Flavones inhibit LPS-induced atrogin-1/MAFbx expression in mouse C2C12 skeletal myotubes

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Running head: Flavones prevent LPS-induced muscle atrophy

Summary

Muscle atrophy is a complex process that occurs as a consequence of various stress events. Muscle atrophy-associated genes (atrogenes) such as atrogin-1/MAFbx and MuRF-1 are induced early in the atrophy process, and the increase in their expression precedes the loss of muscle weight. Although antioxidative nutrients suppress atrogene expression in skeletal muscle cells, the inhibitory effects of flavonoids on inflammation-induced atrogin-1/MAFbx expression have not been clarified. Here, we investigated the inhibitory effects of flavonoids on lipopolysaccharide (LPS)-induced atrogin-1/MAFbx expression. We examined whether nine flavonoids belonging to six flavonoid categories inhibited atrogin-1/MAFbx expression in mouse C2C12 myotubes. Two major flavones, apigenin and luteolin, displayed potent inhibitory effects on atrogin-1/MAFbx expression. The pretreatment with apigenin and luteolin significantly prevented C2C12 myotube diameter caused by LPS stimulation. Importantly, the pretreatment of LPS-stimulated myoblasts with these flavones significantly inhibited LPS-induced JNK phosphorylation in C2C12 myotubes, resulting in the significant suppression of atrogin-1/MAFbx promoter activity. These results suggest that apigenin and luteolin, prevent LPS-mediated atrogin-1/MAFbx expression through the inhibition of the JNK signaling pathway in C2C12 myotubes. Thus, these flavones, apigenin and luteolin, may be promising agents to prevent LPS-induced muscle atrophy.

***Key words*:** Flavones, LPS, muscle atrophy, JNK

Introduction

Skeletal muscle is approximately 40–50% of human body weight, making this muscle the largest tissue mass and the most important protein reservoir in the body. Muscle mass maintenance is dependent on the balance between synthesis and breakdown of myofibrillar proteins (*1*). Signal transduction pathways promote the synthesis and/or degradation of muscle proteins and mediate the regulation of muscle homeostasis as well as muscle hypertrophy or atrophy. Muscle atrophy, characterized by the progressive loss of muscle mass and strength, is a complex process that occurs as a consequence of various stress events, including neural inactivity, mechanical unloading, inflammation, metabolic stress, and elevated glucocorticoids (*2*).

The molecules and cellular pathways regulating skeletal muscle atrophy are still being discovered; however, multiple studies have shown that muscle atrophy in sepsis is primarily the result of increased protein breakdown (*3,4*) via the ubiquitin-proteasome pathway (*5,6*). Specifically, ubiquitin-protein ligases (E3S), atrogin-1/MAFbx and MuRF-1, are critical in the development of muscle atrophy (*7,8*). Atrogin-1/MAFbx and MuRF-1 are induced in the early atrophy process, and the increase in their expression precedes the loss of muscle weight (*9*). Lipopolysaccharide (LPS)-activated toll-like receptor-4 (TLR4) induces C2C12 myotube atrophy by up-regulating the expression of the ubiquitin ligase atrogin-1/MAFbx (*10*). Based on these findings, we hypothesized that the inhibition of

atrogin-1/MAFbx and MuRF-1 expression may prevent or reduce muscle atrophy.

Dietary flavonoids are ubiquitously included in plant foods and have attracted considerable attention regarding their health effects (*11,12*). Previously, periodic injection of the flavonol-type flavonoid quercetin into the gastrocnemius muscle was shown to be effective in preventing muscle weight in mice that underwent tail suspension (*11*). Furthermore, the principal flavonoid prenylation enabled naringenin which is commonly found in grapefruit and sour oranges to prevent muscle atrophy induced by disuse in denervated mice (*13*). In addition, flavonoids prevent muscle atrophy induced by inflammatory conditions such as sepsis. Flavonoids are categorized into the following six major subclasses based on their range and structural complexity: flavonols, flavones, flavan-3-ols, flavanones, anthocyanins and isoflavones.

In the present study, we investigated the inhibitory effects of nine flavonoids belonging to these various categories on the expression of lipopolysaccharide (LPS)-induced atrogin-1/MAFbx to shed light on the prevention of muscle atrophy caused by sepsis.

Material and methods

Cell culture. Murine C2C12 cells with a myoblast-like phenotype were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). The cells were seeded in growth medium containing Dulbecco's modified Eagle medium (DMEM, Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, JRH Biosciences,

Kansas, MO, USA) and 100 U/mL penicillin G + 0.1 mg/mL streptomycin (Nacalai Tesque, Tokyo, Japan) and grown in an incubator at 37°C and 5% CO_2. At a confluence of 95-100%, differentiation medium containing DMEM supplemented with 2% horse serum (Biological Industries, Haemek, Israel) and 100 U/mL penicillin G + 0.1 mg/mL streptomycin (Nacalai Tesque) was used to culture the cells. After three days, C2C12 cells completely differentiated into myotubes.

Identification of possible flavonoids. To identify possible flavonoids with inhibitory effects on atrogin-1/MAFbx expression, nine different flavonoids (naringenin, hesperetin, epicatechin, apigenin, luteolin, keampferol, daizein, genistein, and delphinidin) dissolved in dimethyl sulfoxide (DMSO) were used. Each flavonoid was added to the culture medium at final a concentration of 1, 10, or 100 μM and after 1 hour, the differentiated myotubes were stimulated with 100 ng/ml LPS isolated from E. coli (E. coli 0111: B4). The control group included cells not cultured with flavonoids but treated with LPS dissolved in DMSO, and the untreated group included cell treated with PBS dissolved in DMSO.

Two hours after LPS stimulation, the cultured myotubes were collected, and the total RNA was extracted from the cell cultures using ISOGEN (Nippon Gene, Tokyo, Japan). The atrogin-1/MAFbx mRNA level was examined by real-time polymerase chain reaction (PCR) using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master Mix (Applied

Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control in each run. Normalized fluorescence was plotted against the cycle number (amplification plot), and the threshold suggested by the software was used to calculate the Ct (cycle at threshold). The real-time PCR results are expressed as Ct, and atrogin-1/MAFbx expression is indicated by the number of cycles required to achieve the threshold amplification level. The following oligonucleotide primers were used for amplification: 5'-GGGCGGACGGCTGGAA-3' and 5'-GGCGGACGGCTGGAA-3' for mouse atrogin-1 cDNA; 5'-ACCCAGAAGACTGTGGATGG-3' and 5'-TTCAGCTCTGGGATGACCTT-3' for mouse GAPDH cDNA.

After selecting possible flavonoids with an inhibitory effect on atrogin-1/MAFbx expression, selected flavonoids were added to the culture medium at a final concentration of 1, 10, 25, 50, 75 or 100 μM. Two hours after LPS stimulation, the cultured cells were collected, the total RNA was extracted from the cell culture, and as described above, the atrogin-1/MAFbx mRNA level was analyzed by real-time PCR.

Myotube diameter measurements. Differentiated myotubes were treated with 100 ng/ml LPS and 25 μM possible flavonoids. The control cultures were treated with only LPS, and the untreated control cultures were not treated with LPS. The medium was changed every 24 hours. After 48 hours, the myotubes were photographed at ×20 magnification using the BIOREVO BZ-9000 fluorescent microscope (KEYENCE, Osaka, Japan). For each group, the

diameters of at least 100 myotubes were measured using the BIOREVO BZ-9000 software (KEYENCE).

Inhibition of JNK phosphorylation. The effect of possible flavonoids and JNK phosphorylation inhibitor treatment on murine C2C12 cells was examined by luciferase assays, real-time PCR, and western blot analysis. The C2C12 cells were transfected using jet PRIME reagent (Polyplus, Illkirch, France) according to the manufacturer's instructions. The cells were seeded at a density of 2×10^4 cells into individual wells of 24-well plates 24 hours prior to transfection with the pGL3-3.4kb atrogin-1 promoter construct and pRL-TK (Promega) (1 μg total DNA/well, 1:1 reporter: pRL-TK). When the cells were grown to confluence, the cells were treated with 25 μM flavonoids or 25 μM JNK inhibitor for 1 hour before LPS stimulation for 8 hours, and the myoblasts were lysed and analyzed using the Dual-luciferase reporter assay system (Promega).

Furthermore, the total RNA was extracted from the cell culture using ISOGEN (Nippon Gene), and the atrogin-1/MAFbx mRNA level was examined by real-time PCR as described above.

Immunoblot analysis. For western blotting analysis of the cultured C2C12 cells, the precipitated cells were lysed with M-PER ® Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Whole-cell extracts (25 μg protein/lane) were subjected to SDS-10%-polyacrylamide gel electrophoresis (PAGE) and transferred onto a PVDF membrane (Millipore Co., Bedford, MA,

USA). The membrane was blocked for 1 hour with 4% skim milk in TBS 0.1% Tween (Sigma-Aldrich, St. Louis, MO, USA) and then incubated overnight at 4°C with JNK antibody and phospho-JNK antibody (Cell Signaling Technology, Danvers, MA, USA) with 5% BSA. After incubation with HRP-conjugated anti-rabbit IgG (Cell Signaling Technology) for 1 hour at room temperature, the bound antibodies were detected with suitable secondary antibodies and enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK). Protein concentrations were measured using Lowry's method (*14*).

Statistical analysis. All the experiments were repeated in triplicate with different batches of cells. The means and standard deviations were calculated from the data and then subjected to ANOVA followed by Scheffe's multiple comparison test as a post hoc test using SPSS version 7.0 (SPSS Japan, Tokyo, Japan). Value of $P < 0.05$ was considered statistically significant.

Results

LPS stimulation induced the significant (P<0.01) upregulation of atrogin-1/MAFbx expression in C2C12 myotubes compared with the untreated control. Several flavonoids significantly (P<0.01) decreased the expression of atrogin-1/MAFbx induced by LPS treatment when the flavonoid concentration was 100 μM (Fig. 1A). Furthermore, two flavones, apigenin and luteolin, significantly inhibited the upregulation of atrogin-1/MAFbx expression. The

flavones treated with LPS had more inhibitory effect than those without LPS treatment (Fig. 1A). Based on this finding, we selected these two flavones for further study because they may have preventive effects on muscle atrophy. To evaluate the effect of these flavones on atrogin-1/MAFbx expression caused by LPS, we examined atrogin-1/MAFbx mRNA level in LPS-treated myotubes by real-time PCR. Both apigenin and luteolin caused the dose-dependent inhibition of atrogin-1/MAFbx expression (Fig. 1B). Specifically, when the concentration of these flavones was greater than 10 μM, LPS-induced atrogin-1/MAFbx significantly reduced ($P<0.01$ or $P<0.05$). These results suggest that the optimal flavone concentration with preventive effects for muscle atrophy is 25 μM; therefore, the flavone concentration of 25μM was used in the further studies. Furthermore, the effects of the common chemical structure of apigenin and luteolin, 5,7-dihydroxychromone, on atrogin-1/MAFbx expression in LPS-treated myotubes were examined. Ultimately, 5,7-dihydroxychromone did not induce changes or any changes greater than the flavones in LPS-induced atrogin-1/MAFbx expression (Fig. 1B).

To investigate whether the flavones protect LPS-induced myotube atrophy, the diameter of C2C12 myotubes treated with LPS and flavones was measured. The diameter of the C2C12 myotubes significantly ($P<0.01$) decreased in the cells treated with LPS; however, pretreatment with 25 μM apigenin or luteolin significantly ($P<0.01$) prevented the reduction of the myotube diameter caused by LPS stimulation (Fig. 2). The diameter of the C2C12 myotubes

treated with flavone was comparable to the diameter of the untreated controls.

Apigenin and luteolin have been previously shown to inhibit JNK phosphorylation in the MAPK signaling pathway (*15*). To investigate whether apigenin and luteolin affect JNK phosphorylation in C2C12 myoblasts and myotubes, JNK phosphorylation in C2C12 myotubes was examined. Western blotting analysis showed that the density ratio of phosphorylated JNK to JNK drastically increased in C2C12 myotubes treated with LPS and significantly ($P<0.01$) decreased due to pretreatment with 25 μM apigenin or luteolin (Fig. 3A). This indicates that pretreatment with apigenin and luteolin significantly inhibited LPS-induced phosphorylation of JNK in C2C12 myotubes. ; However, treatment with the JNK inhibitor caused the ratio to reduce to approximately zero. Similarly, treatment with a JNK inhibitor significantly ($P<0.05$) suppressed atrogin-1/MAFbx expression in C2C12 myotubes (Fig. 3B).

To further examine whether these flavones affect the expression of atrogin-1/MAFbx transcriptionally or post-transcriptionally, luciferase reporter assay was utilized. The C2C12 myoblasts were transiently transfected with pGL3 as a reporter of the atrogin-1/MAFbx transcriptional promoter. The treatment of C2C12 myoblasts with LPS induced a significant increase in atrogin-1/MAFbx-luciferase activity compared with the activity level in the untreated control ($P<0.01$) (Fig. 3C). In contrast, the pretreatment of LPS-stimulated myoblasts with apigenin or luteolin resulted in the significant suppression of atrogin-1/MAFbx promoter activity

compared with the LPS-treated control cells ($P<0.01$). Treatment of the LPS-stimulated myoblasts with JNK inhibitor also showed a significant reduction in atrogin-1/MAFbx-luciferase activity. This finding indicates that the flavones, apigenin and luteolin, prevent the upregulation of atrogin-1/MAFbx expression via JNK phosphorylation inhibition.

Discussion

Apigenin (4′,5,7,-trihydroxyflavone) and luteolin (3′,4,5,7-tetrahydroxyflavone) are naturally occurring plant flavones abundantly present in common vegetables and herbs such as parsley, celery, and sweet peppers (*16*). These molecules have been shown to possess numerous anti-inflammatory, antiangiogenic, and anticarcinogenic effects in cell culture and in various animal models (*17-19*) although the mechanisms underlying their chemopreventive effects still remain unclear. In this study, we showed that luteolin and apigenin cause the dose-dependent inhibition of ubiquitin ligase atrogin-1/MAFbx expression via the LPS-TLR4-MAPK/JNK signaling pathway, resulting in the prevention of muscle atrophy caused by the ubiquitin-dependent proteolytic pathway. Furthermore, 5,7-dihydroxychromone, a common apigenin and luteolin chemical structure did not induced any change or induced less change than the flavones in atrogin-1/MAFbx expression in LPS-treated myotubes(Fig. 1B). This finding implies that the chemical side chain may be involved in the inhibition of atrogin-1/MAFbx.

Flavonoids are categorized into six classes by chemical structures. The slight structural differences in flavonoids may disrupt the inflammatory response in different manners, which may be due to the differences in the direction of the effect on MAPK pathway activation (*15*). Nevertheless, two flavones, luteolin and apigenin, are promising agents to prevent and treat LPS-induced muscle atrophy mediated by the JNK signaling pathway and may be useful therapeutic candidates for the prevention of the ubiquitin-dependent proteolytic pathway in skeletal muscle atrophied by inflammatory conditions such as sepsis. In contrast, recent studies indicate that autophagy particularly contributes skeletal muscle atrophy caused by atrophic stimuli, such as starvation and cahexia (*20, 21*). However, apigenin suppressed starvation-induced autophagy and promoted apoptosis in various type of cells, such as malignant neuroblastoma cells (*22*). Because apoptosis generally stimulates muscle atrophy (*23*), it is unlikely that the flavones contribute to prevent muscle atrophy by their autophagy suppression. Further experiments are necessary to elucidate it.

The proliferation of several cells is mediated by growth factors or cytokine-induced MAPK pathway members, a family of serine-threonine proteins (*24*). Although the three MAPK pathways modules, JNK, ERK, and p38, run in parallel, a considerable degree of cross-talk occurs, creating multiple opportunities to modulate and fine-tune responses to various signals (*24*). The activation of the JNK signaling cascade generally results in apoptosis, although this pathway has also been shown to promote cell survival under specific

conditions (*25*). In addition, the cyclic mechanical stretching of human patellar tendon fibroblasts activates JNK and modulates apoptosis (*26*). Although the effects of flavonoids on the NF-κB inflammatory pathway have received considerable attention, the expression of the inflammatory cytokine IL-6 is mediated by an activator protein-1 (AP-1) regulatory element in addition to NF-κB (*27*). Furthermore, Jang et al. reported that luteolin inhibited LPS-induced IL-6 production in microglia by inhibiting JNK phosphorylation (*28*). Our results also showed that pretreatment with apigenin and luteolin significantly inhibits LPS-induced JNK phosphorylation in C2C12 myotubes. Furthermore, our preliminary study showed that ERK and p38 do not downregulate of atrogin-1/MAFbx expression (data not shown). Since we recently reported that isoflavones prevented MuRF-1-mediated muscle atrophy in C2C12 myotubes through SIRT1 activation (*29*), we also considered whether apigenin and luteolin affect MuRF-1-mediated protein degradation. However, the LPS treatment in our study did not change the MuRF-1 expression (data not shown). Taken together, these findings suggest that flavones prevent LPS-mediated muscle atrophy by the downregulation of atrogin-1/MAFbx expression, but not MuRF-1 expression.

The antioxidant properties of flavonoids are widely recognized (*30,31*). The three classical antioxidant structural features of flavonoids are the presence of a B ring catechol group, the presence of a C2-C3 double bond in conjugation with an oxo group at C4 and the presence of both 3-OH and 5-OH (*32-34*). On the basis of this

finding, the most effective antioxidant flavonoids in the nine flavonoids are assumed to be epicatechin, luteolin, keampferol, and delphinidin; however, these candidates with inhibitory effect of expression atrogin-1 were not matched up to the two flavones selected in the present study (Fig. 1A). This indicates that the antioxidant properties of flavonoids might not be involved in the inhibition of expression atrogin-1.

The Foxo3a transcription factor activates the expression of the ubiquitin ligase atrogin-1/MAFbx and is critical for muscle atrophy. JNK mediates Foxo3a activity via the phosphorylation of 14-3-3, which releases Foxo3a from its interaction with 14-3-3 (*35*). The released Foxo3a localizes within the nucleus and, stimulates atrogin-1/MAFbx expression, thereby inducing muscle atrophy. In this scenario, the flavones suppress LPS-induced JNK phosphorylation, which suppresses Foxo3a localization to the nucleus and decreases atrogin-1/MAFbx expression, leading to the inhibition of muscle atrophy. Nevertheless, when the PI3K/AKT pathway is inhibited, JNK signaling stimulation induces Foxo3a nuclear export via the CRM1 nuclear export protein and partly prevents muscle atrophy by decreasing atrogin-1/MAFbx promoter activity (*36*). Controversy still surrounds the nuclear export mechanism of Foxo3a; therefore, further study is required to clarify the role of JNK in LPS-mediated Foxo3a regulation.

The ability of sepsis and LPS to impair muscle protein synthesis results, at least in part, from a decreased mTOR (mammalian target of rapamycin) kinase activity (*37,38*). Park et al. reported that

luteolin inhibited phosphorylation of Akt in LPS-stimulated RAW 264.7 cells (*39*). Therefore, the flavones did not only prevent the ubiquitin-dependent protein degradation, but also stimulated the mTOR-mediated protein synthesis, so that they could prevent the LPS-induced muscle atrophy.

In conclusion, the present study demonstrated the inhibitory effect of two major flavones, apigenin and luteolin, on LPS-induced atrogin-1/MAFbx expression via the MAPK/JNK signaling pathway. Although a role of Foxo3a in atrogin-1/MAFbx inhibition via the LPS-TLR4-MAPK/JNK signaling pathway needs to be explored further, our results indicate that apigenin and luteolin are promising agents to prevent LPS-induced muscle atrophy.

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FIGURE LEGENDS

Figure 1 Flavonoid-mediated effects on LPS-treated C2C12 myotubes. The C2C12 cells were cultured in differentiation medium for 72 hours. After 1 hour pretreatment with flavonoids (1, 10, and 100 μM), the cells were stimulated with LPS (A). The cells treated with flavone apigenin, luteolin, and 5,7-dihydroxychromone (1, 10, 25, 50, 75, and 100 μM) for 1 hour were stimulated with LPS (B). The total RNA was extracted from the C2C12 cells, and real-time RT-PCR for atrogin-1/MAFbx and GAPDH was performed. The intensity ratios of the atrogin-1/MAFbx and GAPDH cDNA were calculated. Each mRNA level was normalized to the GAPDH level. The values are expressed as the means ± standard deviations (n=3). **P<0.01, *P<0.05 compared with the LPS-treated control cells. $^{\#\#}$P<0.01 compared with the untreated control. A.U., arbitrary units.

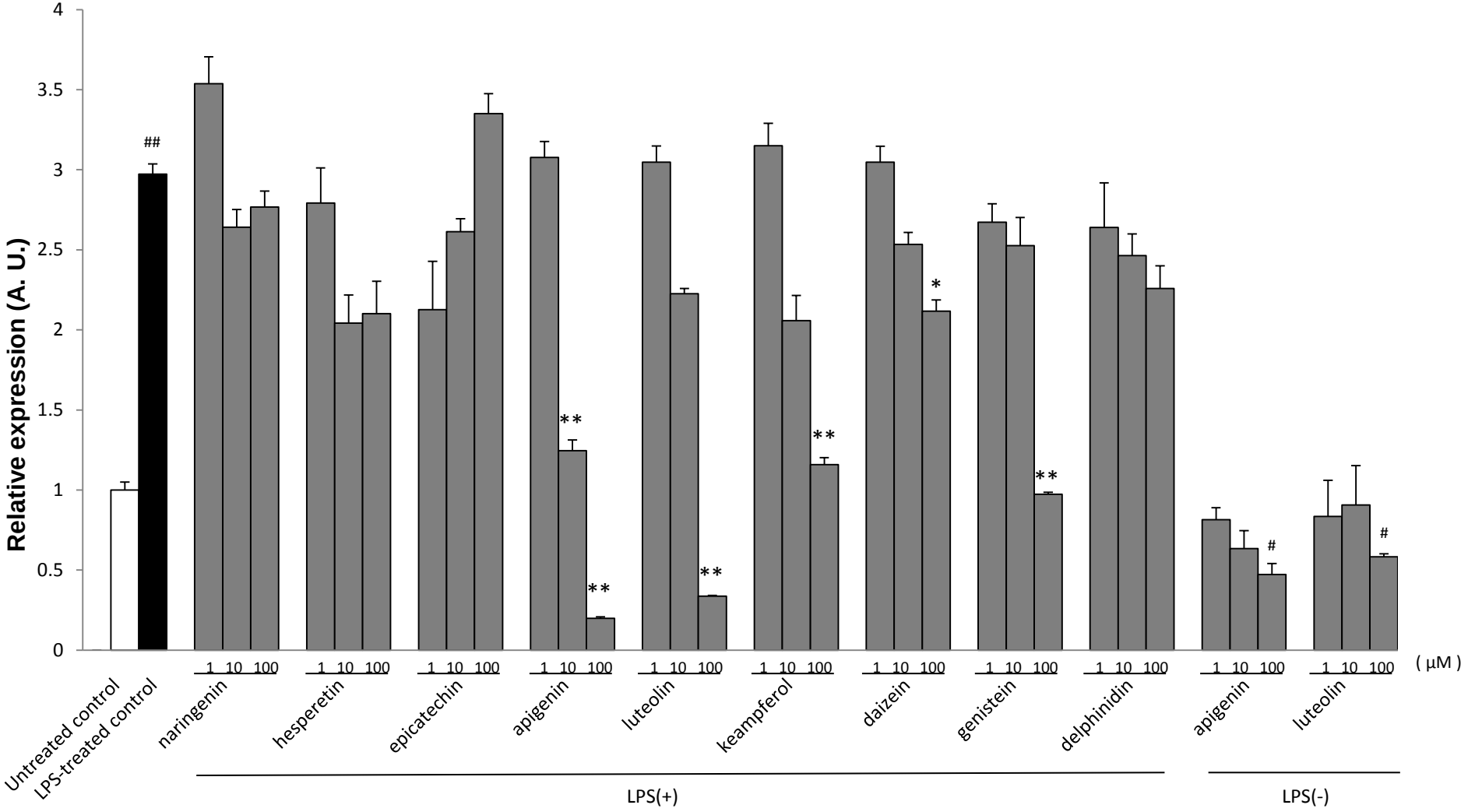
Figure 2 Flavone-mediated effects on LPS-induced C2C12 myotube atrophy. The C2C12 myotubes were treated with 100 ng/ml LPS or LPS and flavones for 48 hours. The myotube diameter was measured to evaluate myotube atrophy. **P<0.01, *P<0.05 compared with the LPS-treated myotubes. $^{\#\#}$P<0.01 compared with the untreated control.

Figure 3 Effects of flavones on JNK phosphorylation in the C2C12 myotubes. After culturing with differentiation medium for 72 hours, the cells were treated with 100 ng/ml LPS. DMSO (0.1%), flavones (25μM) or JNK inhibitor (25μM) was added to the cell culture medium 1 hour before LPS stimulation. The proteins (25 μg/lane) extracted

from the C2C12 myotubes were subjected to immunoblotting to detected phosphorylated JNK and JNK. (A) Western blotting analysis. (B) Real-time PCR analysis. (C) C2C12 myoblastic cells were tranfected with the pGL3-3.4kb atrogin-1 promoter and pRL-TK. Extracts from the myoblasts were subjected to the luciferase reporter assay. Data are expressed as the means ± standard deviations (n=3). **P<0.01, *P<0.05 compared with the LPS-treated cells. $^{\#\#}$P<0.01 compared with the untreated control. A.U., arbitrary units.

Figure 4 Schematic illustration of the signaling pathway mechanisms induced by LPS stimulation. Apigenin and luteolin may inhibit atrogin-1/MAFbx via JNK phosphorylation inhibition, resulting in the prevention of muscle atrophy.

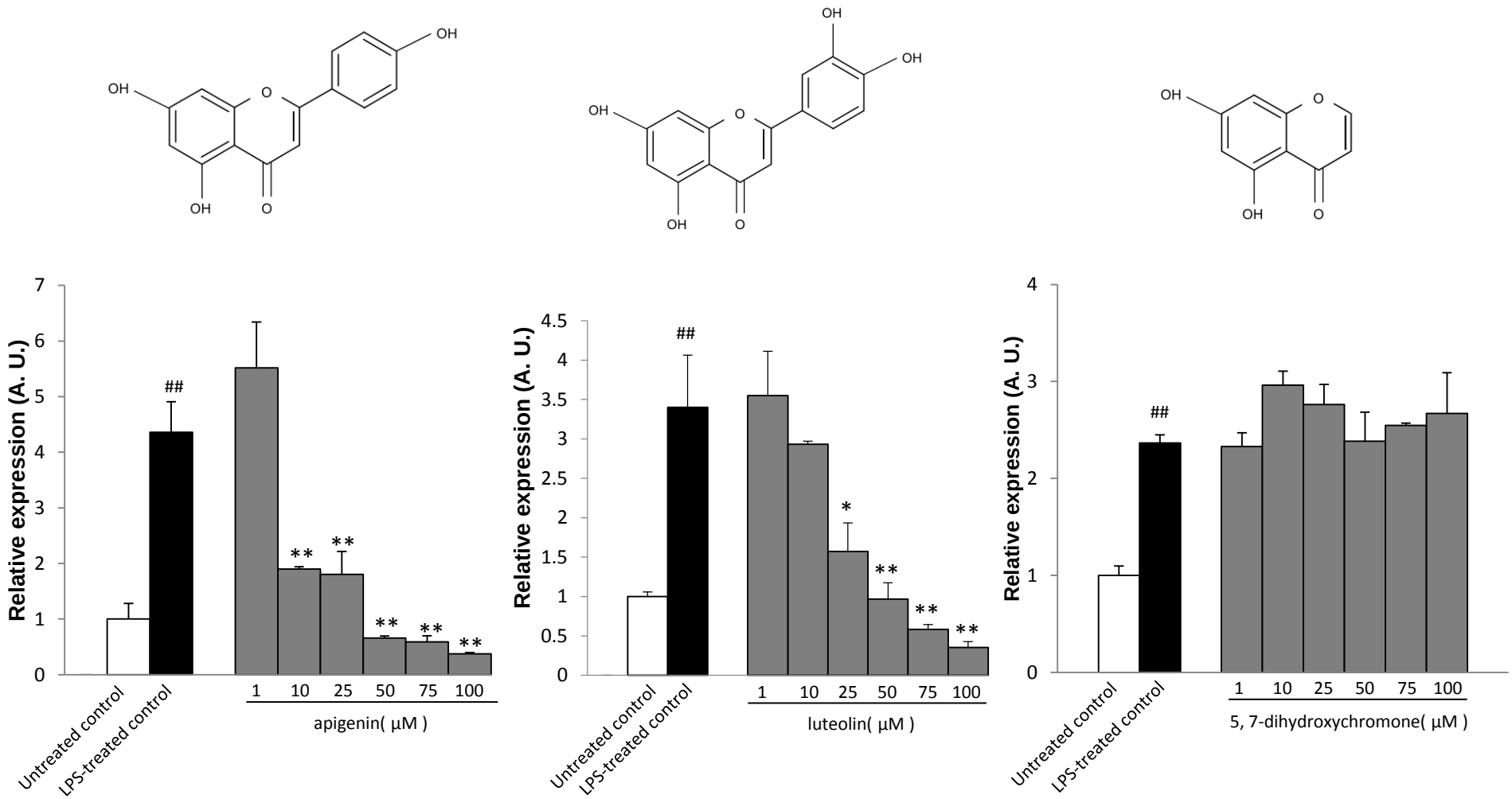
A



Shiota C. *et al.* 1

Figure 1A

B



Shiota C. *et al.* 2

Figure 1B

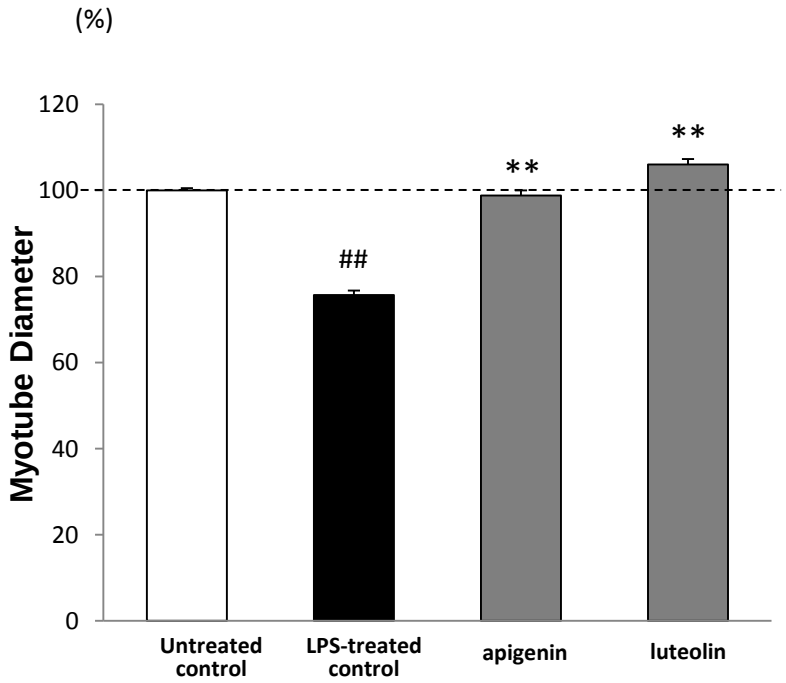

(%)
120
100
80
60
40
20
0
Myotube Diameter
##
**
**
Untreated control
LPS-treated control
apigenin
luteolin

Figure 2

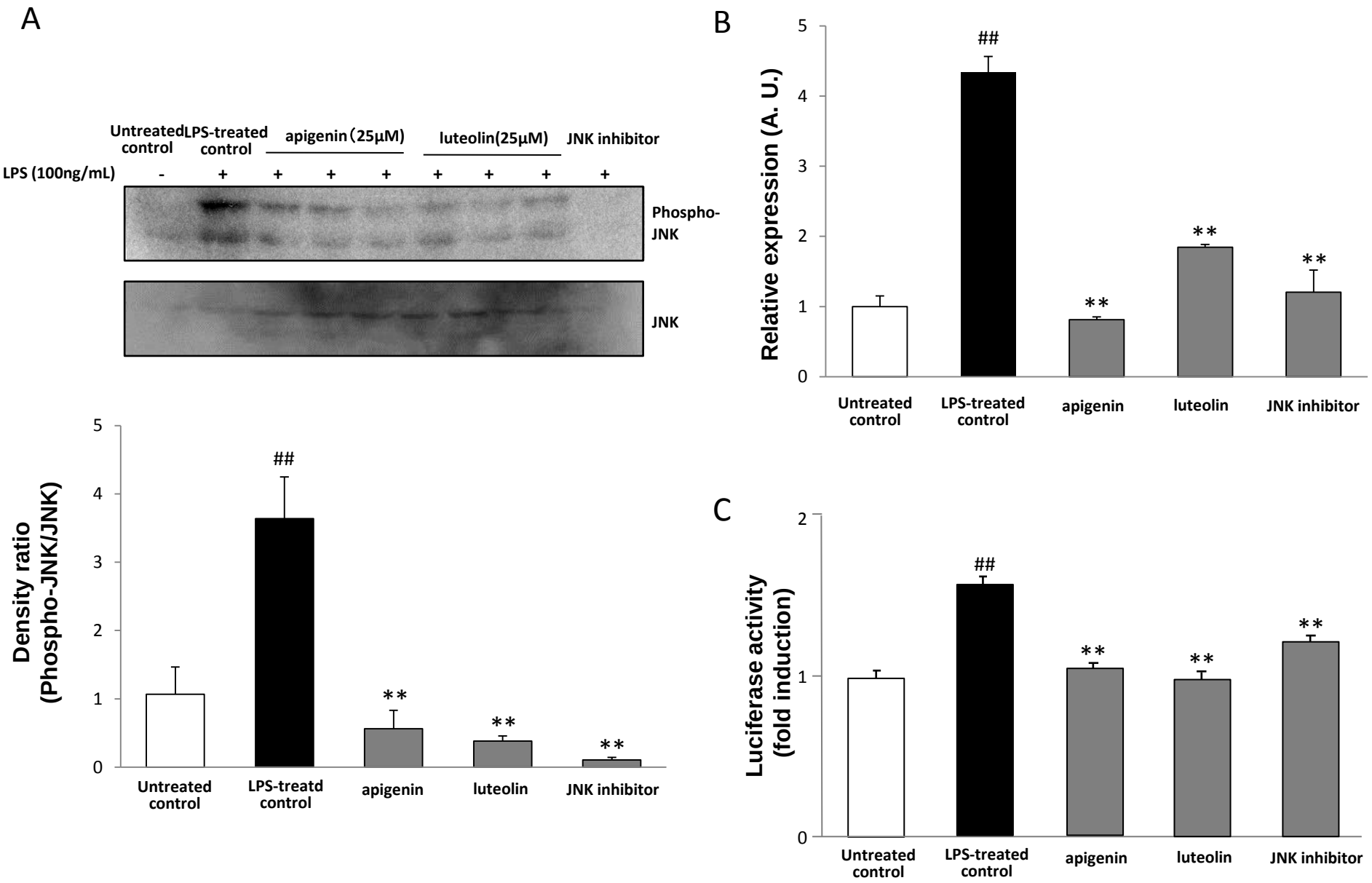

A
Untreated control
LPS-treated control
apigenin (25μM)
luteolin(25μM)
JNK inhibitor
LPS (100ng/mL)
- + + + + + + + +
Phospho-JNK
JNK
Density ratio (Phospho-JNK/JNK)
##
**
Untreated control
LPS-treatd control
apigenin
luteolin
JNK inhibitor
B
Relative expression (A. U.)
##
**
Untreated control
LPS-treated control
apigenin
luteolin
JNK inhibitor
C
Luciferase activity (fold induction)
##
**
Untreated control
LPS-treated control
apigenin
luteolin
JNK inhibitor

Figure 3

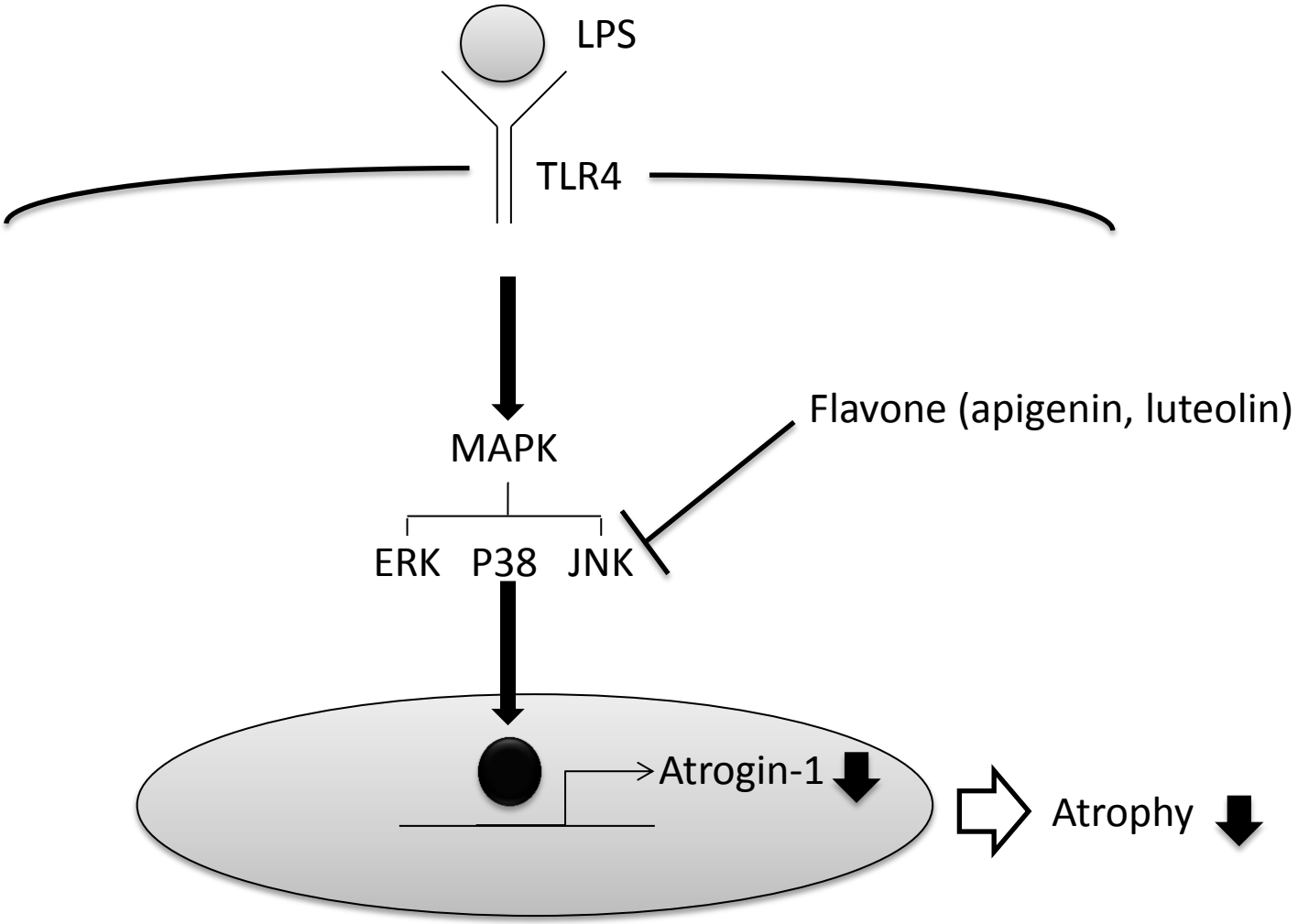

LPS
TLR4
MAPK
Flavone (apigenin, luteolin)
ERK
P38
JNK
Atrogin-1
Atrophy

Figure 4