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Anti-inflammatory potency of FR167653, a p38 mitogen-activated protein kinase inhibitor, in mouse models of acute inflammation

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Abstract

The anti-inflammatory effect of FR167653 (1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate), a p38 mitogen-activated protein (MAP) kinase inhibitor, was examined in two mouse models of acute inflammation. Carrageenan-induced paw edema was inhibited by pretreatment with FR167653, anti-tumor necrosis factor (TNF)-α antibody, and NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide), a selective cyclooxygenase-2 inhibitor. Carrageenan increased TNF-α and prostaglandin E_2 levels in the paw, both of which were suppressed by FR167653. Subcutaneous injection of lipopolysaccharide at the back of mouse caused local increase in vascular permeability determined by leakage of Pontamine sky blue. FR167653 dose-dependently inhibited the lipopolysaccharide-induced plasma leakage. FR167653 also inhibited lipopolysaccharide-induced increases in serum TNF-α level, and skin TNF-α and prostaglandin E_2 levels at the injection site. On the other hand, FR167653 did not reduce arachidonic acid-induced plasma leakage which is not mediated by cyclooxygenase-2. FR167653 exhibits anti-inflammatory effects against both carrageenan-induced paw edema and lipopolysaccharide-induced plasma leakage through inhibiting the synthesis of inflammatory mediators that are regulated by p38 MAP kinase.

Keywords: FR167653; p38 MAP (mitogen-activated protein) kinase inhibitor; Carrageenan; Paw edema; Lipopolysaccharide; Vascular permeability

1. Introduction

FR167653 (1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate) was originally reported to be a cytokine suppressive agent (Yamamoto et al., 1996, 1997), and has recently been shown to be a specific inhibitor of p38 mitogen-activated protein (MAP) kinase (Takahashi et al., 2001). p38 MAP kinase regulates the synthesis of cytokines such as tumor necrosis factor (TNF)-α and interleukin-1-β, and also regulates the induction of cyclooxygenase-2 protein (Lee et al., 1993, 1994; Widmann et al., 1999; McGinty et al., 2000). Since these cytokines play important roles in inflammation, inhibitors of p38 MAP kinase are potential anti-inflammatory agents categorized as cytokine-suppressive anti-inflammatory drugs (CSAIDs) (Lee et al., 1994). SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole), another specific inhibitor of p38 MAP kinase, has been reported to improve adjuvant-induced arthritis in rats, and reduce mortality in a murine model of endotoxin (lipopolysaccharide) shock by inhibiting TNF-α release (Badger et al., 1996). The anti-inflammatory effect of SB203580 has also been reported in allergic airway inflammation (Escott et al., 2000). FR167653 improved the lipopolysaccharide-induced disseminated intravascular coagulation syndrome in rats and lipopolysaccharide-induced septic shock in rabbits (Yamamoto et al., 1996, 1997). However, the anti-inflammatory effects of p38 MAP kinase inhibitors in local inflammation models have not been investigated.

Injection of carrageenan into the hind paw of mice or rats induces an acute swelling of the paw. This model has long been used to assess the anti-inflammatory properties of drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit prostaglandin production. Tissue

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TNF-α and prostaglandin E_2 contents are increased in the carrageenan-injected paw without elevation of serum TNF-α level (Sekut et al., 1994).

Subcutaneous (s.c.) injection of pro-inflammatory agents into the back skin of mice, pre-loaded with dye through the tail vein, induces dye leakage at the site of injection at the back (Fujii et al., 1994, 1996). This acute inflammation model examines local vascular permeability changes. The lipopolysaccharide-induced increase in vascular permeability is mediated through many inflammatory mediators including TNF-α, interleukin-1α, prostanoids synthesized by cyclooxygenase-2, nitric oxide (NO) derived from inducible NO synthase, platelet-activating factor, and histamine (Fujii et al., 1996, 2000; Wada et al., 2000). The present study examined the anti-inflammatory effects of a p38 MAP kinase inhibitor, FR167653, in these local inflammation models.

2. Materials and methods

2.1. Animals

Male ddY mice were obtained from Sankyo Laboratory Service (Tokyo, Japan) and were housed in an air-conditioned room (22 ± 1 °C and 55 ± 5% humidity) with a controlled light–dark cycle (6:00–20:00 light on) and free access to standard chow and tap water. Animals aged between 6 and 8 weeks were used in the experiment. All protocols of the animal experiments were approved by the Animal Care Committee of Tokyo Women's Medical University.

2.2. Chemicals

Carrageenan lambda, lipopolysaccharide (*Salmonella typhimurium*) and arachidonic acid sodium salt were purchased from Sigma (St. Louis, MO, USA), Pontamine sky blue 6B (PSB) from Tokyo Kasei Kogyo (Tokyo, Japan), and SB203580 hydrochloride and NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide) from Calbiochem (San Diego, CA, USA). Rabbit anti-mouse TNF-α polyclonal antibody was obtained from Genzyme (Cambridge, MA, USA), mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit from BioSource (Camarillo, CA, USA), and prostaglandin E_2 ELISA kit from Cayman Chemical (Ann Arbor, MI, USA). FR167653 was kindly provided by Fujisawa Pharmaceutical (Osaka, Japan).

Carrageenan was dissolved in sterile physiological saline (0.9% NaCl) as 1% solution, autoclaved and stored in sterile tubes at 4 °C until use. Lipopolysaccharide (4 mg/ml) was dissolved in phosphate-buffered saline and stored at 4 °C. PSB (5 mg/ml) was dissolved in physiological saline, filtered through membrane filter (pore size 0.22 μM), and stored in sterile tubes. NS-398 was dissolved in a small volume of absolute ethanol, and then diluted with 50% propylene glycol to obtain a 1 mg/ml stock solution. Immediately prior to use, the stock solution was diluted to 0.1 mg/ml with physiological saline. Saline containing 0.01% ethanol and 5% propylene glycol was used as vehicle control for NS-398. Other drugs were dissolved in sterile physiological saline.

2.3. Determination of paw edema

Mice were randomly assigned to experimental groups at the start of the experiments. Twenty-five microliters of 1% carrageenan was injected into the right hind paw under light ether anesthesia. Paw volume was measured before and after carrageenan injection up to 6 h, using a plethysmograph (Ueno et al., 1998). Enzyme inhibitors (10 ml/kg) were injected s.c. at the back 1 h prior to carrageenan injection. Anti-TNF-α antibody (1:400 dilution, 10 ml/kg) was injected s.c. at the back 24 h prior to carrageenan injection.

2.4. Determination of plasma leakage in mouse skin

The microvascular permeability was assessed by extravasation of PSB dye in the skin as previously described (Fujii et al., 1994). Briefly, PSB (50 mg/kg) was injected into the tail vein, and 5 min later, lipopolysaccharide (400 μg/site) or arachidonic acid (200 μg/site) was administered s.c. at the back of the mouse. FR167653 was injected s.c. at the abdominal site 1 h prior to PSB. One (arachidonic acid treatment) or two (lipopolysaccharide treatment) hours later, the mice were killed by cervical dislocation and the stained area of the skin at the site of injection was excised. Approximately 1 g of skin was minced and dispersed in 6 ml of 0.5% Na_2SO_4 solution and the dye was extracted by addition of 14 ml of acetone. After 3.5 h of extraction, the dye concentration was determined using a spectrophotometer at OD_{590}.

2.5. Tissue TNF-α content

Mice were killed by cervical dislocation 3 h after carrageenan injection for measurement of paw TNF-α level, because the local TNF-α level peaked at 3 h after an intraplantar injection of carrageenan in a previous report (Sekut et al., 1994). The paws were separated at the calcaneus bone, snap frozen in liquid nitrogen, and stored at −80 °C until use. For determination of TNF-α, the paw was crushed and ground to powder using a mortar and pestle kept under liquid nitrogen. Using a glass homogenizer, a spoonful of powder was homogenized in 0.5 ml of standard diluent supplied with the TNF-α ELISA kit. The homogenate was centrifuged at $8000 \times g$ for 5 min at 4 °C. The supernatant was used for TNF-α assay and the precipitate for protein determination by Lowry's method.

To determine skin TNF-α level, mice were killed by cervical dislocation 2 h after s.c. lipopolysaccharide injection. The skin was excised at the injected site, frozen,

crushed with a hammer, and stored at −80 °C until assay. Several pieces (50 mg) of crushed tissue was ground with a mortar and pestle kept under liquid nitrogen, and then homogenized in 0.5 ml of buffer as described.

2.6. Serum TNF-α concentrations

In the model of lipopolysaccharide-induced vascular permeability change, serum TNF-α level increased transiently with a peak at 1 h after injection (Irie et al., 2001). In this study, mice were killed by decapitation for the measurement of serum TNF-α level 1 h after lipopolysaccharide treatment, and blood sample was collected from the neck. Serum was separated and stored at −20 °C until assay. Serum TNF-α was measured using a mouse TNF-α ELISA kit according to the manufacturer's specification.

2.7. Measurement of tissue prostaglandin E_2 level

Previous report demonstrated that the peak paw prostaglandin E_2 level was observed at 2 h after an intraplantar injection of carrageenan in rat hind paw (Nantel et al., 1999). Therefore, mice were killed at 2 h after carrageenan injection for determining paw prostaglandin E_2 level. The paws were separated at the calcaneus bone, degloved, frozen in liquid nitrogen, and stored at −80 °C until use. The frozen sample was ground as described above, suspended in 0.5 ml of acetone, and incubated at 4 °C overnight with gentle agitation. For determination of skin prostaglandin E_2 level, 50 mg of crushed tissue was ground into powder, suspended in 0.5 ml acetone, and incubated at 4 °C overnight. After centrifugation at 2000 × *g* for 5 min, the supernatant was removed and evaporated by vacuum centrifugation. The residues were then solubilized with sample buffer supplied with the prostaglandin E_2 ELISA kit for measuring prostaglandin E_2 levels.

2.8. Statistics

All data are expressed as means ± S.E.M. The data was analyzed using one or two-way analysis of variance (ANOVA) followed by Bonferroni/Dunn's test for multiple comparison.

3. Results

3.1. Effect of FR167653 on mouse paw edema induced by carrageenan

Intraplantar injection of 25 μl of 1% carrageenan induced on average 40% increase in paw volume 1 h after carrageenan injection and the increase was sustained for the whole observation period of 6 h (Fig. 1A). Saline injection did not change the paw volume. Pretreatment with FR167653 (30 mg/kg, s.c.) significantly attenuated the carrageenan-induced paw edema during the observation period (Fig. 1A). As the reduction was most remarkable at 3 h after carrageenan injection, we determined the dose effect of FR167653 (3, 10, and 30 mg/kg) in reducing carrageenan-induced paw edema at 3 h. FR167653 dose-dependently decreased the carrageenan-induced edema (Fig. 1B). SB203580 (30 mg/kg, s.c.), another inhibitor of p38 MAP kinase, also significantly inhibited the edema induced by carrageenan (data not shown).

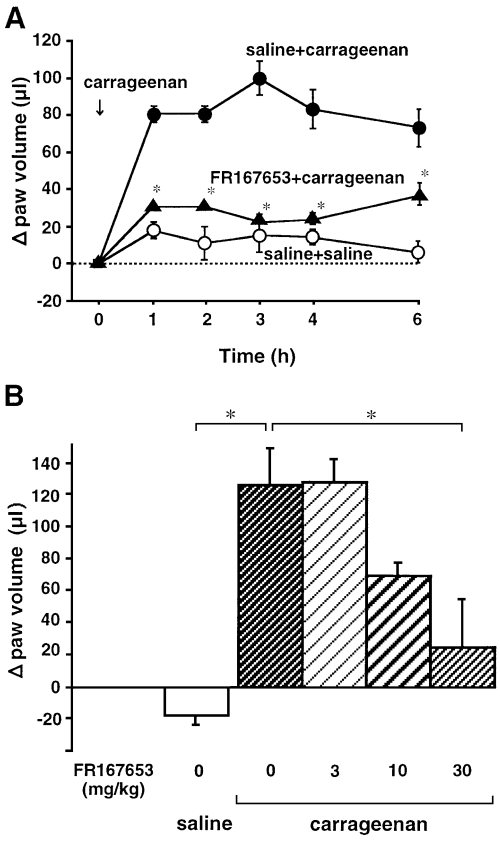

Fig. 1. Effect of FR167653 on carrageenan-induced mouse paw edema. (A) Time course study. FR167653 (30 mg/kg, s.c.) or saline was administered at the back. Paw edema was induced 1 h later by an intraplantar injection of 25 μl of 1% carrageenan. Saline control mice received saline instead of carrageenan. Paw volume was measured plethysmographically before and 1, 2, 3, 4, and 6 h after intraplantar carrageenan or saline injection. Each point represents the mean change in paw volume compared to the baseline control volume before carrageenan/saline injection ($n=4$). * $P<0.01$ vs. saline + carrageenan at the same time point. (B) Dose dependency study. Indicated doses of FR167653 were administered. Then mice were treated with intraplantar carrageenan or saline 1 h later. Changes in paw volume were determined 3 h after the intraplantar injection. Columns and bars represent means ± S.E.M. of five mice.

3.2. Effect of anti-TNF-α antibody and NS-398 on carrageenan-induced paw edema

In mice pretreated with rabbit anti-mouse TNF-α polyclonal antibody 24 h prior to carrageenan, the edema induced by carrageenan was significantly attenuated throughout the observation period (Fig. 2A). NS-398 reduced the

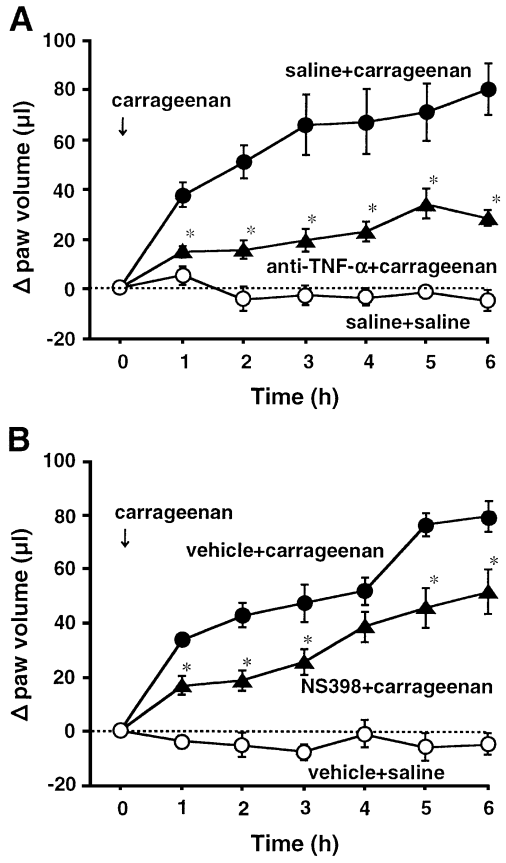

Fig. 2. Effects of pretreatment with anti-mouse TNF-α antibody (A) and a selective cyclooxygenase-2 inhibitor, NS-398 (B) on carrageenan-induced paw edema in mice. (A) Mice received anti-mouse TNF-α polyclonal antibody (1:400 dilution, 10 ml/kg, s.c.) or saline (10 ml/kg, s.c.) 24 h prior to intraplantar injection of carrageenan (1%, 25 μl). Saline control mice received saline (10 ml/kg, s.c.) followed by intraplantar saline 24 h later. Points and bars represent means ± S.E.M. of six to eight mice. * $P<0.01$ vs. saline + carrageenan at the same time point. (B) Mice received NS-398 (1.0 mg/kg, s.c.) or vehicle, then intraplantar carrageenan 1 h later. Control mice received vehicle followed by intraplantar saline. Points and bars represent means ± S.E.M. of four to eight mice. * $P<0.05$ vs. vehicle + carrageenan at the same time point.

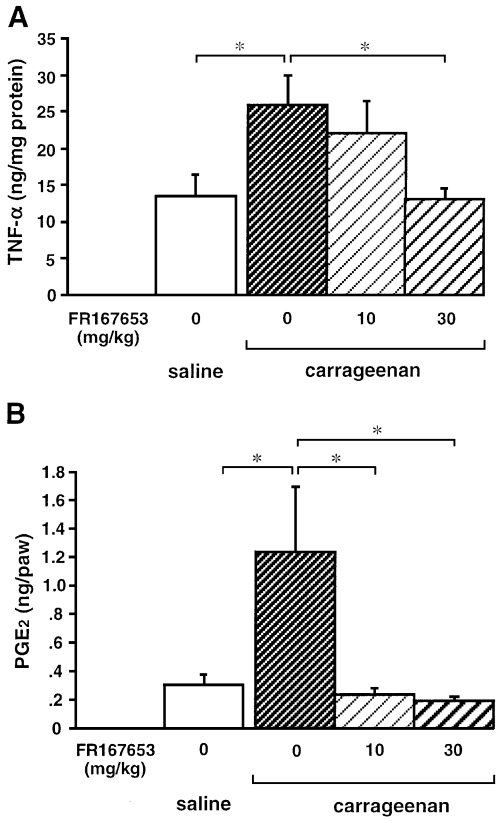

Fig. 3. Effect of FR167653 on TNF-α (A) and prostaglandin E_2 (B) levels in carrageenan-injected paw. Mice received indicated doses of FR167653 or saline followed by intraplantar carrageenan or saline 1 h later. (A) Three hours after intraplantar injection of carrageenan, the paw was homogenized and TNF-α in the supernatant was determined by ELISA. (B) Prostaglandin E_2 content of the paw was determined by ELISA 2 h after intraplantar injection of carrageenan. Columns and bars represent mean ± S.E.M. of five to six mice. * $P<0.05$.

carrageenan-induced paw edema up to 6 h (Fig. 2B). The results of these experiments collectively suggest that both TNF-α and cyclooxygenase-2 play a role in carrageenan-induced paw edema.

3.3. Effect of FR167653 on paw TNF-α and prostaglandin levels

We then investigated whether FR167653 inhibits edema formation through inhibition of TNF-α and prostaglandin E_2. Three hours after carrageenan injection, the TNF-α level in the paw was elevated more than two times than that of saline controls. Pretreatment with FR167653 (10 and 30 mg/kg) dose-dependently suppressed the elevation of tissue TNF-α level (Fig. 3A).

The inhibitory effect of FR167653 was also observed in the tissue prostaglandin E_2 level. Two hours after

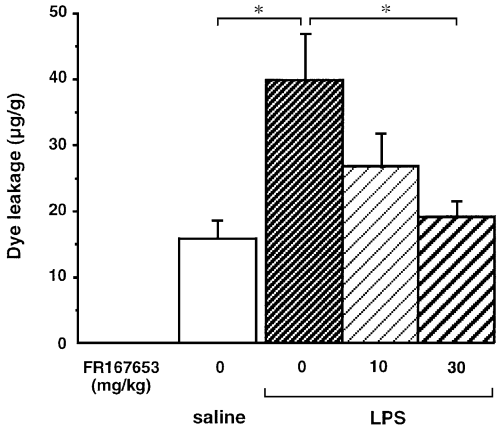

Fig. 4. Effect of FR167653 on lipopolysaccharide-induced increase in vascular permeability of mouse skin. Mice were pretreated with FR167653 (10 or 30 mg/10 ml/kg, s.c.) at the abdomen 1 h prior to Pontamine sky blue 6B (PSB; 50 mg/10 ml/kg, i.v.). Five minutes after PSB, lipopolysaccharide (400 μg/0.1 ml/site, s.c.) was injected at the back. Two hours later, dye in the skin was extracted and determined colorimetrically. Columns and bars represent means ± S.E.M. of five mice. * $P<0.05$.

carrageenan injection, the prostaglandin E_2 level in the paw increased almost four times than that of saline-treated paw. FR167653 at doses of 10 and 30 mg/kg suppressed the paw prostaglandin E_2 level to the level of saline control (Fig. 3B).

3.4. Effect of FR167653 on lipopolysaccharide-induced dye leakage in mice

Subcutaneous injection of lipopolysaccharide (400 μg/site) at the back of mice induced a significant increase in dye leakage at the site of injection (Fig. 4). Pre-administration of FR167653 (10 and 30 mg/kg) dose-dependently prevented the lipopolysaccharide-induced increase in dye leakage in the skin (Fig. 4).

3.5. Effect of FR167653 on TNF-α and prostaglandin E_2 levels in skin injected with lipopolysaccharide

The local TNF-α level in the skin increased significantly 2 h after lipopolysaccharide injection (Fig. 5A). Pretreatment with FR167653 (30 mg/kg) significantly inhibited the local increase in TNF-α (Fig. 5A). An injection of lipopolysaccharide increased the prostaglandin E_2 level in the skin to almost two times that of saline-treated skin (Fig. 5B). FR167653 at a dose of 30 mg/kg significantly prevented the lipopolysaccharide-induced increase in prostaglandin E_2 content (Fig. 5B).

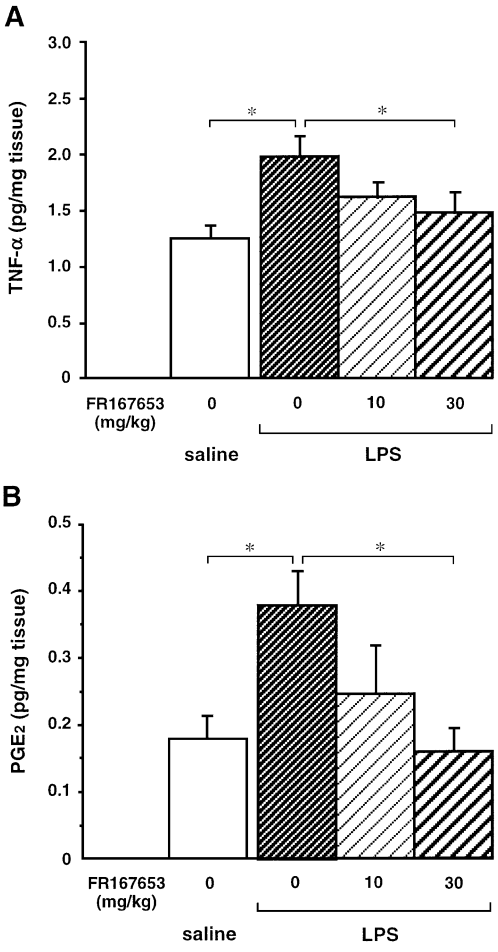

Fig. 5. Effect of FR167653 on TNF-α (A) and prostaglandin E_2 (B) levels in the skin at the site lipopolysaccharide injection. Experimental conditions are the same as Fig. 4. Both TNF-α and prostaglandin E_2 levels in the skin were determined 2 h after s.c. lipopolysaccharide. Columns and bars represent mean ± S.E.M. of five to six mice. $*P<0.05$.

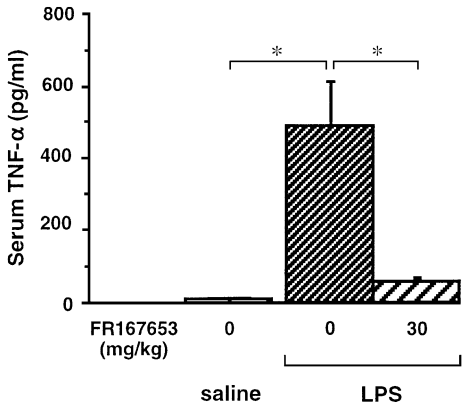

Fig. 6. Effect of FR167653 on lipopolysaccharide-induced increase in serum TNF-α level in mice. FR167653 was administered 1 h prior to s.c. lipopolysaccharide. Blood samples were collected 1 h after lipopolysaccharide injection. Serum TNF-α was measured by ELISA. Columns and bars represent mean ± S.E.M. of six to seven mice. $*P<0.001$.

3.6. Effect of FR167653 on lipopolysaccharide-induced elevation of serum TNF-α

Lipopolysaccharide significantly increased serum TNF-α level (Fig. 6). The lipopolysaccharide-induced increase in serum TNF-α was abolished by pretreatment with 30 mg/kg of FR167653 (Fig. 6).

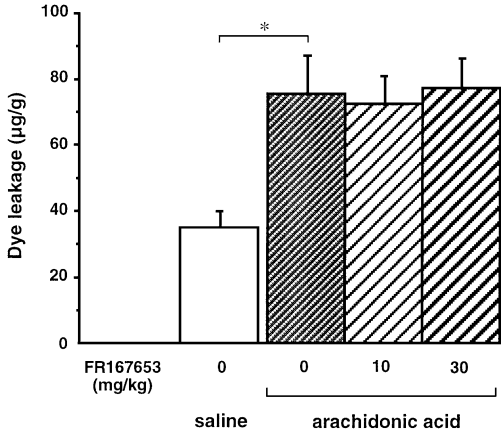

Fig. 7. Effect of FR167653 on arachidonic acid-induced increase in vascular permeability. Mice were pretreated with FR167653 (10 and 30 mg/10 ml/kg, s.c.) at the abdomen 1 h prior to i.v. Pontamine sky blue 6B (PSB). Five minutes after PSB, arachidonic acid 200 μg/0.1 ml/site was injected s.c. at the back. Dye leakage was determined at 1 h after arachidonic acid injection. Columns and bars represent means ± S.E.M. of five to six mice. $*P<0.05$.

3.7. Effect of FR167653 on arachidonic acid-induced dye leakage in mice

Arachidonic acid-induced dye leakage is a model of vascular permeability change not related to cyclooxygenase-2. We examined whether FR167653 attenuates dye leakage in this model. Arachidonic acid significantly increased the dye leakage in the mouse skin (Fig. 7). Pretreatment with FR167653 had no inhibitory effect on the arachidonic acid-induced dye leakage (Fig. 7).

4. Discussion

Pyridinyl-imidazoles that inhibit interleukin-1 and TNF-α production were initially called CSAIDs (Lee et al., 1993, 1994), but later studies showed that the target molecule of these compounds was p38 MAP kinase (Lee et al., 1993, 1994). p38 MAP kinase regulates the synthesis of cytokines, cyclooxygenase-2, and inducible nitric oxide synthase (iNOS) (Badger et al., 1996, 1998; Widmann et al., 1999; McGinty et al., 2000; Faour et al., 2001). Since all these factors (cytokines, cyclooxygenase-2, and iNOS) are mediators of inflammatory processes, inhibitors of p38 MAP kinase may act as anti-inflammatory agents. Our results indicated a potential of FR167653 as an anti-inflammatory agent.

TNF-α plays an important role in carrageenan-induced paw edema (Sekut et al., 1994). In the present study, tissue TNF-α level increased in the carrageenan-injected paw, and neutralization with anti-TNF-α antibody prevented the carrageenan-induced paw edema. FR167653 suppressed carrageenan-induced paw edema, accompanied with a decrease in tissue TNF-α. Thus, suppression of TNF-α production is involved in the mechanism by which FR167653 inhibits paw edema induced by carrageenan.

NS-398, a specific inhibitor of cyclooxygenase-2, prevented the carrageenan-induced edema formation in this study, supporting an important role of cyclooxygenase-2 (Seibert et al., 1994; Zhang et al., 1997). In this study, tissue prostaglandin E_2 level increased in the carrageenan-injected paw, consistent with previous reports (Zhang et al., 1997; Nantel et al., 1999), and the increased prostaglandin E_2 level was again suppressed by FR167653 pretreatment. Thus, the p38 MAP kinase–cyclooxygenase-2–prostaglandin E_2 pathway contributes to the edema formation, in addition to the p38 MAP kinase–cytokine pathway. A recent study reported that carrageenan-induced pleurisy was inhibited by FR167653, accompanied by a decrease in number of leukocyte accumulation, and suppression of cytokine and prostanoids in the pleural exudate (Hatanaka et al., 2001).

Lipopolysaccharide is a well-known activator of p38 MAP kinase (Han et al., 1994). In a previous study using anti-TNF-α antibody, we have shown that TNF-α mediates the lipopolysaccharide-induced plasma leakage (Fujii et al., 2000; Wada et al., 2000). In the present study, FR167653 inhibited the lipopolysaccharide-induced increase in plasma leakage, accompanied by suppression of tissue TNF-α. Prostanoids synthesized by cyclooxygenase-2 are also involved in lipopolysaccharide-induced plasma leakage (Fujii et al., 1996). The results of this study suggest that FR167653 effectively blocks the activation of p38 MAP kinase induced by lipopolysaccharide, and suppresses the increases in synthesis of TNF-α and possibly cyclooxgenase-2.

FR167653 has been shown to have no inhibitory effect on cyclooxygenase-1 and-2 activities, whereas SB203580 inhibits these enzymes (Takahashi et al., 2001). Arachidonic acid-induced increase in dye leakage was inhibited by indomethacin but not by NS-398 (unpublished data), which indicates that it is a cyclooxygenase-1-mediated inflammation model. FR167653 did not inhibit arachidonic acid-induced dye leakage, indicating that the action of FR167653 is not related to cyclooxygenase-1. The anti-inflammatory effects of FR167653 may be due to inhibition of induction of cytokines and cyclooxygenase-2 enzyme but not to an inhibition of cyclooxygenase enzymatic activity.

FR167653 showed anti-inflammatory effects in a carrageenan-induced paw edema model and a lipopolysaccharide-induced dye leakage model in mice. Although the exact mechanism of activation of p38 MAP kinase by carrageenan remains to be determined, this inhibitor of p38 MAP kinase has potential use as an anti-inflammatory agent.

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