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Naringenin has anti-inflammatory properties in macrophage and *ex vivo* human whole-blood models

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Background and Objective: Periodontitis is a chronic inflammatory disease of bacterial etiology, affecting tooth-supporting tissues. The host inflammatory response to periodontopathogens, notably the high and continuous production of cytokines, is considered a major factor causing the local tissue destruction observed in periodontitis. The aim of the present study was to investigate the effect of naringenin, a major flavanone in grapefruits and tomatoes, on the lipopolysaccharide-induced pro-inflammatory cytokine production by host cells, using two different models.

Material and methods: The effect of naringenin was characterized using macrophages stimulated with the lipopolysaccharide of either *Aggregatibacter actinomycetemcomitans* or *Escherichia coli* and using whole blood stimulated with *A. actinomycetemcomitans* lipopolysaccharide, in the presence or absence of naringenin. Lipopolysaccharide-induced interleukin-1β, interleukin-6, interleukin-8 and tumor necrosis factor-α production by macrophages and whole-blood samples treated with naringenin were evaluated using an enzyme-linked immunosorbent assay. Changes in the phosphorylation states of macrophage kinases induced by *A. actinomycetemcomitans* lipopolysaccharide and naringenin were characterized by immunoblot screening.

Results: Our results clearly indicated that naringenin is a potent inhibitor of the pro-inflammatory cytokine response induced by lipopolysaccharide in both macrophages and in whole blood. Naringenin markedly inhibited the phosphorylation on serines 63 and 73 of Jun proto-oncogene-encoded AP-1 transcription factor in lipopolysaccharide-stimulated macrophages.

Conclusion: The results from the present study suggest that naringenin holds promise as a therapeutic agent for treating inflammatory diseases such as periodontitis.

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Periodontitis is an inflammatory disease of tooth-supporting tissues. The disease is initiated by an overgrowth of specific gram-negative anaerobic bacteria that leads to gingival connective tissue destruction and irreversible alveolar bone resorption. The continuous high secretion of various cytokines, including interleukin-1β, interleukin-6, interleukin-8 and tumor necrosis factor-α, by host cells following stimulation with periodontopathogens and their products, is a critical determinant of periodontal tissue destruction (1,2). The lipopolysaccharides of gram-negative bacteria are potent inducers of pro-inflammatory mediators and can initiate a number of host-mediated destructive processes (3). In particular, the lipopolysaccharide of *Aggregatibacter actinomycetemcomitans*

(formerly *Actinobacillus actinomycetemcomitans*), a major etiological agent in localized aggressive periodontitis (4,5), is thought to be involved in processes leading to alveolar bone loss and connective tissue destruction (6,7). It is also one of the most potent inducers of inflammatory mediator production by macrophages (8).

Active compounds endowed with a capacity to modulate the host inflammatory response have received considerable attention as they may be potential new therapeutic agents for the treatment of periodontal diseases (9). Naringenin (4′,5,7-trihydroxyflavanone), a major flavanone in grapefruits and tomatoes, has a wide range of pharmacological properties, including anticancer, antimutagenic and antiatherogenic activities (10–12). It also exerts an anti-inflammatory effect by inhibiting nitric oxide and prostaglandin E_2 production (13,14).

While all types of blood cells are present in normal gingival connective tissue (15), an accumulation of inflammatory cells is observed during periodontitis (16), and the number of leukocytes migrating to the gingival sulcus and periodontal pockets increases during the progression of inflammation (17,18). Monocytes and macrophages, which are found in higher numbers in active periodontal lesions than in inactive sites (19), play an important role in the host inflammatory response to periodontopathogens (20). The aim of this study was to investigate the effect of naringenin on the production of inflammatory mediators (interleukin-1β, tumor necrosis factor-α, interleukin-6 and interleukin-8) by macrophages stimulated with *A. actinomycetemcomitans* and *Escherichia coli* lipopolysaccharide. In addition, the changes in phosphorylation state of macrophage intracellular kinases induced by *A. actinomycetemcomitans* lipopolysaccharide and naringenin were characterized by immunoblot screening. We also used an *ex vivo* human whole-blood model to investigate the effect of naringenin on the *A. actinomycetemcomitans* lipopolysaccharide-induced inflammatory response of a mixed leukocyte population from periodontitis patients. This model has the advantage of taking into consideration the interactions between different immune cell types that contribute to the inflammatory response. Moreover, whole blood represents a more realistic physiological environment for investigating the effect of compounds on the production of inflammatory mediators in response to lipopolysaccharide stimulation because cellular interactions are preserved in the presence of various plasma proteins (soluble CD14, lipopolysaccharide-binding proteins, hormones, soluble cytokine receptors, etc.).

Material and methods

Bacterial growth and lipopolysaccharide preparation

A. actinomycetemcomitans ATCC 29522 was grown in Todd–Hewitt broth supplemented with 1% yeast extract. The bacterial culture was incubated at 37°C under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) for 24 h. A lipopolysaccharide preparation was produced using the procedure described by Darveau & Hancock (21), which is based on protein digestion of a whole-cell extract by proteinase K and successive solubilization and precipitation steps. The lipopolysaccharide preparation was freeze dried and stored at −20°C. Contaminating protein in the lipopolysaccharide preparations was evaluated to be $< 0.005\%$ (w/w), using a protein assay kit (DC protein assay; Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin as the control. A standard lipopolysaccharide preparation from *E. coli* O55:B5 (Sigma-Aldrich Canada, Oakville, ON, Canada) was also used as a control.

Culture of monocytes and differentiation into macrophages

U937 cells (ATCC CRL-1593.2, human monoblastic leukemia cell line) were cultured at 37°C in a 5% CO_2 atmosphere in RPMI-1640 medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/mL of penicillin–streptomycin. Monocytes (2×10^5 cells/mL) were incubated for 48 h in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and containing 10 ng/mL of phorbol myristic acid (Sigma-Aldrich) to induce differentiation into adherent macrophage-like cells, as previously reported (22). Following the treatment with phorbol myristic acid, the RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum was replaced with fresh medium and differentiated cells were incubated for an additional 24 h prior to use. Adherent macrophages were suspended in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and then centrifuged at 200 *g* for 8 min. The cells were washed, suspended in RPMI supplemented with 1% heat-inactivated fetal bovine serum, seeded in six-well plates (2×10^6 cells/well/2 mL) and incubated at 37°C in a 5% CO_2 atmosphere for 2 h prior to the naringenin and lipopolysaccharide treatments.

Treatment of macrophages

A stock solution of naringenin, obtained from Sigma-Aldrich, was prepared in 95% ethanol. Various concentrations of naringenin (0, 5, 10, 25, or 50 μg/mL) were added to macrophages, which were then incubated at 37°C in 5% CO_2 for 2 h prior to adding the *A. actinomycetemcomitans* or *E. coli* lipopolysaccharide at a final concentration of 1 μg/mL. After 24 h of incubation (37°C in 5% CO_2), the culture medium supernatants were removed and stored at −20°C until used. Cells incubated in culture medium with or without naringenin, but not stimulated with lipopolysaccharide, were used as controls.

Determination of cytotoxicity

A 3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test, performed according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany), was used to test the cytotoxicity of naringenin and the lipopolysaccharide towards macrophages.

Whole-blood collection and stimulation

Samples of venous blood were collected from the antecubital vein of six periodontitis patients using the Vacutainer™ system and sterile endotoxin-free blood-collection tubes containing 150 IU of sodium heparin (Becton Dickinson, Franklin Lakes, NJ, USA). Informed consent was obtained from all the donors prior to the experiments. The protocol was approved by the ethics committee of Université Laval. The periodontitis patients were non-smokers and had clinically diseased gingiva with at least three periodontal pockets of ≥5 mm in depth. Patients did not receive any medication (antibiotics, anti-inflammatory agents) or periodontal therapy for 3 mo prior to sampling. Blood samples were taken before any treatment for periodontitis. Hematological analyses of whole-blood samples performed at the Centre Hospitalier de l'Université Laval (Quebec City, Canada) showed that all the subjects had normal leukocyte counts. Whole-blood samples were diluted 1:3 in RPMI-1640 medium (HyClone) and seeded in 4-mL aliquots in six-well plates. Various concentrations of naringenin (0, 5, 10, 25, or 50 μg/mL) were added to the blood samples, which were then incubated at 37°C in 5% CO_2 for 2 h prior to the addition of *A. actinomycetemcomitans* lipopolysaccharide at a final concentration of 1 μg/mL. Following 6 h of incubation at 37°C in a 5% CO_2 humidified atmosphere with occasional gentle shaking, the blood samples were centrifuged at 2000 ***g*** for 5 min. The supernatants were collected and stored at −20°C until used. Samples incubated in the absence of lipopolysaccharide were used as a control.

Determination of cytokine production

Commercial enzyme-linked immunosorbent assay kits (R & D Systems, Minneapolis, MN, USA) were used to quantify the interleukin-1β, tumor necrosis factor-α, interleukin-6 and interleukin-8 concentrations in the cell-free supernatants, according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader with the wavelength correction set at 550 nm. The sensitivities of the commercial enzyme-linked immunosorbent assay kits were 3.9 pg/mL for interleukin-1β, 15.6 pg/mL for tumor necrosis factor-α, 9.3 pg/mL for interleukin-6 and 31.2 pg/mL for interleukin-8.

Analysis of phosphorylation states of kinases

The phosphorylation states of selected protein kinases was determined by Kinexus Bioinformatics (Vancouver, BC, Canada) using the Kinetworks™ Phosphosite Screen 1.0 (KCPS-1.0). For the initial screening procedure, macrophages treated with 1 μg/mL of *A. actinomycetemcomitans* lipopolysaccharide for 1 h were compared with unstimulated control cells. For the second screening procedure, the macrophages were treated with 50 μg/mL of naringenin at 37°C in 5% CO_2 for 90 min and then stimulated with 1 μg/mL of lipopolysaccharide for 1 h. Macrophages stimulated with 1 μg/mL of lipopolysaccharide for 1 h in the absence of naringenin were used as a control. After the incubation period, cell lysates were prepared according to the manufacturer's protocol (Kinexus). In brief, the macrophages were washed twice with ice-cold phosphate-buffered saline and homogenized at 4°C in a buffer containing 20 mM 3-(N-Morpholino) propanesulfonic acid (MOPS) (pH 7.0), 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate (pH 7.2), 1 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 5 μM pepstatin A, 10 μM leupeptin and 0.5% (v/v) Nonidet P-40. Then, the macrophages were broken by sonication on ice and the cell lysates were centrifuged at 136,000 ***g*** for 30 min at 4°C in an LE-80K ultracentrifuge (Beckman, Mississauga, ON, Canada). The protein concentrations of the supernatant fractions were estimated using the Bradford assay (Bio-Rad) and adjusted to 2 mg/mL. KCPS-1.0 analyses of the supernatants were performed by Kinexus. The signals of the phosphorylated proteins were expressed as counts per minute normalized to correct for differences in the amount of protein. Differences greater than 25% were considered significant.

Statistical analysis

Differences between means were analyzed for statistical significance using the Student's *t*-test and were considered significant at $p < 0.05$.

Results

Effect of naringenin on lipopolysaccharide-induced pro-inflammatory cytokine production by macrophages

Macrophages were treated with naringenin prior to being stimulated with lipopolysaccharide to investigate the effect of naringenin on pro-inflammatory cytokine production. An MTT test revealed no obvious cytotoxic effects following treatment of the macrophages with 50 μg/mL of naringenin for 24 h, indicating that any decrease in inflammatory mediator production was probably not related to cell toxicity (data not shown).

The *A. actinomycetemcomitans* lipopolysaccharide-induced interleukin-1β response of the macrophages was significantly inhibited by naringenin at 10, 25 and 50 μg/mL (Fig. 1A), and the tumor necrosis factor-α and interleukin-8 responses were significantly reduced by 25 and 50 μg/mL of naringenin (Fig. 1B,D). Antagonist effects of naringenin on *A. actinomycetemcomitans* lipopolysaccharide-induced interleukin-6 release were also observed (Fig. 1C). At low concentrations (5 and 10 μg/mL), naringenin and *A. actinomycetemcomitans* lipopolysaccharide had a synergistic effect on interleukin-6 production. However, 25 and 50 μg/mL of naringenin caused a significant reduction in lipopolysaccharide-induced interleukin-6 secretion by macrophages. This concentration-dependent effect on interleukin-6 production was also observed with *E. coli* lipopolysaccharide-stimulated macro-

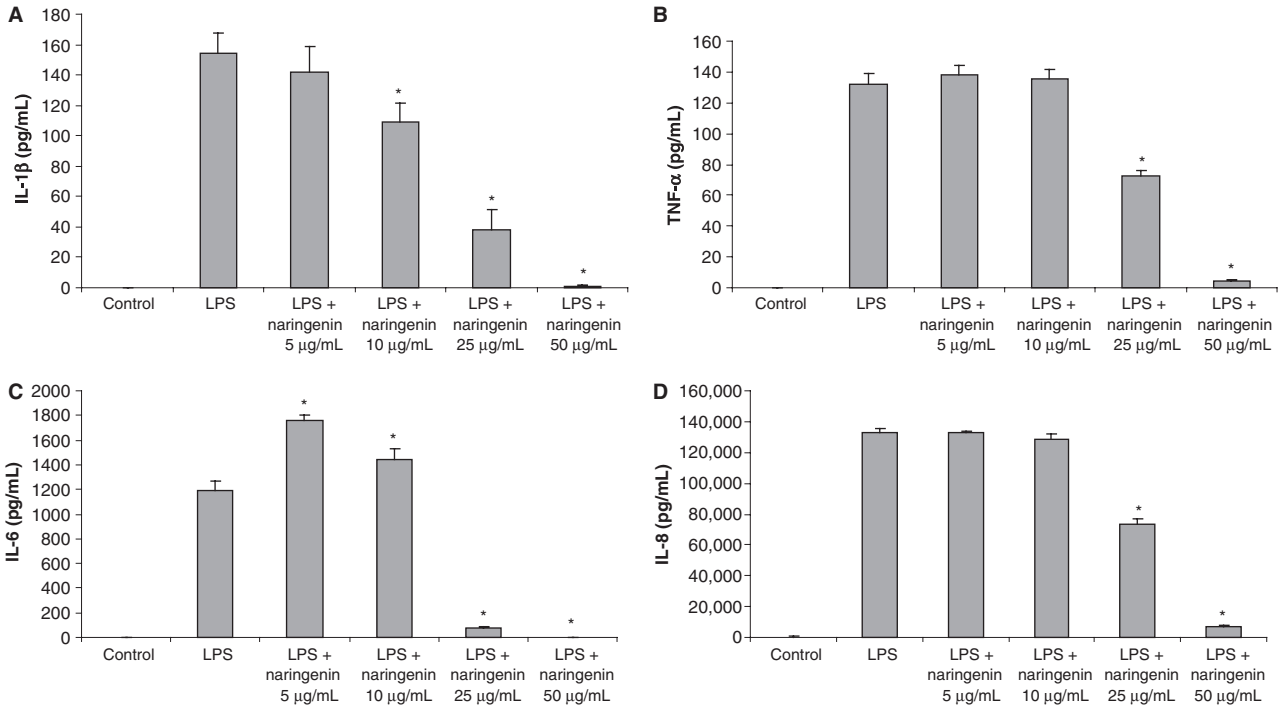

Fig. 1. Effect of treating macrophages with naringenin on the secretion of interleukin-1β (A), tumor necrosis factor-α (B), interleukin-6 (C) and interleukin-8 (D) induced by *Aggregatibacter actinomycetemcomitans* ATCC 29522 lipopolysaccharide (1 μg/mL) for 24 h. Macrophages were treated with naringenin (5, 10, 25 and 50 μg/mL) for 2 h prior to being stimulated with the lipopolysaccharide. Cytokine secretion was assessed by enzyme-linked immunosorbent assay. The data are the means ± standard deviations of triplicate assays for two independent experiments. *, $p < 0.05$ compared with the untreated control. IL, interleukin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

phages (Fig. 2C). *E. coli* lipopolysaccharide-induced interleukin-6 secretion was stimulated by 5 μg/mL of naringenin, whereas higher concentrations (25 and 50 μg/mL) of naringenin significantly reduced the amount of interleukin-6 secreted. *E. coli* lipopolysaccharide-induced stimulation of interleukin-1β, tumor necrosis factor-α and interleukin-8 secretion was also significantly inhibited by 10, 25 and 50 μg/mL of naringenin (Fig. 2A, B,D).

Changes in the phosphorylation states of macrophage signaling protein kinases, induced by *A. actinomycetemcomitans* lipopolysaccharide and naringenin, were characterized by immunoblot screening. *A. actinomycetemcomitans* lipopolysaccharide induced an increase in the phosphorylation of extracellular-regulated protein-serine kinase 5 (Erk5, +65%), mitogen-activated protein-serine kinase p38 alpha (p38a MAPK, +26%) and Jun proto-oncogene-encoded AP-1 transcription factor (Jun) (+2301% on serine 63 and +724% on serine 73) (Table 1). Pretreating macrophages with naringenin before stimulating them with *A. actinomycetemcomitans* lipopolysaccharide resulted in enhanced phosphorylation of Erk5 (+50%) and p38a MAPK (+205%). On the other hand, naringenin strongly inhibited the phosphorylation of Jun on serines 63 and 73 by 62% and 70%, respectively.

Effect of naringenin on the lipopolysaccharide-induced inflammatory response of the *ex vivo* whole-blood model

The ability of naringenin to reduce the production of pro-inflammatory cytokines was further investigated using whole-blood samples from periodontitis subjects. *A. actinomycetemcomitans* lipopolysaccharide induced the secretion of tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8 by the mixed leukocyte population in the whole blood from all six periodontitis subjects (Table 2). The tumor necrosis factor-α and interleukin-1β responses induced by *A. actinomycetemcomitans* lipopolysaccharide were strongly reduced by naringenin in all subjects (Table 2). Lipopolysaccharide-induced interleukin-1β secretion in the samples from all patients, and lipopolysaccharide-induced tumor necrosis factor-α secretion in the samples from patients 3, 4 and 5, were significantly inhibited by 5 μg/mL of naringenin, whereas the lipopolysaccharide-induced tumor necrosis factor-α secretion in the samples from patients 1, 2 and 6 was inhibited by 10 μg/mL. Lipopolysaccharide-induced interleukin-6 secretion in the sample from patient 4 was significantly inhibited by 10 μg/mL of naringenin, whereas 50 μg/mL was required to cause a significant effect in the samples from patients 1, 5 and 6. Lipopolysaccharide-induced interleukin-8 secretion was significantly inhibited in the samples from patients 4, 5 and 6 at naringenin concentrations of 5, 25 and 50 μg/mL, respectively.

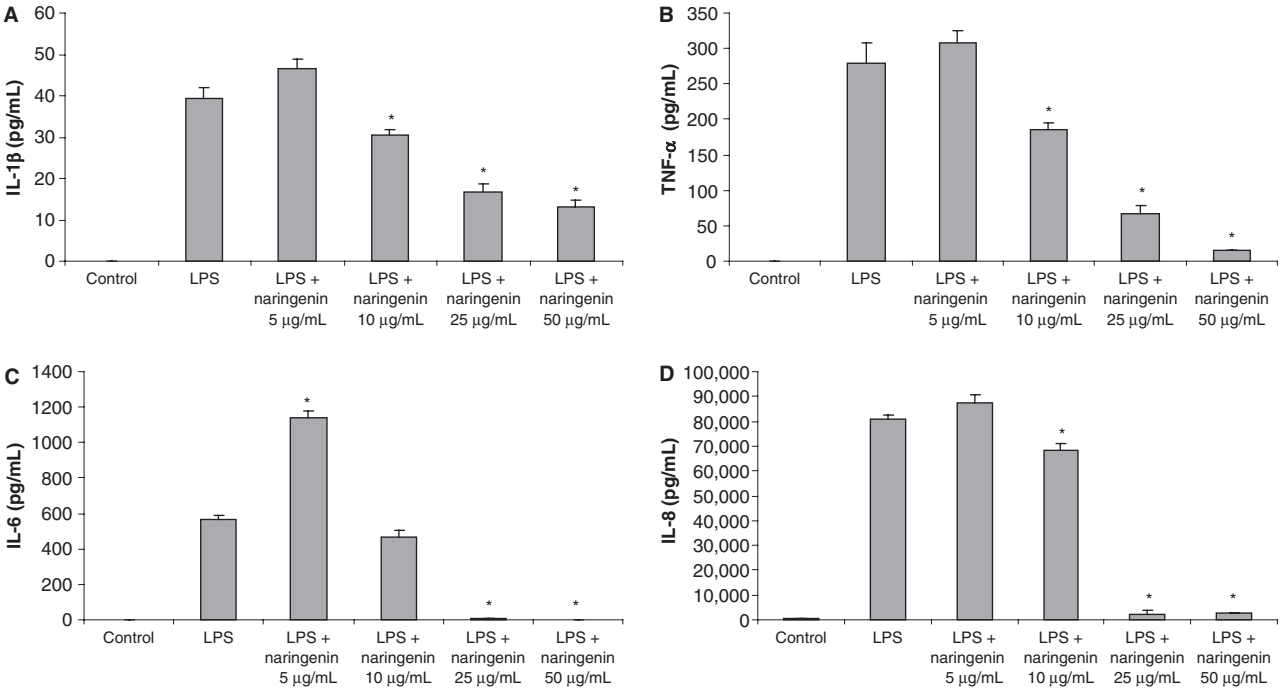

Fig. 2. Effect of treating macrophages with naringenin on the secretion of interleukin-1β (A), tumor necrosis factor-α (B), interleukin-6 (C) and interleukin-8 (D) induced by *Escherichia coli* O55:B5 lipopolysaccharide (1 μg/mL) for 24 h. The macrophages were treated with naringenin (5, 10, 25 and 50 μg/mL) for 2 h prior to being stimulated by the lipopolysaccharide. Cytokine secretion was assessed by enzyme-linked immunosorbent assay. The data are the means ± standard deviations of triplicate assays for two independent experiments. *, $p < 0.05$ compared with the untreated control. IL, interleukin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

Table 1. Changes induced by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide and naringenin in the phosphorylation states of macrophage protein kinases

Protein				Signal (% change from control)	
Full name	Abbreviation	Epitope	MW	Unstimulated vs. LPS	LPS vs. LPS + naringenin
Extracellular regulated protein-serine kinase 5 (Big MAP kinase 1)	Erk5	T218 + Y220	108	+65	+50
Jun proto-oncogene-encoded AP-1 transcription factor	Jun	S63	39	+2301	−62
Jun proto-oncogene-encoded AP-1 transcription factor	Jun	S73	39	+724	−70
Mitogen-activated protein-serine kinase p38 alpha	P38a MAPK	T180 + Y182s	39	+26	+205

Kinexus phosphosite kinase screen (KCPS-1.0) was used to detect these changes. First, unstimulated control macrophages were compared to macrophages stimulated with *A. actinomycetemcomitans* lipopolysaccharide (1 μg/mL) for 1 h; and, second, control macrophages stimulated with *A. actinomycetemcomitans* lipopolysaccharide (1 μg/mL) for 1 h were compared to macrophages treated with naringenin (50 μg/mL) for 90 min prior to being stimulated with *A. actinomycetemcomitans* lipopolysaccharide (1 μg/mL) for 1 h.
LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MW, molecular weight (kDa).

Discussion

The host inflammatory response to periodontopathogens, notably the excessive production of cytokines, is considered a major factor contributing to the local tissue destruction observed in periodontitis. Consequently, therapeutic approaches that inhibit cytokine production are receiving increasing attention as an option in managing chronic periodontitis. We used two different models – *in vitro* cultured macrophages and an *ex vivo* whole-blood model – to investigate the capacity of naringenin to inhibit the lipopolysaccharide-induced inflammatory response.

Pro-inflammatory cytokines, such as interleukin-1β, tumor necrosis factor-α and interleukin-6, are thought to be involved in the initiation and amplification of the inflammatory process. They contribute to the pathophysiology

Table 2. Effect of naringenin on the *Aggregatibacter actinomycetemcomitans* lipopolysaccharide-induced secretion of interleukin-1β, tumor necrosis factor-α, interleukin-6 and interleukin-8 in human whole blood from six periodontitis patients

Conditions of stimulation	Amount of cytokine secreted (pg/mL)			
	TNF-α	IL-1β	IL-6	IL-8
Patient 1				
Control	ND	ND	ND	ND
LPS	2656 ± 132	696 ± 23	8141 ± 531	5550 ± 229
LPS + naringenin 5 μg/mL	2260 ± 191	579 ± 19*	8345 ± 209	5329 ± 363
LPS + naringenin 10 μg/mL	2314 ± 77*	563 ± 17*	7685 ± 231	5295 ± 293
LPS + naringenin 25 μg/mL	1542 ± 81*	389 ± 11*	7558 ± 419	5530 ± 372
LPS + naringenin 50 μg/mL	1086 ± 57*	132 ± 7*	6593 ± 302*	5378 ± 232
Patient 2				
Control	ND	ND	ND	ND
LPS	3471 ± 141	169 ± 11	6593 ± 315	2896 ± 247
LPS + naringenin 5 μg/mL	3195 ± 123	115 ± 5*	6438 ± 159	2548 ± 264
LPS + naringenin 10 μg/mL	2412 ± 86*	86 ± 8*	6238 ± 195	2323 ± 196
LPS + naringenin 25 μg/mL	2102 ± 43*	84 ± 3*	6337 ± 250	2312 ± 244
LPS + naringenin 50 μg/mL	934 ± 21*	13 ± 1*	5927 ± 283	2262 ± 295
Patient 3				
Control	ND	ND	ND	ND
LPS	4870 ± 162	126 ± 12	9410 ± 456	4475 ± 273
LPS + naringenin 5 μg/mL	3577 ± 112*	82 ± 6*	9592 ± 336	4467 ± 178
LPS + naringenin 10 μg/mL	3166 ± 108*	76 ± 8*	9405 ± 195	4634 ± 109
LPS + naringenin 25 μg/mL	2287 ± 55*	72 ± 5*	9108 ± 287	4366 ± 297
LPS + naringenin 50 μg/mL	1750 ± 32*	33 ± 2*	9060 ± 230	3994 ± 260
Patient 4				
Control	ND	ND	ND	ND
LPS	4274 ± 183	317 ± 18	7886 ± 304	1759 ± 162
LPS + naringenin 5 μg/mL	3035 ± 128*	94 ± 8*	7512 ± 315	1103 ± 64*
LPS + naringenin 10 μg/mL	2858 ± 114*	78 ± 4*	6928 ± 156*	1031 ± 56*
LPS + naringenin 25 μg/mL	2849 ± 89*	67 ± 9*	6941 ± 208*	991 ± 103*
LPS + naringenin 50 μg/mL	1025 ± 41*	12 ± 1*	6744 ± 202*	757 ± 54*
Patient 5				
Control	ND	ND	ND	ND
LPS	3452 ± 261	431 ± 26	7261 ± 247	3900 ± 151
LPS + naringenin 5 μg/mL	2565 ± 139*	186 ± 12*	6784 ± 319	4200 ± 116
LPS + naringenin 10 μg/mL	2349 ± 166*	115 ± 17*	6554 ± 432	3694 ± 290
LPS + naringenin 25 μg/mL	1912 ± 64*	62 ± 5*	6607 ± 360	3069 ± 106*
LPS + naringenin 50 μg/mL	480 ± 18*	ND*	6002 ± 282*	2655 ± 66*
Patient 6				
Control	ND	ND	ND	ND
LPS	3328 ± 191	799 ± 31	8016 ± 255	777 ± 52
LPS + naringenin 5 μg/mL	2960 ± 170	469 ± 27*	8071 ± 151	744 ± 32
LPS + naringenin 10 μg/mL	1835 ± 85*	369 ± 15*	7658 ± 325	785 ± 11
LPS + naringenin 25 μg/mL	1742 ± 67*	282 ± 14*	7535 ± 285	713 ± 78
LPS + naringenin 50 μg/mL	1354 ± 34*	199 ± 13*	7043 ± 247*	427 ± 42*

Whole-blood samples were treated with naringenin (5, 10, 25 and 50 μg/mL) for 2 h prior to being stimulated with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide (1 μg/mL) for 6 h. The cytokine concentrations in the supernatants of the whole-blood cultures were assessed by enzyme-linked immunosorbent assay. Control whole-blood samples were incubated without lipopolysaccharide. The data are the means ± SD of triplicate assays.
*, $p < 0.05$ compared with the lipopolysaccharide control.
IL, interleukin; LPS, lipopolysaccharide; ND, not detected (below the detection level); TNF-α, tumor necrosis factor-α.

of many inflammatory diseases, including rheumatoid arthritis, atherosclerosis and periodontitis. More specifically, interleukin-1β and tumor necrosis factor-α play an active role in the progression of periodontitis by inducing the production of adhesion molecules and other mediators that facilitate and amplify the inflammatory response, stimulating matrix metalloproteinases and enhancing bone resorption (23). In the present study, naringenin exhibited a strong potential to reduce the production of interleukin-1β and tumor necrosis factor-α by macrophages. This is consistent with a previous observation that naringenin inhibits tumor necrosis factor-α production by lipopolysaccharide-stimulated mouse macrophages (24). On the contrary, Hougee *et al*. (25) failed to demonstrate an anti-inflammatory property of naringenin using human peripheral blood mononuclear cells. This may be related to the model used, or more likely to the fact that they used a concentration of naringenin much lower than the one used in the present study.

Interleukin-6, a multifunctional cytokine that plays an important role in regulating the immune response and bone resorption during periodontal disease, was inhibited by 25 μg/mL of naringenin. This suggests that naringenin may contribute to reducing the impact of host-destructive processes mediated by these cytokines. Interleukin-8 is a potent chemokine that directs the migration of polymorphonuclear leukocytes, monocytes and macrophages to sites of inflammation. Higher levels of interleukin-8 are found in the gingival crevicular fluid of inflamed periodontal sites than of healthy sites (26). Periodontal therapy reduces immune cell numbers and the levels of interleukin-8 in infiltrates, suggesting that there is a relationship between this chemokine and periodontal status (27). In our study, naringenin reduced lipopolysaccharide-induced interleukin-8 production by macrophages, indicating that naringenin may help to reduce the influx of inflammatory cells into disease sites.

The effect of naringenin on the *A. actinomycetemcomitans* lipopolysaccharide-induced inflammatory response was also studied using whole-blood samples from six periodontitis patients. The *ex vivo* whole-blood model contains all the relevant cell populations that are likely to come into contact with periodontopathogens during the progression of periodontal disease and also takes into consideration the complex cell–cell interactions that occur *in vivo*. This model more closely recreates the *in vivo* situation

because gingival crevicular fluid, which bathes the periodontal pocket, is derived from gingival capillary beds and contains resident and emigrating inflammatory cells. This model is thus relevant for studying inflammatory reactions elicited by periodontopathogens (28,29). In the present study, naringenin exhibited a strong capacity to inhibit the lipopolysaccharide-induced production of interleukin-1β and tumor necrosis factor-α by a mixed leukocyte population in whole-blood samples from periodontitis patients. Those data indicated that the anti-inflammatory property of naringenin is still active in a complex environment such as blood. Interleukin-1β is a potential marker of the progression and severity of periodontitis, as well as an indicator of an appropriate response to treatment (30). It has been reported that inhibitors of tumor necrosis factor-α synthesis can reduce bone resorption in experimental periodontitis in rats (31). Moreover, local inhibition of both interleukin-1 and tumor necrosis factor-α production in periodontal tissues significantly inhibits the inflammatory response and bone loss in ligature-induced periodontitis in monkeys (32). This suggests that local inhibition of these cytokines may be a successful approach for inhibiting bone resorption in periodontitis. Naringenin may thus be a potential therapeutic compound that could be used to inhibit the periodontopathogen-induced interleukin-1β and tumor necrosis factor-α responses of leukocytes in periodontitis patients. Naringenin did not inhibit the production of interleukin-6 and interleukin-8 in all patients, reflecting an interindividual variability of its anti-inflammatory effectiveness against these cytokines. At low concentrations, naringenin enhanced interleukin-6 production by lipopolysaccharide-stimulated macrophages, whereas this effect was not observed in whole-blood samples stimulated by lipopolysaccharide, indicating that it does not stimulate interleukin-6 production in a mixed leukocyte population.

Immunoblot analyses showed that naringenin modulates the phosphorylation states of some macrophage intracellular signaling proteins that are induced by *A. actinomycetemcomitans* lipopolysaccharide. AP-1 complexes are heterodimers of proteins from the Jun and Fos proto-oncogene families that are involved in the transcriptional regulation of many pro-inflammatory mediators such as interleukin-1β, tumor necrosis factor-α, interleukin-6 and interleukin-8 (33–35). *A. actinomycetemcomitans* lipopolysaccharide induced the phosphorylation of Jun on serines 63 and 73, suggesting that it enhances AP-1 activity, because the transcriptional activity and protein stability of Jun is enhanced by the phosphorylation of serines 63 and 73 (36,37). In the present study, naringenin significantly reduced the phosphorylation of Jun on serines 63 and 73, indicating that naringenin may inhibit the transcription of inflammatory cytokine genes. On the other hand, naringenin increased the phosphorylation of Erk5 and p38a MAPK in lipopolysaccharide-stimulated macrophages. This is consistent with a previous study which reported that naringenin increases the phosphorylation of p38 MAPK in hepatocytes (38). The increase in Erk5 phosphorylation is further proof of the anti-inflammatory effect of naringenin because ERK5 activation has been reported to inhibit inflammatory responses (39).

Many pathological states of inflammatory diseases appear to be linked to an imbalance in the cytokine network and to an excessive recruitment of leukocytes to sites of inflammation (2,40). Given that naringenin significantly inhibited several inflammatory cytokines produced by lipopolysaccharide-stimulated macrophages and produced in whole-blood samples from periodontitis patients, it holds promise as a therapeutic agent for treating inflammatory diseases such as periodontitis. Further studies are required to investigate the effect of local application of naringenin as an adjunctive treatment to conventional therapy for periodontitis patients.

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