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European Journal of Pharmacology 368 (1999) 245–250

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Suppression of lipopolysaccharide-induced tumor necrosis factor-release and liver injury in mice by naringin

Kiichiro Kawaguchi [a], Seiichi Kikuchi [a], Hirohide Hasegawa [a,c], Hiroko Maruyama [b], Hiroyuki Morita [a], Yoshio Kumazawa [c,*]

[a] *Medicinal Plant Garden, School of Pharmaceutical Sciences, Kitasato University, Sagamihara 228-8555, Japan*
[b] *Department of Pathology, School of Allied Health Sciences, Kitasato University, Sagamihara 228-8555, Japan*
[c] *Department of Biosciences, School of Science, Kitasato University, Sagamihara 228-8555, Japan*

Received 27 July 1998; revised 16 November 1998; accepted 20 November 1998

Abstract

Suppressive effects of naringin on lipopolysaccharide-induced tumor necrosis factor (TNF) release followed by liver injury were investigated. Intraperitoneal (i.p.) treatment with naringin prior to an intravenous (i.v.) challenge of lipopolysaccharide significantly reduced serum TNF levels in a dose-dependent manner and was the most effective when administered 60 min prior to lipopolysaccharide challenge. Treatment with naringin 3 h prior to lipopolysaccharide challenge resulted in complete protection from lipopolysaccharide lethality in D-galactosamine-sensitized mice. Histological estimation revealed that massive cell infiltration followed by severe injury developed in the livers of lipopolysaccharide-treated and D-galactosamine-treated mice unless they had been pretreated with naringin. Appearance of apoptotic cells was also found to decrease by treatment with naringin. Increases in serum levels of aspartate aminotransferase, alanine aminotransferase and creatine kinase, responsible for lipopolysaccharide-induced liver injury, blocked by naringin administration and the levels were nearly to the normal level. These results indicate that action of naringin is mediated through suppression of lipopolysaccharide-induced TNF production.

Keywords: TNF (tumor necrosis factor); Lipopolysaccharide; Naringin; Flavonoid

1. Introduction

The treatment for purpose of rescue from septic shock has been the subject of basic and clinical research for several decades. Endotoxin which is lipopolysaccharide consisting of outer membrane of Gram-negative bacteria plays a critical role for pathogenesis in septic shock. The pathogenic activities of lipopolysaccharide are induced via endogenous mediators formed on interaction of lipopolysaccharide with cellular targets, especially with macrophages (Rosenstreich and Vogel, 1980; Freudenberg et al., 1986). It is a general agreement that the main pathogenic mediator implicated in lethal shock is tumor necrosis factor (TNF)-α produced by lipopolysaccharide-elicited macrophages (Beutler and Cerami, 1985; Old, 1985; Tracey et al., 1986) and that D-galactosamine-sensitized mice are hypersensitive to lethal toxicity of lipopolysaccharide and to TNF-α produced by the elicited macrophages (Galanos et al., 1979; Lehmann et al., 1987). It has therefore occurred to us that the reduction of TNF levels leads toward protection and rescue from lipopolysaccharide lethality.

Attempts to find out components capable of suppressing TNF-α production have been carried out for the last decade. Since the active center of lipopolysaccharide is the lipid A moiety (Galanos et al., 1985; Homma et al., 1985; Kotani et al., 1985), a number of lipid A derivatives have been synthesized to clarify the structure-activity relationship and to discover antagonists. Recently, it has been demonstrated that diphosphorylated lipid A from *Rhodobacter sphaeroides* is potent antagonist which blocks the release of the lipopolysaccharide-induced proinflammatory cytokines TNF-α and interleukin-1β (Weckesser and Mayer, 1988; Golenbock et al., 1991; Christ et al., 1995). Among medicinal plants, an inhibitory substance(s) from cinnamon bark could bind directly to the lipid A moiety to

* Corresponding author. Tel.: +81-42-778-9534; Fax: +81-42-778-9534; E-mail: kumazawa@jet.sci.kitasato-u.ac.jp

0014-2999/99/$ - see front matter
PII: S0014-2999(98)00867-X

suppress TNF-α release and the lethal shock (Azumi et al., 1997). There is little information on the suppressive substances from flavonoids. We have been studying flavonoids capable of suppressing the release of lipopolysaccharide-induced TNF-α and blocking the lethal shock. The present study shows that the citrus flavonoid naringin, known as the main component of grapefruit, orange and Unshu orange, is able to inhibit the lipopolysaccharide-induced TNF-α release and to block the lethal toxicity in D-galactosamine-sensitized mice.

2. Materials and methods

2.1. Reagents

Naringin (Tokyo Chemical Industry, Tokyo, Japan), hesperidin (Tokyo Chemical Industry), diosmin (Sigma, St. Louis, MO, USA), rutin (Wako, Osaka, Japan), actinomycin D-mannitol (Sigma), D-galactosamine hydrochloride (Wako) and dexamethasone sodium phosphate (Banyu Pharmaceutical, Tokyo, Japan) were used.

2.2. Mice

Both sexes of C57BL/10ScSn (B10Sn) mice, obtained as breeding pairs from the Max-Planck-Institut für Immunbiologie (Freiburg im Breisgau) and raised from the Animal Facility of School of Science, Kitasato University, and female C57BL/6 mice purchased from Clea Japan, Tokyo, Japan, were housed under specific pathogen free conditions and used at 10–16 weeks of age.

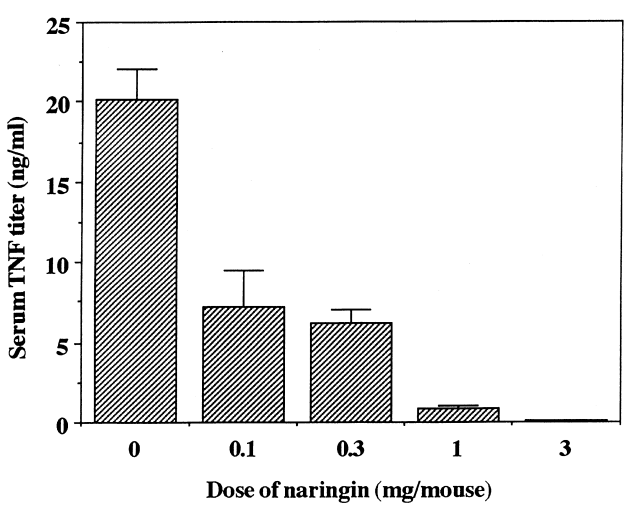

Fig. 1. Dose effects of naringin on lipopolysaccharide-induced TNF release. B57BL/6 mice were injected i.p. with the indicated dose of naringin 3 h before an i.v. challenge of 0.1 μg *S. abortusequi* lipopolysaccharide. Sera were prepared from mouse blood samples 60 min after the lipopolysaccharide challenge. The TNF titer for individuals was determined by a bioassay using L929 (C5F6) cells in the presence of actinomycin D in triplicate cultures (Morita et al., 1997).

Table 1
Suppression of lipopolysaccharide-induced TNF production by flavonoids

Compounds tested	Lipopolysaccharide-induced TNF production[a]	
	Serum TNF (ng/ml)[b]	Suppression (%)
Diosmin	3.57 ± 2.20	74.3
Hesperidin	4.42 ± 1.29	68.2
Naringin	0.80 ± 0.51	94.3
Rutin	2.09 ± 1.38	84.9

[a] Compounds were suspended in saline and injected i.p. at a dose of 1 mg into C57BL/6 mice 1 h before 0.1 μg *S. abortusequi* lipopolysaccharide.
[b] Results represent arithmetic mean ± SD of five mice/group. Serum TNF in positive controls injected with *S. abortusequi* lipopolysaccharide alone was 13.88 ± 3.16 ng/ml.

2.3. Preparation of serum samples

Following deep anesthesia with diethyl ether, blood (0.7–1.2 ml) was taken from mice by cardiac puncture using a 1-ml syringe with 26 gauge needle. After coagulation at room temperature, blood was centrifuged at 3000 rpm for 5 min. Serum samples were collected carefully and kept at −80°C until used.

2.4. Determination of TNF titer

Serum TNF titers were estimated by a cytotoxicity test using TNF-sensitive L929 (C5F6) cells at the presence of actinomycin D as described previously (Morita et al., 1997). Four hours before the termination of cultures, aliquots (10 μl) of Cell Counting Kit, consisting of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)2*H*-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazium methylsulfate (1-Methoxy PMS) (Dojindo, Kumamoto, Japan), were added to each well of 96-well microplates. The resultant formation of formazan by viable cells was estimated by measuring the absorbance

Table 2
Protective effect of naringin on lipopolysaccharide-induced lethality in D-galactosamine-sensitized mice

Compounds tested[a]	Dose (μg)	Dead/mice tested		Lethality (%)[b]
		5 h	24 h	
Saline	–	6/8	8/8	100
Naringin	300	4/6	6/6	100
	1000	0/6	1/6	17
	3000	0/12	0/12	0
Dexamethasone	1	1/4	4/4	100
	10	0/4	3/4	75
	100	0/4	0/4	0

[a] Naringin suspended in saline was injected i.p. at the indicated dose into B10Sn mice 3 h prior to challenge with 0.1 μg *S. abortusequi* lipopolysaccharide. Dexamethasone in saline was injected i.p. at 10 min before challenge.
[b] Lethality was assessed 24 h later.

Table 3
Increased serum levels of aspartate aminotransferase, alanine aminotransferase and creatine kinase responsible for lipopolysaccharide/D-galactosamine-induced liver injury

Enzyme activity	Control	D-Galactosamine + lipopolysaccharide	D-Galactosamine + lipopolysaccharide + naringin
Espartate aminotransferase (GOT)	86 ± 38	4,160 ± 1,472[a]	279 ± 129[b]
Alanine aminotransferase (GPT)	48 ± 13	5,830 ± 2,679[a]	172 ± 125[b]
Creatine kinase	277 ± 172	8,473 ± 3,159[a]	398 ± 105[b]

[a] $P < 0.001$ vs. control.
[b] $P < 0.001$ vs. D-galactosamine + lipopolysaccharide (Student's *t*-test).

at 450 nm (reference at 690 nm). The TNF titer in plasma samples was determined by multiplying the sample dilution and the value of a recombinant murine TNF-α (Genzyme, Boston, MA, USA) which was able to give the 50% cytotoxicity.

2.5. Lipopolysaccharide lethality in D-galactosamine-sensitized mice

B10Sn mice were injected i.v. with 0.1 μg *S. abortusequi* lipopolysaccharide, donated from Dr. Chris Galanos (Max-Planck-Institut für Immunbiologie) and subsequently i.p. with 20 mg D-galactosamine in saline (Galanos et al., 1979). The lethality of lipopolysaccharide was assessed by counting the numbers of dead mice 24 h later. Dexamethasone was injected i.p. 10 min before lipopolysaccharide challenge.

2.6. Biochemical analysis of serum samples

Serum samples were collected from B10Sn mice at 5 h after lipopolysaccharide/D-galactosamine injection. To determine the serum levels of aspartate aminotransferase, alanine aminotransferase, and creatine kinase, aliquots (0.5 ml) of undiluted or diluted samples were analyzed using test reagents, purchased from Sinotest (Sagamihara, Japan), with a Hitachi 9370 automatic analyzer (Tokyo, Japan).

2.7. Histology

Samples from liver of B10Sn mice 5 h after lipopolysaccharide challenge with D-galactosamine were fixed with 10% formalin in 0.01 M phosphate buffer (pH 7.4) and embedded in paraffin. Sections were stained with hematoxylin and eosin.

2.8. Detection of apoptotic cells in liver

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method using the in situ apoptosis detection kit from Takara Shuzo, Kyoto, Japan. Staining of liver sections was performed according to the manufacturer's recommendation. Briefly, sections were deparaffinized and then treated with proteinase K (20 μg/ml) in phosphate buffered saline (PBS). Endogenous peroxidase was blocked by a subsequent incubation with 3% H_2O_2 in methanol for 5 min. After washing with PBS, TUNEL reaction mixture was dropped on slides and incubated for 90 min at 37°C. Apoptotic cells were then detected with horse radish peroxidase-conjugated anti-fluorescein isothiocyanate antibodies (Takara MK503) and 0.26 mg/ml diaminobenzidine (Sigma) followed by counterstaining with 1% methylgreen.

2.9. Statistical analysis

Statistical significance of the data was determined by Student's *t*-test. A P value of less than 0.05 was taken as significant.

3. Results

3.1. Suppression of lipopolysaccharide-induced TNF production by flavonoids

Along with experiments to screen for medicinal plants possessing colony stimulating factor- and TNF-inducing

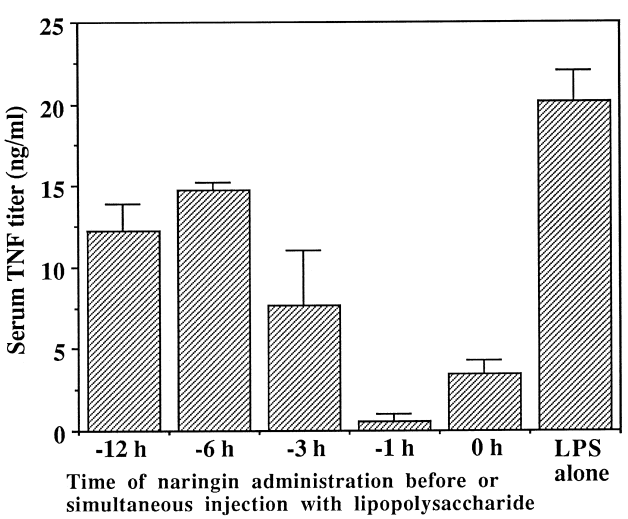

Fig. 2. Timing of naringin administration for inducing suppression of TNF release. B57BL/6 mice were injected i.p. with 1 mg naringin different time points before lipopolysaccharide challenge. See legends of Fig. 1 for other details.

activities (Kawaguchi et al., 1998), TNF-inducing activity of four flavonoids, diosmin, hesperidin, naringin and rutin, was tested using pooled sera 60 min after i.p. administration. None of the compounds tested was able to induce TNF release from macrophages. To evaluate the suppressive effects of these flavonoids on lipopolysaccharide-induced TNF production, compounds were administered at a dose of 1 mg/mouse 60 min before an i.v. injection with 0.1 μg *S. abortusequi* lipopolysaccharide (see Fig. 1 for details). As listed in Table 1, all samples showed significant suppressive activity. Among them, naringin exhibited the strongest activity.

3.2. Protection from lipopolysaccharide lethality in D-galactosamine-sensitized mice by naringin

Administration of D-galactosamine induces hypersensitive conditions to lethal toxicity of lipopolysaccharide as well as TNF-α (Galanos et al., 1979; Lehmann et al., 1987; Freudenberg and Galanos, 1991). Lethal activity of

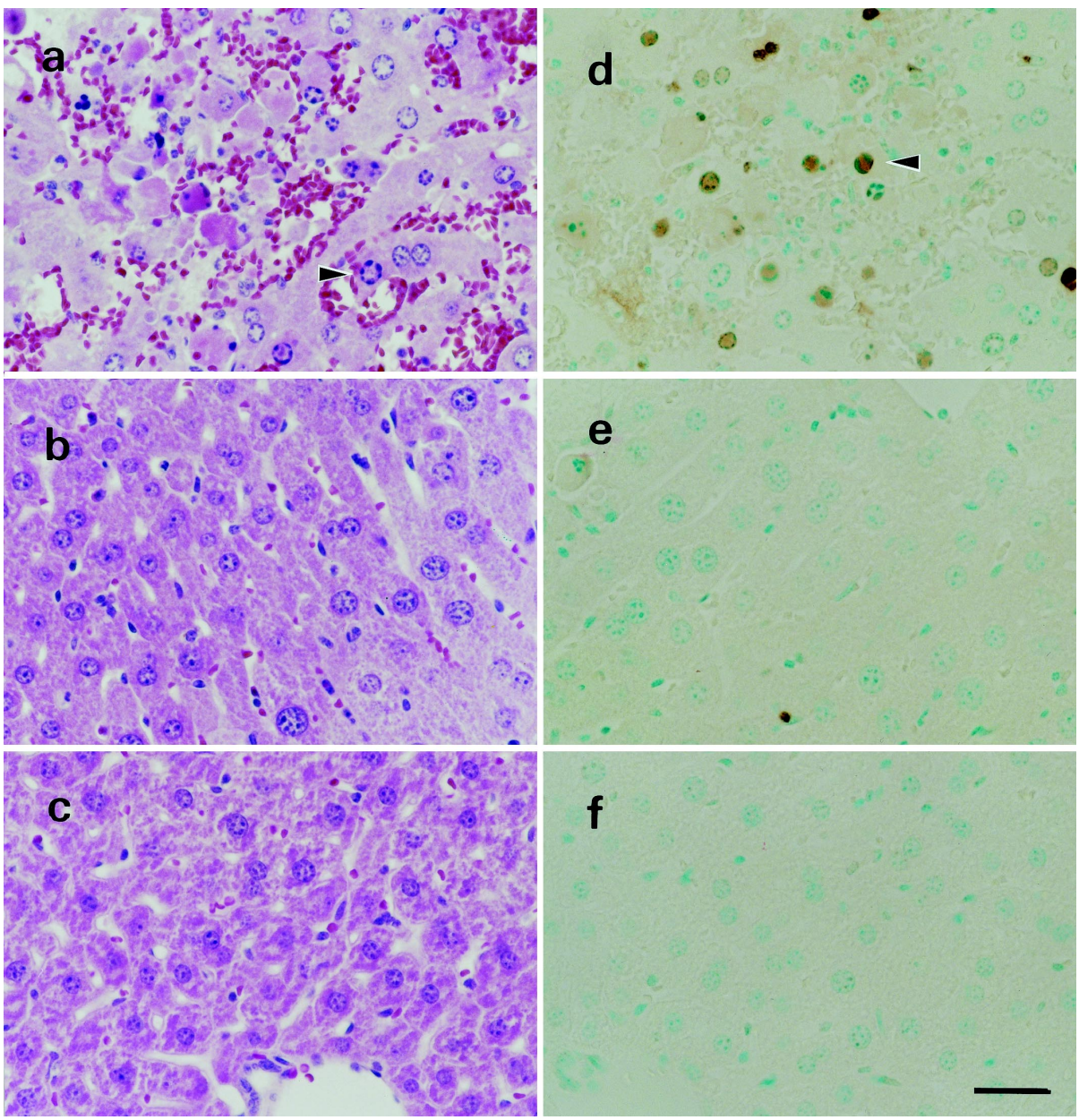

Fig. 3. Histological changes in liver of lipopolysaccharide/D-galactosamine-treated mice. Three hours after treatment with naringin or saline (positive controls), B10Sn mice were injected i.v. with 0.1 μg *S. abortusequi* lipopolysaccharide followed by an i.p. injection with 20 mg D-galactosamine. For negative controls, untreated B10Sn mice were used. Livers were removed from the mice 5 h after the challenge, fixed with 10% formalin and embedded in parafin. Successive sections were stained with either hematoxylin-eosin or the TUNEL method. Fig. 3a–c and d–f shows result of the hematoxylin-eosin staining and the TUNEL method, respectively. The arrowhead and the black bar in figures show apoptotic cells and the 30-μm length, respectively.

lipopolysaccharide developed via the action of TNF-α released by lipopolysaccharide-stimulated macrophages. Sensitization with D-galactosamine were employed to evaluate whether or not naringin showed protective activity against lipopolysaccharide lethal toxicity. Mice were treated at different doses with naringin 3 h before a challenge with 0.1 μg lipopolysaccharide followed by an i.p. injection with 20 mg D-galactosamine. As shown in Table 2, naringin did not show any protective activity at a dose of 0.3 mg/mouse, but at doses of 1 mg and 3 mg naringin markedly reduced the lethality to 17 and 0% levels, respectively. The strength of suppressive activity of naringin was compared with that of dexamethasone which was administered at various doses 10 min before the lipopolysaccharide challenge. The action of dexamethasone was weak at a dose of 10 μg, but at 100 μg/mouse, dexamethasone completely blocked lethality of lipopolysaccharide in D-galactosamine-sensitized mice. The activity of 3 mg naringin was comparable to that of 100 μg dexamethasone.

3.3. Suppressive effect of naringin on enzyme release responsible for lipopolysaccharide-induced hepatocyte injury

In order to evaluate whether or not naringin suppresses release of different enzymes from injured hepatocytes into the circulation, serum samples were collected from D-galactosamine-sensitized mice treated with or without 3 mg naringin 3 h before challenge with 0.1 μg lipopolysaccharide. As listed in Table 3, levels of aspartate aminotransferase, alanine aminotransferase and creatine kinase were dramatically increased in sera of D-galactosamine-sensitized mice 5 h after lipopolysaccharide challenge. Treatment with naringin before lipopolysaccharide challenge resulted in marked reduction of enzyme levels, suggesting that naringin inhibits injury of hepatocytes mediated by TNF.

Fig. 2 shows timing of naringin administration for inducing suppression of TNF release.

3.4. Naringin reduces histological change of liver induced by lipopolysaccharide

To histologically demonstrate inhibition of lipopolysaccharide-induced liver injury by naringin, liver specimen removed from mice 5 h after lipopolysaccharide/D-galactosamine challenge were fixed with 10% formalin and stained with hematoxylin and eosin. As shown in Fig. 3a–c, positive controls which received the D-galactosamine plus lipopolysaccharide challenge showed massive cell infiltration in wide area of liver and appearance of apoptotic cells (Fig. 3a). On the other hand, livers of mice pretreated with naringin (Fig. 3b) resemble to those of untreated controls (Fig. 3c). To confirm apoptosis in liver cells, successive sections were stained using a detection kit for apoptotic cells based on the TUNEL method. As shown in Fig. 3d–f, the positive control showed the positive staining in wide area (Fig. 3d), while apoptotic cells were not apparent in the group treated with naringin (Fig. 3e).

4. Discussion

The present study is the first report that the citrus flavonoid naringin suppresses the release of the lipopolysaccharide-induced TNF-α and blocks the lethal shock in D-galactosamine-sensitized mice. Naringin which is known as the main ingredient of grapefruit juice accounts for the principal bitter taste. One of the pharmacological activities of grapefruit juice is the regulation of the human cytochrome P-450 isozyme (1A2) whose activity was evaluated using caffeine as a probe substrate (Fuhr et al., 1995). Although naringin was the candidate for the enzyme inhibitor, it has recently demonstrated that the most potent inhibitor in grapefruit juice was not primarily naringin but coumarin (Edward and Bernier, 1996). Naringin has the following activities: (1) gastro-protection against ethanol-induced mucosal injury in rat which appears to be mediated by the non-prostaglandin-dependent mechanism (Martin et al., 1994); (2) inhibition of lung metastasis after injection with B16F10 melanoma cell in mice (Menon et al., 1995); (3) protection from okadaic acid-induced inhibition of autophagy (Gordon et al., 1995); and (4) anti-mutagenicity (Calomme et al., 1996; So et al., 1996).

The inhibitory substance from cinnamon is hydrophobic, binds to lipid A and has an inhibitory effect in the chromogenic Limulus test (Azumi et al., 1997). Although naringin is hydrophobic, it is not inhibited in the chromogenic Limulus test after mixing together. Thus, the action of naringin would be mediated via interaction with target cells elicited with lipopolysaccharide. In addition, treatment with naringin inhibited nitric oxide synthesis in peritoneal macrophages stimulated in vitro with lipopolysaccharide (unpublished data). The mechanisms by which naringin inhibits macrophage functions are not yet unclear.

Acknowledgements

We would like to thank Dr. Roland Ryll for his helpful discussion, Dr. T. Nishikawa and Mr. A. Hasegawa, Kitasato Clinical Laboratories (Sumitomo Metal Bio-Sciences), as well as Miss K. Takayanagi, Medicinal Plant Garden, for their technical assistance. This work was supported in part by grant-in-aid for scientific research (The Japan Health Sciences Foundation, #5112-22).

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