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The açaí flavonoid velutin is a potent anti-inflammatory agent: blockade of LPS-mediated TNF-α and IL-6 production through inhibiting NF-κB activation and MAPK pathway☆

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Abstract

Recent studies have shown that some flavonoids are modulators of proinflammatory cytokine production. In this study, velutin, a unique flavone isolated from the pulp of açaí fruit (*Euterpe oleracea* Mart.), was examined for its effects in reducing lipopolysaccharide-induced proinflammatory cytokine tumor necrosis factor (TNF)-α and interleukin (IL)-6 production in RAW 264.7 peripheral macrophages and mice peritoneal macrophages. Three other structurally similar and well-studied flavones, luteolin, apigenin and chrysoeriol, were included as controls and for comparative purposes. Velutin exhibited the greatest potency among all flavones in reducing TNF-α and IL-6 production. Velutin also showed the strongest inhibitory effect in nuclear factor (NF)-κB activation (as assessed by secreted alkaline phosphatase reporter assay) and exhibited the greatest effects in blocking the degradation of inhibitor of NF-κB as well as in inhibiting mitogen-activated protein kinase p38 and JNK phosphorylation; all of these are important signaling pathways involved in production of TNF-α and IL-6. The present study led to the discovery of a strong anti-inflammatory flavone, velutin. This compound effectively inhibited the expression of proinflammatory cytokines TNF-α and IL-6 in low micromole levels by inhibiting NF-κB activation and p38 and JNK phosphorylation.

Keywords: Açaí; IL-6; MAPK; NF-κB; TNF-α; Velutin

1. Introduction

The flavonoids are members of a class of natural compounds widely distributed in the plant kingdom [1]. Epidemiological studies indicated that a diet rich in flavonoids, especially those from fruits and vegetables, is associated with a lowered incidence of chronic diseases such as cardiovascular disease and cancer [2–7]. Flavonoids possess various biological/pharmacological activities with anti-inflammatory activity as an important underlying mechanism [1,8,9]. Chronic inflammation is linked to a wide range of progressive diseases [10–12]. It has been widely accepted that inflammation plays a critical role in the pathogenesis of atherosclerosis. Leukocyte recruitment and expression of proinflammatory cytokines characterize early atherogenesis [11].

Açaí (*Euterpe oleracea* Mart.) belongs to the family Arecaceae (palm tree), and it is indigenous to South America especially in the Amazon flood plains. The pulp from the açaí fruit has received much attention in recent years as one of the new "super fruits" due to its high antioxidant capacity and potential anti-inflammatory activities [13,14]. In a recent paper from our group [15], a diet containing 5% freeze-dried açai juice powder was found to attenuate atherosclerosis in apolipoprotein E-deficient mice. The levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 were significantly lower in sera and in the residential macrophage with and without lipopolysaccharide (LPS) stimulation from mice fed the diet containing freeze-dried açaí juice powder. Tumor necrosis factor-α and IL-6 are key players in the vascular inflammation underlying atherosclerosis. Increased production of these cytokines has been associated with the incidence of heart failure as well as with insulin resistance, dyslipidemia and obesity.

Abbreviations: LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; IκB, inhibitor of NF-κ; SEAP, secreted alkaline phosphatase; TNF-α, tumor necrosis factor-α; RT-PCR, reverse transcription–polymerase chain reaction; IL-6, interleukin-6.

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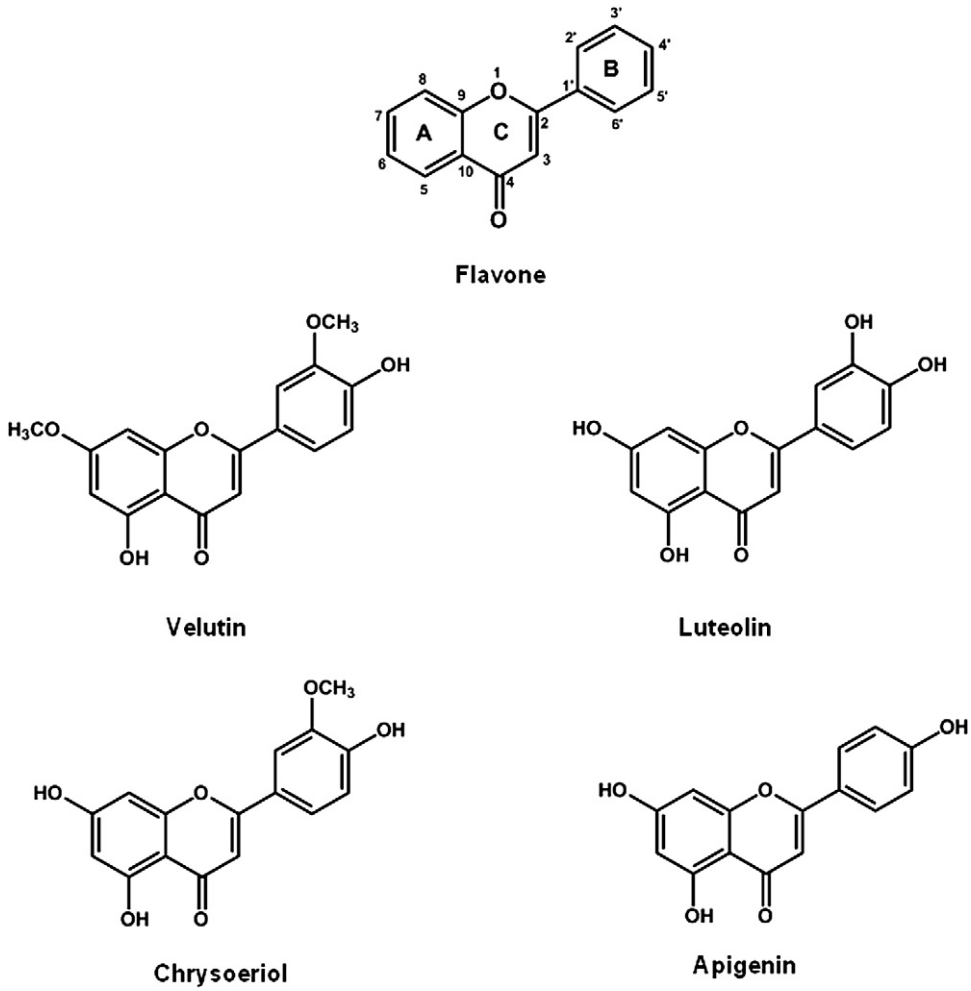

Fig. 1. Chemical structures of four flavones (velutin, luteolin, apigenin and chrysoeriol) included in this study. Basic structure of flavone with position labeled is also included.

Increases in TNF-α and IL-6 are used as predictive biomarkers of current, as well as future, cardiovascular disease and cardiovascular mortality [16,17]. Thus, reducing TNF-α and IL-6 production has been suggested as an important underlying mechanism of the atheroprotective effects of açai juice [15]. Açaí pulp has been found to contain various flavonoids, including luteolin and apigenin, which have been demonstrated to have anti-inflammatory effects [18,19]. Velutin, a unique flavone found in açai pulp, was also found to exhibit superior inhibitory effect in nuclear factor (NF)-κB activation [20].

In the present study, we further examined the anti-inflammatory activities of velutin, with the primary focus on LPS-induced proinflammatory cytokines TNF-α and IL-6 production in macrophages. Three other structurally similar and well-studied flavones, luteolin, apigenin and chrysoeriol, were included as controls and for comparative purposes (Fig. 1). Our results demonstrated that velutin decreases expression of TNF-α and IL-6, and the mechanisms by which these effects occur appear to be through inhibition of the LPS-stimulated activation of mitogen-activated protein kinase (MAPK) and NF-κB pathways.

2. Materials and methods

2.1. Chemicals and reagents

Antibodies to phosphorylated forms of extracellular signal-regulated kinase (Erk) 1/2, stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK), p38 MAP kinase (p38), inhibitor of NF-κB (IκB) and their native forms, and RIPA buffer were from Cell Signaling (Beverly, MA, USA). Mouse anti-β-actin antibody was from Sigma (St. Louis, MO, USA). Lipopolysaccharide, zeocin and QuantiBlue (an alkaline phosphatase substrate) were purchased from InvivoGen (San Diego, CA, USA). Velutin, luteolin and chrysoeriol were isolated from açaí (*E. oleracea* Mart.) fruit pulp [20,21], and their purities were all higher than 99% (by high-performance liquid chromatography). Apigenin (97%) was obtained from Indofine (Hillsborough, NJ, USA). All solvents were obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Animals

The animal protocol was approved by the Animal Care and Use Committee of the Arkansas Children's Hospital Research Institute. C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Animals were maintained in sterile microisolator cages and fed autoclaved-pelleted diet and water *ad libitum*.

AIN-93G diet was provided by Harlan Teklad (Madison, WI, USA). Diet formulation was presented in an earlier paper [15].

C57BL/6 mice were fed AIN-93G diet as control diet (CD) for 5 weeks. Three days prior to sacrifice, 3% thioglycolate was injected intraperitoneally into the mice for peritoneal macrophage collection. At the end of 5 weeks, animals were euthanized using CO_2. The peritoneal macrophages from C57BL/6 mice were collected and cultured.

2.3. Cell culture

RAW 264.7 and RAW-Blue mouse macrophages were obtained from InvivoGen. RAW-Blue cells are derived from RAW 264.7 macrophages with chromosomal integration of a secreted embryonic alkaline phosphatase (SEAP) reporter construct inducible by NF-κB and AP-1. RAW-Blue and RAW 264.7 macrophages were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and zeocin (200 μg/ml).

Thioglycollate-elicited peritoneal macrophages were plated in RPMI-1640 supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, penicillin, streptomycin

and sodium pyruvate. Nonadherent cells were removed after 2 h, and macrophages were used after 48 h.

2.4. SEAP reporter assay

The SEAP reporter assay was conducted in RAW-Blue macrophages. They stably express a SEAP gene inducible by NF-κB. The SEAP protein secreted to the culture media is measured by a SEAP assay kit as a way to evaluate NF-κB activation. The assay was performed in accordance with the method described in our previous paper [20].

2.5. Real-time reverse transcription–polymerase chain reaction analysis

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and treated with RNase-free DNase. Reverse transcription (RT) reaction and quantitative real-time polymerase chain reaction (PCR) were described previously [22]. Real-time PCR primers (Integrated DNA Technologies, Coralville, IA, USA) were as follows: β-actin sense (GGCTATGCTCTCCCTCACG), β-actin antisense (CGCTCGGTCAGGATCTTCAT), TNF-α sense (ACAAGGCTGCCCCGACTAC), TNF-α antisense (TGGAAGACTCCTCCCAGGTATATG), IL-6 sense (TGGAGTCACAGAAGGAGTGGCTAAG) and IL-6 antisense (TCTGACCACAGTGAGGAATGTCCAC). A two-step PCR with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min for 40 cycles was conducted in iCycler (BioRad, Hercules, CA, USA) to determine the threshold cycle (Ct) value. Expression of TNF-α and IL-6 was calculated using the $^{\Delta\Delta}$Ct method of threshold cycles for β-actin as the normalization reference. All real-time PCRs were carried out at least twice from independent complementary DNA preparations. RNA without reverse transcriptase served as a negative control.

2.6. TNF-α and IL-6 enzyme-linked immunosorbent assay

RAW-Blue cells or peritoneal macrophages (5×10^6 cells/well) were pretreated with various concentrations of the indicated reagents for 1 h before LPS stimulation. After 18 h of stimulation, supernatant was collected; TNF-α and IL-6 in the supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using Duoset ELISA kits (R&D, Minneapolis, MN, USA) according to the manufacturer's instructions. The optical density was determined using a BMG Polarstar microplate reader (Cary, NC, USA) at 450 nm. Mean values of triplicate samples for each experiment and two separate experiments were used for analysis.

2.7. Western blot analysis

RAW-Blue cells treated with indicated reagents were lysed in RIPA buffer with protease and phosphatase inhibitors (Cell Signaling, Danvers, MA, USA). Cell lyses were centrifuged at 10,000 rpm for 15 min to remove cell debris. Protein concentrations were determined by Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA, USA). The lysate (10 μg protein/lane) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 5% bovine serum albumin/phosphate-buffered saline containing 0.05% Tween-20 and probed with antibodies against IκB, β-actin, SAPK/JNK (p54/p46), p-SAPK/JNK, Erk1/2 (p44/p42), p-Erk1/2 and p38 MAP. Bands were detected using ECL reagents (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions.

2.8. Statistical analysis

Data were expressed as mean value±S.E.M. unless otherwise mentioned. Student's *t* test was used to analyze differences between groups. One-way analysis of variance (ANOVA) with the Student–Newman–Keuls method was used to compare more than two groups. A value of $P<.05$ was considered as a significant difference unless otherwise mentioned. Statistic analyses were performed by SigmaStat statistical software (SigmaStat 3.5).

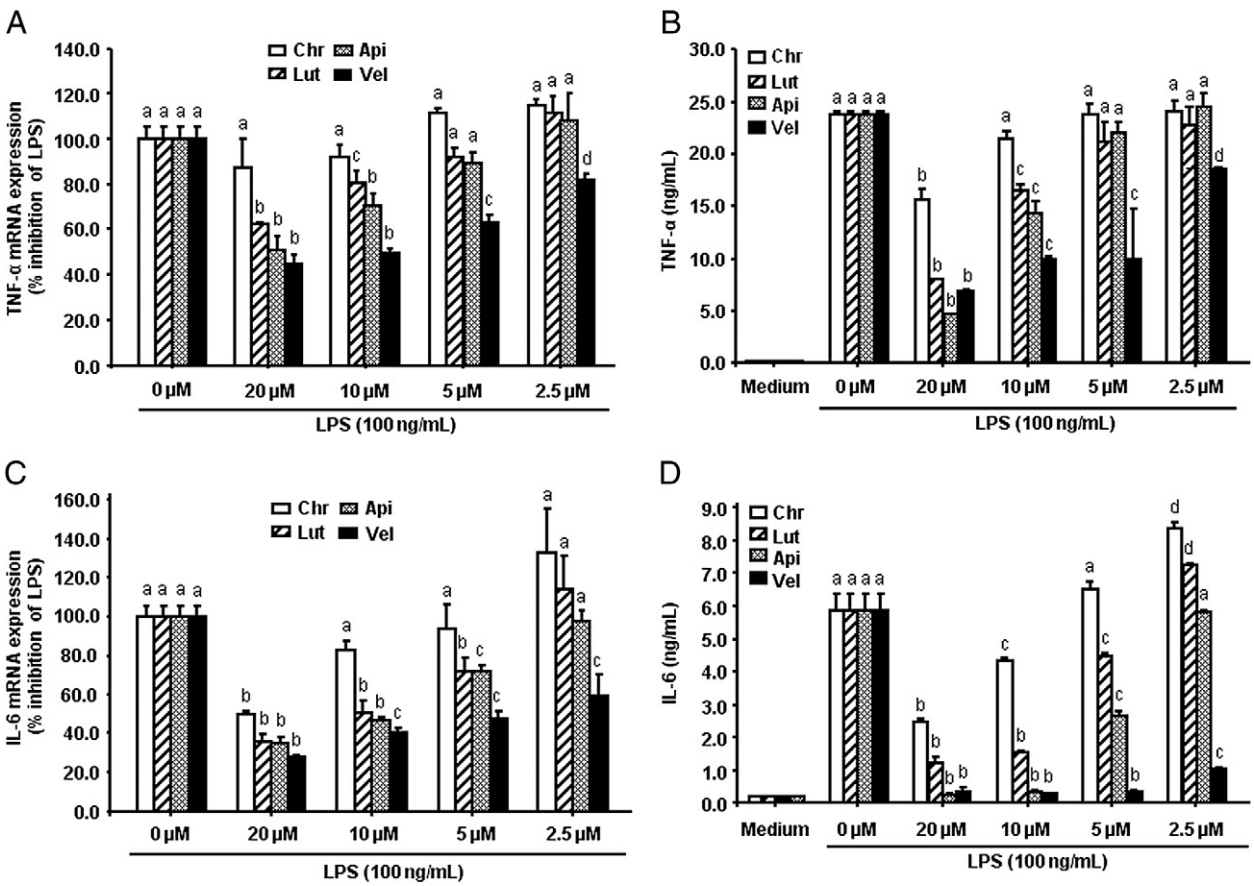

Fig. 2. Suppression of LPS-induced TNF-α and IL-6 mRNA expression and protein levels in RAW 264.7 macrophages. RAW 264.7 macrophages were pretreated with four flavones at concentrations ranging from 2.5 to 20 μM for 1 h and then stimulated with 100 ng/ml LPS for an 18-h incubation period. Control cells were incubated in the absence of flavones. The values are expressed as percentages of TNF-α (A) and IL-6 (C) stimulation at mRNA level. The protein level of TNF-α (B) and IL-6 (D) was measured by ELISA. The data are means±S.E.Ms. of three independent experiments. One-way ANOVA with the Student–Newman–Keuls method was used to compare each flavone-treated group at different concentrations. Means with different letters are different ($P<.05$).

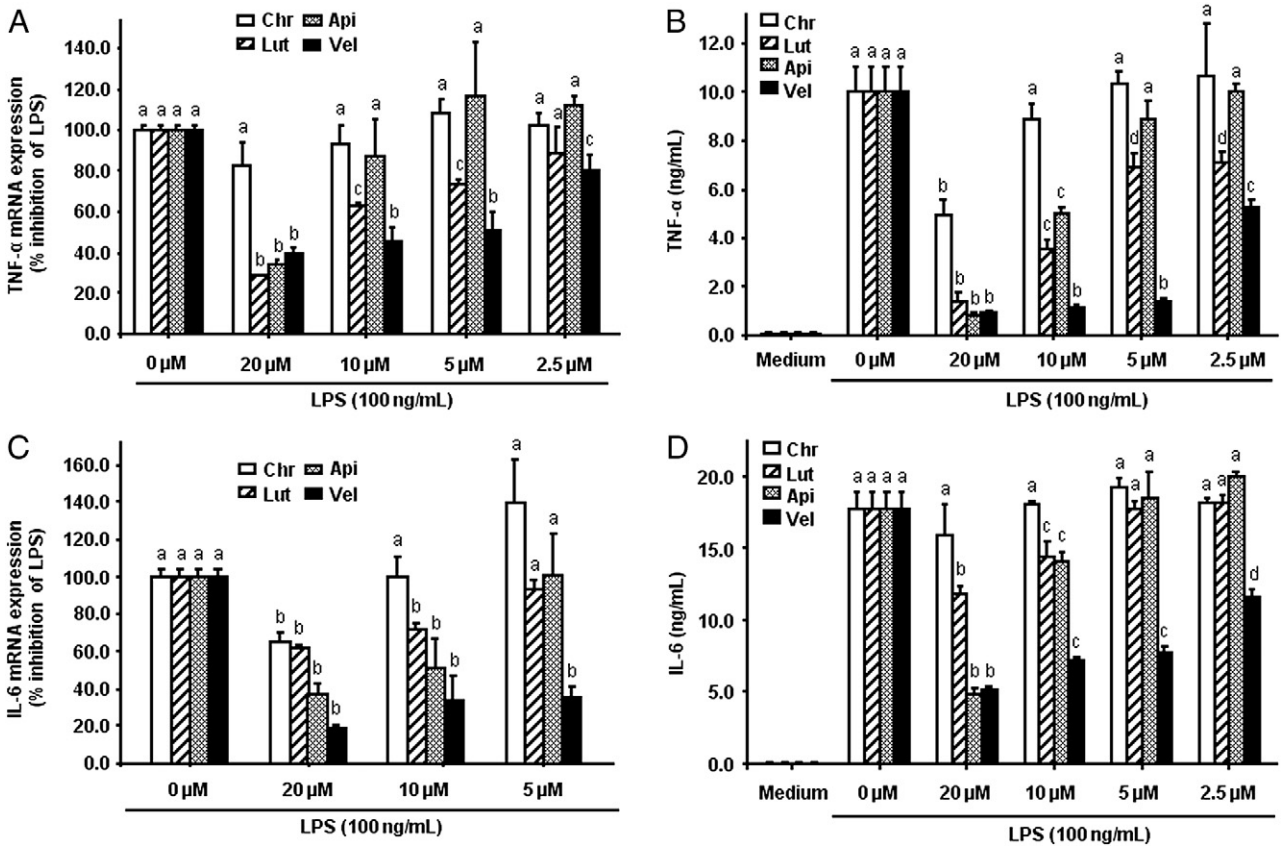

Fig. 3. Suppression of LPS-induced TNF-α and IL-6 mRNA expression and protein levels in mice peritoneal macrophages. Cultured peritoneal macrophages were pretreated with four flavones at concentrations ranging from 2.5 to 20 μM for 1 h and then stimulated with 100 ng/ml LPS for an 18-h incubation period. Control cells were incubated in the absence of flavones. The values are expressed as percentages of TNF-α (A) and IL-6 (C) stimulation at mRNA level. The protein level of TNF-α (B) and IL-6 (D) was measured by ELISA. The data are means±S.E.Ms. of three independent experiments. One-way ANOVA with the Student–Newman–Keuls method was used to compare each flavone-treated group at different concentrations. Means with different letters are different (P<.05).

3. Results

3.1. Flavones inhibit LPS-induced TNF-α and IL-6 production in RAW 264.7 macrophages

RAW 264.7 macrophages were pretreated with four flavones at concentrations ranging from 2.5 to 20 μM for 1 h and then stimulated with 100 ng/ml LPS for an 18-h incubation period. Both TNF-α and IL-6 protein levels were significantly increased upon LPS stimulation (Fig. 2). In general, all four flavones inhibited TNF-α and IL-6 production. For TNF-α production, apigenin displayed the strongest inhibitory effect at 20 μM, whereas velutin showed similar effect to luteolin. Chrysoeriol only showed a marginal inhibitory effect at 20 μM (Fig. 2). But at lower concentrations from 10 to 2.5 μM, velutin showed greater inhibitory effect. For IL-6 production, both velutin and apigenin strongly inhibited IL-6 production at 20 and 10 μM to about the same extent. At 5 and 2.5 μM, the inhibitory effect of apigenin was much weaker at 5 μM and was lost at 2.5 μM. Luteolin showed weaker inhibitory effects than apigenin and velutin from 10 to 5 μM. Chrysoeriol showed mild inhibitory effects only at 20 and 10 μM. Similar to the effects on TNF-α, velutin had the most potent IL-6 inhibitory effects at low doses. Notably, both luteolin and chrysoeriol slightly induced IL-6 production at the low concentration of 2.5 μM (Fig. 2).

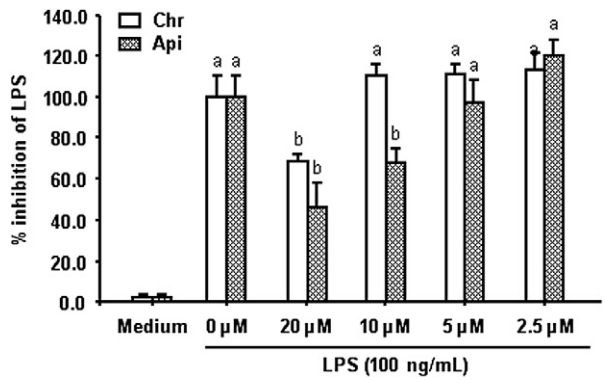

Fig. 4. Dose–response experiments of apigenin (Api) and chrysoeriol (Chr) from SEAP reporter assay induced by LPS. The SEAP reporter assay was conducted in RAW-Blue cells according to our previous paper [20]. Bars represent the mean±S.D. from three independent experiments. One-way ANOVA with the Student–Newman–Keuls method was used to compare each flavone-treated group at different concentrations. Means with different letters are different (P<.05).

To determine whether the decreases of LPS-induced TNF-α and IL-6 might be associated with transcriptional inhibition, messenger RNA (mRNA) levels of TNF-α and IL-6 were measured by RT-PCR. In line with protein levels, velutin was the most potent in reducing TNF-α and IL-6 mRNA levels. Apigenin and luteolin also attenuated LPS-induced TNF-α and IL-6 mRNA expression, but their effects were not as great as velutin. Chrysoeriol only showed a weak effect to IL-6 mRNA expression at 20 μM (Fig. 2).

3.2. Flavones inhibition of LPS-induced TNF-α and IL-6 production in mice peritoneal macrophages

Cultured peritoneal macrophages were pretreated with velutin and three other flavones at concentrations ranging from 2.5 to 20 μM

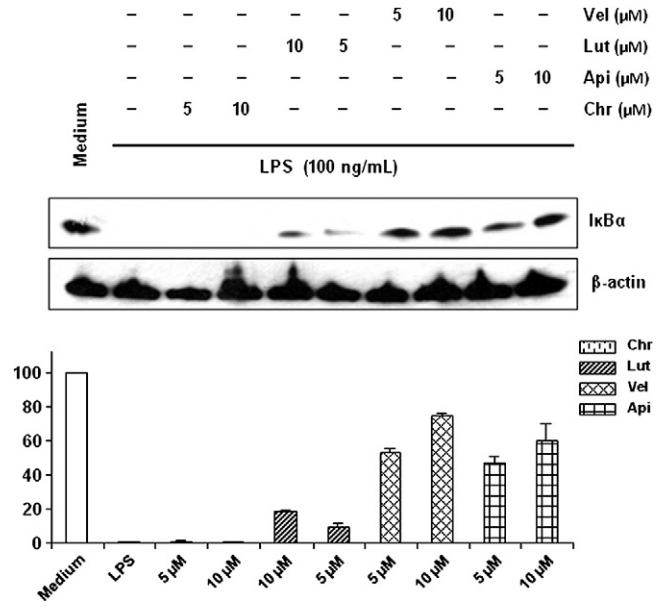

Fig. 5. Velutin, luteolin and apigenin inhibit LPS-induced IκB degradation in RAW264.7 macrophages. Cell lysates were analyzed by immunoblotting by reacting with antibodies against IκB-α compared with β-actin. Results are expressed as ratios of band density of IκB-α to β-actin. Data are means±S.E.Ms. of three replicate experiments.

for 1 h and stimulated with 100 ng/ml LPS during an 18-h incubation period. Similar to the results observed in RAW 264.7 cells, velutin showed comparable inhibitory effects with apigenin and/or luteolin in reducing LPS-induced TNF-α and IL-6 protein levels at 20 μM, but velutin was the most potent of the flavonoids as demonstrated by greater inhibitory effects at lower doses. Chrysoeriol, again, only showed weak inhibition against TNF-α production at 20 μM (Fig. 3).

An almost identical pattern of mRNA expression was observed in this cell line as we obtained from RAW 264.7 cells (Fig. 3).

3.3. Inhibition of NF-κB activation

The effects of apigenin and chrysoeriol in inhibiting SEAP secretion were measured. Apigenin was found to dose-dependently inhibit SEAP secretion, and its IC_{50} was estimated at 17.9 μM. Chrysoeriol only displayed a weak inhibitory effect at 20 μM (Fig. 4).

3.4. Effects of four flavones on IκB degradation

RAW 264.7 cells were pretreated with 5 and 10 μM of velutin and the other three flavones for 1 h and stimulated with LPS for 30 min. Adding LPS resulted in IκB degradation (Fig. 5). Both velutin and apigenin effectively inhibited LPS-induced IκB degradation (Fig. 5) at 5 and 10 μM. Velutin showed the greatest inhibitory effect, while luteolin only showed mild inhibition against LPS-induced IκB

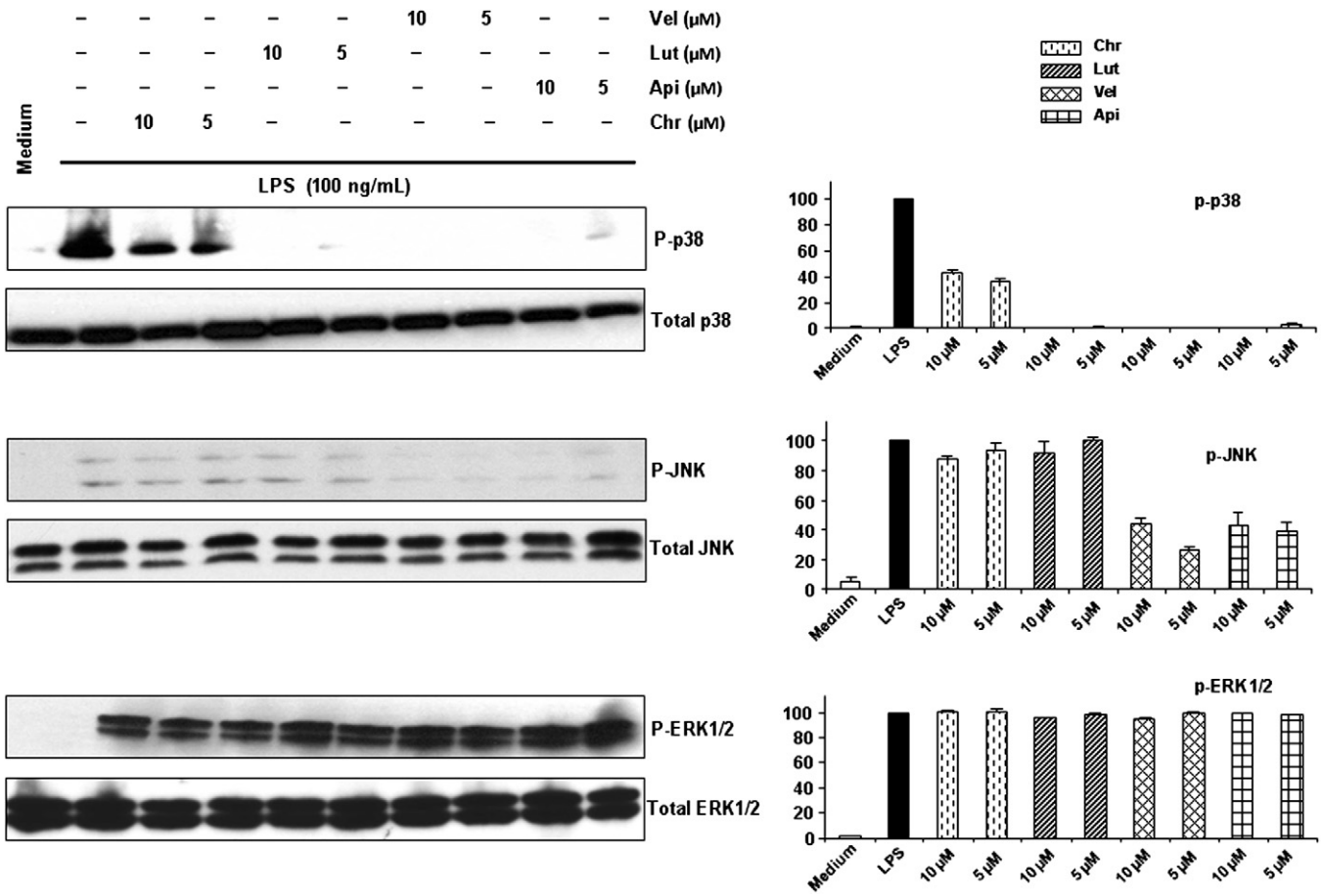

Fig. 6. Effects of four flavones in inhibiting LPS-induced phosphorylation of JNK, ERK and p38. Cells were treated as described in Materials and methods. Western blot analysis was conducted using 30 μg of cell lysate protein/lane and reacted with antibodies against *p*-p38 and p38, *p*-JNK and JNK, and *p*-Erk1/2 and Erk1/2. Results are expressed as relative ratios of band density of phosphorylated forms of p38, JNK and Erk1/2 to the respective total proteins. Data are means±S.E.Ms. of three replicate experiments.

degradation (Fig. 5) at 10 μM, and chrysoeriol did not inhibit LPS-induced IκB degradation (Fig. 5).

3.5. Effects of four flavones on phosphorylation of MAPK

Lipopolysaccharide simulation resulted in phosphorylation of JNK, ERK1/2 and p38 (Fig. 6). Velutin, apigenin and luteolin all significantly inhibited p38 and JNK phosphorylation at 5 and 10 μM. Velutin showed the greatest effect among all four compounds. Chrysoeriol only slightly inhibited p38 phosphorylation. All four flavones failed to inhibit ERK1/2 phosphorylation (Fig. 6).

4. Discussion

Studies have shown that certain flavonoids, such as luteolin, apigenin, quercetin, genistein and hesperetin, belonging to different subgroups of flavonoid, inhibit production of pivotal inflammatory cytokines such as IL-6 and TNF-α. Notably, of all flavonoids being studied, the two flavone compounds, luteolin and apigenin, were reported to be among the most potent agents in inhibiting inflammatory cytokine production in a variety of cell lines [23–27]. In this study, for the first time, we showed that velutin, an uncommon flavone isolated from the pulp of the edible açaí fruit (*E. oleracea* Mart.), displayed even stronger effects in inhibiting both TNF-α and IL-6 mRNA expression and protein levels in two macrophages. Luteolin and apigenin were also found to dose-dependently inhibit production of TNF-α and IL-6, in agreement with previous findings. Chrysoeriol showed only marginal effects at a higher concentration (20 μM). Compared to luteolin and apigenin, velutin exhibited similar inhibitory effects at high concentrations (20 or 10 μM), but showed dramatically greater inhibitory effects at lower concentrations (5 and 2.5 μM) against the production of TNF-α and IL-6 in two macrophages. This is of significant therapeutic relevance since flavonoids are generally presented at low concentrations in the body from dietary sources. These results suggested that velutin may be an attractive natural anti-inflammatory agent.

In addition, for the two most studied flavones, apigenin and luteolin, we found that apigenin tended to show stronger effects at high concentrations (20 and/or 10 μM), while at lower concentrations (5 and 2.5 μM), the effects of these two flavones were similar.

Several mechanisms underlying the inhibition of LPS-induced inflammatory cytokine production by flavonoids have been investigated, of which blocking of NF-κB and MAPK pathways has been proposed as the two major mechanisms [8,28]. Previous studies have shown that exposure of the murine macrophage cell line RAW 264.7 to LPS increases phosphorylation of the MAPK family members ERK1/2, p38 and JNK1/2 in a time-dependent manner [29]. Lipopolysaccharide stimulation also elicits a cascade leading to the activation of NF-κB [30]. Activation of MAPK and NF-κB leads to the production of inflammatory cytokines [31–33]. Thus, inhibition of the LPS-stimulated signal transduction cascades has been proposed as a promising target for the treatment of inflammation.

The SEAP reporter assay was conducted to evaluate the inhibitory effects of velutin and luteolin in inhibiting NF-κB activation in our previous report [20]. The IC_{50} values of these two compounds were estimated at 2.0 μM for velutin and 12.4 μM for luteolin. In this study, the same procedure was performed on apigenin and chrysoeriol. The IC_{50} of apigenin was estimated at 17.4 μM, and chrysoeriol only showed weak inhibitory effects. By comparison, velutin exhibited the greatest inhibitory effects towards NF-κB activation, followed by luteolin and apigenin. In the classical "canonical" activation pathway, the phosphorylation and degradation of the NF-κB inhibitor, IκB-α, resulted in subsequent activation of NF-κB [34]. Pretreatment of velutin resulted in strong blockage of IκB-α degradation (Fig. 5), which may partly explain its strong inhibitory effect in attenuating NF-κB activation. Apigenin showed slightly weaker effects than velutin, whereas luteolin showed an even weaker effect, while chrysoeriol showed no effect.

Lipopolysaccharide stimulates the activation of various MAPK pathways, including ERK, JNK and p38 pathways. These pathways are

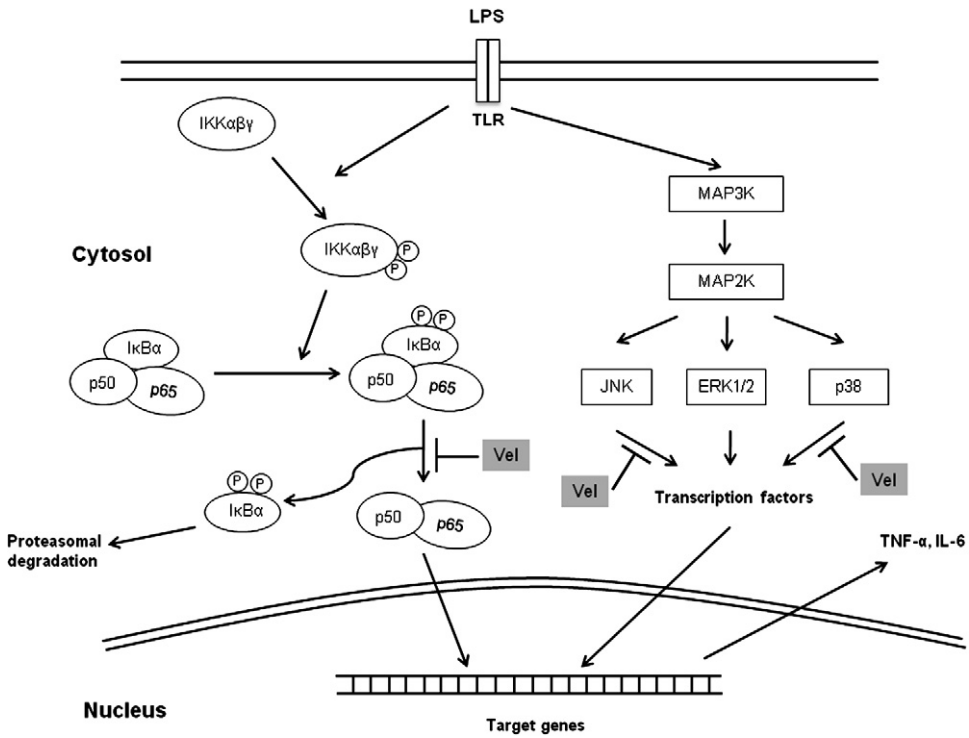

Fig. 7. Proposed mechanisms of velutin in inhibiting TNF-α and IL-6 production in macrophages. TLR, toll like receptor; IKK, IκB kinase; ERK, extracellular signal-related kinases; JNK, c-Jun amino-terminal kinases; p38, p38 mitogen-activated protein kinase.

used to directly or indirectly phosphorylate transcription factors such as Elk-1, c-Jun and ATF-1. Lipopolysaccharide also activates NF-κB through the "canonical" IKK-NF-κB pathway or "noncanonical" pathways [35]. Velutin, luteolin and apigenin all strongly inhibited p38 phosphorylation (Fig. 6). These data suggest that this might be a common pathway affected by flavone structures. Velutin and apigenin also inhibited JNK phosphorylation (Fig. 6), with velutin showing greater effects than apigenin. Neither luteolin nor chrysoeriol had inhibitory effect against JNK phosphorylation, and none of the four flavones affected the ERK1/2 pathway (Fig. 6).

Based on the above-mentioned results, the mechanisms by which velutin inhibits TNF-α and IL-6 production are summarized (Fig. 7). It is worth mentioning that the mechanisms seem to differ by the types of flavonoids, cell and stimulus. For instance, luteolin inhibited LPS-induced phosphorylation of ERK1/2 and p38, but not JNK, in murine macrophage cell line RAW 264.7 [29]. But in murine microglial cells, luteolin inhibited LPS-induced JNK, but not ERK1/2 and p38 phosphorylation [36]. In addition, our data do not rule out the possibility that other mechanisms are involved.

The structure–activity relationship of flavonoids and their anti-inflammatory effects is an important area of research as discussed in various publications [24,26,27,37,38]. Some general principles proposed to date are as follows: (a) flavonoid aglycones are effective forms, and adding sugar moieties in the structures (glycosides) abolishes the inhibitory effect; (b) a C-2,3 double bond with oxo function at position 4, providing a flavone backbone, is necessary for the high anti-inflammatory effect; (c) four hydroxylations at positions 5, 7, 3′, 4′ are necessary for optimal anti-inflammatory effects [24,26,27,37,38]. The structure of luteolin, 3′,4′,5,7-tetrahydroxylflavone, was suggested to be most suitable for oral anti-inflammatory activity [25]. In a most recent paper, flavones were found as promising inhibitors of JNK3, while being less effective against p38α [39]. And in contrast to above-mentioned observations, luteolin-7-glucoside was found to be the most effective p38α inhibitor. Structure–activity relationship evaluation in this paper suggested that, for flavones, substitution with hydroxyl and methoxy groups at the B-ring improved the inhibitory activity. Methylation of the hydroxyl group at 3′-position is well tolerated by JNK3, whereas a methoxy combination at 7- and 4′-positions was ineffective. However, in our study, we found that velutin, whose structure is similar to luteolin but bears two methoxy groups at 7- and 3′-positions (Fig. 1), showed a much stronger inhibitory effect compared to the four flavones. Chrysoeriol, on the contrary, was almost ineffective, although it is structurally similar to luteolin but with only one methoxy group at 3′-position. We do not have a good explanation for the different results observed, and we feel that it is a fertile area for further study. It seems that, in order to be effective, methoxy combination at 7- and 3′-positions is necessary. Since apigenin also exhibited high inhibitory effect, 4′-hydroxylation, but not necessarily 3′-hydroxylation, is required. In future studies, flavones with greater diversity are needed for in-depth structure–function relationship studies. On the other hand, whether and how the methoxy or other acylated groups conjugated with different hydroxyl groups of luteolin or apigenin would increase the anti-inflammatory activities of these compounds would be of great research interest.

Most studies conducted so far examining the anti-inflammatory activities of flavonoids are performed in cell lines. However, cell culture data do not always predict the anticipated outcomes seen in mammals *in vivo*. One key factor is the bioavailability. Issues like absorption, metabolism and distribution must be addressed to evaluate the *in vivo* anti-inflammatory activities of target flavonoids of interest. Moreover, it is well known that polyphenols undergo extensive degradation by gut microflora to form various lower-molecular-weight catabolites that are absorbed after biotransformation [40]. As a result of this biotransformation, some of these compounds can reach a considerable concentration *in vivo*. A complete bioavailability study about velutin is currently ongoing in our laboratory to assess the absorption, metabolism, distribution and possible biotransformation of this compound.

In conclusion, the present study led to the discovery of a strong anti-inflammatory flavone, velutin. This compound effectively inhibited the expression of proinflammatory cytokines TNF-α and IL-6 in very low micromole levels by inhibiting NF-κB activation and p38 and JNK phosphorylation in macrophages. Nonetheless, our data did not rule out the possibilities that other mechanisms may be involved. In addition, a complete pharmacokinetic study of velutin is needed to precisely address its bioavailability.

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