

# Esculetin suppresses lipopolysaccharide-induced inflammatory mediators and cytokines by inhibiting nuclear factor- $\kappa$ B translocation in RAW 264.7 macrophages

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Received January 7, 2014; Accepted October 2, 2014

DOI: 10.3892/mmr.2014.2613

**Abstract.** Although previous studies have demonstrated that the natural coumarin compound esculetin possesses various pharmacological properties, the molecular mechanism of esculetin-mediated anti-inflammatory potential is not fully understood. In this study, we determined the effects of esculetin on lipopolysaccharide (LPS)-induced inflammatory responses of murine RAW 264.7 macrophages. The results indicate that esculetin inhibits LPS-induced nitric oxide and prostaglandin E<sub>2</sub> production in a concentration-dependent manner, and inhibits inducible nitric oxide synthase and cyclooxygenase-2 expression in RAW 264.7 cells. Esculetin also significantly suppresses the production of inflammatory cytokines, including tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$ , which was concomitant with a decrease in their expression levels. Furthermore, it was observed that esculetin attenuated the LPS-mediated nuclear factor- $\kappa$ B (NF- $\kappa$ B) translocation associated with the blocking of inhibitor of NF- $\kappa$ B (I $\kappa$ B)- $\alpha$  degradation as well as reactive oxygen species (ROS) production, without any significant cytotoxicity. These data suggest that, by blocking NF- $\kappa$ B activation, esculetin suppresses LPS-elicited inflammatory events, and this is mediated, at least in part, by inhibiting the generation of ROS. Collectively, these findings provide mechanistic insights into the anti-inflammatory action of esculetin in macrophages.

## Introduction

Inflammation is a complex physiological and pathological process accompanied by the activation of the immune system

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**Key words:** esculetin, RAW 264.7 macrophages, inflammation, nuclear factor- $\kappa$ B, reactive oxygen species

following foreign invasion or tissue damage. Although acute inflammation is a part of the defense response by organisms to remove injurious stimuli, chronic inflammation is associated with a wide variety of diseases, including cardiovascular, autoimmune and pulmonary diseases, Alzheimer's disease, diabetes, arthritis and cancer (1-3). Macrophages are key players in the immune response to foreign invaders, including infectious microorganisms. However, macrophages activated during chronic inflammation play an essential role in inflammation-related diseases through excessive production of inflammatory mediators, nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), reactive oxygen species (ROS) and other inflammatory cytokines including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) (1,4,5).

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a transcription factor that plays a central role in the onset of inflammation, which normally presents in the cytosol and exists as an inactive complex with a class of inhibitory proteins, known as inhibitor of NF- $\kappa$ B (I $\kappa$ B) proteins. The NF- $\kappa$ B dimer dissociates from I $\kappa$ B and translocates to the nucleus following an inflammatory stimulus, where it binds to promoter regions and induces the expression of a wide variety of genes involved in inflammation (6,7). Due to its ubiquitous role in the pathogenesis of inflammatory gene expression, treatments aimed at inhibiting NF- $\kappa$ B may have potential therapeutic advantages in inflammatory diseases. In addition, the generation of ROS by activated macrophages initiates cascades of pro-inflammatory reactions. Since overproduction of ROS by macrophages causes oxidative damage to membrane lipids, DNA, proteins and lipoproteins, the excessive generation of intracellular ROS is one of the most notable hallmarks in the inflammatory process. A number of previous studies have demonstrated that ROS participate in the modulation of NF- $\kappa$ B activation and that a broad range of antioxidants also abolish NF- $\kappa$ B activation and intracellular ROS generation (8,9). These observations indicate that the redox status of cells participates in modulating NF- $\kappa$ B activation (10,11). Therefore, inhibition of ROS production associated with inactivation of NF- $\kappa$ B is a common therapeutic target for numerous inflammatory diseases.

Recently, natural medicinal plants as sources of natural products have been further developed for use in functional

health foods and are typically used for preventing disease rather than curing it, or promoting health. Coumarin compounds are the major components of medicinal plants and are attracting interest due to their beneficial effects on human health (12). Among them, esculetin is a natural coumarin derivative identified in various medicinal plants used as folk medicines. This compound has multiple benefits, including anti-inflammatory (13,14), antioxidative (15-17), neuro-protective (18), immunomodulatory (19) and antitumor activities (20,21). However, the detailed molecular anti-inflammatory mechanism of esculetin has not yet been studied. In the present study, we demonstrate for the first time that esculetin inhibits the production of inflammatory mediators and cytokines as well as ROS generation via the inhibition of the NF- $\kappa$ B activation pathway in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The findings suggest that esculetin has therapeutic potential against inflammatory diseases.

## Materials and methods

**Materials.** LPS (*Escherichia coli* 026:B6), Griess reagent, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and fetal bovine serum (FBS) were obtained from Gibco-BRL (Gaithersburg, MD, USA). Specific antibodies against inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF- $\alpha$ , IL-1 $\beta$ , NF- $\kappa$ B p65 and I $\kappa$ B- $\alpha$  were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against nucleolin and actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Esculetin (6,7-dihydroxycoumarin, 98% purity, Fig. 1) was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (vehicle). All other chemicals not specifically cited were purchased from Sigma-Aldrich.

**Cell culture and cell viability assay.** The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin at 37°C in a 5% CO<sub>2</sub> humidified air environment. Cell viability was evaluated using the MTT assay, which is based on the conversion of MTT to MTT-formazan by mitochondria.

**Nitrite measurement.** RAW 264.7 macrophages were plated in 24-well plates at 5x10<sup>5</sup> cells/well, and treated with various concentrations of esculetin in the absence or presence of 500 ng/ml LPS. Twenty-four hours later, the supernatant of cell cultures was collected, mixed with an equal volume of Griess reagent and then incubated at room temperature for 10 min. NaNO<sub>2</sub> was used to generate a standard curve, and nitrite production was determined by measuring optical density at 550 nm (22).

**Enzyme-linked immunosorbent assay (ELISA).** The levels of PGE<sub>2</sub>, TNF- $\alpha$  and IL-1 $\beta$  were determined by ELISA. ELISA kits from R&D Systems (Minneapolis, MN, USA) were employed for the measurement of TNF- $\alpha$  and IL-1 $\beta$ , and a kit

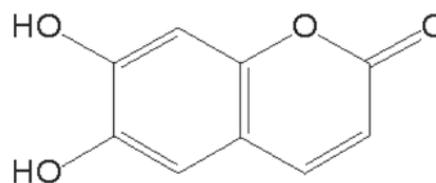


Figure 1. Chemical structure of esculetin.

from Cayman Chemical (Ann Arbor, MI, USA) was employed for the measurement of PGE<sub>2</sub> (23).

**Western blot analysis.** The total cellular proteins were extracted with lysis buffer (20 mM sucrose, 1 mM EDTA, 20  $\mu$ M Tris-l, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM MgCl<sub>2</sub> and 5  $\mu$ g/ml aprotinin) for 30 min. In a parallel experiment, nuclear and cytosolic proteins were prepared using nuclear extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Equal amounts of protein were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) by electroblotting. After 2 h blocking with 5% (w/v) nonfat milk in TBST (1.5 M NaCl, 20 mM Tris-Cl, 0.05% (v/v) Tween-20, pH 7.4), the membranes were incubated overnight at 4°C with the desired antibodies. The blots were then washed with TBST 2 h prior to incubation at room temperature with peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL, USA).

**Measurement of ROS generation.** To measure intracellular ROS, the cells were incubated for 4 h at 37°C in phosphate-buffered saline containing 20 mM DCFH-DA to label intracellular ROS. The ROS production in the cells was monitored with a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA, USA) using CellQuest Pro software (Becton Dickinson).

**Statistical analysis.** The data are expressed as the means  $\pm$  standard deviation. Statistical comparisons were performed using SPSS 12.0 (SPSS, Inc., Chicago, IL, USA) followed by Fisher's test. Significant differences between the groups were determined using the unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant result.

## Results

**Esculetin inhibits the production of pro-inflammatory mediators in RAW 264.7 macrophages.** In order to test whether esculetin was capable of reversing LPS-induced accumulation of NO and PGE<sub>2</sub>, RAW 264.7 macrophages were treated with various concentrations of esculetin for 1 h before being stimulated with LPS for 24 h. As demonstrated in Fig. 2A and B, treatment of RAW 264.7 cells with LPS caused a marked increase in the production of NO and PGE<sub>2</sub>. When the cells were incubated with esculetin (12  $\mu$ g/ml) alone, the concentration of NO and PGE<sub>2</sub> remained at a background level similar to that in the unstimulated culture. However, treatment with

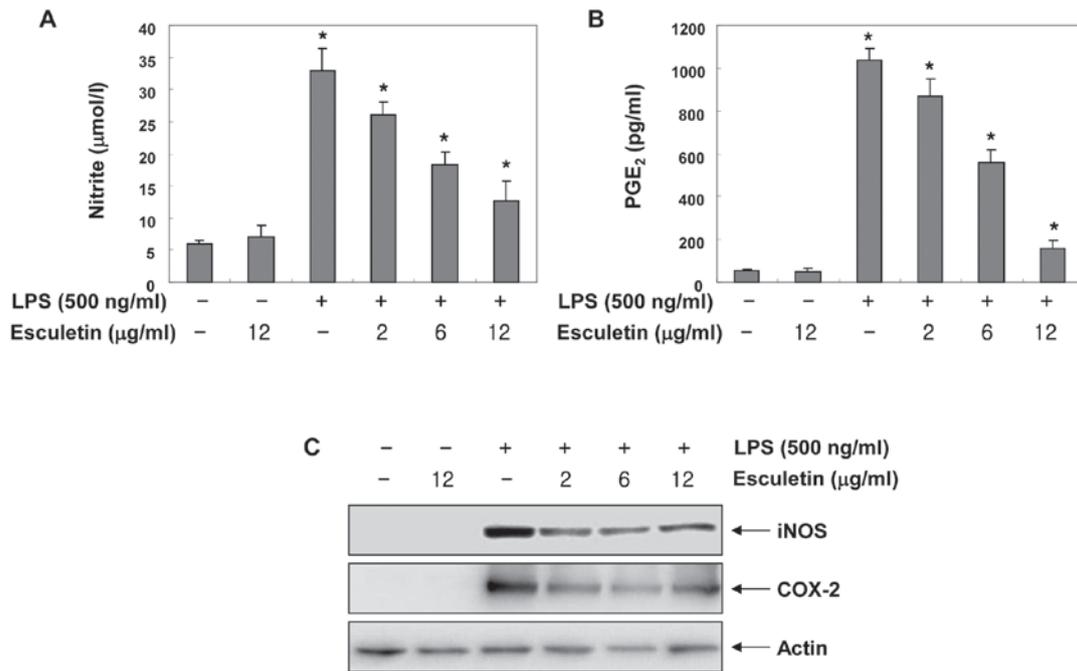


Figure 2. Inhibition of nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production, and inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression by esculletin in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. RAW 264.7 cells were pre-treated with various concentrations of esculletin for 1 h prior to incubation with LPS (500 ng/ml) for 24 h. Nitrite content was measured using the Griess reaction (A) and PGE<sub>2</sub> concentration was measured in culture media using a commercial enzyme-linked immunosorbent assay kit (B). Each value indicates the mean ± standard deviation and is representative of results obtained from three independent experiments. \*P<0.05 compared with cells treated with LPS in the absence of esculletin. (C) Cell lysates were prepared from cells grown under the same conditions and western blot analysis was performed using antibodies specific to murine iNOS and COX-2, and an enhanced chemiluminescence detection system. Actin was used as an internal control.

esculetin for 1 h prior to being incubated with LPS resulted in a dose-dependent inhibition of the LPS-induced NO and PGE<sub>2</sub> production in RAW 264.7 cells.

*Esculetin inhibits the expression of iNOS and COX-2 in RAW 264.7 macrophages.* To determine whether esculletin decreases NO and PGE<sub>2</sub> production via the suppression of iNOS and COX-2 expression, the levels of iNOS and COX-2 proteins in cells treated with LPS in the presence of various concentrations of esculletin were determined by western blot analysis. As shown in Fig. 2C, RAW 264.7 cells expressed high levels of iNOS and COX-2 proteins when stimulated with LPS alone; however, pretreatment with esculletin down-regulated the expression of these LPS-stimulated proteins in a concentration-dependent manner. The results suggest that esculletin-induced reductions in the expression of iNOS and COX-2 were the cause of the inhibition of NO and PGE<sub>2</sub> production.

*Esculetin inhibits the production and expression of pro-inflammatory cytokines.* We next attempted to determine the potential effects of esculletin on the LPS-induced release of pro-inflammatory cytokines, including TNF-α and IL-1β, using ELISA. The results revealed that the levels of the two cytokines were increased in the culture media of LPS-stimulated RAW 264.7 cells, and these increases were significantly decreased in a concentration-dependent manner by pre-treatment with esculletin (Fig. 3A and B). In a parallel experiment, immunoblotting was performed to determine whether esculletin inhibited the expression of these cytokines.

From the results, it was observed that esculletin decreased the levels of TNF-α and IL-1β proteins in LPS-stimulated cells in a concentration-dependent manner (Fig. 3C). These results also indicate that esculletin acts primarily by preventing the accumulation of pro-inflammatory cytokines through alteration of their expression.

*Esculetin inhibits LPS-induced nuclear translocation of NF-κB and degradation of IκB-α in RAW 264.7 macrophages.* Since NF-κB is a central transcription factor that regulates the expression of a large number of inflammatory-related genes, we next examined the effect of esculletin on LPS-induced NF-κB translocation from the cytosol to the nucleus and IκB-α degradation. As shown in Fig. 4, the amount of NF-κB p65 subunit in the nucleus was markedly increased upon exposure to LPS alone, but esculletin inhibited LPS-mediated nuclear translocation of NF-κB p65 in a time-dependent manner. Under the same conditions, esculletin inhibited LPS-mediated IκB-α degradation, suggesting that esculletin may block LPS-induced nuclear translocation of NF-κB p65 by suppression of IκB-α degradation.

*Esculetin inhibits the generation of ROS in LPS-stimulated RAW 264.7 macrophages.* Since previous studies have demonstrated that LPS-induced ROS generation is associated with the activation of NF-κB (11), we examined the intracellular levels of ROS in order to determine whether intracellular redox states are involved in the inhibition of NF-κB signaling by esculletin. As shown in Fig. 5A, the levels of ROS were rapidly increased upon exposure to LPS alone;

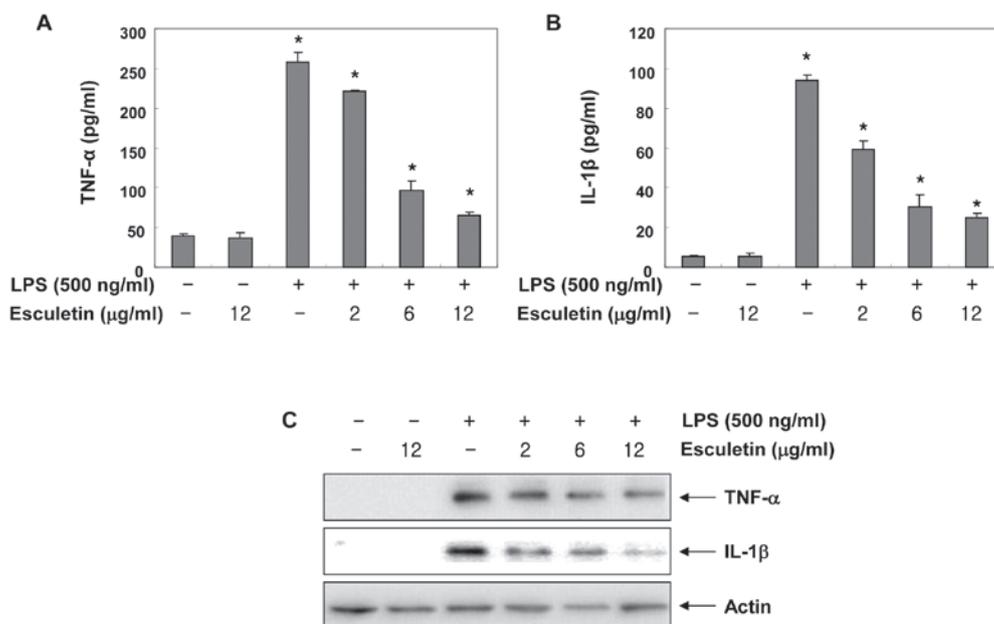


Figure 3. Effects of esculletin on production and expression of pro-inflammatory cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) in lipopolysaccharide (LPS)-challenged RAW 264.7 macrophages. RAW 264.7 cells were pre-treated with the indicated concentrations of esculletin for 1 h prior to LPS treatment (500 ng/ml). Following incubation for 24 h, the levels of TNF- $\alpha$  (A) and IL-1 $\beta$  (B) present in the supernatants were measured. Each value indicates the mean  $\pm$  standard deviation and is representative of results obtained from three independent experiments. \* $P < 0.05$  compared with cells treated with LPS in the absence of esculletin. (C) Total proteins were isolated and subjected to SDS-polyacrylamide gel electrophoresis followed by western blot analysis using anti-TNF- $\alpha$  and anti-IL-1 $\beta$  antibodies, and an enhanced chemiluminescence detection system. Actin was used as an internal control.

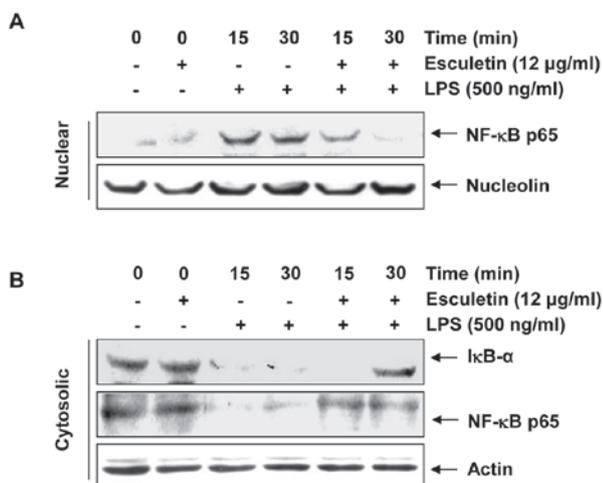


Figure 4. Effects of esculletin on lipopolysaccharide (LPS)-induced nuclear translocation of nuclear factor-kappa B (NF- $\kappa$ B) and degradation of inhibitor of NF- $\kappa$ B (I $\kappa$ B)- $\alpha$  in RAW 264.7 macrophages. Cells were treated with 12  $\mu$ g/ml esculletin for 1 h prior to LPS treatment (500 ng/ml) for the indicated times. Nuclear (A) and cytosolic (B) proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis followed by western blot analysis using anti-NF- $\kappa$ B p65 and anti-I $\kappa$ B- $\alpha$  antibodies. Nucleolin and actin were used as internal controls for the nuclear and cytosolic fractions, respectively.

however, pre-incubation with esculletin for 30 min attenuated the ROS generation in a dose-dependent manner in the LPS-activated RAW 264.7 macrophages (Fig. 5A). These results suggest that the inhibitory effects of esculletin on the NF- $\kappa$ B signaling pathway are at least partially correlated with a decrease in ROS generation. To examine whether esculletin is cytotoxic to RAW 264.7 cells, the cells were exposed to various concentrations of 7,8-DHF for 24 h in the presence or

absence of LPS, and cell viability was then measured by the MTT assay. Within our tested concentrations, no cytotoxic effect of esculletin was observed in RAW 264.7 cells (Fig. 5B). These results clearly indicated that anti-inflammatory activity of esculletin in LPS-stimulated RAW 264.7 macrophages was not due to the cytotoxic action of esculletin.

## Discussion

Coumarins including esculletin, fraxetin and daphnetin are fragrant organic chemical compounds in the benzopyrone chemical class, and are found naturally in a number of medicinal plants. Among them, esculletin is well known as a potent non-competitive inhibitor of lipoxygenase (24). Although this compound has multiple beneficial effects including anti-oxidant activity (15-17), to date, there is no data to demonstrate the anti-inflammatory effects of esculletin in activated macrophages. In this study, we examined the effects of esculletin on the release of several pro-inflammatory mediators and cytokines as well as on ROS generation in LPS-stimulated murine macrophage RAW 264.7 cells. It was demonstrated that esculletin inhibited the expression of iNOS and COX-2 as well as NO and PGE<sub>2</sub> production in LPS-treated RAW 264.7 macrophages. Esculetin also attenuated LPS-induced production and expression of pro-inflammatory cytokines including TNF- $\alpha$  and IL-1 $\beta$  as well as the generation of ROS, and these effects are mediated through the inhibition of nuclear translocation of NF- $\kappa$ B.

Accumulated evidence indicates that NO and PGE<sub>2</sub> are significant mediators of inflammation. Although three distinct isoforms of NOS have been isolated and cloned, a number of cell types express iNOS in their function in the host defense against microbial and viral pathogens, and this leads to the

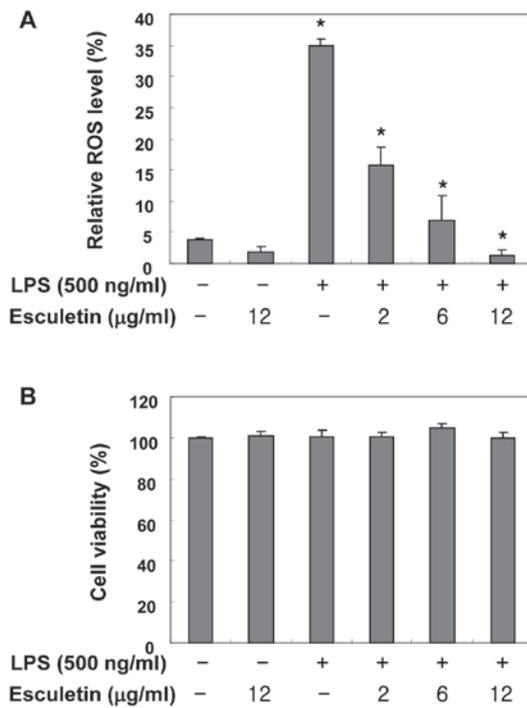


Figure 5. Effect of esculetin on lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) generation and cell viability in RAW 264.7 macrophages. (A) Cells were pre-treated with the indicated concentrations of esculetin for 1 h, then LPS (500 ng/ml) was added and cells were incubated for 30 min. ROS generation was assessed using a ROS-sensitive fluorescence indicator, DCFH-DA, and flow cytometry. (B) Cells were treated with the indicated concentrations of esculetin or LPS alone, or pre-treated with various concentrations of esculetin for 1 h prior to LPS treatment. After 24 h, cell viability was assessed using MTT reduction assays. The values are expressed as the mean  $\pm$  standard deviation from three experiments. \* $P < 0.05$  compared with unstimulated control group.

formation of NO radicals. As a result, abnormally high levels of NO may contribute to the pathogenesis of inflammatory diseases (25,26). COXs are enzymes that catalyze the conversion of arachidonic acid to  $\text{PGH}_2$ , a precursor for a variety of biologically active mediators, including  $\text{PGE}_2$ , prostacyclin and thromboxane A<sub>2</sub>. Of the two COX isoforms, COX-2 is induced in response to numerous stimulants and is activated at sites of inflammation (27,28). Therefore, endotoxin-induced NO and  $\text{PGE}_2$  production may be used as a measure of the progression of inflammation, and inhibition of their production may have potential therapeutic value for preventing inflammatory reaction and disease. In addition, it is well known that iNOS and COX-2 are rapidly and highly expressed in LPS-activated macrophages and contribute to the pathogenesis of septic shock (29,30). In this study, we observed that esculetin inhibits LPS-induced NO and  $\text{PGE}_2$  synthesis in a concentration-dependent manner in RAW 264.7 macrophages. Consistently, this suppression was correlated with the downregulation of iNOS and COX-2 expression (Fig. 2).

Conversely, it has been reported that the dysregulation of cytokines including TNF- $\alpha$  and IL-1 $\beta$ , commonly released by macrophages, plays an essential role in a number of undesirable inflammatory lesions. The production of these cytokines by activated macrophages is rapidly increased in acute inflammatory responses associated with infection, injury, trauma and other stress (31,32). This production is then

further increased by autocrine and paracrine routes, greatly increasing the severity of the immune response which causes inflammation (33,34). Moreover, the production of TNF- $\alpha$  and IL-1 $\beta$  is crucially required for the synergistic induction of NO and  $\text{PGE}_2$  production in LPS-stimulated macrophages (35,36). Thus, the selective inhibition of cytokine production and function may be effective therapeutically in the control of inflammatory disorders. In the present study, our data indicated that esculetin significantly inhibited the production of TNF- $\alpha$  as well as IL-1 $\beta$ , which was in parallel with the comparable inhibition of their expression in RAW 264.7 cells stimulated by LPS (Fig. 3). In conjunction with inhibiting LPS-induced NO and  $\text{PGE}_2$  production, these findings provide evidence that esculetin possesses useful anti-inflammatory activity.

NF- $\kappa$ B transcription factor has been proven to play a key role in inflammatory responses through the regulation of genes encoding various inflammatory cytokines and inducible enzymes including COX-2 and iNOS in macrophages (6,7,37). NF- $\kappa$ B is normally present in the cytosol due to its association with I $\kappa$ B. Once activated by inflammatory stimulants, I $\kappa$ B is rapidly phosphorylated and degraded by the 26S proteasome. I $\kappa$ B degradation liberates NF- $\kappa$ B, which then translocates to the nucleus, resulting in transcriptional induction of inflammation-associated genes (10,38,39). This study indicates that esculetin strongly inhibited the nuclear translocation of NF- $\kappa$ B and blocked the degradation of I $\kappa$ B in LPS-stimulated RAW 264.7 macrophages (Fig. 4). These observations suggest that the novel anti-inflammatory mechanisms mediated by esculetin are based on the inhibition of the LPS-mediated activation of NF- $\kappa$ B.

Oxidative stress occurs when the balance of the intracellular redox state is disrupted by the excessive production of ROS. Accumulating evidence indicates that antioxidant compounds inhibit the production of inflammatory cytokines by suppressing the expression of the corresponding genes by suppressing NF- $\kappa$ B activation through the removal of ROS (11,40,41). The suppressive effects of these compounds on the production of the associated inflammatory mediators are also associated with their antioxidant activities, and thus reduce inflammation (2,42). Therefore, ROS is thought to be involved in inflammatory gene expression through the redox-based activation of the NF- $\kappa$ B signaling pathway. In this study, our data demonstrated that ROS production was markedly increased in LPS-stimulated RAW 264.7 cells and treatment with esculetin attenuated ROS production in a concentration-dependent manner (Fig. 5). These results indicate that esculetin has intracellular radical scavenging activity in RAW 264.7 cells and suggest that the potential inhibition of ROS generation by esculetin is consistent with the inhibition of the expression of NF- $\kappa$ B-dependent cytokines and mediators.

In summary, we have demonstrated that the treatment of mouse macrophage RAW 264.7 cells with esculetin decreased the levels of pro-inflammatory mediators including NO and  $\text{PGE}_2$  following LPS stimulation through downregulation of iNOS and COX-2 expression. Esculetin also significantly inhibited the formation of pro-inflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , which was associated with the suppression of expression of their corresponding genes. In addition, the anti-inflammatory properties of esculetin were mediated by the downregulation of NF- $\kappa$ B activation and the

inhibition of ROS accumulation. Although further *in vivo* investigation is necessary to qualify the anti-inflammatory activity of esculetin, our data suggest that esculetin has a strong antioxidant capacity to inhibit inflammation-associated gene expression by suppressing the redox-dependent NF- $\kappa$ B signaling pathway.

### Acknowledgements

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) grant funded by the Korean government (no. 2012046358) and the R&D program of MOTIE/KIAT (10040391, Development of Functional Food Materials and Device for Prevention of Aging-Associated Muscle Function Decrease), Republic of Korea.

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