Article

The Inhibitory Effect of Quercetin on IL-6 Production by LPS-Stimulated Neutrophils

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Quercetin is a herbal flavonoid derived from various foods of plant origin and plays a role in anti-inflammation. Although a number of researches in the field have been done, the mechanism of anti-inflammatory effect of quercetin should be further clarified. In the present study, we investigated the effects of quercetin on IL-6 production by LPS-stimulated neutrophils in human. Neutrophils were were pre-treated with quercetin at the final concentrations of ranging from 0-80 μM for 30 min, or not treated, and then incubated in the presence or absence of lipopolysaccharide (LPS) at a final concentration of 100 ng/ml for indicated time. The secretion level of IL-6 in the culture supernatants was assayed by ELISA, the intracellular level of IL-6 was detected by flow cytometry and the expression of IL-6 mRNA was analyzed by RT-PCR. The experiment results showed that neutrophils cultured with medium or quercetin alone did not express IL-6, but LPS (100 ng/ml) induced IL-6 expression of neutrophils. However, after pre-treatment of neutrophils with quercetin (40 μM) for 30 min, the inducible effects of LPS on the increase of IL-6 secretion, intracellular IL-6 level and IL-6 mRNA expression by neutrophils were abrogated. IL-6 is one of the important pro-inflammatory factors, especially in early phage of inflammation. Thus, our data suggested that quercetin might exert its anti-inflammatory effect through negatively modulating pro-inflammatory factors, such as IL-6. The inhibitory effects of quercetin on IL-6 production by neutrophils may provide a theoretical basis on future therapy of inflammation. *Cellular & Molecular Immunology*. 2005;2(6):455-460.

Introduction

Flavonoids are polyphenolic compounds found in various foods of plant origin. It has been estimated that humans consuming high fruit and vegetable diets may ingest up to 1 g of these compounds daily (1-3). Quercetin (3, 3', 4', 5, 7-tetrasulphate, molecular structure shown in Figure 1), the major representative of the flavonol subclass of flavonoids, is an integral part of the human diet and the average human intake has been estimated to be ~25 mg/day. Much of the evidence supporting the beneficial role of quercetin for health comes from epidemiological literature. Quercetin (Que), as an antioxidants, enhance some parameters of immune function when added to isolated immune cells *in vitro* or when given as supplements to humans or animals *in vivo*

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(4-6). One potential mechanism is the effect of antioxidants on the production of immunoregulatory molecules such as cytokines. Cytokines, such as interleukin-1 (IL-1), tumor necrosis factor (TNF), transforming growth factor-α (TGF-α), and IL-6 are induced in response to invading pathogenic bacteria and can mediate and inhibit cellular injury and repair (7, 8).

In the late 1960s, Kishimoto et al. discovered activities in the culture supernatant of T cells that induced proliferation and differentiation of B cells. The cytokine was named as IL-6. IL-6 is derived from not only immunocompetent cells but also other cells, such as neoplastic cells, endothelial cells, and muscle fibers (9). IL-6 turned out to play additional roles, including myeloma growth factor and hepatocyte-stimulating factor activities. More importantly, IL-6 was involved in a number of chronic inflammatory diseases, such as rheumatoid arthritis and Castleman's disease. Palma et al. reported that polymorphonuclear neutrophils (PMN) also produced IL-6 after lipopolysaccharide (LPS) stimulation (10). IL-6 is an immunoregulatory cytokine that activates a cell-surface signaling assembly composed of IL-6, the IL-6 α-receptor (IL-6Rα), and the shared signaling receptor gp130 (11). It is well known that IL-6 plays an important role in acute-phase inflammatory reaction through accelerating release of PMN from the bone marrow, enhancing PMN adhesiveness and recruitment, activating PMN, and enhancing release of
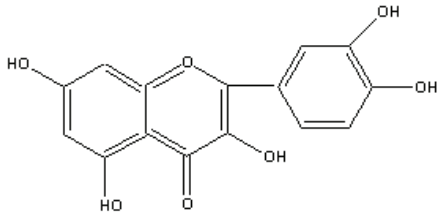
Figure 1. The molecular structure of quercetin.

cytotoxic contents (12-15). Neutrophils are terminally differentiated cells and most important inflammatory cells. On one hand, activated neutrophils defense against pathogenic micro-organs; on the other hand, activated neutrophils may aggravate constitutive damage of auto-tissues through producing various inflammatory mediators (16, 17). Therefore, the level of IL-6 in body or in local inflammatory sites may affect the solution of inflammation. In normal situation of body, bone marrow plasma and peripheral blood serum have no or very low levels of IL-6. However, in infectious situation of pathogenic bacteria, IL-6 producing cells synthesize and release IL-6 in response to the stimuli. LPS, a potential pro-inflammatory factor derived from Gram-negative bacteria, induces IL-6 production by neutrophils and expedites neutrophil recruitment by activating Toll-like receptor 4 (TLR4) (18). LPS-activated neutrophils release harmful mediators - cytotoxic contents, leading to the exacerbation of inflammation in body or in local inflammatory sites (19-23). Since quercetin plays a role in anti-inflammation, it might be reasonable to speculate upon the inhibitory effects of quercetin on LPS-induced IL-6 production by neutrophils. Thus, we conducted the present study to evaluate the effects of quercetin on LPS-induced IL-6 production by neutrophils in human.

Materials and Methods

Reagents
Que, LPS (*Escherichia coli* O26:B6), and Dextran T-500 were purchased from Sigma. PE-conjugated rat anti-human IL-6 and PE-conjugated isotype control rat IgG1 were purchased from PharMingen. ELISA detection kit for IL-6 was purchased from R&D Systems. RNeasy kits were purchased from Qiagen. Advantage RT-for-PCR for cDNA application was purchased from Clontech.

Cell isolation
Heparinized peripheral blood was obtained from healthy volunteers. Neutrophils were isolated using 3% dextran sedimentation followed by density gradient centrifugation with Ficoll-Paque (Phamacia). Erythrocytes were eliminated by hypotonic lysis in Gey's solution. After washing with phosphate-buffered saline (PBS), neutrophils were suspended in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Bioserum), 10 mM N-2-

hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The neutrophils were 96% pure on morphologic examination and 98% viability on the trypan blue dye exclusion assay.

IL-6 concentrations of cultured supernatant
Neutrophils were cultured with or without 100 ng/ml LPS for indicted time. Some wells with neutrophils were pre-treated with quercetin at various concentrations (ranging from 0-80 μM) for 30 min and then 100 ng/ml LPS was added into the cultures for indicted time. The supernatant of cultures was collected and aliquots of IL-6 were snap-frozen and stored at -80°C until analyzed by ELISA.
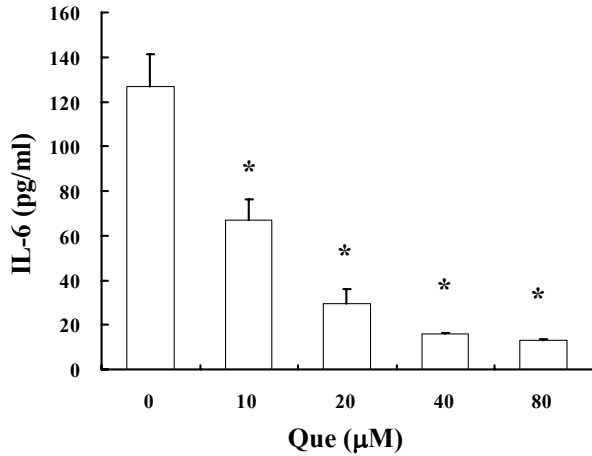
Detection of cellular IL-6 by FACS
Neutrophils were incubated with or without LPS at a final concentration of 100 ng/ml for 6 h. Some were pre-treated with quercetin (40 μM) for 30 min and then 100 ng/ml LPS was added into the cultures for further 6 h. The cells were washed twice and 4×10^5 cells were resuspended in PBS containing 2.5% FCS. The intracellular level of IL-6 was determined by FACS analysis. Briefly, the cultured neutrophils were collected and fixed by 4% formaldehyde. The neutrophil membrane was perforated by 95% cold ethanol. Then, the neutrophils were labeled with PE-conjugated anti-IL-6 (rat anti-human) or isotype control rat IgG1. Data were acquired from 10,000 gated events in each assay, and mean cell fluorescence of forward and side angle scatter-gated neutrophils was assessed using a FACScan flow cytometer (BD Biosciences).

RT-PCR
Neutrophils were incubated with or without LPS at a final concentration of 100 ng/ml for 6 h. Some wells with neutronphils were pre-treated with quercetin (40 μM) for 30 min and then 100 ng/ml LPS was added into the cultures for further 3 h. IL-6 mRNA expression of the cultured neutrophils was measured by RT-PCR using sets of primers specific for human IL-6, as previously described (24). Briefly, total cellular RNA was isolated from cultured neutrophils using RNeasy kits, and cDNA was prepared using Advantage RT-for-PCR. IL-6 RNA was amplified through 30 cycles of 60 s at 95°C, 30 s at 63°C, and 30 s at 72°C. Every PCR tube contained 50 pmol/L each of the following primers: 5'-TCA ATG AGG AGA CTT GCC TG-3' plus 5'-GAT GAG TTG TCA TGT CCT GC-3' (for IL-6) and 5'-CTC ACG TCA TCC AGC AGA GA-3' plus 5'-CAA GCT TTG AGT GCA AGA GA-3' (for β_2 microglobulin control). The expected PCR product obtained with these IL-6 primers was 261 base pairs (bp) in size. The PCR products were electrophoresed on 2% agarose gels and the gels were stained with ethidium bromide.

Statistical analysis
Results are expressed as the mean ± standard deviation. Statistical analysis was performed using the paired Student's
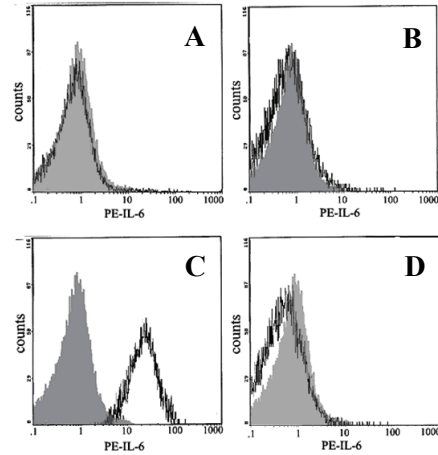
Figure 2. Effect of various concentrations of quercetin on IL-6 secretion by LPS-stimulated neutrophils. Neutrophils were pre-treated with quercetin at the final concentration ranging from 0-80 μM for 30 min and the cells were incubated with LPS at a final concentration of 100 ng/ml for 16 h. Subsequently, the culture supernatants were collected and IL-6 concentrations were detected by ELISA. The results are mean ± SD of four separate experiments using neutrophils isolated from four different healthy donors. *Compared with LPS alone, $p < 0.05$.

Figure 3. Detection of intracellular IL-6 level of LPS-stimulated PMN by FACS. Neutrophils were incubated with or without LPS at a final concentration of 100 ng/ml for 6 hours. Some wells with neutrophils were pre-treated with quercetin (40 μM) for 30 min and then 100 ng/ml LPS was added into the cultures for further 6 h. The cells were harvested and labeled with PE-conjugated anti-IL-6 (rat anti-human) or isotype control rat IgG1. The intracellular level of IL-6 was determined by FACS analysis. The results were representative of four separate experiments using neutrophils isolated from four different healthy donors. (A) PMN + medium alone, (B) PMN + Que, (C) PMN + LPS, (D) PMN + Que + LPS.

t-test. $p < 0.05$ was considered significant.

Results

Effect of quercetin on LPS-induced IL-6 secretion by neutrophils
Neutrophils were incubated in the presence or absence of LPS at a final concentration of 100 ng/ml for 4, 6, 8, 16, 24 h. Some were pre-treated with quercetin at the final con-centrations of ranging from 0-80 μM. As shown in Figure 2, the optimum concentration of quercetin that inhibited LPS-induced IL-6 secretion by neutrophils is 40 μM. As shown in Table 1, LPS-stimulated neutrophils secreted IL-6 from 4 to 24 h and the peak of IL-6 secretion by LPS-stimulated neutrophils was at 16 h. However, the inducible effect of LPS on IL-6 production by neutrophils was abrogated after the pre-treatment of neutrophils with quercetin (40 μM) for 30 min ($p < 0.05$). Quercetin alone had no effect on IL-6

production by neutrophils.

Effect of Quercetin on intracellular IL-6 by LPS-stimulated neutrophils
In order to confirm the inhibitory effect of quercetin on IL-6 production by LPS-stimulated neutrophils, we further detected the intracellular IL-6 level by using FACS analysis. Neutrophils were incubated in the presence or absence of LPS at a final concentration of 100 ng/ml for 6 hours. Some wells with neutrophils were pre-treated with quercetin at the final concentration of 40 μM for 30 min and then 100 ng/ml LPS was added into the cultures for further 6 h. The cultured neutrophils were harvested and the membrane was perforated by 95% cold ethanol. Then, the intracellular level of IL-6 was determined by FACS analysis. As shown in Figure 3, the cultured neutrophils without stimulation of LPS did not express IL-6 and quercetin alone had no effect on IL-6

Table 1. Time course of effect of quercetin on IL-6 secretion by LPS-stimulated PMN

Group	4 h	6 h	8 h	16 h	24 h
PMN + medium	1.34 ± 0.16	1.41 ± 0.18	1.87 ± 0.20	1.19 ± 0.22	1.30 ± 0.12
PMN + Que	1.95 ± 0.18	1.23 ± 0.27	1.31 ± 0.14	2.12 ± 0.24	1.40 ± 0.16
PMN + LPS	10.30 ± 1.22*	63.34 ± 5.94*	82.18 ± 10.36*	130.04 ± 13.84*	130.55 ± 14.08*
PMN + Que + LPS	5.98 ± 0.66[#]	6.03 ± 0.54[#]	6.32 ± 0.72[#]	5.98 ± 0.61[#]	6.15 ± 0.78[#]

n = 6. No IL-6 was detected in fresh PMN. *compared with negative control, $p < 0.05$; [#]compared with LPS 100 ng/ml alone, $p < 0.05$.
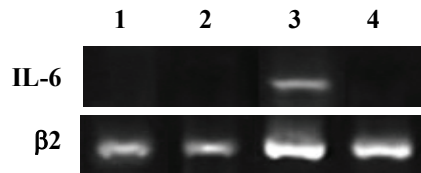
Figure 4. Analysis of IL-6 mRNA expression of LPS-stimulated PMN by RT-PCR. Neutrophils were incubated with or without LPS at a final concentration of 100 ng/ml for 6 h. Some wells with neutrophils were pre-treated with quercetin (40 μM) for 30 min and then 100 ng/ml LPS was added into the cultures for further 3 h. IL-6 mRNA expression of the cultured neutrophils was measured by RT-PCR using sets of primers specific for human IL-6. The results were representative of four separate experiments using neutrophils isolated from four different healthy donors. Lane 1, control; Lane 2, Que; Lane 3, LPS; Lane 4, Que + LPS.

expression (Figures 3A and 3B). After LPS stimulation, the neutrophils expressed intracellular IL-6 (Figure 3C). However, pre-treatment of neutrophils with quercetin abrogated the inducible effect of LPS on IL-6 production by the neutrophils (Figure 3D).

Effect of quercetin on IL-6 mRNA expression of LPS-stimulated neutrophils
We further confirmed the IL-6 mRNA expression of the neutrophils by using RT-PCR. As shown in Figure 4, each experiment groups of neutrophils expressed mRNA of β_2 microglobulin, as a positive control. The neutrophils without LPS stimulation or with stimulation of quercetin alone did not express IL-6 mRNA. The neutrophils treated with LPS (100 ng/ml) alone for 3 h expressed IL-6 mRNA, but the effects of LPS on the expression of IL-6 mRNA were inhibited after pre-treatment of the neutrophils with quercetin.

Discussion

Cytokines, secreted from activated immune cells and other cells, play important roles in the regulation of inflammatory responses by controlling proliferation, differentiation, and effective function of immune cells (25). IL-6, a pro-inflammatory cytokine, has been implicated in many inflammatory diseases in both adults and neonates (26). One of the mechanisms of IL-6 that regulate inflammatory reaction is mediated by affecting the functions and life-span of neutrophils (27-30). Neutrophils play a very important role in the cellular pathology of inflammatory diseases. Being stimulated by pro-inflammatory factors, such as LPS, accumulated neutrophils are activated and exert the function of defence against pathogenic micro-organs through the secretion of harmful mediators, such as oxygen radicals, lysosomal enzymes, and cytokines. In the process, the surrounding tissue injury may occur if the harmful mediators are excessively produced. High levels of inflammatory cytokines and neutrophil-derived toxic factors in circulation or in local inflammatory sites may lead to the aggravation of

local inflammation and the occurrence of severe diseases, including systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), etc. Therefore, it is important for the strategy of anti-inflammation to reduce the production of pro-inflammatory cytokines and mediators, and the susceptibility of inflammatory cells to stimuli.

The present investigation exhibits the inhibitory effect of quercetin on IL-6 production by LPS-stimulated neutrophils. These results indicate that quercetin might decrease the susceptibility of neutrophils to pro-inflammatory factors (e.g., LPS), or interfere with the response of neutrophils to pro-inflammatory factors. The inhibitory effect of quercetin on LPS-induced IL-6 production by neutrophils might partially eliminate the accumulation of neutrophils into the inflamed sites through inhibiting neutrophil activation and neutrophil adhesion to endothelial cells. Thus, this process is much beneficial to the resolution of inflammation. p38-mitogen activated protein kinase (p38-MAPK) has been implicated as an important regulator of the coordinated release of cytokines by immunocompetent cells and the functional response of neutrophils to inflammatory stimuli (31-33). Many different stimuli can activate p38 MAPK, including LPS, cytokines such as TNF-α and IL-1, growth factors, and stresses such as heat shock, hypoxia, and ischemia/reperfusion (34). In addition, p38 MAPK positively regulates a variety of genes involved in inflammation, such as TNF-α, IL-1, IL-6, IL-8, cyclooxygenase-2, and collagenase-1 and -3 (35, 36). LPS is also capable of delaying spontaneous apoptosis and prolonging functional lifespan of neutrophils through activating of MAPK (32). Recently, Schroeter et al. reported that one of the effects of quercetin is likely to involve an apoptotic mode of cell death in which members of the MAPK family may play a role (37, 38). Thus, it is likely that quercetin might inhibit LPS-induced IL-6 production by neutrophils through preventing LPS-stimulated activation of MAPK, which may contribute to the anti-inflammatory effect of quercetin.

Fruits and vegetables are rich in quercetin. High fruit and vegetable diets influence the appearance of pro-inflammatory cytokines in the cerebellum of aged rats, inducing an anti-inflammatory response in the aged rat cerebellum (39). Furthermore, the data shown in the present study suggest that quercetin may decrease the susceptibility of neutrophils to pro-inflammatory factors. Our data raise the possibility that some of the beneficial effects of diets rich in fruits and vegetables are related to the prevention of body from pro-inflammatory cytokines and cytotoxin mediators.

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