

MAPKs-NF κ B Pathway Plays a Crucial Role in the Antiinflammatory Effects of Amentoflavone in Lipopolysaccharide-treated BV2 Microglia

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Houng, *et al.*: Amentoflavone reduced LPS-stimulated BV2 microglia via MAPKs-NFκB

Amentoflavone also known as didemethyl-ginkgetin, 3',8"-biapigenin, is a plant bioflavonoid found in several plants, with a number of pharmacological effects including antiinflammatory, antioxidant, antiviral, neuroprotective, and anticancer. The present study revealed that secretion of prostaglandin E2 and nitric oxide were inhibited by amentoflavone in a concentration-dependent manner in the lipopolysaccharide/interferon γ -stimulated BV2 microglial cells. Meanwhile, protein expression of inducible nitric oxide synthase and cyclooxygenase-2 induced by lipopolysaccharide/gamma interferon were inhibited by amentoflavone in the same concentration range. Moreover, amentoflavone not only reduced the phosphorylation of nuclear factor- κ B but also inhibited the phosphorylation of mitogen-activated protein kinases, including extracellular-signal-regulated kinase, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases induced by lipopolysaccharide. In addition, a parallel concentration-dependent manner was observed in the inhibition of secretion of prostaglandin E2 and nitric oxide, expression of inducible nitric oxide synthase and cyclooxygenase-2, and phosphorylation of mitogen-activated protein kinases and nuclear factor- κ B pathway. These results suggested that amentoflavone possessed the potential to act against lipopolysaccharide/interferon γ -induced secretion of prostaglandin E2 and nitric oxide via downregulation of inducible nitric oxide synthase and cyclooxygenase-2 expressions by blocking the activation of nuclear factor- κ B pathway via phosphorylation of mitogen-activated protein kinases.

Key words: Amentoflavone, microglial BV2 cells, MAPKs, NFκB, COX-2, iNOS

Microglia, the resident macrophages in the central nervous system, play a critical role in the innate immunological responses and constitute about 15 % of the total glial cell population in the brain^[1,2]. In response to injury and infection induced by pathogens, microglia are activated immediately and secrete proinflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2), and proinflammatory cytokines^[3,4]. Neuroinflammation is mediated by activated microglia, which lead to neuronal cell death in several neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, prion diseases, amyotrophic lateral sclerosis, Huntington's disease, and Pick's disease^[5]. Therefore, microglial activation-associated neuroinflammation serves as an important target in searching potential therapeutic reagents for neurodegenerative disorders.

Lipopolysaccharide (LPS), also called endotoxin, is the major outer membrane component of Gram-negative bacteria and often applied to induce microglia activation in *in vitro* assay^[6]. However, over-activated microglial cells induced by LPS generate proinflammatory mediators and result in dramatic neurotoxicity^[7].

Nuclear factor (NF)- κ B is the transcription factor for regulating expressions of numerous proinflammatory genes, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)^[8]. Activation of NF- κ B is regulated by mitogen-activated protein kinases (MAPK), including p38 MAPK, extracellular signal-regulated kinases (ERK) 1/2, and c-Jun N-terminal kinase (JNK) subfamilies^[9].

Amentoflavone, also named didemethyl-ginkgetin or 3',8"-biapigenin, is a bioflavonoid found in several plants, such as *Ginkgo biloba*, *Chamaecyparis obtusa*, *Hypericum perforatum*, *Selaginella tamariscina*, *Torreya nucifera*, and *Xerophyta plicata*. Amentoflavone reported to possess a variety of activities that include antiinflammatory, antioxidant, antiviral, neuroprotective, and anticancer^[10-12]. In macrophages, amentoflavone effectively suppresses

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Accepted 30 November 2017

Revised 18 April 2017

Received 16 November 2016

Indian J Pharm Sci 2018;80(1):204-210

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LPS-induced NO and PGE₂ production by inhibiting the activation of activator protein-1^[13]. However, the protective effect and molecular mechanism of amentoflavone on microglia activation caused by LPS has not been reported. The present study reported an antiinflammatory effect and possible molecular mechanism of amentoflavone in BV2 microglial cells after LPS administration.

Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Carlsbad, CA). Interferon- γ was purchased from R&D Systems (Minneapolis, MN). LPS (*Escherichia coli*, serotype 0111:B4), dimethyl sulfoxide (DMSO), foetal bovine serum (FBS), amentoflavone, and other reagents, unless specifically stated, were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against COX-2, iNOS, phospho-ERK, ERK, phospho-p38, p38, phospho-JNK, JNK, phospho-p65, p65, and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were obtained from Jackson Immuno Research Laboratories. (Baltimore, MD). Amentoflavone was dissolved in DMSO and the final concentration of DMSO never exceeded 0.5 %.

The murine BV2 microglia cell line was cultured in DMEM supplemented with 10 % FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37° in a humidified incubator with 5 % CO₂ at 37°. In all experiments, cells were treated with amentoflavone for 30 min at 0, 30, 50, 100, or 200 μ M, respectively before treating with or without LPS (100 ng/ml)/gamma interferon (IFN γ ; 10 U/ml) in serum-free DMEM.

Generation of NO was determined by Griess reaction^[3]. BV2 cells treated with vehicle DMSO only was acted as control group, while experimental groups were treated with amentoflavone at various concentrations of either 30, 50, 100, or 200 μ M for 30 min after DMSO pre-treatment. Afterwards, an additional incubation with or without LPS/INF γ for 16 h followed. The culture medium was reacted with equal volume of 0.1 % naphthylethylenediamine hydrochloride and 1 % sulfanilamide in 5 % phosphoric acid at room temperature in the dark. The absorbance at 540 nm was determined using a microplate reader.

The content of PGE₂ was determined using an enzyme-linked immunosorbent assay (ELISA)^[14]. After treatment, the culture medium was collected for measurement of PGE₂ concentration using ELISA kit (R&D Systems, Minneapolis, MN). The concentrations

were interpolated from the calibration curve generated by the recombinant PGE₂ standard.

After treatment, the cells were washed with phosphate-buffered saline (PBS) and harvested in Laemmli sample buffer. Protein concentration of cellular lysates was determined by Bradford assay. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and then incubated with 5 % nonfat milk for 1 h. The membranes were washed with PBS containing 0.1 % Tween-20, and incubated for 2 h with the indicated primary antibodies. After washing again, a 1:10 000 (v/v) dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 h and the blots were then developed using enhanced chemiluminescence Western blotting reagents^[15].

Statistical analyses were performed using ANOVA followed by the Bonferroni *t* test for multigroup comparisons; *p*<0.05 was considered significant for all tests.

Generation of PGE₂ and NO is the critical feature in LPS/INF γ -stimulated BV2 cells activation^[8]. To

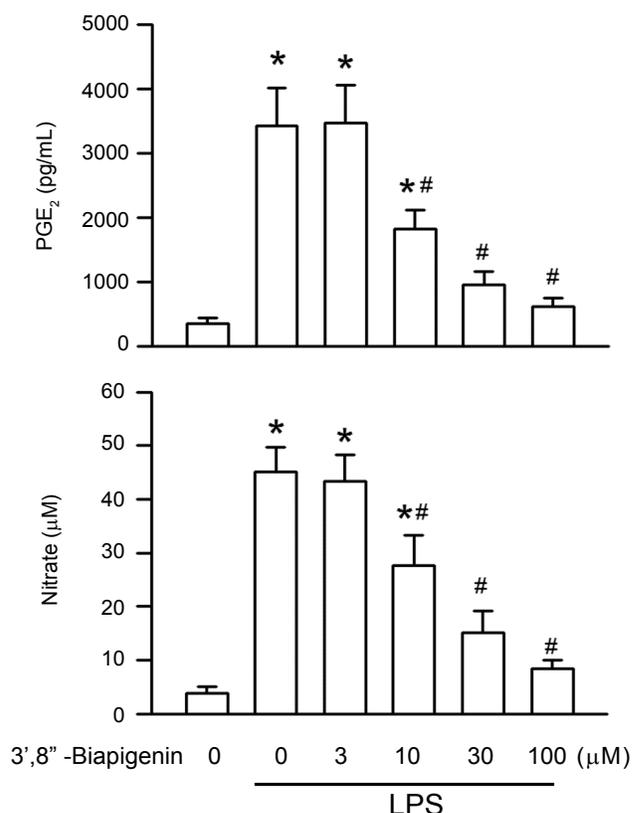


Fig. 1: Effect of amentoflavone on LPS/INF γ -induced NO (A) and PGE₂ (B) production

Results are expressed as mean \pm SD (n=5). *Significant difference from control values with *p*<0.05. #Significant difference from LPS/INF γ values with *p*<0.05

evaluate the antiinflammatory effect of amentoflavone on BV2 cells, secretion of PGE2 and NO was measured by ELISA assay and Griess assay, respectively. Administration of LPS for 16 h significantly increased PGE2 level as compared to the control. The level of PGE2 generated was inhibited by amentoflavone in LPS/INF γ -stimulated BV2 cells in a concentration-dependent manner with an IC₅₀ value of 15.02 \pm 4.81 μ M (fig. 1). In addition, the contents of NO secretion were increased from initial level after LPS/INF γ treatment for 16 h. Amentoflavone also concentration-dependently induced the inhibition of NO secretion with an IC₅₀ value of 16.04 \pm 1.88 μ M (fig. 1). These results suggested the LPS/INF γ -induced activation of microglia were inhibited by amentoflavone.

NO and PGE2 were expressed by iNOS and COX-2, respectively. The effect of amentoflavone on protein expression of iNOS and COX-2 was analysed by western blot assay. Treatment of LPS/INF γ significantly increased the protein expression of iNOS and COX-2 (fig. 2). Amentoflavone reduced LPS/INF γ -induced protein expression of COX-2 and iNOS in a concentration-dependent manner with an IC₅₀ value of 11.69 \pm 5.88 and 9.31 \pm 5.19 μ M, respectively.

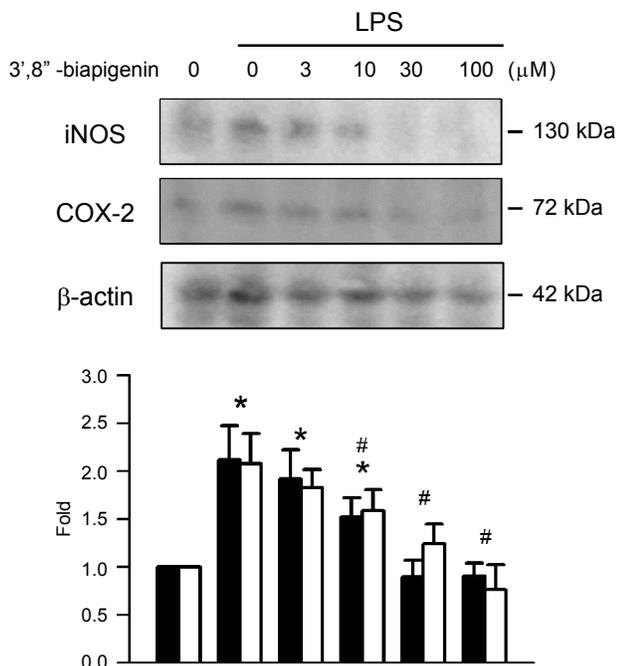


Fig. 2: Effect of amentoflavone on LPS/INF γ -induced iNOS and COX2 expression

The BV2 cell lysates were isolated and subjected to western blotting for measurement of iNOS, COX-2, and β -actin. The differences in fold of COX-2 (filled columns) and iNOS (empty columns) expression between the treated and control groups were calculated. Values are expressed as mean \pm SD (n=3 in each group). *Significant difference from control values with p<0.05. #Significant difference from LPS/INF γ values with p<0.05

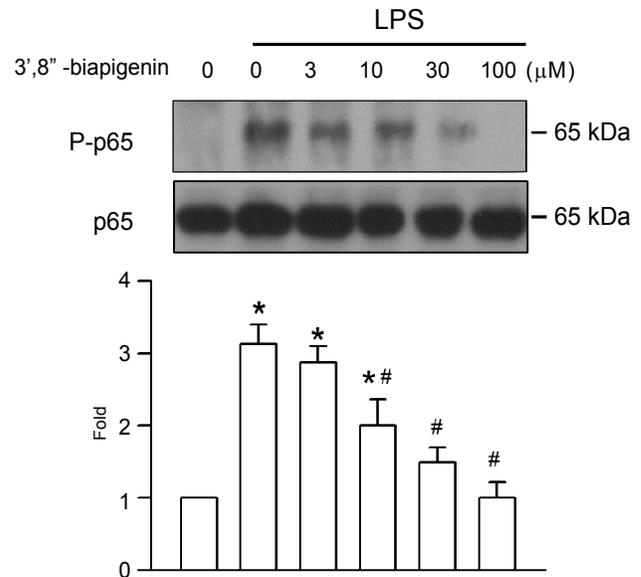


Fig. 3: Effect of amentoflavone on LPS/INF γ -induced phosphorylation of p65

The BV2 cell lysates were isolated and subjected to western blotting for measurement of phospho-p65 and p65. The differences in fold of p65 and I κ B phosphorylation between the treated and control groups were calculated. Values are expressed as mean \pm SD (n=3 in each group). *Significant difference from control values with p<0.05. #Significant difference from LPS/INF γ values with p<0.05

Phosphorylation of NF- κ B, which prompts transcription of iNOS and COX-2, has a central role in inflammation. The effect of amentoflavone on LPS/INF γ -induced NF- κ B phosphorylation was analysed by western blot assay. After LPS/INF γ administration, NF- κ B phosphorylation was increased markedly. However, it was significantly suppressed by amentoflavone pretreatment in a concentration-dependent manner with an IC₅₀ value of 11.97 \pm 4.91 μ M (fig. 3).

Each of the three MAPK pathways, ERK, p38 MAPK, and JNK, plays an important role in NF- κ B activation induced by LPS/INF γ in neurons. Therefore, the effect of amentoflavone on MAPK activation was assessed by phosphorylation of ERK, p38 MAPK, and JNK. Activation of BV2 cells induced by LPS/INF γ resulted in the phosphorylation of ERK, p38 MAPK, and JNK (fig. 4). Amentoflavone decreased the phosphorylation intensity of ERK, p38 MAPK, and JNK in a concentration-dependent manner with an IC₅₀ value of 12.38 \pm 2.61, 10.79 \pm 1.14, and 15.04 \pm 3.58 μ M, respectively. In addition, there was no influence on the expression of ERK, p38 MAPK, and JNK in all treatment group (fig. 4). These results suggested that amentoflavone reduced the LPS/INF γ -stimulated inflammatory effect in BV2 cells via inhibition of the MAPK pathway.

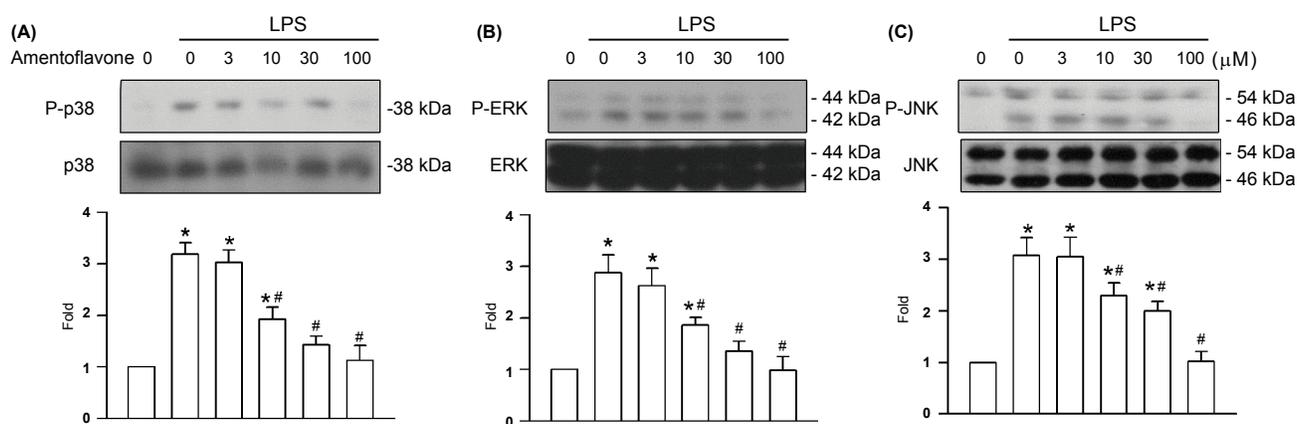


Fig. 4: Effect of amentoflavone on LPS/INF γ -induced (A) p38 MAPK, (B) JNK, and (C) ERK phosphorylation
BV2 cell lysates were isolated and subjected to western blotting for measurement of phospho-p38/p38 MAPK, phospho-JNK/JNK, and phospho-ERK/ERK. The differences in fold of p38, JNK, and ERK phosphorylation between the treated and control groups were calculated. Values are expressed as mean \pm SD (n=3 in each group). *Significant difference from control values with p<0.05. #Significant difference from LPS/INF γ values with p<0.05

The present study was designed to explore the protective effect of amentoflavone on LPS/INF γ -induced NO and PGE2 generation via MAPK-NF- κ B pathway in BV2 microglial cells. Microglia is macrophage-like glial cells located throughout the brain and spinal cord with a diverse role in the innate immunity and in inflammatory neurological diseases^[16]. Evidence has demonstrated that BV2 microglial cells appear to be a valid substitute for primary microglia in inflammatory experiments in *in vitro* assay^[17]. At present study, primary neurons were simulated by BV2 microglial cells. During neuroinflammation, microglia plays the central role in several immunopathological responses involved in the production of proinflammatory mediators such as NO and PGE2. NO is one of the major proinflammatory mediators generated by iNOS, which is highly expressed in activated microglia and macrophages after treating with LPS. PGE2 is another important proinflammatory mediator converted from arachidonic acid by COX-2^[3]. According to our results, the secretion of NO and PGE2 was inhibited by amentoflavone at the similar IC₅₀ in LPS/INF γ -stimulated BV2 cells. In addition, amentoflavone presented parallel inhibition of COX-2 and iNOS protein expressions in LPS/INF γ -stimulated cells. Similar observation has been proclaimed in previous study, which also shows that generation of NO and PGE2 is inhibited by amentoflavone via protein expression of iNOS and COX-2, respectively in LPS-treated RAW264.7 macrophages^[13]. These evidences implied that the blockade of protein expression has a critical role in the antiinflammatory activity of amentoflavone (fig. 5).

NF- κ B is the essential transcription factor for regulating

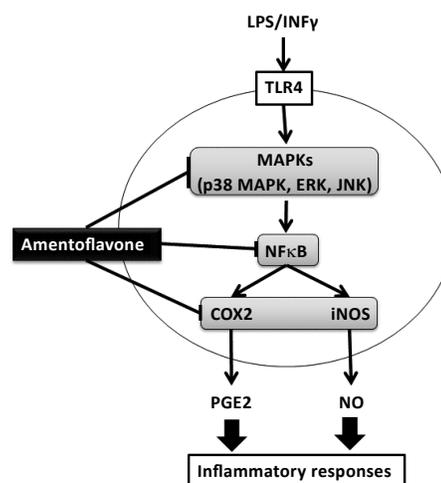


Fig. 5: Schematic diagram of the protective mechanism of amentoflavone on LPS/INF γ -induced BV2 cells activation

expression of a number of proinflammatory genes, such as NO and PGE2, in the LPS-stimulated BV2 microglial cells^[8]. In mammalian cells, the NF- κ B family contains five structurally related proteins, which are p50, p52, p65 (RelA), RelB, and c-Rel. There are two forms, homodimer and heterodimer, of NF- κ B. The NF- κ B heterodimer is composed of p50 and p65, which is the most abundant and functionally most important form of NF- κ B. Under normal condition, NF- κ B dimers keep the inactive form associated with the I κ B, which is the inhibitory protein in the cytosol. After cellular activation with different stimulants, phosphorylation of I κ B is induced by I κ B kinase. Subsequently, 26S proteasome degrades the I κ B phosphorylation via ubiquitination leading to NF- κ B dimers activation and is phosphorylated at p65. The active form of NF- κ B translocates to the nucleus, binds to transcription site,

and induces the array of proinflammatory genes^[18]. Previous study has demonstrated amentoflavone inhibits LPS-induced NF- κ B activity in RAW264.7 macrophages^[19]. In murine model of ulcerative colitis, amentoflavone inhibits expression of iNOS, COX-2, and proinflammatory cytokines via NF- κ B pathway^[20]. At present, LPS-induced p65 phosphorylation was inhibited by amentoflavone in BV2 microglial cells in a concentration-dependent manner. In addition, our data had shown parallel concentration-dependent inhibition of NF- κ B phosphorylation and iNOS and COX-2 expression. Taken together, amentoflavone reduced the LPS/IFN γ -induced expression of iNOS and COX-2 via NF- κ B pathway.

Activation of NF- κ B pathway is mediated by the phosphorylation of MAPKs in LPS/IFN γ -stimulated BV2 cells^[3]. Up to now, at least three distinct subfamilies of MAPKs, ERK, p38 MAPK, and JNK, have been clearly characterized in mammals. Dual phosphorylation of a tyrosine and a neighbouring threonine residue within the conserved tripeptide motif Thr-X-Tyr located in the activation loop of the kinase domain are required for MAPK activation^[21]. The current study demonstrated that LPS/IFN γ -induced phosphorylation of ERK, p38 MAPK, and JNK were inhibited by amentoflavone in a concentration-dependent manner. Amentoflavone exhibited parallel inhibition of LPS/IFN γ -induced phosphorylation of MAPK and NF- κ B p65 with similar IC₅₀ value. These results suggested that the generation of NO and PGE2 was inhibited by amentoflavone through MAPKs, including ERK, p38 MAPK, and JNK, in LPS/IFN γ -stimulated BV2 microglial cells.

Neuroinflammation leads to not only generation of reactive oxygen species and proinflammatory cytokines but also formation of activated microglia and astrocytes; and at the same time, contributes to neuronal and oligodendrocytic death in neurodegenerative patients^[22,23]. MAPKs-NF- κ B activation, especially the constitutively activated NF- κ B, plays an important role in neuroinflammation, has been found to possess a critical linkage with a wide variety of neurodegenerative patients^[24-26]. At present, we found pre-treatment with amentoflavone significantly reduced the secretion of PGE2 and NO via protein expression of iNOS and COX-2 in BV2 microglial cells stimulated by LPS/IFN γ . The mechanism of inhibition was attributed mainly to the down-regulation of I κ B-NF κ B pathway activation by blocking the upstream activator MAPKs, which included ERK, p38 MAPK,

and JNK. Experimental results supported the use of amentoflavone as a potential preventive reagent for neuroinflammatory diseases associated with direct infection caused by Gram-negative bacteria.

Acknowledgments:

The authors would like to thank the Ministry of Science and Technology of the Republic of China, Taiwan, for financially supporting this research under Contract No. MOST 104-2320-B-040 -006 -, 105-2320-B-040 -022-, and 106-2320-B-040 -022 -MY3.

Conflict of interest:

All authors declare no conflict of interest.

Financial support and sponsorship:

Nil.

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