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Effect of kaempferol on the production and gene expression of monocyte chemoattractant protein-1 in J774.2 macrophages

Jan Kowalski[1], Arkadiusz Samojedny[1], Monika Paul[2], Grażyna Pietsz[3], Tadeusz Wilczok[2]

[1]Department of Genomics, Medical University of Silesia; [2]Department of Molecular Biology and Genetics, [3]Department of Microbiology and Immunology, Medical University of Silesia, Warszawska 14, PL 40-006 Katowice, Poland

Correspondence: Jan Kowalski, e-mail: genomika@slam.katowice.pl

Abstract:
Monocyte chemoattractant protein-1 (MCP-1) is produced by activated macrophages, and is involved in pathogenesis of cardiovascular and neurodegenerative disorders. There is a need to develop drugs that inhibit excessive infiltration of monocytes and lymphocytes to the arterial wall and central nervous system. The aim of this study was to evaluate the effect of kaempferol on the (MCP-1) gene expression and MCP-1 protein release by J774.2 macrophage cultures *in vitro*. Kaempferol given both before and after lipopolysaccharide (LPS) administration reduced secretion of MCP-1. Kaempferol administered before LPS stimulation significantly decreased the number of copies of MCP-1 mRNA. The results suggest that kaempferol inhibits MCP-1 production at the transcriptional level, and that this is an additional anti-inflammatory mechanism of action of this flavonoid.

Key words:
kaempferol, J774.2 macrophages, MCP-1

Introduction

Bioactive compounds are extranutritional constituents that typically occur in small quantities in foods. They are being intensively studied to evaluate their effects on health. The results of many epidemiologic studies have shown protective effects of plant-based diets on cardiovascular and cancer disease [2, 11, 14]. Flavonoids are present in all plants. They have been studied in apple, cocoa, onion, cereals, legumes, nuts, olive oil, fruits, tea and red wine. One of the most important flavonoids is kaempferol. There is evidence to suggest that kaempferol inhibits both oxidative susceptibility of low density lipoprotein (LDL) *in vitro*, and eicosanoid synthesis as well as platelet aggregation [7, 17, 25]. Several *in vitro* experiments have shown that kaempferol functions as chemopreventive agent as well. It has been shown to inhibit cellular events associated with initiation, promotion and progression of carcinogenesis [18, 28].

Monocyte chemoattractant protein (MCP-1) is the most important chemotactic factor involved in this process. It is produced in response to the action of lipopolysaccharide (LPS), oxidized LDL and such cytokines as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) [22, 23]. It has been shown that MCP-1 is a very important mole-

cule in the initial steps of atherosclerotic plaque formation [4, 12]. It seems valuable for developing drugs that inhibit inflammatory process, thus preventing, for example, cardiovascular and autoimmune disease. Such a role would be played by drugs that can inhibit MCP-1 release from macrophages, especially those located in the arterial wall. At present, there is no literature data on the effect of kaempferol on macrophage MCP-1 synthesis and secretion. The abovementioned data prompted us to evaluate the effect of kaempferol on MCP-1 gene expression and release in J.774.2 macrophage cell line.

Materials and Methods

Chemicals

LPS (from *Escherichia coli*, serotype O111:B4), kaempferol, 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, 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 passed three times a week. For the MCP-1 secretion experiments, cells were detached by vigorous pipeting, and after centrifugation, plated using fresh medium. Macrophages, at a density of 5×10^4 cells/ml, were plated in 96-well plates (Becton Dickinson and Company, Franklin Lakes, USA) and incubated 24 h before experiment. Then, the culture medium was replaced with a fresh medium. In one series of experiments, dilutions of kaempferol (0.3, 1, 3, 10, 30 μM) were added to cell cultures 20 min before LPS (1 μg/ml), in other experiment, single concentration of kaempferol (30 μM) was introduced to the cultures 20 min after LPS stimulation. The concentrations of kaempferol used in this investigation were similar to those previously used by other authors [3, 20]. Stimulation was conducted for 24 h, then supernatants were harvested, centrifuged at 2000 rpm for 5 min, and assayed for MCP-1. For the 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 30 μM of kaempferol was added to cultures before LPS administration. After 24 h, total RNA from such cultures was extracted. Cells not treated with LPS or kaempferol were used as control. Kaempferol was dissolved in 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 kaempferol on cell viability was assessed by trypan blue exclusion test. Cell viability was greater than 95% in all performed experiments.

Determination of mRNA for MCP-1

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 described by Chomczyński [6]. RNA concentration was determined by measuring spectrophotometrically the absorbance ($A_{260/280}$) using a range of dilutions (Beckman DU^R 530 Spectrophotometer).

RT-QPCR for detection 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 MCP-1 mRNA was calculated as a number of target cDNA copies per 100 ng of total RNA used in RT-QPCR reaction. The samples were processed twice, and the mean value obtained from the replicate determination 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 house-keeping gene is an appropriate internal standard.

MCP-1 production

The concentration of chemokine in cell culture supernatants was determined using a sandwich ELISA according to manufacturer's instructions. The Mouse MCP-1 Immunoassay Kit was obtained from Biosource International, Inc. Camarillo (USA). The absorbance at 450 nm was read using a microplate reader. This assay has a detection sensitivity of 9 pg/ml. The intra-assay coefficient of variation for MCP-1 was 2.5%.

Statistical analysis

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

Results

To study the effect of LPS on the production of MCP-1 by J774.2 macrophages, cultures were treated with various concentrations of LPS (0.1–2 μg/ml). The culture supernatants were collected 24 h after the stimulation. LPS increased the levels of MCP-1 in a concentration-dependent manner, which indicates that LPS-activated macrophages produce MCP-1 (data not shown).

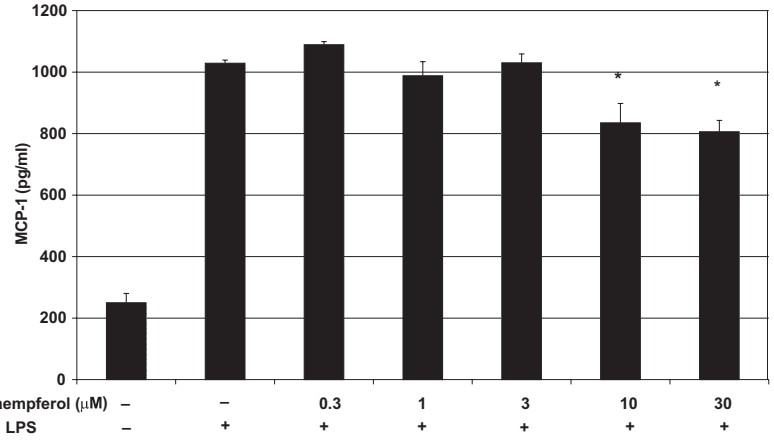

Fig. 1. Concentration-dependent effects of kaempferol given 20 min before LPS (1 μg/ml) on the release of MCP-1 in the J774.2 cell cultures. The cultures were incubated with the indicated concentrations of kaempferol for 24 h. The values are the mean ± SE of two independent experiments, each with four determinations. * – significantly different from the vehicle (LPS + medium)-treated cultures, assessed with ANOVA followed by Newman-Keuls multiple comparison test, $p < 0.05$

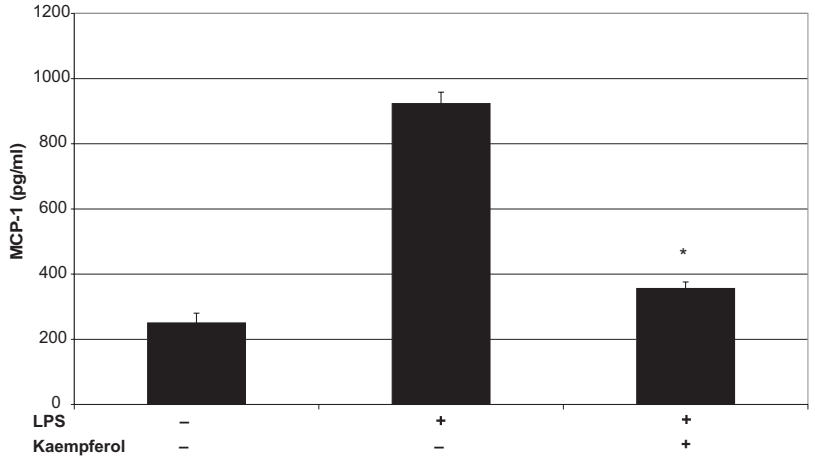

Fig. 2. The effect of kaempferol given 20 min after LPS (1 μg/ml) administration on the release of MCP-1 in the J774.2 macrophage cultures. The cultures were incubated with kaempferol at a dose of 30 μM for 24 h. The values are the mean ± SE of two independent experiments, each with four determinations. * – significantly different from the vehicle (LPS + medium)-treated cultures, assessed with ANOVA followed by Newman-Keuls multiple comparison test, $p < 0.05$

Effect of kaempferol on LPS-induced MCP-1 release

In the first study, kaempferol was added to cultures 20 min before administration of LPS. Twenty-four hour exposure to the higher concentrations of kaempferol (30 and 10 μM) caused a dose-dependent decrease in MCP-1 secretion, while 3, 1 and 0.3 μM doses were ineffective (Fig. 1). In the next experiments, kaempferol was investigated at a single 30 μM concentration using one time point. Similarly, MCP-1 release decreased after 24-h exposure to 30 μM of kaempferol added to cultures 20 min after LPS treatment (Fig. 2).

Effect of kaempferol on LPS-induced MCP-1 mRNA expression

Kaempferol at a 30 μM dose added to the J774.2 macrophage cultures caused significant decrease in the number of MCP-1 mRNA copies (Fig. 3).

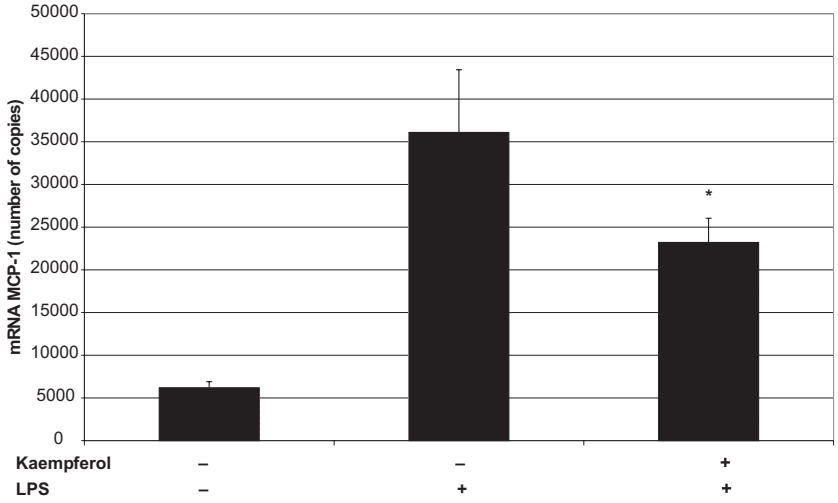

Fig. 3. The effect of kaempferol given 20 min before administration of LPS (1 μg/ml) on the MCP-1 gene expression in J774.2 macrophages cultures. The cultures were incubated with 30 μM of kaempferol for 24 h. The values are the mean ± SE of two independent experiments, each with three determinations. * – significantly different from the vehicle (LPS + medium)-treated cultures, assessed with ANOVA followed by Newman-Keuls multiple comparison test, $p < 0.05$

Discussion

The discovery of molecules released by activated macrophages in the course of inflammatory, autoimmune and neurodegenerative disorders has prompted the search for agents inhibiting the release of these molecules [8, 9, 13]. Kaempferol used in this study decreased both MCP-1 gene expression and protein secretion by LPS-activated macrophages. As MCP-1 strongly induces inflammatory process, the results of this study suggest that kaempferol has an anti-inflammatory potential. In the available literature there is no data concerning direct effects of kaempferol on MCP-1 synthesis and production.

It is not surprising that kaempferol inhibits macrophage activity. A number of reports have indicated that flavonoids are immunosuppressive for lymphocytes and macrophages. Blonska et al. [4] found that kaempferol and other flavone derivatives such as chrysin and quercetin supressed IL-1β, nitric oxide (NO) release and gene expresion in J774A.1 macrophages. Krol et al. [16] observed diminution of free radical and nitrite production in neutrophils and macrophages by flavonoids. Many authors documented inhibitory effect of kaempferol on the inducible nitric oxide synthase expression in J774.2 macrophages [17, 20] and cyclooxygenase-1,2 expression in RAW 264.7 cells [26]. Okamoto et al. [19] found that kaempferol inhibited release of Th1 cytokine IFN-γ and IL-2 in T lymphocyte cultures and shifted TH1/TH2 to TH2 activation. Such flavonoids as kaempferol and quercetin inhibited release of hydrogen peroxide (H_2O_2) from neutrophils [29].

The mechanism by which kaempferol inhibits MCP-1 release and gene expression is unknown. The production and release of various cytokines (TNF-α, IL-1β, MCP-1) by LPS-activated macrophages is mediated by the induction of such transcriptional factors as nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) [10, 21]. NF-κB is sequestered in the cytoplasm as an inactive complex with the inhibitory protein IκB family. Upon stimulation its inhibitory subunit is phosphorylated and degraded. Then, the active NF-κB is translocated to the nucleus [1]. It was found that flavones (chrysin, luteolin, oroxylin A and myricetin) inhibited NF-κB activation [3, 5, 24, 27]. Thus, it is likely that kaempferol inhibits MCP-1 production and synthesis by inhibiting NF-κB activation.

The role of MCP-1 in atherogenesis is well documented. On the other hand, some epidemiologic studies have reported association between protective action of flavonoids and cardiovascular disease. The results of the present study may support the view about health benefits of flavonoids and flavonoid-rich diet.

Summing up, in our study we demonstrate for the first time that kaempferol is an inhibitor of LPS-induced MCP-1 release in J774.2 macrophages. This action is mediated by MCP-1 gene transcription. The influence on MCP-1 gene transcription and protein release constitutes an additional mechanism of the anti-inflammatory action of kaempferol.

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