Pharmacological Reports
2005, 57, 390–394
ISSN 1734-1140

Short communication

Effect of apigenin, kaempferol and resveratrol on the expression of interleukin-1β and tumor necrosis factor-α genes in J774.2 macrophages

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Abstract :
Flavonoids have been reported to bring benefits in lowering inflammation, oxidative stress and exert positive effects in cancer and cardiovascular and chronic inflammatory diseases. Apigenin, kaempferol and resveratrol present in fruits, vegetables and grain were investigated for their effect on the synthesis of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) at transcriptional level in lipopolysaccharide (LPS)-stimulated J774.2 macrophages. Apigenin (30 μM), kaempferol (30 μM) and resveratrol (50 μM) significantly decreased the number of TNF-α mRNA copies in LPS-activated J774.2 macrophages. Apigenin and kaempferol caused inhibition of IL-1β gene expression in J774.2 macrophages, but resveratrol was ineffective.
These results indicate that apigenin, kaempferol and resveratrol exert inhibitory effects on the TNF-α and except for of resveratrol on IL-1β gene expression in J774.2 macrophages at the transcriptional level. In addition, the studied compounds may be the mediators responsible for protective role of a diet high in fruits and vegetables in the cardiovascular and inflammatory diseases.

Key words:
apigenin, kaempferol, resveratrol, IL-1β mRNA, TNF-α mRNA, J774.2 macrophages

Introduction

Many epidemiological studies have shown that an increased intake of polyphenolic phytochemicals such as flavonoids and phenolic acids found in a number of vegetables and fruits may contribute to low incidence of cardiovascular diseases [14, 19]. Dietary intakes of flavonoids are inversly correlated with the plasma low-density lipoprotein (LDL)-cholesterol concentration [1]. Flavonoids and polyphenolics have a great potential to delay LDL oxidation through their radical-scavenging capacity [16]. Wine flavonoids have been shown to protect against atherosclerosis by inhibiting the accumulation of oxidized LDL in atherosclerotic lesions, and removing atherogenic lesions in apolipoprotein E-deficient mice [3]. In addition some flavonoids inhibit platelet aggregation *in vitro* and tromboxane synthesis *in vivo* [24]. This observation demonstrates that flavonoids may confer protection against early events in atherogenic lesion formation.

Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are well known cytokines, secreted in great amounts by the activated macrophages. It was demonstrated that IL-1β and TNF-α may contribute to the

development of bacterial sepsis [2], hypercholesterolemia [21], hypertension [25], and multiplex sclerosis [10].

Many investigations demonstrated inhibiting activity of flavonoids on synthesis of prostanoids, nitric oxide or free radicals, but less attention has been paid to the effect of flavonoids on the synthesis of proinflammatory cytokines (IL-1β and TNF-α) at the transcriptional level. Therefore, in the present study, we assessed the antiinflammatory activity of three flavonoid substances: apigenin, kaempferol and resveratrol on IL-1β and TNF-α gene expression in J774.2 macrophages.

Materials and Methods

Chemicals

LPS (from *Escherichia coli*, serotype O111:B4), apigenin, kaempferol, resveratrol, dimethyl sulfoxide (DMSO) and trypan blue were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Cell culture

The mouse macrophage cell line J.774.2 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cells were maintained in an atmosphere of 5% CO_2, at 37°C in DMEM supplemented with 10% FCS, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10 μg/ml of Fungizone (Gibco BRL Life Technologies, Paisley, UK). The cells were cultured in 75 cm^2 plastic flasks (Nunc A/S Roskilde, Denmark) and passaged three times a week. For experiments, cells were detached by vigorous pipeting, and, after centrifugation, plated using fresh medium. For study of gene expression, 1×10^6 macrophages were plated in 35 mm Petri dishes and incubated for 24 h. Then the culture medium was replaced with fresh medium and 50 μM of resveratrol, 30 μM of apigenin and 30 μM of kaempferol was added to cultures 20 min before LPS administration. We chose the above-mentioned doses of the studied compounds because they caused an inhibition of MCP-1 gene expression in an earlier experiment [17, 18, Kowalski et al., unpublished data]. After 24 h, total RNA from such cultures was extracted. Cells not treated with LPS or the studied compounds were used as control. Resveratrol, apigenin and kaempferol were dissolved in dimethyl sulfoxide (DMSO) and diluted in complete cell culture medium in order to obtain appropriate concentrations. The final concentration of DMSO was adjusted to 0.1% (v/v). The control cells received the same amount of DMSO. The effect of the studied compounds on cell viability in culture was assessed by trypan blue exclusion test. Cell viability was greater than 95% in all performed experiments.

Determination of IL-1β and TNF-α mRNAs

Preparation of total cellular RNA

Total cellular RNA was extracted from J774.2 cells using Tri Reagent (Sigma Chemical Co. MO, USA). J774.2 macrophages were washed and lysed by addition of Tri Reagent to each Petri dish. After complete dissociation of nucleoprotein complexes, RNA was isolated according to the Tri Reagent protocol of Chomczyński [8]. RNA concentration was determined by measuring spectrophotometric absorbance ($A_{260/280}$) of a range of dilutions (Beckman DU^R 530 Spectrophotometer).

RT-QPCR for detection of mRNAs

RNA (100 ng) was reverse-transcribed into cDNA using the Reverse Transcription System (Applied Biosystems, USA) with random hexamers and Multiscribe Reverse Transcriptase, according to the manfacturer's instructions. The reverse transcriptase reaction was carried out at 48°C for 30 min, followed by deactivation of the enzyme for 5 min at 95°C.

For quantitative PCR, The TaqMan Universe PCR Master Mix and Target Primers and Probe from Applied Biosystems were used. Amplification of cDNA was performed in Micro Amp Optical 96-well Reaction Plates (Applied Biosystems) on an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). The reaction mixture (20 μl) was composed of 4 μl of RNase-free water, 10 μl of 2× TaqMan Universal PCR Master Mix, 1 μl of 20× Target Primers and Probe, 25 ng of cDNA according to manufacturer's recommendation. The reaction conditions were: initially 2 min at 50°C, followed by 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. The quantity of amplified cDNA fragments was determined from Ct value (cycle threshold : threshold

value of fluorescence) with a reference to the standard curve generated by amplification of five known concentrations of β-actin gene (1×10^2 to 10^4 copies) (β-actin Control Reagent Kit, Applied Biosystems). The amount of IL-1β mRNA or TNF-α mRNA was calculated as a number of target cDNA copies per 1 μg of total RNA used in RT-QPCR reaction. The mean value obtained from replicate determinations was used in subsequent calculations. Moreover, in order to normalize the differences in efficiencies of extraction and purification of RNA and cDNA synthesis among tubes, the GAPDH density, amplified from the same RT product, was used as an internal standard. No significant differences were observed in GAPDH signals between pre- and post-LPS treatment, suggesting that this housekeeping gene is an appropriate internal standard.

Statistical analysis

The data in figures are expressed as the arithmetic mean ± SE of two independent experiments (six measurements). Differences were analyzed with ANOVA and then with the Newman-Keuls test for multiple comparisons beetwen group means using Graph Pad Prism software (version 2.01). Differences were considered statistically significant if $p < 0.05$.

Results

Unstimulated J774.2 macrophages expressed very low number of mRNA copies for IL-1β and TNF-α. In contrast, incubation of cells with LPS at a dose of 1 μg/ml for 24 h induced the appearance of high number of IL-1β copies and moderate number of TNF-α mRNA copies.

Effect of kaempferol, apigenin and resveratrol on LPS-induced IL-1β gene expression

Apigenin and kaempferol at a dose of 30 μM added to the J774.2 macrophage cultures caused significant decrease in the number of IL-1β mRNA copies. Exposure of J774.2 macrophages to resveratrol (50 μM) did not change IL-1β expression (Fig. 1).

Effect of kaempferol, apigenin and resveratrol on LPS-induced TNF-α gene expression

Twenty-four-hour exposure to 50 μM of resveratrol or to 30 μM of apigenin as well as to kaempferol caused significant decrease in the number of TNF-α mRNA copies (Fig. 2).

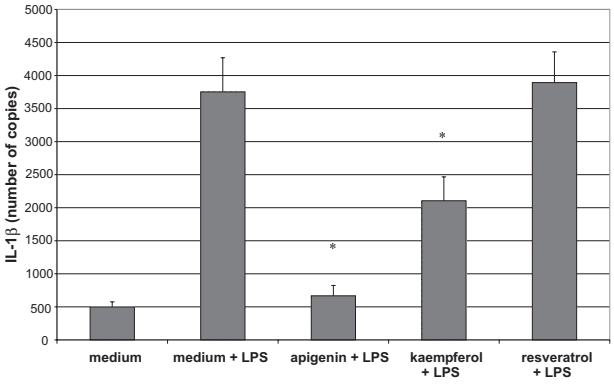

Fig. 1. The effect of resveratrol, apigenin and kaempferol given 20 min before administration of LPS (1 μg/ml) on the IL-1β gene expression in J774.2 macrophages cultures. The cultures were incubated with 50 μM of resveratrol, 30 μM of apigenin and kaempferol for 24 h. The values are the mean ± SE of two independent experiments, each with three determinations. * – significantly different from the vehicle (LPS + medium)-treated cultures, assessed with ANOVA followed by Newman-Keuls multiple comparison test, $p < 0.05$

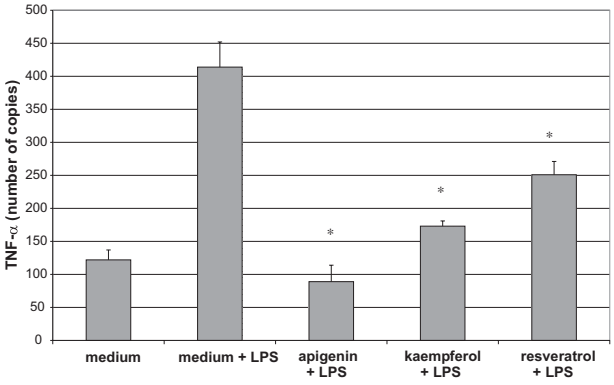

Fig. 2. The effect of resveratrol, apigenin and kaempferol given 20 min before administration of LPS (1 μg/ml) on the TNF-α gene expression in J774.2 macrophages cultures. The cultures were incubated with 50 μM of resveratrol, 30 μM of apigenin and kaempferol for 24 h. The values are the mean ± SE of two independent experiments, each with three determinations. * – significantly different from the vehicle (LPS + medium)-treated cultures, assessed with ANOVA followed by Newman-Keuls multiple comparison test, $p < 0.05$

Discussion

The course of inflammation depends on the production of many inflammatory proteins. An important source of these mediators are macrophages. Macrophages activated by LPS produce many cytokines, including IL-1β and TNF-α [21]. IL-1β and TNF-α are strong regulatory proteins, which induce several genes thought to participate in tissue destruction [11], infection [2], inflammation and shock [29].

The present study examined the effect of kaempferol, apigenin and resveratrol, on LPS-induced IL-1β and TNF-α gene expression in J774.2 macrophages. We found that treatment of macrophages with kaempferol, apigenin and resveratrol significantly suppressed TNF-α gene expression. However, the expression of IL-1β gene was inhibited only by treatment with kaempferol and apigenin. Why resveratrol was capable to inhibit TNF-α gene expression but not that of IL-1β gene is difficult to explain. These results suggest that apigenin, kaempferol and, partly, resveratrol act on gene expression at the transcriptional level. These observations are in agreement with previously published data. Pretreatment of macrophages with luteolin, quercetin and genistein inhibited LPS-induced TNF-α and IL-6 secretion [30]. Quercetin suppressed TNF-α-induced increase in the IL-8 and MCP-1 mRNA levels in human synovial cells [26]. Bioflavonoids extracted from the bark of *Pinus maritima* significantly inhibited IL-1β gene expression in macrophages [7]. Kaempferol and apigenin exhibited the inhibitory effects on TNF-α-induced E-selectin expression in human endothelial cells [28]. Apigenin inhibited cytokine-induced intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 VCAM-1 and E-selectin expression in human endothelial cells [15]. Additionally, oral administration of luteolin and apigenin could suppress serum TNF-α production in mice. Resveratrol, used at the concentrations present in human plasma following moderate wine consumption, was demonstrated to be an inhibitor of (VCAM-1) gene expression by TNF-α-induced human endothelial cells [5]. Resveratrol also can inhibit the production of interferon-γ (IFN-γ) and IL-2 by splenic lymphocytes and the production of TNF-α and IL-12 by peritoneal macrophages [13].

The mechanism by which apigenin, kaempferol and resveratrol block macrophagic IL-1β gene expression is not fully understood. The positive regulatory domains required for induction of IL-1β and TNF-α by LPS have been defined in their promoters. DNA binding studies demonstrate a requirement for nuclear factor kappa B (NFκB), a pivotal transcription factor in chronic inflammatory diseases [4, 9]. Some flavonoids block cytokine-induced ICAM, VCAM, and E-selectin expression in human endothelial cells by acting on NFκB transcriptional activation [15]. Chrysin supresses IκB degradation and decreases the NFκB level in nuclei of LPS/IFN-γ-stimulated RAW 264.7 macrophages [6]. Luteolin was effective in inhibiting IκB degradation, resulting in suppression of TNF-α and IL-6 production [30]. Resveratrol-increased tyrosine phosphorylation in IκB-α, p50-NFκB, and p65- NFκB suggests the involvement of such alterations in the modulation of NFκB transcriptional activity in human endothelial cells [24]. Resveratrol blocked TNF-α-induced NFκB activation in myeloid (U-9370, lymphoid (Jurkat), and epithelial (HeLa) cells [22]. Apigenin inhibited TNF-α-induced activation of NFκB *via* the IκB pathway in human prostate carcinoma PC-3 cells [27]. In addition, apigenin is proposed as an inhibitor of the mitogen-activated protein-kinase (MAPK) pathway [12]. Thus, it is likely that apigenin, resveratrol and kaempferol reduce transcription of IL-1β and TNF-α in J774.2 macrophages by inhibiting NFκB activation. Additionally, the most potent inhibitory action of apigenin may be a result of simultaneous inhibition of NFκB activation and MAPK activity.

In conclusion, we demonstrated that apigenin, kaempferol and resveratrol inhibited TNF-α gene, but only apigenin and kaempferol suppressed IL-1β gene expression. These results indicate that antiinflammatory action of these compounds, at least partly, may be mediated by transcriptional inhibition of IL-1β and TNF-α gene expression in macrophages.

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Received:
November 4, 2004; in revised form: February 28, 2005.