

Fisetin Suppresses Macrophage-Mediated Inflammatory Responses by Blockade of Src and Syk

Jun Ho Kim^{1,†}, Mi-Yeon Kim^{2,†}, Jong-Hoon Kim^{3,*} and Jae Youl Cho^{1,*}

¹Department of Genetic Engineering, Sungkyunkwan University, Suwon 440-746, ²School of Systems Biological Science, Soongsil University, Seoul 156-743, ³Department of Veterinary Physiology, College of Veterinary Medicine, Biosafety Research Institute, Chonbuk National University, Jeonju 561-756, Republic of Korea

Abstract

Flavonoids, such as fisetin (3,7,3',4'-tetrahydroxyflavone), are plant secondary metabolites. It has been reported that fisetin is able to perform numerous pharmacological roles including anti-inflammatory, anti-microbial, and anti-cancer activities; however, the exact anti-inflammatory mechanism of fisetin is not understood. In this study, the pharmacological action modes of fisetin in lipopolysaccharide (LPS)-stimulated macrophage-like cells were elucidated by using immunoblotting analysis, kinase assays, and an overexpression strategy. Fisetin diminished the release of nitric oxide (NO) and reduced the mRNA levels of inducible NO synthase (iNOS), tumor necrosis factor (TNF)- α , and cyclooxygenase (COX)-2 in LPS-stimulated RAW264.7 cells without displaying cytotoxicity. This compound also blocked the nuclear translocation of p65/nuclear factor (NF)- κ B. In agreement, the upstream phosphorylation events for NF- κ B activation, composed of Src, Syk, and I κ B α , were also reduced by fisetin. The phospho-Src level, triggered by overexpression of wild-type Src, was also inhibited by fisetin. Therefore, these results strongly suggest that fisetin can be considered a bioactive immunomodulatory compound with anti-inflammatory properties through suppression of Src and Syk activities.

Key Words: Fisetin, Anti-inflammatory effect, NF- κ B, Src, Syk

INTRODUCTION

Inflammation is one of the common biological reactions that protect our body from infection of bacteria, virus, and fungi. When inflammation occurs, the body responds by activating many different types of immune cells to clear out pathogens. Macrophages, the most common inflammatory cells, play a critical role in removing infectious materials and activate other immune cells. For these processes, activated macrophages produce pro-inflammatory cytokines and inflammatory mediators such as nitric oxide (NO) (Yang *et al.*, 2014a; Soler Palacios *et al.*, 2015). New production of these molecules is regulated at the transcriptional level by controlling nuclear translocation and DNA binding activity of inflammation-regulatory transcription factors such as nuclear factor (NF)- κ B and activator protein (AP-1). By the action of these proteins, mRNA levels of inflammatory genes, such as inducible NO synthase (iNOS), cyclooxygenase (COX)-2, and tumor necrosis factor

(TNF)- α can be dramatically increased (Van Den Berg and Bresnihan, 1999; Vilahur and Badimon, 2014).

Flavonoids are plant pigments that possess various physiological effects including antioxidant, antiviral, anti-cancer, anti-bacterial and anti-inflammatory activities (Rice-Evans *et al.*, 1996; Jang *et al.*, 2005; Khan *et al.*, 2013; Jeong *et al.*, 2014b). Fisetin (Fig. 1) is one of the polyphenolic flavonoids, which are widely found in many fruits and vegetables such

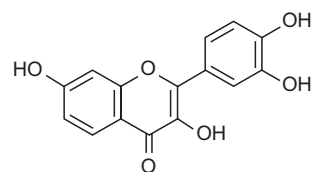


Fig. 1. Chemical structure of fisetin.

Open Access <http://dx.doi.org/10.4062/biomolther.2015.036>

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received Mar 30, 2015 Revised Apr 19, 2015 Accepted Apr 27, 2015

Published online Sep 1, 2015

*Corresponding Authors

E-mail: jhkim1@chonbuk.ac.kr (Kim JH), jaecho@skku.edu (Cho JY)

Tel: +82-63-270-2563 (Kim JH), +82-31-290-7868 (Cho JY)

Fax: +82-31-290-7870 (Kim JH), +82-63-270-3780 (Cho JY)

[†]The first two authors contributed equally to this work.

as strawberry, mango, and onion (Khan *et al.*, 2013; Pal *et al.*, 2015). It has also been reported that fisetin displays anti-oxidative, anti-inflammatory, and anti-proliferative activities (Khan *et al.*, 2013). In particular, several groups have suggested the anti-inflammatory role of fisetin occurs via suppression of NF- κ B and AP-1 activation (Kim *et al.*, 2012), however, how fisetin blocks these pathways is not fully elucidated in terms of identifying direct target enzymes. In this study, therefore, we sought to identify the molecular targets of fisetin involved in the negative regulation of lipopolysaccharide (LPS)-stimulated macrophages.

MATERIALS AND METHODS

Materials

Fisetin (purity: >98%), polyethylenimine (PEI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (a tetrazole) (MTT), and LPS (*E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). RAW264.7 (a mouse macrophage-like cell line) and HEK293 (a human embryonic kidney cell line) cells were purchased from ATCC (Rockville, MD, USA). All other chemicals used in this study were of analytical grade from Sigma Chemical Company. Phospho-specific or total-protein antibodies recognizing p65, p50, inhibitor of κ B α (I κ B α), Src, spleen tyrosine kinase (Syk), lamin A/C and β -actin were obtained from Cell Signaling Technology (Beverly, MA, USA).

Expression vectors and DNA transfection

Wild-type Src (Src-WT) was used as reported previously (Yang *et al.*, 2008). All constructs were confirmed by automated DNA sequencing. Overexpression experiments were performed with HEK293 cells (1×10^6 cells/mL) by transfection with Src-WT using the PEI method in 12-well plates as reported previously (Shen *et al.*, 2011; Song *et al.*, 2012). The cells were utilized for the experiments 24 h post-transfection. Fisetin was added to cells 24 h before termination.

Cell culture and drug preparation

RAW264.7 cells were cultured in RPMI1640 with 10% heat-inactivated FBS and 1% penicillin/streptomycin at 37°C in 5% CO₂. HEK293 cells were maintained in DMEM media supplemented with 5% heat-inactivated FBS and 1% penicillin/streptomycin at 37°C in 5% CO₂. The stock solutions of fisetin for the *in vitro* experiments were prepared in dimethylsulfoxide (DMSO).

Determination of NO production

After pre-incubation of RAW264.7 cells (1×10^6 cells/mL) for 18 h, fisetin was added to the cells for 30 min. After that, the cells were treated with LPS (1 μ g/mL) for 24 h. The effect of fisetin (0 to 30 μ M) on the production of NO was determined by analyzing NO levels using Griess reagents (Kim *et al.*, 2013a; Youn *et al.*, 2013).

Cell viability test

The cytotoxic effects of fisetin (0 to 30 μ M) were then evaluated using a conventional MTT assay as previously reported

Table 1. PCR primers used in this study

Name		Sequence (5' to 3')
iNOS	F	GGAGCCTTTAGACCTCAACAGA
	R	TGAACGAGGAGGGTGGTG
TNF- α	F	TGCCTATGTCTCAGCCTCTTC
	R	GAGGCCATTTGGAACTTCT
COX-2	F	GGGAGTCTGGAACATTGTGAA
	R	GCACATTGTAAGTAGGTGGACTGT
GAPDH	F	CAATGAATACGGCTACAGCAAC
	R	AGGGAGATGCTCAGTGTGG

(Pauwels *et al.*, 1988; Yayeh *et al.*, 2012; Oh *et al.*, 2013). For the final 3 h of culture, 10 μ l of MTT solution (10 mg/mL in phosphate-buffered saline, pH 7.4) were added to each well. Reactions were stopped by the addition of 15% sodium dodecyl sulfate (SDS) into each well, solubilizing the formazan. The absorbance at 570 nm (OD₅₇₀₋₆₃₀) was measured using a Spectramax 250 microplate reader (BioTex, Bad Friedrichshall, Germany).

mRNA analysis using semi-quantitative and quantitative reverse transcriptase-polymerase chain reactions

In order to determine cytokine mRNA expression levels, total RNA was isolated from LPS-treated RAW264.7 cells using TRIzol Reagent, according to the manufacturer's instructions. Total RNA was stored at -70°C until use. Semi-quantitative RT reactions were conducted as previously reported (Lee *et al.*, 2009). Quantification of mRNA was performed by real-time reverse transcriptase polymerase chain reaction (RT-PCR) with SYBR Premix Ex Taq according to the manufacturer's instructions (Takara, Shiga, Japan) using a real-time thermal cycler (Bio-Rad, Hercules, CA, USA) as reported previously (Kang *et al.*, 2013). Semi-quantitative RT-PCR was conducted as previously reported with minor modifications (Sohn *et al.*, 2013). All of the primers (Bioneer, Daejeon, Korea) used are listed in Table 1.

Preparation of cell lysates and nuclear fractions for immunoblotting

RAW264.7 or HEK293 cells (5×10^6 cells/mL) were washed three times in cold phosphate buffered saline (PBS) and lysed in lysis buffer as reported previously (Kim *et al.*, 2014b). Nuclear lysates were prepared using a three-step procedure (Byeon *et al.*, 2008). After treatment, the cells were collected with a rubber policeman, washed with 1 \times PBS, and lysed in 500 μ l of lysis buffer on ice for 4 min. During the second step, the pellet (the nuclear fraction) was washed once with wash buffer without Nonidet P-40. During the final step, the nuclei were resuspended in an extraction buffer consisting of the lysis buffer plus 500 mM KCl and 10% glycerol. The nuclei/extraction buffer mixture was frozen at -80°C then thawed on ice and centrifuged at 14,000 rpm for 5 min. The supernatant was collected as the nuclear extract. Whole-cell or nuclear lysates were then analyzed by a conventional immunoblotting method (Yang *et al.*, 2014b). The total and phosphorylated levels of p65, p50, I κ B α , Src, Syk, HA, lamin A/C, and β -actin were visualized using an ECL system (Amersham, Little Chalfont, Buckinghamshire, UK) as reported previously (Lee *et al.*, 2012).

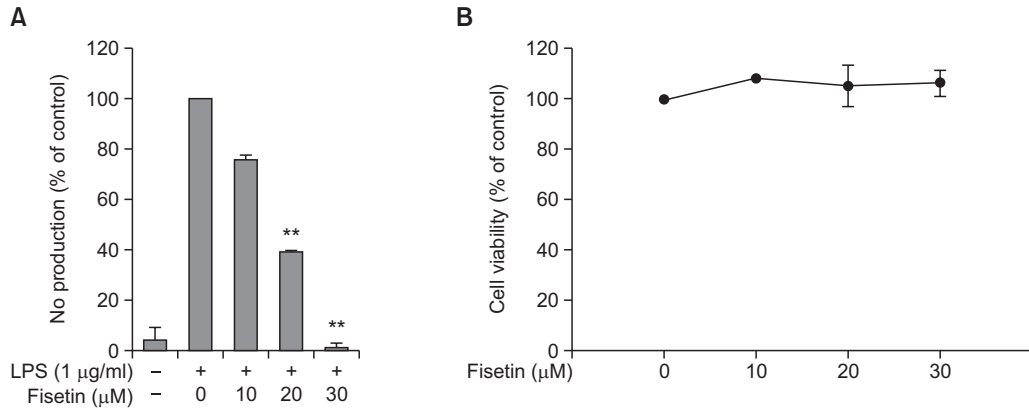


Fig. 2. The effect of fisetin on the production of NO and cell viability in LPS-stimulated RAW 264.7 cells. (A) RAW264.7 cells (1×10^6 cells/mL) were treated with LPS (1 µg/mL) in the presence or absence of fisetin (0 to 30 µM) for 24 h. The supernatants were then collected, and the NO concentration in the supernatants was determined using the Griess assay. (B) RAW264.7 cells (1×10^6 cells/mL) were treated with fisetin for 24 h, and cell viability was evaluated using the MTT assay. All data are expressed as the mean \pm SD of experiments, which were performed with six samples. ** $p < 0.01$ compared to normal or control groups.

In vitro kinase assay with purified enzymes

In order to evaluate the inhibition of the kinase activities of Src or Syk using purified enzymes, the kinase profiler service from Millipore (Billerica, MA, USA) was used. Purified Src or Syk (human) (1-5 mU) were incubated with the reaction buffer in a final reaction volume of 25 µl. The reaction was initiated by the addition of Mg-ATP. After incubation for 40 min at room temperature, the reaction was stopped by the addition of 5 µl of a 3% phosphoric acid solution. Ten microliters of the reaction were then spotted onto a P30 Filtermat that was washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

Statistical analyses

All of the data presented in this paper are expressed as means \pm SD. For statistical comparisons, results were analyzed using either ANOVA/Scheffe's *post-hoc* test or the Kruskal-Wallis/Mann-Whitney test. A p -value < 0.05 was considered to be statistically significant. All statistical tests were carried out using the computer program, SPSS (SPSS Inc., Chicago, IL, USA).

RESULTS

Effect of fisetin on inflammatory responses

First, to confirm the function of fisetin as an inflammatory-response suppressor in macrophage cells, we tested its capability to suppress NO production in LPS-treated RAW264.7 cells. As expected, production of NO was dose-dependently decreased by fisetin in RAW264.7 cells stimulated by LPS (Fig. 2A). It was found that there was no cytotoxic activity of fisetin at its effective anti-inflammatory concentrations (Fig. 2B).

Effect of fisetin on transcriptional activation in LPS-treated RAW264.7 cells

To check whether the anti-inflammatory effect of fisetin occurs at the transcriptional level, we determined the mRNA expression levels of inflammatory genes such as inducible nitric

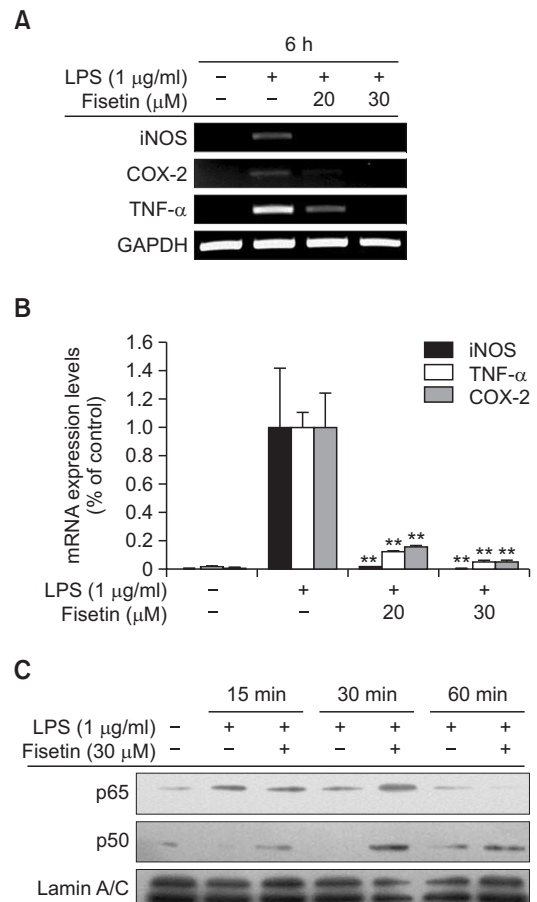


Fig. 3. The effect of fisetin on transcriptional activation in LPS-stimulated RAW264.7 cells. (A and B) The mRNA levels of iNOS, COX-2, and TNF-α expressed in LPS (1 µg/mL)-treated RAW264.7 cells in the presence or absence of fisetin (20 and 30 µM) were measured by RT-PCR (A) or real-time PCR (B). (C) The nuclear levels of p65 and p50 in RAW264.7 cells treated with LPS (1 µg/mL) in the presence or absence of fisetin (30 µM) were analyzed by immunoblotting analysis. Data (B) are expressed as the mean \pm SD of experiments, which were performed with six samples. ** $p < 0.01$ compared to normal or control groups.

oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor- α (TNF- α). As expected, the expression of inflammatory mediator genes was suppressed in cells treated with either 20 or 30 μ M of fisetin according to both semi-quantitative (Fig. 3A) and real-time RT-PCR (Fig. 3B) analyses. We also examined transcription factor levels in nuclear extracts by immunoblotting analysis and found that fisetin treatment was capable of inhibiting the nuclear translocation of p65/NF- κ B at

60 min but not of p50/NF- κ B (Fig. 3C).

Effect of fisetin on the upstream signaling of NF- κ B activation

Many studies have demonstrated that the NF- κ B pathway is regulated by intracellular signaling cascades (Byeon *et al.*, 2012; Yi *et al.*, 2014). These signaling cascades include Src, Syk, and I κ B α . To identify fisetin-targeted molecules in this pathway, we confirmed the expression levels of these molecules by immunoblotting analysis. Expectedly, the phospho-I κ B α level in fisetin-treated cells was clearly decreased at 5, 30, and 60 min (Fig. 4A). Also, the autophosphorylation levels of Src and Syk were strikingly suppressed by fisetin at 2 min, without decreasing total levels of Src and Syk (Fig. 4B).

Inhibition of Src and Syk kinase activity and overexpression of Src by fisetin

In order to confirm inhibitory activity of Src/Syk phosphorylation, we checked whether these enzymes can directly block the kinase activity of Src and Syk. As expected, the kinase activities of purified Src and Syk were completely inhibited by fisetin (30 μ M). This result indicates that fisetin can act as a direct inhibitor of these enzymes (Fig. 5A). To further validate fisetin-mediated suppression of Src activity, we employed an overexpression strategy using HA-Src. We found that overexpressed Src increased the phospho-Src level and fisetin suppressed the phosphorylation of Src in a dose-dependent manner (Fig. 5B).

DISCUSSION

It was reported that fisetin exhibits anti-inflammatory, anti-oxidative, and anti-proliferative activities (Pal *et al.*, 2015). In our study, this compound was revealed to significantly inhibit NO production in RAW264.7 cells without affecting their cell viability (Fig. 2A, 2B). In addition, mRNA expression levels of pro-inflammatory genes were decreased by fisetin (Fig. 3A, 3B). Additionally, the translocation of transcription factor p65, a subunit of NF- κ B, into the nucleus was remarkably inhibited at 60 min (Fig. 3C), implying that NF- κ B upstream signaling

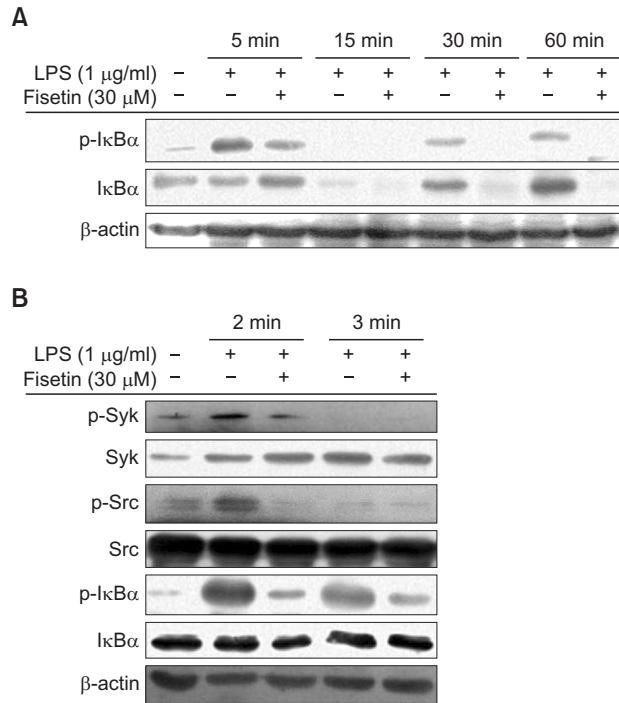


Fig. 4. The effect of fisetin on the upstream signaling cascade of NF- κ B. (A and B) RAW264.7 cells were incubated with LPS (1 μ g/mL) in the presence or absence of fisetin (30 μ M) for 30 min. The total phospho-protein levels of Src, Syk, and I κ B α from whole lysates were determined by immunoblotting analysis.

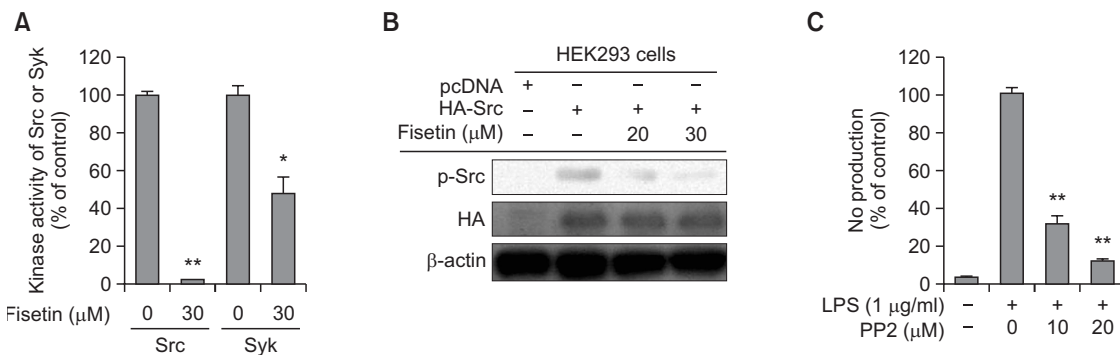


Fig. 5. The effect of fisetin on the activity of Src and Syk. (A) Inhibitory activity of fisetin (30 μ M) on the kinase activity of purified Src or Syk was examined by kinase assay. (B) Inhibitory activity of fisetin (20 and 30 μ M) on the phosphorylation of overexpressed Src was determined by immunoblotting analysis. (C) RAW264.7 cells (1×10^6 cells/mL) were treated with LPS (1 μ g/mL) in the presence or absence of PP2 (10 and 20 μ M) for 24 h. The supernatants were then collected, and the NO concentration in the supernatants was determined using the Griess assay. Data (A and C) are expressed as the mean \pm SD of experiments, which were performed with six samples. * $p < 0.05$ and ** $p < 0.01$ compared to normal or control groups.

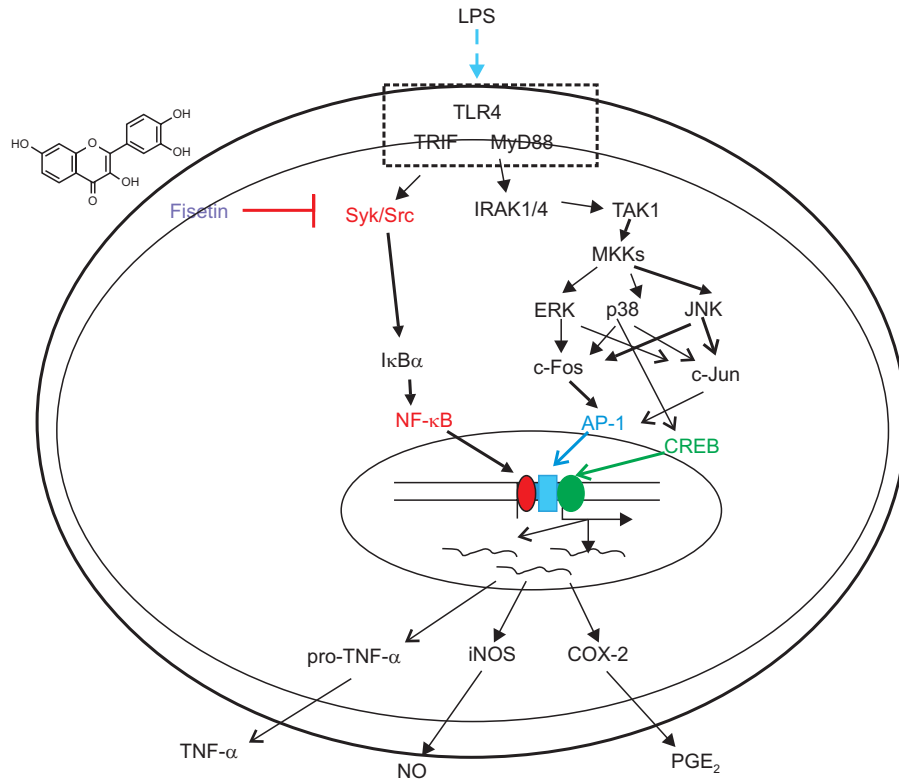


Fig. 6. Putative inhibitory pathway of fisetin in LPS-activated inflammatory events.

pathway may be targeted by fisetin treatment. Indeed, the phosphorylation of NF-κB regulatory upstream protein (IκBα), a key event for p65 translocation (Courtois and Gilmore, 2006), was decreased by fisetin (Fig. 4A). Similarly, it was also reported that fisetin exerts downregulation of interleukin-6 and TNF-α in LPS-activated RAW264.7 cells via suppression of NF-κB, although exact target protein was not fully identified (Kim *et al.*, 2012). Previous papers and our present results led us to hypothesize that early enzymes, activated within 5 min under LPS stimulation, could be regulated by fisetin.

Previously, we determined that Src and Syk are major NF-κB regulatory protein tyrosine kinases (Byeon *et al.*, 2012; Yi *et al.*, 2014). In parallel, inhibitory compounds, such as carnosic acid and quercetin, and plant-derived extracts from *Evo-dia lepta*, *Artemisia asiatica*, and *Rhodomlyrtus tomentosa*, which show anti-inflammatory activities, were found to suppress Src and Syk (Oh *et al.*, 2012; Endale *et al.*, 2013; Jeong *et al.*, 2013; Yoon *et al.*, 2013). These enzymes' active forms and pro-inflammatory cytokines were increased in response to LPS stimulation at early time points (Lee *et al.*, 2009). In particular, activated Src and Syk are known to be involved in regulation of IκBα-phosphorylation (Lee *et al.*, 2009). Therefore, we also tested whether fisetin is able to suppress the phosphorylation of Src and Syk, a hallmark to determine activated forms of Src and Syk (Byeon *et al.*, 2012; Yi *et al.*, 2014). As expected, the phosphorylation of Src and Syk at 2 min was strongly suppressed by fisetin (Fig. 4B). Since the phosphorylation of these enzymes is generated by their catalytic enzyme activities (Byeon *et al.*, 2012; Yi *et al.*, 2014), it is supposed that fisetin can directly diminish the enzyme activity

of Src and Syk. To prove this possibility, we determined the kinase activity by a conventional enzyme assay with purified Src and Syk as reported previously (Kim *et al.*, 2013b; Jeong *et al.*, 2014b). As Fig. 5A shows, the kinase activity of Src was strongly reduced by fisetin (30 μM), while Syk was suppressed up to 52%, indicating that Src is the more preferred target of fisetin. Validation of this was also performed by overexpression work with HA-Src-expressing construct and by exploration of NO inhibitory activity under the treatment of PP2, a Src inhibitor (Byeon *et al.*, 2013). In fact, it was confirmed that transfection of Src cDNA increased the phosphorylation level of Src (Byeon *et al.*, 2013; Dung *et al.*, 2014). Similarly, fisetin treatment dose-dependently inhibited the phosphorylation of Src raised by Src overexpression (Fig. 5B). PP2 also strongly blocked the release of NO in LPS-treated RAW264.7 cells (Fig. 5C) as reported previously (Yang *et al.*, 2014a). Therefore, these results, and other previously reported results, strongly support that Src could be a major direct target of fisetin in its anti-inflammatory actions.

Our data, demonstrating fisetin's inhibitory potency and its anti-inflammatory mechanisms, strongly suggest that it can be developed as an anti-inflammatory drug. However, development of a single compound from natural plants as an anti-inflammatory drug is difficult due to high production cost and low purification yield. Although the amount of this compound is very low, it has been demonstrated that fisetin is one of the major anti-inflammatory components in various plants, such as *Muntingia calabura*, *Toxicodendron vernicifluum*, and *Rhus verniciflua*, with *in vitro* and *in vivo* anti-inflammatory activity (Park *et al.*, 2013; Kim *et al.*, 2015). A recent trend in the

pharmaceutical industry is discovering strategies to develop the natural plant itself as the new drug. Indeed, STILLÉN™ from Dong-A Pharmaceutical Co. in Korea, was developed from *Artemisiae argyi* Folium 14 years ago. The activity of this compound against gastric ulcer and gastritis is promising, even in the extract (Jeong *et al.*, 2014a). The active components of this extract are flavonoids eupatilin, jaceosidin, and luteolin (Seo *et al.*, 2003). Considering these, it is also proposed that fisetin-containing plants can be chosen as anti-inflammatory candidate herbal medicines. Numerous reports have proved that fisetin-containing *Rhus verniciflua* is orally effective against 2,4-dinitrofluorobenzene-induced allergic contact dermatitis and carrageenan-induced edema (Jung *et al.*, 2011; Park *et al.*, 2013; Kim *et al.*, 2014a). A chemical synthetic approach for mass production of fisetin and its derivatives was also reported previously (Chiruta *et al.*, 2012). Using this method, it is expected that fisetin can be prepared in a low-cost way. Although synthetic fisetin can be applied in industrial fields, extract formulas seem to be easier to develop and display more potent efficacy due to a mixture form with other active ingredients. Therefore, we will continue screening fisetin-rich plants by HPLC analysis and will prove their anti-inflammatory efficacy by using *in vitro* and *in vivo* experimental models.

In summary, we have demonstrated that fisetin can suppress I κ B α phosphorylation, p65 translocation, inflammatory gene expression, and NO production by direct blockade of Src and Syk kinase activities as summarized in Fig. 6. Because fisetin shows potent anti-inflammatory activity, we suggest that fisetin-rich plants from edible sources could be applied to the development of therapeutic remedies to cure various inflammatory symptoms. Therefore, relevant works will be further proved in the future projects.

ACKNOWLEDGMENTS

This work was carried out with the support of the Cooperative Research Program for Agriculture Science & Technology Development (Project no. PJ009241), Rural Development Administration, Korea.

REFERENCES

- Byeon, S. E., Lee, Y. G., Kim, B. H., Shen, T., Lee, S. Y., Park, H. J., Park, S. C., Rhee, M. H. and Cho, J. Y. (2008) Surfactin blocks NO production in lipopolysaccharide-activated macrophages by inhibiting NF-kappaB activation. *J. Microbiol. Biotechnol.* **18**, 1984-1989.
- Byeon, S. E., Yi, Y. S., Oh, J., Yoo, B. C., Hong, S. and Cho, J. Y. (2012) The role of Src kinase in macrophage-mediated inflammatory responses. *Mediators Inflamm.* **2012**, 512926.
- Byeon, S. E., Yu, T., Yang, Y., Lee, Y. G., Kim, J. H., Oh, J., Jeong, H. Y., Hong, S., Yoo, B. C., Cho, W. J., Hong, S. and Cho, J. Y. (2013) Hydroquinone regulates hemeoxygenase-1 expression via modulation of Src kinase activity through thiolation of cysteine residues. *Free Radic. Biol. Med.* **57**, 105-118.
- Chiruta, C., Schubert, D., Dargusch, R. and Maher, P. (2012) Chemical modification of the multitarget neuroprotective compound fisetin. *J. Med. Chem.* **55**, 378-389.
- Courtois, G. and Gilmore, T. D. (2006) Mutations in the NF-kappaB signaling pathway: implications for human disease. *Oncogene* **25**, 6831-6843.
- Dung, T. T., Kim, S. C., Yoo, B. C., Sung, G. H., Yang, W. S., Kim, H. G., Park, J. G., Rhee, M. H., Park, K. W., Yoon, K., Lee, Y., Hong, S., Kim, J. H. and Cho, J. Y. (2014) (5-Hydroxy-4-oxo-4H-pyran-2-yl)methyl 6-hydroxynaphthalene-2-carboxylate, a kojic acid derivative, inhibits inflammatory mediator production via the suppression of Syk/Src and NF-kappaB activation. *Int. Immunopharmacol.* **20**, 37-45.
- Endale, M., Park, S. C., Kim, S., Kim, S. H., Yang, Y., Cho, J. Y. and Rhee, M. H. (2013) Quercetin disrupts tyrosine-phosphorylated phosphatidylinositol 3-kinase and myeloid differentiation factor-88 association, and inhibits MAPK/AP-1 and IKK/NF-kappaB-induced inflammatory mediators production in RAW 264.7 cells. *Immunobiology* **218**, 1452-1467.
- Jang, H.-S., Kook, S.-H., Son, Y.-O., Kim, J.-G., Jeon, Y.-M., Jang, Y.-S., Choi, K.-C., Kim, J., Han, S.-K. and Lee, K.-Y. (2005) Flavonoids purified from *Rhus verniciflua* Stokes actively inhibit cell growth and induce apoptosis in human osteosarcoma cells. *Biochim. Biophys. Acta* **1726**, 309-316.
- Jeong, D., Yang, W. S., Yang, Y., Nam, G., Kim, J. H., Yoon, D. H., Noh, H. J., Lee, S., Kim, T. W., Sung, G. H. and Cho, J. Y. (2013) In vitro and in vivo anti-inflammatory effect of *Rhodomyrtos tomentosae* methanol extract. *J. Ethnopharmacol.* **146**, 205-213.
- Jeong, D., Yi, Y. S., Sung, G. H., Yang, W. S., Park, J. G., Yoon, K., Yoon, D. H., Song, C., Lee, Y., Rhee, M. H., Kim, T. W., Kim, J. H. and Cho, J. Y. (2014a) Anti-inflammatory activities and mechanisms of *Artemisia asiatica* ethanol extract. *J. Ethnopharmacol.* **152**, 487-496.
- Jeong, H. Y., Sung, G.-H., Kim, J. H., Yoon, J. Y., Yang, Y., Park, J. G., Kim, S. H., Yi, Y.-S., Yang, W. S. and Yoon, D. H. (2014b) Syk and Src are major pharmacological targets of a *Cerbera manghas* methanol extract with kaempferol-based anti-inflammatory activity. *J. Ethnopharmacol.* **151**, 960-969.
- Jung, C. H., Kim, J. H., Kim, J. H., Chung, J. H., Choi, H. S., Seo, J. B., Shin, Y. C., Kim, S. H. and Ko, S. G. (2011) Anti-inflammatory effect of *Rhus verniciflua* Stokes by suppression of iNOS-mediated Akt and ERK pathways: in-vitro and in-vivo studies. *J. Pharm. Pharmacol.* **63**, 679-687.
- Kang, G. J., Han, S. C., Ock, J. W., Kang, H. K. and Yoo, E. S. (2013) Anti-inflammatory effect of quercetagenin, an active component of immature *Citrus unshiu*, in HaCaT human keratinocytes. *Biomol. Ther.* **21**, 138-145.
- Khan, N., Syed, D. N., Ahmad, N. and Mukhtar, H. (2013) Fisetin: a dietary antioxidant for health promotion. *Antioxid. Redox Signal.* **19**, 151-162.
- Kim, D. H., Chung, J. H., Yoon, J. S., Ha, Y. M., Bae, S., Lee, E. K., Jung, K. J., Kim, M. S., Kim, Y. J., Kim, M. K. and Chung, H. Y. (2013a) Ginsenoside Rd inhibits the expressions of iNOS and COX-2 by suppressing NF-kappaB in LPS-stimulated RAW264.7 cells and mouse liver. *J. Ginseng Res.* **37**, 54-63.
- Kim, J. H., Lee, Y. G., Yoo, S., Oh, J., Jeong, D., Song, W. K., Yoo, B. C., Rhee, M. H., Park, J., Cha, S. H., Hong, S. and Cho, J. Y. (2013b) Involvement of Src and the actin cytoskeleton in the anti-tumorigenic action of adenosine dialdehyde. *Biochem. Pharmacol.* **85**, 1042-1056.
- Kim, J. H., Shin, Y. C. and Ko, S. G. (2014a) Integrating traditional medicine into modern inflammatory diseases care: multitargeting by *Rhus verniciflua* Stokes. *Mediators Inflamm.* **2014**, 154561.
- Kim, K. H., Moon, E., Choi, S. U., Pang, C., Kim, S. Y. and Lee, K. R. (2015) Identification of cytotoxic and anti-inflammatory constituents from the bark of *Toxicodendron vernicifluum* (Stokes) F.A. Barkley. *J. Ethnopharmacol.* **162**, 231-237.
- Kim, M. Y., Yoo, B. C. and Cho, J. Y. (2014b) Ginsenoside-Rp1-induced apolipoprotein A-1 expression in the LoVo human colon cancer cell line. *J. Ginseng Res.* **38**, 251-255.
- Kim, S.-C., Kang, S.-H., Jeong, S.-J., Kim, S.-H., Ko, H. S. and Kim, S.-H. (2012) Inhibition of c-Jun N-terminal kinase and nuclear factor κ B pathways mediates fisetin-exerted anti-inflammatory activity in lipopolysaccharide-treated RAW264.7 cells. *Immunopharmacol. Immunotoxicol.* **34**, 645-650.
- Lee, J. A., Lee, M. Y., Shin, I. S., Seo, C. S., Ha, H. and Shin, H. K. (2012) Anti-inflammatory effects of *Amomum compactum* on RAW 264.7 cells via induction of heme oxygenase-1. *Arch. Pharm. Res.* **35**, 739-746.

- Lee, Y. G., Chain, B. M. and Cho, J. Y. (2009) Distinct role of spleen tyrosine kinase in the early phosphorylation of inhibitor of kappaB alpha via activation of the phosphoinositide-3-kinase and Akt pathways. *Int. J. Biochem. Cell Biol.* **41**, 811-821.
- Oh, C. T., Park, J. I., Jung, Y. R., Joo, Y. A., Shin, D. H., Cho, H. J., Ahn, S. M., Lim, Y. H., Park, C. K. and Hwang, J. S. (2013) Inhibitory effect of Korean red ginseng on melanocyte proliferation and its possible implication in GM-CSF mediated signaling. *J. Ginseng Res.* **37**, 389-400.
- Oh, J., Yu, T., Choi, S. J., Yang, Y., Baek, H. S., An, S. A., Kwon, L. K., Kim, J., Rho, H. S., Shin, S. S., Choi, W. S., Hong, S. and Cho, J. Y. (2012) Syk/Src pathway-targeted inhibition of skin inflammatory responses by carnosic acid. *Mediators Inflamm.* **2012**, 781375.
- Pal, H. C., Athar, M., Elmets, C. A. and Afaq, F. (2015) Fisetin inhibits UVB-induced cutaneous inflammation and activation of PI3K/AKT/NFkB signaling pathways in SKH-1 hairless mice. *Photochem. Photobiol.* **91**, 225-234.
- Park, D. K., Lee, Y. G. and Park, H. J. (2013) Extract of *Rhus verniciflua* bark suppresses 2,4-dinitrofluorobenzene-induced allergic contact dermatitis. *Evid. Based Complement Alternat. Med.* **2013**, 879696.
- Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J. and De Clercq, E. (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J. Virol. Methods* **20**, 309-321.
- Rice-Evans, C. A., Miller, N. J. and Paganga, G. (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* **20**, 933-956.
- Seo, J. M., Kang, H. M., Son, K. H., Kim, J. H., Lee, C. W., Kim, H. M., Chang, S. I. and Kwon, B. M. (2003) Antitumor activity of flavones isolated from *Artemisia argyi*. *Planta Med.* **69**, 218-222.
- Shen, T., Lee, J., Park, M. H., Lee, Y. G., Rho, H. S., Kwak, Y. S., Rhee, M. H., Park, Y. C. and Cho, J. Y. (2011) Ginsenoside Rp1, a ginsenoside derivative, blocks promoter activation of iNOS and COX-2 genes by suppression of an IKKbeta-mediated NF-kB pathway in HEK293 cells. *J. Ginseng Res.* **35**, 200-208.
- Sohn, S. H., Kim, S. K., Kim, Y. O., Kim, H. D., Shin, Y. S., Yang, S. O., Kim, S. Y. and Lee, S. W. (2013) A comparison of antioxidant activity of Korean white and red ginsengs on H2O2-induced oxidative stress in HepG2 hepatoma cells. *J. Ginseng Res.* **37**, 442-450.
- Soler Palacios, B., Estrada-Capetillo, L., Izquierdo, E., Criado, G., Nieto, C., Municio, C., González-Alvaro, I., Sánchez-Mateos, P., Pablos, J. L. and Corbí, A. L. (2015) Macrophages from the synovium of active rheumatoid arthritis exhibit an activin A-dependent pro-inflammatory profile. *J. Pathol.* **235**, 515-526.
- Song, S. B., Tung, N. H., Quang, T. H., Ngan, N. T., Kim, K. E. and Kim, Y. H. (2012) Inhibition of TNF-alpha-mediated NF-kappaB transcriptional activity in HepG2 cells by dammarane-type saponins from *Panax ginseng* leaves. *J. Ginseng Res.* **36**, 146-152.
- van den Berg, W. B. and Bresnihan, B. (1999) Pathogenesis of joint damage in rheumatoid arthritis: evidence of a dominant role for interleukin-1. *Baillieres Best Pract. Res. Clin. Rheumatol.* **13**, 577-597.
- Vilahir, G. and Badimon, L. (2014) Ischemia/reperfusion activates myocardial innate immune response: the key role of the toll-like receptor. *Front. Physiol.* **5**, 496.
- Yang, K.-J., Shin, S., Piao, L., Shin, E., Li, Y., Park, K. A., Byun, H. S., Won, M., Hong, J. and Kweon, G. R. (2008) Regulation of 3-phosphoinositide-dependent protein kinase-1 (PDK1) by Src involves tyrosine phosphorylation of PDK1 and Src homology 2 domain binding. *J. Biol. Chem.* **283**, 1480-1491.
- Yang, W. S., Jeong, D., Yi, Y. S., Lee, B. H., Kim, T. W., Htwe, K. M., Kim, Y. D., Yoon, K. D., Hong, S., Lee, W. S. and Cho, J. Y. (2014a) *Myrsine seguinii* ethanolic extract and its active component quercetin inhibit macrophage activation and peritonitis induced by LPS by targeting to Syk/Src/IRAK-1. *J. Ethnopharmacol.* **151**, 1165-1174.
- Yang, Y., Yang, W. S., Yu, T., Yi, Y. S., Park, J. G., Jeong, D., Kim, J. H., Oh, J. S., Yoon, K., Kim, J. H. and Cho, J. Y. (2014b) Novel anti-inflammatory function of NSC95397 by the suppression of multiple kinases. *Biochem. Pharmacol.* **88**, 201-215.
- Yayeh, T., Jung, K. H., Jeong, H. Y., Park, J. H., Song, Y. B., Kwak, Y. S., Kang, H. S., Cho, J. Y., Oh, J. W., Kim, S. K. and Rhee, M. H. (2012) Korean red ginseng saponin fraction downregulates pro-inflammatory mediators in LPS stimulated RAW264.7 cells and protects mice against endotoxic shock. *J. Ginseng Res.* **36**, 263-269.
- Yi, Y. S., Son, Y. J., Ryou, C., Sung, G. H., Kim, J. H. and Cho, J. Y. (2014) Functional roles of Syk in macrophage-mediated inflammatory responses. *Mediators Inflamm.* **2014**, 270302.
- Yoon, J. Y., Jeong, H. Y., Kim, S. H., Kim, H. G., Nam, G., Kim, J. P., Yoon, D. H., Hwang, H., Kim, T. W., Hong, S. and Cho, J. Y. (2013) Methanol extract of *Evodia lepta* displays Syk/Src-targeted anti-inflammatory activity. *J. Ethnopharmacol.* **148**, 999-1007.
- Youn, C. K., Park, S. J., Lee, M. Y., Cha, M. J., Kim, O. H., You, H. J., Chang, I. Y., Yoon, S. P. and Jeon, Y. J. (2013) Silibinin inhibits LPS-induced macrophage activation by blocking p38 MAPK in RAW 264.7 Cells. *Biomol. Ther.* **21**, 258-263.