Formononetin inhibits neuroinflammation and increases estrogen receptor beta (ERβ) protein expression in BV2 microglia

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Abstract

Formononetin is a bioactive non-steroidal polyphenol found in a variety of plants. In this study we evaluated the effects of formononetin on neuroinflammation in LPS-stimulated BV2 microglia. Results showed that formononetin significantly reduced the production of TNF-α, IL-6 and IL-1β, nitrite and PGE_2, as well as protein levels of iNOS and COX-2. Reporter gene assays showed that formononetin produced inhibition of NF-κB luciferase activity in HEK293 cells stimulated with TNF-α. Immunoblotting experiments revealed an inhibition of IKKα phosphorylation, with the resultant attenuation of phosphorylation and degradation of IκBα following LPS stimulation. Formononetin also produced an inhibition of nuclear translocation and DNA binding by NF-κB following LPS stimulation. RNAi experiments showed that transfection of BV2 microglia with ERβ siRNA resulted in the loss of anti-inflammatory action of formononetin. MTT assay and MAP2 immunoreactivity experiments showed that formononetin produced significant neuroprotective activity by preventing BV2 microglia conditioned media-induced toxicity to HT22 neurons. Investigations on the effect of formononetin on MCF7 breast cancer cells revealed that, while the compound significantly increased ER-luciferase activity, its effects on proliferation were modest. This study has established that formononetin inhibits neuroinflammation by targeting NF-κB signalling pathway in BV2 microglia, possibly through mechanisms involving ERβ. Formononetin appears to modulate ERβ in MCF7 breast cancer cells with limited proliferative effect. Formononetin could therefore serve as a chemical scaffold for the development of novel compounds which have selective neuroprotective actions in the brain.

Keywords

Formononetin, Neuroinflammation, NF-κB, ERβ

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1. Introduction

Neuroinflammation is an important component of the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS). This has been demonstrated by accumulating evidence which shows that the β-amyloid deposits in AD patients are surrounded by reactive inflammatory mediators like acute phase reactant proteins, pro-inflammatory cytokines and other complement components [1]. Similarly, the substantia nigra and striatum in the PD patients were observed to have high levels of proinflammatory cytokines, inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2) and activated microglia [2].

Microglia are the resident immune cells in the central nervous system (CNS), and their activation has been linked to the release a host of pro-inflammatory cytokines including interleukin-1 beta (IL-1β), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), as well as production of nitric oxide, reactive oxygen species and other mediators associated with neurodegeneration [3]. It is now well established that microglia-mediated neuroinflammation is a phenomenon that is shared by various neurodegenerative diseases [4].

Several reports have suggested that sex hormones might be a potential treatment for achieving neuroprotection in neurodegenerative disorders. The primary female hormone estrogen, and the primary male hormone testosterone have shown neuroprotective effects in the brain, and these have been found to be important in the prevention of AD [5]. For example, estrogen has been shown to protect cultured neurons and neural cell lines from neurotoxicity induced by amyloid beta [5-8]. Several studies have also shown that estrogen and selective etsrogen receptor modulators (SERMs) produce anti-inflammatory activity in activated microglia and *in vivo* [9-11]. However, considering the potential for unwanted peripheral activity of estrogens and some SERMs, the discovery and

development of novel neuroprotective estrogen derivatives which do not impact negatively on peripheral tissues, is a promising future therapeutic option for the treatment of neurodegenerative disorders.

Formononetin (7-hydroxy-4'-methoxyisoflavone) (Figure 1) is a plant-derived non-steroidal isoflavone with biological activities similar to those of estrogen [12, 13]. Some studies have shown that formononetin protects neurons from oxidative stress and toxicity induced by L-glutamate or amyloid beta [14, 15]. The compound was also shown to inhibit the production of TNF-α, NO and superoxide in mesencephalic neuron-glia cultures and microglia-enriched cultures [16]. However, little is known about effects of formononetin on neuroinflammation induced by LPS in pure microglia cultures. Also, there is a clear gap in our knowledge regarding the biochemical and molecular targets involved in the actions of this compound in the microglia and neurons. In this study, we explored the roles of microglia nuclear factor-kappa B (NF-κB) signalling pathway in the anti-inflammatory action of this compound. We also elucidated a possible involvement of microglia ERβ in the inhibition of neuroinflammation by formononetin.

2. Materials and methods

2.1 Materials

Formononetin was obtained from Sigma, dissolved in dimethylsulfoxide (DMSO) and aliquots stored at -20°C. The following reagents were used: RPMI1640 (Sigma), Fetal bovine serum (FBS; Sigma), sodium pyruvate (Sigma), streptomycin/penicillin (Sigma), Eagles Minimum Essential Medium (MEM) (Life Technologies). Lipopolysaccharide (LPS) derived from *Salmonella enterica* serotype typhimurium was purchased from Caltag Medsystems (UK).

2.2 Cell culture

BV2 mouse microglia cell line ICLCATL03001 (Interlab Cell Line Collection Banca Biologica e Cell Factory, Italy) were cultured in RPMI1640 medium, supplemented with 2 mM glutamine, 10% (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were maintained at sub-confluence in a CO_2 incubator at 37ºC.

The human embryonic kidney cell line 293 (HEK293; ATCC) was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 2mM glutamine.

HT22 mouse hippocampal neuronal cells were a kind gift from Dr Jeff Davies, and cultured in DMEM supplemented with 10 % FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a 5% CO_2 incubator at 37°C.

The human breast cancer cells MCF7 (ECACC 86012803), which express both the wild type and variant estrogen receptors were purchased from ECACC. The cells were grown in Eagele's Modified Essential Mediaum (EMEM), containing 2 mM Glutamine, 1% Non Essential Amino Acids (NEAA) and 10% FBS.

2.3 Determination of TNF-α IL-1β, IL-6, PGE_2 and nitrite in culture supernatants

BV2 cells were cultured for 48 h and incubated with or without LPS (100 ng/ml) in the absence or presence of formononetin (2.5, 5 and 10 µM) for 24 h. Thereafter, supernatants were collected and analysed for levels of TNF-α and IL-6 using mouse ELISA kits (R&D Systems). Levels of IL-1β in culture supernatants were measured with mouse IL-1β ELISA kit (Biolegend, UK). All experiments were carried out according to the manufacturers' instructions. Absorbance was measured in a plate reader (Infinite F50) at a wavelength of 450 nm.

Levels of PGE_2 in culture supernatants were measured using an EIA kit (Arbor assay, USA), according to the manufacturer's instructions. Absorbance was measured in a plate reader (Infinite F50) at wavelength of 450 nm.

Nitrite concentration in the culture medium was measured using Griess assay kit (Promega, UK), and the calculated concentration was taken as an indicator of NO production. Absorbance was read at 540 nm and calculated against a sodium nitrite standard curve.

2.4 Immunoblotting

Equal amounts of protein (20 µg) were separated on a polycryamide electrophoresis gel and transferred onto a polyvinylidine fluoride (PVDF) membrane. Membranes were incubated in blocking buffer for 1 h at room temperature, washed 3 times for 10 min each in Tris-buffered saline containing 0.1% Tween 20 (TBS-T), and incubated with primary antibodies overnight at 4°C. Primary antibodies used were rabbit anti-COX-2 (Santa Cruz; 1:500), rabbit anti-iNOS (Santa Cruz, 1:500), rabbit anti-IκBα (Santa Cruz; 1:250), rabbit anti-phospho-IκBα (Santa Cruz, 1:250), rabbit anti-IKKα (Santa Cruz; 1:250), rabbit anti-phospho-IKKα (Santa Cruz, 1:250), rabbit anti-phospho-p65 (Santa Cruz, 1:500), rabbit anti-p65 (Santa Cruz 1:500), rabbit anti-microtubule-associated protein-2 (MAP2) (Santa Cruz, 1:500) , rabbit anti-ERβ (Santa Cruz 1:250) and rabbit anti-actin (Sigma Aldrich, 1:500). Primary antibodies were diluted in TBS-T and 1% bovine serum albumin (BSA).

After extensive washing (three times for 15 min each in TBS-T), proteins were detected by incubation with Alexa Fluor 680 goat anti-rabbit secondary antibody (1:10000; Life Technologies) at room temperature for 1 h. Detection was done using a LICOR Odyssey Imager. All Western blot experiments were carried out at least three times.

2.5 Reporter gene assays

In order to determine the effect of formononetin on NF-κB mediated gene expression, a luciferase reporter gene assay was carried out. HEK293 cells were seeded out at a concentration of 4×10^5 cells/ml. Twenty-four hours later, medium was changed to Opti-MEM and cells transfected with a Cignal NF-κB reporter (SA Biosciences), using TransIT-LT1 transfection reagent (Mirus Bio LLC) and incubated for a further 16 h at 37ºC in 5% CO_2. After this period, culture media was changed to DMEM and cells incubated for a further 8 h. Thereafter, transfected HEK293 cells were stimulated with TNF-α (1 ng/ml) in the presence or absence of formononetin (2.5, 5, and 10 μM) for 6 h. NFκB-mediated gene expression was measured with ONE-Glo luciferase assay kit (Promega, Southampton, UK) according to the manufacturer's instructions using a Polarstar Optima Plate reader.

ERβ activation in MCF7 cells was also evaluated using a reporter gene assay, following a similar procedure. In the case of MCF7 cells, treatment was done with formononetin (2.5-10 μM) only.

2.6 Electrophoretic mobility shift and DNA binding assays

Electrophoretic mobility shift assay (EMSA) was used to investigate the effects of formononetin on LPS-induced DNA binding by NF-κB. BV2 microglia were treated with 2.5, 5 and 10 μM formononetin 30 min prior to stimulation with LPS (100 ng/ml). After 1 h nuclear extracts were prepared. NF-κB oligonucleotides end-labeled with IRDye 700 provided (LI-COR Biosciences) were used for binding reactions, according to the manufacturer's instructions.

An ELISA-based DNA binding assay (EMSA) was also used to investigate the effects of formononetin on DNA binding of NF-κB. BV2 microglia were treated with formononetin (2.5–10 μM). Thirty minutes later, cells were stimulated with LPS (100 ng/ml) for a further

1 h. Thereafter, DNA binding assays were carried on nuclear extracts using the TransAM NF-κB transcription factor EMSA kit containing immobilised NF-κB consensus site (5′ GGGACTTTCC-3′) (Activ Motif, Belgium) according the manufacturer's instructions and described previously [17].

2.7 Immunofluorescence microscopy

Treated cells were fixed with ice-cold 100% methanol at −20°C for 15 min and washed 3 times with phosphate buffered saline (PBS) for 5 min. Non-specific binding was blocked by incubating cells in 5% BSA blocking solution (containing 10% horse serum in 1X TBS-T) for 60 min at room temperature. This was followed by washing with PBS. Thereafter, the cells were incubated with the following primary antibodies overnight at 4°C: rabbit anti-NF-κB p65 (Santa Cruz; 1:100), rabbit anti-ERα (Abcam; 1:100), rabbit anti-ERβ (Santa Cruz; 1:100) and rabbit anti-MAP2 (Santa Cruz; 1:100). Following overnight incubation, cells were washed thrice with PBS and incubated for 2 h in the dark with Alexa Fluor 488-conjugated donkey anti-rabbit IgG secondary antibody (Life Technologies; 1:500). Thereafter, cells were washed with PBS and counterstained with DAPI for 5 min. After rinsing cells with PBS, excess buffer was removed and gold antifade reagent (Invitrogen) was added. All staining procedures were performed at room temperature. Fluorescence images were obtained using EVOS® FLoid® cell imaging station.

2.8 RNA interference

Small interfering RNAs (siRNAs) targeting ERβ (Santa Cruz Biotechnology) were used to silence ERβ gene. BV2 cells were cultured until they were 70% confluent. Transfections of ERβ and control siRNA duplexes onto the cells were carried out as earlier described [18]. Following transfection, cells were treated with formononetin (10 μM), followed by LPS (100 ng/ml). Levels of nitrite, PGE_2 and TNF-α were measured in culture supernatants of both ERβ-silenced and control cells. Also, protein levels of phospho-p65 were measured

using immunofluorescence.Transfection efficiency was determined by preparing whole cell extract from both control siRNA and ERβ-siRNA-transfected BV2 cells, and western blot carried out to measure levels of ERβ protein.

2.9 MTT assay for cell viability

BV2 cells were cultured for 48 h and incubated with or without LPS (100 ng/ml) in the absence or presence of formononetin (2.5, 5 and 10 µM) for 24 h. 3-(4, 5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) solution (5 mg/ml; Sigma) was then added to each well. The plate was then incubated for 4 h at 37ºC, followed by removal of 190 µl of medium from each well. Therefater, 190 µl of DMSO solution was added to each well. After the formazan crystals had dissolved, absorbance was read at 540 nm with Tecan F50 microplate reader.

2.10 BV2 microglia conditioned media-induced neurotoxicity

BV2 microglial cells were pre-treated with formononetin (2.5–10 µM) prior to stimulation with LPS (1 µg/ml). After 24 h incubation, supernatants were collected and centrifugated (conditioned media). Cultured HT22 hippocampal neuronal cells were then incubated with BV2 microglia conditoned media for 24 h. At the end of the experiment, 190 µl MTT solution (5 mg/ml) was added to each well containing HT22 neurons and incubated at 37ºC for 4 h. Then, 190 µl of the medium was removed and 150 µl of DMSO solution was added to wells to dissolve the formazan crystals. Thorough mixing of the preparation was facilitated by shaking the plate for a few seconds before absorbance was read at 540 nm with a plate reader.

2.11 Neuron-BV2 microglia transwell cultures

Neuron-BV2 microglia transwell cultures were also used to investigate microglia-mediated neurotoxicity. HT22 mouse hippocampal neurons were seeded out in 96-well dishes. BV2

microglia were seeded out in transwell chambers (0.4 µm; Corning). Inserts were placed on top of the wells containing HT22 neuronal culture. The microglia layer was then treated with formononetin (2.5, 5 and 10 µM), followed by LPS (1 µg/ml) for 24 h. Neuronal viability was determined using MTT assay.

2.12 E screen assay for proliferation of MCF7 human breast cancer cells

The E screen assay was used to determine the estrogenic effect of formononetin by measuring its ability to induce proliferation of MCF7 human breast cancer cells in an estrogen-free medium. MCF7 cells are known to respond to estrogenic compounds which act by binding to estrogen receptor, resulting in cell proliferation. This experiment was carried out as described by Resende et al. (2013), with some modifications [19]. Briefly, MCF7 cells were seeded in 24-well plates. After 24 h, cells were washed with PBS, and Serum Replacement 2 was added to the cells. Cells were then treated with formononetin (2.5, 5 and 10 µM) or 17β-estradiol and incubated for 6 days. Thereafter, Serum Replacement 2 was removed from the cells, followed by sulforhodamine B assay. Proliferative effect was calculated as:

Proliferative Effect of Treatment/17β-estradiol = Absorbance of treated cells/Absorbance of untreated control.

Relative Proliferative Effect (%) = [Proliferative Effect of Treatment / Proliferative Effect of 17β-estradiol] × 100

2.13 Statistical Analysis

All experiments were performed at least three times and in triplicates unless otherwise stated. Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one way ANOVA with post-hoc Tukey test (multiple comparisons).

3. Results

3.1 Formononetin did not effect BV2 cell viability

In order to determine whether formononetin affected the viability of BV2 cells, an MTT assay was carried out after incubating the cells with the compound for 24 h. Results showed that there was no significant difference in the viability of cells treated with formononetin (2.5, 5 and10 μM) when compared with control (untreated) cells (Figure 2).

3.2 Formononetin reduced TNF-α, IL-1β and IL-6 production in LPS-activated BV2 microglia

The release of pro-inflammatory cytokines is now known to be a major process in neuroinflammation. Consequently, we were interested to know if pre-treatment with formononetin would suppress the release of TNF-α, IL-1β and IL-6 in LPS-activated BV2 microglia. Stimulation of the cells with LPS (100 ng/ml) resulted in a significant increase in the secretion of TNF-α in comparison with unstimulated cells (Figure 3a). Similar results were obtained following measurements of IL-6 levels in culture supernatants (Figure 3b). On pre-treating the cells with formononetin (2.5, 5 and 10 μM), there was a significant reduction in TNF-α production, in comparison with LPS control (Figure 3a). Similarly, levels of IL-6 secreted into culture supernatants was significantly ($p<0.05$) reduced by 5 and 10 μM formononetin (Figure 3b). Similar results were obtained following analysis of supernatants for levels of IL-1β (Figure 3c).

3.3 Formononetin inhibited nitrite production through suppression of iNOS protein in LPS-activated BV2 microglia

We evaluated the effect of formononetin on nitrite production in LPS-stimulated microglia, and found that the compound dose-dependently inhibited nitrite production (Figure 4a). Further investigation using western blotting showed that formononetin (2.5, 5 and 10 μM)

produced significant ($p<0.001$) suppression of iNOS protein levels (Figure 4b). These results suggest that formononetin suppressed NO production in LPS-activated BV2 microglia through inhibition of iNOS protein expression.

3.4 Formononetin prevented COX-2-mediated PGE_2 production in LPS-activated BV2 microglia

As shown in Figure 5a, LPS-stimulated BV2 cells produced detectable levels of PGE_2 compared with unstimulated cells. Formononetin (2.5, 5 and10 μM) significantly reduced PGE_2 production in a concentration-dependent manner. Also results in Figure 5b show marked increase in COX-2 protein in LPS stimulated cells. However, pre-treatment with formononetin (2.5, 5 and 10 μM) significantly attenuated LPS-induced COX-2 protein expression.

3.5 Formononetin inhibits neuroinflammation by targeting NF-κB signalling pathway

NF-κB is a transcription factor that plays important roles in the immune system. NF-κB regulates the expression of cytokines, (COX-2). To determine whether formononetin shows any general effect on NF-κB-mediated gene transcription, a luciferase reporter gene assay was used. HEK293 cells were transfected with a vector containing NF-κB regulated luciferase reporter construct, and the experiment revealed that formononetin (2.5, 5 and 10 μM) significantly ($p<0.001$) inhibited NF-κB regulated luciferase reporter gene expression following stimulation with TNF-α (1 ng/ml) (Figure 6a).

We next investigated the effects of formononetin on NF-κB signalling activated following treatment of BV2 microglia with LPS (100 ng/ml). One of the early steps in NF-κB signalling is the phosphorylation of IKKα. Results in Figure 6b shows the expected increase in phosphorylation of following stimulation with LPS, which was reduced by pre-

treatment with formononetin 30 min prior to LPS stimulation (Figure 6b). Following IKK activation, the kinase phosphorylates IκB, followed by its subsequent proteasomal degradation. We were able to show that formononetin pre-treatment prevented both phosphorylation and degradation of IκB protein as a result of stimulation with LPS (100 ng/ml) (Figure 6c). The next step involved using western blotting and immunofluorescence to demonstrate an inhibition of LPS-induced phosphorylation (Figure 6d) and nuclear localisation (Figure 6e) of NF-κB p65 sub-unit with formononetin (2.5-10 μM) pre-treatment.

Encouraged by the results showing that formononetin inhibits neuroinflammation by targeting NF-κB activation pathway in BV2 microglia, we were interested in determining the effects of this compound on DNA binding by NF-κB. Two methods were used to evaluate this activity. Results of EMSA experiments in Figure 6f clearly shows that stimulation with LPS resulted in the binding of NF-κB in the nuclear extract to its consensus site on the DNA. This phenomenon was clearly reduced in BV2 cells that were pre-treated with formononetin. These observations were further confirmed using quantitative NF-κB DNA binding assays, which show a similar trend of LPS-induced DNA binding of NF-κB (Figure 6g). However, pre-treatment with formononetin (2.5-10 μM) resulted in inhibition of DNA binding by NF-κB. All these results appear to suggest that formononetin interferes with multiple steps in the signalling pathway involving NF-κB in BV2 microglia.

3.6 Formononetin increases levels of ERβ protein in BV2 microglia

Experiments were conducted to determine whether formononetin would increase the expression of ER protein in BV2 microglia. Initial immunofluorescence experiments revealed that ERα protein was undetectable in BV2 microglia, while appreciable levels of

basal ERβ were detected in these cells (Figures 7a). Encouraged by this outcome, we evaluated the effect of formononetin treatment on protein levels of ERβ in BV2 microglia. Results of immunoblotting experiments revealed that at 2.5 μM, there was no significant increase in the levels of ERβ. On increasing the concentrations of formononetin to 5 and 10 μM, there was a significant ($p<0.01$) increase in ERβ expression (~1.5 and ~1.75-fold increase, respectively) (Figure 7b). Interestingly, immunofluorescence results showed an increase in immunostaining for ERβ at all the concentrations tested (Figure 7c), an outcome that may be as a result of differences in sensitivities of the two methods.

3.7 Inhibition of neuroinflammation by formononetin was reversed by knockdown of ERβ in LPS-activated BV2 microglia

In order to understand any possible roles played by activation of ERβ in the anti-inflammatory effects of formononetin, we investigated the effects of the compound on the production of inflammatory mediators in ERβ siRNA-transfected BV2 microglia. Results showed that in control siRNA-tranfected cells which were treated with formononetin (10 μM) prior to stimulation with LPS (100 ng/ml), there was a significant ($p<0.05$) reduction in the production of nitrite, PGE_2 and TNF-α, in comparison with LPS control (Figures 8a-c). These anti-inflammatory effects were significantly ($p<0.01$) reversed in ERβ siRNA-transfected cells, in comparison with control-siRNA cells (Figures 8a-c).

Similarly, Figure 8d shows that LPS stimulation resulted in nuclear translocation of the NF-κB p65 subunit in control siRNA transfected BV2 cells, in comparison with unstimulated cells. This process was blocked in the presence of formononetin (10 μM). However, when cells were transfected with ERβ siRNA, formononetin lost its ability to prevent LPS-induced nuclear translocation of NF-κB p65 subunit. Transfection efficiency using western blotting revealed presence of ERβ protein in control siRNA transfected cells, while there was a downregulation of the protein in ERβ knockdown cells (Figure 8e).

3.8 Formononetin protects HT22 neurons from BV2 microglia conditioned media-mediated toxicity

Our results showing that formononetin inhibits neuroinflammation encouraged us to ask whether this compound could prevent neuroinflammation-mediated neurotoxicity. BV2 microglia were pre-treated with formononetin and then stimulated with LPS for 24 h. Cultured HT22 cells were then exposed to conditioned media from the stimulated BV2 microglia for a further 24 h. MTT assay results showed a significant ($p<0.01$) reduction in the viability of HT22 neurons following addition of conditioned media from LPS-stimulated BV2 cells (Figure 9a). However, neuronal viability was significantly ($p<0.05$) increased following exposure to conditioned media from BV2 cells that were pre-treated with formononetin (2.5-10 µM) prior to LPS stimulation, in comparison with LPS alone.

The microtubule associated protein 2 (MAP2) is a protein that functions to maintain neuronal morphology and used as a marker of neuronal integrity. Western blotting and immunostaining for MAP2 protein confirmed an increase in MAP2 protein in HT22 neurons as a result of pre-treating BV2 microglia with formononetin (2.5-10 µM) prior to exposing the neurons to microglia conditioned media (Figures 9b and 9c).

Further experiments to evaluate the neuroprotective effect of formononetin using BV2 microglia/HT22 neuron transwell co-culture revealed a similar trend as the conditioned media-induced neurotoxicity. Figure 9d shows a reduction in viability of HT22 neurons that were co-cultured with BV2 microglia stimulated with LPS, in comparison with neurons co-cultured with unstimulated BV2 cells. However, pre-treatment of BV2 microglia with formononetin (2.5-10 µM) prior to LPS stimulation resulted in improved viability of respective adjacent neurons.

3.9 Effects of formononetin on MCF7 human breast cancer cells

Firstly, we wanted to determine the effect of formononetin on the transcriptional activity of estrogen receptors in MCF7 breast cancer cells. Our results revealed that treatment of these cells with 17β-oestradiol (10 nM) resulted in significant ($p<0.01$) increase in estrogen response elements (ERE) luciferase activity (Figure 10a). Similarly, on treating cells with formononetin (2.5-10 μM), there was a significant ($p<0.05$) and concentration-dependent increase in luciferase activity. Our results also revealed that treatment with 17-β-oestradiol (10 nM) resulted in significant ($p<0.001$) proliferation of MCF7 cells (Figure 10b and 10c). Interestingly, there was no difference in proliferation between control cells and cells treated with formononetin (2.5 and 5 μM). On increasing the concentration of the compound to 10 μM, there was a slight but insignificant increase in the proliferation of MCF7 cells (Figures 10b and c).

4. Discussion

Recent studies have clearly shown that estrogen-like compounds which inhibit neuroinflammation provide therapeutic opportunities in neurodegenerative disorders like AD. In this study we investigated inhibition of neuroinflammation by formononetin in BV2 microglia stimulated with LPS, and found that the compound suppressed production of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β from the cells. Our results also demonstrated an attenuation of iNOS-mediated NO and COX-2-mediated PGE_2 production from LPS-activated BV2 microglia. These results suggest that formononetin prevents neuroinflammation resulting from stimulating BV2 microglia with LPS. We also showed that pre-treatment of BV2 microglia with formononetin prevented microglia conditioned medium- and BV2 microglia/neuron transwell co-culture-induced neurotoxicity in HT22 hippocampal neurons, further suggesting that the anti-inflammatory activity of this compound could be useful in neuroinflammation-mediated neurodegeneration.

Neuroinflammation has been strongly linked to the aetiopathogenesis of neurodegenerative disorders such as AD [20] and PD [21]. While it is widely recognised that diverse neuroinflammatory signals contribute to the pathogenesis of these conditions, our results seem to suggest that the anti-inflammatory action of formononetin could be exploited in AD and PD therapeutics. Previously, formononetin isolated from various natural sources has been reported to have inhibitory effects on LPS-stimulated nitric oxide release in LPS-stimulated RAW264.7 cells [22] as well as pro-inflammatory cytokine production in bone marrow-derived dendritic cells [23]. Furthermore, formononetin was reported to inhibit both TNF-α and NO production from neuron-glia cultures and microglia-enriched cultures [16]. Our results have confirmed these results on anti-inflammatory effects of formononetin in peripheral inflammatory cells, and showed for the first time that the compound blocks the production of a variety of pro-inflammatory mediators and proteins in pure microglia culture stimulated with LPS.

It is now widely accepted that the transcription factor, NF-κB plays a significant role in neuroinflammation and neurodegeneration. Furthermore accumulating evidence suggests that disease modifying drugs for neurodegenerative disorders which inhibit neuroinflammation act by inhibiting NF-κB activation either directly or indirectly [24]. Consequently, the molecular mechanism of anti-neuroinflammatory action of formononetin in BV2 microglia was elucidated by investigating its effects on important steps in the NF-κB signalling pathway. Firstly, using HEK293 cells transfected with a plasmid construct bearing a luciferase reporter gene under the control of NF-κB, we showed that formononetin inhibited the transcriptional activity of NF-κB in general. It was further established that in LPS-stimulated BV2 microglia, formononetin inhibited ciritical steps involving IKK-mediated phosphorylation and degradation of IκB, nuclear translocation as well as DNA binding of NF-κB.

Several studies have reported NF-κB inhibitory activity of formononetin in various cellular models. Inhbition of IL-1β-induced NO/iNOS and apoptosis in INS1 panceratic beta cells by formononetin has been attributed to its ability to inhibit the activation of NF-κB [25]. Inhibition of H_2O_2-induced apoptosis in retinal ganglion cells by formononetin has also been linked to inhibition of NF-κB activation [26]. Furthermore formononetin was reported to inhibit NF-κB signalling pathway in bone-marrow-derived macrophages stimulated with receptor activator of NF-κB ligand (RANKL) [27]. Biochanin A is a closely related compound to formononetin which has been reported to inhibit neuroinflammation in the microglia [28-30]. However, to our knowledge this is the first report showing inhibition of neuroinflammation through NF-κB signalling pathway in LPS-activated microglia by formononetin, and provides further evidence on the anti-neuroinflammatory potential of the compound.

Several studies have suggested anti-neuroinflammatory actions of estrogens in animal models and *in vitro*. It has also been suggested that this action may account for their neuroprotective activity in the brain. However, the risk of tumours associated with estrogen has led to investigations of neuroprotective selective estrogen receptor modulators (SERMs) which may act as selecetive estrogen enzyme modulators (SEEMs). Considering our results showing inhibition of neuroinflammation by this compound, we were interested in establishing the role of interaction with estrogen receptors in its anti-inflammatory activity in the microglia.

In order to establish whether formononetin has an effect on protein levels of estrogen receptors in the microglia, firstly we were interested in confirming the subtype of esterogen receptors that are expressed in these cells. Interestingly, our results showed that BV2 microglia express ERβ estrogen receptor rather than ERα. This is consistent with earlier reports showing that estrogen reduces production of pro-inflammatory mediators from BV2

microglia through interactions with ERβ [31, 32]. We further showed that treatment of BV2 microglia with formononetin resulted in an increase in the levels of ERβ protein, further suggesting that the compound possibly modulates the activity of the estrogen enzyme in the microglia.

The results showing that formononetin increased the level of ERβ in the microglia are quite intriguing. We therefore hypothesised that this action of the compound contribute to its anti-inflammatory activity in the microglia. We tested this hypothesis by investigating anti-inflammatory activity of formononetin in ERβ knockdown BV2 cells. We were able to show that silencing ERβ resulted in the loss of anti-inflammatory activity of formononetin in BV2 microglia. We further showed that silencing ERβ resulted in the loss of the ability to prevent translocation of p65 sub-unit. These results clearly suggest a significant contribution by ERβ in NF-κB-mediated inhibition of neuroinflammation by formononetin, an outcome that is being reported for the first time.

Studies have suggested that oestrognic compounds may be interfering with NF-κB-mediated neuroinflammation by targeting translocation of NF-κB. For example, oestradiol has been a targets. Future studies will need to confirm this hypothesis.

A major limitation with estrogenic compounds is their tendency to induce proliferation of certain cancer cells, especially breast cancer cells. In fact some studies have aimed to establish the potential effects of formononetin on breast cancer cells. A study by Wang et al. reported estrogenic receptor mediated proliferation of mammary glands in mice treated with formononetin [36]. Similar observations were made *in vitro* in MCF7 breast cancer cells by Ji et al. [37]. However, recent studies have reported that formononetin showed activities which were consistent with anticancer activity in MCF7 cells [38-40]. Interestingly, our results showed that formononetin increased transcriptional activity of estrogen receptors in MCF7 cells. However, its effect on the proliferation of these cells

was found to be insignificant. On the other hand, significant transcriptional activity and proliferative effects were shown by 17β-oestradiol treatment.

This study has established that formononetin inhibits neuroinflammation by targeting NF-κB signalling pathway in BV2 microglia. Formononetin interacts with ERβ in the microglia, an action that possibly contributes to its anti-inflammatory activity. We further demonstrated that this compound interacts with estrogen receptors in MCF7 breast cancer cells with limited proliferative effect. While it is not clear whether this compound has a cancer risk when used in neurodegenerative disorders, it serves as an excellent chemical scaffold for the development of novel compounds which have selective actions in the brain.

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Conflict of interest

There are no conflicts of interest.

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Figure Legends

Figure 1

Chemical structure of formononetin.

Figure 2

Pre-treatment with formononetin (2.5, 5 and10 μM) did not affect the viability of BV2 microglia stimulated with LPS (100 ng/ml) for 24 h. All values are expressed as mean ± SD for 3 independent experiments.

Figure 3

Formononetin reduced TNF-α (a), IL-6 (b) and IL-1β (c) production in LPS-activated BV2 microglia. Cells were stimulated with LPS (100 ng/ml) in the presence or absence of formononetin (2.5, 5 and 10 μM) for 24 h. At the end of the incubation period, supernatants were collected and pro-inflammatory cytokines analysed with ELISA. Data are expressed as mean ± SD for 3 independent experiments. Statistical analysis was performed using one way ANOVA with post-hoc Tukey test (multiple comparisons). **p<0.01, ***p<0.001; in comparison with LPS control.

Figure 4

Formononetin produced iNOS-mediated reduction in NO production in BV2 cells stimulated with LPS. BV2 cells were stimulated with LPS (100 ng/ml) in the presence or absence of formononetin (2.5, 5 and 10 μM) for 24 h. Culture supernatants were collected to measure levels of nitrite using Griess assay (a) and iNOS protein using immunoblotting (b). Data are expressed as mean ± SD for 3 independent experiments. Statistical analysis was performed using one way ANOVA with posthoc Tukey test (multiple comparisons). ***p<0.001; in comparison with LPS control.

Figure 5

Formononetin produced COX-2-mediated reduction in PGE_2 production in BV2 cells stimulated with LPS. BV2 cells were stimulated with LPS (100 ng/ml) in the presence or absence of formononetin (2.5, 5 and10 μM) for 24 h. Culture supernatants were collected to measure levels of PGE_2 using EIA (a) and COX-2 protein using immunoblotting (b). Data are expressed as mean ± SD for 3 independent experiments. Statistical analysis was performed using one way ANOVA with posthoc Tukey test (multiple comparisons). *p<0.05, ***p<0.001; in comparison with LPS control. (ns: not significant).

Figure 6

Inhibition of TNF-α-induced NF-κB-dependent luciferase activity in HEK293 cells by formononetin (a). Formononetin suppressed IKK-mediated phosphorylation and degradation of IκB. BV2 microglia were stimulated with LPS (100 ng/ml) in the presence or absence of formononetin (2.5, 5 and10 μM) for 30 min. Immunoblotting was carried out using anti-IKKα, anti-phospho-IκBα and anti-IκBα antibodies (b,c). Formononetin interfered with nuclear translocation of NF-κB p65 subunit through inhibition of p65 phosphorylation as determined by immunoblotting (d) and nuclear accumulation of p65 evaluated with immunofluorescence (e). Binding of NF-κB to the DNA was suppressed by formononetin in EMSA (f) and DNA binding assays (g). All values are expressed as mean±SD for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Tukey test.**p<0.01, ***p<0.001; in comparison with TNF-α or LPS control.

Figure 7

Cultured BV2 microglia express ERβ, but not ERα protein, as determined by immunofluorescence (a). Formononetin treatment resulted in upregulation of ERβ protein in BV2 microglia (b, c). All values are expressed as mean±SD for 3

independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Tukey test. **p<0.01, ***p<0.001; in comparison with LPS control. (ns: not significant).

Figure 8

Inhibition of neuroinflammation by formononetin was abolished with ERβ siRNA transfection in LPS-activated BV2 microglia. Control siRNA- and ERβ siRNA-transfected BV2 cells were treated with formononetin (10 μM) for 30 min prior to stimulation with LPS (100 ng/ml) for 24 h. Supernatants were collected to measure levels of nitrite (a), PGE_2 (b) and TNF-α (c). Immunofluorescence detection for p65 (d). Immunoblotting for ERβ was carried out to evaluate transfection efficiency (e). All values are expressed as mean±SD for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Tukey test. **p<0.01, ***p<0.001; comparing control siRNA- and ERβ siRNA-transfected BV2 cells.

Figure 9

Neuroprotection against microglia conditioned media-induced toxicity to HT22 cells by formononetin. BV2 microglia were treated with formononetin (2.5-10 μM) followed by stimulation with LPS (1 μg/ml) for 24 h. Cultured HT22 mouse hippocampal neurons were then exposed to conditioned media and viability determined using MTT assay (a) and MAP2 immunodetection (b, c). MTT viability assay showing neuroprotective effect of formononetin in BV2 microglia/HT22 neuron transwell co-culture (d). All values are expressed as mean±SD for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Tukey test. **p<0.01, ***p<0.001; in comparison with conditioned media from LPS-stimulated BV2 microglia only. (ns: not significant).

Figure 10

Effect of formononetin in MCF7 breast cancer cells. Formononetin (2.5-10 μM) and 17-β-oestradiol significantly increased ER-mediated luciferase activity in MCF7 cells (a). 17-β-oestradiol, but not formononetin produced significant proleiferation of MCF7 cells as determined using the E screen assay (b, c).

Proliferative Effect of Treatment/17β-estradiol = Absorbance of treated cells/Absorbance of untreated control.

Relative Proliferative Effect (%) = [Proliferative Effect of Treatment / Proliferative Effect of 17β-estradiol] × 100

All values are expressed as mean±SD for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Tukey test. *p<0.05, **p<0.01, ***p<0.001; in comparison with control treatment. (ns: not significant).

Figure 1

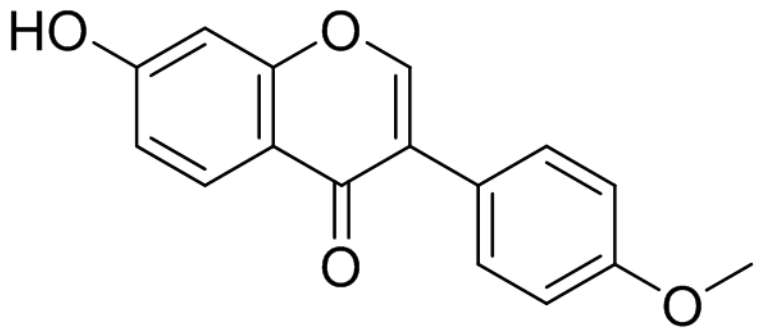


Figure 2

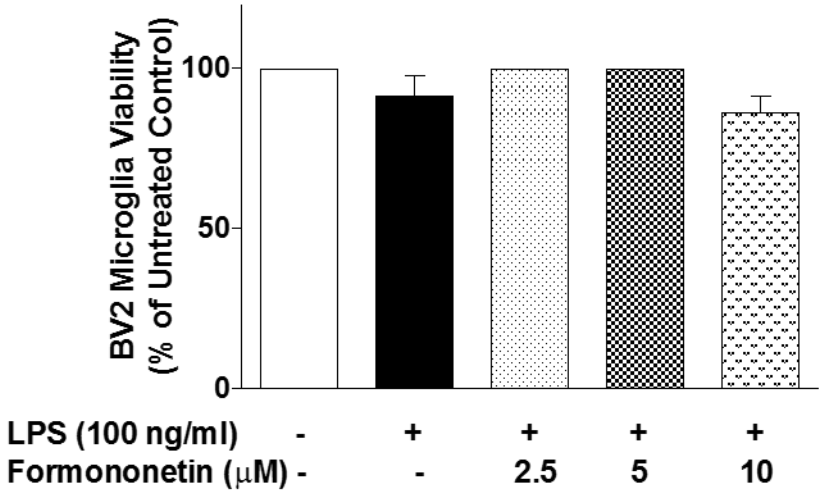

BV2 Microglia Viability
(% of Untreated Control)
100
50
0
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

Figure 3

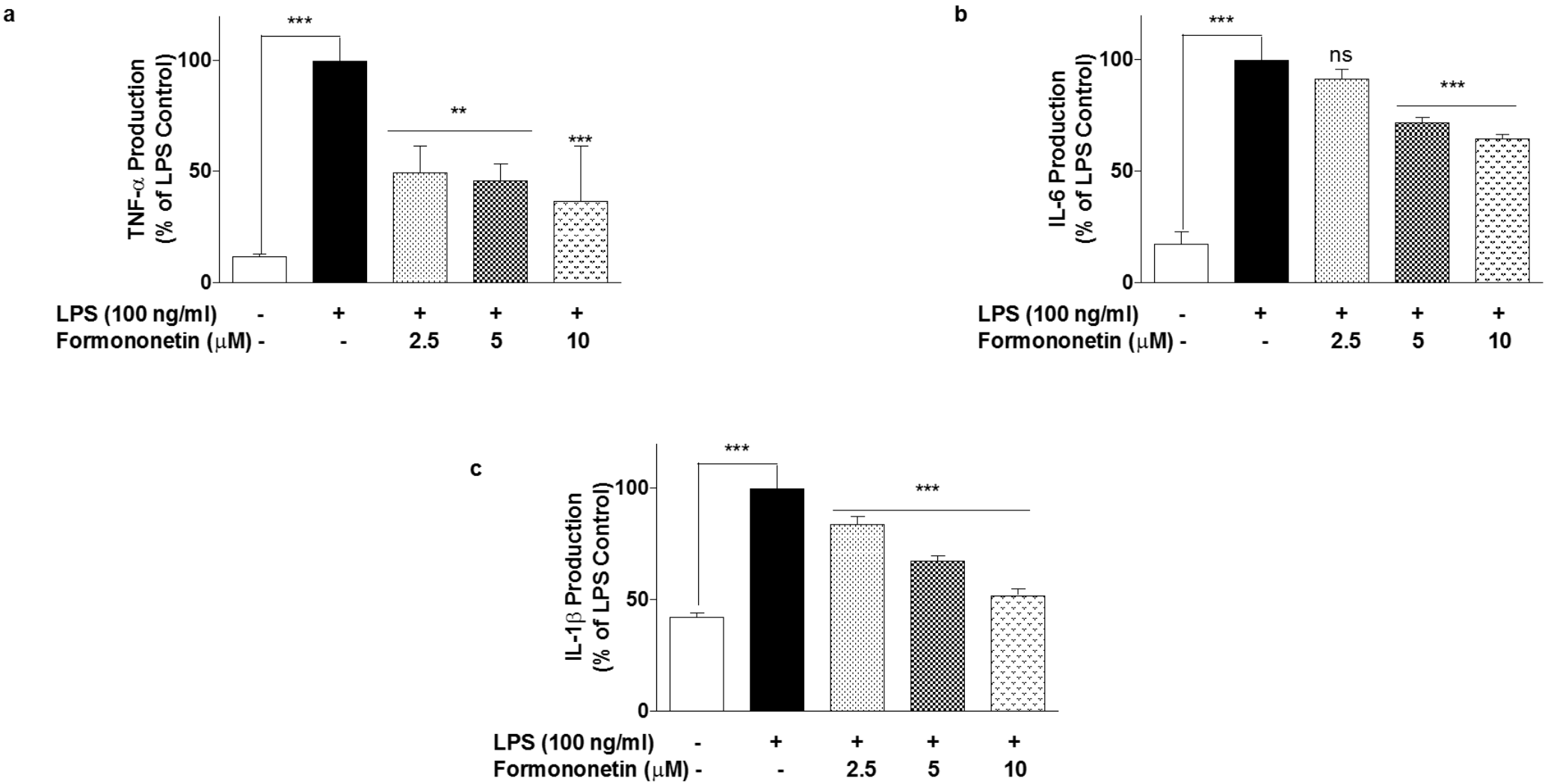

a
TNF-α Production
(% of LPS Control)
100
50
0
***
**
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10
b
IL-6 Production
(% of LPS Control)
100
50
0
***
ns
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10
c
IL-1β Production
(% of LPS Control)
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

Figure 4

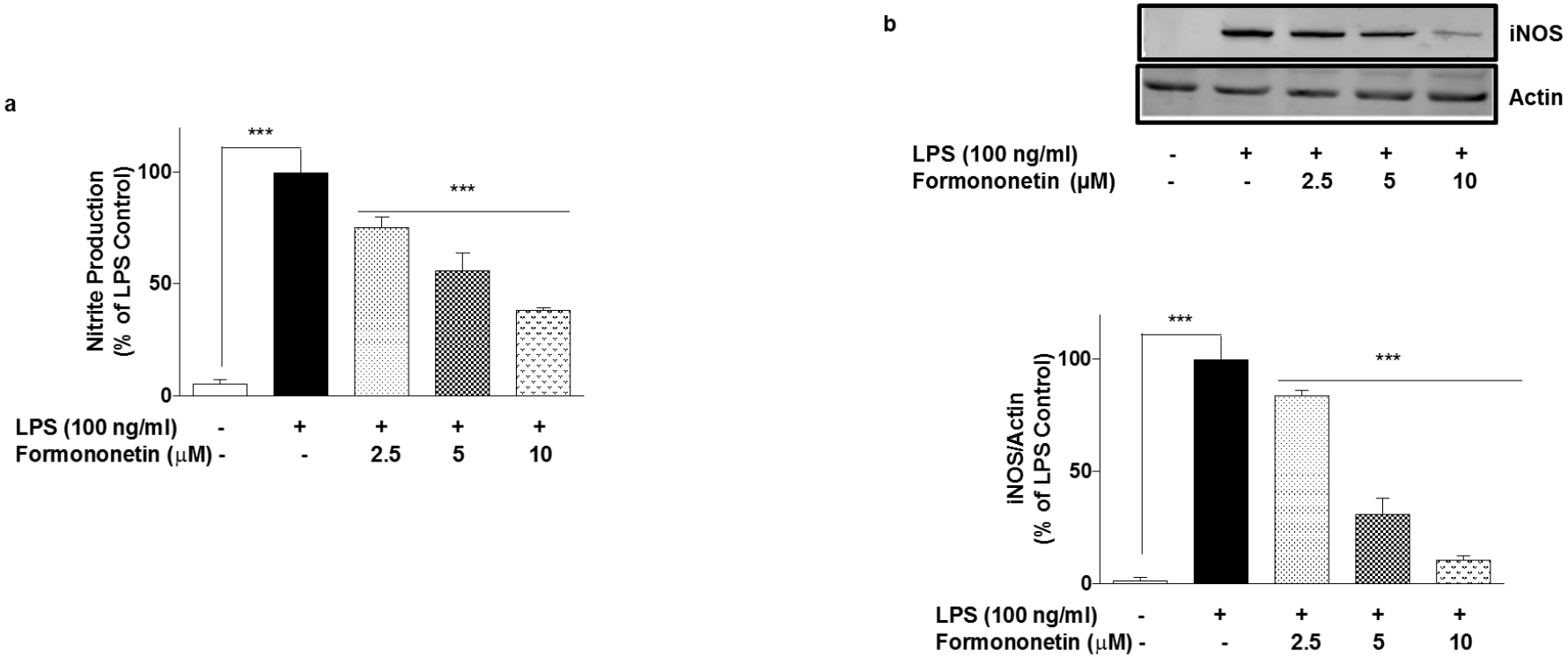
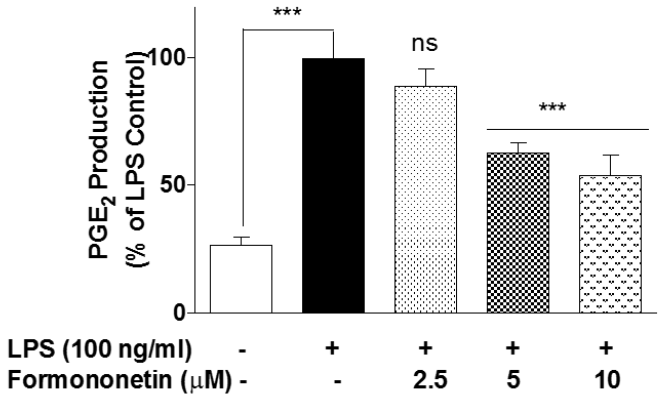

a
Nitrite Production
(% of LPS Control)
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10
b
iNOS
Actin
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10
iNOS/Actin
(% of LPS Control)
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

Figure 5

a


***
ns
***
100
50
0
PGE2 Production
(% of LPS Control)
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

b

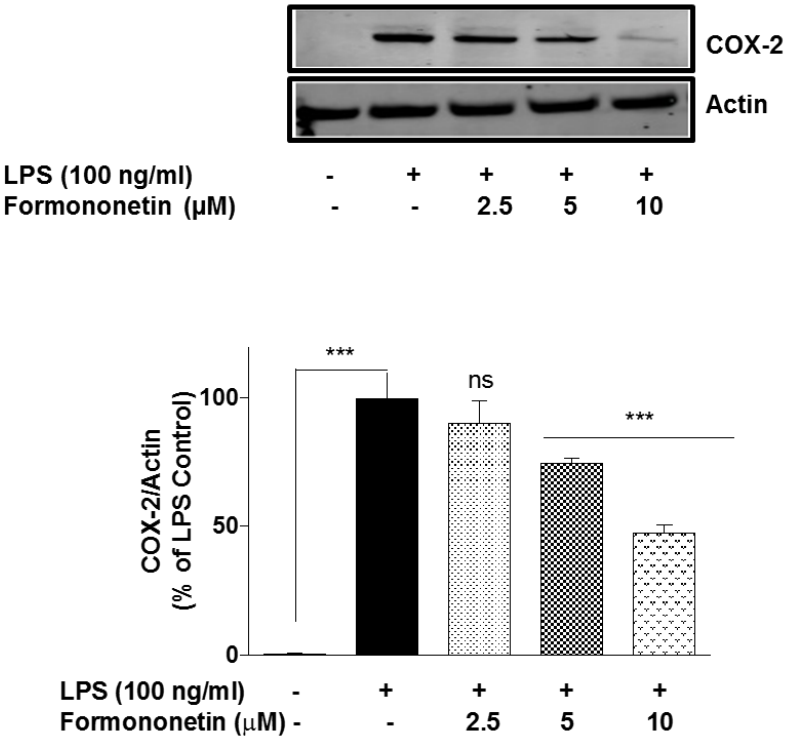

COX-2
Actin
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10
***
ns
***
100
50
0
COX-2/Actin
(% of LPS Control)
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

Figure 6

a

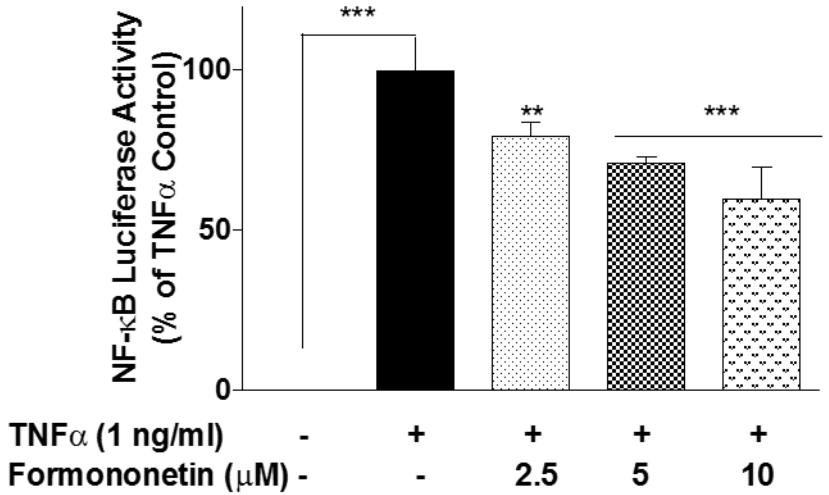
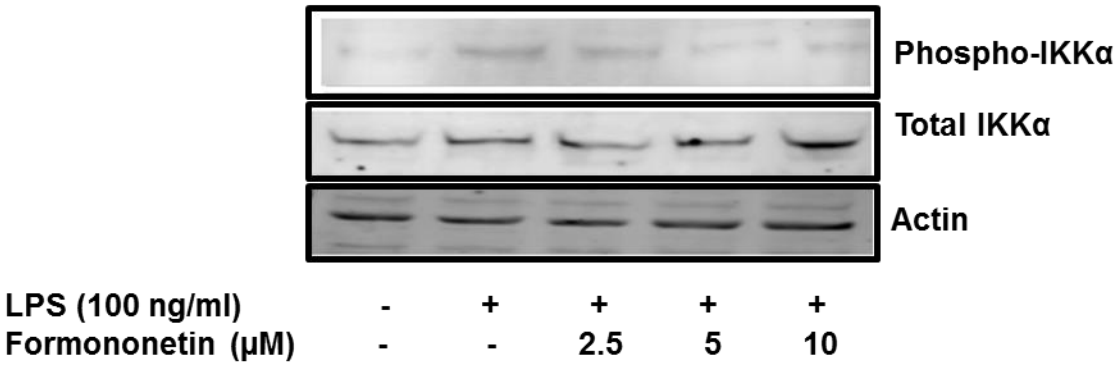

NF-κB Luciferase Activity
(% of TNFα Control)
100
50
0
***
**
***
TNFα (1 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

Figure 6

b


Phospho-IKKα
Total IKKα
Actin
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

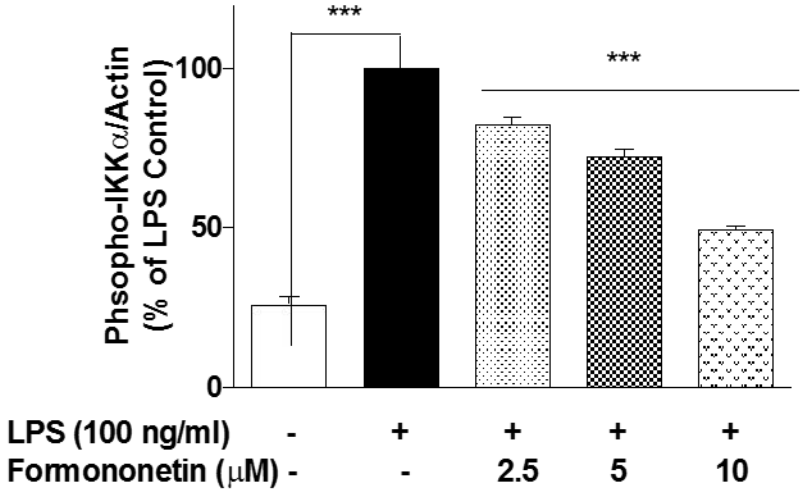

***
***
Phsopho-IKKα/Actin
(% of LPS Control)
100
50
0
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

Figure 6

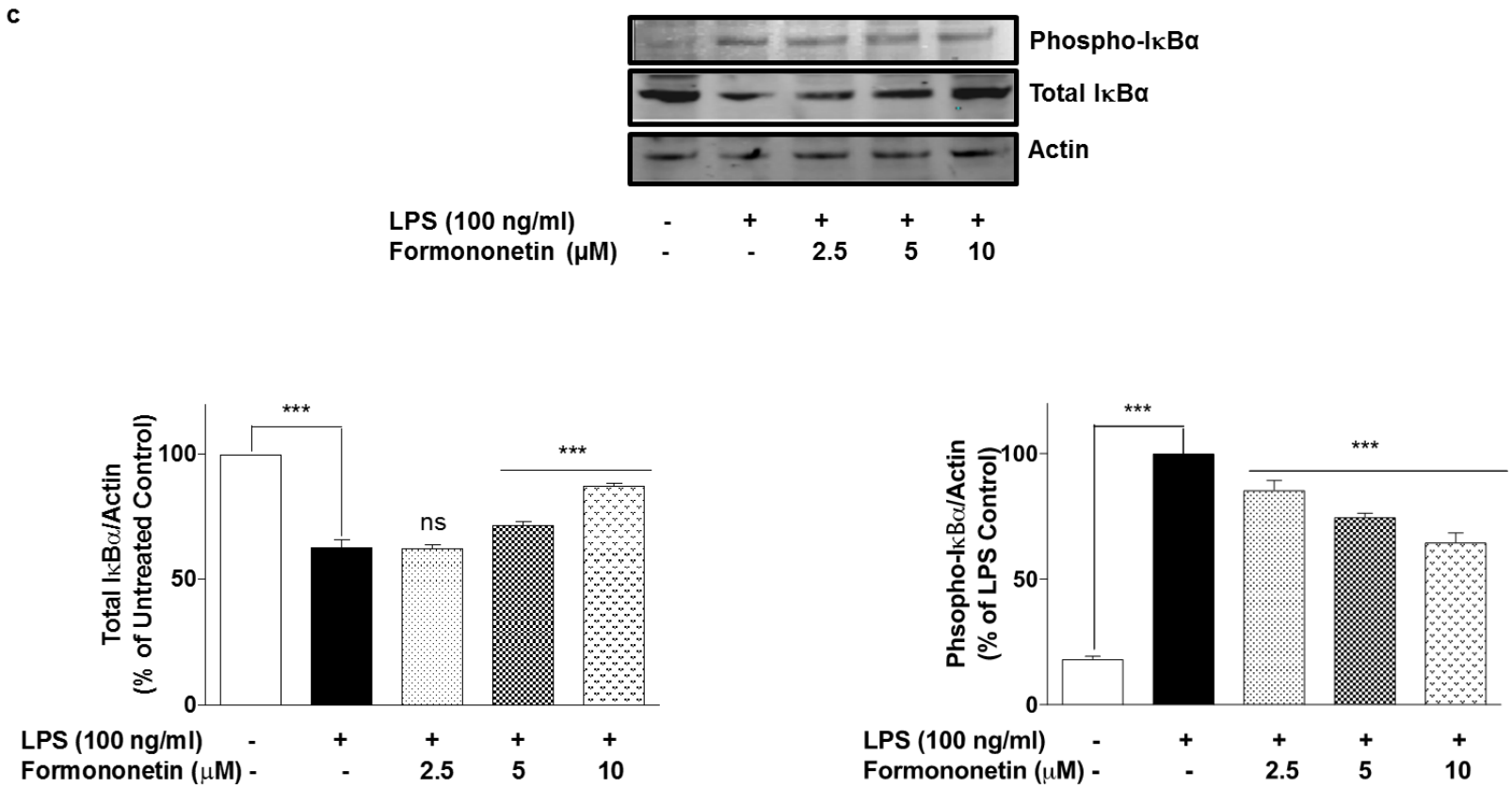

c
Phospho-IκBα
Total IκBα
Actin
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10
Total IκBα/Actin (% of Untreated Control)
100
50
0
***
ns
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10
Phsopho-IκBα/Actin (% of LPS Control)
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

Figure 6

d

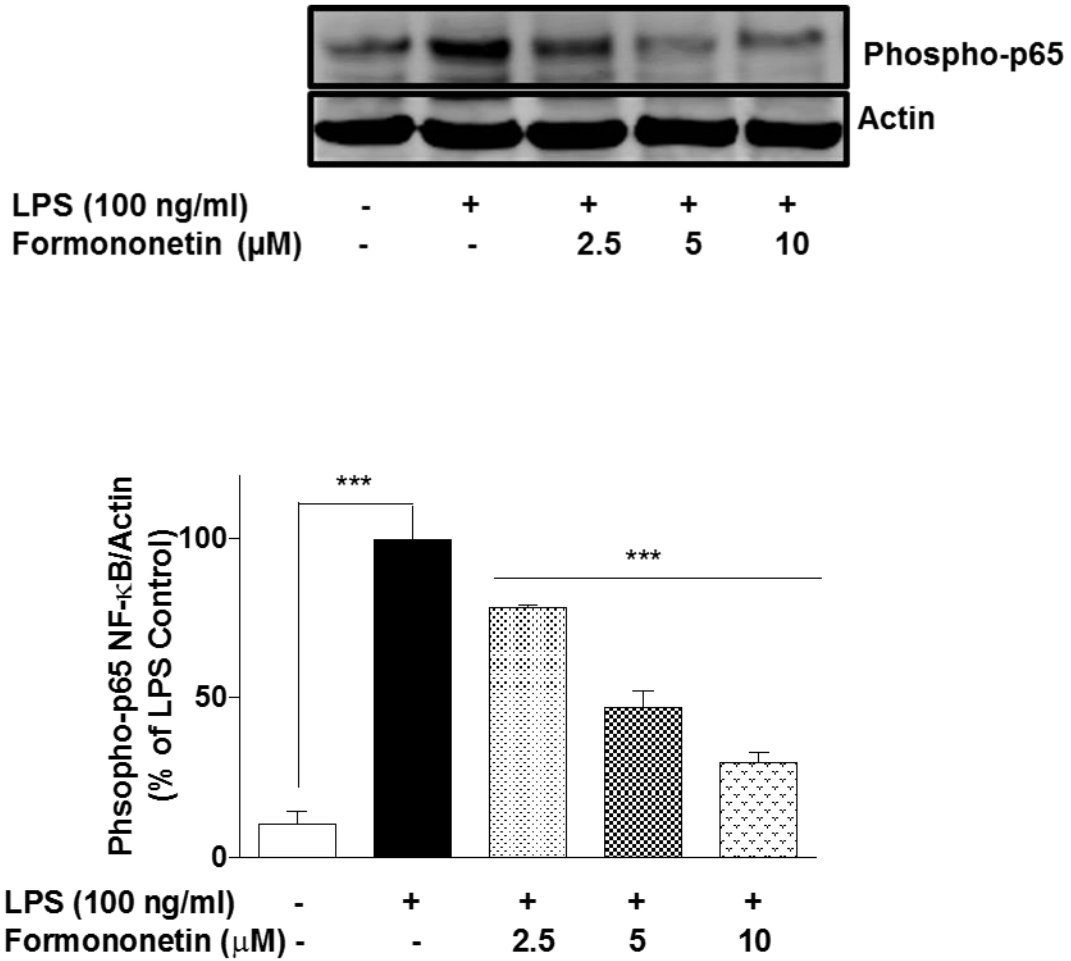

Phospho-p65
Actin
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10
Phsopho-p65 NF-κB/Actin (% of LPS Control)
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

Figure 6

e

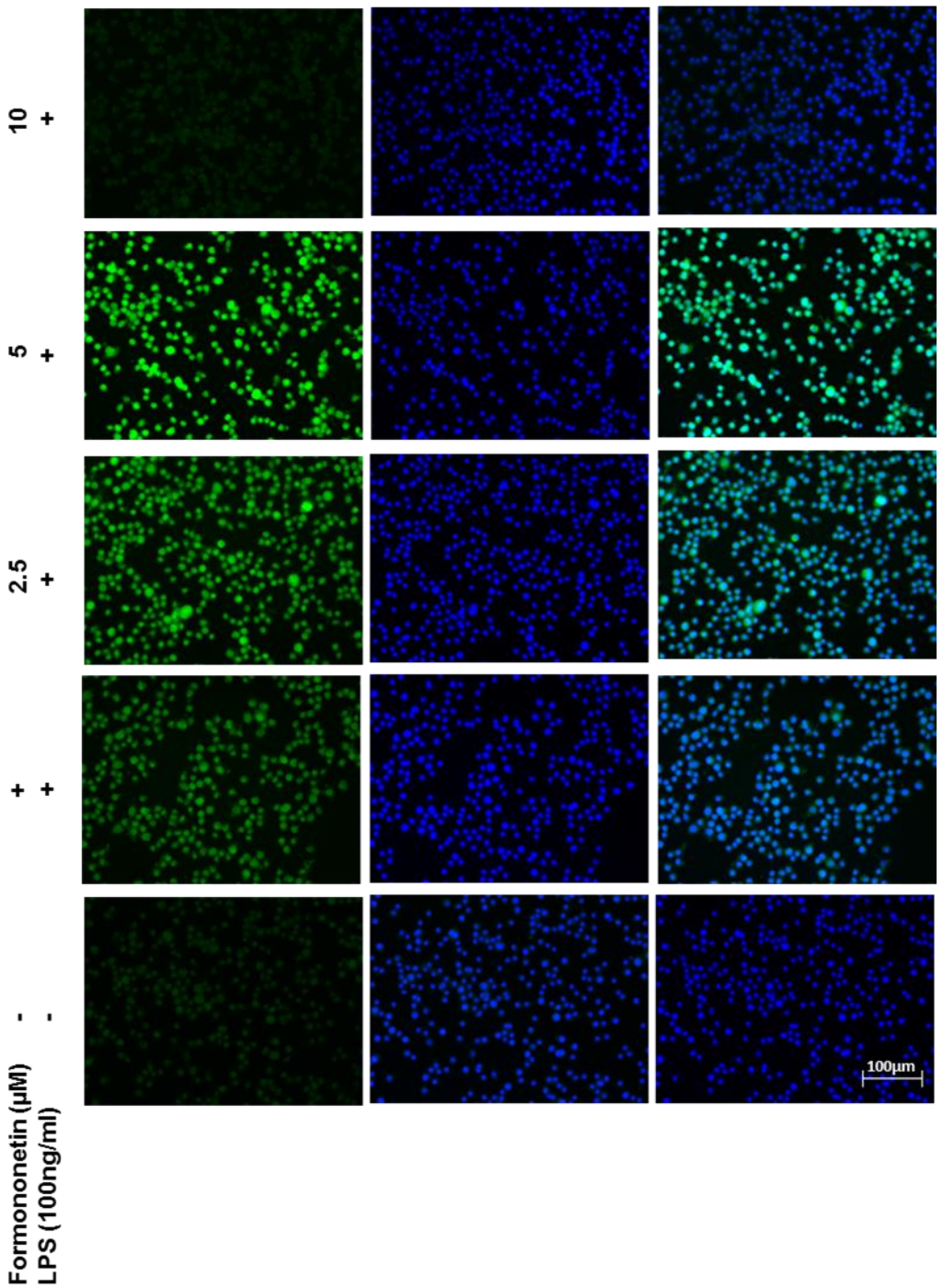
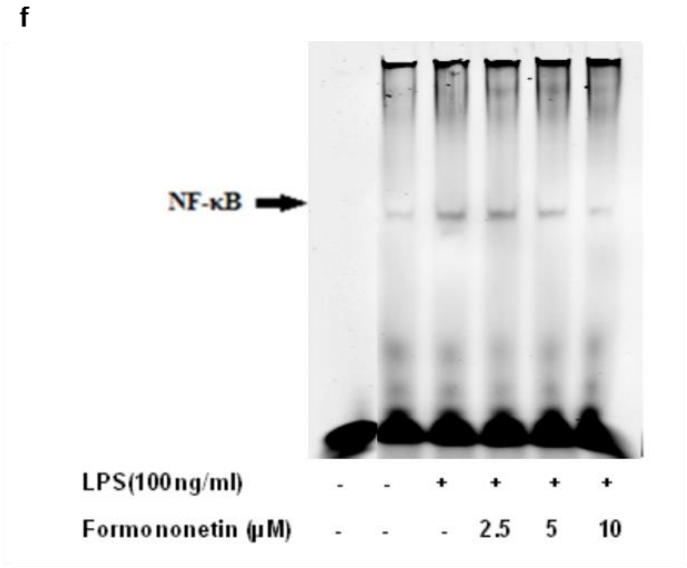


Figure 6


f
NF-κB
LPS(100ng/ml) - - + + + +
Formononetin (μM) - - - 2.5 5 10

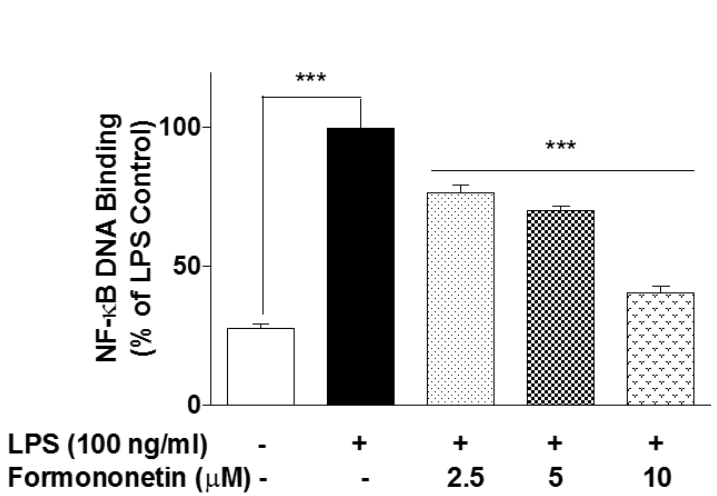

g
NF-κB DNA Binding
(% of LPS Control)
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

Figure 7

a

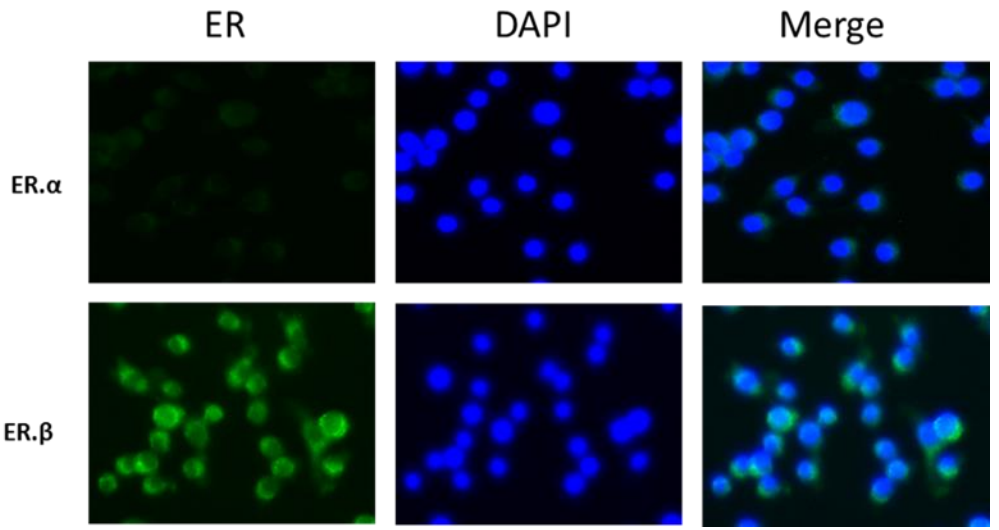
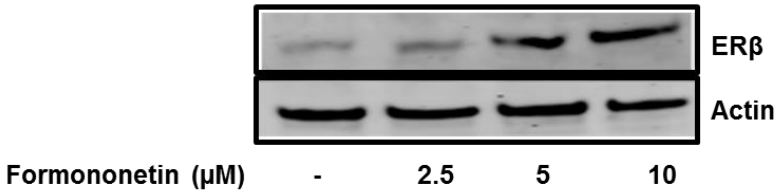
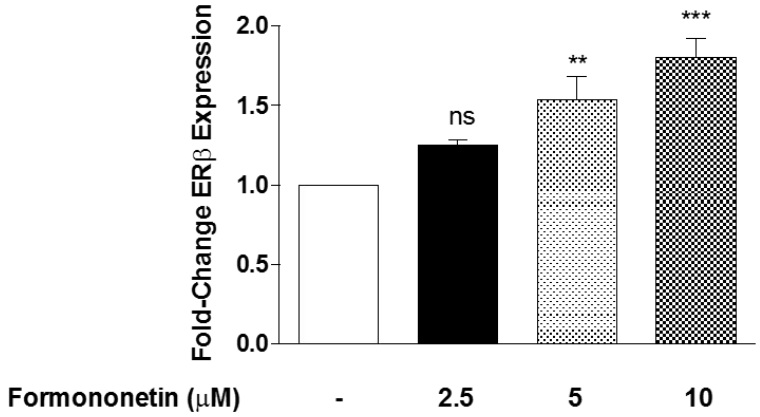


Figure 7

b


ERβ
Actin
Formononetin (μM) - 2.5 5 10


Fold-Change ERβ Expression
2.0
1.5
1.0
0.5
0.0
ns
**
***
Formononetin (μM) - 2.5 5 10

c

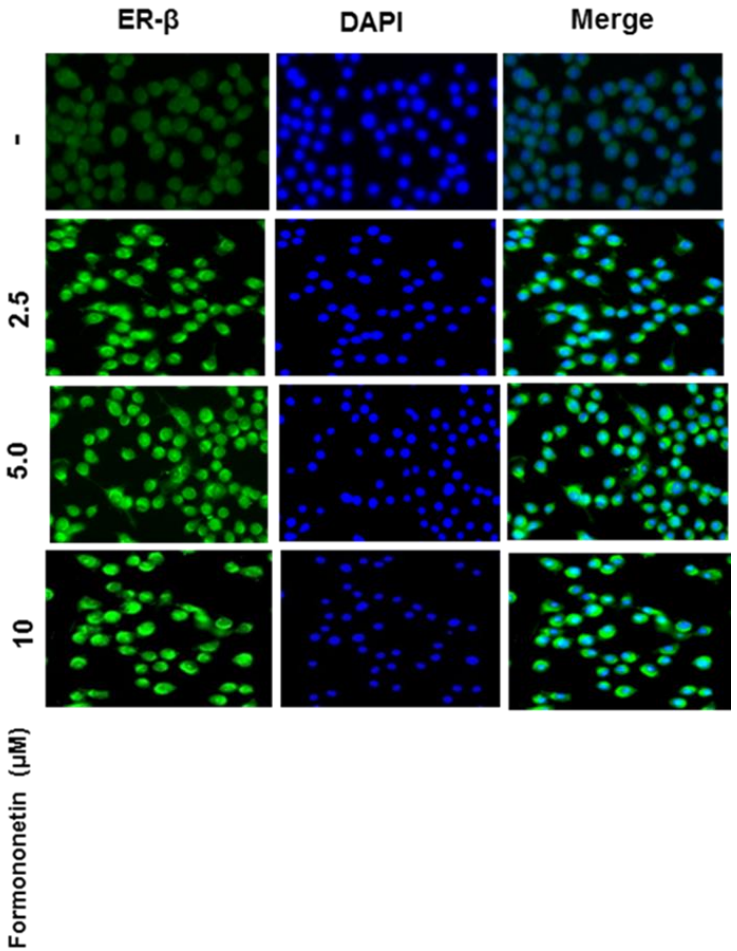

ER-β
DAPI
Merge
-
2.5
5.0
10
Formononetin (μM)

Figure 8

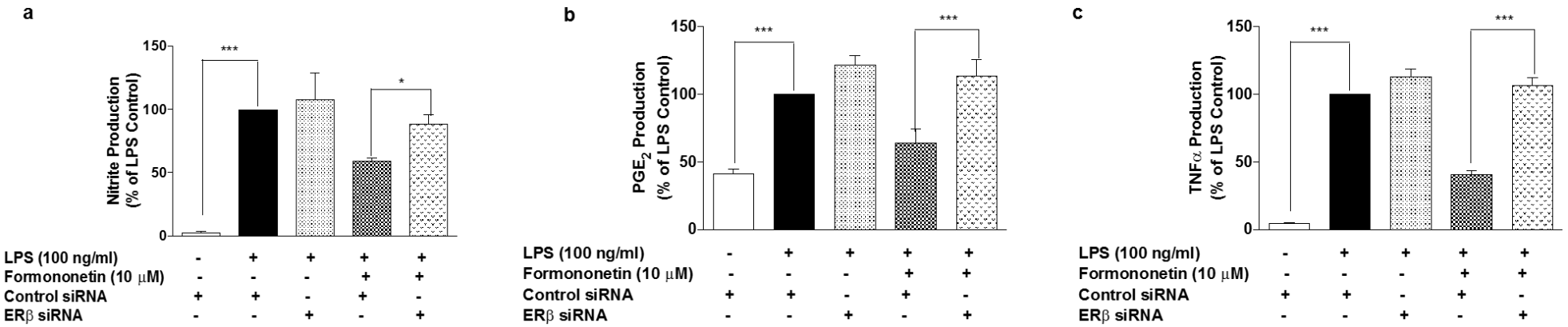

a
Nitrite Production (% of LPS Control)
150
100
50
0
***
*
LPS (100 ng/ml) - + + + +
Formononetin (10 μM) - - - + +
Control siRNA + + - + -
ERβ siRNA - - + - +
b
PGE2 Production (% of LPS Control)
150
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Formononetin (10 μM) - - - + +
Control siRNA + + - + -
ERβ siRNA - - + - +
c
TNFα Production (% of LPS Control)
150
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Formononetin (10 μM) - - - + +
Control siRNA + + - + -
ERβ siRNA - - + - +

Figure 8

d

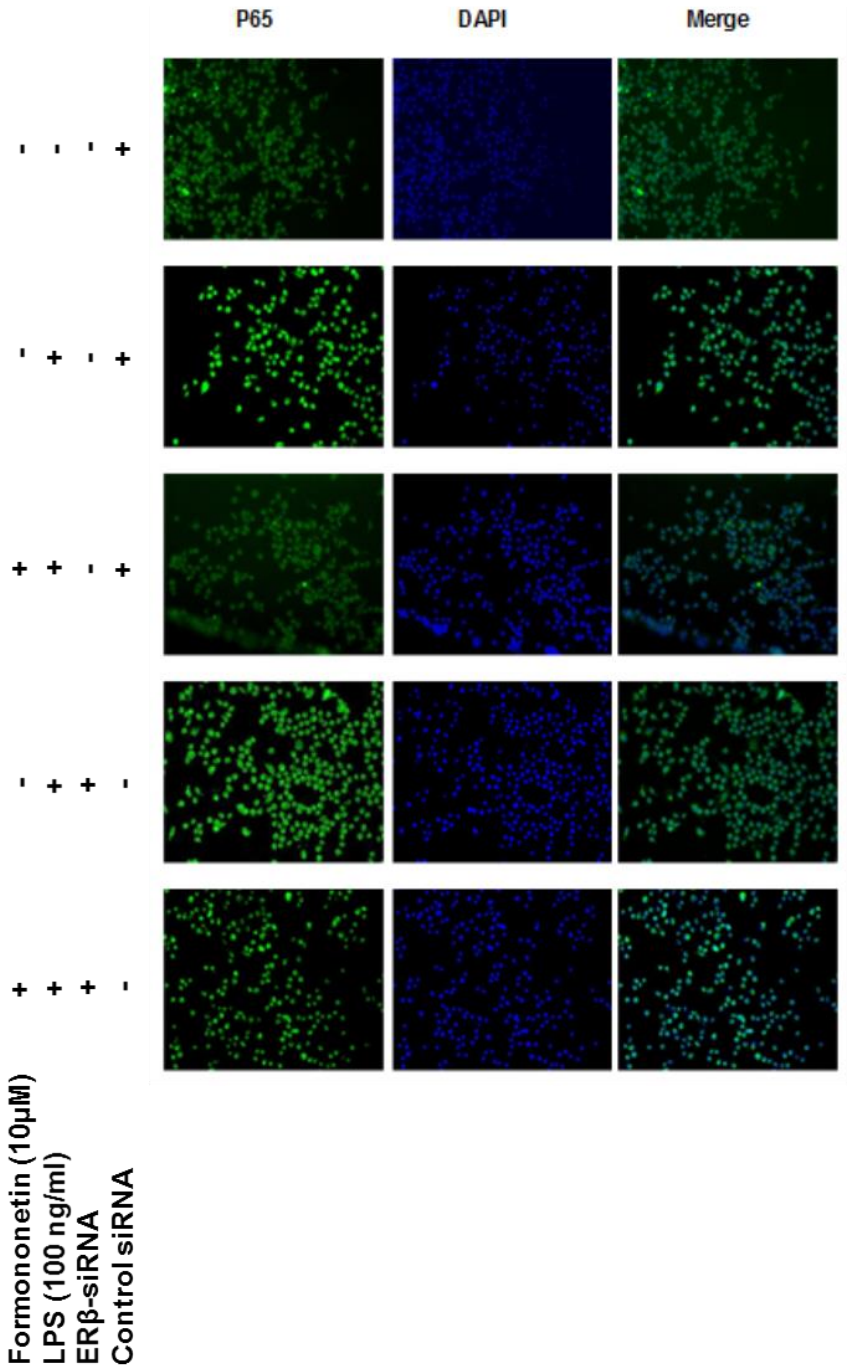

P65
DAPI
Merge
- - - +
- + - +
+ + - +
- + + -
+ + + -
Formononetin (10μM)
LPS (100 ng/ml)
ERβ-siRNA
Control siRNA

Figure 8

e

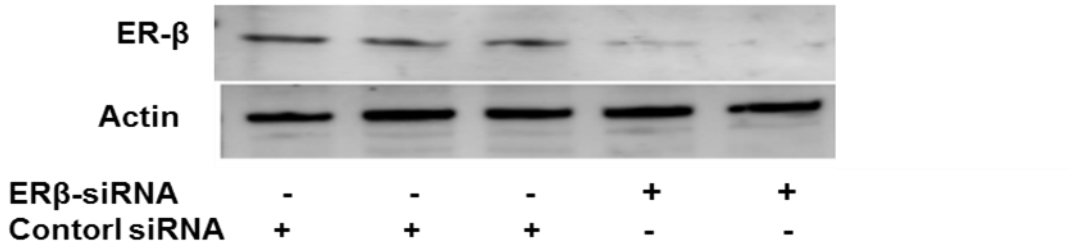


Figure 9

a

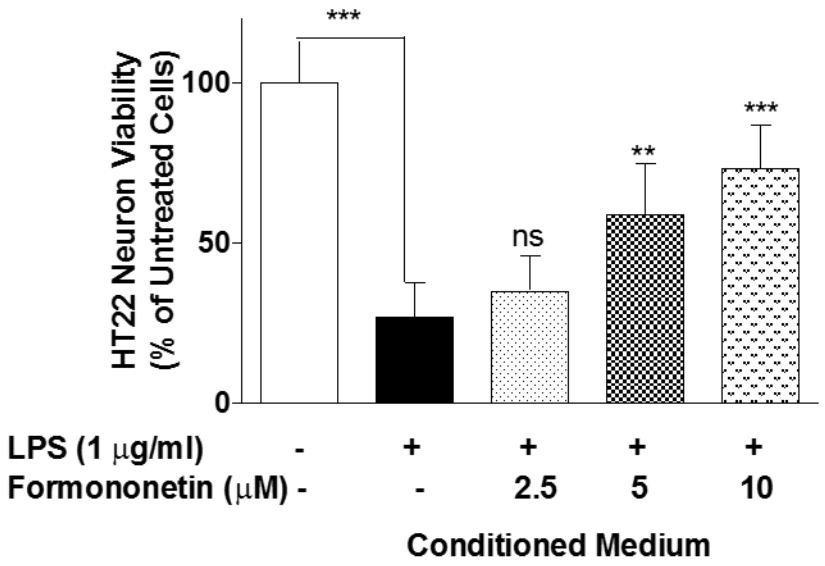
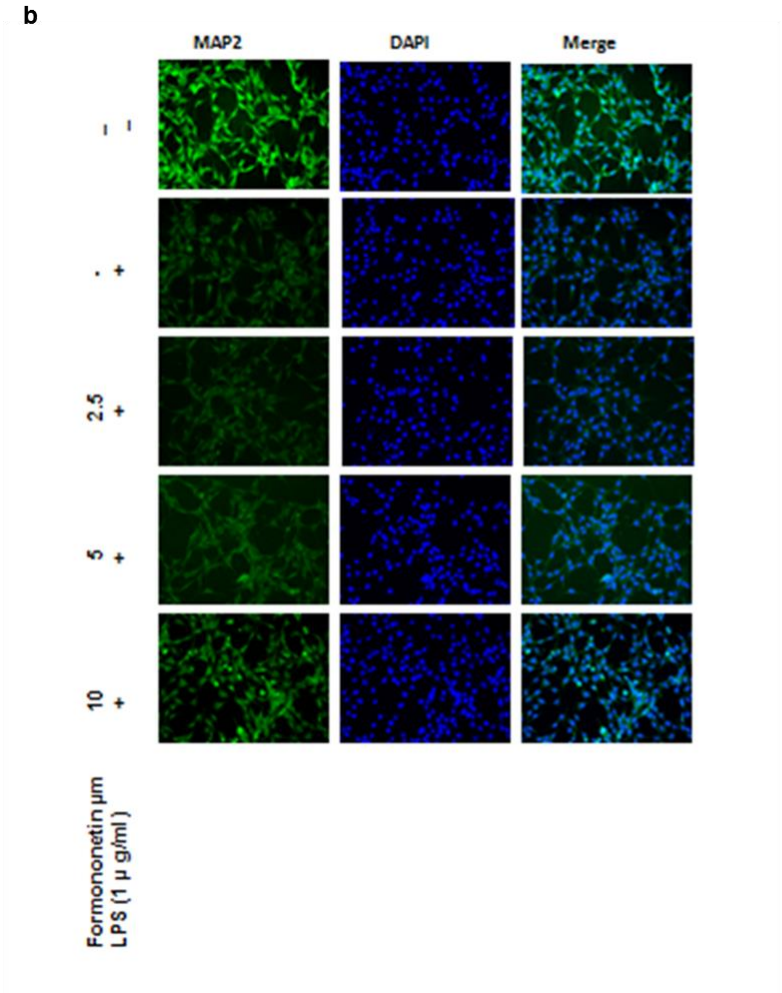

HT22 Neuron Viability
(% of Untreated Cells)
100
50
0
***
ns
**
***
LPS (1 μg/ml) - + + + +
Formononetin (μM) - - 2.5 5 10
Conditioned Medium

Figure 9


b
MAP2
DAPI
Merge
- -
- +
2.5 +
5 +
10 +
Formononetin µm
LPS (1 µ g/ml)

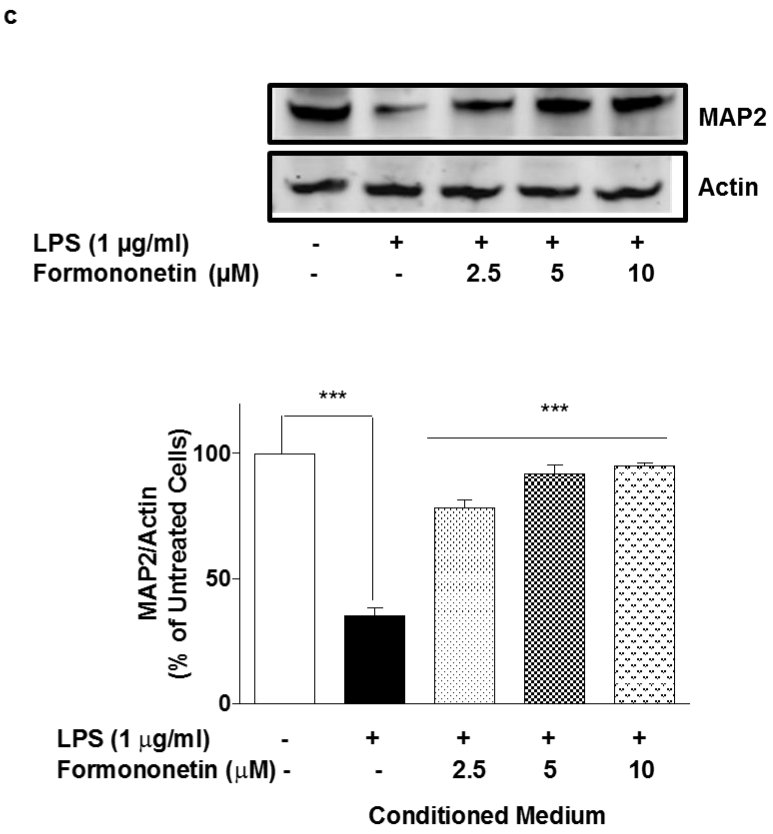

c
MAP2
Actin
LPS (1 µg/ml) - + + + +
Formononetin (µM) - - 2.5 5 10
***
***
MAP2/Actin
(% of Untreated Cells)
100
50
0
LPS (1 µg/ml) - + + + +
Formononetin (µM) - - 2.5 5 10
Conditioned Medium

Figure 9

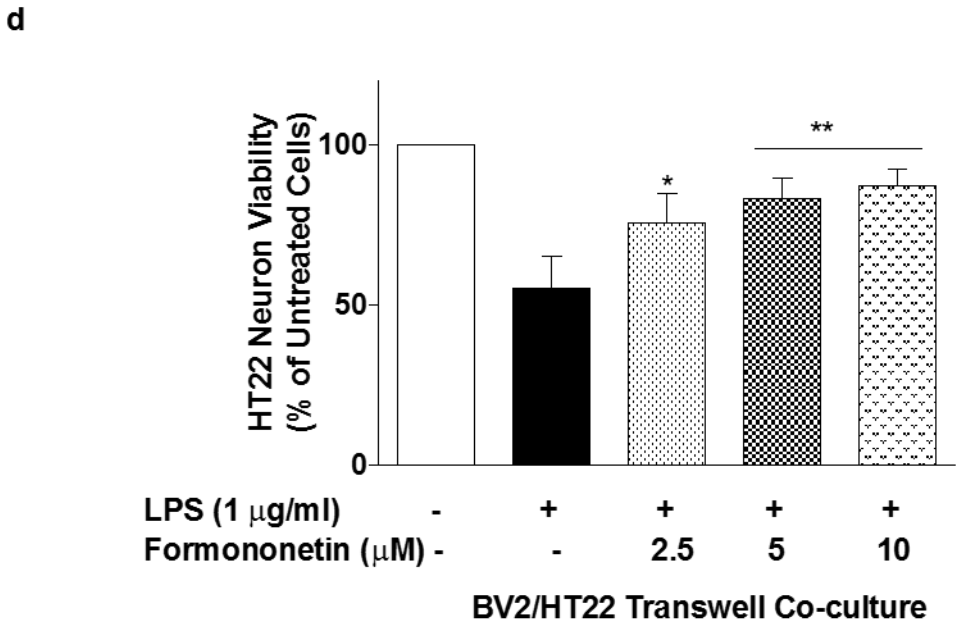
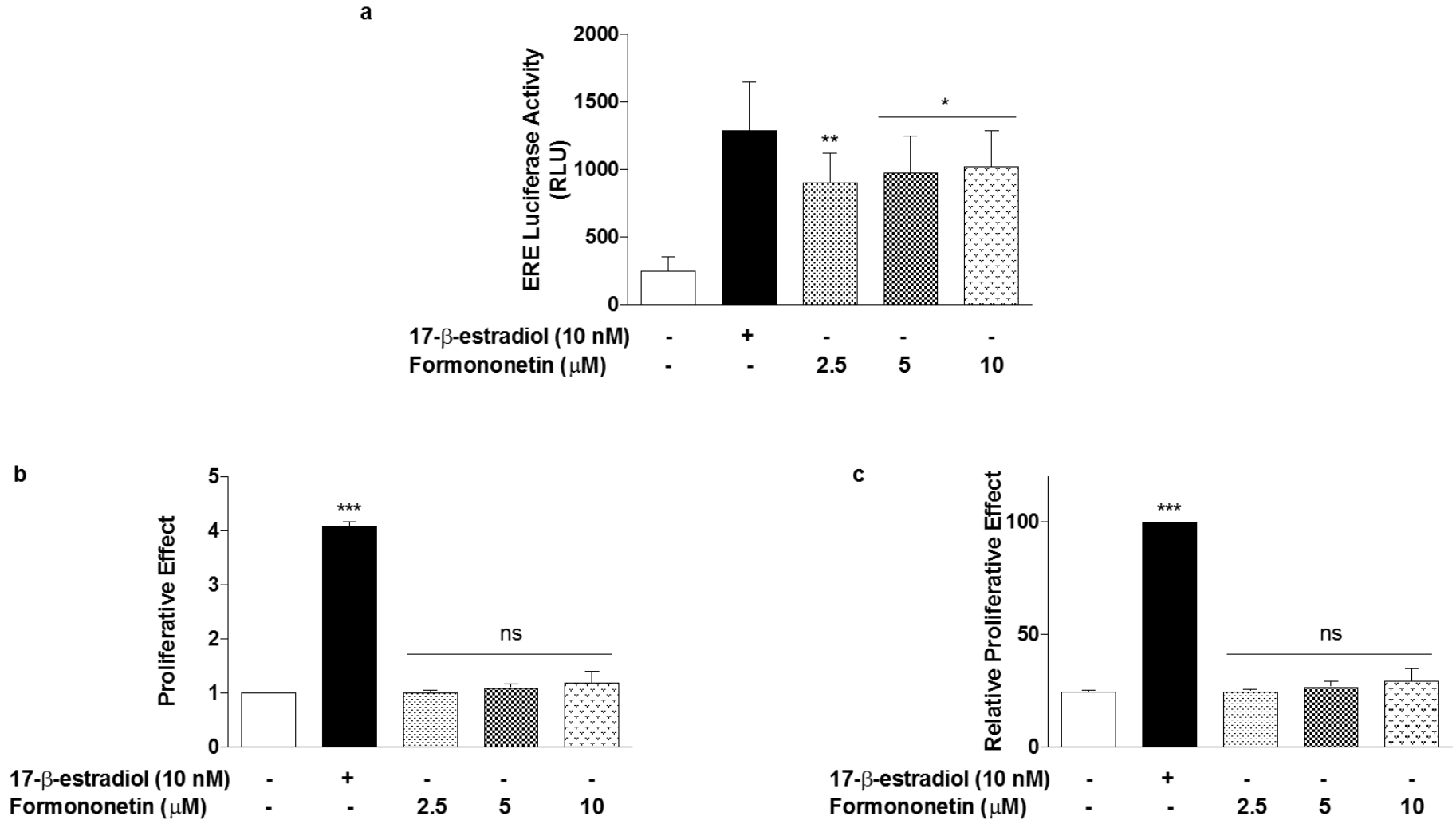

d
HT22 Neuron Viability
(% of Untreated Cells)
100
50
0
*
**
LPS (1 μg/ml) - + + + +
Formononetin (μM) - - 2.5 5 10
BV2/HT22 Transwell Co-culture

Figure 10


a
ERE Luciferase Activity (RLU)
2000
1500
1000
500
0
**
*
17-β-estradiol (10 nM) - + - - -
Formononetin (μM) - - 2.5 5 10
b
Proliferative Effect
5
4
3
2
1
0
***
ns
17-β-estradiol (10 nM) - + - - -
Formononetin (μM) - - 2.5 5 10
c
Relative Proliferative Effect
100
50
0
***
ns
17-β-estradiol (10 nM) - + - - -
Formononetin (μM) - - 2.5 5 10