



Quercetin-3-O-(2"-galloyl)- α -L-rhamnopyranoside inhibits TNF- α -activated NF- κ B-induced inflammatory mediator production by suppressing ERK activation

Chung Soo Lee ^{a,*}, Eun Byul Jeong ^a, Yun Jeong Kim ^a, Min Sung Lee ^b, Seong Jun Seo ^c, Kwan Hee Park ^d, Min Won Lee ^d

^a Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, South Korea

^b Department of Internal Medicine, Soonchunhyang University Hospital, Bucheon City, Kyung-Gi-Do 420-767, South Korea

^c Department of Dermatology, Chung-Ang University Hospital, Seoul 156-755, South Korea

^d Pharmacognosy Laboratory, College of Pharmacy, Chung-Ang University, Seoul 156-756, South Korea

ARTICLE INFO

Article history:

Received 7 January 2013

Accepted 1 May 2013

Available online 14 May 2013

Keywords:

Keratinocytes

Tumor necrosis factor- α

QGR

Inflammatory mediator production

ERK pathway

ABSTRACT

Quercetin and its derivatives have anti-inflammatory and anti-oxidant effects. However, the effect of quercetin-3-O-(2"-galloyl)- α -L-rhamnopyranoside (QGR), a new quercetin derivative, on the tumor necrosis factor (TNF)- α -stimulated production of inflammatory mediators in keratinocytes is unclear. In addition, the effect of QGR on the ERK and NF- κ B-mediated inflammatory process has not been studied. In human keratinocyte HaCaT cells, we investigated the effect of QGR on the TNF- α -stimulated production of inflammatory mediators in relation to the nuclear factor (NF)- κ B, which regulates the transcription genes involved in immune and inflammatory responses. QGR inhibited the TNF- α -stimulated production of cytokines and chemokines in HaCaT cells. QGR, dexamethasone, cyclosporine A, Bay 11-7085 (an inhibitor of NF- κ B activation) and cell signaling ERK inhibitor attenuated the TNF- α -induced formation of inflammatory mediators and activation of the NF- κ B and ERK. Unlike other compounds, dexamethasone and cyclosporine A did not reduce formation of reactive oxygen species. The results show that QGR may attenuate TNF- α -stimulated inflammatory mediator production in HaCaT cells by suppressing the activation of the ERK-mediated NF- κ B pathway that is mediated by reactive oxygen species. Additionally, QGR may exhibit a preventive effect against the proinflammatory mediator-induced skin diseases by inhibiting the activation of the ERK and NF- κ B pathways.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Tumor necrosis factor (TNF)- α is involved in the inflammation and immune response in the skin [1,2]. TNF- α induces the activation of T cells, production of cutaneous T cell-attracting chemokines in keratinocytes and prolongation of skin inflammation [3,4]. The effect of TNF- α is mediated by the Ras/Raf/MEK/ERK signaling pathway [5]. The activation of ERK pathway induces nuclear factor (NF)- κ B activation by phosphorylating and activating inhibitory κ B kinase [5,6]. NF- κ B regulates genes responsible for the innate and adaptive immune responses as well as inflammation [7]. The activation of NF- κ B is triggered by a variety of agents, including cytokine TNF- α , oxidative stress and DNA damage [8,9]. Reactive oxygen species play a critical role in the physiological regulation of cellular functions and are involved in pathologic conditions, such as inflammation and cell

death [10]. They have been shown to induce the activation of NF- κ B [8,11,12].

The flavonoid quercetin and its derivatives have anti-oxidant and anti-inflammatory effects [13,14]. Quercetin and quercetin hydrate reduce the oxidative stress-induced cell death in keratinocytes through their anti-oxidant action [13]. Quercetin inhibits the UV irradiation-induced inflammatory cytokine production in primary human keratinocytes by suppressing the NF- κ B pathway [14]. The derivative quercetin 3-O- α -L-arabinofuranoside inhibits the lipopolysaccharide-stimulated expression of nitric oxide synthase and cyclooxygenase-2 and the production of PGE₂ in HaCaT cells [15]. However, flavonoids are demonstrated to exhibit a different effect on the cytokine-induced productions of the proinflammatory mediator IP-10 and IL-8 [16]. Flavonoid rutin and its aglycon quercetin significantly inhibit the TNF- α and IFN- γ -induced release of IP-10, whereas they do not affect cytokine-induced IL-8 release. Quercetin attenuates the H₂O₂-induced mitochondrial and cell damage and death in the human keratinocyte cell line NCTC 2544 cells and HaCaT cells [13,17]. On the contrary, it has been shown that

* Corresponding author. Tel.: +82 2 820 5659; fax: +82 2 813 5387.

E-mail address: leecs@cau.ac.kr (C.S. Lee).

polyphenols, including quercetin does not prevent UVC-induced cell death in human HaCaT cells [18].

Quercetin and its derivatives have anti-inflammatory and anti-oxidant effects. Nevertheless, they exhibit a variable effect on the cytokine production and cell damage induced by toxic insults. Furthermore, the effect of quercetin-3-O-(2''-galloyl)- α -L-rhamnopyranoside (QGR), a new quercetin derivative, on the TNF- α -stimulated production of inflammatory mediators in relation to the NF- κ B-mediated inflammatory process has not been studied. In addition, it is also unclear whether the effect of QGR on the TNF- α -induced activation of NF- κ B is mediated by its effect on the ERK pathway. Therefore, using the human keratinocyte HaCaT cells, we investigated the effect of QGR on the TNF- α -induced inflammatory mediator production in relation to the ERK and NF- κ B pathways. Then, we assessed the effect and action of QGR as a preventative compound to treat inflammatory skin diseases, including atopic dermatitis.

2. Materials and methods

2.1. Materials

Human tumor necrosis factor (TNF)- α (recombinant *E. coli*), Bay 11-7085 ((2E)-3-[[4-(1,1-Dimethylethyl)phenyl]sulfonyl]-2-propenenitrile), ERK activation inhibitor peptide (type I, Ste-MEK1₁₃), dexamethasone, cyclosporine A and horseradish peroxidase-conjugated anti-mouse IgG were purchased from EMD-Calbiochem. Co. (La Jolla, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for human CXCL1/IL1 β , human IL6, human thymus and activation-regulated chemokine (CCL17), human CTACK/CCL27, and human/mouse/rat phospho-ERK1/ERK2 were purchased from R&D systems, Inc. (Minneapolis, MN, USA). Antibodies for NF- κ B p65 (F-6), NF- κ B p50 (4D1), phospho-I κ B- α (B-9) and β -actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). TransAM™ NF- κ B assay kit was purchased from Active Motif® (Carlsbad, CA, USA). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), N^G-methyl-L-arginine acetate salt (L-NMMA) and other chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

2.2. Extraction, isolation and structural identification of quercetin-3-O-(2''-galloyl)- α -L-rhamnopyranoside

Quercetin-3-O-(2''-galloyl)- α -L-rhamnopyranoside (QGR) was isolated from the leaves of *Acer ginnala* Maxim collected from Korea National Arboretum (Pocheon, Korea, Fig. 1). The leaves (1 kg) of *A. ginnala* were extracted several times with 80% methanol at room temperature. After the removal of methanol under vacuum, aqueous solution was

filtered through filter paper (Tokyo Roshi Kaisha Ltd., Japan). The solution filtered was concentrated (227.5 g), applied to the column filled with Sephadex LH-20 (25–100 μ m, 2 kg, 10 $\times$ 80 cm, GE Healthcare Bio-Science AB, Uppsala, Sweden) and the solution then was eluted with H₂O contained methanol (30 to 100% gradient), which afforded 3 sub-fractions. The fraction 3 (43 g) was applied to the columns filled with MCI-gel CHP20P (75–150 μ m, 600 g, 5 $\times$ 60, Mitsubishi Chemical, Tokyo, Japan) with a gradient elution system of water–methanol. From these procedures, quercetin-3-O-(2''-galloyl)- α -L-rhamnopyranoside (QGR, 850 mg) was yielded. Purity of QGR was analyzed by high performance liquid chromatography (Waters 600 system, Milford, MA, USA). The yield had approximately 98% purity.

The structural identity of QGR was characterized by spectral analyses. The 1D nuclear magnetic resonance (NMR) findings such as, ¹H (600 MHz) and ¹³C (150 MHz) NMR were recorded with Gemini 2000 (Varian, Palo Alto, CA, USA). Low-resolution fast atom bombardment mass spectrum (LRFAB-MS) was recorded with JMS-600W (JEOL, Tokyo, Japan).

Yellow amorphous powder, negative FAB-MS: m/z 599 [M-H][−]. ¹H NMR (600 MHz, CD₃OD): δ 1.03 (3H, d, J = 5.4 Hz, H-6''), 3.46–3.48 (2H, m, H-4'',5''), 4.02 (1H, dd, J = 3.6, 9.0 Hz, H-3''), 5.49 (1H, d, J = 1.5 Hz, H-1''), 5.62 (1H, dd, J = 1.5, 3.6 Hz, H-2''), 6.19 (1H, d, J = 1.8 Hz, H-6), 6.37 (1H, d, J = 1.8 Hz, H-8), 6.94 (1H, d, J = 8.4 Hz, H-5''), 7.07 (2H, s, H-2'', 6''), 7.34 (1H, dd, J = 2.4, 8.4 Hz, H-6'), 7.37 (1H, d, J = 2.4 Hz, H-2'). ¹³C NMR (125 MHz, CD₃OD): δ 16.4 (C-6''), 69.3 (C-3''), 70.8 (C-5''), 72.1 (C-2''), 72.4 (C-4''), 93.3 (C-8), 98.5 (C-6), 99.1 (C-1''), 104.4 (C-10), 108.9 (C-2'', 6''), 115.1 (C-5'), 115.5 (C-2'), 119.8 (C-1'''), 121.4 (C-6'), 121.5 (C-1'), 134.2 (C-3), 138.5 (C-4'''), 145.0 (C-3''', 5''), 148.4 (C-4'), 157.1 (C-9), 157.9 (C-2), 161.7 (C-5), 164.4 (C-7), 166.1 (C-7'''), 178.0 (C-4).

2.3. Keratinocyte culture

HaCaT cells, immortalized human keratinocyte cell lines, were provide from Dr. Seo (Chung-Ang University Hospital, Seoul, South Korea) and cultured in keratinocyte-SFM supplemented with DMEM medium (high glucose) supplemented with 2 mM L-glutamine and 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin.

2.4. Immunoassays for cytokines and chemokines

HaCaT cells (1 $\times$ 10⁵ cells/300 μ l for the cytokine assay or 5 $\times$ 10⁵ cells/400 μ l for the chemokine assay) were grown in a 24-well plate and treated with 10 ng/ml TNF- α for 24 h. After centrifugation at 412 $\times$ g for 10 min, the amounts of IL-1 β , IL-6, CCL17 and CCL27 in the culture supernatants were analyzed using the corresponding ELISA kits, according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader (Magellan, TECAN, Salzburg, Austria).

2.5. Preparation of cytosolic and nuclear extracts

HaCaT cells (5 $\times$ 10⁶ cells/ml) were treated with TNF- α for 15 min at 37 °C. Keratinocyte cytosolic and nuclear extracts were prepared according to the previously reported method [19]. HaCaT cells were harvested by centrifugation at 412 $\times$ g for 10 min and washed twice with PBS. The cells were suspended in 400 μ l lysis buffer (10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 1 mM sodium orthovanadate, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin and 10 mM HEPES-KOH, pH 7.8) and were allowed to swell on ice for 15 min. Next, 25 μ l of a 10% Nonidet NP-40 solution (final concentration approximately 0.6%) was added, and the tubes were vigorously vortexed for 10 s. The homogenates were centrifuged at 12,000 $\times$ g for 10 min at 4 °C. The supernatants were stored as cytoplasmic extracts and kept at −70 °C. The nuclear pellets were resuspended in 50 μ l of an ice-cold hypertonic solution containing 5% glycerol and 0.4 M NaCl

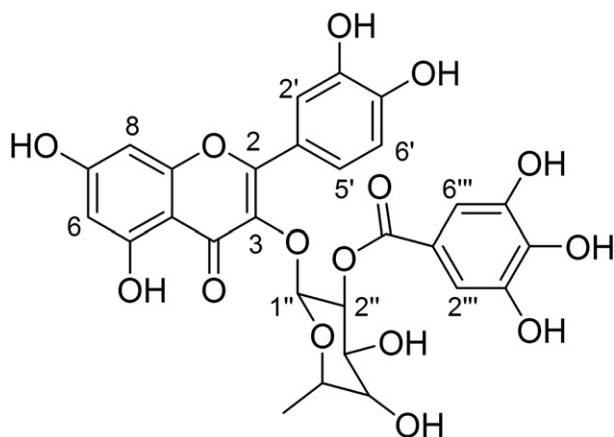


Fig. 1. Chemical structure of QGR.

lysis buffer. The tubes were incubated on ice for 30 min and then centrifuged at $12,000 \times g$ for 15 min at 4°C . The supernatants were collected as the nuclear extracts and stored at -70°C . Protein concentrations were determined using the Bradford method according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Western blot for phospho-I κ B and NF- κ B levels

The cytosolic and nuclear extracts were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min. Samples ($30 \mu\text{g}$ protein/well) were loaded onto each lane of 12% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (GE Healthcare Chalfont St. Giles, Buckinghamshire, UK). Membranes were blocked for 2 h in TBS (50 mM Tris-HCl, pH 7.5 and 150 mM NaCl) containing 0.1% Tween 20 and 5% non-fat dried milk. Each membrane was labeled with the appropriate antibody (NF- κ B p65, NF- κ B p50, phospho-I κ B- α , ERK 1/2 or β -actin) overnight at 4°C with gentle agitation. After 4 washes in TBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG for 2 h at room temperature. Membranes were treated with SuperSignal® West Pico chemiluminescence substrate and protein bands were visualized by detecting the enhanced chemiluminescence in an appropriate image analyzer (Lite for Las-1000 plus version 1.1; Fuji Photo Film Co., Tokyo, Japan).

2.7. Assay for DNA binding activity of NF- κ B

The binding of NF- κ B p65 to DNA was determined according to the user's manual for the transAM™ NF- κ B kit. HaCaT cells (2×10^6 cells/ml) were treated with 10 ng/ml TNF- α for 15 min. Nuclear extracts were prepared according to the procedure described in the Active Motif® protocol and added to a 96-well plate to which oligonucleotides containing an NF- κ B consensus binding site (5'-GGGACTTCC-3') are immobilized. The active NF- κ B p65 bound to DNA was exposed to a primary antibody specific for NF- κ B p65, which was reacted with anti-rabbit horseradish peroxidase-conjugated IgG. Next, the color developer and stop solutions were added to the plate. The absorbance of samples was measured at 450 nm with a reference wavelength of 655 nm in a microplate reader.

2.8. Enzyme-linked immunosorbent assays for ERK

HaCaT cells (1×10^6 cells/ml) were treated with TNF- α for 4 h. Cells were harvested by centrifugation at $412 \times g$ for 10 min, washed twice with PBS and suspended in the lysis buffer provided by R&D systems for whole cell lysates. The homogenates were centrifuged at $2000 \times g$ for 5 min and the supernatant was used for the ELISA. The amounts of phosphorylated ERK1/2 were determined according to the manufacturer's instructions for the immunoassays. The supernatants were exposed first to the antibody for the phosphorylated form of the kinase, next to the biotinylated detection antibodies, and finally to streptavidin-horseradish-peroxidase. The absorbance was measured at 405 nm.

2.9. Measurement of intracellular reactive oxygen species formation

The dye DCFH₂-DA, which is oxidized to fluorescent dichlorofluorescein (DCF) by hydroperoxides, was used to measure relative levels of cellular peroxides [20]. HaCaT cells (1×10^5 cells/400 μl in 24-well plate) were treated with TNF- α for 24 h at 37°C . The cells were washed, suspended in fetal bovine serum-free RPMI-1640, incubated with $50 \mu\text{M}$ dye for 30 min at 37°C and washed with phosphate buffered saline. The cell suspensions were centrifuged at $412 \times g$ for 10 min and medium was removed. The cells were dissolved with 1% Triton X-100 and fluorescence was measured at an excitation wavelength of 485 nm and an emission

wavelength of 530 nm using a fluorescence microplate reader (SPECTRAFLUOR, TECAN, Salzburg, Austria).

2.10. Statistical analysis

The data are expressed as the mean $\pm$ SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA). When significance was detected, the post hoc comparisons between the different groups were made using Duncan's test for multiple comparisons. A probability value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. QGR inhibits production of inflammatory mediators

The effect of QGR on the production of cytokines and chemokines in HaCaT cells exposed to TNF- α was investigated. Treatment with 1–25 μM QGR reduced the TNF- α -induced productions of IL-1 β and IL-6 in a concentration-dependent manner (Fig. 2). At 15 μM , QGR inhibited the cytokine production by approximately 58–63%. Treatment with QGR alone, up to 25, did not induce cytokine production in cells not treated with TNF- α . We examined the effect of corticosteroid dexamethasone and immunosuppressant cyclosporine A on the cytokine production and compared the effect of two drugs with that of QGR. Treatment with 5 μM dexamethasone and 0.5 nM cyclosporine A attenuated the TNF- α -induced productions of cytokines, which was somewhat

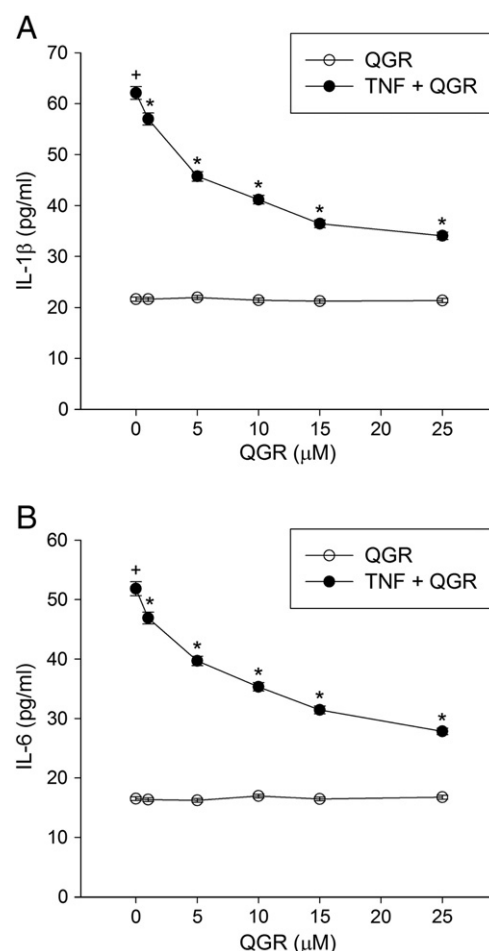


Fig. 2. Effect of QGR on cytokine production. HaCaT cells were pre-treated with 1–25 μM QGR for 20 min and exposed to 10 ng/ml TNF- α in combination with QGR for 24 h. Then the amounts of IL-1 β (A) and IL-6 (B) were measured by using ELISA. Data represent mean $\pm$ SEM ($n = 6$). * $P < 0.05$ compared to control and * $P < 0.05$ compared to TNF- α alone.

less than the effect of 15 μ M QGR (Fig. 3). Next, we examined whether the TNF- α -induced production of inflammatory mediators was mediated by the ERK and NF- κ B signaling pathways. Treatment with 2.5 μ M Bay 11-7085 (an irreversible inhibitor of I κ B- α phosphorylation) or 0.5 μ M ERK inhibitor reduced the TNF- α -induced production of cytokines (Fig. 3). Alone, the inhibitors did not induce cytokine production.

The effect of QGR on the TNF- α -induced production of chemokines was examined. QGR attenuated the TNF- α -induced production of CCL17 and CCL27 in a concentration-dependent manner (Fig. 4). At 15 μ M, QGR inhibited the chemokine production by approximately 54–55%. As observed in the cytokine production, treatment with 5 μ M dexamethasone or 0.5 nM cyclosporine A attenuated the TNF- α -induced productions of chemokines, which was somewhat less than the effect of 15 μ M QGR (Fig. 5). Next, the involvement of the ERK and NF- κ B signaling pathways in the TNF- α -induced production of chemokines was examined. Treatment with Bay 11-7085 or ERK inhibitor reduced the TNF- α -induced production of chemokines (Fig. 5). Alone, the inhibitors did not induce chemokine production.

3.2. QGR inhibits activation of NF- κ B

We measured whether the effect of QGR on the TNF- α -induced production of inflammatory mediators in HaCaT cells resulted from altered NF- κ B activation. Treatment with TNF- α produced an increase in NF- κ B p65, NF- κ B p50 and phospho-I κ B levels in HaCaT cells (Fig. 6A). Treatment with QGR, dexamethasone, Bay 11-7085 or ERK inhibitor attenuated TNF- α -induced I κ B- α phosphorylation and NF- κ B activation.

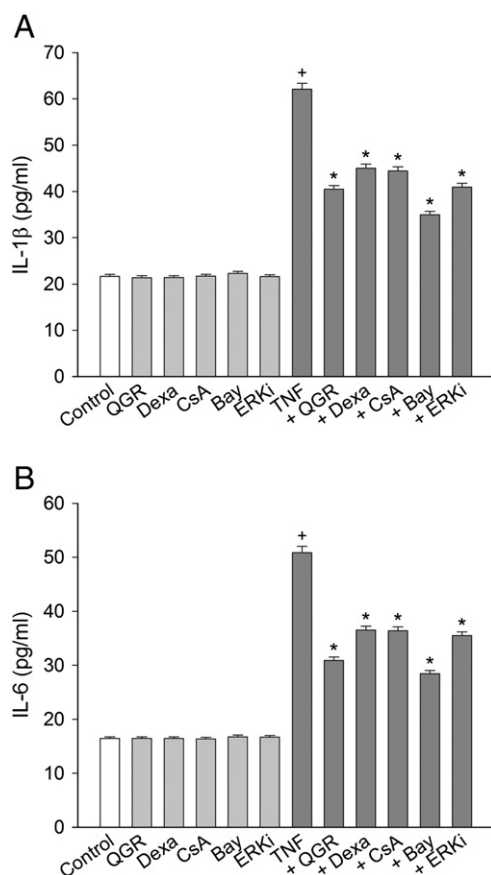


Fig. 3. Effect of dexamethasone, Bay 11-7085 and ERK inhibitor on cytokine production. HaCaT cells were pre-treated with compounds (15 μ M QGR, 5 μ M dexamethasone, 0.5 nM cyclosporine A, 2.5 μ M Bay 11-7085 or 0.5 μ M ERK inhibitor) for 20 min and exposed to 10 ng/ml TNF- α in combination with compounds for 24 h. Then the amounts of IL-1 β (A) and IL-6 (B) were measured. Data represent mean $\pm$ SEM ($n = 6$). $^+P < 0.05$ compared to control and $^*P < 0.05$ compared to TNF- α alone.

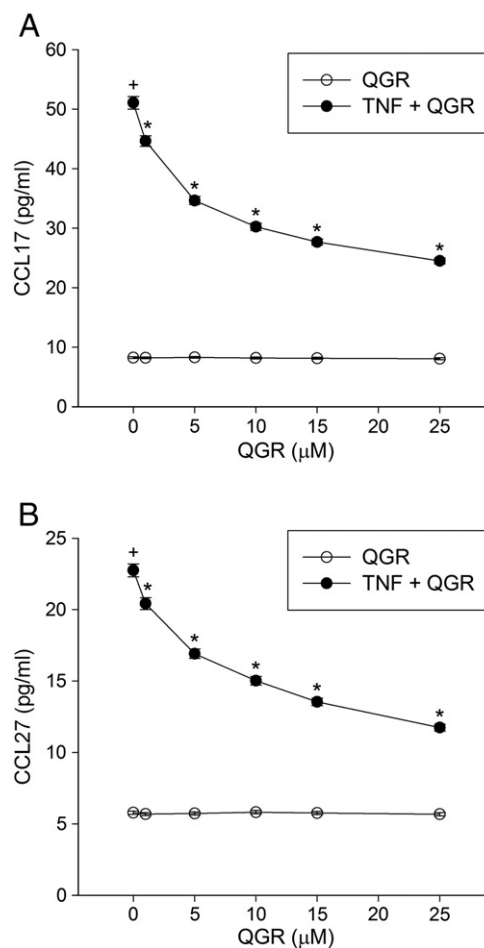


Fig. 4. Effect of QGR on chemokine production. HaCaT cells were pre-treated with 1–25 μ M QGR for 20 min and exposed to 10 ng/ml TNF- α in combination with QGR for 24 h. Then the amounts of CCL17 (A) and CCL27 (B) were measured by using ELISA. Data represent mean $\pm$ SEM ($n = 6$). $^+P < 0.05$ compared to control; and $^*P < 0.05$ compared to TNF- α alone.

The inhibitory effect of QGR on TNF- α -induced NF- κ B activation was confirmed by monitoring the binding of NF- κ B to DNA. Non-stimulated cells exhibited a small increase in NF- κ B-DNA binding activity. TNF- α produced a marked increase in the NF- κ B-DNA binding activity, which was attenuated by the addition of QGR, dexamethasone, cyclosporine A, Bay 11-7085 or ERK inhibitor (Fig. 6B).

3.3. QGR inhibits ERK activation

Whether the TNF- α -induced production of inflammatory mediators was regulated by ERK pathway was examined. The TNF- α -induced activation of ERK was confirmed by the preventive effect of specific ERK inhibitor. Treatment with QGR, dexamethasone, cyclosporine A or ERK inhibitor attenuated the TNF- α -induced increase in the phospho-ERK1/2 levels and the phosphorylation of ERK (Fig. 7). Compounds alone did not induce phosphorylation of ERK.

3.4. QGR inhibits formation of reactive oxygen species

The effect of QGR on the formation of reactive oxygen species as the response of stimulated keratinocytes was examined. The formation of reactive oxygen species within cells was determined by monitoring a conversion of DCFH₂-DA to DCF. HaCaT cells treated with TNF- α for 24 h showed a significant increase in DCF fluorescence, which was inhibited by the addition of 1 mM thiol compound *N*-acetylcysteine or 30 μ M trolox (a scavenger of hydroxyl radicals and peroxynitrite) or

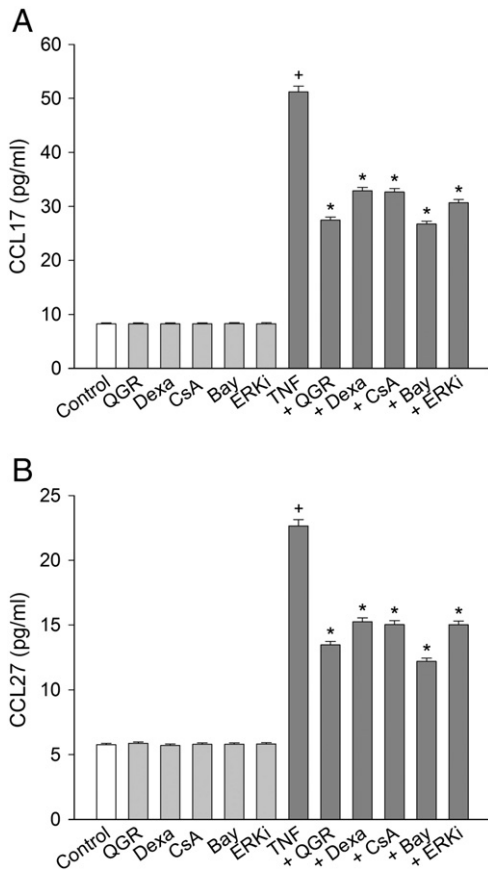


Fig. 5. Effect of dexamethasone, Bay 11-7085 and ERK inhibitor on chemokine production. HaCaT cells were pre-treated with compounds (15 μ M QGR, 5 μ M dexamethasone, 0.5 nM cyclosporine A, 2.5 μ M Bay 11-7085 or 0.5 μ M ERK inhibitor) for 20 min and exposed to 10 ng/ml TNF- α in combination with compounds for 24 h. Then the amounts of CCL17 (A) and CCL27 (B) were measured. Data represent mean $\pm$ SEM ($n = 6$). $^+P < 0.05$ compared to control; and $^*P < 0.05$ compared to TNF- α alone.

100 μ M ascorbic acid (Fig. 8A). We examined whether the TNF- α -induced formation of reactive oxygen species was regulated by the ERK and NF- κ B pathways. Except cyclosporine A, treatment with QGR, dexamethasone, Bay 11-7085 or ERK inhibitor attenuated the TNF- α -induced increase in DCF fluorescence (Fig. 8B).

4. Discussion

Quercetin and its derivatives have been shown to have anti-inflammatory and anti-oxidant effects. Nevertheless, they exhibit a variable effect on the cytokine production and cell damage induced by toxic insults [14,16–18]. In addition, the effect of a new quercetin derivative QGR on the TNF- α -stimulated production of inflammatory mediators, which may be involved in the skin inflammation and immune response, has not been studied. Therefore, in order to evaluate the effect and action of QGR as a preventive compound in the disease process of inflammatory skin diseases, we investigated the effect of QGR on stimulated responses in keratinocytes. The cytokine TNF- α stimulates the production of other cytokines, such as IL-1 β and IL-6, and chemokines, such as CCL2/MCP-1 and CCL27 in keratinocytes [1,3,21]. Consistent with these reports, the HaCaT cells treated with TNF- α produced a significant amount of IL-1 β , IL-6, CCL17 and CCL27. Proinflammatory cytokines elicit the amplification and persistence of inflammatory and immune responses in skin [1]. Chemokines are involved in the recruitment of T cells into skin and in the induction of skin inflammation [1]. Therefore, we examined changes in the levels of CCL17 and CCL27 as these chemokines are well expressed in keratinocytes exposed to

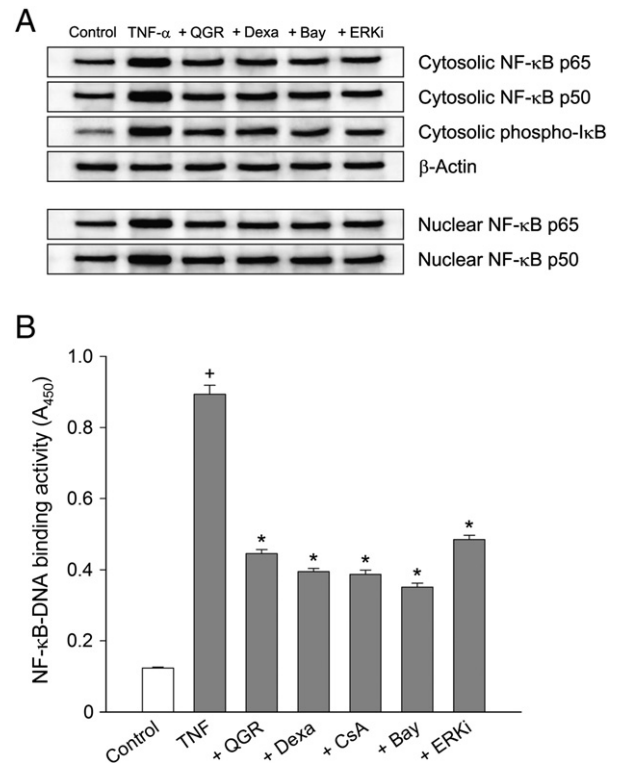


Fig. 6. Effect of QGR on NF- κ B activation. HaCaT cells were pre-treated with compounds (15 μ M QGR, 5 μ M dexamethasone, 0.5 nM cyclosporine A, 2.5 μ M Bay 11-7085 or 0.5 μ M ERK inhibitor) for 20 min and exposed to 10 ng/ml TNF- α in combination with compounds for 15 min. In A, The levels of NF- κ B p65, NF- κ B p50 and phospho-I κ B- α were analyzed by western blotting with antibodies for NF- κ B p65, NF- κ B p50, phospho-I κ B- α and β -actin. Data are representative of three different experiments. In B, the NF- κ B DNA binding activity was measured by assay kit. Data represent mean $\pm$ SEM ($n = 3$). $^+P < 0.05$ compared to control; and $^*P < 0.05$ compared to TNF- α alone.

proinflammatory cytokines [1,21,22]. In this study, QGR significantly inhibited the TNF- α -induced production of cytokines and chemokines in HaCaT cells. These results suggest that QGR may reduce the infiltration of T cells and neutrophils into skin through the inhibition of chemokine production. QGR may prevent the cytokine- and chemokine-mediated immune cell function and inflammatory reaction. The inhibitory effect of QGR at the designated concentrations on the production of inflammatory mediators was similar to those of dexamethasone and cyclosporine. This finding suggests that QGR may exhibit an inhibitory effect on the production of inflammatory mediators compatible with those of immune suppressants.

It is unclear whether the effect of QGR on the TNF- α -stimulated production of inflammatory mediators is mediated by its effect on the NF- κ B activation. In this respect, we investigated alterations of the NF- κ B activation. TNF- α binds to the TNF receptor-1 and activates the NF- κ B, which regulates the transcription genes involved in immune and inflammatory responses [23,24]. The aberrant activation of NF- κ B in both keratinocytes and lymphocytes may be involved in the development of inflammatory skin diseases [25]. The basal NF- κ B-DNA binding activity in peripheral blood mononuclear cells was found to be significantly higher in atopic eczema patients than healthy age-matched control patients [26]. TNF- α induces production of cytokines, chemokines and reactive oxygen species in keratinocytes through the activation of NF- κ B [11,23,27]. The phosphorylation and proteolytic degradation of I κ B induce the release of NF- κ B dimers [28]. The translocation of active NF- κ B dimers to the nucleus elicits the activation of specific target genes. In this study, TNF- α increased phospho-I κ B and NF- κ B p65/50 levels, and induced the binding of NF- κ B to DNA in HaCaT cells. In agreement with previous reports, TNF- α treatment increased the phospho-

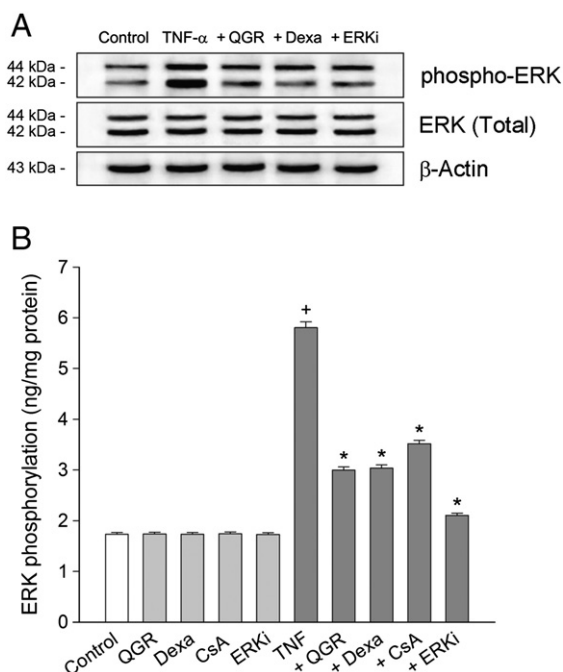


Fig. 7. Effect of QGR on activation of ERK. In A, HaCaT cells were treated with 10 ng/ml TNF- α in the presence of compounds (15 μ M QGR, 5 μ M dexamethasone, 0.5 nM cyclosporine A or 0.5 μ M ERK inhibitor) for 4 h. The levels of phosphorylated-ERK and total ERK were analyzed by western blotting (A) and ELISA (B). Data for A representative of three to four different experiments and data for B represent the mean $\pm$ S.E.M., $n = 5-6$. ⁺ $P < 0.05$ compared to control; and ^{*} $P < 0.05$ compared to TNF- α alone.

I κ B and NF- κ B p65/50 levels, and the binding of NF- κ B to DNA in keratinocytes. These results and the inhibitory effect of Bay 11-7085 suggest that the TNF- α -induced inflammatory mediator production may be mediated by NF- κ B pathway activation, i.e., the nuclear translocation and specific DNA binding of activated NF- κ B dimers. Then, we examined whether the preventive effect of QGR on the TNF- α -induced production of inflammatory mediators in HaCaT cells was due to an inhibitory effect on NF- κ B activation. The results suggest that QGR may inhibit the TNF- α -induced production of inflammatory mediators by suppressing the activation of the NF- κ B. The transcription of the proinflammatory genes leads to the production of mRNAs responsible for the synthesis of cytokines and chemokines [7,29]. NF- κ B regulates the transcription genes involved in the inflammatory response [7]. Based on these reports, QGR may inhibit the TNF- α -induced production of inflammatory mediators by suppressing the production of mRNAs responsible for producing chemokines and cytokines. The inhibitory effect of dexamethasone and cyclosporine suggests that they inhibit the production of inflammatory mediators by suppressing the activation of the NF- κ B. The QGR may exhibit an inhibitory effect on the NF- κ B activation compatible with immune suppressants.

Proinflammatory cytokines induce NF- κ B activation by activating the Ras/Raf/MEK/ERK pathway [5,6]. Therefore, we examined whether TNF- α -induced NF- κ B activation and subsequent production of inflammatory mediators in HaCaT cells was mediated by ERK activation. In this study, HaCaT cells exposed to TNF- α had activated (phosphorylated) ERK. The result suggests that TNF- α induces activation of NF- κ B by activating the ERK pathway. The inhibitory effect of QGR on the phosphorylation of ERK suggests that QGR may inhibit the TNF- α -induced production of inflammatory mediators by suppressing NF- κ B activation, which is regulated by the ERK pathway. The inhibitory effect of dexamethasone and cyclosporine suggests that they inhibit the production of inflammatory mediators by suppressing the activation of the ERK-mediated NF- κ B. The QGR may exhibit an inhibitory effect on the ERK pathway compatible with immune suppressants.

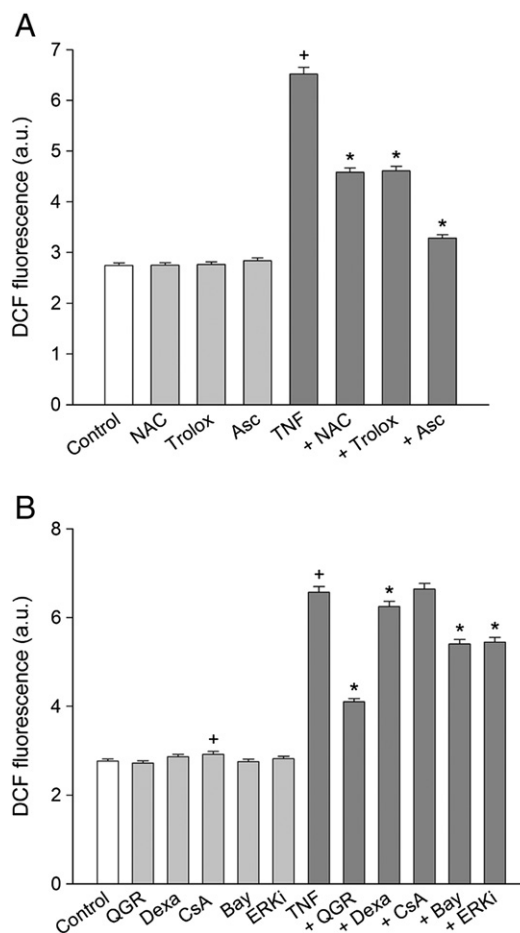


Fig. 8. Effect of QGR on formation of reactive oxygen species. HaCaT cells were treated with 10 ng/ml TNF- α in the presence of compounds (1 mM *N*-acetylcysteine, 30 μ M trolox, 100 μ M ascorbic acid, 15 μ M QGR, 5 μ M dexamethasone, 0.5 nM cyclosporine A, 2.5 μ M Bay 11-7085 or 0.5 μ M ERK inhibitor) for 24 h. Then, the changes in DCF fluorescence, which indicates formation of reactive oxygen species formation, were measured and data are expressed as arbitrary units (a.u.) of fluorescence. Data represent mean $\pm$ SEM ($n = 6$). ⁺ $P < 0.05$ compared to control; and ^{*} $P < 0.05$ compared TNF- α alone.

Reactive oxygen species are suggested to be involved in the TNF- α -induced signaling pathways [9,27]. Reactive oxygen species elicit the activation of NF- κ B [8,11,12]. Therefore, we investigated the formation of reactive oxygen species in the TNF- α -stimulated keratinocytes, which may be involved in the NF- κ B activation. Treatment with TNF- α elicited the formation of reactive oxygen species in HaCaT cells, which was inhibited by the addition of oxidant scavengers *N*-acetylcysteine, trolox and ascorbic acid, and QGR. *N*-Acetylcysteine is suggested to inhibit the TNF- α -induced cytokine production by suppressing reactive oxygen species formation [27,30]. Therefore, the TNF- α -induced activation of the ERK and NF- κ B pathways may be achieved by formation of reactive oxygen species. QGR may prevent the TNF- α -induced production of proinflammatory mediators by suppressing the ERK and NF- κ B pathways that is regulated by reactive oxygen species. In contrast to QGR, the cyclosporine A may attenuate the ERK and NF- κ B pathways-mediated production of inflammatory mediators without the mediation of reactive oxygen species.

A previous report suggests there is a mutual cross-talk reaction between formation of reactive oxygen species and activation of NF- κ B [31]. It has been shown that inhibition of NF- κ B may attenuate oxidative stress and improve cardiac mitochondrial structural integrity [32]. In this study, ERK inhibitor and Bay 11-7085 attenuated the TNF- α -induced formation of reactive oxygen species. On the basis of the previous reports, the results suggest that the TNF- α -induced production of reactive

oxygen species may be mediated by activation of the ERK and NF- κ B pathways.

Overall, the results show that QGR may attenuate TNF- α -stimulated inflammatory mediator production in HaCaT cells by suppressing the activation of the ERK-mediated NF- κ B pathway that is mediated by reactive oxygen species. Additionally, QGR may exhibit a preventive effect against the proinflammatory mediator-induced skin diseases by inhibiting the activation of the ERK and NF- κ B pathways.

Acknowledgments

This study was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A091121).

References

- Pastore S, Mascia F, Girolomoni G. The contribution of keratinocytes to the pathogenesis of atopic dermatitis. *Eur J Dermatol* 2006;16(2):125–31.
- Tsuruta D. NF- κ B links keratinocytes and lymphocytes in the pathogenesis of psoriasis. *Recent Pat Inflamm Allergy Drug Discov* 2009;3(1):40–8.
- Vestergaard C, Johansen C, Otkjaer K, Deleuran M, Iversen L. Tumor necrosis factor- α -induced CTACK/CCL27 (cutaneous T-cell-attracting chemokine) production in keratinocytes is controlled by nuclear factor kappaB. *Cytokine* 2005;29(2):49–55.
- Murakawa M, Yamaoka K, Tanaka Y, Fukuda Y. Involvement of tumor necrosis factor (TNF)- α in phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin edema in mice. *Biochem Pharmacol* 2006;71(9):1331–6.
- Chang F, Steelman LS, Shelton JG, Lee JT, Navolanic PM, Blalock WL, et al. Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway. *Int J Oncol* 2003;22(3):469–80.
- McCubrey JA, Steelman LS, Abrams SL, Lee JT, Chang F, Bertrand FE, et al. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv Enzyme Regul* 2006;46:249–79.
- Ghosh S, Hayden MS. New regulators of NF- κ B in inflammation. *Nat Rev Immunol* 2008;8(11):837–48.
- Schreck R, Albersmann K, Baeuerle PA. Nuclear factor κ B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic Res Commun* 1992;17(4):221–37.
- Hughes G, Murphy MP, Ledgerwood EC. Mitochondrial reactive oxygen species regulate the temporal activation of nuclear factor κ B to modulate tumour necrosis factor-induced apoptosis: evidence from mitochondria-targeted antioxidants. *Biochem J* 2005;389(Pt 1):83–9.
- Pourova J, Kottova M, Voprsalova M, Pour M. Reactive oxygen and nitrogen species in normal physiological processes. *Acta Physiol (Oxf)* 2010;198(1):15–35 [Epub 2009 Sep 3. Review].
- Köhler HB, Huchzermeyer B, Martin M, De Bruin A, Meier B, Nolte I. TNF- α dependent NF- κ B activation in cultured canine keratinocytes is partly mediated by reactive oxygen species. *Vet Dermatol* 2001;12(3):129–37.
- Haddad JJ. Redox regulation of pro-inflammatory cytokines and I κ B- α /NF- κ B nuclear translocation and activation. *Biochem Biophys Res Commun* 2002;296(4):847–56.
- Potenza L, Calabrini C, De Bellis R, Mancini U, Cucchiari L, Dachà M. Effect of quercetin on oxidative nuclear and mitochondrial DNA damage. *Biofactors* 2008;33(1):33–48.
- Vicentini FT, He T, Shao Y, Fonseca MJ, Verri Jr WA, Fisher GJ, et al. Quercetin inhibits UV irradiation-induced inflammatory cytokine production in primary human keratinocytes by suppressing NF- κ B pathway. *J Dermatol Sci* 2011;61(3):162–8.
- Choi SE, Park KH, Han BH, Jeong MS, Seo SJ, Lee DI, et al. Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by phenolic compounds from roots of rhododendron mucronulatum. *Phytother Res* 2011. <http://dx.doi.org/10.1002/ptr3376>.
- Pastore S, Potapovich A, Kostyuk V, Mariani V, Lulli D, De Luca C, et al. Plant polyphenols effectively protect HaCaT cells from ultraviolet C-triggered necrosis and suppress inflammatory chemokine expression. *Ann N Y Acad Sci* 2009;1171:305–13.
- Wang XY, He PY, Du J, Zhang JZ. Quercetin in combating H₂O₂ induced early cell apoptosis and mitochondrial damage to normal human keratinocytes. *Chin Med J (Engl)* 2010;123(5):532–6.
- Kostyuk V, Potapovich A, Suhan T, De Luca C, Pressi G, Dal Toso R, et al. Plant polyphenols against UV-C-induced cellular death. *Planta Med* 2008;74(5):509–14.
- Schreiber E, Matthias P, Müller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* 1989;17(15):6419.
- Fu W, Luo H, Parthasarathy S, Mattson MP. Catecholamines potentiate amyloid β -peptide neurotoxicity: involvement of oxidative stress, mitochondrial dysfunction, and perturbed calcium homeostasis. *Neurobiol Dis* 1998;5(4):229–43.
- Kanda N, Koike S, Watanabe S. IL-17 suppresses TNF- α -induced CCL27 production through induction of COX-2 in human keratinocytes. *J Allergy Clin Immunol* 2005;116(5):1144–50.
- Kanda N, Watanabe S. Leukotriene B(4) enhances tumour necrosis factor- α -induced CCL27 production in human keratinocytes. *Clin Exp Allergy* 2007;37(7):1074–82.
- Baldwin Jr AS. Series introduction: the transcription factor NF- κ B and human disease. *J Clin Invest* 2001;107(1):3–6.
- Lind MH, Rozell B, Wallin RP, van Hogerlinden M, Ljunggren HG, Toftgard R, et al. Tumor necrosis factor receptor 1-mediated signaling is required for skin cancer development induced by NF- κ B inhibition. *Proc Natl Acad Sci U S A* 2004;101(14):4972–7.
- Rebholz B, Haase I, Eckelt B, Paxian S, Flaig MJ, Ghoreschi K, et al. Crosstalk between keratinocytes and adaptive immune cells in an I κ B α protein-mediated inflammatory disease of the skin. *Immunity* 2007;27(2):296–307.
- Angelini F, Di Matteo G, Balestrero S, Brunetti E, Mancino G, Rossi P, et al. Nuclear factor κ B activity is increased in peripheral blood mononuclear cells of children affected by atopic and non-atopic eczema. *Int J Immunopathol Pharmacol* 2007;20(1):59–67.
- Young CN, Koepke JI, Terlecky LJ, Borkin MS, Boyd SL, Terlecky SR. Reactive oxygen species in tumor necrosis factor- α -activated primary human keratinocytes: implications for psoriasis and inflammatory skin disease. *J Invest Dermatol* 2008;128(11):2606–14.
- Henkel T, Machleidt T, Alkalay I, Krönke M, Ben-Neriah Y, Baeuerle PA. Rapid proteolysis of I κ B- α is necessary for activation of transcription factor NF- κ B. *Nature* 1993;365(6442):182–5.
- Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. *J Clin Invest* 2001;107(1):7–11.
- Sato M, Miyazaki T, Nagaya T, Murata Y, Ida N, Maeda K, et al. Antioxidants inhibit tumor necrosis factor- α mediated stimulation of interleukin-8, monocyte chemoattractant protein-1, and collagenase expression in cultured human synovial cells. *J Rheumatol* 1996;23(3):432–8.
- Bubuci C, Papa S, Dean K, Franzoso G. Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. *Oncogene* 2006;25(51):6731–48.
- Mariappan N, Elks CM, Sriramula S, Guggilam A, Liu Z, Borkhsenius O, et al. NF- κ B-induced oxidative stress contributes to mitochondrial and cardiac function in type II diabetes. *Cardiovasc Res* 2010;85(3):473–83.