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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.

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 of a host of pro-inflammatory cytokines including interleukin-1 beta (IL-1β), tumour necrosis factor-α (TNF-α), and 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 estrogen 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) (Fig. 1) is a plant-derived non-steroidal isoflavone with biological activities similar to those of estrogen [12,13]. Some studies have shown that formononetin

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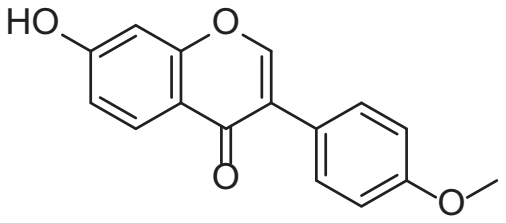

Fig. 1. Chemical structure of formononetin.

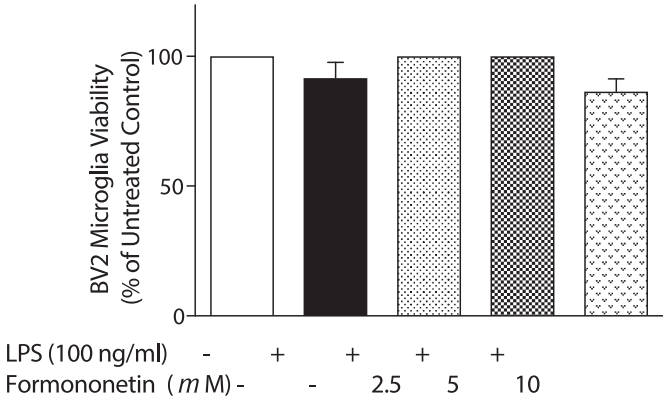

Fig. 2. Pre-treatment with formononetin (2.5, 5 and 10 μM) did not affect the viability of BV2 microglia stimulated with LPS (100 ng/ml) for 24 h. All values are expressed as mean ± SD for 3 independent experiments.

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 37 °C.

The human embryonic kidney cell line 293 (HEK293; ATCC) was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 2 mM 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 Eagle's Modified Essential Medium (EMEM), containing 2 mM Glutamine, 1% Non Essential Amino Acids (NEAA) and 10% FBS.

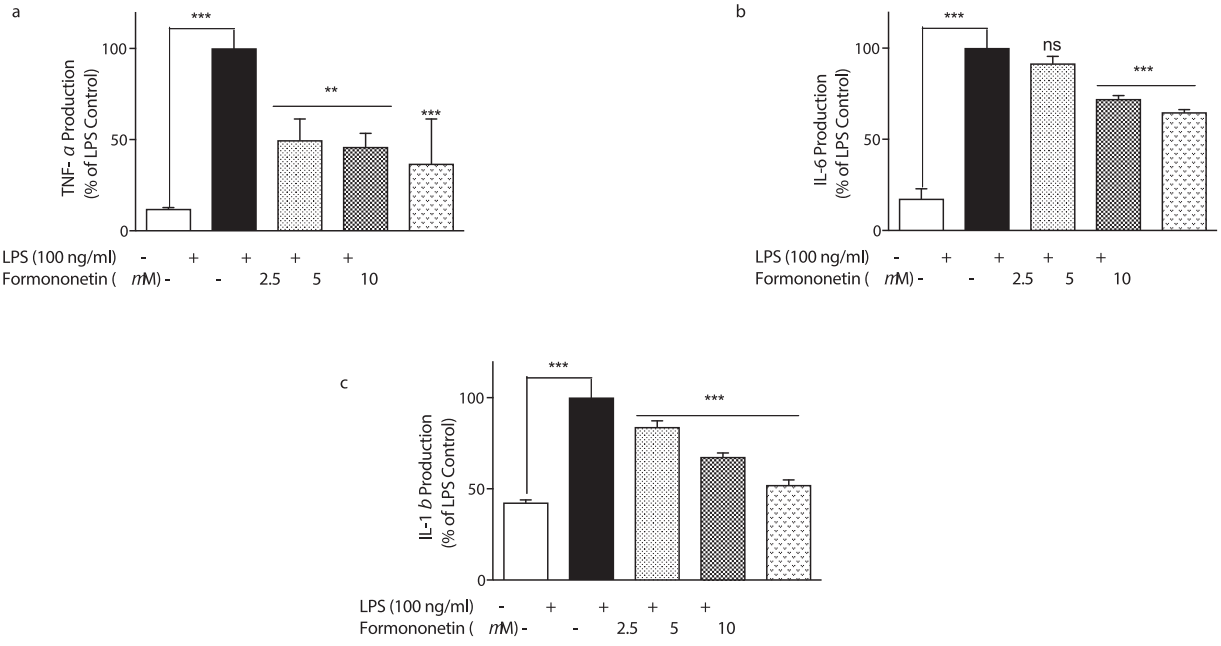

Fig. 3. Formononetin reduced TNF-α (a), IL-6 (b) and IL-1β (c) production in LPS-activated BV2 microglia. Cells were stimulated with LPS (100 ng/ml) in the presence or absence of formononetin (2.5, 5 and 10 μM) for 24 h. At the end of the incubation period, supernatants were collected and pro-inflammatory 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.