The citrus flavonone naringenin reduces lipopolysaccharide-induced inflammatory pain and leukocyte recruitment by inhibiting NF-κB activation⁎

Felipe A. Pinho-Ribeiroa, Ana C. Zarpelona, Sandra S. Mizokamia, Sergio M. Borghi, Rangel L. Silva, Thiago M. Cunha, Jose C. Alves-Filho, Fernando Q. Cunha, Rubia Casagranded, Waldiceu A. Verri Jr. a, b, c, d, e

⁎Corresponding author. Pathology Department, State University of Londrina, Rod. Celso Garcia Cid Km 380, PR445, 86057-970, Londrina, Paraná, Brazil
Corresponding author. Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, Avenida Bandeirantes, 14050–490 Ribeirão Preto, São Paulo, Brazil
Corresponding author. Pathology Department, State University of Londrina, Rod. Celso Garcia Cid Km 380, PR445, 86057-970, Londrina, Paraná, Brazil
Corresponding author. Department of Pharmaceutical Sciences, Centro de Ciências da Saúde, Universidade Estadual de Londrina, Avenida Robert Koch, 60, Hospital Universitário, 86038–350 Londrina, Paraná, Brazil
Corresponding author. Pathology Department, State University of Londrina, Rod. Celso Garcia Cid Km 380, PR445, 86057-970, Londrina, Paraná, Brazil

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Research Articles

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Lipopolysaccharide (LPS) is the major structural component of Gram-negative bacteria cell wall and a highly pro-inflammatory toxin. Naringenin is found in Citrus fruits and exhibits antioxidant and anti-inflammatory properties through inhibition of NF-κB activation but its effects in LPS-induced inflammatory pain and leukocyte recruitment were not investigated yet. We investigated the effects of naringenin in mechanical hyperalgesia, thermal hyperalgesia and leukocyte recruitment induced by intraplantar injection of LPS in mice. We found that naringenin reduced hyperalgesia to mechanical and thermal stimuli, and leukocyte recruitment were not investigated yet. We investigated the effects of naringenin in mechanical hyperalgesia, thermal hyperalgesia and leukocyte recruitment.

1. Introduction

Naringenin (NGN; 4′,5,7-trihydroxyflavanone) is a flavonone that occurs naturally in vegetables, fruits, herbs and nuts widely consumed by humans, including Lycopersicum esculentum (tomato) [1], Citrus paradise (grapefruit) [2], Citrus sinensis (orange) [3,4], Lippia graveolens (mexican oregano) [5] and nuts like pistachio [6]. An increasing number of pre-clinical studies suggest that NGN presents great potential to treat a wide range of pathological conditions that are related to inflammation and oxidative stress, including ultraviolet B-induced skin damage [7], daunorubicin-induced nephrotoxicity [8], diabetic neuropathy [9], and neuropathic pain [10,11]. Its anti-inflammatory activity is of great interest due to the role of inflammatory mediators in diseases that significantly reduce patients’ quality of life, mainly because of inflammatory pain.

Pain is a clinical sign of inflammation and an important complaint of patients with infectious diseases [12–14]. Lipopolysaccharide (LPS), the major constituent of gram-negative bacterial cell walls, is released in large amounts during infection by gram-negative bacteria due to bacteriolytic activity of mediators produced by the immune system. Neutrophils represent important sources of these and other mediators that, despite killing of bacteria, increase the release of LPS that can lead to additional postinfectious sequelae or even to endotoxic shock [15]. Additionally, LPS and inflammatory mediators act synergistically to increase pain sensitivity [16–18]. Therefore, targeting the effects of LPS is of major clinical importance. NGN inhibits the synthesis of inflammatory mediators by macrophages infected with a gram-negative bacteria [19] and kills gram-positive bacteria [20], but the efficacy of naringenin in reducing LPS-induced pain and leukocyte recruitment were not described previously. Therefore, we aimed to evaluate the effects of NGN in LPS-induced inflammatory pain, leukocyte recruitment in mice and its mechanisms of action.
2. Materials and methods

2.1. General In Vivo experimental procedures

Mice were treated by oral (p.o.) gavage with naringenin (NGN; 16.7, 50, or 150 mg/kg, 100 μL) or vehicle (sterile saline, 100 μL) 1 h before LPS injection. Hyperalgesia to mechanical and thermal stimuli was evaluated 1–5 h after intraplantar (i.pl.) injection with 100 ng of LPS. Neutrophil and macrophage recruitment to the plantar tissue was evaluated 5 h after LPS injection by myeloperoxidase (MPO) and by N-acetyl-β-D-glucosaminidase (NAG) assays, respectively. Oxidative stress, cytokine production, and NF-κB activity in paw skin tissue were determined 3 h (peak of hyperalgesia) after LPS i.pl. injection. In another approach, LPS (100 ng) was administered by intraperitoneal (i.p.) injection. Leukocyte migration to the peritoneal cavity, oxidative stress, and cytokine levels were evaluated 4 h after LPS i.p. injection.

2.2. Test compounds

The compounds used in this study were saline (NaCl 0.9%; Fresenius Kabi Brasil Ltda. Aquiraz, CE, Brazil), naringenin (≥95% purity, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and LPS (lipopolysaccharides from E. coli, Santa Cruz Biotechnology, Santa Cruz, CA, United States). Immediately before the p.o. administration, naringenin was diluted in saline solution.

2.3. Animals

Male Swiss mice (25–30 g) from the Universidade Estadual de Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12–12 h at 21 °C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled (21 °C) room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 32.186.2012.64). All efforts were made to minimize animals’ suffering and to reduce the number of animals used.

2.4. Mechanical hyperalgesia test

Mechanical hyperalgesia was measured by an electronic version of von Frey filaments [21]. The test consisted of evoking a hind paw reflex with a hand-held force transducer (electronic anesthetizer; Insight Equipamentos, Ribeirão Preto, SP, Brazil). After paw withdrawal, the pressure intensity was recorded automatically, and the values were an average of three measurements. Mice were tested before (basal) and after stimulus injection. The results are expressed as delta (Δ) withdrawal threshold (in g), calculated by subtracting the mean measurements obtained at 1, 3, or 5 h after i.pl. stimulus with LPS from the basal mean measurements.

2.5. Hot plate test

Mice were placed on a hot plate apparatus (Hot Plate HP-2002, Insight Equipamentos, Ribeirão Preto, SP, Brazil) maintained at 55 °C. The paw withdrawal reaction was registered. A maximum latency (cut-off) was set at 20 s to avoid tissue damage [22,23].

2.6. MPO activity

The neutrophil recruitment to the paw tissue was evaluated by the MPO kinetic-colorimetric assay [24,25]. Samples were homogenized using a tissue-tearor (Biospec®) in ice-cold K2HPO4 buffer (400 μL, 50 mM, pH 6.0) containing HTAB (0.5% weight/ volume), and the homogenates were centrifuged (16,100 g; 2 min; 4 °C). The supernatants (30 μL) were mixed with K2HPO4 buffer (200 μL, 50 mM, pH 6.0) containing o-dianisidine dihydrochloride (0.0167 M; w/v) and hydrogen peroxide (0.015%, v/v). The absorbance was determined after 5 min at 450 nm (Multiskan GO, Thermo Scientific, Vantaa, Finland). The results of MPO activity are expressed as the number of neutrophils per mg of tissue by using a standard curve of neutrophils (196–400,000 cells).

2.7. NAG activity

NAG (N-acetyl-β-D-glucosaminidase) activity was determined by an adapted colorimetric method previously described [26]. Briefly, the supernatants (20 μL) obtained in the MPO activity assay, were placed in a 96-well plate and mixed with K2HPO4 buffer (80 μL, 50 mM, pH 6.0). The reaction was initiated by the addition of K2HPO4 buffer (100 μL, 50 mM, pH 6.0) containing 4-nitrophenyl N-acetyl-β-D-glucosaminide substrate (2.24 mM). The plate was incubated at 37 °C for 10 min, and glycine buffer (100 μL, 0.2 M pH 10.6) was added. The enzymatic activity was determined spectrophotometrically at 400 nm (Multiskan GO Thermo Scientific). The results of NAG activity are expressed as the number of macrophages per mg of tissue by using a standard curve of macrophages (196–400,000 cells).

2.8. ABTS and FRAP assays

The ability of samples to resist oxidative damage was determined by its free radical scavenging (ABTS [2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay] and ferric reducing (FRAP assay) properties. The tests were adapted to a 96-well microplate format as previously described [26]. Plantar tissue samples were collected 3 h after i.pl. stimulus with LPS and homogenized immediately in ice-cold KCl buffer (500 μL, 1.15% w/v). The absorbance of ABTS and FRAP assays were measured at 730 and 595 nm (Multiskan GO Thermo Scientific), respectively, and the results were equated against a standard Trolox curve (0.02–20 nmol).
of relative luminescence units (RLU). LDH leakage was measured as described previously [20,30] using supernatants to determine cytotoxicity by Cytotoxicity Detection KitPLUS (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s directions.

2.13. Data analyses

Results are means ± SEM of 6 mice per group per experiment, each experiment was performed twice. In the in vitro experiments are means ± SEM of three independent experiments performed in triplicate. Two-way ANOVA was used to compare the groups and doses at all times when the parameters were measured at different times after the stimulus injection. The analyzed factors were treatments, time and time versus treatment interaction. One-way ANOVA followed by Tukey’s test was performed for each time-point. P < 0.05 was considered significant.

3. Results

3.1. Naringenin (NGN) inhibits LPS-induced inflammatory pain

Mice were treated with NGN (16.7–150 mg/kg, p.o.) 1 h before i.pl. injection with LPS. Treatment with NGN in the doses of 50 and 150 mg/kg inhibited hyperalgesia induced by LPS to mechanical (Fig. 1A) and thermal stimuli (Fig. 1B). No effects were observed at the dose of 16.7 mg/kg of NGN. LPS also induced neutrophil (MPO activity) (Fig. 1C) and macrophage (MPO and NAG activity) (Fig. 1D) recruitment in the paw skin, while NGN inhibited these effects (Fig. 1C–1D) at the doses of 50 and 150 mg/kg with no statistically significant differences between these two doses. Thus, we chose to use the dose of 50 mg/kg was in the following experiments.

3.2. NGN inhibits oxidative stress induced by LPS

Neutrophils and macrophages are important sources of reactive oxygen species that contribute to inflammatory pain [31–36]. Therefore, we next evaluated the antioxidant status of paw skin tissue 3 h after LPS i.pl. stimulus and the effects of NGN treatment (50 mg/kg, p.o., 1 h before) in this system. LPS reduced ABTS radical scavenging and the ferric reducing (FRAP) abilities of paw skin tissue (Fig. 2A–B). Furthermore, treatment with NGN inhibited LPS-induced oxidative stress (Fig. 2A–B).

3.3. NGN reduces LPS-induced cytokine production in the paw skin

Mice received NGN (50 mg/kg, p.o., 1 h before LPS), and paw skin concentrations of pro-inflammatori/hyperalgesic cytokines TNF-α, IL-1β, IL-6, and IL-12 were determined 3 h after LPS i.pl. injection. LPS injection induced the production of all evaluated cytokines (Fig. 3). On the other hand, NGN treatment (50 mg/kg) reduced significantly the LPS-induced production of TNF-α, IL-1β, IL-6, and IL-12 (Fig. 3).

3.4. NGN reduces leukocyte recruitment, superoxide anion production, lipid peroxidation, and cytokine production induced by LPS in the peritoneal cavity

Mice were treated with NGN (16.7–150 mg/kg, p.o.) 1 h before peritonitis induced by intraperitoneal (i.p.) injection of LPS (100 ng). Peritoneal lavage fluid was harvested 5 h after LPS injection. LPS induced leukocyte recruitment into the peritoneal cavity (Fig. 4A–C). Treatment with naringenin reduced total leukocyte (Fig. 4A), neutrophil (Fig. 4B) and mononuclear cell (Fig. 4C) recruitment to the peritoneal cavity at the doses of 50 and 100 mg/kg. Furthermore, NGN abrogated LPS-induced increases in MPO (Fig. 4D) and NAG (Fig. 4E) activities, which lines up well with the results obtained in the paw skin (Fig. 1). Treatment with NGN (50 mg/kg) inhibited LPS-induced superoxide anion production (NBT assay) (Fig. 5A) and malondialdehyde (MDA) formation, a product of lipid peroxidation (Fig. 5B). Moreover, LPS injection increased TNF-α, IL-1β, IL-6, and IL-12 concentration in the

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**Fig. 3. Naringenin (NGN) inhibits LPS-induced production of hyperalgesic cytokines in paw skin tissue.** Samples of the paw skin tissue were collected 3 h after LPS injection and the levels of TNF-α (A), IL-1β (B), IL-6 (C), and IL-12 (D) were determined by ELISA. Results are means ± SEM, n = 6 mice per group per experiment, two independent experiments [P < 0.05 vs. saline control; #P < 0.05 vs. 0 mg/kg (vehicle)]. (ANOVA followed by Tukey’s test).}
peritoneal cavity, and NGN (50 mg/kg) treatment lowered the concentration of all these cytokines (Fig. 6). These data corroborate that NGN modulates immune responses by inhibiting leukocyte activation and recruitment.

3.5. NGN reduces NF-κB activation, cytokine production, and superoxide anion production by RAW 264.7 macrophages stimulated with LPS

LPS induces the expression of pro-inflammatory cytokines in a NF-κB-dependent manner. Considering this, we hypothesized that NGN may affect NF-κB signaling. The effects of NGN were evaluated in LPS-stimulated RAW 264.7 macrophages stably expressing luciferase under a NF-κB responsive promoter. Cells were pre-treated with NGN (30–1000 nM) 1 h before LPS stimulus (1 μg/mL). Supernatants were collected 6 h after LPS stimulus and cell lysates were used to evaluate NF-κB activation by a luciferase activity assay (Fig. 7A). Additionally, the production of superoxide anion (Fig. 7B) and cytokines (Fig. 7C-F), as well as LDH concentration/cell viability (Fig. 7G) were also evaluated. LPS-induced NF-κB activation was reduced by NGN treatment at 300 and 1000 nM (Fig. 7A). Moreover, NGN reduced LPS-induced superoxide anions (Fig. 7B), TNF-α (Fig. 7C), IL-1β (Fig. 7D), IL-6 (Fig. 7E), and IL-12 secretion (Fig. 7F). These inhibitory effects showed a similar profile as for NF-κB activation inhibition (Fig. 7A), except that NGN inhibited LPS-induced superoxide anion production only at the concentration of 1000 nM (Fig. 7B) and the concentrations of 30 and 100 nM also inhibited IL-12 production (Fig. 7F). No cytotoxicity was induced by treatments (Fig. 7G). Therefore, we next evaluated whether NGN could also inhibit NF-κB activity in the paw skin.

3.6. NGN inhibits LPS-induced NF-κB activation in vivo

NF-κB activity in the paw skin was then investigated by measuring the ratio of total and phosphorylated NF-κB subunit p65. Mice were treated with 50 mg/kg of NGN 1 h before i.pl. injection of LPS (100 ng), and paw skin tissue samples were collected 3 h after stimulus. The concentration of total and phosphorylated NF-κB p65 levels were measured by ELISA. Corroborating the results of Fig. 7, NGN inhibited LPS-induced NF-κB activation in the paw skin as observed by a reversal of LPS-induced decreases in the ratio of total NF-κB/phosphorylated NF-κB (Fig. 8). The same dose of NGN also reduced inflammatory pain (Fig. 1), leukocyte recruitment (Figs. 1 and 4).
and cytokines [31] and sensitizing nociceptive neurons, including reactive oxygen species
neutrophils and macrophages produce mediators capable of activating
describe the effects of host-related mediators. During in
overall quantity of LPS is not increased due to the action of recruited
by beta-lactam antibiotics, despite of killing bacteria also contributes
detrimental response to the host due to its pro-in
composition, and the release of LPS after bacteriolysis could be a
response can be harmful as the induction of bacteriolysis also
In fact, the bacteriolysis induced by neutrophil-derived mediators or
Myeloperoxidase (MPO) [15]. In a sense, this in
neutrophils degranulate and release bacteriolysis-inducing mediators
In conclusion, the present study has demonstrated the analgesic
producing inflammatory pain in several animal models, we aimed to
evaluate whether NGN treatment could modulate oxidative stress and
cytokine production as mechanisms of blocking hyperalgesia.
It has been described that NGN is an antioxidant molecule [4]
which may be responsible for its protective effects observed here in
reducing oxidative stress induced by LPS. However, the inhibitory
effects of NGN on neutrophil and macrophage recruitment to the paw
skin observed here also support the contribution of an anti-
flammatory activity resulting in inhibition of oxidative stress in this
model. NGN also reduced TNF-α, IL-1β, IL-6, and IL-12 production
in the mice paw, indicating that inhibiting cytokine production is an
important additional mechanism of this flavonoid. These cytokines are
produced by neutrophils and macrophages, and play important roles
in driving inflammatory pain [35,39,40]. Since previous data indicated
the relationship between cytokines and oxidative stress systems in the
induction of inflammatory hyperalgesia [41]. NGN mediated control of
both may contribute to the inhibition of inflammatory hyperalgesia.

Macrophages are tissue-resident cells of the innate immune
system. These cells respond rapidly to LPS by releasing pro-
flammatory mediators that activate endothelial cells and promote
the recruitment of neutrophils to the site of injury in a NF-κB-
dependent manner [42–44]. Moreover, macrophage-derived prod-
ucts, such as IL-1β and TNF-α, together with LPS, extend the survival of
recruited neutrophils and, thus, contribute to tissue damage and
hyperalgesia [45]. Taking into account the above described key role of
macrophages during the initial steps that lead to an inflammatory
response, the effects of naringenin was evaluated during LPS-induced
macrophage activation. We observed that NGN treatment inhibited
NF-κB activation in vitro in RAW 264.7 macrophages without inducing
cytotoxicity or compromising cell viability as well as in vivo in the paw
skin of mice. Taken together, these results suggest that NGN targets
NF-κB to inhibit inflammatory pain.

It was demonstrated that TLR4 activation by intraplantar injection
with LPS in mice induces acute hyperalgesia in a MyD88-dependent
and TRIF-independent manner [18]. We demonstrated that NGN
inhibits LPS-induced NF-κB activity which correlates with reduced
levels of hyperalgesic mediators (TNF-α, IL-1β, IL-6, IL-12, and
superoxide anion). Thus, it is plausible that NGN inhibits LPS-induced
hyperalgesia and inflammatory cell recruitment by inhibiting the
production of these mediators by macrophages. Activated neutrophils
also release superoxide anion and hyperalgesic cytokines in large
amounts in a NF-κB-dependent manner, and inhibiting the recruit-
ment of these cells by NGN treatment is also a mechanism that
certainly contributes to reducing inflammatory pain. It was demon-
strated recently that LPS activates the transient receptor potential
cation channel TRPA1 directly on nociceptive neurons to induce
urogenic inflammation [46]. NGN can enhance the activity of TRPA1
in enteroendocrine cells [47], however, it remains to be determined
whether NGN affects nociceptive neuron activity through TRPA1
modulation. The ability of NGN to inhibit LPS-induced inflammatory
pain and leukocyte recruitment was related to inhibition of macro-
phages by targeting superoxide anion release, cytokine production
(TNF-α, IL-1β, IL-6, and IL-12), and NF-κB activation. Moreover, NGN
partially inhibited the recruitment of leukocytes to the peritoneal
cavity, which represents an important effect of reducing inflamm-
ation without abolishing the immune response during infectious
diseases.

In conclusion, the present study has demonstrated the analgesic
and anti-inflammatory potential of the flavonoid NGN both in vivo and
in vitro. The antiinflammatory action of NGN observed here in models of
mechanical and thermal hyperalgesia may depend on inhibition of
leukocyte recruitment and activation (macrophages and neutrophils),
oductive stress (increased antioxidant capacity), and cytokine
production (TNF-α, IL-1β, IL-6 and IL-12). Furthermore, the anti-
flammatory effect of NGN was demonstrated in peritoneal exudate
(hubination of recruitment and activation of leukocytes, superoxide

**Fig. 6.** Naringenin (NGN) inhibits LPS-induced cytokine production in peritoneal cavity. Peritoneal exudate was collected 6 h after LPS injection, and the levels of TNF-α (A), IL-
1β (B), IL-6 (C), and IL-12 (D) were determined by ELISA. Results are means ± SEM, n = 6 mice per group per experiment, two independent experiments [*P*<.05 vs. saline control; #P<.05 vs. 0 mg/kg (vehicle). [ANOVA followed by Tukey’s test]].

oxidative stress (Figs. 2 and 5), and cytokine production (Figs. 3 and 6) in the paw skin and peritoneal cavity.

4. Discussion

Pain is one of the most prominent complaints of patients with
infectious diseases. It has been described that even small doses of LPS,
which is found in large quantities in Gram-negative bacteria, increase
pain sensation in humans [17]. Our results suggest a role for neutrophil
and macrophage recruitment and activation in hyperalgesia induced
by LPS, and that naringenin (NGN) reduces inflammatory pain through
inhibition of these inflammatory events. To kill bacteria, activated
neutrophils degranulate and release bacteriolysis-inducing mediators
such as myeloperoxidase (MPO) [15]. In a sense, this inflammatory
response can be harmful as the induction of bacteriolysis also
promotes the release of large amounts of bacterial cell wall
components. LPS represents 70% of gram-negative bacterial cell wall
composition, and the release of LPS after bacteriolysis could be a
detrimental response to the host due to its pro-inflammatory activity.
In fact, the bacteriolysis induced by neutrophil-derived mediators or
by beta-lactam antibiotics, despite of killing bacteria also contributes
to sustain inflammation and to post-infectious sequelae [37,38]. Thus,
these results suggest that NGN exhibits a clinically relevant activity
that deserves to be investigated in detail. In the present study, the
overall quantity of LPS is not increased due to the action of recruited
cells because we do not use living bacteria, permitting us to better
describe the effects of host-related mediators. During inflammation,
neutrophils and macrophages produce mediators capable of activating
and sensitizing nociceptive neurons, including reactive oxygen species
and cytokines [31–36]. Considering the key role of these mediators in
anion production and lipid peroxidation and TNF-α, IL-1β, IL-6 and IL-12 production) and in RAW 264.7 macrophage cell lineage cultures (inhibition of NF-κB activation, superoxide anion, TNF-α, IL-1β, IL-6 and IL-12 production without cytotoxicity association in tested concentrations). The observed phenotype was related to NGN inhibition of NF-κB activation. These data indicate that NGN may be a promising compound to treat inflammatory pain in patients with inflammatory diseases, and consequently, merits further preclinical and clinical investigations.

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References


[31] Ginsburg I. Bacterial cationic peptides can also function as bacteriolysis-inducing agents mimicking beta-lactam antibiotics?: it is enigmatic why this concept is consistently disregarded. Med Hypotheses 2004;62:367–74.


[34] Ginsburg I. Bactericidal cationic peptides can also function as bacteriolysis-inducing agents mimicking beta-lactam antibiotics?: it is enigmatic why this concept is consistently disregarded. Med Hypotheses 2004;62:367–74.


