Protection of burn-induced skin injuries by the flavonoid kaempferol

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Thermal burn injury induces inflammatory cell infiltrates in the dermis and thickening of the epidermis. Following a burn injury, various mediators, including reactive oxygen species (ROS), are produced in macrophages and neutrophils, exposing all tissues to oxidative injury. The anti-oxidant activities of flavonoids have been widely exploited to scavenge ROS. In this study, we observed that several flavonoids-kaempferol, quercetin, fisetin, and chrysin-inhibit LPS-induced IL-8 promoter activation in RAW 264.7 cells. In contrast with quercetin and fisetin, pretreatment of kaempferol and chrysin did not decrease cell viability. Inflammatory cell infiltrates in the dermis and thickening of the epidermis induced by burn injuries in mice was relieved by kaempferol treatment. However, the injury was worsened by fisetin, quercetin, and chrysin. Expression of TNF-α induced by burn injuries was decreased by kaempferol. These findings suggest the potential use of kaempferol as a therapeutic in thermal burn-induced skin injuries. [BMB reports 2010; 43(1): 46-51]

INTRODUCTION

Thermal injury provokes tissue damage, immunosuppression, susceptibility to bacterial infection as a result of the activation of inflammatory responses, and induction of immune dysfunction (1-4). The exposure of skin tissue to thermal burns is known to preferentially induce augmented recruitment of neutrophils and macrophages (5-8), T lymphocyte dysfunction (9, 10), production of Th2 cytokine profiles such as IL-4 and IL-10 (11), production of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) (8, 12), and expression of inflammatory mediators (13, 14). Researchers have shown that the neutrophil chemoattractant genes KC (GROα) and MIP-2 are expressed in different cell populations at sites of surgical injury in the skin (8). Further, Shallo et al., have reported that thermal injury to mouse skin upregulates the expression of MCP-1, leading to infiltration of macrophages into the skin (12). Evidence has been presented that thermal injury leads to production of inflammatory cytokines such as TNF-α, IL-4, and IL-6, and these cytokines have been shown to play roles in the development of life-threatening post-burn complications such as multiple organ failure (1-4, 15).

Although the mechanisms involved in tissue damage and immune dysfunction have yet to be elucidated, it is likely that a wide variety of mediators, including reactive oxygen species (ROS), are produced in macrophages and neutrophils after exposure to burn injury (16-18). All tissues, including the burned skin, are subsequently exposed to oxidative injury, so researchers have accordingly applied antioxidant defense systems to enhance burn healing (19, 20).

Flavonoids have remarkable and diverse biological activities, including powerful antioxidant and anti-inflammatory effects, and inhibition of cell-signaling proteins (21-23). The antioxidant activities of flavonoids are widely exploited to scavenge ROS, and have attracted increasing attention as useful therapeutics for a variety of diseases including cancers as well as cardiovascular, autoimmune, and infectious diseases (24). We recently reported that the generation of ROS in response to TNF-α was also reduced by flavonoids via inhibition of phosphorylation and degradation of IκBα and translocation of NF-κB p65 (25). Here, we show that the flavonoid kaempferol acts as a potent therapeutic, reducing inflammation in thermal burn-induced skin injuries.

RESULTS AND DISCUSSION

Inhibitory effects of flavonoids on LPS-induced IL-8 promoter activity in RAW 264.7 cells

In a previous study, we showed that IL-8 promoter activation and gene expression are differentially regulated by each flavonoid in TNF-α-stimulated human embryonic kidney (HEK) 293 cells
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In this study, we first investigated the effects of flavonoids on LPS-induced IL-8 promoter activation in mouse cells. To evaluate whether flavonoids inhibit LPS-inducible gene expression in mouse cells, the IL-8 promoter-reporter construct was transiently transfected into a mouse macrophage cell line, RAW 264.7 cells, and pretreated with flavonoids (40 μM) at 37°C for 1 h. The cells were then cultured with LPS (200 ng/ml) for 12 h, and luciferase activity was monitored. Each flavonoid had a different effect on LPS-stimulated RAW 264.7 cells. The activation of IL-8 promoter was dramatically reduced in the presence of fisetin and chrysin, whereas kaempferol and quercetin were less effective in suppressing IL-8 promoter activation (Fig. 1A).

Differential effect of flavonoids on viability of RAW 264.7 cells
In a previous study (25), we showed that treatment of HEK 293 cells with quercetin or chrysin decreased viability in a dose-dependent manner. However, the viability of the cells was not affected in kaempferol-treated HEK 293 cells. To explore whether the treatment of RAW 264.7 cells with flavonoids induced cell death and influenced LPS-induced IL-8 promoter activation, we investigated the effects of flavonoids on the viability of RAW 264.7 cells, using an MTT assay. As shown in Fig. 1B, the viability of the cells dramatically decreased in a dose-dependent manner with treatment by quercetin and fisetin. However, kaempferol and chrysin induced death of RAW 264.7 cells only at high concentrations, such as 160 μM. Therefore, kaempferol and chrysin were selected as candidate flavonoids that specifically inhibit inflammation in mouse cells without cytotoxicity.

Protective effect of kaempferol upon thermal injury
It is well known that flavonoids have antioxidant and anti-inflammatory effects (24, 25). Therefore, we first examined whether flavonoids could protect the skin from thermal injury-induced inflammation. To examine the effects of flavonoids on thermal injuries, flavonoids were topically applied to dorsal skin for 30 days (daily) after thermal injury. Macroscopic appearances of lesions induced by thermal injury spontaneously healed after 21 days by regeneration and repair (scar formation) (Fig. 2A). During the healing process, the macroscopic appearances of wound closure showed greater progress from kaempferol treatment than from treatments of quercetin, fisetin, or chrysin (Figs. 2A, 3A); the healing kinetics was much faster in the case of kaempferol (see 10 days after thermal injury). We also observed inflammatory cell infiltrates in the dermis at 10 days post-induction of thermal injury by a microscopic evaluation of hematoxylin and eosin (H&E). At days 21 and 30 post-burn, the burned area of the skin revealed significant thickening of the epidermis (Figs. 2B, 3B). Treatment of the burned area of skin with kaempferol clearly reduced inflammatory cell infiltrates in the dermis and thickening of the epidermis. In contrast, more abundant inflammatory cell infiltrates in the dermis and thickening of the epidermis were observed in the quercetin, fisetin, and chrysin-treated skin at 21 days post-burn (Fig. 2B). Therefore, it is confirmed that kaempferol has unique anti-inflammatory therapeutic effects on thermal injury-induced burned area of skin in mice.

Inhibitory effect of kaempferol on thermal injury-induced TNF-α gene expression
It is known that inflammatory cytokines such as TNF-α and IL-4 induced by thermal injury trigger marked immune dysfunction and multiple organ failure (1-4, 15). Therefore, we evaluated TNF-α mRNA expression in the burned area of skin to determine if it was modulated by kaempferol treatment.
Fig. 2. Effects of flavonoids on thermal burn injury-induced skin. Thermal injury in BALB/c mice was induced as described in Materials and Methods. For 21 days (daily), 100 μl of each flavonoid (100 μM) was applied to the back skin. The results are representative of 3 mice in each group. (A) Macroscopic appearance of skin lesions in burn injury-induced mice with or without flavonoids treatment. (B) Examination of burn injury histology. Skin paraffin sections were processed for H&E staining. The photographs were taken at an original magnification of ×100 and ×200.

Fig. 3. Effects of kaempferol on thermal burn injury-induced skin. Thermal injury in BALB/c mice was induced and 100 μl kaempferol (100 μM) was applied to the back skin every day for the indicated time periods. The results are representative of 3 mice in each group. (A) Macroscopic appearance of skin lesions in burn injury-induced mice with or without kaempferol treatment. (B) Examination of burn injury histology. Skin paraffin sections were processed for H&E staining. The photographs were taken at an original magnification of ×100.

RT-PCR results revealed the expression of TNF-α mRNA in the burned area of skin at 10 days post-burn. The TNF-α mRNA expression in the burned area of skin was specifically reduced in the presence of kaempferol (Fig. 4).

Flavonoids, which are known to be antioxidants and to have multiple biological activities, have attracted considerable attention as useful therapeutics for several diseases (24). Flavonoids inhibit the activation of NF-κB (26, 27) and the expression of proinflammatory genes in response to inflammatory mediators such as TNF-α (23, 28). In a previous study, we demonstrated that kaempferol, quercetin, fisetin, and chrysin block TNF-α-induced IL-8 promoter activation and gene expression in HEK 293 cells. Generation of reactive oxygen species (ROS) in response to TNF-α was also reduced by these flavonoids (25). In the present study, we found that flavonoids (kaempferol, quercetin, fisetin, and chrysin) block LPS-induced IL-8 promoter activation and gene expression in HEK 293 cells.
activation in RAW 264.7 cells with differential potency (Fig. 1). As quercetin and fisetin induced robust cell death, reduction of IL-8 promoter activation by these two flavonoids appears to partly result from cytotoxic side effects.

Previously, thermal burn injury was shown to induce inflammatory cell infiltrates in the dermis and thickening of the epidermis (1-8). Macrophages and neutrophils are recruited into the dermis and produce ROS, resulting in exposure to oxidative injury (16-18). In the present study, we observed that inflammatory cell infiltrates in the dermis and thickening of the epidermis are relieved by kaempferol treatment on the thermal injury-induced burned area of skin (Figs. 2B, 3B). Although fisetin, quercetin, and chrysin showed strong inhibitory activity on IL-8 promoter activation by LPS stimulation, inflammatory cell infiltrates in the dermis and thickening of the epidermis were increased by these flavonoids (Fig. 2B). Along with cytotoxicity, this harmful effect in vivo excludes these flavonoids as therapeutics. Notably, TNF-α mRNA expression induced by burn injury was specifically reduced in the presence of kaempferol (Fig. 4). Considering these findings, among the several flavonoids studied here, kaempferol has the highest potential for use as a therapeutic for thermal injury of the skin. Intensive studies on the properties and therapeutic effects or side effects of kaempferol should therefore be undertaken in the future.

MATERIALS AND METHODS

Cell culture and reagents
RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were placed in a complete medium for 24 h before being treated with LPS (200 ng/ml) for 12 h. To detect the effects of flavonoids on LPS-induced IL-8 promoter activation, we pre-incubated the cells with flavonoids at the indicated concentrations for 1 h prior to LPS treatment. We confirmed equivalent transfection efficiency by cotransfecting the promoterless Renilla luciferase vector pRL-null (Promega, Madison, WI, USA). The luciferase activities were measured by using a Dual-Luciferase Reporter Assay System (Promega) with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA).

Cell viability measurement by MTT assay
The cell growth and viability of RAW 264.7 cells treated with flavonoids were determined by an MTT assay with a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) solution, as described previously (25, 29). RAW 264.7 cells were seeded in 96-well plates and treated with each flavonoid at the indicated concentration. After cells were treated with flavonoids for the indicated times, MTT solution was added to each well, and the plates were incubated for an additional 4 h at 37°C. After removal of the medium, the formazan crystals were solubilized in DMSO. The color development was monitored at 595 nm with a reference wavelength of 650 nm.

RT-PCR analysis
We performed RT-PCR by using total RNA isolated from the skin by means of an RNasey RNA isolation kit (Qiagen, Valencia, CA, USA). Five micrograms of total RNA was reverse-transcribed in the first-strand synthesis buffer, which contained 6 μg/ml of oligo (dT) primer, 50 U of reverse transcriptase, 4 mM of dNTP, and 40 U of RNase inhibitor. One microliter of the cDNA mixture was subjected to the standard PCR reaction for 25 cycles with the following primer sets: mouse TNF-α, 5'-TCTTCATCACTTCTAGGCCC-3' (sense), 5'-GGGATAGACCAAGGTACAC-3' (anti-sense); mouse GAPDH, 5'-ATGGTGGAAGGTCGGGTGAACG-3' (sense), 5'-GGGAGTAGAAGGTCGGGTGAAAG-3' (anti-sense). The PCR products were visualized with UV light after being resolved on a 1% agarose gel.

Animals and induction of thermal injury
Seven-week-old male BALB/c specific-pathogen-free (SPF) mice were obtained from Orient Bio Inc. (Gyeonggi, Korea). Animal studies were approved by the Institutional Animal Care and Use Committee of Hallym University (Hallym2009-49).
Mice were anesthetized with isoflurane. For induction of thermal injury, the hair on the skin from the back of the animals (back skin) was shaved off with electronic clippers three days before each experiment. All mice were subjected to a 20% total body surface area (TBSA) dorsal scald using a modified procedure of Walker and Mason (30). The mice were placed into a plastic template and exposed to 20% TBSA and then immersed in 90°C water for 7 s. To avoid further scalding, the mice were immediately dried off using a towel. We then applied 100 μl of flavonoids in ethanol (100 μM) to the back skin for the indicated periods (daily).

Histology
Mice were sacrificed at the indicated days of post burn. An area of approximately 1.5 × 1.5 cm² was excised. Specimens of back skin from each animal were fixed in 4% paraformaldehyde solution, embedded in paraffin by the conventional procedure of back skin from each animal were fixed in 4% paraformaldehyde solution, embedded in paraffin by the conventional method, and cut into 4-μm-thick sections. The deparaffinized sections were stained with hematoxylin-eosin for a gross histological examination.

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