a dose of 10 μg/kg. This dose of PAF was shown to produce 100% mortality in rats. FR128998 was administered intravenously with PAF or orally 1 h prior to PAF injection. Mortality was determined 24 h after PAF injection.

2.5 Experimental disseminated intravascular coagulation model

An experimental model of disseminated intravascular coagulation in rats was carried out according to the method of Imura et al. (1986) with slight modifications. Male Wistar rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and right femoral veins were cannulated for infusion of lipopolysaccharide and FR128998. Lipopolysaccharide at 0.25 mg/kg/h was infused at a flow rate of 0.6 ml/h for 4 h.
Blood samples were withdrawn from the abdominal aorta at 2 or 4 h after the lipopolysaccharide infusion for the determination of TNF production or the coagulation parameters of disseminated intravascular coagulation, respectively. The samples used for the coagulation parameters were anticoagulated with 3.8% sodium citrate (1/10 volume). The coagulation parameters were estimated: platelet counts, blood leukocyte counts, prothrombin time, activated partial thromboplastin time, fibrinogen and fibrinogen/fibrin degradation products. Platelets and leukocytes were counted using an automatic counter (Sysmex PL 110, ToyoMedenshi Co., Japan). Fibrinogen/fibrin degradation products were measured with a commercial kit (Teikoku Kogyo Pharmaceutical Co., Japan), which utilizes a latex agglutination test. Prothrombin time, activated partial thromboplastin time and fibrinogen were determined using an autoanalyzer for disseminated intravascular coagulation parameters (Amelung-Coagulometer KC10A, Amelung, Germany).

2.6 Assay of serum TNF

Blood was allowed to clot in plastic tubes kept on ice for 30 min. Serum was obtained by centrifugation at 3000 rpm for 20 min at 4°C and stored at −70°C until assayed. TNF activity was measured by using the cytolytic assay on TNF-sensitive L-929 fibroblast cells as described previously (Inamura et al., 1992), with a slight modification. Briefly, 5 × 10^4 mouse L-929 fibroblast cells were grown in wells of a 96-well Microtest III plate in the presence of 1 μg/ml actinomycin D and the diluted serum sample. After 18 h, the supernatant was removed and the live cells were stained with MTT solution (final concentration, 0.5 mg/ml). The degree of cell lysis was quantitated by using an automatic Titertek Multiscan autoreader set for absorption at 610 nm. Diluted samples of natural TNF (specific activity, 4 × 10^5 JPU/mg protein, Hayashibara Biochemical Laboratories, Okayama, Japan) also were incubated with L-929 cells and used to prepare a standard curve for determining the unit of TNF (U/ml).

2.7 TNF production in vitro

Blood was collected from the abdominal aorta in anesthetized rats and thoroughly mixed with a dextran solution (6.0%). After standing for 2 h at 37°C, the separated leukocytes were collected by centrifugation at 2000 rpm for 20 min. The cells were washed with RPMI 1640 medium and suspended with complete RPMI 1640 medium (C-RPMI) containing 10% fetal bovine serum at a concentration of 2.5 × 10^6 cells/ml.

Alveolar macrophages were prepared as described by Sone et al. (1980). Briefly, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and exsanguinated by severing both renal arteries. After opening the chest cavity to produce pneumothorax, the trachea was cannulated with a polyethylene tube. The lungs were lavaged with 5 ml of Ca^2+-Mg^2+-free phosphate buffered saline (PBS) prewarmed to 37°C. The process was repeated several times to yield a recovered total of 50 ml lavage fluid per rat. The cells were collected by centrifugation at 2000 rpm for 10 min, washed and adjusted to 3 × 10^5 cells/ml with C-RPMI.

Various amounts of FR128998 were simultaneously added to the cultures of leukocytes/macrophages incubated with lipopolysaccharide or PAF at 37°C for 16 h. The cell-free supernatants were then harvested, filtered through a 0.45 μm Millipore membrane and stored at −20°C until use. The TNF activity of the supernatant was examined as described in section 2.6.

2.8 Statistical analysis of data

The results are expressed as means ± S.E.M. Comparison between groups of parametric data was made by Student's t-test (two-tailed). The level of significance was chosen as P < 0.05.

3. Results

3.1 PAF receptor antagonist activity of FR128998 in vitro and in vivo

PAF receptor antagonist activity was evaluated from the inhibition of PAF-induced rabbits or human platelet aggregation in vitro and the protective effect against PAF-induced mortality in rats. As shown in Table 1, FR128998 inhibited the PAF-induced platelet aggrega-

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<tr>
<td>Platelet aggregation</td>
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<td>IC₅₀</td>
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<tr>
<td>Rabbit (μM)</td>
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<td>Human (μM)</td>
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<td>PAF-induced mortality in rats</td>
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<td>ED₅₀</td>
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<td>i.v. (mg/kg)</td>
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Platelet aggregation was performed according to the method of Terashita et al. (1983). Human and rabbit platelets were stimulated by PAF at 0.35 μM or 35 nM, respectively. In PAF-induced mortality experiments, PAF was injected intravenously into male Wistar rats at a dose of 10 μg/kg FR128998 was administered intravenously with PAF or orally 1 h prior to PAF injection. Mortality was determined 24 h after PAF injection.
tion. The IC\textsubscript{50} values were 17.0 and 2.8 \textmu M for rabbits and human platelet, respectively. However, FR128998 did not inhibit arachidonic acid or ADP-induced aggregation, suggesting that it acts specifically against PAF (data not shown).

PAF (10 \textmu g/kg) produced 100\% mortality within 1 h of injection in rats. Under these conditions, FR128998 suppressed lethality in a dose-dependent manner with complete inhibition at 10 mg/kg (p.o. and i.v.) of this drug. The ED\textsubscript{50} values were 1.0 and 2.8 mg/kg for intravenous and oral administration, respectively (Table 1).

3.2 Effect of FR128998 on leukopenia and thrombocytopenia caused by lipopolysaccharide infusion

Lipopolysaccharide at 0.25 mg/kg/h was infused continuously for 4 h for the development of the disseminated intravascular coagulation-like syndrome according to the serum parameters. Leukocytes were clearly decreased from $4.1 \times 10^3$ to $1.5 \times 10^3$ even at 1 h after infusion of lipopolysaccharide and the low level of blood leukocytes was sustained for 4 h. The kinetics of platelet number during the disseminated intravascular coagulation model showed a different

Table 2

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<th>Treatment</th>
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<td>PT (s)</td>
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<tr>
<td>Normal</td>
<td>10</td>
<td>13.7 ± 0.1 \textsuperscript{c}</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>19.7 ± 0.6</td>
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<tr>
<td>FR 128998 (mg/kg/h)</td>
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<tr>
<td>0.25</td>
<td>10</td>
<td>19.8 ± 1</td>
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<tr>
<td>1.0</td>
<td>10</td>
<td>16.6 ± 0.6</td>
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Lipopolysaccharide was infused for 4 h with or without simultaneous treatment with FR128998. Blood samples were collected at the end of the infusion. PT, APTT and FDP represent prothrombin time, activated partial thromboplastin time, and fibrinogen/fibrin degradation products, respectively. The results are expressed as means ± S E M.

\textsuperscript{c} P < 0.05, \textsuperscript{b} P < 0.01, \textsuperscript{c} P < 0.001, significantly different from value for the control.
pattern from that of leukocytes. At 3 h after lipopolysaccharide infusion, the platelets only decreased from 5.5 × 10⁵ cells/mm³ to 3.8 × 10⁵ cells/mm³ FR128998 (0.25 or 1.0 mg/kg/h) was infused simultaneously from the start of the experiment. As shown in Fig. 2, the treatment with FR128998 has no effect on the leukopenia at 4 h after lipopolysaccharide infusion. In contrast, the lipopolysaccharide-induced reduction in blood platelet count was significantly inhibited by the treatment with FR128998 (1.0 mg/kg/h).

3.3. Effect of FR128998 on coagulation parameters

An experimental disseminated intravascular coagulation model induced by lipopolysaccharide infusion produced abnormal activation of the blood coagulation and fibrinolytic systems as follows. The changes in coagulation parameters occurred at 2 h and peaked at 4 h after the lipopolysaccharide infusion (data not shown). Therefore, FR128998 was infused simultaneously for 4 h and the effect of FR128998 on the disseminated intravascular coagulation model was examined at the end of the infusion.

As shown in Table 2, in the control group, prothrombin time and activated partial thromboplastin time were significantly prolonged from 13.7 to 19.7 s (P < 0.001) and 21.0 to 57.4 s (P < 0.001), respectively. The level of fibrinogen was pronouncedly decreased from 2.12 to 0.35 g/l (P < 0.001) and that of fibrinogen/fibrin degradation products was increased from 0.2 to 3.4 μg/ml (P < 0.001). The treatment with FR128998 improved the coagulation parameters associated with disseminated intravascular coagulation in a dose-dependent manner and significant improvement

Fig. 3 Effect of FR128998 on serum TNF level at 2 h of lipopolysaccharide infusion in rats. Serum samples were collected at the peak TNF level (2 h) and TNF activity was determined by bioassy using L-929 cells. The results are expressed as means ± S.E.M., and asterisks show the significance of the difference from control (⁎ P < 0.05, ⁎⁎⁎ P < 0.001).

Fig. 4 Effect of FR128998 on TNF production by peripheral blood leukocytes stimulated with lipopolysaccharide in rats. Peripheral blood leukocytes were incubated with lipopolysaccharide in the presence or absence of FR128998 for 16 h. The supernatants were collected and TNF activity was determined by bioassy using L-929 cells. The results are expressed as means ± S.E.M., and asterisks show the significance of the difference from control (⁎ P < 0.05, ⁎⁎⁎ P < 0.001).

Fig. 5 Inhibition of TNF production by FR128998 in alveolar macrophages and peripheral blood leukocytes stimulated with lipopolysaccharide in rats. Alveolar macrophages and peripheral blood leukocytes were incubated with lipopolysaccharide in the presence or absence of FR128998 at 10 μM for 16 h. The supernatants were collected and TNF activity was determined by bioassy using L-929 cells. The results are expressed as means ± S.E.M., and asterisks show the significance of the difference from control (⁎⁎ P < 0.01, ⁎⁎⁎ P < 0.001).
of these parameters was observed on treatment with 1.0 mg/kg/h FR128998

3.4 Effect of FR128998 on serum TNF levels in rats

The basal level of TNF was 51 ± 11 U/ml Serum TNF was detected at 1 h, reaching a peak of 3163 ± 940 U/ml at 2 h after lipopolysaccharide infusion, and gradually declined to the basal level. This seems similar to the kinetics described by Rabnovici et al. (1990) who studied the effect of PAF receptor antagonist (BN50739) on endotoxin shock induced by bolus injection of lipopolysaccharide in rats. To evaluate the effect of FR128998 on the TNF production accompanied with disseminated intravascular coagulation, therefore, the serum samples were obtained at 2 h after the start of lipopolysaccharide infusion. The treatment with FR128998 (1.0 mg/kg/h) significantly inhibited the production of serum TNF from 3163 ± 940 to 974 ± 351, as shown in Fig. 3.

3.5 Effect of FR128998 on TNF production in vitro

The role of PAF in the mechanism of TNF production induced by lipopolysaccharide was investigated in vitro. Peripheral blood leukocytes at 1.25 × 10⁶ cells/ml were incubated with lipopolysaccharide in the presence or absence of FR128998 for 16 h. The TNF production after stimulation with lipopolysaccharide increased significantly from 1.2 to 20.0 U/ml (Fig. 4). On treatment with FR128998 at 10⁻⁷ to 10⁻⁵ M, the TNF level was reduced in a dose-related way by 17–72%.

We examined the effect of FR128998 on TNF production in alveolar macrophages stimulated with lipopolysaccharide (Fig. 5). Alveolar macrophages at 5 × 10⁶ cells/ml were stimulated with lipopolysaccharide in the presence or absence of FR128998 at 10 μM for 16 h. Alveolar macrophages, even in smaller numbers than blood leukocytes, produced TNF at 41.1 U/ml, similar to the level of TNF produced by blood leukocytes. FR128998 (10 μM) showed similar inhibitory actions on TNF production in alveolar macrophages and blood leukocytes.

When leukocytes were cultured for 16 h in the presence of PAE, TNF production was observed for concentrations from 10⁻⁷ M (Fig. 6). FR128998 could block the PAF-induced increase in TNF production for all concentrations of PAF tested (Fig. 6).

4. Discussion

FR128998, a novel PAF receptor antagonist, inhibits PAF-induced platelet aggregation and mortality in rats, and has a characteristic structure different from those of previous PAF receptor antagonists.

In this investigation, the infusion of lipopolysaccharide (0.25 mg/kg/h) for 4 h induced leukopenia, thrombocytopenia, prolongation of prothrombin time and activated partial thromboplastin time, reduction of fibrinogen and increase of fibrinogen/fibrin degradation products. This result is consistent with the data presented by Imura et al. (1986). Treatment with FR128998 significantly inhibited the changes in the parameters associated with blood coagulation/fibrinolytic systems induced by the infusion of lipopolysaccharide for 4 h. These results indicate that PAF receptor antagonists play an important role in the protection against the development of the lipopolysaccharide-induced disseminated intravascular coagulation in rats. PAF is generated and released in the plasma of rats experimentally exposed to endotoxin (Chang et al., 1987, Doebber et al., 1985), and induces changes in vascular permeability, thrombocytopenia and leukopenia in animal models (Hannahan, 1986). However, the mechanism of the development of thrombocytopenia induced by the infusion of lipopolysaccharide in rats is not through a direct effect of PAF, because rat platelets are devoid of high-affinity binding sites for PAF and cannot be activated by PAF. Whether FR128998 exerts its inhibitory effect on the lipopolysaccharide-induced disseminated intravascular coagulation in rats directly through PAF receptor antagonistic activity or indirectly by the inhibition of other mediators associated with the pathophysiology of disseminated intravascular coagulation remains unclear.

Like PAF, TNF was implicated as a major mediator of various phenomena induced by lipopolysaccharide...
inasmuch as elevated plasma level of TNF was found in lipopolysaccharide-injected animals and humans (Ziegler, 1988). Little is known, however, about the interaction of TNF and the development of disseminated intravascular coagulation induced by lipopolysaccharide infusion in rats. In the present study we found that the level of serum TNF peaked at 2 h and returned to basal levels at 4 h after lipopolysaccharide infusion. This observation is in agreement with results of previous studies (Rabinovici et al., 1990) on kinetics of serum TNF after the bolus injection of lipopolysaccharide. FR128998 attenuated the elevation of serum TNF production and exerted an inhibitory effect on the changes in the coagulation parameters induced by lipopolysaccharide infusion, except for leukopenia. The kinetics of the coagulation parameters showed that leukocytes decreased rapidly, but other parameters did not at 1 h after lipopolysaccharide infusion. It is conceivable that the phenomenon observed in leukopenia is mediated by different mediators such as adhesion molecule and therefore no inhibitory effect can be expected from treatment with FR128998. The kinetics of platelet, prothrombin time, activated partial thromboplastin time, fibrinogen and fibrinogen/fibrin degradation products revealed that the changes of these parameters started at 2 h and accelerated during lipopolysaccharide infusion for 4 h, accompanied by the development of disseminated intravascular coagulation in rats. The present data prompt us to suggest that TNF is the initiator of the development of disseminated intravascular coagulation and causes the changes in the coagulation parameters associated with coagulation/fibrinolysis.

The in vivo prevention of lipopolysaccharide-induced TNF production by PAF receptor antagonist is still controversial. BN50739, PAF receptor antagonist, attenuated the lipopolysaccharide-induced elevation of TNF in rats (Rabinovici et al., 1990), but not in rabbits (Yue et al., 1990). To clarify the mechanism of the inhibitory action of FR128998 on TNF production, in vitro studies were performed. TNF production of lipopolysaccharide-stimulated rat blood leukocytes was blocked by FR128998 in a dose-dependent manner. Similar inhibition was obtained with rat alveolar macrophages which produced more TNF than did blood monocytes (Okubo et al., 1990). In addition, the in vivo elevation of PAF levels after the administration of lipopolysaccharide (Chang et al., 1987) precedes the production of TNF (Rabinovici et al., 1990), and moreover the addition of PAF to monocyte cultures markedly enhances TNF production (Ziegler-Heitbrock et al., 1992). Therefore, it is conceivable that the inhibitory effect of FR128998 on TNF production is associated with the interaction of these mediators. This postulate is supported by our finding that PAF augmented TNF production in rat peripheral blood leukocytes. This result is also in agreement with other reports, in which the release of TNF was stimulated by PAF in human monocyte cultures (Rus et al., 1991; Bonavida and Braquet, 1988). Although PAF has less capacity to induce TNF production by leukocytes than lipopolysaccharide, FR128998 inhibited TNF production of rat leukocytes stimulated by lipopolysaccharide as well as PAF. This result indicates that PAF plays a pivotal role in the regulation of TNF production.

In conclusion, FR128998, a novel PAF receptor antagonist, inhibited the development of disseminated intravascular coagulation induced by lipopolysaccharide infusion in a dose-dependent manner. FR128998 also inhibited lipopolysaccharide-induced TNF production in vivo. Furthermore, TNF production was also blocked by the addition of FR128998 to cultures of blood leukocytes stimulated with lipopolysaccharide or PAF. It is expected that FR128998 exerts a potential therapeutic capacity on the rat disseminated intravascular coagulation model via an indirect activity such as the inhibition of TNF production, in addition to the direct anti-PAF activity.

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