University of Huddersfield

Modulation of neuroinflammation with the phytoestrogen, formononetin

A thesis submitted in partial fulfilment for the degree of Doctor of Philosophy

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Copyright Statement

"If the human brain were so simple that we could understand it, we would be so simple that we couldn't."

Emerson M. Pugh

Abstract

Neuroinflammation and pro-inflammatory mediators play key roles in the pathogenesis of neurodegenerative diseases such as Alzheimer disease (AD). Studies have suggested that oestrogen has anti-inflammatory and neuroprotective activities. However, risks of tumour development, heart disease, stroke and blood clots associated with oestrogen replacement therapy limit its application in neurodegenerative disorders. This has switched interest to the use of alternatives such as the selective oestrogen receptor modulators (SERMs) and phytoestrogens which retain the neuroprotective effects of oestrogen with fewer side effects. Formononentin (FMN) is a phytoestrogen which showed significant anti-inflammatory activity through inhibition of nitric oxide (NO) released from lipopolysaccharide-stimulated mouse macrophage RAW cells. However, the ability of formononetin to produce anti-neuroinflammatory activity in LPS-activated microglia is unknown. In addition, the molecular mechanisms involved in the action of the compound in neuroinflammation is unknown. It is also not clear if the anti-inflammatory and neuroprotective effects of formononetin are mediated through estrogen receptors. This study has evaluated the effects of formononetin on neuroinflammation in BV2 microglia. Also the roles of ERβ activation in the anti-inflammatory activity of formononetin in the microglia was investigated.

BV2 microglia were activated with lipopolysaccharide (LPS) with or without formononetin (2.5, 5 and 10 μM), followed by measurement of TNFα, IL-6, nitrite and PGE_2 levels in culture supernatants. Also, protein levels of iNOS, COX-2, total IκBα, phospho-IκBα, phospho-p65 subunit, phospho-IKKα, phospho-p38, phospho-JNK, phospho-ERK 1/2, phospho-MLK3, phospho-TAK1 and ERβ were detected with immunoblotting. A reporter gene assay was used to evaluate NF-κB and ERβ transcriptional activation in HEK293. Electrophoretic mobility shift assay (EMSA) and DNA binding assays were used to examine the effect of formononetin on DNA binding of NF-κB. HT22 neuronal cells were used to study the neuroprotective activity of formononetin. The role of estrogen receptor in the anti-inflammatory activity of formononetin was investigated with western blotting for ERα and ERβ expression in BV2 microglia The effect of formononetin on transcriptional activity of oestrogen-response element (ERE) was also investigated. Small interfering RNAs (siRNAs)

targeted at ERβ were used to silence ERβ geneand levels of TNFα, nitrite and PGE_2 were detected in ERβ-silenced BV2 microglia, while immunofluorescence was used to detect NF-κB p65 subunit.

Results show that formononetin significantly suppressed TNFα, IL-6 and IL-1β without affecting viability of BV2 microglia cells. Furthermore, formononetin inhibited the production of nitrite and PGE_2, and reduced protein levels of COX-2 and iNOS in LPS-activated microglia. In addition, formononetin prevented HTT22 neuronal toxicity produced by microglia-conditioned medium. Formononetin targeted NF-κB activity by inhibiting the phosphorylation of IKKα, IκBα and nuclear translocation of the p65 subunit. The compound also suppressed NF-κB/DNA binding activity in LPS stimulated BV2 microglia. Further results showed that formononetin inhibited p-38, JNK and ERK1/2 MAPKs activation by targeting upstream proteins TAK-1 and MLK3. The study revealed that BV2 microglia express ERβ, but not ERα. The study also demonstrated that formononetin increased transcriptional activity of ERE in HEK293 cells. Results further showed that this compound increased protein levels of ERβ in BV2 microglia. RNA interference experiments revealed that NF-κB-mediated anti-inflammatory activity of formononetin was abolished following transfection of BV2 microglia with ERβ siRNA. Taken together, this study has demonstrated for the first time, that formononetin inhibits neuroinflammation in BV2 microglia , and neuroinflammation-mediated neurotoxicity in HT22 mouse hippocampal neurons by targeting upstream convergence proteins in both the NF-κB and MAPK signalling pathways. This study also established that the ability of the compound to interact with microglial ERβ possibly contributes to the anti-inflammatory action of formononetin.

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Abbreviations

AD	Alzheimer disease
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BBB	Blood brain barrier
BCRs	B-cell receptors
CM	Conditioned media
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal fluid
CX3CL1	Fractalkine
CXCL12	Stromal cell–derived factor 1
DDT	Dithiothreitol
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dsRNA	Double stranded Ribonucleic acid
E2	Estradiol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic Mobility Shift Assay
ERE	estrogen-response element
ERK	Extracellular signal-regulated kinases
ERs	Oestrogen receptors
ERß-KO	Oestrogen receptor ß knockout
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
FBS	Fetal bovine serum
FMN	Formononetin
HRP	Horseradish peroxidase
IF	Immunofluorescence
IFNγ	Gamma-interferon
IL-6	Intrlukine-6
INOs	Inducible nitric oxide synthase
IS	Immune system
ISS	Inner immune system
JNK	C-Jun N-terminal kinases
KDa	kilo Dalton
LDS	Lithium dodecyl sulfate

LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
MC	Mast cells
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic acid
MS	Multiple sclerosis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response 88
NES	Nuclear export signal
NFAT	Nuclear factor of activated T cells
NF-Kb	Nuclear factor kappa b
NLR's	NOD-like receptors
NLS	Nuclear localization signal
NO	Nitric oxide
O2-	Superoxide
PAMPs	Pathogen-associated molecular patterns
PAR2	Proteinase activated receptor 2
PBS	Phosphate buffered saline
PD	Parkinson disease
PGE2	Prostaglandin E2
PRR	Pattern recognition receptor
PVDF	Polyvinylidene difluoride
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SERMs	Selective Oestrogenic receptor modulators
siRNA	Small interfering RNA
siRNA	Small interference Ribonucleic acid
SOCS	Suppressor of cytokine signalling proteins
TACE	Tumour necrosis factor-alpha-matrix metalloproteinases converting enzyme
TAK1	Transforming growth factor beta-activated kinase 1
TBS	Tris-buffered saline
TBS- T	Tris-buffered saline containing Tween
TC	T. cells
TCRs	T-cell receptors
TGF-β	Transforming growth factor beta
TLR's	Toll-like receptors
TLR4	Toll like receptor-4
TNFα	Tumour necrosis factor-α
Tpl2	Tumour progression locus 2
UCPs	Uncoupling protein

Chapter One

Introduction

1.1. Background

The immune system is a complex biological system, which has a role in defending the body against attacks such as pathogens and their toxins. The immune system has three lines of defence. First, the anatomical and physiological barriers such as the skin, mucus and clearance mechanisms, the stomach pH and lysozymes, bacteriolytic tears, saliva and other secretions and the blood-brain barrier (BBB) are the first lines of defence the body against pathogens (Delves et al., 2000; Langman, 2014).

The innate immune response implicates nonspecific immune defence mechanisms that start immediately or within hours after an antigen or chemical passes in to the body. Innate immunity is congenital; macrophages recognise pathogens via receptors pattern recognition receptors (PRRs) including toll like receptors (TLRs) and nucleotide oligomerization domain (NOD) receptors. Recognition of these patterns by macrophages initiates cellular defence mechanisms (LeClair, 2003; Han et al., 2005; Sterka et al., 2006; Lotze et al., 2007). The primary role of this inflammatory response is to eliminate foreign substances by phagocytosis. The resolution of inflammation and tissue repair is a second step in the innate immune response. To resolve infection or repair injury, the innate immune response requires the substitution of lost or damaged cells and restructuring of the extracellular matrix (Barton, 2008; Koenderman et al., 2014).

The third line of defence is acquired immunity or adaptive immunity. It is non-congenital. The immune system reacts with antigens and the acquired immunity's components learn how to attack each antigen and create a memory by generating specific antibodies for it. This way it can deal with the antigen if they re-attack the body because it learns, adapts, and remembers them (Koenderman et al., 2014).

The central nervous system (CNS) has long been considered to be an immune privileged organ. This concept results from the presence of the blood-brain barrier (BBB), anatomophysiological barrier, and tight junctions, which are impermeable to antibodies and immune cells. Research in psychoneuroimmunology in the years 1980 and 1990 have revealed more complex relationships between IS and CNS. Indeed, an infectious episode is accompanied by physiological and behavioural changes such as fever, fatigue, decreased food intake and withdrawal, grouped under the term status

disease (Konsman et al., 2001).. The detection of a system of innate immunity within the CNS has allowed a better understanding of the interactions between IS and CNS and the role of these interactions in the host response to infection and associated behavioural changes (Ransohoff et al., 2012).

1.2. Neuroinflammation

Neuroinflammation as a term was first used in the 1990s to describe the inflammatory process and its role in the pathophysiology of most neurodegenerative diseases. Neuroinflammation is now frequently seen as deleterious for neurological function, and is common to a large number of situations, from normal aging to neurological conditions such as neurodegenerative diseases, autoimmune diseases or epilepsy and stroke (Hanisch et al., 2007). The definition of the term ‘neuroinflammation’ is controversial. Some scientists think the term should be restricted to responses involving two immune cells T and B lymphocytes from the periphery (Bird et al., 2011). Others use a broader definition that includes activation and proliferation of microglial cells (Glass et al., 2010). Graeber and co-workers exclude neuroinflammation in the definition of immune responses involving microglial cells observed during Alzheimer's and Parkinson's diseases, since these conditions do not show infiltration of peripheral cells, so they were grouped under the term ‘microglial activation or neuroinflammtion’ (Graeber et al., 2011). Neuroinflammation is associated with the CNS in several neurodegenerative disorders such as Alzheimer's disease, multiple sclerosis, and Parkinson's disease (Sheng et al., 2001; McGeer et al., 2004; Block et al., 2005; Phillis et al., 2006). It involves activation of microglia (Perry et al., 2013) which are considered as the resident immune cells of the CNS. (Schwartz et al., 2013). In response to tissue damage or pathogen invasion there is a developing inflammatory response which is amided to protect the CNS and initiates tissue repair (Wyss et al., 2002). However, sustained inflammation or failure in normal resolution mechanisms in the brain may result in the production of neurotoxic factors increasing the disease states (Lull et al., 2010; Sarma, 2014).(Figure 1.1)

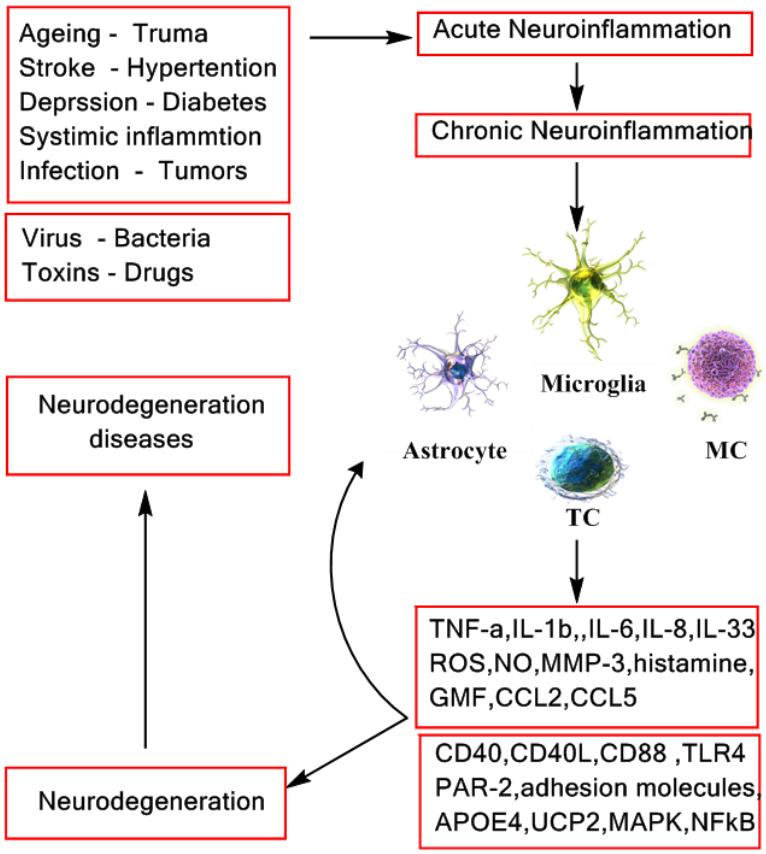

Figure 1.1 Schematic diagram showing neuroinflammation-mediated neurodegeneration in the brain.

Several factors induce neuroinflammation such as aging, dementia, stroke, brain injuries, obesity and local and systemic infections by activating microglia, astrocytes and neurons in the brain. These factors also activate immune and inflammatory cells such as T-cells and mast cells in the brain. Because of this activation, many proinflammatory and neurotoxic mediators are released and increasess expression of inflammation related receptor proteins in the brain. These inflammatory mediators and enhanced protein expressions will further increase neuroinflammation and neurodegeneration, which develop into progressive neurodegenerative diseases. MC=mast cells, PAR-2=proteinase activated receptor-2, TC=T-cells, UCPs=mitochondrial uncoupling proteins. Modified from (Duraisamy et al., 2017)

1.3. Neuroinflammation in Alzheimer's disease

Alzheimer's disease (AD) is the most common form of age-related neurodegenerative diseases which cause of dementia (Heneka, et al. 2015). AD is a result of changes in brain areas especially the cortex and hippocampus, resulting in a progressive and irreversible changes in the morphology and biochemistry of the brain. The symptoms

of AD are characterised by memory loss, general mental dysfunction and several behavioural and neuropsychiatric disturbances (Morales, et al. 2014).

The pathology of AD is based on the role of amyloid-β (Aβ) in the formation of extracellular amyloid aggregates which in turn results in the formation of Tau aggregates and neurofibrillary tangles which contribute to neuronal loss, synaptic dysfunction, and diseased neurons characteristic of AD (Minter et al. 2016). In addition, amyloid-β (Aβ) also causes an activation of microglia and astrocytes, resulting in the release of several pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6 which may act directly on neurones to induce neurotoxicity and neurodegeneration (Guerriero et al. 2017) (Figure 1.2) .

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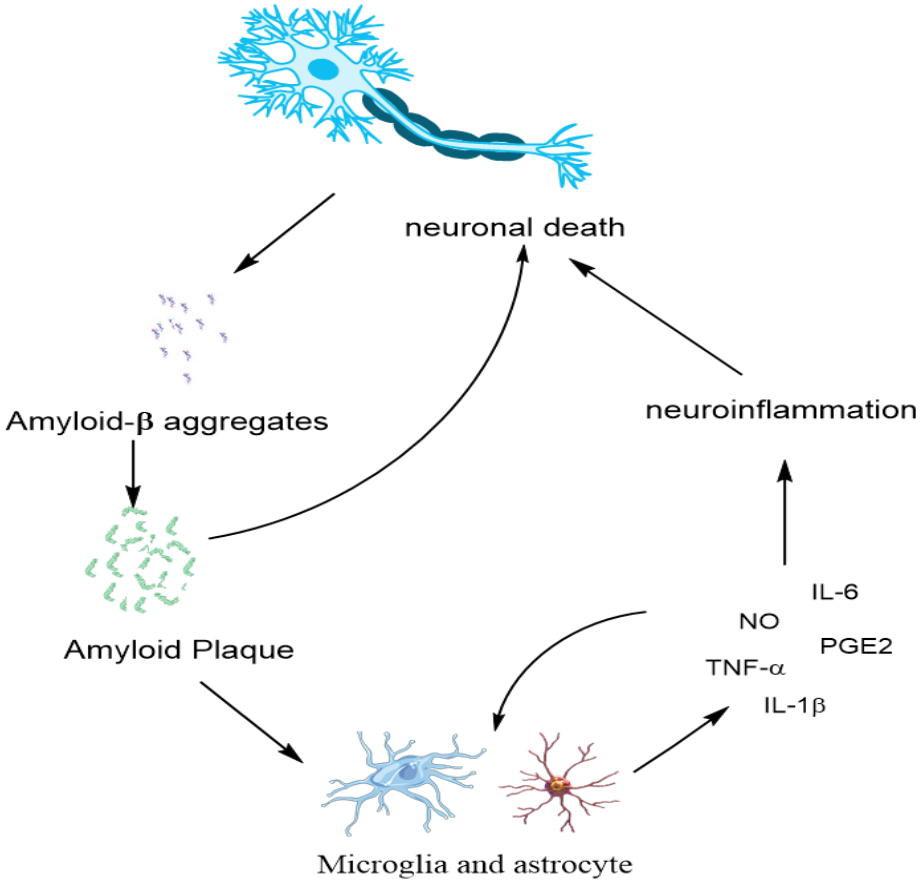


Figure 1.2 Neuroinflammation in Alzheimer's disease

Modified from (Castellani et al., 2008)

1.4. . Natural compounds with anti-inflammatory effects

The field of drug discovery has paid a lot of attention to the neuroprotective activities of natural compounds from traditional medicinal herbs, which have antioxidant, anti-inflammatory, anti-apoptotic effects.

Genistein, one of the principal active components of Soybean isoflavone, is reported to produce anti-inflammatory activity in BV2 microglia cells, through inhibition of TLR4/NF-kB signal pathway (Zhou et al., 2014).

Biochanin A, an O-methylated isoflavone, is a natural organic compound that has shown anti-inflammatory property in LPS-activated BV2 microglia through inhibition of mitogen-activated protein kinase (MAPK) signalling pathways (Wu et al., 2015).

Another study has revealed that ginseng an ancient herb used in traditional Chinese medicine has anti-inflammatory action in LPS-activated BV2 microglia cells by inhibiting the release of the pro-inflammatory factors such as iNOS and COX2 (Lee et al., 2012)

1.5. . Phytoestrogens

Phytoestrogens are naturally occurring components of many plants. As the name suggests, they show oestrogen-like properties. A slightly broader definition also includes effects that suggest an oestrogen mechanism. These include binding to the oestrogen receptor, and induction of specific oestrogen responsive gene products (Sirotkin et al., 2014).

Chemically, phytoestrogens can be classified into five groups, chalcones, flavonoids (flavones, flavonols, flavanones, isoflavonoids), lignans, stilbenoids, and miscellaneous classes as shown in figure 1.3. Particularly, isoflavonoids are a subgroup of flavonoids which include amongst others the chemical groups of isoflavones, isoflavanones, pterocarpanes, and coumestans (Michel et al., 2013; Sirotkin et al., 2014).

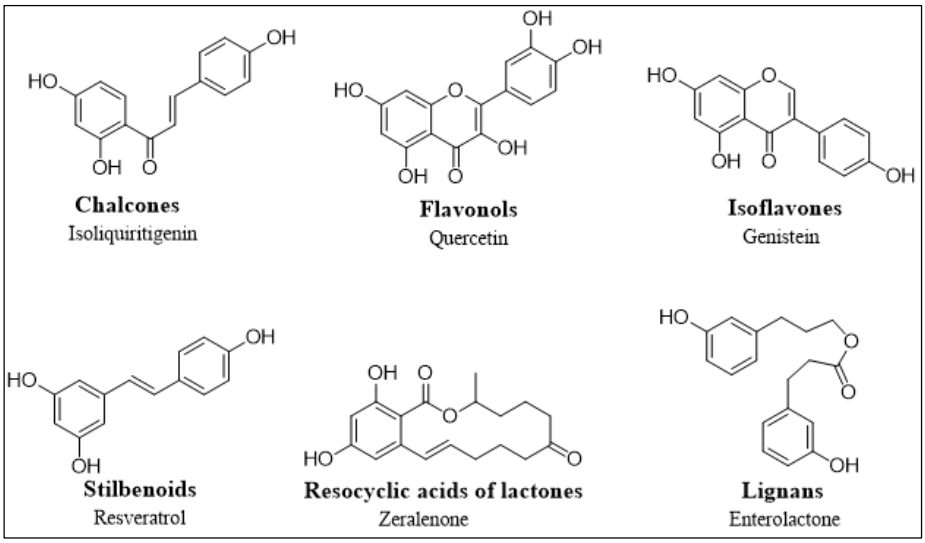


Figure 1.3 Molecular structures of the most ubiquitous phytoestrogens

(Michel et al., 2013).

Isoflavones (3-phenylchromone derivatives) are the most-studied phytoestrogens. They differ from the flavones commonly found in plants only by the position of the linkage of chromone and phenyl ring. Isoflavones are predominantly found in legumes and are especially found in high amounts in soybeans. (Poluzzi et al., 2014).The most important isoflavones found in plants are biochanin A, daidzein, glycitein, genistein, and formononetin. Biochanin and formononetin are derivatives of genistein and daidzein with an additional methyl group (Zhao et al., 2010).

In plants, isoflavones are usually present in glucoside form, which is the biologically inactive form. They are converted to the active form by intestinal bacteria and enzymes (Adlercreutz et al., 1987) .

Phytoestrogens have been reported to show many pharmacological activities. In the reproductive system, exposure of women to phytoestrogens in both pre- and post-menopausal period may prevent the menopausal symptoms because of the decline in endogenous oestrogen levels, hot flashes and vasomotor symptoms, (Kronenberg et al., 2002; Branca et al., 2005; Bedell et al., 2014). Phytoestrogens have been therapeutically used for diminishing skin aging (Thornton, 2013). Pytoestrogens also play an important role in the immune system; soy phytoestrogens inhibit the intracellular signalling pathway related to NF-κB transcription factor, activating inflammation and immune response (Liao et al., 2014; Shukla et al., 2015)

Animal experiments established the neuroprotective effect of phytoestrogens in terms of their ability to prevent oxidative stress-induced degenerative changes in these neurons (Chao et al., 2012).

1.5.1. Formononetin

Formononetin (7-hydroxy-4'-methoxy isoflavone) is also known as Biochanin B. It is present in a number of plants like the red clover along with other phytoestrogens. It largely occurs in leguminous plants, particularly in beans, such as green beans, lima beans, soy (Jian-Hong et al., 2010). As shown in figures 1.4 and 1.5formononetin has a structural similarity to oestrogen (Yang et al., 2012). It shows oestrogenic effects (Mu et al., 2009b) and hypolipidemic properties (Siddiqui et al., 1976). Formononetin showed significant anti-inflammatory activity through inhibition of nitric oxide (NO) released from lipopolysaccharide-stimulated mouse macrophage RAW cells (Lai et al., 2014). Also, formononetin showed antioxidant effects *in vivo* (Mu et al., 2009a). Several studies proved that formononetin has anti-cancer activity through initiation of growth-inhibitory, pro-apoptotic activities as well as inhibition of tumour cell invasion (Chen et al., 2011; Auyeung et al., 2012). Other studies revealed that formononetin target JNK, ERK and p38 MAPK signalling pathways to inhibit enterovirus 71 replication (Wang et al., 2015) and produced wound healing activities (Huh et al., 2011).

It is reported that formononetin has potent antioxidant effects and oestrogenic effect (Mu et al., 2009b). Furthermore, some researchers have shown that formononetin protect neurons from oxidative stress and toxicity induced by L-glutamate or amyloid-β (Occhiuto et al., 2009). However, little is known about the effects of formononetin on the neuroinflammation induced by LPS in microglia cells.

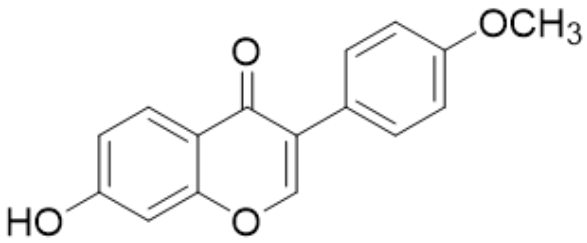


Figure 1.4 Chemical structure of formononetin.

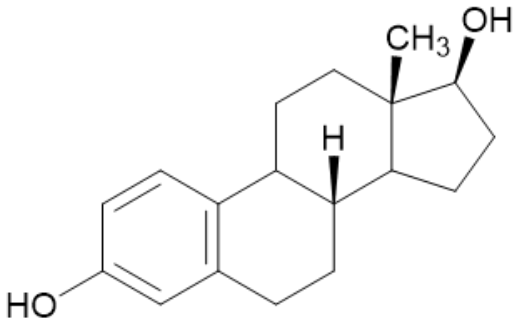


Figure 1.5 Chemical structure of 17β-Estradiol.

1.6. Gap in knowledge

The effect of formononetin in LPS activated BV2 microglia cells remains unclear. It is also not known if any potential inhibition of neuroinflammation by this compound would translate into neuroprotective activity. The molecular targets of action of this compound is also unclear, especially those relating to activation of microglia oestrogen receptors.

1.6.1. Aim of the study

The aim is to investigate inhibition of neuroinflammation by formononetin in LPS activated BV2 microglial cells.

1.6.2. Specific objective

Specifically, the following objectives would be achieved

1. To investigate the effects of formononetin in LPS-activated BV2 microglia.
2. To determine whether formononetin would protect neurons from neuroinflammation-mediated toxicity.
3. To elucidate the roles of NF-κB and MAPKs in the inhibition of neuroinflammation by formononetin.
4. To explore the roles played by activation of microglia oestrogen receptor beta (ERβ) in the anti-neuroinflammatory and neuroprotective activities of formononetin.

Chapter Two

Inhibition of neuroinflammation and neuroinflammation-mediated neurotoxicity by formononetin

2.1. Introduction

Microglia, which are the local immune cells in the central nervous system (CNS), contribute to CNS inflammation in many pathological conditions.

Microglia cells are not inactivated under normal conditions. However, they become activated following brain injury and migrate to the injured area .They also phagocytose cells and cellular debris. . In the process microglia, produce inflammatory mediators such as cytokines, chemokines, and reactive oxygen species. Even though an acute inflammation is beneficial after an inflammatory insult, there is evidence that excessive microglial activation can have damaging consequences on healthy cells such as neurons.

Bacterial lipopolysaccharide (LPS) has the ability to induce microglia activation, leading to the release of inflammatory mediators (Thameem et al., 2007) These inflammatory mediators, such as TNF-α, IL-1β, IL-6 and PGE_2 , play an important role in the pathological processes involved in neurodegeneration (Smith et al., 2012).

Neurons are particularly susceptible to inflammation-related injury, suggesting that neuroprotective and anti-inflammatory agents (Hirsch et al., 2009; Singhal et al., 2015) may achieve the prevention of neuronal cell damage. It is widely well-known neuroinflammation is associated with many forms of neuronal cell damage that occurs in many neurological diseases and disorders, such as stroke, Alzheimer's disease and Parkinson's disease (Min et al., 2003; Kyoung et al., 2004). Therefore, the identification of agents to inhibit neuroinflammation might be an effective approach for the treatment of neurodegenerative diseases.

2.2. The microglia in neuroinflammation

An integral part of human immune defence is the microglial cells which are found in the CNS, where they constitute 20% of the total amount of glial population (Kreutzberg, 1996). Following examination of the brains of adult mice, these cells were found extensively in the grey matter, hippocampus, olfactory telencephalon, basal ganglia and substantia nigra (Block et al., 2007). They have a branched structure and are formed in the mesodermal tissues of the CNS, from monocyte-macrophage lineage. Microglia cells are locomotive in ordinaryconditions; they move around and detect changes in their surroundings (Gao et al., 2008). If they sense any alarming situation,

they become activated and behave like macrophages. By functionality, the microglia are classified into:

- Phagocytic phenotype, characterised by innate activation.
- Antigen presenting phenotype, characterised by adaptive activation and the ability to determine the production of cytokines (Town et al., 2005).

On activation, microglial cells undergo a morphological change i.e. changes to amoeboid shape accompanied with shrinking of cellular components and enlargement of the soma. The activated microglia are able to ingest and destroy cellular waste and other foreign materials. In addition, they also attract more microglia, cytokines and other elements that may help in microglial proliferation, by producing chemokines such as fractalkine/neurotactin (CX3CL1) and stromal cell–derived factor 1 (CXCL12) (Wolf et al., 2017). Neuroinflammation in microglial cells can be useful for recovery purposes as stimulating myelin repair, eliminating toxic proteins and averting neurodegeneration (Gao et al., 2008). However, the disruption in the inflammatory process might cause many problems. In any such case, several cytotoxic factors like superoxide (O_2^-), nitric oxide (NO) and tumour necrosis factor-α (TNFα) produced by activated microglia will lead to chronic neuroinflammation (Boche et al., 2013) .

2.3. Microglial activation

The concept of microglial activation has been known since the discovery of microglia as a source of inflammatory factors that are not normally expressed in CNS (Graeber et al., 1988; Graeber et al., 1990). As a defence mechanism, microglia mediate the immune response in the CNS because of infection, brain injury, and stress. There is a release of pro-inflammatory cytokines and generation of reactive oxygen species. These secretions of microglia are beneficial to the host as a protection from infection in the CNS, but they also can be harmful when inflammatory process is chronic. Activated microglia can be divided into two phenotypes (M1 and M2) (figure 2.1) based on the kind of the activator. The classic activated phenotype of microglia (M1) involved in the production of the pro-inflammatory cytokines such as IL-1β, iL-6, TNFα and reactive oxygen species. Whereas (M2) the alternatively activated microglia is an anti-inflammatory phenotype, which release anti-inflammatory cytokines such as IL-4 and IL-10,as well as neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Doens et al., 2014). Activated microglia have

been detected in the CNS in most of neurodegenerative disorders, and they are believed to play a critical role in the development of these diseases.

Several experimental studies have showed that the inhibition of microglial activation leads to reduced levels of neurotoxins and improved neuronal survival in neurodegenerative diseases (Tang et al., 2016).

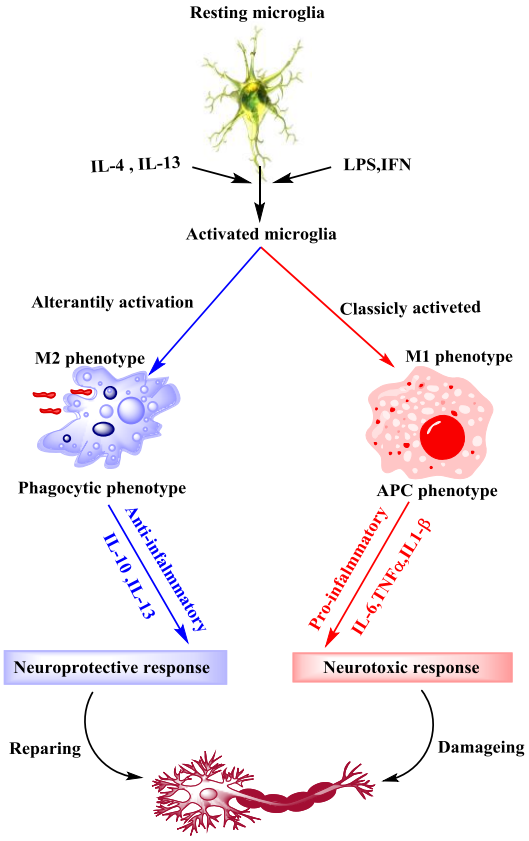


Figure 2.1 Microglia activation phyenotypes

Microglia have two states of activation (classical activation M1 phenotype) and (alternative activation M2 phenotype) depending on stimulant. M1 phenotype produce various pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, as well as superoxide, ROS and nitic oxide. These factors lead to neurotoxic response. M2 microglia induced by IL-4/IL-13 and IL-10. M2 phenotype facilitate phagocytosis of cell debris and misfolded proteins and tissue repair, and support neuron survival by neurotrophic factors. Modified from (Carniglia et al., 2017).

2.3.1. The role of toll-like receptors (TLRs) in neuroinflammation

The toll protein receptors are pattern recognition receptors, first discovered in *Drosophila*. They were shown to be essential for determining the dorsal–ventral patterning during embryogenesis (Morisalo et al., 1995; Belvin et al., 1996). They also

play a critical role in the early innate immune response to invading pathogens by sensing microorganisms (Cherry et al., 2006; Ray et al., 2013).In the inflammation aspect a particular interest is in TLR4 which could be activated by lipopolysaccharide (LPS) component of the Gram-negative bacteria (Hanke et al., 2011) resulting in serial activation and phosphorylation of proteins Nuclear Factor kappa B (NFκB) and Mitogen-Activated Protein Kinases (MAPKs). These pathways regulate a wide array of genes in different cell types (Smale, 2010). Some of these genes are responsible for the production of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-β (IL-1β), and interleukin-6 (IL-6) which amplify the inflammatory response (Takeuchi et al., 2010). Furthermore, the generation of reactive oxygen or nitrogen species (ROS or RNS) further amplifies the process.

2.3.1.1. Toll-like receptor 4 (TLR4)

The innate immune receptor toll-like-receptor 4 (TLR4), localised on the surface of microglia, and is a first-line host defence receptor against invading microorganisms (Yao et al., 2013). TLR4 is expressed in the microglia and its activation produces the inflammatory and innate immune responses (Marsh et al., 2009; Pascual et al., 2011). Lipopolysaccharide (LPS) is the main component of the outer membrane of Gram-negative bacteria bind to the CD14 and TLR4/MD2 complex leading an activation of TLR4 (Hyakkoku et al., 2010).

TLR4 activation results in the activation of the myeloid differentiation protein 88 (MyD88), the interleukin-1 (IL-1) receptor-associated kinase, the tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6), and the transforming growth factor-beta-activated kinase 1 (TAK1). Subsequently, I kappa B kinase (IKK) complex is activated. The activated IKK complex phosphorylates I kappa Bα, which triggers its ubiquitination/degradation and subsequent release of nuclear factor kappa B (NF-κB), which then translocate to the nucleus and activates the transcription of kappa B-dependent genes, such as IL-1, IL-6, and TNF-α (Figure 2.2) (Ock et al., 2007; Lehnardt, 2010).

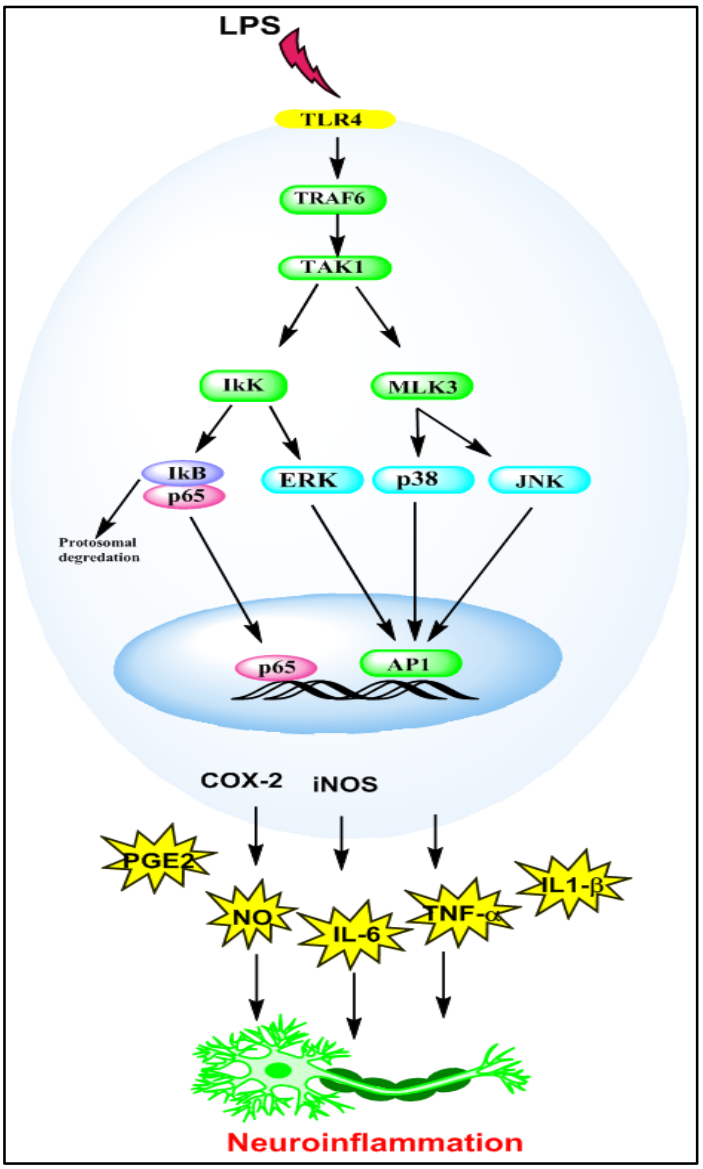


Figure 2.2 Schematic overview of Toll-like receptor (TLR) 4 signalling pathway.

Lipopolysaccharide (LPS) binds to TLR4 on the cell membrane, followed by activation of the TNF receptor-associated factor 6 (TRAF6) which leads to activation of transforming growth factor beta-activated kinase 1 (TAK1), This in turn leads to activation of transcription factors nuclear factor (NF)-κB and MAPKs including p38, JNK and ERK, and controls the expression of pro-inflammatory cytokines and other immune-related genes(O'Neill et al., 2000; Guo et al., 2010).

2.4. Roles of pro-inflammatory factors in neuroinflammation

2.4.1. Pro inflammatory cytokines

Pro-inflammatory cytokines are proteins which control cellular immunity and the inflammatory response (Borish et al., 2003; Murphy et al., 2008). IL-1β, IL-6 and TNF-α are cytokines produced in the periphery and the CNS (typically by microglia) (Brough et al., 2011). These cytokines have been thoroughly investigated in studies involving neuroinflammation and neurodegenerative diseases (Wang & Tan, 2015; Kim et al., 2016).

2.4.1.1. Tumour necrosis factor (TNF-α)

Tumour necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine which has been linked to innate immune response (Clark, 2007). The biological activities of TNF-α are mediated by two receptors, which though are similar structurally but are functionally different. These receptors are (TNFRI or p55) and (TNFRII or p75) (Vitkovic et al., 2000). TNF-α receptors are important in host defence against pathogens (Dempsey et al., 2003). Most of the inflammatory responses, which are linked to TNF-α, are mediated by the p55 TNFRI. For example, studies of TNF-α receptor-deficient mice reveal that p55 plays a critical role in mediating endotoxic shock (Pfeffer et al., 1993; Waters et al., 2013). Consequently, a thorough understanding of TNF-α signalling pathway in neurodegenerative disorders would be useful in the development of novel bioactives in the treatment of these conditions.

2.4.2. Interleukin-6 (IL-6)

Interleukin-6 (IL-6, also known as IFN-β2) is involved in the pathogenesis of inflammatory disorders. Significant neuropathological changes in conditions such as multiple sclerosis (MS), Parkinson's and Alzheimer's diseases are strongly linked with increased IL-6 expression in the brain (Rothaug et al., 2016). It is important to note that IL-6 expression is regulated by transcription factors such as nuclear factor κB (NF-κB), activator protein 1 (AP-1), cAMP response element binding protein (CREB) and CCAAT/ enhancer-binding protein β (C/EBPβ) (Dendorfer et al., 1994).

2.4.2.1. IL-1 Beta (IL-1β)

The activation of IL-1-β is via Toll-like receptors (TLR) with the production of pro-IL-1-β. Subsequently, pro-IL-1β is converted into the mature form via the enzyme caspase-1(Gibson et al., 2004; Shaftel et al., 2007).

2.4.3. Pro-inflammatory enzymes

2.4.3.1. Cyclooxygenase (COX)

Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins (PGs) which are mediators that play a major role in several physiological and pathological processes, including inflammation (Farooqui et al., 2007). Two distinct COX isoforms are known as COX-1 and COX-2. These isoforms differ in mechanisms, tissue distribution and the mechanisms involved in their coupling to upstream and downstream proteins in the CNS (Grösch et al., 2017). COX-1 is constitutively expressed in most tissues, and has been shown to be responsible for PG synthesis in homeostasis (Phillis et al., 2006). On the other hand, COX-2 is mainly induced in response to inflammatory stimuli. The property of COX-2 led to the belief that selective inhibition of the enzyme can reduce inflammation without affecting the physiological functions of COX-1-derived PGs, resulting in the development of COX-2 selective inhibitors. In terms of the microglia, COX-2 mRNA and protein as well as prostaglandin E2 formation were not detected in unstimulated microglia. However, following stimulation with lipopolysaccharide their levels and expression were elevated (Hoozemans et al., 2001).

2.4.3.2. Nitric oxide synthases

The synthesis of NO in inflammation depends on an inducible form of NO synthase (iNOS), which is synthesised in leucocytes, vasculature and possibly parenchymal cells at sites of injury, in response to cytokines or microbial products.

2.5. Theme of this chapter

The focus of the study in this chapter is the investigation into the possible protective effects of formononetin in the LPS-induced BV2 microglia inflammation. This chapter

also highlights the neuroprotective effect of formononetin in the mouse hippocampal HT22 neurons.

2.6. Materials and methods

2.6.1. Materials

The following materials were used: tissue culture flasks (75 mL), tissue culture plates (96-, 24- and 6-well plates), 50 ml and 15 ml centrifuge tubes, serological pipettes, micro-test tubes 0.5 ,1.5 ml and RNA free Eppendorf tubes 1.5 ml , 1000 ,200 and 10 µl tips, pipetting reservoir (55 ml) were acquired from Sarstedt (Nürnbrecht, Germany). The supplier for the other materials like reagents, chemicals, buffers, ELISA kits, gels, membranes, antibodies and instruments will be described.

2.6.2. Formononetin

- Formononetin was obtained from Sigma-Aldrich, dissolved in DMSO, and a stock solution of 0.1 M frozen at -80°C. The purity of formononetin was confirmed using proton NMR.One milligram of formononetin was dissolved in DMSO-d6.NMR spectra were recorded on a Bruker Ascend 400 MHz spectrometer, with chemical shifts being quoted in ppm referenced relative to the residual solvent signal (, DMSO δ = 2.5) as shown in figure 2.3.

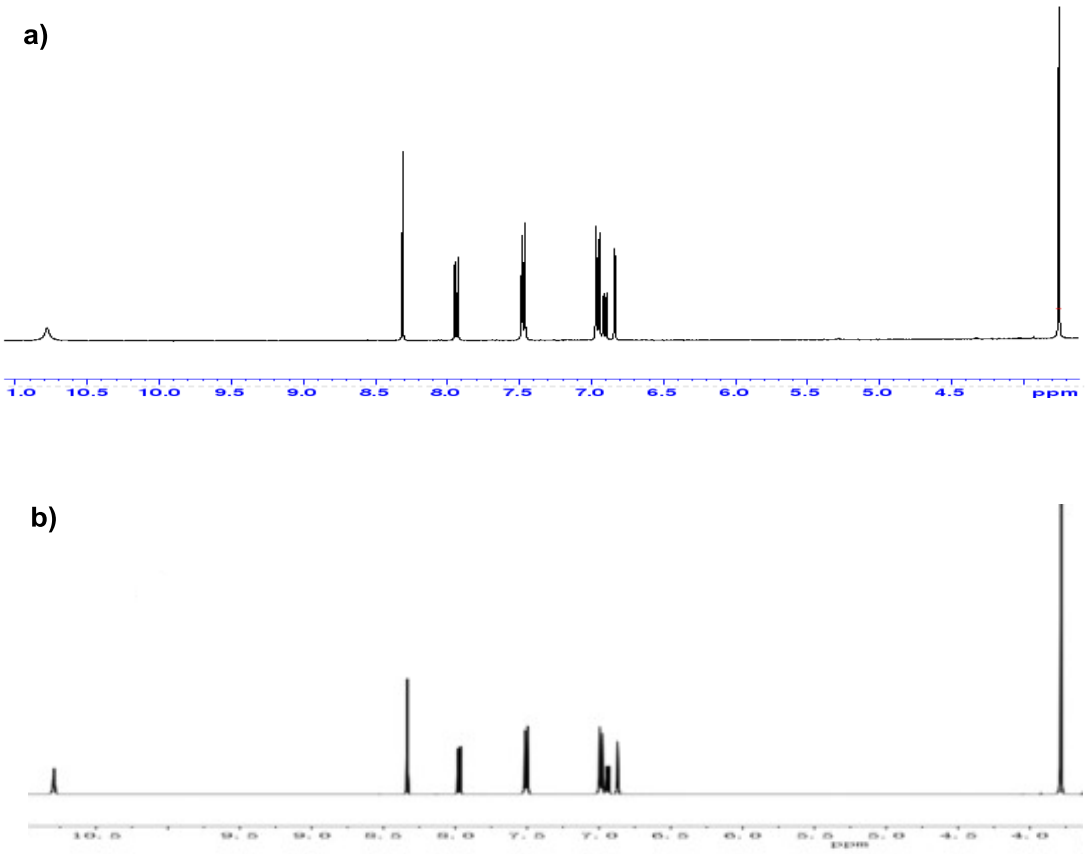


Figure 2.3 NMR spectra for formononetin which used in the whole study

(a) ^{1}H NMR spectra of FMN which used in the study (b)^{1}H NMR spectra of FMN from different study as reference (b) (Guo et al., 2017).

2.6.3. Culture media

Roswell Park Memorial Institute medium RPMI 1640, which already contained L-glutamine (2 mM) was purchased from Sigma (UK). This was supplemented with 10% foetal bovine serum (Sigma), 100 mM sodium pyruvate (Sigma), streptomycin (100 U/ml) and penicillin G (100 mg/ml) (Sigma).

Opti-MEM reduced-serum medium which is an improved Minimal Essential Medium (MEM) was supplemented with 5% FBS (Sigma), 100 mM sodium pyruvate (Sigma), streptomycin (100 U/ml) and penicillin (100 mg/ml) (Sigma).

DMEM–Dulbecco's Modified Eagle Medium (Life Technologies) was supplemented with 10% FBS (Sigma), 100 mM sodium pyruvate (Sigma), streptomycin (100 U/ml) and penicillin G (100 mg/ml) (Sigma)

2.6.4. Lipopolysaccharide

Lipopolysaccharide (LPS) from *Salmonella typhimurium* (Innaxon UK) was used and stored at 4°C. A 100 µg/ml concentration of LPS was prepared using sterile deionised double distilled water just before stimulating cells and discarded after the stimulation.

In order to identify the concentration of LPS which produces optimum activation of BV2 microglia, cells were stimulated with different concentrations of LPS (10, 50, 100, 250, 500 and 1000 ng/ml) followed by incubation for 24 hours. At the end of the incubation period, supernatant was collected. Nitrite assay was preformed to detect nitritic oxide production as an indicator for microglia activation. Results showed a concentration-dependent increase in nitrite production up to 100 ng/ml of LPS (Figure 2.4).

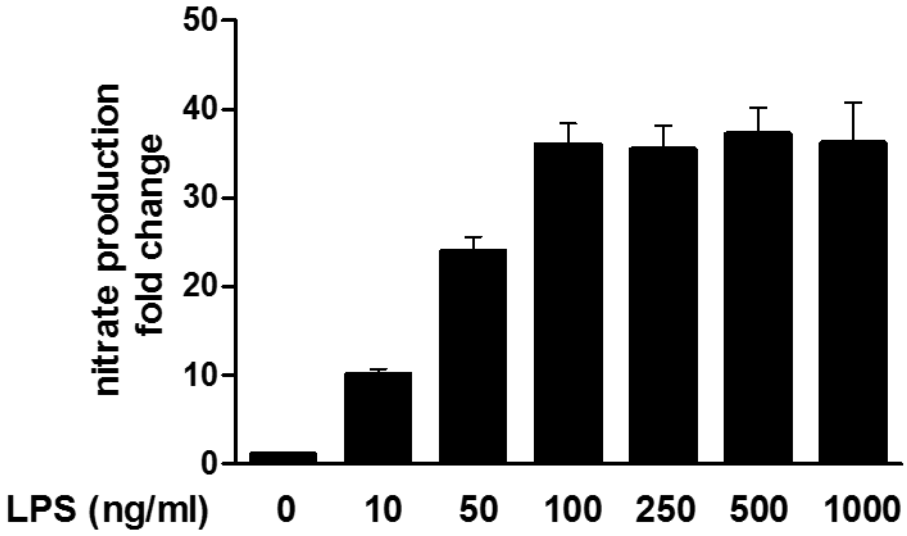


Figure 2.4 LPS concentration to stimulates BV2

2.6.5. Cell culture

2.6.5.1. BV2 cell culture

BV2 mouse microglia cell line ICLCATL03001 was purchased from Interlab Cell Line Collection Banca Biologicae Cell Factory, Italy. Cells were cultured in 75 mL filter-capped vented flasks using complete RPMI 1640 medium in a 5% CO_2 incubator at 37ºC. Culture medium was changed with fresh Complete RPMI 1640 medium every 48 hours. Confluent cells were washed with phosphate-buffered saline (PBS) and

trypsinised with 0.25% trypsin-EDTA solution. RPMI medium was added to terminate the action of trypsin. Thereafter, cells were collected in 50 ml sterile centrifuge tubes and centrifuged at 1200 rpm for 5 minutes. The medium was then aspirated, and cells were re-suspended in fresh complete medium. The suspended cells were counted and sub-cultured in a new flask or seeded out at a concentration of 2×10^5 cell/ml in various cell culture plates.

Viability experiments using MTT assay revealed that treatment of BV2 cells with formononetin (2.5, 5.0 and 10 µM) in the presence or the absence of LPS did not induce cytotoxicity figure 2.4.

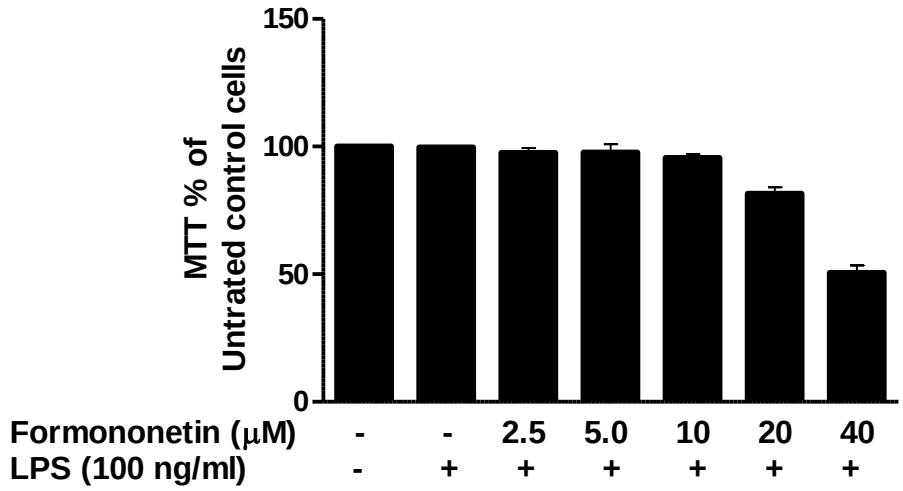


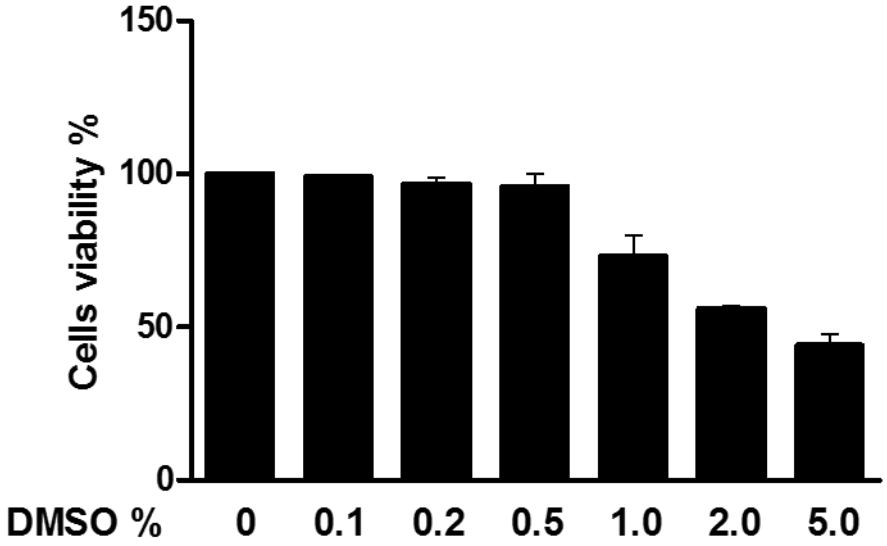
Figure 2.5 Cell viability assay for different concentrations of formononetin.

BV2 cells were treated with different concentrations of formononetin followed by stimulation with LPS (100 ng/ml) and incubated for 24 hours. After the incubation MTT assay was carried out. Results showed that formononetin at concentration of 2.5, 5 and 10 µM did not affect cell viability whereas at a concentration of 20 µM and 40 µM, cell viability decreased to 80% and ~50%, respectively.

In order to investigate the effect of DMSO (used as a vehicle in dissolving formononetin) on cell viability an MTT assay was carried out.

Confluent cultures of BV2 microglia cells in 96-well plates were treated with DMSO (0.1, 0.2, 0.5, 1, 2 and 5%), and incubated for 24 hrs, followed by MTT assay. The results showed that the concentration of DMSO from 0.1 to 0.5% did not affect the

viability of BV2 microglia. However, treatment with 1% of DMSO produced toxicity to microglia (Figure 2.6).



2.6 MTT assay for BV2 cells toxicity with DMSO.

2.6.5.2. Mouse hippocampal neuronal cells (HT22) cell culture

HT22 neuronal cells were obtained from Dr-Jeff Davis through the (Salk instite for biological studies, California,USA). These cells were derived from HT4 cells (Davis & Maher, 1994). HT22 cells were cultured in DMEM supplemented with 10 % FBS, 100 U/ml penicillin and 100 mg/ml streptomycin in a 5 % CO2 incubator at 37 ºC.

2.6.6. MTT assay for cell viability

The MTT assay measures the reduction of yellow 3-(4, 5-dimethythiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) in the mitochondria. The yellow colour of MTT solution is reduced to an insoluble coloured (dark purple) formazan product (Figure 2.7). Cells are solubilised with an organic solvent (e.g., DMSO) and the released, solubilised formazan reagent is measured spectrophotometrically. Since the reduction of MTT can only occur in metabolically active cells , the level of activity is a measure of the viability of the cells. MTT viability assay was conducted to confirm that the different concentrations of formononetin used in the whole study were not reducing viability of the cells, indicating that any observed reduction in signals was not as a result of reduced cell numbers (Mosmann, 1983).

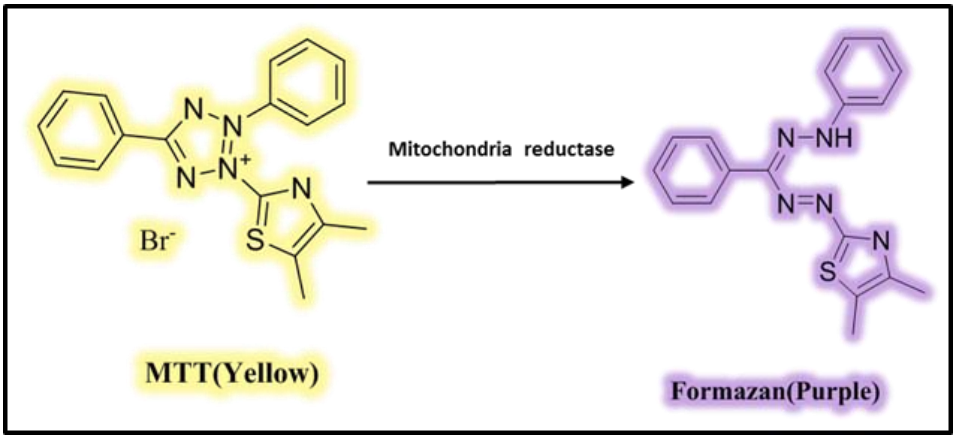


Figure 2.7 The principle of MTT assay, the water-soluble yellow MTT reduced to purple insoluble formazan can be solubilised in DMSO. (Abe et al., 2000).

BV2 cells were cultured in 96 well plate for 48 hours. This was followed by medium change to serum-free RPMI medium. Cells were then incubated with or without LPS (100 ng/ml) in the absence or presence of formononetin (2.5, 5 and 10 µM) for 24 hours. The medium was replaced with addition of 200 µl of MTT solution (0.5 mg/ml; Sigma) to each well. The plate was then incubated for 4 hours at 37ºC. After incubation, 200 µl of medium was removed from each well without disturbing cells clusters. Therafter 150 µl of DMSO solution was added to each well to dissolve formazan crystals. After all the crystals had dissolved, absorbance was read at 540 nm with a microplate reader (Tecan F50).

2.6.7. Determination of nitrite production (Griess assay)

The principle of the assay is based on the conversion of sulfanilic acid to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to N-(1-naphthyl) ethylenediamine, forming an azo dye (Figure 2.8) that can be quantified through colorimetry.

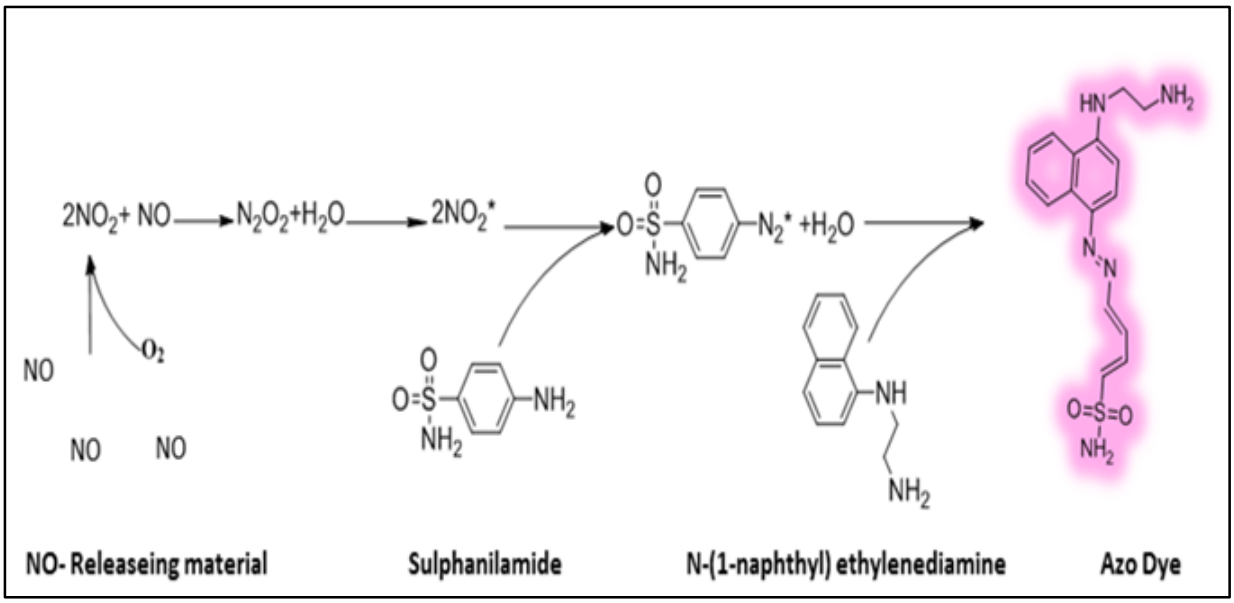


Figure 2.8 Principle of the Griess assay

The reaction of nitrite (NO2−) with Griess reagents forms an azo dye that is easily detected spectrophotometrically (Coneski et al., 2012).

BV2 cells were seeded out in a 96-well plate at a density of 2 x 10^5 cells/ml for 48 hours. Cells were then stimulated with LPS (100 ng/ml) in the absence or presence of formononetin (2.5, 5 and 10 µM) for 24 hours. Cell supernatants were collected and centrifuged for 5 minutes at 1500 rpm. Nitrite concentration in the culture medium was measured using Griess assay kit (Promega). Culture supernatants (50 µl) were added to a 96-well microtest plate. Thereafter, 50 µl of sulphanilamide added to the plate and incubated in dark for 10 minutes. Then, 50 µl of N-(1-naphthyl) ethylenediamine (NED) was added and incubated for 10 minutes. Absorbance was read at 540 nm in a microplate reader (Infinite F50, Tecan) and calculated against a sodium nitrite standard curve.

2.6.8. Enzyme-linked immunosorbent assay (ELISA)

BV2 cells were cultured in an incubator at 37ºC with 5% CO_2. The cell density was 2 x 10^5 cells/ml. Thereafter, the cells were pre-treated with formononetin (2.5-10 µM) for 30 minutes followed by stimulation with LPS (100 ng/ml) for a further 24 hours. Then, culture supernatants were collected and centrifuged for 5 minutes at 1500 rpm. The concentration of TNFα, IL-6, and IL-1β were measured using ELISA kits (Biolegend), according to the manufacturer's instructions.

Firstly, 96-well microplate was pre-coated by adding 100 µl of 1:200 diluted capture antibody to each well and incubated overnight at 4ºC. This was followed by adding 200 µl of blocking buffer for 1 hour at room temperature. After blocking, 100 µl of standard and samples were added in triplicates and incubated at room temperature for 2 hours Then detection antibody was added to the plate for 1 hour, followed by addition of 100 µl of an Avidin-HRP solution to each well for 30 minutes. Thereafter, 100 µl of TMB substrate was added followed by incubation in the dark for 15 minutes. Finally, the reaction was terminated by the addition of 100 µl of stop solution (0.1 M sulphuric acid) to each well. Absorbance was measured at 450 nm with a Tecan Infinite F50 microplate reader. The concentration of cytokines in samples was calculated from a standard curve. At the end of each step up to stopping the reaction, the plate was washed for 4 times using washing buffer.

2.6.9. Prostaglandin E2 (PGE2) detection enzyme immunoassay (EIA)

Enzyme immunoassay (EIA) is a reliable method for measuring levels of PGE_2 in a culture medium. PGE_2 EIA test kit operates by competition between the enzyme conjugate and the PGE_2 in the sample for a limited number of binding sites on the antibody-coated plate. This approach allows for detection of low concentrations of PGE_2 in a culture medium.

BV2 cells were cultured at a density of 2 x 10^5 cells/ml in 5% CO_2 incubator at 37ºC. Thereafter, cells were treated with formononetin (2.5-10 µM) 30 minutes prior to stimulation with LPS (100 ng/ml) for further 24 hours. Levels of PGE_2 in culture supernatants were measured using an EIA kit (Arbor Assays, Ann Arbor, Michigan, USA). This was done by adding 50 µl of PGE_2 standards and samples in triplicates to pre-coated 96-well plate with a PGE_2 capture antibody, followed by addition of of PGE_2 conjugate (25 µl) and PGE_2 antibody (25 µl). The plate was incubated at room temperature for 2 hours. Thereafter, plate was washed four times using washing buffer and100 µl of TMB substrate added to each well and incubated in dark for 30 minutes. The reaction then was terminated using a stop solution. Absorbance was measured in a microplate reader (Infinite F50, Tecan) at a wavelength of 450 nm. The concentration of PGE_2 was calculated comparing to the standard curve.

2.6.10. Cell Lysis and Protein Extraction

Cell lysis is the first step in cell fractionation and protein extraction and purification. As such, cell lysis opens the door to a myriad of proteomics research methods.

2.6.10.1. Preparation of whole cell extracts

Cell lysis buffer is used to lyse cells under non-denaturing conditions to study protein levels in the cell. Whole cell lysates were prepared by washing cells with ice-cold PBS, followed by addition of 20µl cell lysis radioimmunoprecipitation assay buffer (RIPA) (Cell Signaling) which contains 1 mM Phenylmethylsulfonyl fluoride (PMSF) (Sigma). The cells were incubated on ice for 10 minutes and sonicated for 1 minute followed by cold centrifugation for 15 minutes at 13500 rpm. The resulting supernatants were collected, quantified and stored at -80 ºC.

2.6.10.2. Protein quantitation

Whole cell extract was diluted with de-ionised double distilled water. Then, 5 µl of protein samples and standards were added to 96-well plate in duplicate. This was followed by adding 250 µl of Coomassie reagent, and incubation at room temperature for 10 minutes. Absorbance was measured at 540 nm and protein concentration was calculated from a protein standard curve.

2.6.11. Immunoblotting

Approximately 20 µg of protein were denatured by heating with 5 µl of lithium dodecyl sulfate (Invitrogen) and 2µl of 500 mM dithiothreitol (DTT) (Invitrogen) at 70ºC for 10 minutes. This was followed by electrophoresis on 4-12% Bis-Tris pre-cast polyacrylamide gels (Invitrogen) at a voltage of 200V for 35 minutes in a NuPAGE SDS running buffer (Fisher Scientific). Separated proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore) using transfer buffer (Fisher Scientific) for 2 hours at 25V. After transferring the protein, membranes were incubated in blocking buffer non-fat dry milk (Santa Cruz) for 1 hour at room temperature and washed three times for 10 minutes each in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and 1% BSA, and incubated with the primary antibody (Table 2.1) which was diluted in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) overnight at 4°C . Proteins were detected by incubation

with Alexa Fluor 680 goat anti-rabbit secondary antibody (1:10000; Life Technologies) at room temperature for 1 hour. Detection was done using a LICOR Odyssey Imager. All western blot experiments were carried out at least three times.

Antibody	supplier	Host	Type	Dilution
Anti-COX-2	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:500**
Anti-iNOS	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:500**
Anti-MAP2	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:500**
Anti-actin	**Sigma Aldrrich**	**Rabbit**	**Polyclonal**	**1:1000**

Table 2.1 Primary antibodies used with details of resources and dilution factor used.

2.6.12. Neurotoxicity of BV2 conditioned medium

Conditioned medium (CM) is a medium that cells have been cultured in for a period to allow these cells to release/ secrete proteins, cytokines and chemicals .Condition media is used to show that specific cell type produces certain mediators that can affect other cells phenotype.

Conditioned medium-induced neurotoxicity has been used to investigate the effect of formononetin in neurotoxicity induced by LPS activated BV2 cells. BV2 microglia cells were pre-treated with formononetin (2.5–10 µM) and then stimulated with LPS (1 µg/ml) for 24 hours. This was followed by centrifugation to obtain cell-free supernatants. The HT-22 hippocampal neuronal cells were serum-starved for 3 hours and then treated with BV2 microglial cell-conditioned medium, followed by 24-hour incubation. At the end of the experiment, 190 µl MTT solution (0.5 mg/ml) was added to each well containing HT22 neurons and incubated at 37 ºC for 4 hours. Then, 190 µl of the medium was removed and 150 µl of DMSO solvent was added to wells to dissolve the formazan crystals. The plate was shaken for a few seconds to achieve through mixing followed by measurement of absorbance at 540 nm with a microplate reader. BV2 microglia culture media were also collected and levels of TNFα, NO, and PGE_2 measured in the samples.

2.6.13. Immunofluorescence

BV2 cells were seeded at a density of 2 x 10^5 cell/ml in a 24-well plate. At confluence, cells were pre-treated with formononetin (10 µM) for 30 minutes followed by stimulation with LPS (100 ng/ml) for 24 hours, followed by collecting the supernatants and centrifugation. HT22 hippocampal neuronal cells were treated with BV2 microglia

cells-conditioned medium , followed by 24 hour incubation After incubation, cells were fixed with ice-cold 100% methanol at −20°C for 15 minutes and washed three times with phosphate buffer saline (PBS) for 5 minutes. This was followed by blocking non-specific binding by incubating cells in 5% BSA blocking solution (containing 10% horse serum in 1X TBS-T) for 60 minutes at room temperature. Thereafter, the cells were incubated with anti-MAP2 antibody (Santa Cruz diluted at 1:100 using 5% BSA blocking solution) overnight at 4°C. Following the incubation, cells were washed three times with PBS and incubated for 2 hours in the dark with Alexa Fluor 488-conjugated donkey anti-rabbit IgG secondary antibody (Life Technologies; 1:500) using 5% BSA blocking buffer Cells were then washed with PBS and counterstained with 4', 6-diamidino-2-phenylindole DAPI (Invitrogen) at concentration of 100 µM for three minutes. Cells were rinsed with PBS, the excess buffer was removed and gold antifade reagent (Invitrogen) was added. Fluorescence images were acquired using EVOS® FLoid® cell imaging station.

2.6.14. Statistical Analysis

All experiments were performed at least three times and in triplicates and the statistical analysis was preformed using one-way analysis of variance (ANOVA). ANOVA allows for comparison of the mean values between all the groups. Analysis was followed by the post-hoc Student-Newman-Keuls test with multiple comparisons. Calculation of statistical analysis were done with Graph Pad Prism software version 5 (San Diego, US). Differences were considered significant at $p<0.05$. Immunofluorescence imaging results are representative of those obtained from three independent experiments.

2.7. Results

2.7.1. Formononetin did not affect BV2 cell viability

In order to determine whether formononetin affected the viability of BV2 cells, MTT assay was carried out after incubating the cells with the compound for 24 hours. Results show that there was no significant difference in the viability of cells treated with formononetin (2.5, 5 and 10 μM) when compared with control (untreated) cells (Figure 2.7).

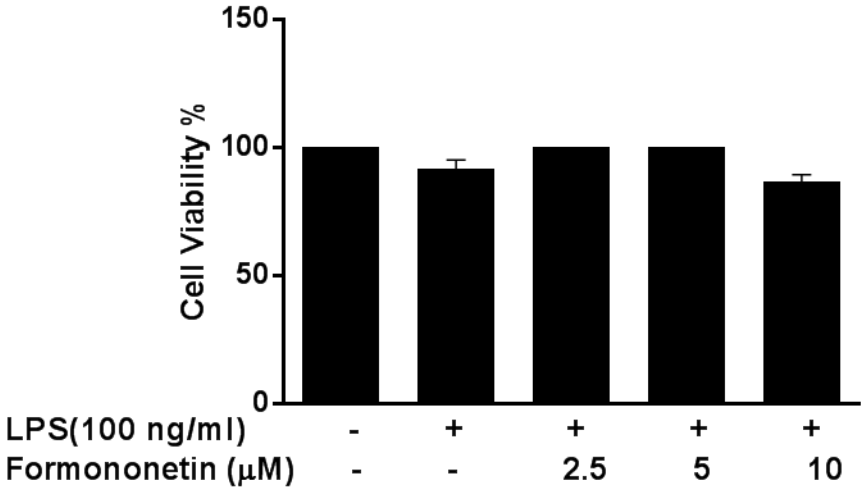


Figure 2.9 Effect of formononetin on cell viability in LPS-activated BV2 microglia.

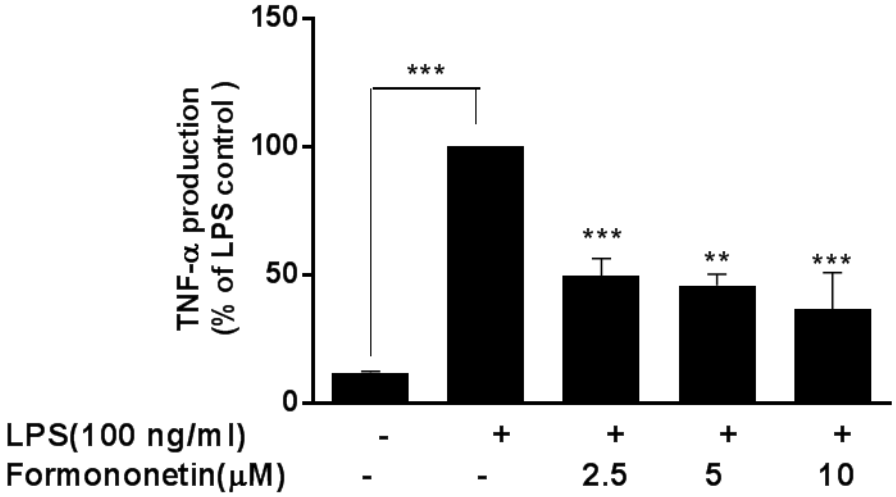
Pre-treatment with formononetin (2.5, 5 and10 μM) did not affect the viability of BV2 microglia stimulated with LPS. Cells were stimulated LPS (100 ng/ml) in the presence or absence of formononetin (2.5, 5 and10 μM) for 24 h. At the end of the incubation period, MTT assay was carried out on cells.

2.7.2. Formononetin reduced the production of pro-inflammatory cytokines TNFα, IL-6 and IL-1β in BV2 microglia stimulated with LPS

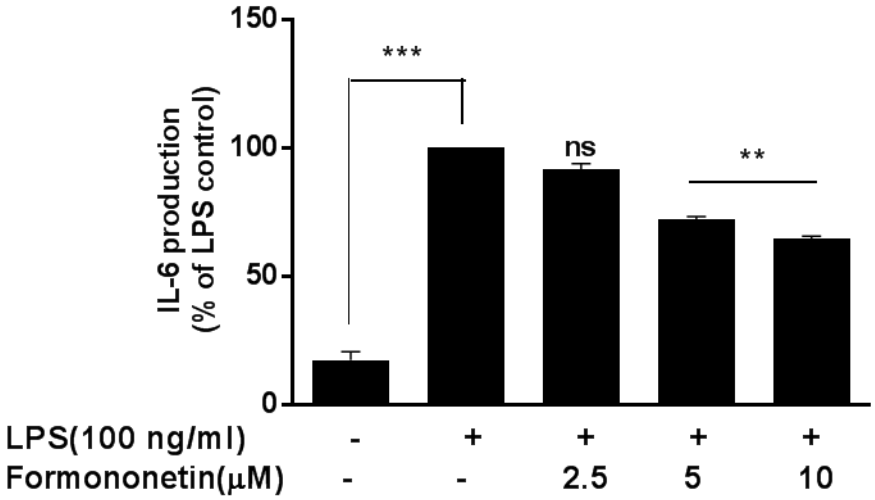
The release of pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β is now known to be a major process in neuroinflammation. Consequently, an investigation was carried out to determine if pre-treatment with formononetin would suppress the release of these cytokines in LPS-activated BV2 microglia. Stimulation of the cells with LPS (100 ng/ml) resulted in a significant increase in the secretion of TNFα, IL-6 and IL-1β in comparison with unstimulated BV2 cells (Figure 2.8). However, when cells were

pre-treated with formononetin (2.5, 5 and 10 µM), there was a significant reduction ($p<0.001$) in TNFα production, in comparison with LPS control (Figure 2.8a). Similarly, increased levels of IL-6 secreted into culture supernatants was significantly ($p<0.05$) reduced by 5 and 10 µM formononetin (Figure 2.8b). Also results in (Figure 2.8c) showed significant reduction of the production of IL-1β ($p<0.001$) compared with LPS control.

a)



b)



c)

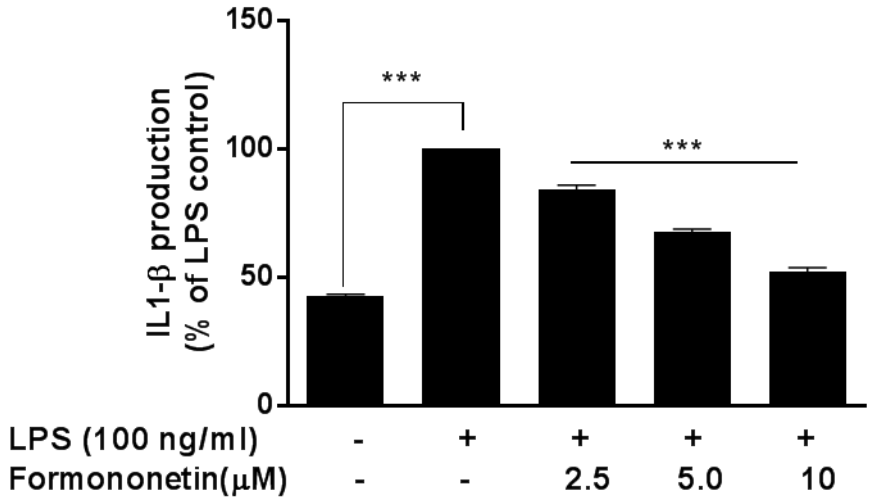


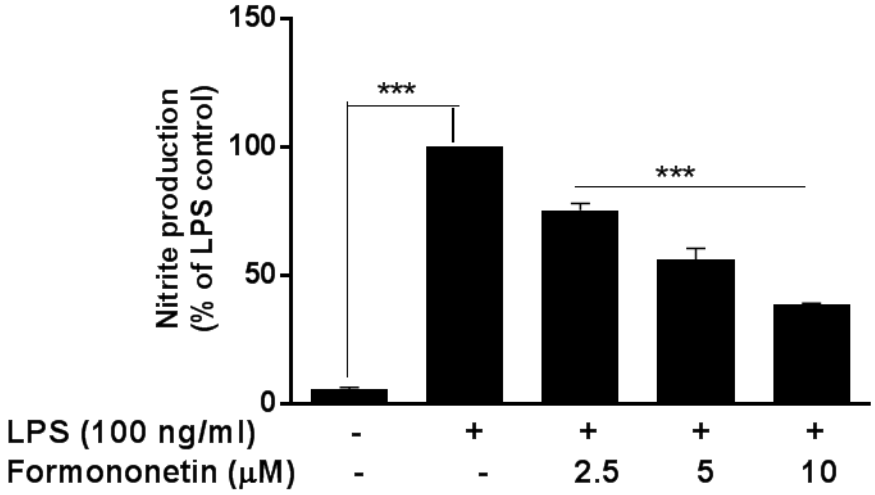
Figure 2.10 Effect of formononetin on TNFα , IL-6 and IL-1β production in LPS-activated BV2 microglia.

Formononetin reduced TNFα (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 and10 μM) for 24 hours. At the end of the incubation period, supernatants were collected for ELISA measurements.

2.7.3. Formononetin inhibited nitrite production through reduction of iNOS protein in LPS-activated BV2 microglia

The effect of formononetin on nitrite production in LPS-stimulated BV2 microglia was evaluated. The compound dose-dependently suppressed the release of nitrite ($p<0.001$) following stimulation with LPS (Figure 2.9a). Further investigations using western blotting showed that formononetin (2.5, 5 and 10 μM) produced significant ($p<0.001$) suppression of elevated levels of iNOS protein following LPS stimulation (Figure 2.7b). These results suggest that formononetin suppressed NO production in LPS-activated BV2 microglia by reducing the levels of iNOS protein.

a)



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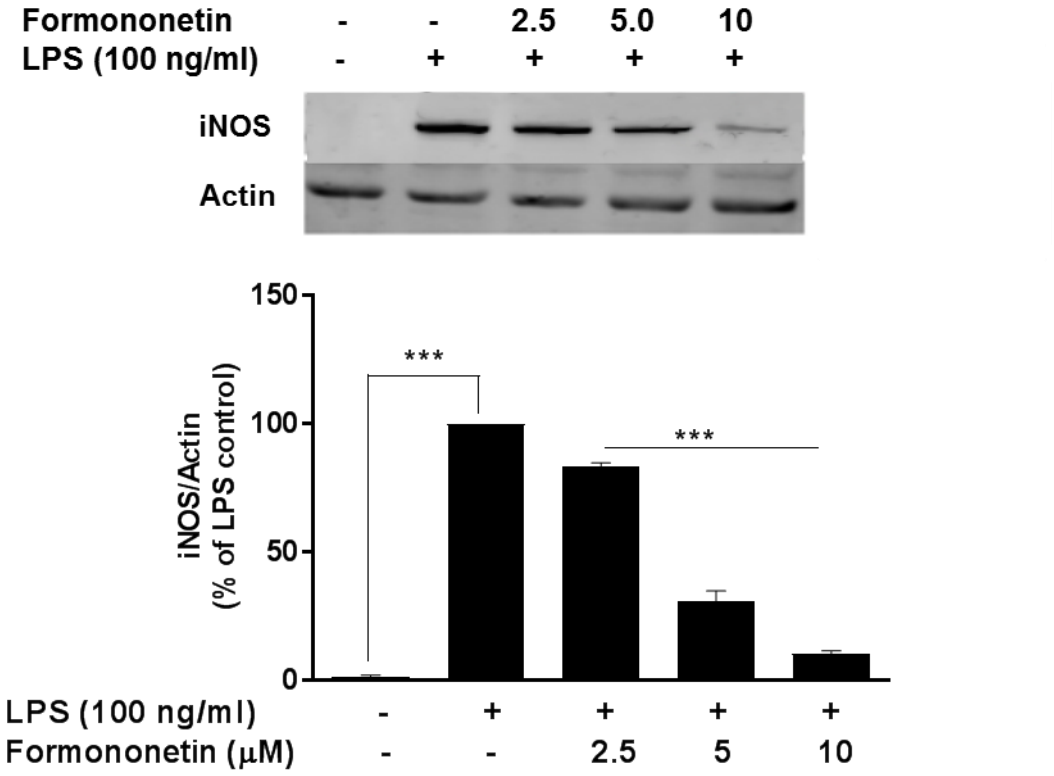


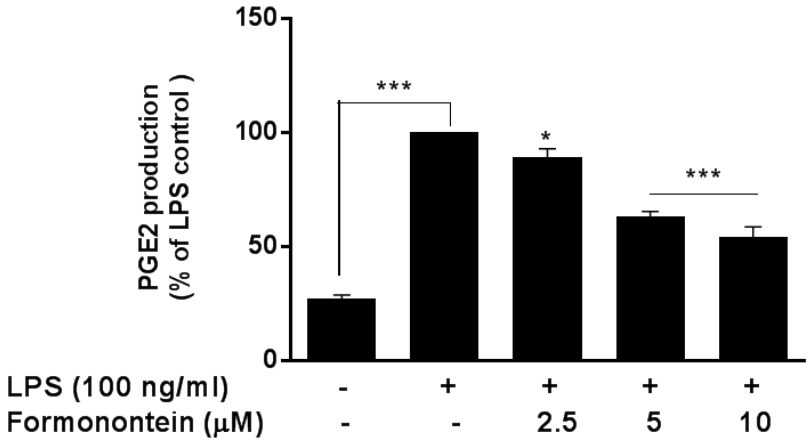
Figure 2.11 Effect of formononetin on nitric oxide (NO) and inducible nitric oxide (iNOS) protein expression in BV2 cells stimulated with LPS.

Formononetin inhibited nitric oxide (NO) release (a) and inducible nitric oxide (iNOS) protein expression (b) 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 hours followed by preforming nitrite assay and iNOS western blot

2.7.4. Formononetin reduced PGE_2 production by reducing levels of COX-2 protein in LPS-stimulated BV2 microglia

The effects of pre-treatment with formononetin (2.5, 5 and10 μM) were tested on PGE_2 production in BV2 microglia which were stimulated with LPS. As shown in Figure 2.10a, stimulation of BV2 cells produced marked elevation of PGE_2 compared to cells, which were not stimulated. However, in the presence of formononetin (5 and10 μM) there was significant ($p<0.001$) and concentration-dependent reduction in PGE_2 production. Following results showing effects of formononetin on PGE2 production, further experiments were performed to determine whether its actions were mediated by the COX-2 enzyme. Results in Figure 2.10b show an increase in the levels of COX-2 protein in LPS stimulated cells, when compared with unstimulated cells. Pre-treatment with formononetin (10 μM) produced significant ($p<0.001$) reduction in LPS-induced elevation in COX-2 protein expression. These results suggest that formononetin inhibits LPS-induced PGE_2 production by suppressing the levels of COX-2 protein in BV2 microglia.

a)



b)

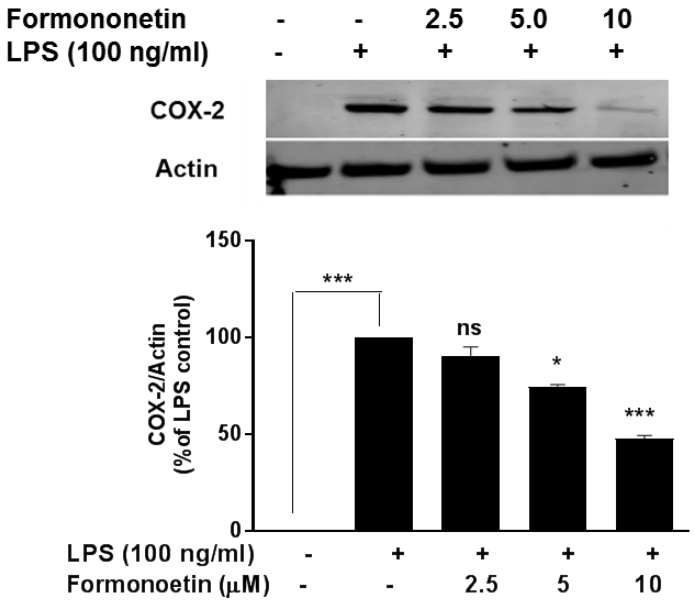


Figure 2.12 Effect of formononetin on PGE_2 production and COX-2 protein levels in BV2 microglia activated with LPS.

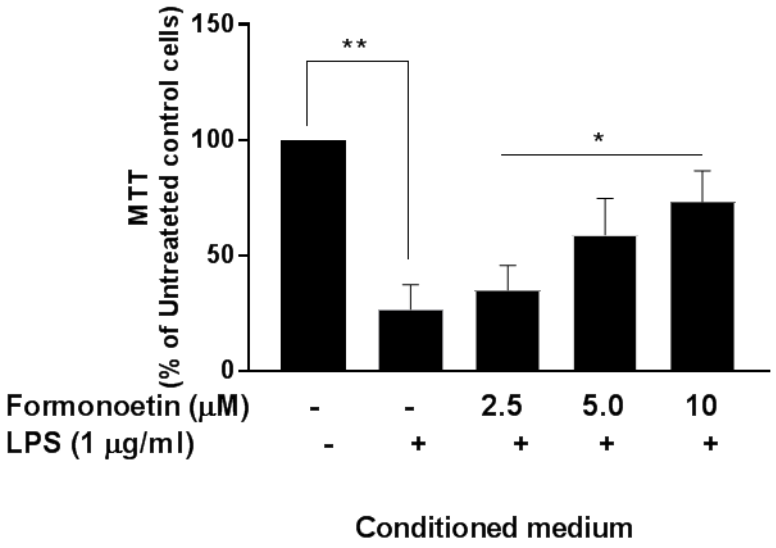
Formononetin attenuated PGE_2 production (a), COX-2 protein expression (b), in LPS-activated BV2 microglia. Microglia were incubated in a medium containing formononetin (2.5, 5 and10 μM) for 30 min and then activated with LPS (100 ng/ml) for 24 h. Formononetin prevents neuroinflammation-mediated HT22 neuronal death by regulating microglial activation.

The neuroprotective effect of formononetin in BV2 microglia/HT22 hippocampal neuron co-culture was investigated. BV2 microglial cells were pre-treated with formononetin (2.5–10 μM) and then stimulated with LPS (1 μg/ml) for 24 hours. HT22 cells were then exposed to conditioned media from the stimulated BV2 microglia for a further 24 hours. At the end of the experiment, viability of neuronal cells (a measure of neurotoxicity) was determined using the MTT assay for cell viability. Also, levels of MAP2 protein was detected by immunofluorescence and western blot.

MTT results showed that formononetin significantly ($p<0.01$) increased HT-22 cell viability in a dose dependant manner compared to LPS control (Figure 2.11a), Furthermore, western blotting was performed for MAP2 protein. MAP2 belongs to a family of proteins known as the microtubule associated proteins and is an excellent marker for neuronal cells. Results show a significant increase ($p \leq 0.001$) in MAP2 expression (Figure 2.11b). These observations were confirmed by double-

fluorescence imaging which showed an increasing in MAP2 expression in HT22 cells which were pre-treated with different concentrations of formononetin (Figure 2.11c).

a)



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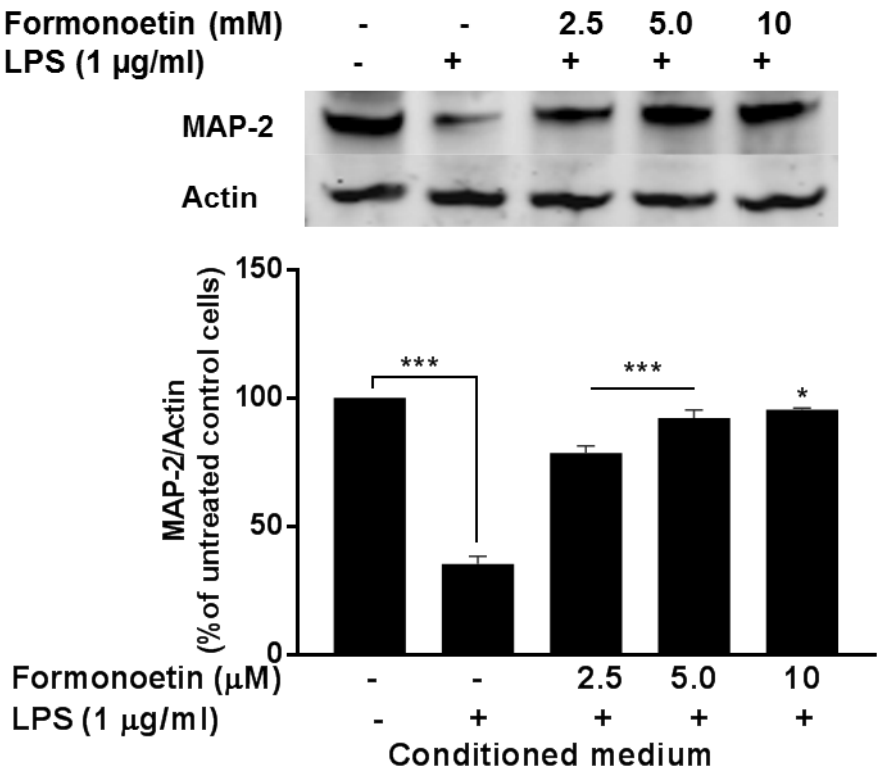

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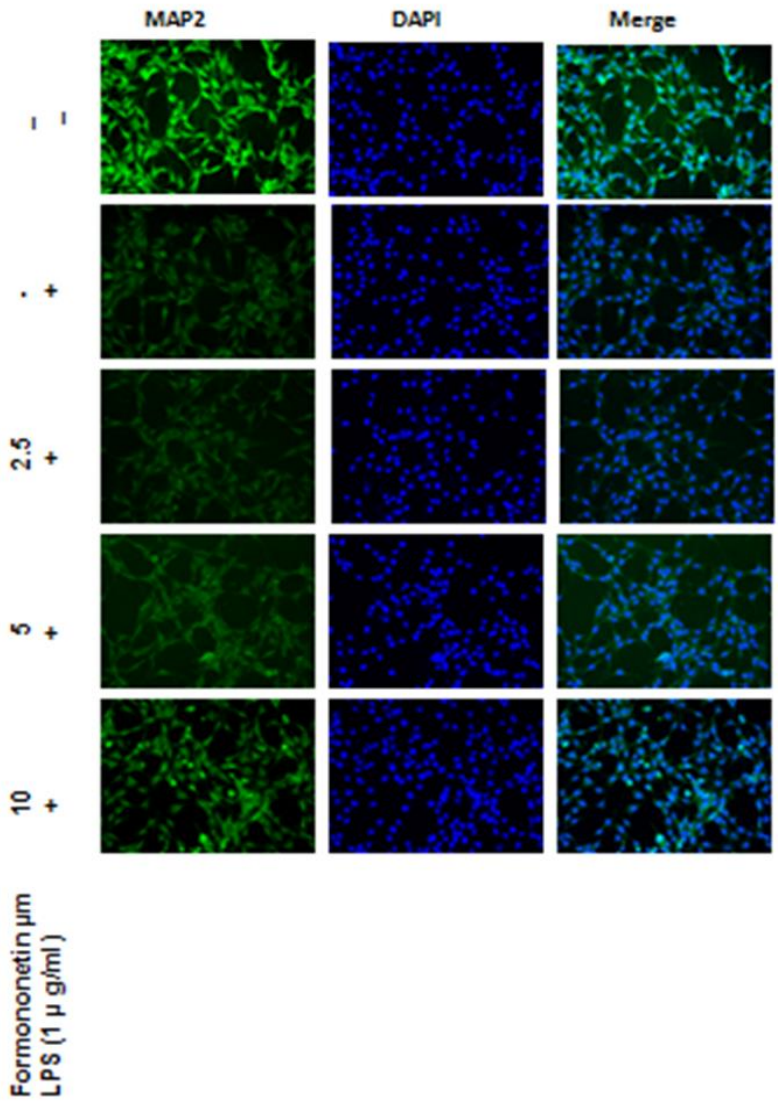


Figure 2.13 Effects of formononetin on the microglia-mediated neurotoxicity.

Mouse hippocampal HT22 cells were treated with conditioned medium (CM) from BV2 microglia exposed to LPS (1 µg/ml) for 24 h with or without formononetin pre-treatment. After 24-hour incubation, the cell viability of HT22 cells was assessed by MTT assay. LPS (1 µg/ml)-treated BV2 cells CM reduces the viability of HT22 cells. However, the decrease in cell viability was ameliorated by formononetin in a dose dependent manner(a). HT22 neuronal cultures were treated for 24 h with cell-free supernatant from LPS-stimulated BV2 in the absence or presence of formononetin. Lysates were collected and analysed for levels of MAP2 protein using immunoblotting (b),followed by immunofluorescence imaging for MAP2 protein (c).Formononetin produced concentration-dependent increase in MAP2 protein in HT22 neuronal cells.

2.8. Discussion

Several lines of evidence from several studies indicate that pro-inflammatory cytokines, notably TNF-α, IL- 6 and IL-1β, participate in the early development of inflammation, and they play a crucial role in neuroinflammation. (Rubio-Perez et al., 2012). Modulation of the pro-inflammatory cytokines production showed to prevent neurotoxicity (Wang & Tan, 2015) Pro-inflammatory cytokines have therefore appropriately received considerable attention as therapeutic targets in neuroinflammation (Linker et al., 2008; Lehnardt, 2010; Kim & Leonard, 2016).

In the present study, induction of neuroinflammation with LPS in BV2 microglia resulted in a significant increase in the levels of TNF-α, IL-6 and IL-1β, in comparison with the control. However, pre-treatment with formononetin reduced LPS-induced elevation in the levels of these pro-inflammatory cytokines.These results provide the first evidence suggesting that formononetin inhibits neuroinflammation in BV2 microglia. These results are also consistent with previous studies which showed that formononetin blocks allergic inflammation via suppression of pro‑inflammatory cytokine production (Xu et al., 2017).

One of the most critical molecules released by activated microglia is nitric oxide (NO). NO produces physiological effects in neuromodulation and neurotransmission in the brain. However, excessive production of NO by microglia induces neuroinflammation and produces neuronal cell injury and death (Ghasemi et al., 2014). Nitric oxide (NO) is produced by the action of nitric oxide synthase (iNOS), which catalyses the reaction of arginine with molecular oxygen to form citrulline and NO (Ljubisavljevic et al., 2014).

This study demonstrated that in the presence of formononetin, there was a reduction in nitrite production caused by LPS stimulation of BV2 microglia. It was further shown that suppression of nitrite production by formononetin was through reduction of inducible nitric oxide synthase (iNOS). Both observations provided further evidence on the inhibition of neuroinflammation by formononetin, and confirmed recent reports showing iNOS-mediated inhibition of NO production form N9 microglia cells (Zhou et al., 2017; Lai et al., 2017).

The role of COX-2-mediated neurotoxicity in neurodegenerative disorders has been well explored (Feng et al., 2002). One of the key downstream products of COX-2 is

prostaglandin E2 (PGE2).COX-2 is upregulated during neuroinflammation in both the neurons and reactive microglia (Zarghi et al., 2012). PGE_2 levels were found to be increased in the CSF of AD patients (Sil et al., 2016). Thus, PGE2 is considered as an important marker of neuroinflammation. Moreover,PGE_2 mediated release of TNF-α, IL-1β, and nitrite may result in neuronal death (Müller et al., 2004). In this study it was shown that formononetin suppressed COX-2 protein expression which might explain its ability to reduce PGE_2 production in LPS-activated BV2 microglia.

A number of studies reported that phytoestrogens like genistein and equol inhibited the expression of COX-2 and reduced PGE_2 levels in microglia cells (Hertrampf et al., 2005; Subedi et al., 2017). It has also been shown that formononetin suppresses COX-2 and PGE_2 in (LPS)-stimulated mouse macrophage RAW 264.7 cells (Lai et al., 2012). *In vivo* studies revealed that mRNA levels of COX-2 in the brain of the TBI rat was significantly down-regulated with formononetin (Li et al., 2014). These outcomes show that formononetin appears to exert modulatory activities on COX-2/PGE_2 in diverse models of inflammation. These results are also consistent with the effects of the compound shown in the current investigation.

In the CNS, chronic microglial activation has been linked to a persistent, continuous production of pro-inflammatory cytokines, interleukins and chemokines. The production of excessive amounts of these neurotoxic factors released by activated microglia has been shown to affect adjacent neurons (Lull et al., 2010). Neuronal cultures treated with conditioned medium from microglia cells that were stimulated with inflammatory agents such as LPS are a useful tool to study neuroinflammation *in vitro*(Haenseler et al., 2017).

Encouraged by results showing that formononetin treatment resulted in a reduction in elevated levels of pro-inflammatory mediators in BV2 microglia following LPS stimulation, neuroinflammation-induced neurotoxicity experiments were carried out using conditioned medium from LPS-activated BV2 microglia. Results of cell viability experiments revealed neuroinflammation-induced neurotoxicity in HT22 hippocampal neurons exposed to conditioned medium from BV2 cells that were stimulated with LPS. Interestingly, neurotoxicity was reduced when BV2 cells were treated with formononetin prior to LPS stimulation, suggesting that this compound is able to prevent neuronal damage and death caused by excessive release of neurotoxic factors from microglia cells.

These observations were confirmed by detecting MAP2 protein expression following exposure of HT22 neurons to conditioned medium. It was shown that conditioned medium that was obtained from LPS-stimulated BV2 microglia cells reduced the expression of MAP2 protein in HT22 neurons, compared to unstimulated control cells, a phenomenon that was reversed in the presence for formononetin. MAP2 is a member of the microtubule-associated family which assemble neuronal microtubules (Ankam et al., 2015). MAP2 is a specific neuronal marker and is known to be highly expressed in healthy neuronal cells.

This study has demonstrated a neuroprotective activity for formononetin. This outcome is consistent with another study showing that formononetin protected dopaminergic neurons against LPS-induced injury through inhibition of microglia activation and pro-inflammatory factors (Chen et al., 2008)These observations are significant as several studies revealed that neuroinflammation is linked with the induction of neuronal death through the release of excessive amounts of pro-inflammatory cytokines (Zhao et al., 2013; de Pablos et al., 2014).

In summary, this study has provided evidence showing that formononetin produces anti-inflammatory effects in LPS-activated BV2 microglia by targeting NO/iNOS and PGE2/COX2 production as well as reducing the production of pro-inflammatory cytokines TNFα, IL-6 and IL-1β. Also it appears that inhibition of neuroinflammation by formononetin may be partially responsible for its neuroprotective activity in neuroinflammation-induced toxicity to HT22 hippocampal neurons. It would be interesting to establish in future if formononetin could modulate other forms of neurotoxicity..

Chapter Three

Cellular signalling involving Nuclear Factor-kappa B (NF-κB) and the Mitogen Activated Protein Kinases (MAPKs) are the molecular targets of anti-inflammatory activity of formononetin in LPS activated BV2 microglial

3.1. Introduction

The activation of glial cells is accompanied by an increase in the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, NO and prostaglandin E2. This production is promoted by activation of inflammatory signalling pathways such as NF-κB and MAPK, which are triggered by the toll-like receptor signalling (Walter et al., 2007; Chen et al., 2012). The mammalian nuclear factor- kappa B (NF-κB) and MAPK regulate a number of important processes, including neuroinflammation and immune responses, cell growth and survival. Moreover, NF-κB and MAPK activities contribute to the pathology of several human diseases, including many cancers and chronic inflammatory diseases by controlling the production of the pro-inflammatory cytokines. In the previous chapter it was shown that formononetin inhibit the production of the pro-inflammatory cytokines IL-6, TNF-α and Il-1β and reduced the expression of iNOS and COX-2 which resulted in an inhibition of NO and PGE2 production. In this chapter, the roles of NF-κB and MAPK pathways as possible molecular targets for anti-inflammatory activity of formononetin are described.

3.1.1. Nuclear Factor-kappa B

Nuclear factor kappa beta (NF-κB) is a transcription factor that plays important roles in the immune system (Portou et al., 2015; Serasanambati et al., 2016) by regulating several gene expressions of cytokines such as IL-6, IL1β ,TNFα, (iNOS (COX-2) and growth factors. NF-κB also plays a role in the development and the activity of a number of tissues including the central nervous system (Caviedes et al., 2017). Moreover, pathological dysregulation of NF-κB is linked to inflammatory and autoimmune diseases as well as cancer (Tafani et al., 2013; Farh et al., 2015).

NF-κB family consists of five related transcription factors: p50, p52, RelA (p65), c-Rel and RelB (Dev et al., 2010).There are two signalling pathways leading to the activation of NF-κB known as the canonical pathway (or classical) and the non-canonical pathway (or alternative pathway) (Gilmore, 2006) (Figure 3.1). The activation of IκB kinase (IKK) complex is the common step in both cascades. There is catalytic kinase subunit for IκB kinase (IKK) complex (IKKα and/or IKKβ) and the regulatory non-enzymatic scaffold protein NEMO (NF-kappa B essential modulator also known as IKKɣ) (Perkins, 2007).NF-κB transcription subunits gets activated by the

phosphorylation of IKK which is followed by phosphorylation and proteasomal degradation of IκB proteasome. Consequently NF-κB unbinds and then translocates into the nucleus to trigger the expression of target genes (Brasier, 2006).

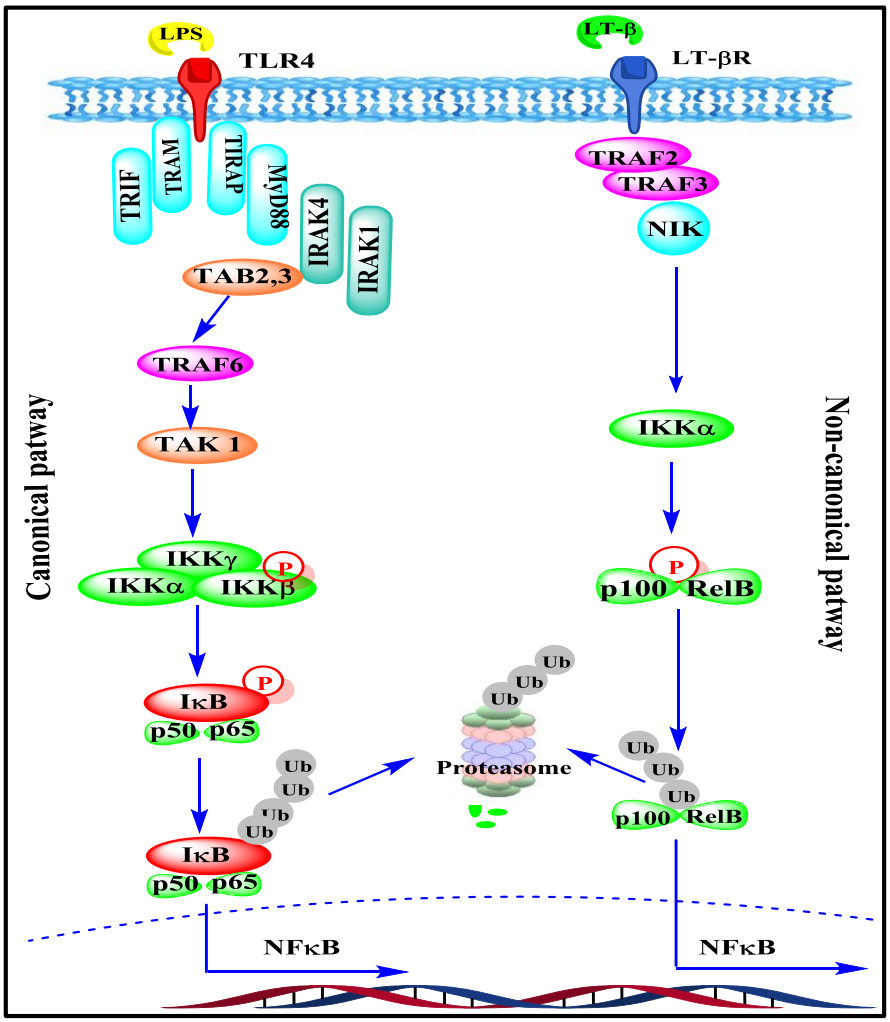


Figure 3.1 Canonical and noncanonical pathways of NF-κB activation.

The canonical NF-κB pathway (**left**) by signals such as TLR ligands (e.g. LPS) activates the IKKβ subunit. Phosphorylation of IκB proteins by IKKβ results in ubiquitination (Ub) of IκB and degradation. This releases NF-κB, which then enters the nucleus to regulate the transcription of inflammatory gene expression. The non-canonical pathway (**right**) requires NIK to activate IKKα, followed by phosphorylation of p100 (NF-κB2). Modified from (Oeckinghaus et al., 2011).

3.1.2. Mitogen-activated protein kinases (MAPK)

The mitogen-activated protein (MAPK) kinases are Ser/Thr kinases divided into tree subfamilies that include extracellular regulated kinase (ERK), c-Jun NH2-terminal kinase, also called stress activated protein kinases or SAPK (JNK) and p38 (Raman et al., 2007). The MAPKs are activated in response to different extracellular stimuli such as stress, growth factors , and pro-inflammatory cytokines (IL-1β, TNF-α) (Huang et al., 2010).

MAPKs pathway consists of several phosphorylation of proteins (Tatevossian et al., 2010). These initiate the phosphorylation of certain transcription factors such as NF-κB, signal transducers and activators of transcription protein (STAT) and nuclear factor of activated T cells (NFAT). Activation of these factors results in a cascade of signalling events that lead downstream to the activation of one or more of the MAP kinases (Figure 3.3). The involvement of multiple signalling proteins within a complex signalling network allows for regulation at various steps in the pathway and integration of signals from a diversity of stimuli (Cargnello et al., 2011).

The activation of MAPK is through two steps and has three protein modules: the MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK (Klip, 2009). The first step involves the phosphorylation of a kinase that has a dual specificity called MAP2K (or MAP/ERK kinase (MEK) or (MKK) by MAP3K. The second step is through phosphorylation of the MAPK by MKKS where MAPK is active. This phosphorylation is carried out by the addition of a phosphate group on Ser or Thr (Kyriakis et al., 2012). There are 12 MAPKs, 7 MKKS, and 14 MKK kinases were identified (Johnson et al., 2002; Morel et al., 2004) (Figure 3.2).

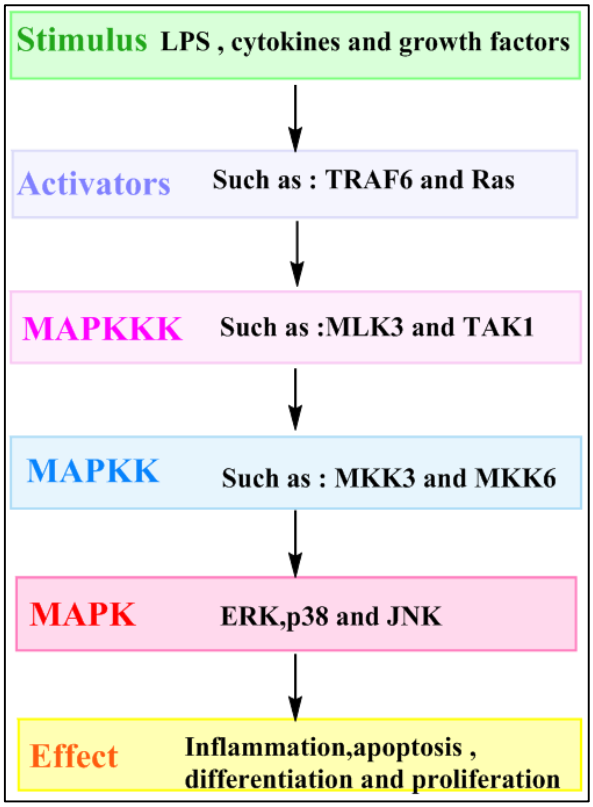


Figure 3.2 Mitogen-activated protein kinases (MAPK) cascade.

Modified from (Dzamko et al., 2014)

3.1.2.1. p38 MAPK

The protein p38 is a "sensor" of cell stress. It is made of four isoforms: p38-α -β -γ , and -δ which have 60% homology (Corrêa et al., 2012). In inflammatory cells, p38α form is the most expressed (Huang et al., 2010). p38 MAPK was identified for the first time as an activated kinase response to bacterial lipopolysaccharide (LPS) (Bode et al., 2012). The p38 MAPKs are activated by several extracellular stimuli such as cytokines (IL-1, IL-2, IL-7, IL-17, IL-18, TGF-β and TNF-α), and some pathogens or their products (LPS, *Staphylococcal peptidoglycan*, *Staphylococcal enterotoxin B*, *Echovirus 1* and *Herpes simplex virus 1)* (Ono et al., 2000). IL-1, TNF-α and LPS are the major inflammatory stimuli inducing p38 MAPKs (Bode et al., 2012). The p38 MAPK contributes significantly to neuroinflammation due to its role in the production of pro-inflammatory cytokines (Chan et al., 2017). Following stress stimulus, p38 activates the mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK-2) (Duraisamy et al., 2008). Taken together, it is hypothesised that suppression of the p38 MAPK activation may be a therapeutic strategy in neuroinflammation (Figure 3.3).

3.1.2.2. C-jun N-terminal kinase (JNK)

C-Jun N-terminal kinases (JNK) are kinases which respond to stress and are activated by various forms of triggers (Varfolomeev et al., 2004). JNK activation results in cell death through inflammation and apoptosis (Cao et al., 2004). Activation of JNK signalling results in pro-inflammatory cytokine production and cell death (Varfolomeev et al., 2004; Gao et al., 2005). JNK plays important roles in neuroinflammation (Tu et al., 2011), and is activated by stimuli such as pro-inflammatory cytokines (IL-1 and TNF-α) (Berenbaum, 2004). It has also been demonstrated to induce the expression of COX-2 (Geng et al., 1996; Hunot et al., 2004).

3.1.2.3. Extracellular signal-regulated kinase (ERK)

The first mammalian MAPKs identified was the extracellular signal-regulated kinases (ERK1/ERK2) (Calvo et al., 2010). The ERKs are activated, by pro-inflammatory stimuli including cytokines such as TNFα, and PAMPs, such as LPS, These mechanisms of ERK activation play an important role in innate immunity and inflammation (Potapovich et al., 2011). The ERK pathway plays a major role in cell proliferation.However there is relatively little information on the role of ERK in neuroinflammation (Wuertz et al., 2012). More recent reports have suggested the role of MAP3K tpl2/cot in ERK-mediated LPS-induced activation of macrophages. Tumour progression locus 2 (Tpl2) knockout mice displayed abrogated LPS-induced ERK activation and TNF-α release (Kawaguchi et al., 2011). ERK activation appears to modulate translocation of TNF-α mRNA from the nucleus to the cytoplasm rather than affecting transcription or stabilisation of TNF-α mRNA (Fan et al., 2010) (Figure 3.3).

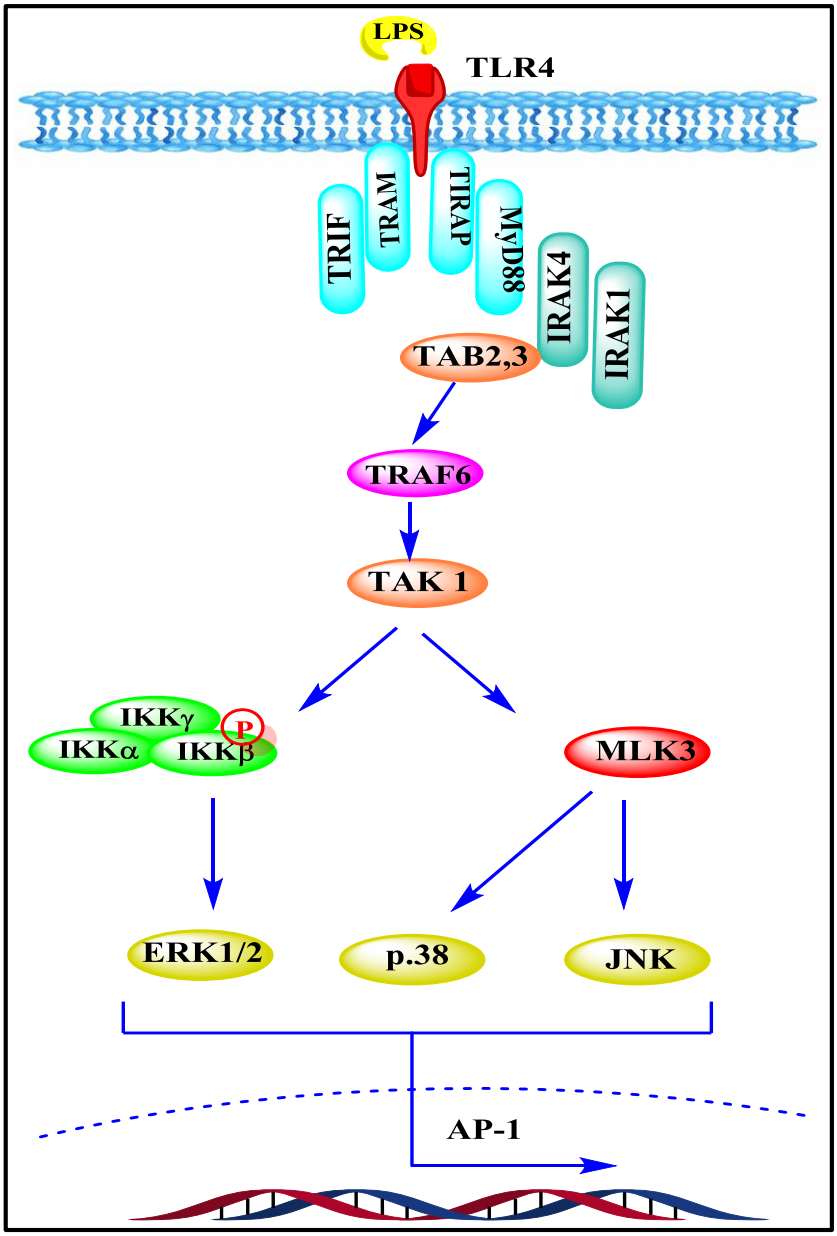


Figure 3.3 Simplified MAPK signaling pathways.

Overview of TLR-activated MAPK signalling pathway. As a result of LPS stimulation, activated signals go down from the TLR4 to the MAPK signalling pathways p.38 ,JNK and ERK1/2 . Modified from (Murshid et al., 2015)

In chapter two of this thesis, the anti-inflammatory effects of formononetin in LPS activated BV2 microglia was described. Furthermore, NF-κB and MAPK signalling pathways are known to be two of the most important signalling pathways responsible in neuroinflammation (Oh et al., 2013; He et al., 2016; Santa-Cecília et al., 2016). In addition, activation of both NF-κB and MAPK signalling pathways results marked

production of pro-inflammatory mediators (Awada et al., 2014; Lai et al., 2017).Therefore, the experiments described in this chapter was aimed at evaluating whether formononetin inhibits neuroinflammation through modulation of the NF-κB and MAPK signalling pathways.

3.2. Materials and methods

3.2.1. Cell culture

3.2.1.1. BV2 cell culture

This has been described in chapter two (section 2.6.5.1)

3.2.1.2. Human embryonic kidney (HEK293) cell culture

HEK293 cells obtained from (European Collection of Cell Cultures (ECACC) were grown in DMEM supplemented with 10% FBS and 2 mM glutamine. Cells were split 1:3 when they reached 85–90% confluence using trypsin/EDTA in PBS.

3.2.1.3. Preparation of whole cell extracts

This has been described in chapter two (section 2.6.10.1).

3.2.1.4. Preparation of nuclear extracts

Nuclear extracts were prepared using EpiSeeker nuclear extraction kit (Abcam), according to the manufacturer's instructions. Cells were washed with cold PBS. Thereafter, 20 µl of pre-extraction buffer was added to the cells and incubated on ice for 10 min. This was followed by centrifugation at 12000 rpm for 1 min. Then, 10 µl of nuclear extraction buffer was added to the resulting pellet and incubated on ice for 15 min, followed by centrifugation at 13500 rpm for 15 min at 4C^{o}. The resulting nuclear extracts in the supernatants were collected and stored at -80C$^{o.}$

3.2.2. Protein quantitation

The procedure for protein quantification was as described in chapter two (section 2.6.10.2).

3.2.3. Immunoblotting

The procedure for immunoblotting has been described in chapter two (section 2.6.11). The antibodies used in these studies are listed in Table 3.1.

Antibody	supplier	Host	Type	Dilution
Anti-IκBα	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:250**
Anti-phospho-IκBα	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:250**
Anti-IKKα	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:250**
Anti-phospho-IKKα	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:250**
Anti-phospho-p65	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:500**
Anti-p.38	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:500**
Anti-phospho-p38	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:500**
Anti-JNK	**Abcam**	**Rabbit**	**Polyclonal**	**1:500**
Anti-phosho JNK	**Abcam**	**Rabbit**	**Polyclonal**	**1:500**
Anti-phosho ERK1/2	**Abcam**	**Rabbit**	**Polyclonal**	**1:500**
Anti phospho-TAK1	**Abcam**	**Rabbit**	**Polyclonal**	**1:500**
Anti-phospho MLK3	**Abcam**	**Rabbit**	**Polyclonal**	**1:500**
Anti-actin	**Sigma Aldrrich**	**Rabbit**	**Polyclonal**	**1:1000**

Table 3.1 Primary antibodies used with details of sources and dilution factor used.

3.2.4. Reporter gene assays

Genetic reporters are employed as indicators for investigating gene expression and cellular mechanisms linked to gene expression.(Cook et al., 2018) In this regard, a reporter gene is cloned with a DNA sequence of interest into an expression vector, which is then transfected into cells. Thereafter, an assay is performed on the cells to detect the presence of the reporter through a direct measurement of the reporter protein itself or its enzymatic activity (Figure 3.4).

HEK293 cell line was used in this study as HEK293 cells have been used extensively as an expression tool for recombinant proteins since it was generated over 25 years ago (Graham et al., 1977). In this study reporter gene assay were used to investigate the effect of formononetin on NFκB activity.

HEK293 cells were cultured in Modified Eagles medium (MEM) supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS). At confluence, the cells were sub-cultured (at a ratio of 1:3) for 24 h before transfection. Thereafter, cells were harvested and re-suspended at 4 x10^5 cells/ml in Opti-MEM medium containing 5% FBS. Cells were seeded out in a solid white 96-well plates and incubated with 0.1 µg of pGL4.32 [luc2P/NF-kB-RE/Hygro] vector (Promega, UK), using Lipofectamine® 2000 (Thermo Fisher Scientific) transfection reagent (diluted 1:3 in serum-free Opti-MEM) and

incubated for a further 16 h at 37ºC in 5% CO_2 incubator. Following transfection, the medium was changed to Opti-MEM and cells incubated for a further 8 h.

Transfected cells were treated with formononetin (2.5–10 µM) and incubated for 30 min at 37ºC followed by TNFα (1 ng/ml) for 6 h. At the end of the stimulation, NF κB-mediated gene expression was measured using Dual-Glo Luciferase Assay System (Promega, Southampton, UK), according to the manufacturer's instructions. 100 µl of luciferase assay reagent added to each well, followed by measurement of firefly luciferase activity. Thereafter 100 µl of Stop & Glo reagent was added to each well, then renilla luciferase activity was measured using FLUOstar OPTIMA Plate reader (BMG LABTECH), and the promoter activity calculated.

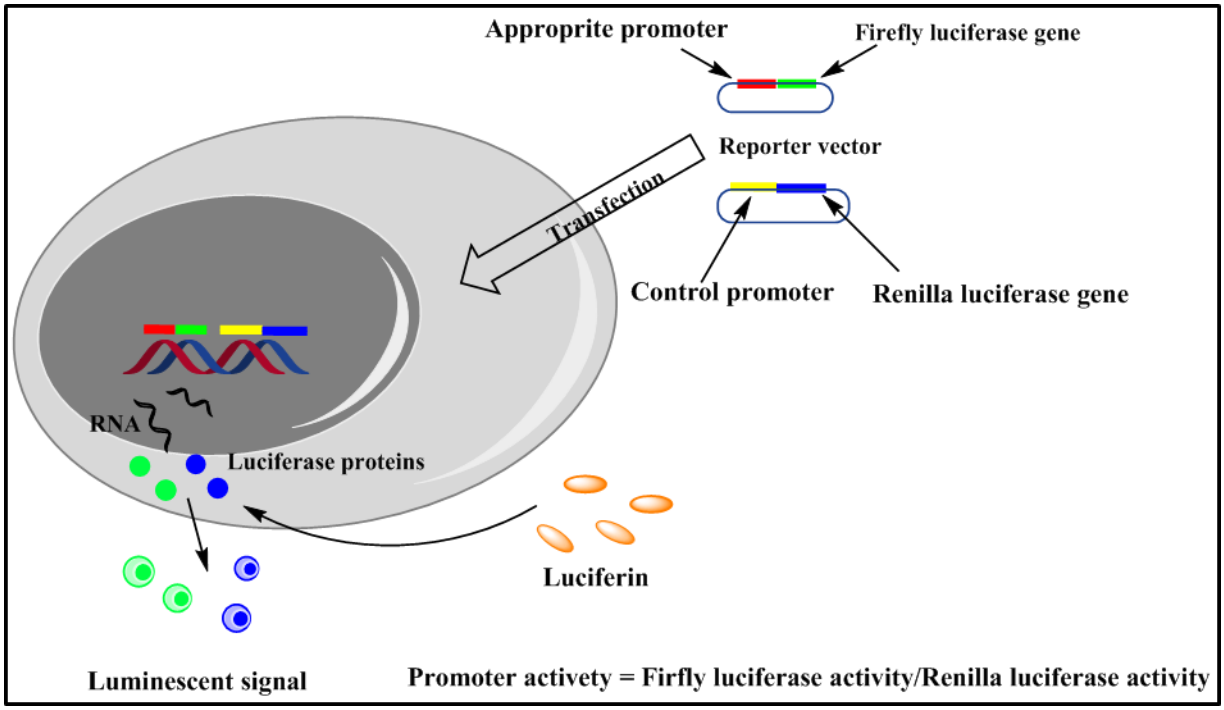


Figure 3.4 Scheme for the mechanism of the dual-reporter system.

(Modified from Bronstein et al., 1994)

3.2.5. DNA binding assays

Activation of NF-κB results in the release of pro-inflammatory cytokines and other mediators involved in the neuroinflammation. LPS stimulation can induce transcription of several inflammatory genes through interactions of NF-κB with the DNA in the microglia (Bellezzo et al., 1996; Hanamsagar et al., 2012; C. Xu et al., 2014).

Therefore, modulation of DNA binding by NF-κB is a critical strategy in evaluation of NF-κB pathway.

3.2.5.1. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) principle is that proteins with different molecular weight will have different electrophoretic mobilities in a nondenaturing gel matrix. In the case of a DNA–protein complex, the presence of DNA-binding protein will cause the DNA to migrate in a characteristic manner, usually more slowly than the free DNA, and will thus cause shift in the DNA mobility visible upon detection (Figure 2.4).

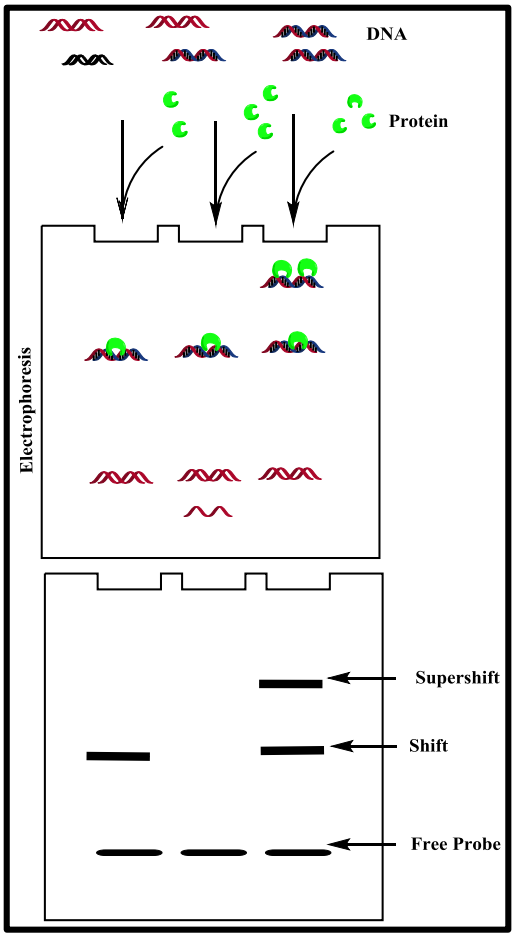


Figure 3.5 The schematic illustration of electrophoretic mobility shift assays (EMSA) (Song et al., 2015)

Electrophoretic mobility shift assay was used to investigate the effect of formononetin on LPS-induced DNA binding of NF-κB. Cultured BV2 cells were treated with formononetin (2.5-10 μM) for 30 minutes prior to stimulation with LPS (100 ng/ml) for a further 1 h. Nuclear extracts were prepared and nuclear protein (5 μg/μl) was incubated with IRDye^{TM} infrared dye end-labeled oligonucleotides containing the consensus binding sequence for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) (Licor Biosciences) in binding buffer (100 mM Tris, 500 mM KCl; pH 7.5, 6.25 mM MgCl2, 0.05 mM EDTA, 1% Nonidet P-40, 25 mM dithiothreitol (DTT), 2.5% tween20 , and 1 μg/μl poly (dl.dC)) for 30 minutes on ice.This was followed by the addition of 2 μl of orange loading dye (IR-labeled probe).The reaction samples were separated on native polyacrylamide gel (5% TBE, Life Technologies). The gel was electrophoresed at 200 V at room temperature for approximately 1.5 hours, and imaged on the Licor Odyssey® Infrared Imaging System.

3.2.5.2. ELISA-based DNA binding assay

NF-κB DNA binding ELISA kit contains 96-well plate with a specific double strand oligonucleotide immobilised on the well surface. NF-κB in the nuclear extract binds specifically to this oligonucleotide, which is then quantified by adding a specific primary and secondary antibodies. The resulting colorimetric outcome can be quantified using a microplate reader (Figure 3.5). Effects of formononetin on DNA binding of NF-κB was confirmed using an ELISA-based DNA binding assay.

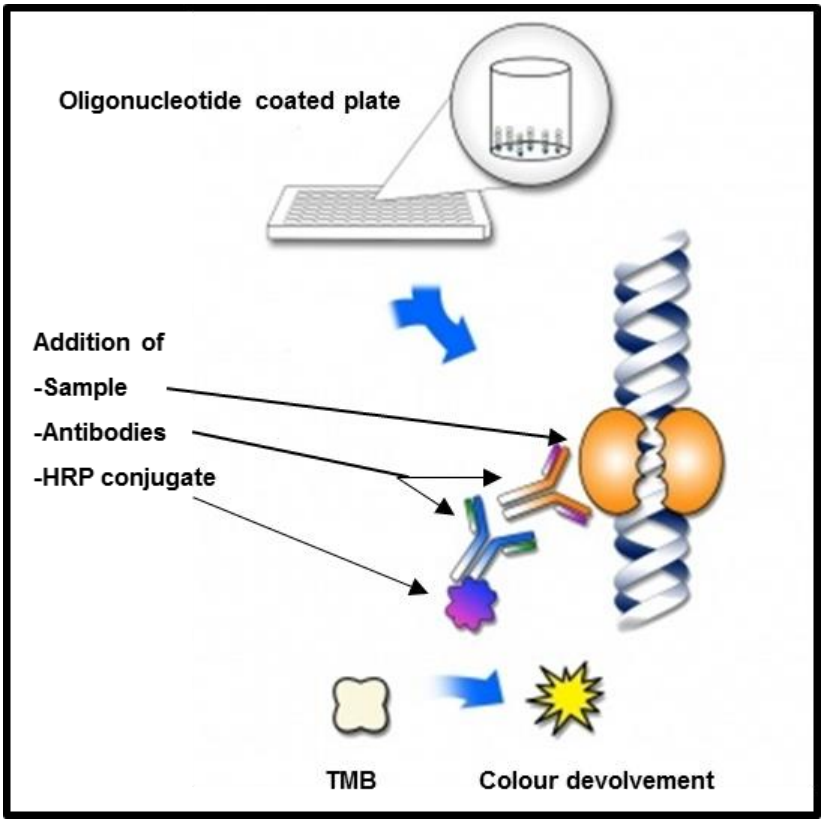


Figure 3.6 Chart for the ELISA-based DNA binding assay principle.

(Wild et al., 2013).

Cultured BV2 microglia were treated with 2.5, 5 and 10 µM formononetin 30 minutes prior to stimulation with LPS (100 ng/ml). One hour later, nuclear extracts were prepared using EpiSeeker Nuclear Extraction Kit (Abcam), according to the manufacturer's instructions. DNA binding assay was carried on nuclear extracts using the TransAM NF-κB transcription factor ELISA kit (Activ Motif, Belgium) according to the manufacturer's instructions. The ELISA kit employs a 96-well plate to which an oligonucleotide containing the NF-κB consensus site (‘5-GGGACTTTCC-3’) has been immobilized. Briefly, 30 µl of complete binding buffer was added to each well, followed by 20 µg nuclear extract samples. The plate was covered and rocked (100 rpm) for 1 hour at room temperature. This was followed by the addition of NF-κB antibody (1:1000; for 1 hour) and HRP-conjugated antibody (1:1000; for 1 hour). Absorbance was read on a Tecan F50 microplate reader at 450 nm.

3.2.6. Immunofluorescence

BV2 cells were seeded at a density of 2 x 10^5 cell/ml in a 24-well plate. At confluence, cells were pre-treated with formononetin (10 µM) for 30 minutes followed by stimulation with LPS (100 ng/ml) for various time points. After treatment, cells were fixed with ice-cold 100% methanol at −20°C for 15 minutes and washed three times with phosphate buffer saline (PBS) for 5 minutes. This was followed by blocking the non-specific binding by incubating cells in 5% BSA blocking solution (containing 10% horse serum in 1X TBS-T) for 60 minutes at room temperature. .Thereafter, the cells were incubated withanti-p65 antibody (Santa Cruz) diluted 1:100 overnight at 4°C.Following the incubation, cells were washed three times with PBS and incubated for 2 hours in the dark with Alexa Fluor 488-conjugated donkey anti-rabbit IgG secondary antibody (Life Technologies; 1:500) using 5% BSA blocking buffer Cells were then washed with PBS and counterstained with 4', 6-diamidino-2-phenylindole DAPI (Invitrogen) at concentration of 100µM for three minutes. Cells were rinsed with PBS, the excess buffer was removed and gold antifade reagent (Invitrogen) was added. Fluorescence images were acquired using EVOS® FLoid® cell imaging station.

3.3. Results

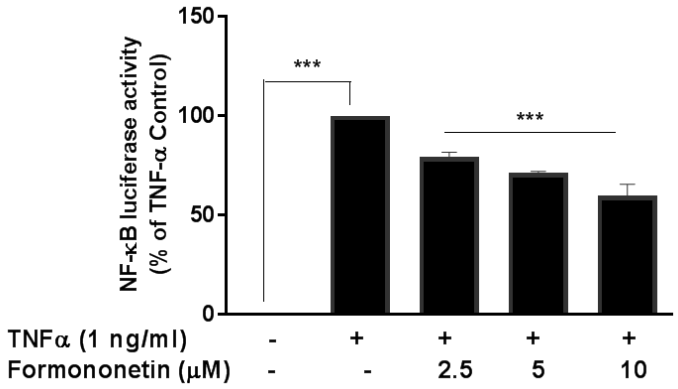
3.3.1.1. Formononetin inhibits neuroinflammation through modulation of NF-κB signalling

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 bearing NF-κB regulated luciferase reporter construct. The cells were then stimulated with TNFα (1 ng/ml) to activate NF-κB gene transcription in the presence or absence of formononetin (2.5, 5 and 10 μM). The experiment showed significant levels of NF-κB activation on cells stimulated with TNFα. However, cells treated with formononetin (2.5, 5 and10 μM) significantly ($p<0.001$) inhibited TNFα-induced NF-κB activation (Figure 3.6a).

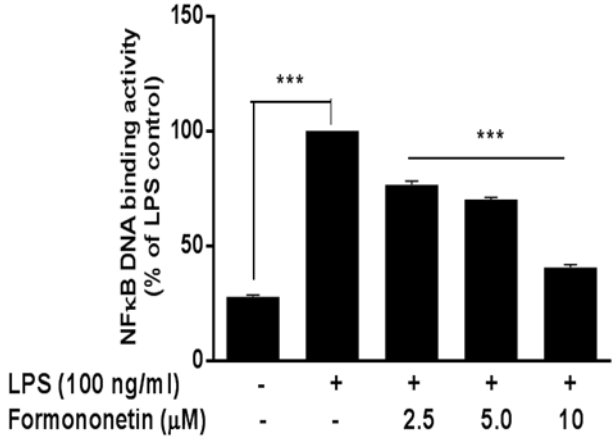
Further experiments were carried out to show that formononetin prevented LPS-induced DNA binding by NF-κB. Two different techniques were used-EMSA and ELISA-based DNA binding assay. BV2 cells were treated with formononetin (2.5, 5 and10 μM) followed by stimulation with LPS (100 ng/ml) for 1 hour. At the end of the incubation, nuclear extracts were collected followed by an ELISA-based DNA binding assay. Formononetin significantly ($p<0.001$) inhibited DNA binding activity of NF-κB (Figure 3.6b).

The same nuclear extracts were used for EMSA assay. Formononetin (2.5, 5 and10 μM) reduced NF-κB DNA binding activity compared with LPS-stimulated BV2 microglia in this assay (Figure 3.6c). All these results suggested that formononetin could interfere with multiple steps in the signalling pathway involving NF-κB in BV2 microglia.

a)



b)



C)

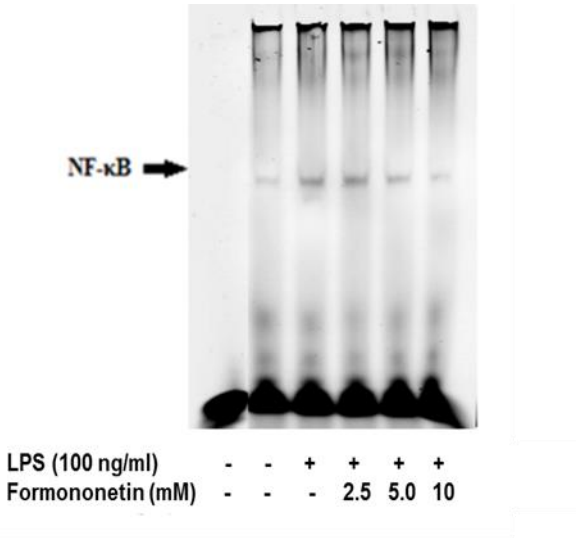


Figure 3.7 Formononetin inhibited NF-κB activity and DNA binding of NF-κB in LPS-stimulated BV2 microglia.

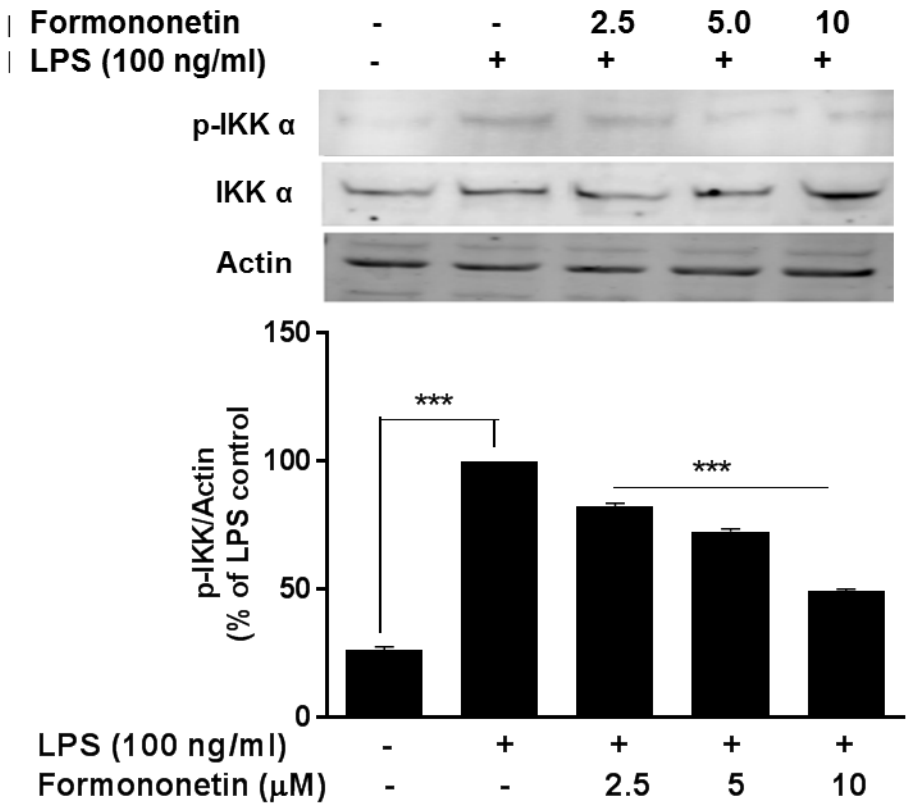
(a)TNFα-induced NF-kB-dependent gene expression in HEK 293 cells was inhibited by formononetin. Transfected cells were incubated with formononetin (2.5-10 μM) followed by stimulation with TNFα (1 ng/ml) for an additional 6 h. Luminescence was then measured.(b)Nuclear extracts from cells were added to 96-well plates to which an oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3') has been immobilised, followed by addition of NF-κB and HRP-conjugated antibodies. Absorbance was read on a microplate reader. (c)Nuclear extract was collected followed by EMSA.

3.3.1.2. Formononetin blocks IKK/ I kappa B phosphorylation and nuclear translocation of p65 in LPS-activated BV2 microglia.

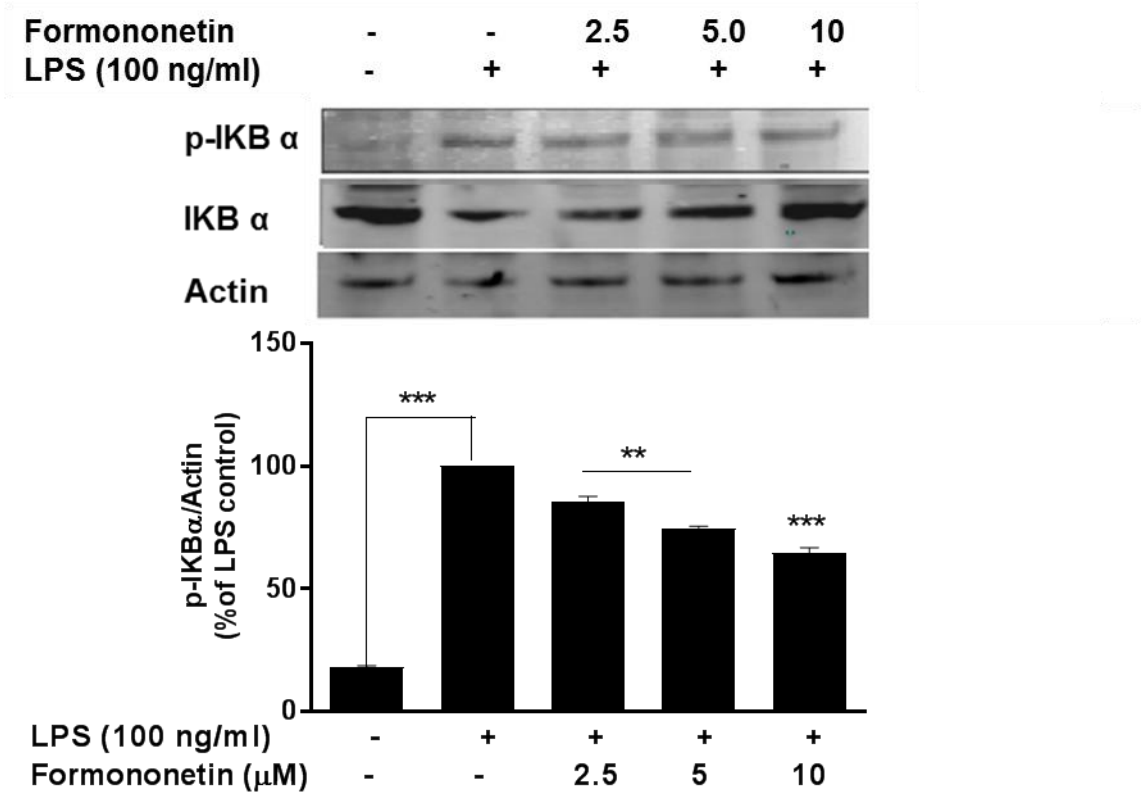
Inactive NF-κB exists in the cytoplasm as a complex with inhibitor of kappa B (IκBα) protein. Following an inflammatory stimulus such as LPS, this complex is phosphorylated by IκB kinase (IKK), resulting in the translocation of active NF-κB to the nucleus. In this study, the effect of formononetin on this process in LPS-activated BV2 microglia was studied. BV2 cells were pre-treated with formononetin (2.5, 5 and 10 µM) for 30 min, followed by LPS (100 ng/ml) stimulation for a further 60 min. Using western blotting, it was observed that stimulation of BV2 microglia with LPS resulted in an increase in the phosphorylation of IKK compared to the unstimulated control cells. Whereas cells pre-treated with formononetin (2.5 µM) showed about 20% reduction of phosphorylated IKK compared to LPS stimulated cells .The degree of reduction increased to 35 % in cells treated with formononetin (5 µM) reaching 50% in pre-treated cells with (10 µM) formononetin. (Figure 3.7a). Further, western blot experiments were performed to investigate the effect of formononetin on the phosphorylation of IκBα. LPS stimulated BV2 cells had higher levels of phospho-IκB compared with unstimulated control cells. However, pre-treated cells with formononetin (10 µM) significantly ($p<0.001$) inhibited LPS-induced phosphorylation of IκB. This was accompanied by a concentration-dependent inhibition of IκB degradation (Figure 3.7 b, c).

Further experiments were performed to study the effects of the compound on nuclear translocation of the p65 sub-unit in LPS-stimulated BV2 microglia. Cells were pre-treated with formononetin (2.5, 5 and 10 µM) for 30 minfollowed by LPS stimulation for 60 min. Then nuclear cell extracts were collected followed by western blot to detect phosphorylated p65. A significant elevation in the levels of phosphorylated p65 in LPS stimulated BV2 was observed in comparison with unstimulated cells. However, formononetin (5 and 10 µM) produced significant inhibition ($p<0.001$) of nuclear translocation of the p65 sub-unit following stimulation of the cells with LPS (Figure 3.7c). These observations were confirmed using immunofluorescence imaging which showed a decrease in p65 sub-unit in LPS-stimulated BV2 microglia which were pre-treated with formononetin (2.5, 5 and 10 µM (Figure 3.7 d).

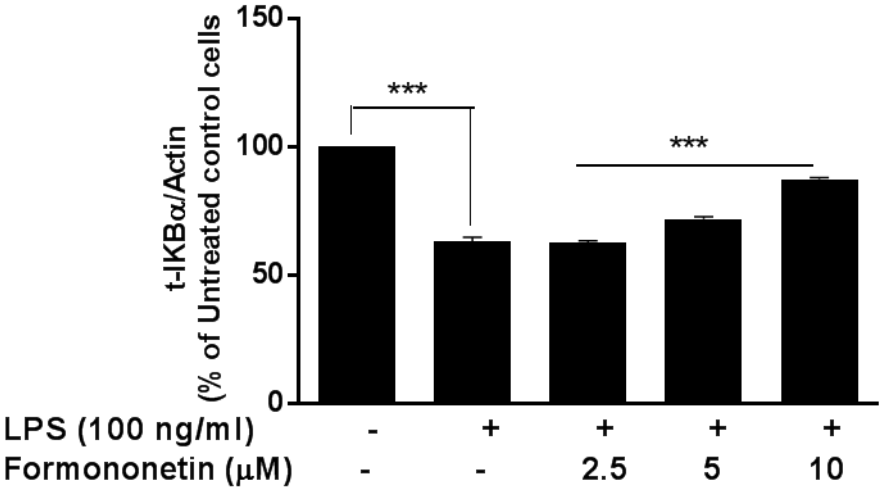
a)


Formononetin - - 2.5 5.0 10
LPS (100 ng/ml) - + + + +
p-IKK α
IKK α
Actin
p-IKK/Actin (% of LPS control)
150
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

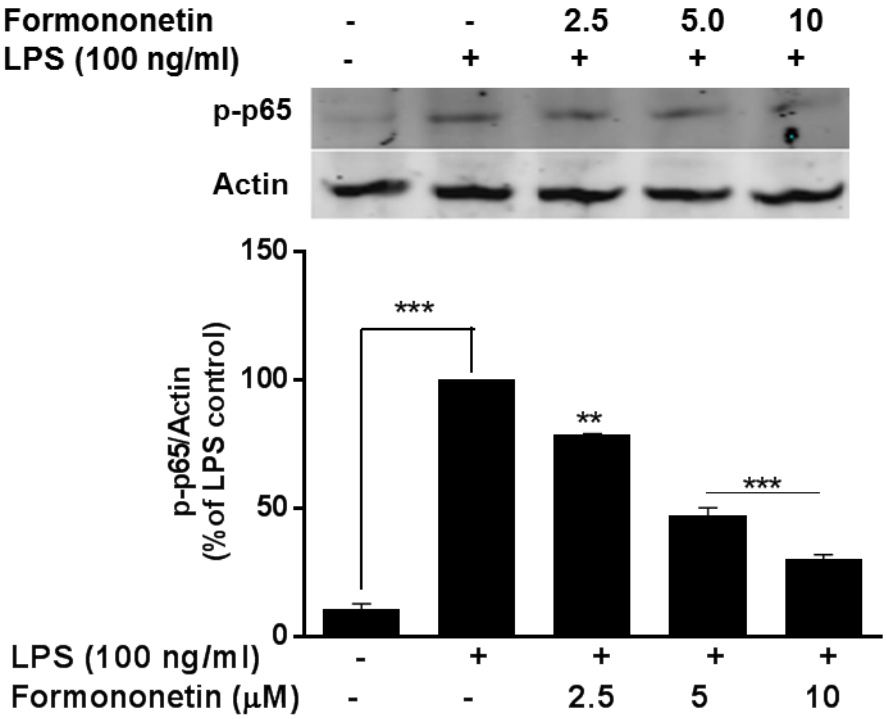
b)


Formononetin - - 2.5 5.0 10
LPS (100 ng/ml) - + + + +
p-IKB α
IKB α
Actin
p-IKBα/Actin (%of LPS control)
150
100
50
0
***
**
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

c)


t-IKBα/Actin
(% of Untreated control cells
150
100
50
0
***
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

d)


Formononetin - - 2.5 5.0 10
LPS (100 ng/ml) - + + + +
p-p65
Actin
p-p65/Actin
(%of LPS control)
150
100
50
0
***
**
***
LPS (100 ng/ml) - + + + +
Formononetin (μM) - - 2.5 5 10

e)

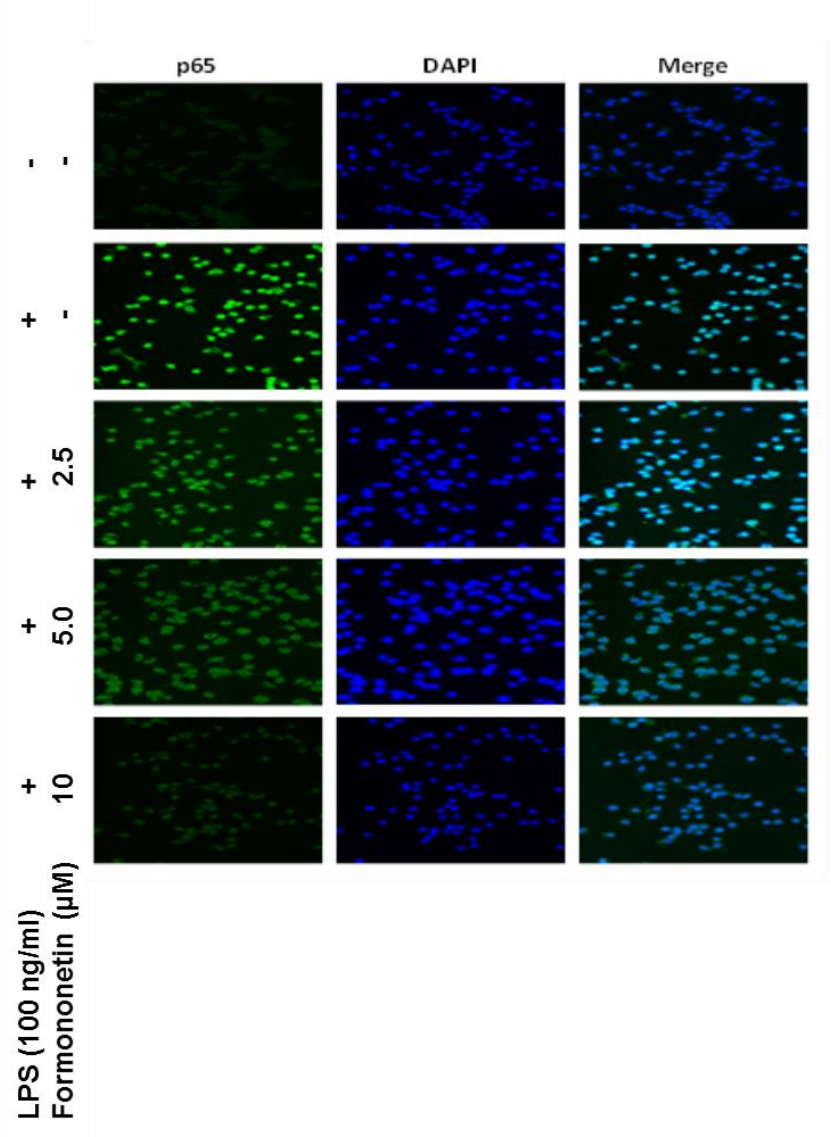


Figure 3.8 Effect of formononetin on IKK/IκB and nuclear translocation of p65 in LPS-activated BV2 microglia.

Formononetin attenuated phosphorylation of IKKα (a) as well as phosphorylation and degradation of IκBα (b and c) in LPS stimulated BV-2 cells. BV2 cells were pre-treated for 30 minutes with formononetin (2.5-10 μM) prior to stimulation with LPS, and then incubated for another 1 hour. At the end of the incubation, whole cell extracts were collected and western blot performed to measure total and phospho-IKKα and phospho-IKKα, phsopho- and total IκBα. Induced p65 subunit nuclear translocation was significantly inhibited by formononetin in BV2 microglia cells. Microglia were incubated in a medium containing formononetin (2.5, 5 and10 μM) for 30 min and then activated with LPS (100 ng/ml) for 60 min, followed by western blot (d) Localisation of NF-κB p65 was visualised with fluorescence microscopy after immunofluorescence staining with NF-κB p65 antibody (green). Cells were stained with DAPI to visualize nuclei (blue) (e).

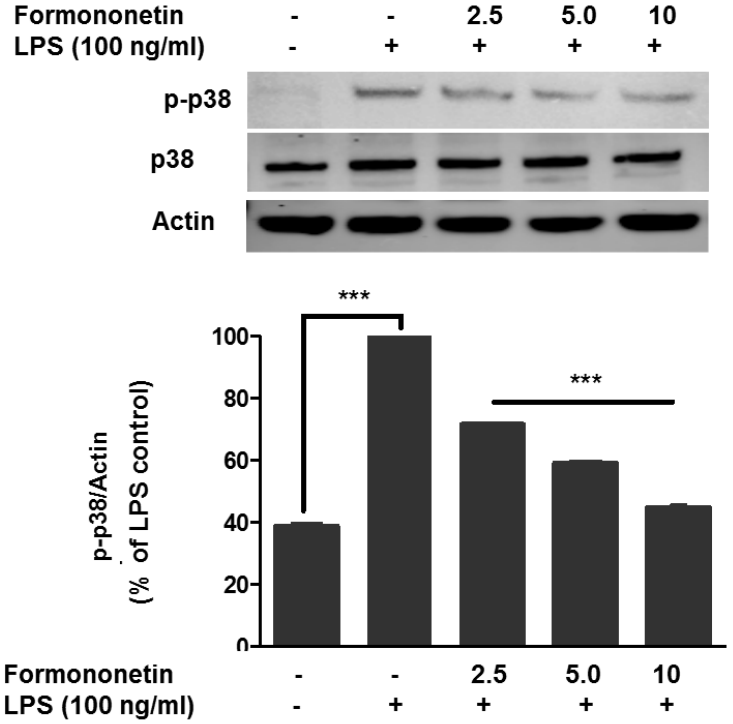
3.3.2. Inhibition of MAPKs contributed to inhibition of neuroinflammation by formononetin

This study assessed the effect of formononetin on LPS-induced phosphorylation of p38, JNK and ERK in BV2 microglial cells. BV2 cells were treated with formononetin (2.5, 5.0 and 10 μM) and incubated for 30 min, followed by stimulation with LPS (100 ng/ml), and incubation for a further 1 h. At the end of the incubation, whole cell lysates were collected and western blot was performed to detect p38, JNK and ERK.

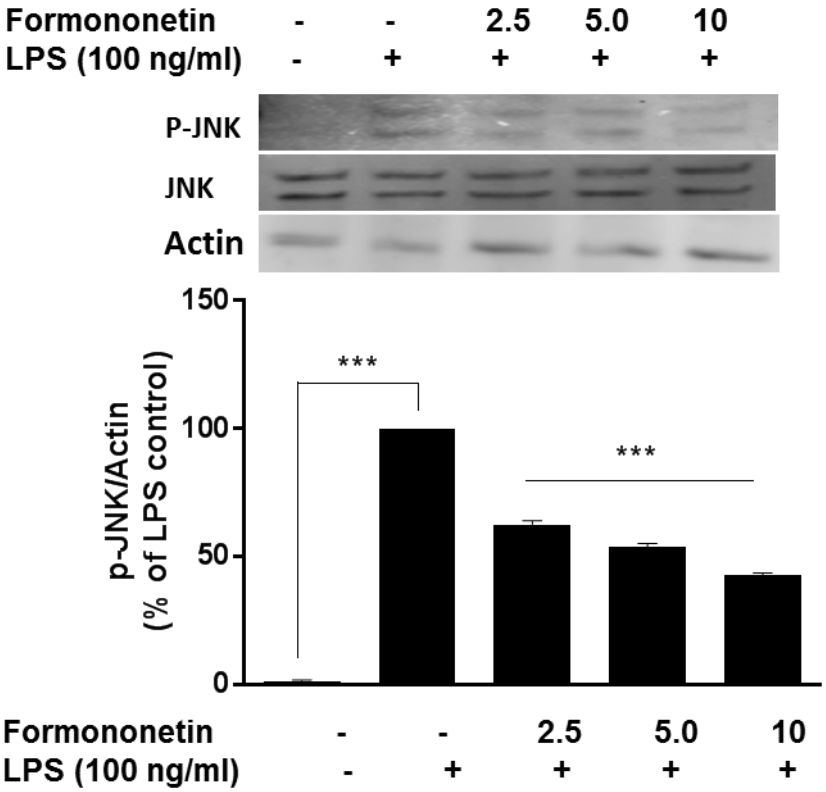
Exposure of BV2 cells to LPS for 1 h significantly increased the levels of phosphorylated p38 compared to unstimulated control cells. However, pre-treatment with formononetin (2.5, 5.0 and 10 μM) markedly ($p<0.001$) reduced the phosphorylation levels p38 compared to LPS control cells (Figure 3.8 a).

Stimulated BV2 cell with LPS (100 ng/ml) for one hour showed a significant elevation in phosphorylated JNK when compared with un-stimulated cells.However pre-treatment with formononetin (2.5, 5.0 and 10 μM) showed significant ($p<0.001$) reduction in p-JNK in LPS stimulated BV2 cells (Figure 3.8 b). Further results revealed that LPS (100 ng/ml) activation of BV2 microglia resulted in an increasing the phosphorylation of ERK1/2, whereas formononetin (2.5, 5.0 and 10 μM) significantly ($p<0.001$) supressed the activation of ERK1/2 in dose dependent manner (Figure 3.8 c).These results demonstrate that LPS induces activation of p38, JNK and ERK; these effects were prevented with formononetin treatment.

a)


Formononetin - - 2.5 5.0 10
LPS (100 ng/ml) - + + + +
p-p38
p38
Actin
***
***
p-p38/Actin
(% of LPS control)
100
80
60
40
20
0
Formononetin - - 2.5 5.0 10
LPS (100 ng/ml) - + + + +

b)


Formononetin - - 2.5 5.0 10
LPS (100 ng/ml) - + + + +
P-JNK
JNK
Actin
***
***
p-JNK/Actin
(% of LPS control)
150
100
50
0
Formononetin - - 2.5 5.0 10
LPS (100 ng/ml) - + + + +

c)

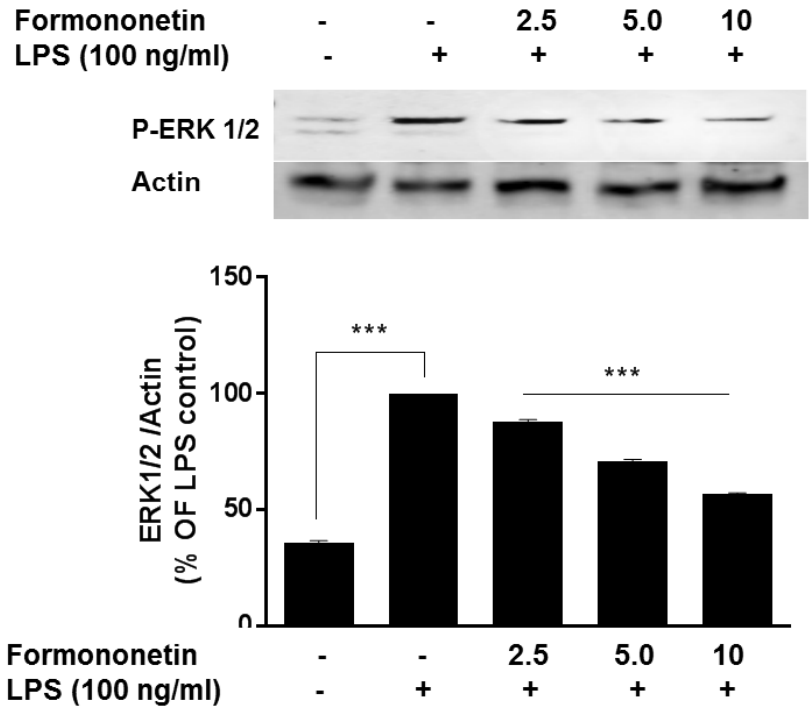


Figure 3.9 Effect of formononetin on MAPK in BV2 cells stimulated with LPS.

Formononetin inhibited LPS (100 ng/ml) induced phosphorylation of p.p38, p.JNK and ERK in BV2 microglia. Cells were pre-treated with formononetin (2.5-10 µM) prior to activation with LPS. At the end of the incubation, whole cell extracts were collected and western blot performed for total and phospho-p38 (a), phospho and total JNK (b) and phospho-ERK.

3.3.2.1. Formononetin supresses the activation of MLK3 in LPS stimulated BV2 cells

Mixed-lineage protein kinase 3 (MLK3) is a member of the mitogen-activated protein (MAP) kinase kinase kinase which has been linked to the NF-κB, as well as the ERK1/2, c-Jun NH2-terminal kinase (JNK), and p38 MAPK athways (Patrick et al., 2001; Kim et al., 2004). Therefore, it becomes necessary to investigate the effect of formononetin on the activation of MLK3 in LPS stimulated BV2 microglia cells. LPS-stimulated BV2 cells expressed higher levels of phosphorylated-MLK3 compared to unstimulated control cells. On pre-treating cells with formononetin (2.5, 5.0 and 10 µM), a significant ($p<0.001$) reduction in phosphorylated -MLK3 was observed (Figure 3.9).

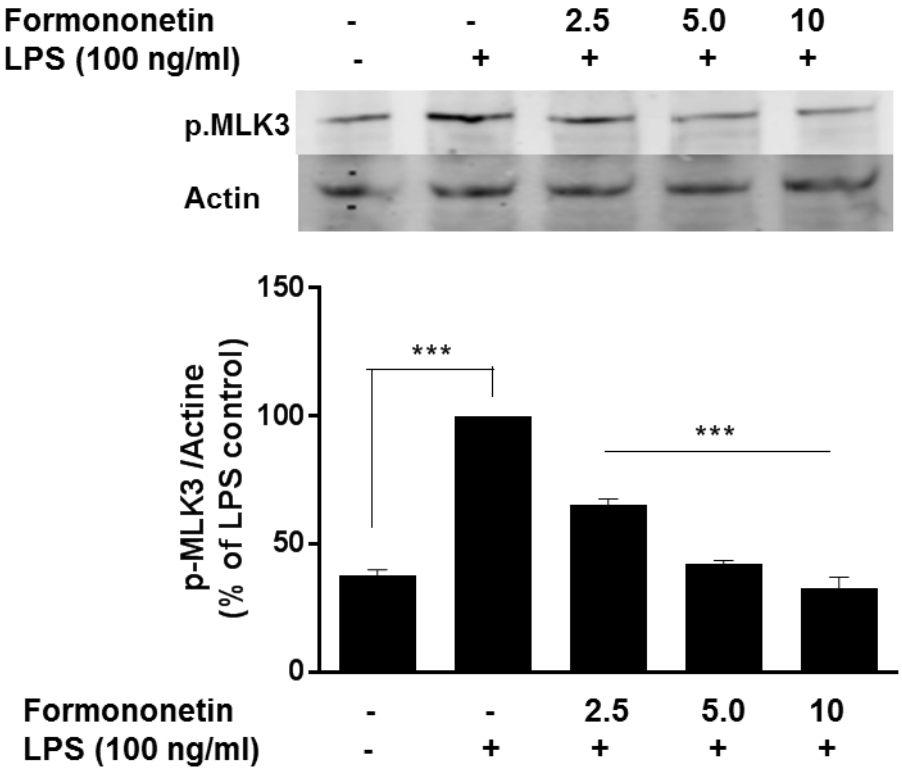


Figure 3.10 Effect of formononetin in MLK3 phosphorylation in LPS activated BV2 microglia.

Formononetin inhibited LPS (100 ng/ml) -induced MLK3 phosphorylation in BV2 microglia. Cells were pre-treated with formononetin (2.5-10 μM) in the presence or absence of LPS (100 ng/ml) and incubated for 30 min. Western blot was performed for phospho-MLK3.

3.3.3. Foromononetin inhibited the activation of TAK1 on LPS stimulated BV2 cell.

Activation of both transcription factors NF-κB and activating protein-1 (AP-1) signalling pathways is through a common upstream kinase transforming growth factor-β-activated kinase 1 (TAK1) (Sakurai et al., 1998; Cejas et al., 2010). TAK1 was originally identified as a mitogen-activated kinase kinase kinase (MAP3K) activated by transforming growth factor-β (TGF-β) (Yamaguchi et al., 1995). However, it has been characterised as a key regulator in inflammatory and immune signaling pathways such as NF-κB and MAPK (Sato et al., 2005; Sakurai, 2012; Goldmann et al., 2013). TAK-1 can be considered as a convergent target protein activating both NF-κB and MAPK

signalling pathways. Therefore, it was thought to investigate the effects of formononetin on TAK1 activation on LPS activated BV2 microglia.

Stimulation of BV2 microglia with LPS resulted in a marked expression of TAK1 protein, compared to un-stimulated control cells. However, cells pre-treated with formononetin (, 5.0 and 10 µM) showed significantly ($p<0.01$) reduced levels of TAK1 phosphorylation (Figure 3.10).

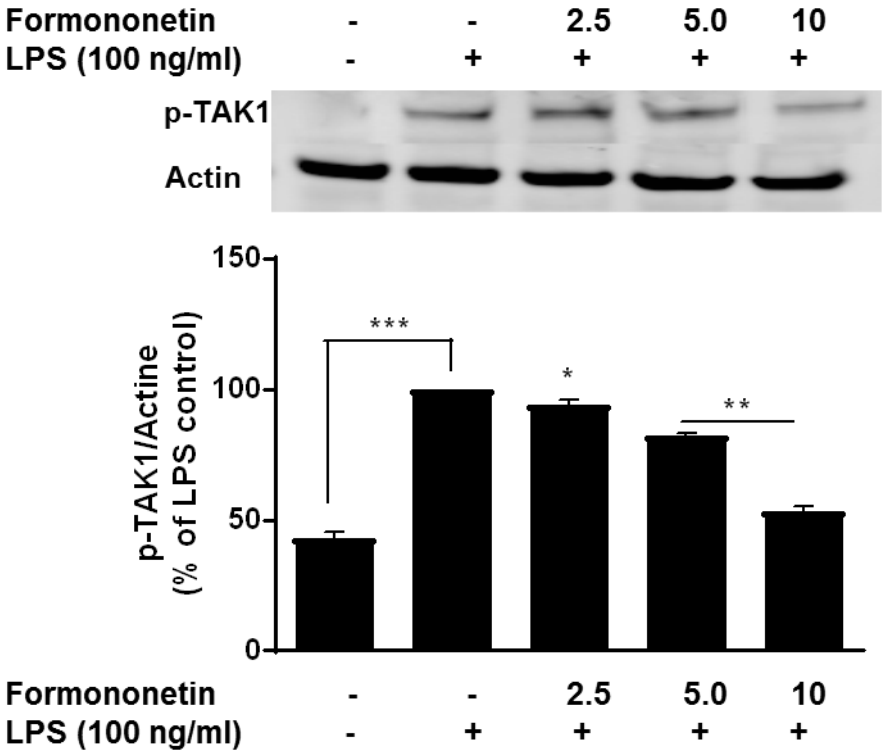


Figure 3.11 Effects of formononetinon TAK1 phosphorylation.

BV2 cells were pre-treated with indicated concentrations of formononetin for 30 minutes prior to incubation of LPS (100 ng/mL) for 30 min. p-TAK1 was determined by western blot. Each immunoreactive band was digitised and expressed as a ratio of β-actin levels.

3.4. Discussion

NF-κB plays an important role in sustaining the inflammatory response (Pugazhenthi et al., 2013). Based on the results highlighted in chapter two of the thesis, which demonstrate inhibition of neuroinflammation by formononetin, it became necessary to investigate whether inhibition of NF-κB contributed to the molecular mechanism of action of the compound.

Results with HEK293 transfected with NF-κB-bearing luciferase vector plasmid revealed suppression of TNFα-induced activation of the NF-ϰB-driven luciferase expression, suggesting that this compound inhibited NF-κB-dependent gene expression in general. Further experiments in BV2 microglia stimulated with LPS revealed that formononetin blocked critical steps involving IKK-mediated phosphorylation and degradation of IκB, nuclear translocation as well as DNA binding of NF-κB. These observations have clearly demonstrated that formononetin prevented the release of pro-inflammatory mediators in BV2 microglia by targeting multiple steps in NF-κB signalling following stimulation with LPS. Formononetin was shown to produce similar NF-κB inhibitory activity in other cells. The compound was reported to prevent apoptosis induced by IL-1β in the rat INS-1 cells through inhibition of NF-κB (Wang et al., 2012). Similar NF-κB inhibitory activity of the compound was reported in retinal ganglion cells (RGCs) (Jia et al., 2014).

The Inflammatory process following NF-κB activation has been closely linked to the onset of various neurodegenerative diseases, including Alzheimer's disease (Zhao et al., 2014) and Parkinson's disease (Anitua et al., 2015). Emerging evidence suggests that inflammatory responses contribute to the progress of AD, accelerating the course of the disease. Interestingly, the non-steroidal anti-inflammatory drugs (NSAIDs) used to reduce inflammation have been shown to inhibit the activation of NF-κB in AD (Altinoz et al., 2004; Deardorff et al., 2017). Natural compounds could also inhibit neuroinflammation by inhibiting NF-κB activity. Examples of such compounds are curcumin (Ambegaokar et al., 2003), resveratrol (Capiralla et al., 2012), pterostilbene (Capiralla et al., 2012), punicalagin (Xu et al., 2014), macranthoin G (Hu et al., 2014), and salidroside (Gao et al., 2015).

The results from this study and evidence from previously published data on formononetin clearly demonstrated that the compound could block pro-inflammatory NF-κB activation in many cells of the body, including brain microglia. Furthermore, inhibition of microglia NF-κB suggested that this compound could be a useful scaffold for novel NF-κB inhibitors for neurodegenerative disorders.

The MAPKs signalling molecules p38, JNK and ERK1/2 play important roles in inflammatory processes (Kyriakis et al., 2012). Specifically, p38 MAPK signalling is strongly linked to neuroinflammation (Fan et al., 2013). Accumulating evidence indicates that p38 plays multiple roles in AD pathophysiology and that patients suffering from AD could benefit from p38 MAPK inhibitors (Munoz et al., 2010). Recent studies have demonstrated that p38 MAPK is capable of mediating neuroinflammation in AD. Consequently, the inhibition of p38 MAPK is proposed as a critical strategy in AD drug development (Lee et al., 2017). Results from this study show an inhibition in LPS-induced activation of p38 MAPK in BV2 microglia. This outcome showed that inhibition of p38 could potentially contribute to anti-neuroinflammatory activity by formononetin. It could also be concluded that this activity of the compound could be useful in AD treatment.

Formononetin was also shown to block the activation of both JNK and ERK1/2 MAPKs in LPS-activated BV2 microglia. This was a significant outcome as JNK is known to mediate LPS-induced neuroinflammation and white matter injury (Wang, et al., 2012). Also, regulating ERK1/2 activation is known to be important in Aβ-induced neuroinflammation in BV2 microglia (Yu et al., 2017). Similar phytoestrogens have been reported to target JNK and ERK1/2 activation in different models (Liu et al., 2014; W. Wang et al., 2015; Lim et al., 2017; Park et al., 2017).

Mixed lineage kinase 3 is expressed in immune effector cells including microglia in the CNS and is activated by cellular and metabolic stress (Dong et al., 2016). Inhibition of MLK3 is a potential strategy for inhibiting neuroinflammation (Ronkina et al., 2010), as it is an important upstream regulator of p38 and JNK MAPKs which have been shown to play major roles in neuroinflammation and neuronal apoptosis (Lin et al., 2013). Results showed that formononetin significantly inhibited the activation of MLK3 induced by LPS in BV2 microglia, which might account for the effects of the compound on p38, and JNK MAPKs. Biochanin-A, a related compound to formononetin, was

previously shown to prevent UV-induced COX-2 expression by targeting MLK3 in Human HaCaT cells (Lim et al., 2013). These related phytoestrogens might be acting upstream to block events resulting in the release of inflammatory mediators.

TAK1 is a member of the MAPK kinase kinase, which functions as an upstream signalling molecule of NF-κB and MAPKs. Activated TAK1 complex phosphorylates IKK, which activates NF-κB. It was therefore hypothesised that targeting TAK1 activation might be an attractive strategy to treat inflammatory responses (Endale et al., 2017). In this study, LPS-induced TAK1 phosphorylation was significantly inhibited by formononetin. It could therefore be postulated that the suppression of LPS-induced TAK1 activity by formononetin might be responsible, in part, for its inhibition of both NF-κB and MAPK signalling pathways.

These findings demonstrated that formononetin could inhibit the production of pro-inflammatory factors in LPS-activated BV2 microglia through dual inhibition of NF-κB and MAPK signalling. This activity of the compound might be related to its ability to target the upstream TAK1 in the microglia (Figure 3.11). This study did not verify these actions of the compound *in vivo*. Further studies would therefore be needed to establish both NF-κB and MAPK inhibition in different transgenic (NF-κB knockout) models of neuroinflammation.

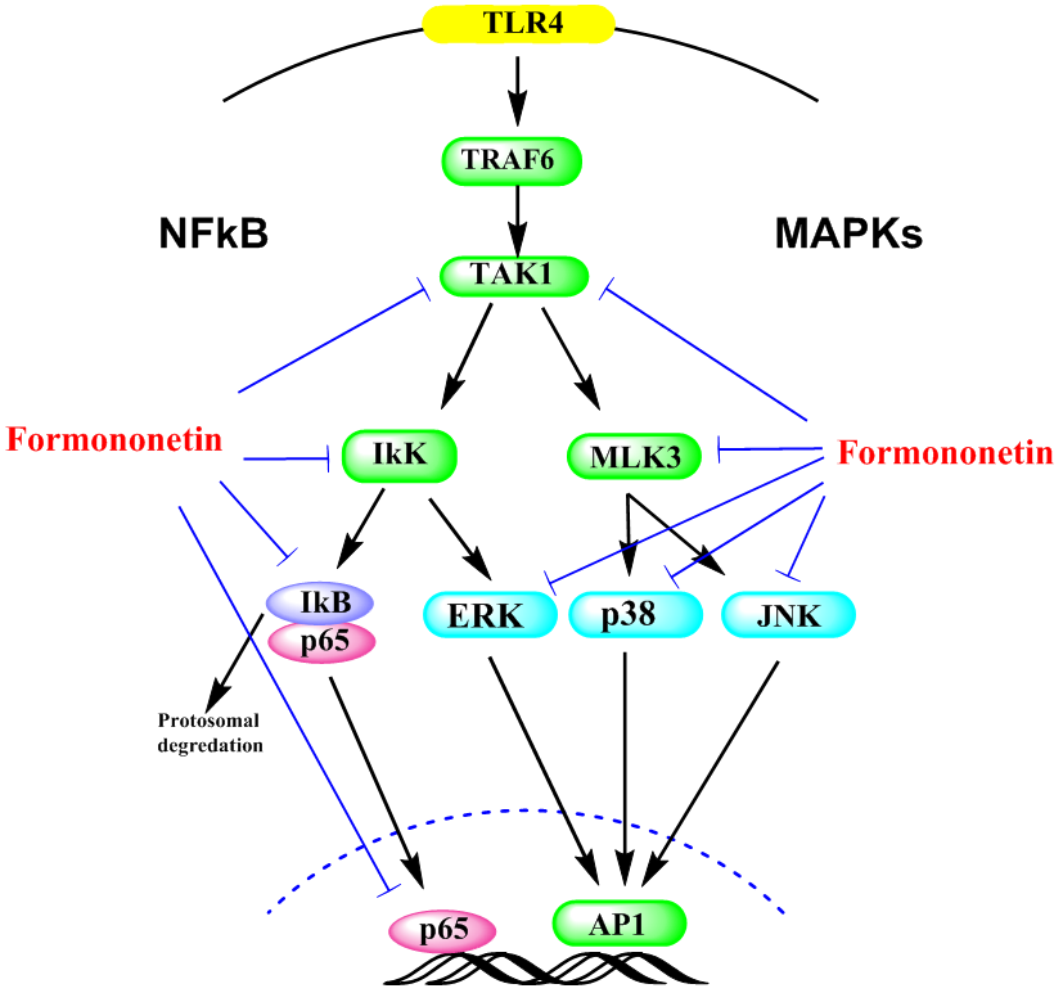


Figure 3.12 Scheme concluding the Molecular mechanisms underlying the anti-inflammatory activities of formononetin in LPS activated BV2 microglial cells.

Chapter Four

The role of oestrogen receptor beta (ERβ) activation in the inhibition of neuroinflammation by formononetin in LPS-activated BV2 microglia

4.1. Introduction

4.1.1. Oestrogen and neuroinflammation

The role of oestrogens in various physiological and pathophysiological mechanisms is dependent on their ability to bind to oestrogen receptors and promote activation of transcription of oestrogen responsive genes (Hewitt et al., 2016; Coyoy et al., 2016). Two different oestrogen receptor (ER) subtypes, are known: ERα, the first ER discovered, and ERβ, which was discovered in 1996 (Kuiper et al., 1997; Lin et al., 2004). The tissue distribution patterns of ERα and ERβ are quite different. ERβ is more highly expressed than ERα in the prostate, ovary, colon, urinary tract, and some brain regions (Gong et al., 2014).

Anti-neuroinflammatory effects of oestrogens and related compounds have demonstrated in experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS) (Vegeto et al., 2001; Tiwari-Woodruff et al., 2007; Gold et al., 2009; Glass & Saijo, 2010).

4.1.2. Mechanisms of action of oestrogen

After diffusion through the plasma membrane, oestrogen can activate the transcription of their target genes by various signalling pathways, genomic or extragenomic (Figure 4.1) (Cornil et al., 2015)

4.1.2.1. The classic mechanism: ERE-dependent genomic transcriptional activity

In the absence of a ligand, the receptor for oestrogen is in monomeric form, complexed with chaperone proteins, Heat Shock Proteins (HSP) HSP70 and HSP90 located in the nucleus or cytoplasm. Binding of a ligand results in a change in conformation of the receptor, which dissociates HSPs, dimerises with another receptor and transferred to the nucleus (Stender et al., 2010). The dimer can then bind to ERE upstream of the target genes and activate transcription (McKenna et al., 2002). Depending on the cell type and promoters involved, the receiver can then engage in a stimulatory or inhibitory activity vis-a-vis the expression of target genes (Tora et al., 1989).

4.1.2.2. Genomic transcriptional activity not related to ERE

The oestrogen receptor is capable of inducing transcription of genes lacking ERE sequence, by indirect binding of the DNA, through interaction with a complex formed by two transcription factors fos and jun also known as AP-1 (Wu et al., 2008). This signalling pathway is involved in the transcription of genes such IGF-1 (Insulin-like Growth Factor-1). The expression of certain genes containing promoter GC-rich sequences bases is activated through interaction with SP1 complex (Stimulating Protein 1) (Hall et al., 2001).

4.1.2.3. Independent transcriptional activity of the ligand

In parallel with a hormone-dependent activation, the oestrogen receptor may be modulated by extracellular signals, in the absence of E2 such as Growth factors (Epidermal Growth Factor) and IGF-1 they are able to activate the receptor and induce expression of target genes (Ignar et al., 1995).

4.1.2.4. Activation from a receiver located in the membrane

Besides the genomic effects, there are effects called "non-genomic", and although they may be at the origin of transcriptional effects and occurring within seconds or minutes after the addition of E2. These effects are mediated through activation of kinases and phosphatases. The membrane receptor is localized in caveolae-associated with proteins as caveolin (Evinger et al., 2005). It has been proposed that the location membrane receptor is permitted through post-translational modifications in particularly the S-palmitoylation of cysteine 447 (Marino et al., 2006). The link ligand involves various signalling cascades such as the activation of MAP kinases, 3-phosphatidyl-inositol kinase (PI3K), phospholipase C (PLC) of protein kinase C (PKC), of endothelial NO synthase (eNOS) or the path of EGFR (Epidermal growth factor receptor) (Levin, 2002).

Oestrogen is indeed able to activate the EGF receptor from stimulating of ERα membrane, leading to the release of EGF. The ERα addressed the cytoplasmic membrane is coupled to a G protein and activates, via the Src protein, the Metalloproteinases MMP-2, and MMP-9, which freed the heparin-binding epidermal growth factor (HB-EGF). The latter will then induce the phosphorylation of its receptor (EGFR) and thereby activate the signalling pathway ERK / MAPK. Activation of EGFR

induces activation cascade of kinases (Razandi et al., 2003). This is one of the non-genomic effects of ERα (Levin, 2003). In addition to these mechanisms of action, in absence of E2, ERα is capable of modulating cell growth and differentiation. For example, ERα inhibits neuronal growth by down-regulation of the MAPK and PI3K / Akt.

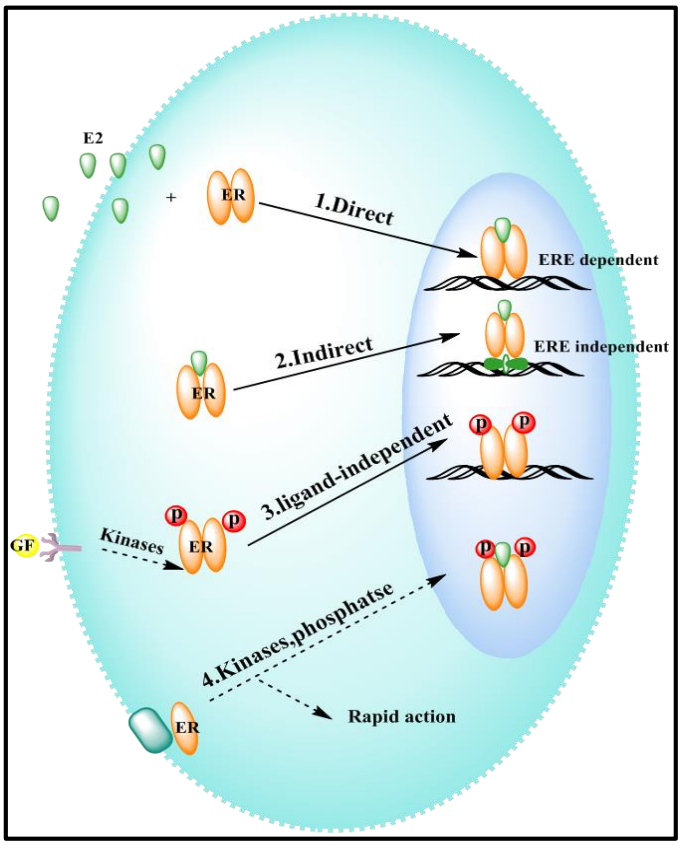


Figure 4.1 Mechanism of action of oestrogen receptors.

1. Classic mechanism of action (direct): after activation by the ligand receptor dimers bind DNA at specific sequences (ERE).
2. Mechanism of action independent ERE (indirect): the receptor dimers bind to DNA via protein interactions.
3. Mechanism of ligand-independent action: growth factors activating kinases that phosphorylate the receptors and attach to the DNA at specific sequences (ERE).
4. Non-genomic mechanism of action localized to the membrane receptors activated kinases, leading to rapid changes in cytoplasmic proteins or to transcriptional regulation.

Modified from (Reid et al., 2002) and (Heldring et al., 2007).

4.1.3. Oestrogen and selective oestrogen receptor modulation

Some studies indicate that oestrogen produces anti-inflammatory and neuroprotective effects (Ramien et al., 2016). However, there is a risk of tumours, heart disease, stroke and blood clots associated with oestrogen treatment (Medicine, 2004). This has re-focused in the selective oestrogen receptor modulators (SERMs) in the therapeutics of neurodegenerative diseases (Bernardi et al., 2003; Pullman, 2003). SERMs, such as tamoxifen and raloxifene, are molecules which bind with high affinity to oestrogen receptors (ERs), acting as oestrogen agonists in some tissue such as bone, liver, and uterine cells and as antagonists in others such as breast (Jordan, 2003; Wood et al., 2003). These agonist/antagonist profiles for individual SERMs may differ also among brain areas (Zhou et al., 2002). Both of the known oestrogen receptors, ERα and ERβ, exist in microglial cells (Keller et al., 2000), and are thought to mediate classic receptor-mediated responses.

Oestrogen exerts anti-inflammatory activity via ERα and ERβ on activated macrophages and activated microglial cells (Dodel et al., 1999; Drew et al., 2000; Keller et al., 2000; Vegeto et al., 2004; Lewis et al., 2008). There is a general agreement on the ability of oestrogens to limit the microglia proinflammatory status after short exposure to bacterial lysates (Kim, 2016), and viruses (Vrachnis et al., 2014). The hypothesis of the anti-inflammatory potential of oestrogen was based initially on the *in vitro* observation that 17-estradiol prevented the morphological changes induced by LPS and the concomitant synthesis of proinflammatory molecules , such as matrix metalloproteinase 9, prostaglandin E2, iNOS with reactive oxygen species production (Paradkar et al., 2017).

Phytoestrogens are a diverse group of plant-derived non-steroidal structural analogues of mammalian oestrogens that can bind to oestrogen receptors (ERs) and exert estrogenic or anti- estrogenic activities (Setchell, 1998; Andres et al., 2012). Interestingly, the soy isoflavones, such as daidzein and genistein, were among the first compounds noted to be ligands with selective affinity for ERβ (Meyers et al., 2001; Muthyala et al., 2004). Another study yielded a novel phytoestrogenic formulation by combining three phytoestrogens genistein, daidzein, and equol referred to as the phyto-β-SERM formulation, which exhibits an 83-fold binding selectivity for the oestrogen receptor subtype β (ERβ) over ERα (Zhao, 2013). The relatively selective

binding of phytoestrogens to ERβ indicated that they might produce clinical effects distinct from oestrogens by either selectively or differentially triggering ERβ-mediated transcriptional activation or repression pathways.

BV2 microglia cell line naturally expresses ERβ but not ERα (Baker et al., 2004). These studies were designed to test the hypothesis that formononetin exerts anti-inflammatory effects in BV2 microglia cells, specifically via interactions with ERβ.

4.2. Materials and methods

4.2.1. Cell culture

4.2.1.1. BV2 cell culture

BV2 cells were cultured as described in chapter two (Section 2.6.5.1).

4.2.1.2. **Human embryonic kidney (HEK293) cell culture**

HEK293 cells were cultured as described in chapter three (Section 3.6.1.2).

4.2.2. Preparation of whole cell extracts

This was carried out as described in chapter two (Section 2.6.10.1).

4.2.3. Preparation of nuclear extracts

This was carried out as described in chapter three (Section 3.2.4.1).

4.2.4. Protein quantitation

This was carried out as described in chapter two (Section 2.6.10.2).

4.2.5. Immunoblotting

The procedure for immunoblotting has been described in chapter two (section 2.6.11). The antibodies used in these studies are listed in Table 4.1.

Antibody	supplier	Host	Type	Dilution
Anti-ERα	**Abcam**	**Rabbit**	**Polyclonal**	**1:500**
Anti-ERβ	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:500**
Anti-phospho-p65	**Santa Cruz**	**Rabbit**	**Polyclonal**	**1:500**
Anti-actin	**Sigma Aldrrich**	**Rabbit**	**Polyclonal**	**1:1000**

Table 4.1Primary antibodies used with details of resources and dilution factor used.

4.2.6. Reporter gene assays

This procedure was similar to that described in chapter three (section 3.2.4), with some modifications. The vector used in this case was the Cignal ERE Reporter (luc) (sabiosciences). ERE reporter kit is designed to monitor the activity of oestrogen receptor-induced signal transduction pathways in cultured cells.

4.2.7. Determination of nitrite production (Griess assay).

Griess assay was performed as described in chaper two (section 2.6.7).

4.2.8. Enzyme-linked immunosorbent assay (ELISA) for TNF-α detection.

This was carried out as described in chapter two (section 2.6.8).

4.2.9. Prostaglandin E2 (PGE2) detection enzyme immunoassay (EIA).

This was carried out as described in chapter two (section 2.6.9).

4.2.10. Immunofluorescence

This was carried out as described in chapter two (section 2.6.13) .In this case after the blocking step cells were incubated with (anti-ERα (Abcam), anti-ERβ (Santa Cruz), and anti-p65 antibody (Santa Cruz)) diluted 1:100 using PBS, following overnight incubation at 4°C.

4.2.11. ERβ siRNA transfection

Small interfering RNA (siRNA) is a double-stranded RNA composed of 19~22 nucleotides. When transfected into cells, siRNAs are associated with a protein complex called RNA-induced silencing complex (RISC) which unwind the double-stranded and recruits targeted mRNA leading to cleavage and subsequently degraded (Figure 4.2).

RNA interference (RNAi) is the best way to effectively knock down gene expression to study protein function. To confirm whether oestrogen receptor beta (ERβ) contributed to the anti-inflammatory actions of formononetin in LPS-activated BV2 microglia, ERβ-siRNA was used in investigations.

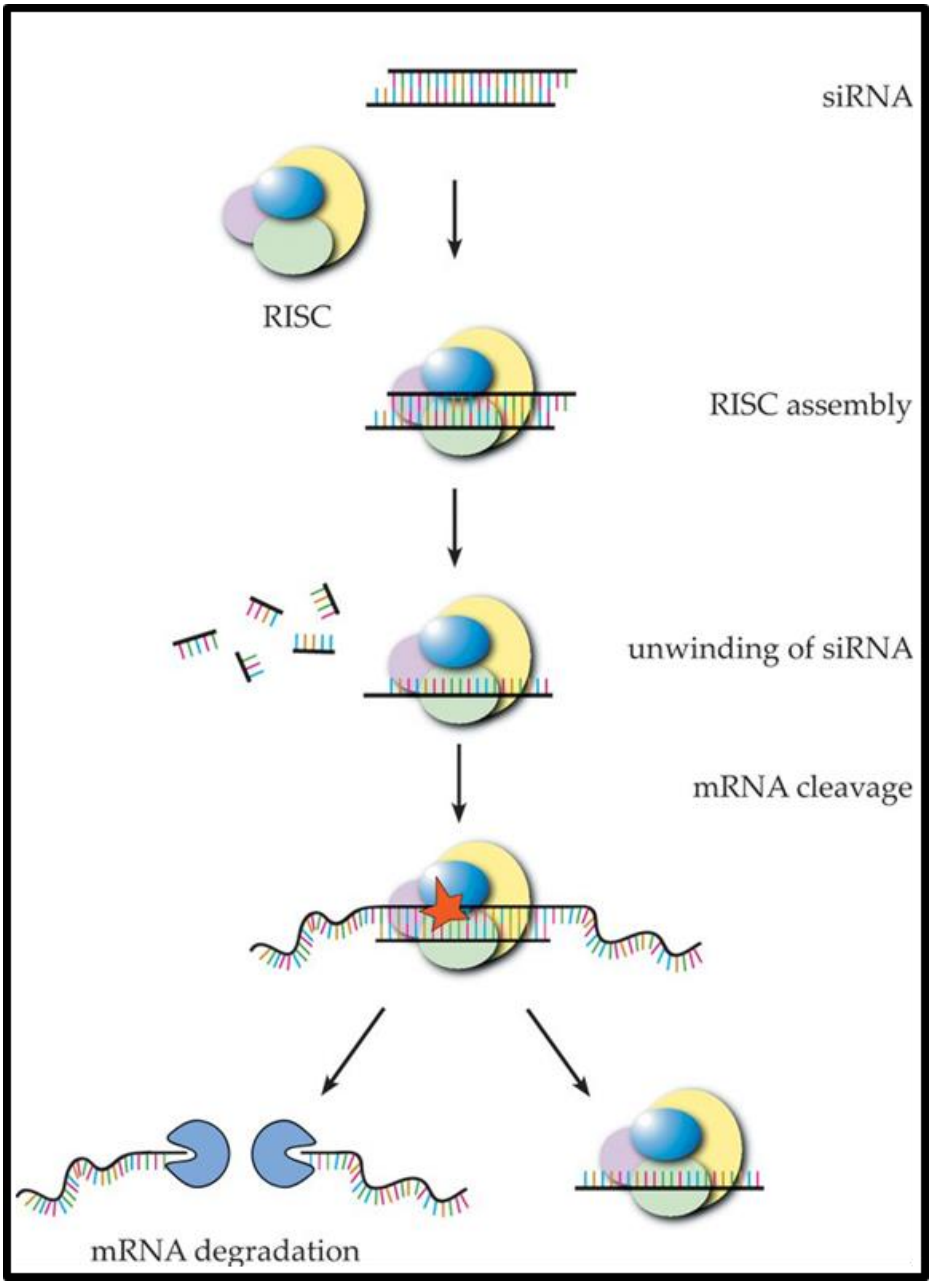


Figure 4.2 mechanism of RNA interference using siRNA.

Small interference RNA (siRNA) is incorporated into RISC, followed by unwinding of the double-stranded molecule by the helicase activity of RISC. The sense strand of siRNA is removed and the antisense strand recruits targeted mRNA, which is cleaved by RISC and subsequently degraded by cellular nucleases. Modified from (Meister et al., 2004).

Small interfering RNAs (siRNAs) targeted at ERβ (Santa Cruz Biotechnology) were used to silence ERβ gene. BV2 cells were cultured in 96 well plate at density of $2x10^5$/ml using antibiotic-free (penicillin and streptomycin) RPMI1640 medium, and incubated at 37°C in a 5% CO_2 incubator until 50% confluent. Thereafter, medium was changed to Opti-MEM reduced serum medium (Thermo Fisher). Then, in an Eppendorf tube (Tube A), 2 µl of ERβ siRNA duplex were diluted into 100 µl of Opti-MEM reduced serum medium. In another tube (Tube B), 2 µl of lipofectamine 2000 transfection reagent (Thermo Fisher) was diluted into 100 µl of Opti-MEM reduced serum medium. The dilutions were mixed gently and incubated for 5 min at room temperature. Next, the contents of tube B were transferred to tube A to prepare the transfection cocktail, which was then incubated for 30 min at room temperature. Next 200 µl of ERβ siRNA transfection cocktail was added to each well and incubated for 6 h at 37°C. Control BV2 microglia was transfected with control siRNA duplex. Following transfection, media was changed in ERβ siRNA and control siRNA-transfected cells to complete media and incubated for a further 18 h. Thereafter, cells were treated with formononetin (10 µM), followed by LPS (100 ng/ml). Levels of nitrite, PGE_2,andTNFα were measured in culture supernatants of both ERβ-silenced and control cells. In addition, protein expression of NFκBp65 sub-unit was 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.

4.3. Results

4.3.1. Effect of formononetin on luciferase-mediated oestrogen-response element activity

In order to investigate the effects of formononetin on ERE-reporter gene activity, HEK293 cells were transfected with ERE reporter plasmid (Cignal ERE Reporter (luc) (Sabiosciences), and then treated with formononetin (2.5, 5.0 and 10µM). Relative luciferase activities were shown as fold change of treated cells. Results revealed that in comparison with control BV2 cells treated with formononetin showed a 5~fold and significantly ($p<0.001$) increased ERE transcriptional activity when the concentration of the compound 10 µM (Figure 4.3) .

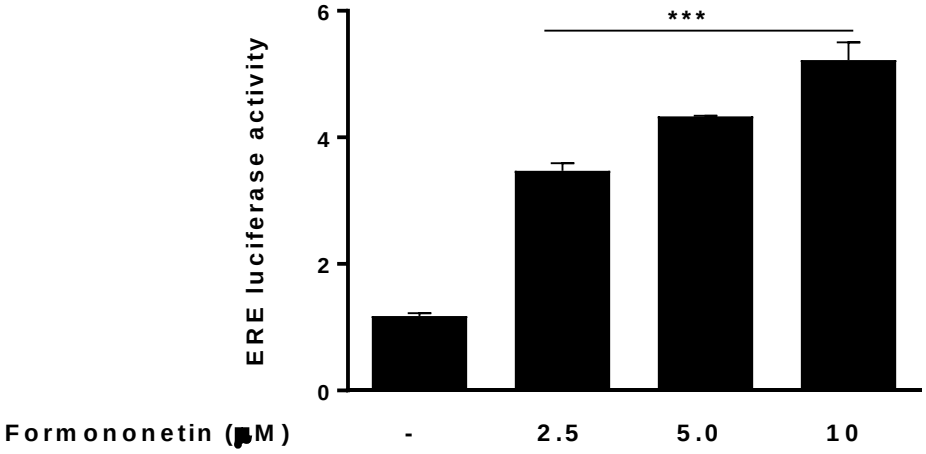


Figure 4.3 Effects of formononetin in the oestrogen-response element ERE transcription activity

Formononetin -induced ERE-dependent gene expression in HEK 293 cells. HEK293 cells were transfected with ERE reporter. After 16 hours of transfection, medium was changed to assay medium (Opti-MEM). After 24 h cells were treated with formononetin (2.5, 5 and 10 µM) for 6 hours. Dual Luciferase assay was performed, and promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization.

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4.4. BV2 microglia cells express high levels of ERβ compared to ERα

To establish whether formononetin has a modulatory action on oestrogen receptors (ERα and ERβ) in BV2 microglia, whole cell lysates were analysed using immunobloting to detect both ERα and ERβ expression. Results in Figure 4.4a show that BV2 microglia expressed ~five times higher ERβ protein than ERα. The expression of ERβ in BV2 cells was further confirmed with immunofluorescence (Figure 4.4b).

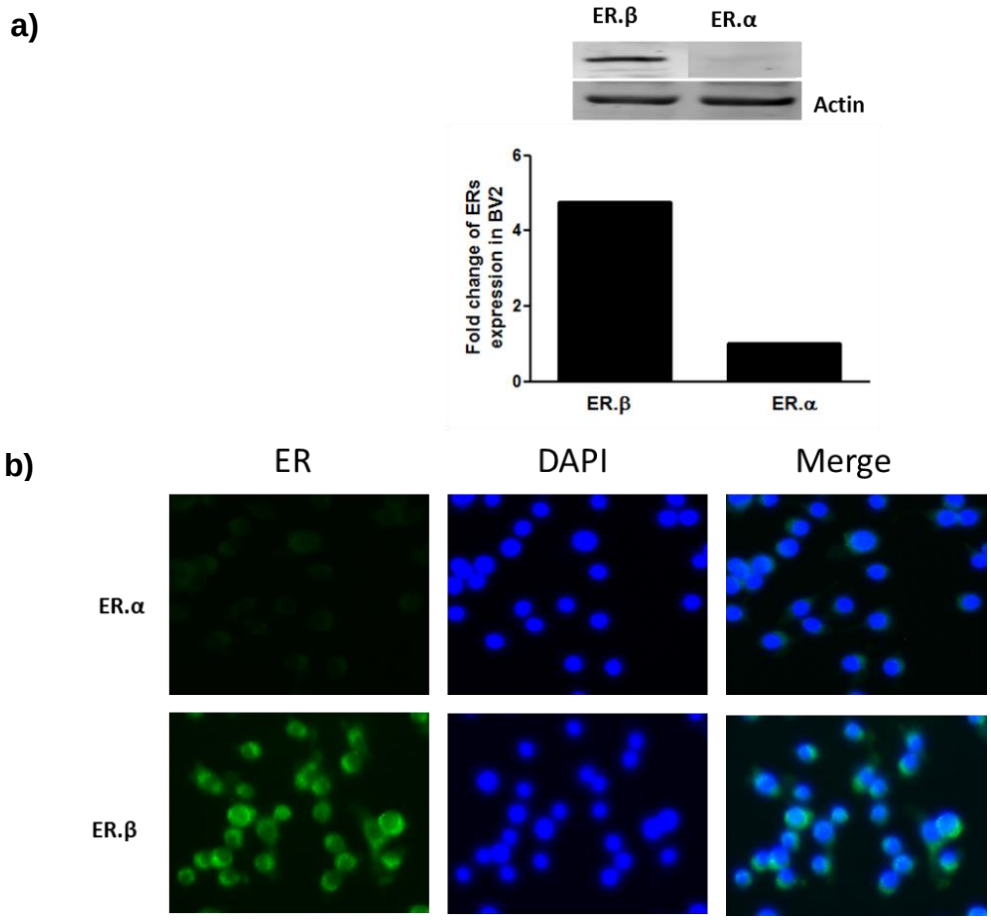


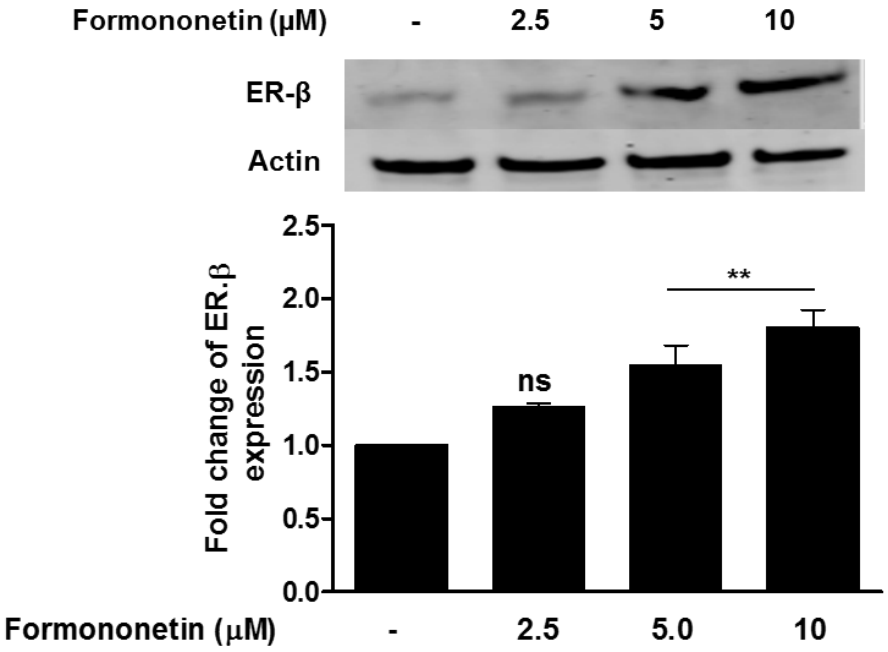
Figure 4.4 Oestrogen receptors ERs expression in BV2 microglia cells.

Identification of ER proteins in BV2 cells by immunoblotting analysis using whole cell extract showed that BV2 microglia express five times more ERβ than ERα (a), which was confirmed by double-fluorescence imaging (b).

4.4.1. Effects of formononetin on ERβ expression in BV2 microglia

To investigate if formononetin increase the level of ERβ protein in BV2 microglia, the cells were treated with different concentrations of the compound followed by immunoblotting to detect ERβ protein. Result show significant ($p<0.01$) increase in the level of ERβ protein with 5.0 and 10 µM concentrations of formononetin, compared to untreated cells (Figure 4.5 a). In addition, double-fluorescence imaging for ERβ expression in BV2 microglia treated with formononetin (2.5, 5.0 and 10 µM) showed an increase in the expression of ERβ compared to untreated control cells (Figure 4.5 b).

a)



b)

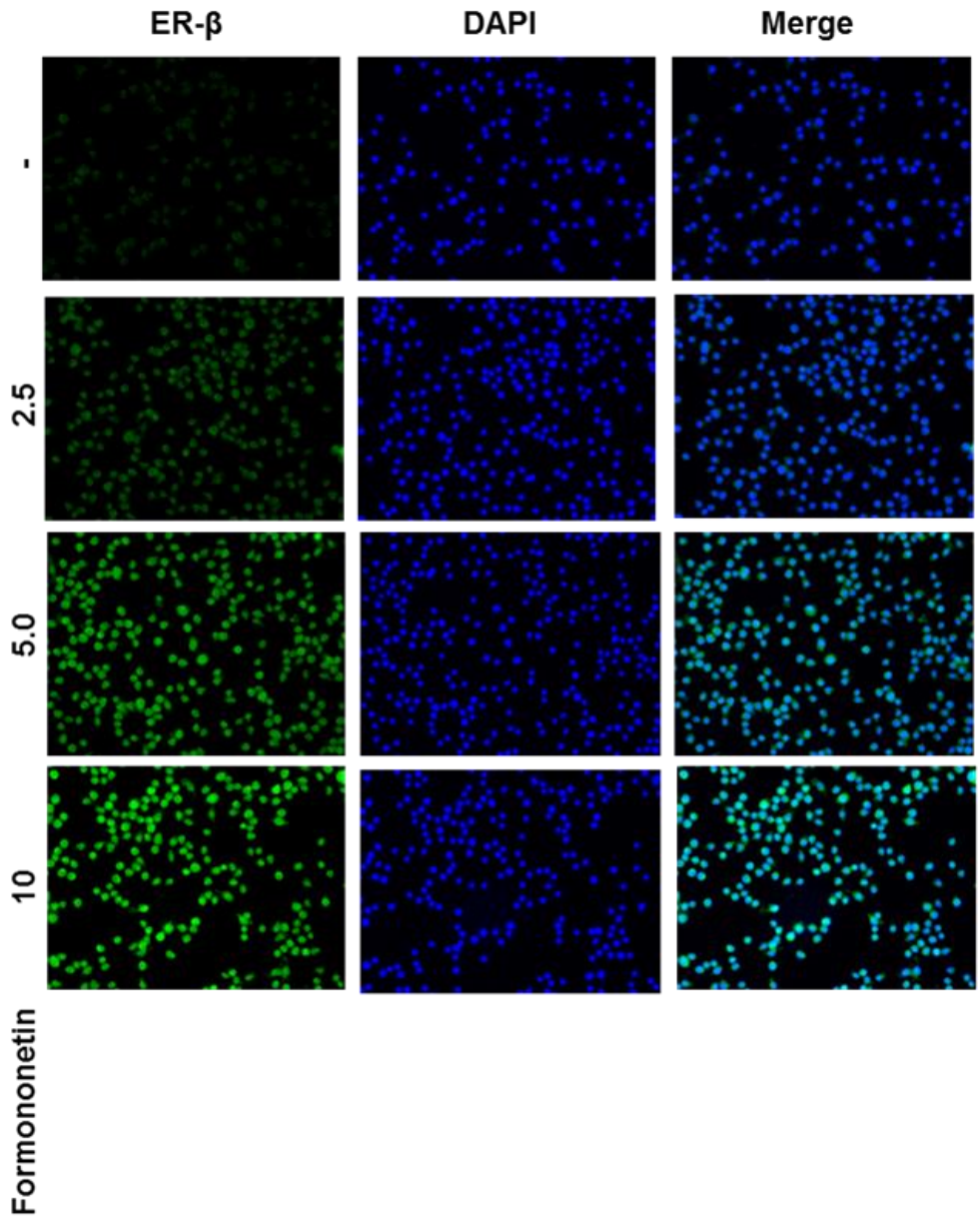


Figure 4.5 Formononetin activate the expression of oestrogen receptor beta (ERβ) in BV2 microglia cells.

(a)Formononetin increased the expression of ERβ in BV2 microglia cells. BV2 cells were treated with formononetin (2.5, 5.0 and 10 μM), followed by immunoblotting for ERβ (b) The expression of ERβ was evaluated by immunofluorescence analysis.

4.4.2. Effects of formononetin on nitrite, PGE_2 and TNFα production in the presence of (ERβ) siRNA in LPS-activated BV2 microglia

In earlier experiments, it was shown that formononetin suppressed the release of pro-inflammatory mediators NO, TNFα and PGE_2 in LPS-stimulated BV2 microglia. Consequently, experiments were performed to find out whether ERβ contributed to these effects.

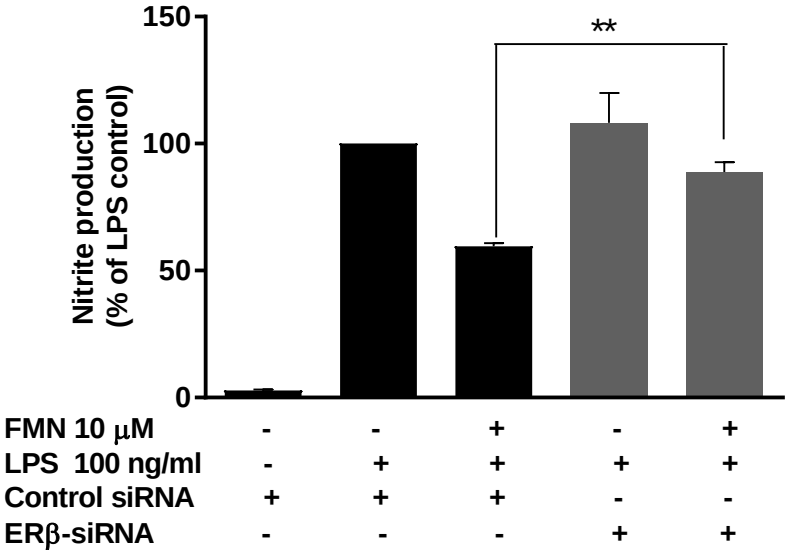
The effect of formononetin (10 μM) on nitrite production in ERβ siRNA-transfected BV2 microglia was investigated, and results show an increase in nitrite production in control siRNA cells stimulated with LPS compared to unstimulated control siRNA cells.However, control siRNA transfected BV2 cells which were pre-treated with formononetin (10 μM) followed by LPS stimulation produced ~ 50% nitrite in comparison with cells stimulated with LPS alone. In the ERβ siRNA transfected cells, which were pre-treated with formononetin (10 μM) there was no inhibition of nitrite production, when compared with control siRNA cells treated with same concentration of formononetin (Figure 4.6a). These suggested that formononetin mightbe suppressing NO production in LPS-activated BV2 microglia through mechanisms linked to ERβ.

The effects of formononetin (10 μM) on TNFα and PGE_2 production were also evaluated in the presence and absence of ERβ gene.

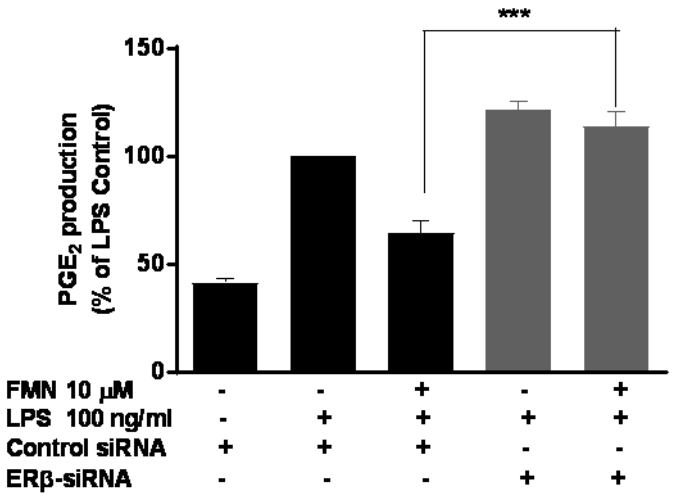
In control siRNA-transfected BV2 cells, which were stimulated with LPS there was a marked release of PGE_2 compared with unstimulated cells. This increase was attenuated when cells were treated with formononetin (10μM). However, formononetin (10 μM) did not affect the production of PGE_2 in ERβ siRNA-transfected BV2 cells (Figure 4.6b).

In addition, the results revealed that formononetin inhibited the production of TNFα in LPS-stimulated control siRNA transfected BV2 cells, however, the same concentration of formononetin (10 μM) lost its ability to suppress the production of TNFα in the absence of ERβ when compared with control cells (Figure 4.6c). These results suggest that the activation of ERβ might contribute to the suppression of pro-inflammatory mediators in LPS-activated BV2 microglial by formononetin.

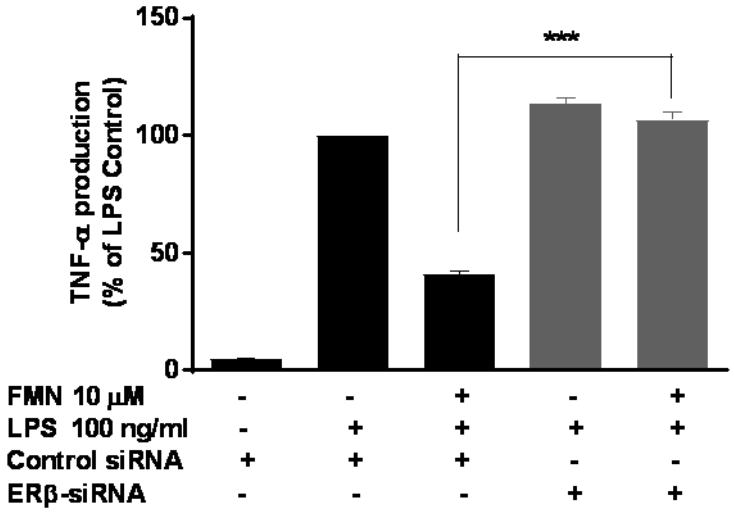
a)


Nitrite production
(% of LPS control)
150
100
50
0
**
FMN 10 μM - - + - +
LPS 100 ng/ml - + + + +
Control siRNA + + + - -
ERβ-siRNA - - - + +

b)


PGE2 production
(% of LPS Control)
150
100
50
0
***
FMN 10 μM - - + - +
LPS 100 ng/ml - + + + +
Control siRNA + + + - -
ERβ-siRNA - - - + +

c)



d)

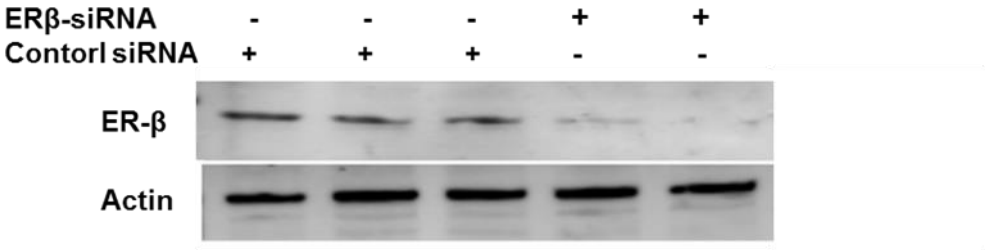


Figure 4.6 Effects of formononetin in nitrite production in the absence of oestrogen receptor beta (ERβ) in LPS-activated BV2 microglia.

Formononetin attenuated nitrite,TNFα and PGE2 production in control siRNA LPS-activated BV2 microglia but not in the ERβ siRNA cells .Control siRNA and ERβ siRNA transfected BV2 microglia were incubated in medium containing formononetin (10 μM) for 30 min and then stimulated with LPS (100 ng/ml) for 24 h. (a) Formononetin diminished nitrite production in the control siRNA and not in ERβ siRNA BV2 microglia. Formononetin did not reduce PGE2 production (b), and TNFα (c), in ERβ siRNA LPS-activated BV2 microglia. (d) Western blot showing ER-β siRNA transfection efficiency.

4.4.3. Effects of formononetin in localisation of NF-κB p65 in the absence of oestrogen receptor beta (ERβ) in LPS-activated BV2 microglia.

In order to understand whether ERβ contributed to the NF-κB inhibitory activity of formononetin in LPS-activated BV2 microglia, immunofluorescence experiments were carried out on cells ,which were transfected with control siRNA or ERβ siRNA followed by treatment with formononetin (10 μM) for 30 min, and then stimulated with LPS (100 ng/ml) for 60 min. At the end of the incubation, cells were stained with antibodies anti-NF-κB p65 antibody. As shown in Figure 4.7, immunofluorescence images revealed that NF-κB p65 was sequestered in the cytoplasm (untreated panel). Furthermore, nuclear accumulation of NF-κB p65 was markedly induced after stimulation with LPS (LPS panel). However, LPS-induced translocation of NF-κB p65 was abolished after pre-treating the cells with formononetin (10 μM) (formononetin panel). On transfecting cells with ERβ siRNA, there was a marked reversal of the anti-inflammatory effect of formononetin on nuclear translocation of NF-□B p65, as shown by an increase in NF □B p65 nuclear translocation compared to control siRNA transfected BV2 cells (Formononetin ERβsiRNA panel).

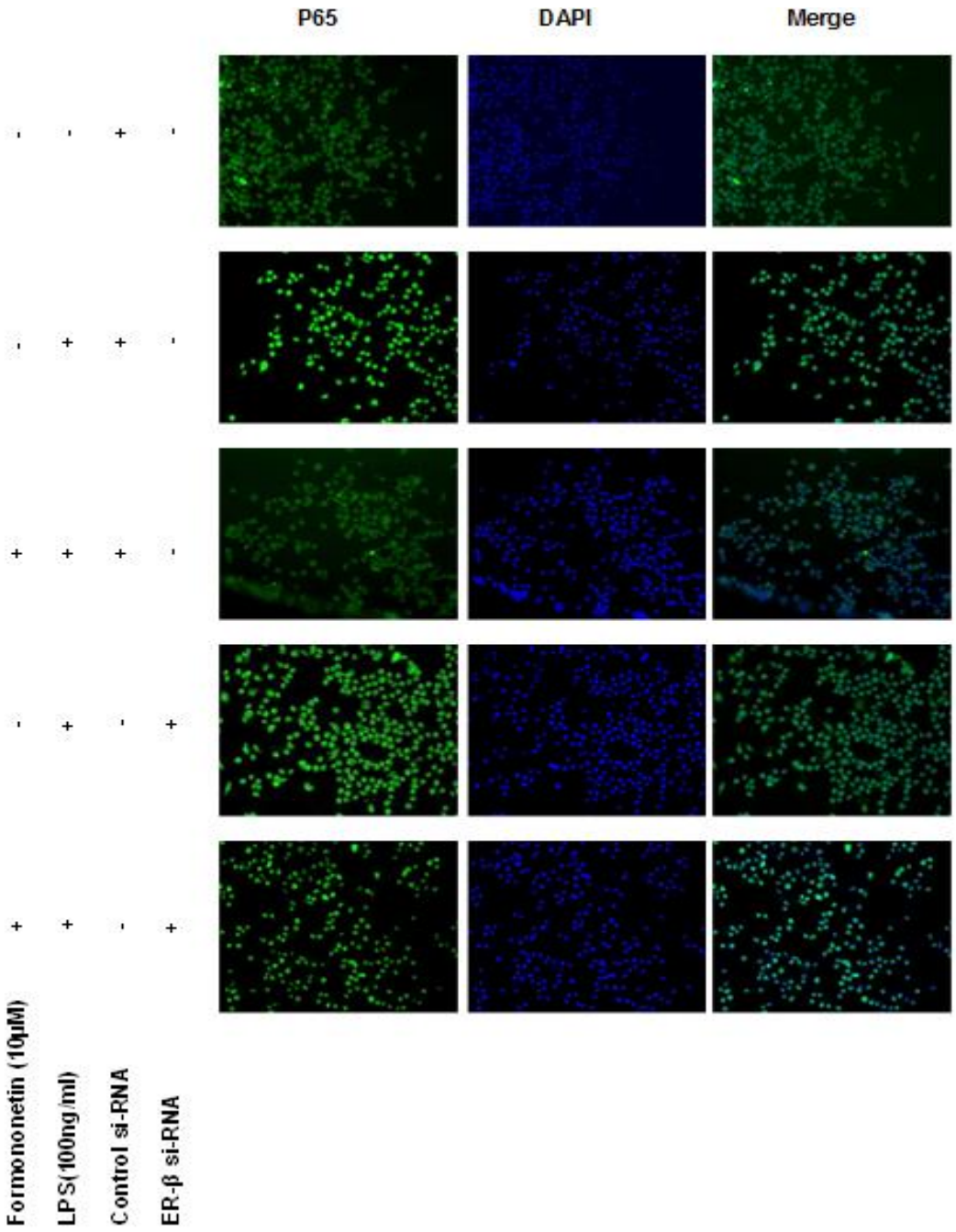


Figure 4.7 Effects of formononetin in localization of NF-κB p65 in the absence of oestrogen receptor beta (ERß) in LPS-activated BV2 microglia.

Localization of NF-κB p65 was visualised with fluorescence microscopy after immunofluorescence staining with NF-κB p65 antibody (green). Cells were stained with DAPI to visualize nuclei (blue). Results are representative of those obtained from three independent experiments.

4.5. Discussion

Some studies showed that the biological actions of formononetin are similar to that of oestrogen (Howes et al., 2002; Wang & Han, 2012). Consequently, the hypothesis in this chapter was to establish a link between the anti-inflammatory activities of the compound in BV2 microglia and its ability to modulate the activity of microglial oestrogen receptor.

Experiments conducted with HEK293 in this study revealed that formononetin activated oestrogen response element dependent transcription. These results suggest that oestrogen responses was potentiated by formononetin through one of the two oestrogen receptors (ERα or ERβ).

To establish whether formononetin has a modulatory action on protein levels of oestrogen receptors in the microglia, firstly the interest was to define the subtype of oestrogen receptors in BV2 microglia. Interestingly, the results showed that BV2 microglia expressed ERβ oestrogen receptor rather than ERα. This was consistent with earlier reports showing that oestrogen reduced production of pro-inflammatory mediators from BV2 microglia through interactions with ERβ (Baker et al., 2004; Wu et al., 2013).

These observations were consistent with results which showed that ERβ was implicated in cell survival in the developing and aging brain, and that ERβ knock-out mouse developed major brain malformations due to neuronal loss during ageing (Wang et al., 2003). Furthermore, ERβ has been reported to be localised to the mitochondria of many cells including neurons, and has been suggested to play a role in neuroprotection (Yang et al., 2004). Interestingly, studies in primary astrocyte revealed that compounds which bind ERβ are effective in preventing the release of pro-inflammatory cytokines (Lewis et al., 2008), further suggesting that this receptor has a potential role in neuroprotection.

Having established in this study that the ERβ subtype of oestrogen was expressed in BV2 microglia, further experiments were conducted and revealed that formononetin increased the levels of ERβ protein in BV2 microglia. Several studies have demonstrated that some phytoestrogens, such as daidzein and genistein, have been shown to be ligands at ERβ (Meyers et al., 2001; Muthyala et al., 2004). In trustingly,

studies in both *in vitro* cell cultures and *in vivo* animal models revealed that the phyto-β-SERM formulation (genistein, daidzein,and equol) produced an 83-fold binding preference for ERβ over ERα and showed neuroprotection in the brain, while being devoid of feminising activity as seen with non-selective estrogenic compounds such as 17β-oestradiol in the reproductive system (Zhao et al., 2009; Zhao, 2013).

Following the observations which established that formononetin increased ERβ protein in BV2 microglia, it became necessary to determine the role played by the receptor in the earlier observed showing inhibition of neuroinflammation by the compound in BV2 microglia (Chapters 2 and 3). Results revealed that inhibition of neuroinflammation by formononetin was clearly lost in the absence of ERβ gene. Further investigations revealed that ERβ-dependent inhibition of neuroinflammation by formononetin was possibly related to cross talk between ERβ and NF-κB.Further investigations revealed that ERβ-dependent inhibition of neuroinflammation by formononetin was possibly related to cross talk between ERβ and NF-κB.

Studies have shown that ERβ functions as a gate-keeper for NF-κB signalling by repressing its expression and nuclear translocation, thus regulating its signalling in prostate cancer (Mak et al., 2015). While a regulatory role of ERβ on NF-κB signalling in the microglia is unclear, it would appear from the data generated in this study that the ability of formononetin to affect the nuclear accumulation of NF-κB depends on some sort of interaction with ERβ. Interestingly, a recent study showed that the seed extract of Cicer microphyllum (which contains biochanin A, formononetin and genistein) produced ERβ-mediated neuroprotective activity in Sprague Dawley rats (Sharma et al., 2017). The exact nature of the interactions between ERβ and NF-κB therefore needs further investigation. Furthermore, it would be interesting to discover the exact step in the NF-κB signalling pathway, that is negatively affected by ERβ, as this would further our knowledge on identifying novel oestrogenic compounds in neuroinflammation and/or neurodegeneration.

Chapter Five

General discussion and conclusion

5.1. Discussion

Recent studies have clearly shown that oestrogen-like compounds, which inhibit neuroinflammation, provide therapeutic opportunities in neurodegenerative disorders like AD. This study investigated the inhibition of neuroinflammation by formononetin in BV2 microglia stimulated with LPS, and found that the compound suppressed production of the pro-inflammatory cytokines TNFα, IL-6 and IL-1β from the cells. 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. It also showed that pre-treatment of BV2 microglia with formononetin prevented microglia conditioned medium-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 (Leyns et al., 2017) and PD (Kaur et al., 2017). While it is widely recognised that diverse neuroinflammatory signals contribute to the pathogenesis of these conditions, results from this research 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 RAW 264.7 cells (Lai et al., 2013) as well as pro-inflammatory cytokine production in bone marrow-derived dendritic cells (W. Li et al., 2014). Furthermore, formononetin was reported to inhibit both TNFα and NO production from neuron-glia cultures and microglia-enriched cultures (Chen et al., 2008). This study 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 BV2 microglia 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 (Srinivasan et al., 2015). 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, a reporter gene assay results showed that formononetin inhibited the transcriptional activity of NF-κB in general. It was further established that in LPS-stimulated BV2 microglia, formononetin inhibited critical 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. Inhibition of IL-1β-induced NO/iNOS and apoptosis in INS1 pancreatic beta cells by formononetin has been attributed to its ability to inhibit the activation of NF-κB (Wang, 2012). Inhibition of H_2O_2-induced apoptosis in retinal ganglion cells by formononetin has also been linked to inhibition of NF-κB activation (Jia et al., 2014). Furthermore formononetin was reported to inhibit NF-κB signalling pathway in bone-marrow-derived macrophages stimulated with RANKL (Huh et al., 2014). Biochanin A is a closely related phytoestrogen to formononetin which has been reported to inhibit neuroinflammation in the microglia (Chen et al., 2007; Wu et al., 2015). However, 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.

Mitogen-activated protein kinases MAPK signalling pathways have been shown to regulate diverse biological functions such as proliferation, differentiation , apoptosis and inflammation in various cell types (Kyriakis et al., 2012). The role of (MAPKs) in neuroinflammation and neurodegenerative diseases is well established (Munoz et al., 2010). The major MAPK pathway subfamilies involved in the regulation of COX-2, iNOS and cytokine synthesis in LPS activated microglia are signalling proteins such as p38 MAPK, extracellular signal regulated kinases (ERK1/2) and c-jun-N terminal kinase (JNK) (Bhatia et al., 2017).

The phosphorylation of p38 MAPK induces the expression of proinflammatory molecules and is one of the important signaling pathways during inflammation (Chaves et al., 2013; Swaroop et al., 2016). Phytoestrogens have been shown to regulate the activation of p38 MAPK in different cell lines (Sobenin et al., 2016; Maxwell et al., 2017). Also several studies have suggested that some phytoestrogens block the

phosphorylation of p38 MAPK in LPS-activated BV2 microglia (Kang et al., 2014; Wu et al., 2015). These findings were confirmed in this study, which demonstrated that formononetin produced significant inhibition of p38 phosphorylation in LPS-activated BV2 microglia.

Activation of MAPKs in microglia leads to the phosphorylation of the transcription factor, cJun, which contributes to the activation of microglia via the transcription of its target genes TNFα, IL-1β, COX-2 and MCP-1 (Waetzig et al., 2005). Furthermore, JNK has been implicated in the pathogenesis of various neurodegenerative diseases such as Parkinson's disease (Brecht et al., 2005) and Alzheimer's disease (Colombo et al., 2007). This study has demonstrated that formononetin inhibits the phosphorylation of JNK in LPS-activated BV2 microglia. The outcome of this study is consistent with a recent study which showed that a phytoestrogen genistein protects PC12 cells from Aβ25–35 induced neurotoxicity by inhibiting JNK activation (You et al., 2017). Another study showed that Biochanin A attenuates LPS-induced pro-inflammatory responses and inhibits the activation of the JNK in BV2 microglial cells (Wu et al., 2015).

There are numerous reports demonstrating that phosphorylation of ERKs, is necessary for up-regulation of iNOS and COX-2 in LPS-stimulated BV-2 cells (Oh et al., 2009; Kim et al., 2013), Kaempferol a phytoestrogen acts through ERK/MAPKs to elicit protection in a model of neuroinflammation in BV2 microglial cells (Park et al., 2011). This study showed that formononetin inhibited the phosphorylation of ERK1/2 in LPS-activated BV2 microglia. Mixed-lineage protein kinase 3 (MLK3) has been associated in multiple signaling cascades, including the extracellular signal-regulated kinase, c-Jun NH2-terminal kinase (JNK), and p38 MAP kinase pathways (Patrick et al., 2001; Kim et al., 2004). Inhibition of MLK3 is a potential strategy for treatment of neuroinflammation (Ronkina et al., 2010). This study proved that formononetin modulates the activation of MLK3, an action which may account for its impact on downstream targets, JNK and p38.

Transforming growth factor b-activated kinase 1 (TAK1) a member of the MAPK kinase kinase functions as an upstream signalling molecule of NF-κB and MAPKs. Activated TAK1 complex phosphorylates IKK, which activates NF-κB. It is thought that targeting TAK1 activation may be an attractive strategy to treat inflammatory responses (Endale

et al., 2017). Results of this study suggest that formononetin blocked TAK1 activation during neuroinflammtion, which explains its dual activities on both NF-κB activation and MAPKs pathways.

Several studies have suggested anti-neuroinflammatory actions of oestrogens 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 and associated with oestrogen has led to investigations of neuroprotective selective oestrogen receptor modulators (SERMs) and phytoestrogens which may act as selecetive oestrogen enzyme modulators (SEEMs). Considering the reported estrogenic nature of formononetin and results from this research, which demonstrated inhibition of neuroinflammation by the compound, the role of oestrogen receptors in its anti-inflammatory activity in the microglia was explored and proven.

The potential of formononetin in neurodegenerative disorders, either as a therapeutic entity in its own right, or as a chemical scaffold for producing novel therapeutic compounds, depends on the ability to cross the blood-brain barrier (BBB) and act in the brain. Although this study clearly demonstrated neuroprotective activity for formononetin *in vitro*, its therapeutic potential will be further enhanced with investigations,which would provide evidence of BBB permeation.

A major limitation with oestrogenic 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 separate line of investigation was initiated in the research group to provide further information on the question of whether formononetin could induce proliferation of human breast cancer cells and ovarian cancer cells (Figure 5.1).

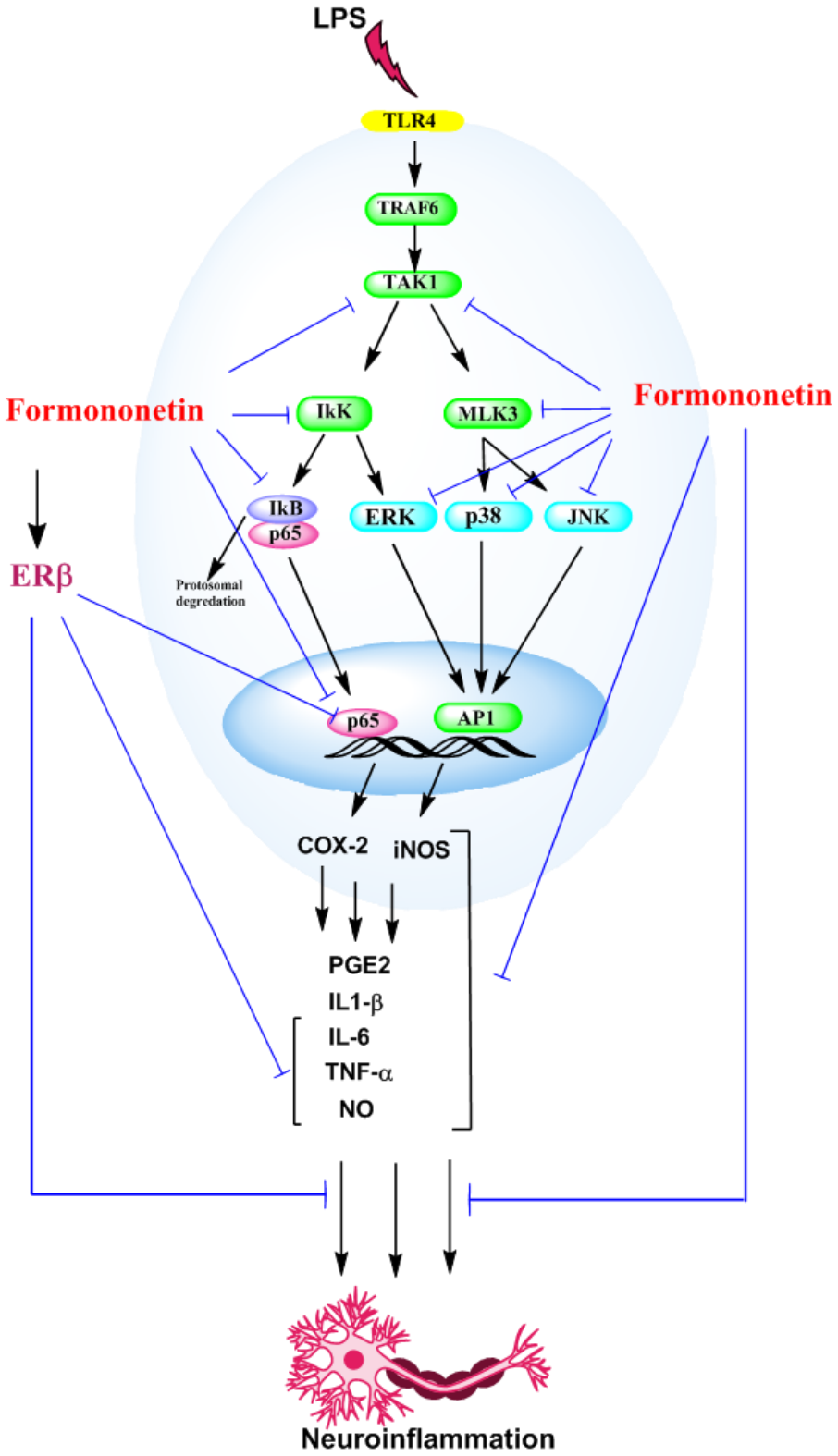


Figure 5.1 Proposed mechanism of action of formononetin in LPS-activated BV2 microglia

5.2. Conclusion

These investigations have provided an enough evidence to conclude that formononetin inhibits neuroinflammation through a dual molecular targeting of NF-κB, and possibly MAPKs signalling pathways in BV2 microglia that have been stimulated with LPS. Also, for the first time this thesis demonstrates a potential link between activation of ERβ and inhibition of NF-κB-mediated neuroinflammation by formononetin.

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Published Papers and posters

Papers:

El-Bakoush, A., & Olajide, O. A. (2018). Formononetin inhibits neuroinflammation and increases estrogen receptor beta (ERβ) protein expression in BV2 microglia. International Immunopharmacology, 61, 325-337.

-Posters

-ABDEL ELBAKOUSH, OLUMAYOKUN OLAJIDE. Formononetin, A Phytoestrogen In Red Clover Inhibits Neuroinflammation In LPS-activated Microglia. 084P Queen Elizabeth II Conference Centre London Pharmacology 2014. http://www.pa2online.org/abstract/abstract.jsp?abid=32436&kw=BV2&author=ELBAKOUSH&cat=-1&period=-1

-Abdel, E. Olumayokun,O, NF-κB-mediated inhibition of neuroinflammation by formononetin: role of ERβ. 235P Queen Elizabeth II Conference Centre London Pharmacology 2015.

http://www.pa2online.org/abstract/abstract.jsp?abid=32894&kw=BV2&author=ELBAKOUSH&cat=-1&period=-1

-Abdel M. Elbakoush and Dr. Olumayokun A. Olajide.Formononetin Prevents Neuroinflammation-Mediated HT22 Neuronal Death. PM053 The British Neuroscience Association (BNA), Birmingham 2017.

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