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THE EFFECT OF PPARγ ACTIVATION BY PIOGLITAZONE ON THE LIPOPOLYSACCHARIDE-INDUCED PGE_2 AND NO PRODUCTION: POTENTIALUNDERLYING ALTERATION OF SIGNALING TRANSDUCTION

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
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Lexington, Kentucky

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Lexington, Kentucky

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Microglia-mediated neuroinflammation plays an important role in the pathogenesis of Parkinson's disease (PD). Uncontrolled microglia activation produces major proinflammatory factors including cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) that may cause dopaminergic neurodegeneration. Peroxisome proliferator-activated receptor γ (PPARγ) agonist pioglitazone has potent anti-inflammatory property. We hypothesize pioglitazone protects dopaminergic neuron from lipopolysaccharide (LPS)-induced neurotoxicity by interacting with relevant signal pathways, inhibiting microglial activation and decreasing inflammatory mediators.

First, the neuroprotection of pioglitazone was explored. Second, the signaling transductions such as jun N-terminal kinase (JNK) and the interference with these pathways by pioglitazone were investigated. Third, the effect of pioglitazone on these pathways-mediated PGE_2 / nitric oxide (NO) generation was investigated. Finally, the effect of PPARγ antagonist on the inhibition of PGE_2 / NO by pioglitazone was explored. The results show that LPS neurotoxicity is microglia-dependent, and pioglitazone protects neurons against LPS insult possibly by suppressing LPS-induced microglia activation and proliferation. Second, pioglitazone protects neurons from COX-2 / PGE_2 mediated neuronal loss by interfering with the NF-κB and JNK, in PPARγ-independent mechanisms. Third, pioglitazone significantly inhibits LPS-induced iNOS / NO production, and inhibition of LPS-induced iNOS protects neuron. Fourth, inhibition p38 MAPK reduces LPS-induced NO generation but no effect is found upon JNK inhibition, and pioglitazone inhibits p38 MAPK phosphorylation induced by LPS. In addition, pioglitazone increases PPARγ phosphorylation, followed by the increased PI3K/Akt phosphorylation. Nevertheless, inhibition of PI3K increases LPS-induced p38 MAPK phosphorylation. Inhibition of PI3K eliminates the inhibitive effect of pioglitazone on the LPS-induced NO production, suggesting that the inhibitive effect of pioglitazone on the LPS-induced iNOS and NO might be PI3K-dependent.

In summary, the results suggested that pioglitazone protects neurons by inhibiting microglia-mediated COX-2 and iNOS, by differentially interacting with LPS-induced JNK, NF-kB, and p38 MAPK signaling. Particularly, pioglitazone reduces JNK-mediated COX-2 / PGE_2 and p38 MAPK-mediated iNOS / NO. It is worth noting that the inhibitive effect of pioglitazone on LPS-induced NO might be dependent on the PI3K activation. In addition, our results suggest that both PPARγ-dependent and PPARγ-independent mechanisms might be involved in the inhibitive effect of pioglitazone on LPS-induced PGE_2 and NO generation.

KEYWORDS: microglia, dopaminergic neurons, cytokines, signaling pathways, pioglitazone

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This dissertation is dedicated to my beautiful wife Qing, whose great patience throughout the duration of my graduate studies enabled me to pursue the research contained herein and whose unconditional support and encouragement enabled me to complete it.

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CHAPTER 1: Introduction

Idiopathic Parkinson's disease (IPD) is a common neurological progressive movement disorder with an incidence of 1-3% among the population (Bennett et al., 1996; Lang and Lozano, 1998). The primary pathology is the significant loss of pigmented dopaminergic neurons in the substantia nigra (SN) and accordingly gradual depletion of striatal dopamine, leading to its cardinal movement symptoms: resting tremor, rigidity, slowed movement (named bradykinesia), and postural instability. As of now, pharmacological treatment of PD such as levodopa and dopamine agonists can only suppress symptoms. Long-term treatment will develop the medication-induced complications such as dyskinesias characterized by involuntary and excessive movement. So it is very important to understand the molecular mechanisms underlying its pathogenesis. Several neurotoxins, including 6-hydroxydopamine (6-OHDA) (Ungerstedt, 1968), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1984), LPS (Castano et al., 1998), and rotenone (Betarbet et al., 2000) have been used to study its pathological process.

Although its etiology is still not completely known, chronic neuroinflammation, oxidative stress, apoptosis, stress, mitochondrial and proteasomal dysfunction are implicated in its pathogenesis (McGeer et al., 1988b; McGeer et al., 1988a; Hirsch et al., 1997; Jenner, 1998; Greenamyre et al., 1999; Olanow and Tatton, 1999). Indeed, up-regulated inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are observed in the postmortem PD brain samples (Knott et al., 2000; Teismann et al., 2003b; Teismann et al., 2003a), and are associated with the death of dopaminergic neurons *in vitro* (Okuno et al., 2005; Lin et al., 2007) and *in vivo* (Liberatore et al., 1999; Teismann and Ferger, 2001; Arimoto and Bing, 2003; Vijitruth et al., 2006).

Peroxisome proliferator-activated receptor γ (PPARγ) activation has been known to negatively mediate microglial and macrophage activation (Jiang et al., 1998; Ricote et al., 1998; Bernardo and Minghetti, 2006; Drew et al., 2006), which may play a central role in the dopaminergic neuronal death. Recently, there have been a few studies demonstrating that activation of PPARγ with its agonists protects dopaminergic neurons against MPTP and LPS insult (Breidert et al., 2002; Dehmer et al., 2004; Hunter et al.,

2007), however, the underlying mechanisms of neuroprotection with PPARγ agonists is not completely known. This part of the dissertation will seek to examine the effect of PPARγ activation with its synthetic ligand pioglitazone on the LPS-induced up-regulation of COX-2 and iNOS, to explore the potentially differential role of its activation in dopaminergic neurons and microglial cells, and to investigate the role of signaling transduction pathways (p38 MAPK, JNK, and PI3K/Akt) involved in the LPS-induced PGE_2 / nitric oxide and their interference with PPARγ agonist. The first specific aim was to investigate whether LPS-induced dopaminergic neuronal loss is microglia-dependent or not by utilizing various primary rat cell culture systems. The second aim focused on the role of PPARγ activation in the signaling pathways associated with the LPS-induced PGE_2 and NO production. This chapter summarizes the relevant studies on the LPS and PPARγ, its interference with cytotoxic molecules including cytokines and proinflammatory factors, and its potential to be used in the neurodegenerative diseases.

Introduction to LPS studies relevant to Parkinson's disease

LPS is a strong inducer of neuroinflammation and oxidative stress. It showed that intranigral LPS injection in rats (2μg, from *E. coli*) induces a decrease in tyrosine hydroxylase (TH) positive immunoreactivity with a decrease in the dopamine level on the sites of both SN and striatum, which are accompanied with microglia activation (Castano et al., 1998). A further study from same group demonstrated that the neurotoxicity of intranigral LPS injection is specific to dopaminergic neurons, compared with serotoninergic and GABAergic neurons (Herrera et al., 2000). All above studies implicated an important role of microglia activation in the dopaminergic neuronal death. An LPS *in vitro* rat model showed that LPS (4μg/ml) did not induce dopaminergic neuronal loss in a primary mesencephalic culture. The presence of added microglia, however, was associated with the significant loss of dopaminergic neurons. In addition, their study suggested that nitric oxide may mediate microglia-induced dopaminergic neuronal death (Le et al., 2001), consistent with the results from intranigral LPS injection study in which the role of microglial iNOS was implicated (Arimoto and Bing, 2003). On the other hand, an LPS infusion PD model provided evidence that activated microglia-induced superoxide may play a critical role in dopaminergic degeneration (Gao et al., 2002). A few *in vitro* studies linked the activated MAPKs cascades with the death

of dopaminergic neurons (Lund et al., 2005; McLaughlin et al., 2006). In their study, pretreatment of CEP1347, an inhibitor for mitogen-activated protein kinase kinase kinase (MAPKKK), suppressed LPS-induced phosphorylation of p38 MAPK and JNK which are downstream to MAPKKK, suggesting the involvement of MAPKs during the inflammatory response in the microglia. Very recently, results from an LPS intranigral model showed that co-injection of LPS with p38 MAPK inhibitor rescued dopaminergic neurons in the SN, accompanied with reduced iNOS expression (Ruano et al., 2006). Two years ago, people observed that rat intranigral LPS injection induces COX-2 expression and PGE_2 synthesis in the SN (de Meira Santos Lima et al., 2006), consistent with previous results from other studies of PD animal models (Feng et al., 2002; Teismann et al., 2003b; Sanchez-Pernaute et al., 2004; Vijitruth et al., 2006). Almost at the same time, a coculture study on mice primary microglia and rat primary neurons upon LPS insult suggested that the LPS-induced PGE_2 level is increased in a microglia density-dependent manner (McLaughlin et al., 2006). The above studies strongly suggested that uncontrolled activated microglia-mediated inflammation can be detrimental to dopaminergic neurons, probably via the activation of MAPKs pathways and increasing iNOS / COX-2 expression. However, the direct relationship between activated microglia-mediated neuroinflammation and the dopaminergic neuronal degeneration is still not completely investigated. Both LPS *in vivo* and *in vitro* studies can not rule out the neuroprotective and / or neurotoxic role of astrocytic cells under certain conditions since either primary mesencephalic cultures or primary mesencephalic-glia cultures were used in their studies. In addition, the biochemical responses of neuronal and microglia cell lines to neurotoxin LPS can not be easily compared with the responses from the primary cells. The following Table 1.1 summarizes LPS in vitro and in vivo studies relevant to Parkinson's disease.

Table 1. 1 Summary of LPS *in vivo* and *in vitro* studies relevant to Parkinson's disease. (page 4-5)

Model	Doses	Relevant findings	Reference
Intranigral injection Wistar rats	2µg	Decreased TH+ cells Decreased DA level Microglia activation	(Castano et al., 1998)
Intranigral injection Wistar rats	2µg	LPS neurotoxicity is specific to DA neurons Microglia activation	(Herrera et al., 2000)
Coculture of primary mesencephalic and primary microglia (rats)	4 µg/ml	TH+ cell loss when microglia added.	(Le et al., 2001)
Intranigral injection SD rats	5µg	iNOS protein increased in SN. iNOS inhibitor L-NNA injection in SN rescued neurons	(Arimoto and Bing, 2003)
Infusion LPS Fisher rats	5ng/hr x 2 weeks	Microglia activation preceded selective neuronal loss. Superoxide might be important	(Gao et al., 2002)
LPS *in vitro* E15 rat	10-80µg/ml	Selective neuronal loss iNOS inhibitor L-NIL did not rescue neurons.	(Gayle et al., 2002)
Single Intraperitoneal injection SD female rats E10.5, pups killed at p21	1mg/kg	Decreased DA neurons Decreased DA level	(Ling et al., 2002)
Intranigral injection into NADPH deficient mice	5µg	NADPH oxidase-generated ROS mediates neurotoxicity	(Qin et al., 2004)
Microglia cell line (BV-2)	100ng/ml	MAPKs pathway might be involved	(Lund et al., 2005)
Primary mesencephalic culture; microglia culture, PC12 cell culture	1-100ng/ml	Caspase-3 activation in dopaminergic neuronal loss	(Wang et al., 2005)

Mouse macrophage cell line RAW 264.7, dopaminergic cell line SH-SY5Y	10ng/ml	NO-dependent neurotoxicity	(Shavali et al., 2006)
Intranigral injection	1 μg	Inhibition of p38 MAPK decreased LPS-induced iNOS	(Ruano et al., 2006)
Intraperitoneal injection TNFR1/R2 deficient mice	5mg/kg	Microglia activation and deceased TH+ cells. TNFR1/R2-/- resistant to the increase of TNFα in brain	(Qin et al., 2007)
Intrastriatal injection SD rats	16-60 μg LPS 20mg/kg; orally administrated pioglitazone	Pioglitazone rescued TH+ neurons with inhibition of iNOS and COX-2. Pioglitazone suppressed protein oxidation	(Hunter et al., 2007)

Table 1.1 continued

* DA = dopamine; L-NNA= N^G-nitro-L-Arginine; L-N^G-Nitroarginine, NOS inhibitor; i.c.v = intracerebroventricular; TNFR1/R2 = Tumor necrosis factor α receptor 1 / 2; L-NIL = dihydrochloride, relative selective inhibitor of iNOS

General introduction to PPARs

PPARs are ligand-inducible transcription factors belonging to the superfamily of nuclear receptors. There are three isotypes identified as of now. They are structurally homologous: PPARα (NR1C1) (Issemann and Green, 1990), PPARβ (NR1C2), and PPARγ (NR1C3) (Michalik and Wahli, 1999; Torra et al., 2001). PPARs play a central role in the regulation of lipid metabolism and glucose homeostasis, well-known effects that have been used to develop drugs for treatment of type II diabetes (Auwerx, 1999).

Their differential physiological functions are related to their distinct expression and localization in a variety of cell types and tissues. Although they are mainly expressed in the tissues with high fatty acid metabolism including liver and adipose tissue, the expression of all three isotypes are observed in neurons. Recently, there has been much interest in the potent inhibitive effects of PPARs on inflammation and oxidative

stress, suggesting their role in treating neurodegenerative diseases (Heneka et al., 1999; Combs et al., 2000; Heneka et al., 2000; Sastre et al., 2003; Bernardo et al., 2005; Heneka et al., 2005).

Distribution of PPARγ and other isotypes

Developmentally, PPARγ transcription was observed on gestation of day 13.5 in the CNS and on 18.5 in the brown adipose tissue in rat and mouse (Heneka and Landreth, 2007). High levels of PPARα expression are found in the liver, kidney, intestine, skeletal muscle, and the pancreas. In contrast, PPARβ is expressed ubiquitously and earlier during fetal development (more detail in Heneka review (Heneka and Landreth, 2007)). In adult rodent including rat and mice, the distribution of PPARγ and PPARα is similar to their expression observed in the fetal period, and PPARβ transcript is more abundant than the PPARα and γ transcripts.

However, the expression of PPARs is less understood during human development. A few studies have showed that human PPARγ is also abundantly expressed in adipose tissue, with lower levels in skeletal muscle, the heart, and the liver. Noticeably, it is present in the macrophages (Huang et al., 1999; Barish et al., 2005). PPARα is most highly expressed in tissues with fatty acid catabolism like PPARγ. Similar to the expression pattern observed in the rodent, PPARβ expression is ubiquitous.

Importantly, expression of all three PPARs is observed in the CNS during late rat embryogenesis and in the adult, of which the PPARγ is found dominantly in microglia. In astrocytes all three PPAR isotypes are found with different degrees. The following table 1.2 summarizes PPAR distribution in the peripheral tissues and in the CNS.

Table 1. 2 Distribution and functions of PPARs in mammal tissues.

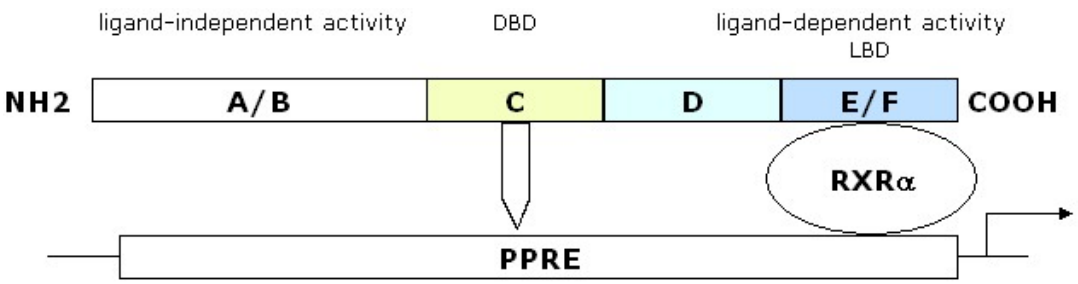
Isotype	Brown adipose tissue	White adipose tissue	Kidney	Intestine	Heart	Muscle	Liver	Pancreas	Rat CNS (all isotypes express in brain except following areas or cells)	Functions
PPARα	+++	+	++	++	++	++	+++	++	Hypothalamus (-) VTA (-) Purkinje cell (-) Granule cell (-) Oligodendrocyte (-)	Lipid Catabolism; Anti-inflammation
PPARβ	++	++	++	++	++	+++	++	No study		Lipid homeostasis; Oligodendrocyte differentiation? Anti-inflammation
PPARγ	++	+++	++	++	++	++	++	No study	VTA (-) Purkinje cell (-) Telencephalon (-) Oligodendrocyte (-)	Lipogenesis; Anti-inflammation

VTA = ventral tegmental area; (-) means not found. Empty cell means no study was found.

Based on: (Moreno et al., 2004)

PPAR structure and putative mechanisms of their function

Although PPAR isotypes are encoded by distinct genes on different chromosomes, they are homologous in structure. Generally, there are four major domains in the PPAR structure. Their N-terminal A/B domain takes part in the ligand-independent regulation of receptor activity, the DNA-binding domain (DBD) is highly conserved among the three isoforms and is linked to the C-terminal ligand-binding domain (LBD). Its E/F domain is responsible for the ligand-dependent transactivation function, and its D domain is characteristic of a variable hinge region (Kersten, 2000, 89:141). (Figure 1.1)



DBD = DNA-binding domain; LBD = ligand-binding domain
RXRα = Retinoic acid receptor
PPRE = peroxisome proliferator response element

Figure 1. 1 PPARs structure

Four main distinct regions are contained in PPARs, from A/B to E/F domain. The N-terminal A/B domain contains ligand-independent activity, C domain contains a DNA-binding domain, D is characteristic of a variable hinge region, and E/F is ligand-binding domain with a ligand-dependent activity. Shown here is its heterodimer retinoic acid receptor (RXRα) binding to the peroxisome proliferator response element on the target genes. Modified from (Boitier et al., 2003).

Although the exact mechanisms of how PPARs regulate gene expression remains elusive, an increasing body of evidence suggested that multiple mechanisms might be involved in these processes.

The first putative mechanism of its transcriptional control of gene expression is named *ligand-dependent transactivation* via heterodimerizing with retinoid-S-receptors (RXRs). As shown in Figure 1.2 (1), coactivators such as CREB-binding protein (CBP)/p300 are recruited, and the PPAR-RXR heterodimers binding to the typical PPAR response elements (PPREs) on the promoter of target genes then activate related transcription (Desvergne and Wahli, 1999; McKenna and O'Malley, 2002).

Noticeably, PPARs can inhibit proinflammatory gene expression by a second putative mechanism named *ligand-dependent transrepression* via antagonizing the activities of other transcription factors, including NF-kB and activator protein-1 (AP-1) (Marx et al., 1998; Staels et al., 1998), without involving in PPREs binding, as shown in Figure 1.2 (2). In this way, their target genes transcription can be *trans*repressed. A line of evidence suggested that anti-inflammation of PPARγ agonists is related to this mechanism (Pascual et al., 2005; Glass and Ogawa, 2006).

The third putative mechanism is named *ligand-independent repression* via interaction of recruited corepressor complexes and PPARs, thus inactivating target genes, as shown in Figure 1.2 (3). Dr. Dowell reported that PPARα can interact with nuclear receptor corepressor directly in human embryonic 293 cells (Dowell et al., 1999). However, whether other PPAR isotypes can function in this way remains to be studied. For more detail see (Ricote and Glass, 2007).

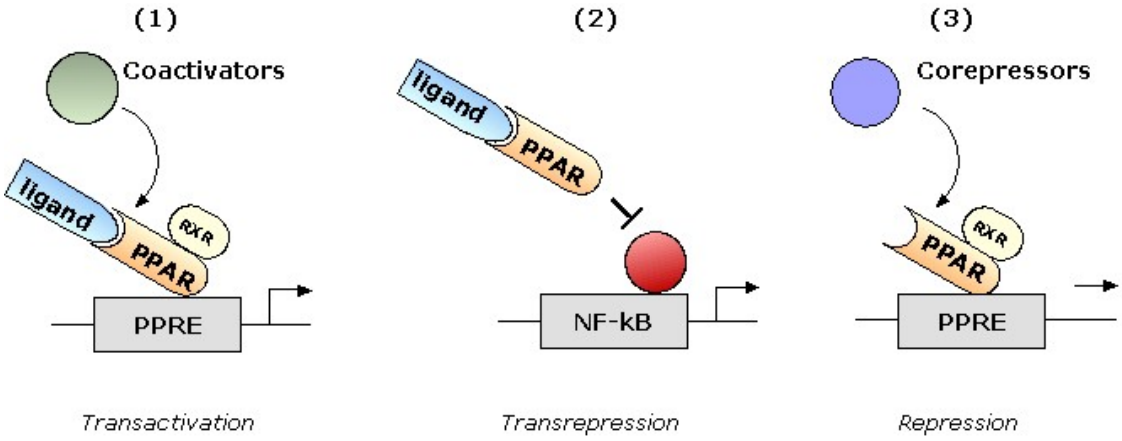


Figure 1. 2 Schematic graph shows gene expression by PPARs.
(1) *Ligand-dependent transactivation*. Ligand-binding recruits coactivator complexes, bind to PPRE, and activates gene expression. (2) *Ligand-dependent transrepression*. Ligand-binding with PPAR antagonizes activity of other transcription factor, shown here is NF-kB inhibition. (3) *Ligand-independent repression*. Binding of PPAR to RXR recruits corepressor complexes, which suppress gene expression. Modified from (Ricote and Glass, 2007).

PPARs and neurodegenerative diseases

Although neurodegenerative diseases are characterized by their distinct pathological localization involved in the different groups of cells, excessive reactive microglia are frequently observed in patients with Alzheimer's disease (AD) (Rogers et al., 1988), PD (McGeer et al., 1988b), multiple sclerosis (MS)(Gobin et al., 2001), and amyotrophic lateral sclerosis (ALS) (Kawamata et al., 1992). An increasing body of evidence from postmortem studies implicates the role of microglia activation-mediated neuroinflammation in the pathogenesis of neurodegenerative diseases. For example, elevated levels of proinflammatory factors such as TNF-α and cytokines PGE_2 in PD (Mogi et al., 1994b; Mogi et al., 1994a; Mogi et al., 1996; Mogi et al., 2007) and AD (Griffin et al., 1995) were found near the degenerating neurons. Animal *in vivo* and *in vitro* studies of various neurodegenerative diseases showed that inhibition of these cytotoxic molecules provides neuroprotection (Misko et al., 1995; Leonard et al., 1997; Drachman and Rothstein, 2000; Zhu et al., 2002; Pompl et al., 2003; Nathan et al., 2005; Ni et al., 2007; Kotilinek et al., 2008).

The anti-inflammation activities of PPARs were first noticed when a prolonged inflammatory response was observed in PPARα null mice (Devchand et al., 1996). As of now, it has been shown that all three PPAR isoforms can participate in the mediation of inflammatory response. PPARβ deficient mice showed delayed would healing and its agonist inhibited cytokine-induced vascular cell adhesion molecule in epithelial cells (Michalik et al., 2001; Rival et al., 2002). PPARγ agonist 15-deoxy-D-prostaglandin J2 (15d-PGJ2) suppresses interferon-γ-induced iNOS protein and its mRNA in mouse peritoneal macrophages (Ricote et al., 1998). Further study demonstrated that its agonists can also inhibit proinflammatory factors such as TNF-α and interleukin-6 in different cell types (Delerive et al., 2001).

Recent evidence indicates that PPAR agonists provide neuroprotection by mediating inflammatory processes in animal models of PD (Dehmer et al., 2004), AD (Combs et al., 2000; Landreth and Heneka, 2001), MS (Feinstein et al., 2002), and ALS (Kiaei et al., 2005).

PPARγ and Parkinson's disease

Although the etiology of Parkinson's disease is still not known, it is generally accepted that unlimited activated microglia mediates dopaminergic neuronal loss via releasing cytotoxic molecules including cytokines and proinflammatory factors. It was shown that PPARγ activation with 15d-PGJ2 inhibits the level of TNF-α and iNOS protein / mRNA expression in mouse peritoneal macrophage (Jiang et al., 1998; Ricote et al., 1998), suggesting the effects of PPARγ activation on preventing inflammatory responses in the brain. Indeed, one PPARγ synthetic agonist, troglitazone, suppresses cerebellar iNOS expression and protected cerebellar granule cells against LPS/IFN-γ insult (Heneka et al., 2000). A few studies from PD animal models have demonstrated that a PPARγ synthetic agonist protects dopaminergic neurons accompanying with inhibited microglia activation in a mice MPTP model (Breidert et al., 2002; Dehmer et al., 2004). Three days of orally administrated pioglitazone prior to MPTP (i.p) treatment significantly protected dopaminergic neurons from death, and iNOS-positive microglial cells were suppressed in the pioglitazone-treated group (Breidert et al., 2002); further study from the same research group suggested that pioglitazone protected TH positive neurons against MPTP insult (30mg/kg i.p.) probably via PPARγ activation, NF-kB activation, and suppression of iNOS expression (Dehmer et al., 2004). Very recently, study on the molecular mechanisms of our intrastriatal LPS-induced dopaminergic neuronal loss rat model suggested that the dopaminergic neuronal protection by pioglitazone is associated with its inhibition of microglia activation, iNOS and COX-2 expression, and restoration of mitochondria function (Hunter et al., 2007). Table 1.3 summarizes current studies of PPARγ agonists relevant to PD. However, 1) the potential inhibitory effect of the PPARγ agonist pioglitazone on LPS-induced microglia activation, and 2) the role and signaling transduction pathways of pioglitazone in activated microglia-mediated dopaminergic neuronal death have not been investigated. The hypothesis is 1) PPARγ agonist pioglitazone inhibits LPS-induced microglia activation; and 2) pioglitazone protects dopaminergic neuronal loss against neurotoxicity of microglia-mediated iNOS and COX-2.

Table 1. 3 Summary of current reference relevant to neuroprotection of PPARγ agonists.

Model	Major results	Reference
MPTP (i.p)	Protect DA neurons Inhibition of glial activation	(Breidert et al., 2002)
MPTP (i.p)	Reduce iNOS positive cells Induce IkBα expression Inhibit NF-kB nuclear translocation	(Dehmer et al., 2004)
Intrastriatal LPS injection	Protect DA neurons Inhibition of microglia activation Inhibition of iNOS / COX-2	(Hunter et al., 2007)
LPS *in vitro*	Protection of DA neurons via inhibiting microglia activation Inhibition of COX-2 via interference with JNK and NF-kB	(Xing et al., 2007)
LPS *in vitro*	Suppresses p38 MAPK activation-mediated iNOS possibly via activation of PPARγ and PI3K / Akt pathway	(Xing et al., 2008)
MPTP (i.p)	Protection of DA neurons Inhibition of MPTP conversion to MPP+ via suppressing MAO-B	(Quinn et al., 2008)

*ip= Intraperitoneal injection; DA= dopaminergic neurons

MAPKs and PI3K in Parkinson's disease

From extracellular stimuli such as proinflammatory cytokines to the target gene expression, intracellular signaling transduction is tightly regulated in the divergent and convergent manner. One of the best known proinflammatory pathways is mitogen-activated protein kinases pathways (MAPKs), which include p38 MAPK, JNK, and ERK1/2. Dependent on different extracellular stimuli and different cell types, differential

biological responses are tightly controlled following complicated sequential activation from MAPKKKs to MAPKs. (Figure 1.3)

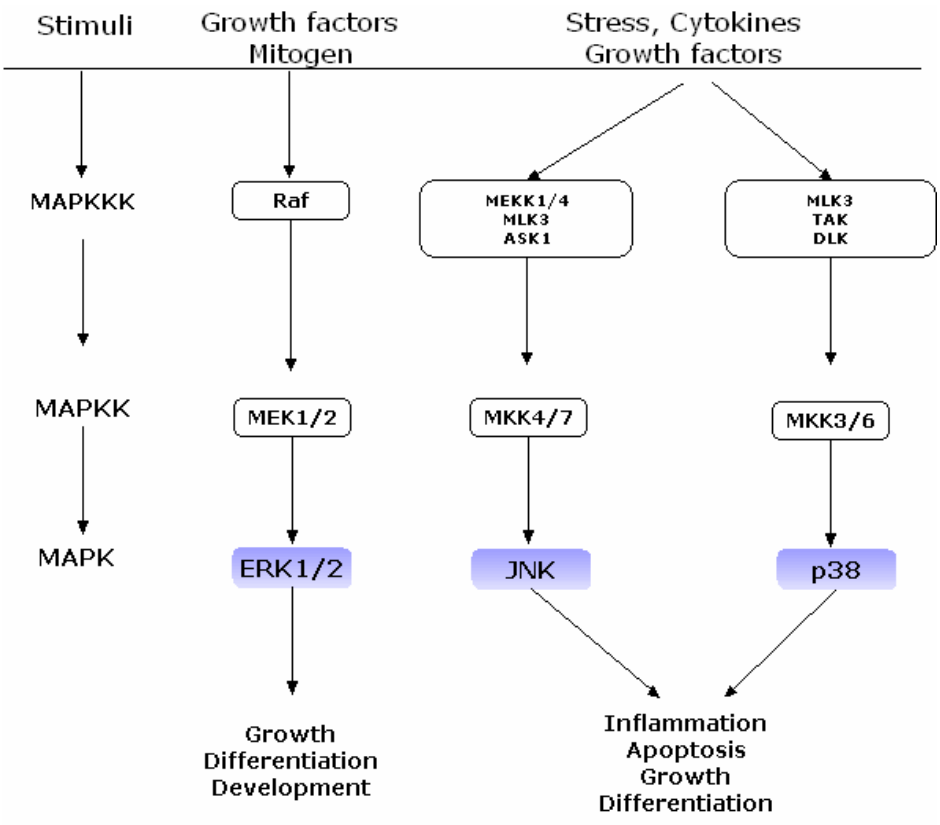


Figure 1. 3 Schematic graph of MAPK pathways.
A variety of extracellular signals such as growth factors and cytokines differentially activate MAPKKKs such as Raf, MEKK, MLK, followed by sequential activation of MAPKKs such as MEK1/2, and activation of MAPKs (ERK1/2, JNK, and p38), inducing different biological responses. Based on (Gallo and Johnson, 2002) and website: http://www.bch.msu.edu/faculty/gallo.htm.

It is generally accepted that p38 MAPK and JNK pathways are two main signaling cascades that modulate inflammatory response and stress (Kyriakis and Avruch, 2001). One small postmortem study on PD brains demonstrated the immunoreactivity of their phosphorylation (Ferrer et al., 2001), and a very recent analysis from the extracts of leukocytes from PD patients provided more evidence that the JNK activity was reduced (White et al., 2007), suggesting these pathways are involved in the pathogenesis of PD.

On the other hand, experimental animal *in vivo* and *in vitro* studies strongly implicated MAPK activation-mediated dopaminergic neuronal death in a MPTP / MPP+ model (Du et al., 2001; Gomez-Santos et al., 2002), 6-OHDA (Choi et al., 2004; Gomez-Lazaro et al., 2008), rotenone (Zhao et al., 2006) and other models (Gomez-Santos et al., 2003; Wilms et al., 2003; Choi et al., 2004; de Bernardo et al., 2004; Lee et al., 2005). Although the involvement of p38 MAPK cascade with LPS-induced neuronal death in the hippocampus was reported (de Bock et al., 1998), the MAPKs signaling transduction associated with LPS-induced dopaminergic neuronal loss is still poorly understood. The result from an *in vitro* study suggested that LPS-induced neuronal loss is prevented by the inhibition of p38 MAPK pathway, accompanied by the decrease of NO production in the primary mesencephalic culture (Jeohn et al., 2002). The other *in vivo* experimental study demonstrated that inhibition of p38 MAPK not only rescues dopaminergic neurons but also decreases iNOS expression (Ruano et al., 2006). The role of the JNK pathway in the LPS model is less studied. The result from a midbrain slice culture study suggested that inhibition of JNK activity rescues LPS/IFNγ-induced dopaminergic neuronal loss without influencing iNOS expression (Shibata et al., 2006), suggesting that the activation of LPS-induced p38 MAPK and JNK pathways potentially mediates differential production of cytotoxic molecules which ultimately lead to neuronal death. Recently, one clinical trial (2007) on 806 patients with early PD using CEP1347, an inhibitor of JNK signaling cascade, was done by the Parkinson Study Group. Although the results did not show beneficial slowing down of the progression of PD, it suggests that the therapeutic strategies should take multiple signaling cascades associated with inflammatory response into account. The hypothesis is that LPS-induced activation of MAPK pathways differentially mediate the iNOS/NO and COX-2 / PGE_2 expression.

PI3K phosphatidylinositol-3-OH kinase (PI3K) / Akt (also known as protein kinase B, PKB) is a well-known cell survival signaling pathway. Neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor, and insulin-like growth factor I activate the PI3K/Akt cascade and promote neuronal survival and / or axonal growth (Halvorsen et al., 2002; Nusser et al., 2002; Bonnet et al., 2004; Zhou et al., 2004; Yoshii and Constantine-Paton, 2007). Neurotoxin studies in animals provided a link of PI3K activity with neuronal survival (King et al., 2001; Halvorsen et al., 2002). Their results demonstrated that inhibition of PI3K by LY294002 promotes MPP^+-induced SH-SY5Y apoptosis, and activation of Akt can protect cells (King et al., 2001), and nerve growth factor treatment prevents SH-SY5Y apoptosis possibly via increasing the phosphorylation of PI3K / Akt (Halvorsen et al., 2002). However, the potentially differential regulation of MAPK and PI3K pathways by PPARγ activation and their differential roles in the LPS-induced iNOS and COX-2 expression is not completely understood. The hypothesis is that the PPARγ agonist pioglitazone protects dopaminergic neurons by the inhibition of iNOS and COX-2 expression via potentially differential mediation of MAPK and PI3K activity.

Overview of experimental aims

Although intensive studies have been done to investigate the molecular mechanisms of the pathogenesis in PD, and several common mechanisms such as neuroinflammation and oxidative stress are generally accepted, the role of neuroinflammation-mediated signaling transduction in dopaminergic neuronal loss and their interference with PPARγ agonist is poorly understood. The experimental studies described in this dissertation primarily address the following general hypotheses: 1) LPS-induced dopaminergic neuronal death is microglia-dependent. It is easy to manipulate different primary cell cultures and differentiate the role of microglial activation in the degeneration of dopaminergic neurons. 2) a PPARγ agonist protects against LPS-induced neurotoxicity via inhibiting p38 MAPK / JNK pathways, which potentially have differential roles in mediating iNOS and COX-2 expression. 3) a PPARγ agonist might also protect dopaminergic neurons via activating the PI3K/Akt pathway, which probably mediates p38 MAPK / JNK activity. The detailed hypotheses and specific aims will be presented in each chapter.

The general profile of PPARγ activation by its synthetic ligand pioglitazone and the influence of LPS on its activation were evaluated by western blot. To measure the effect of PPARγ activation by pioglitazone on LPS-induced microglial activation and dopaminergic neuronal loss, primary microglia-enriched cultures, primary neuron-microglia mixed culture and primary neuron-enriched cultures were used, and immunocytochemistry for microglia and TH positive neurons was done. The effect of PPARγ activation on the activity of MAPKs (p38 MAPK and JNK) and the PI3K/Akt pathway was evaluated by western blot, and the selective inhibitors of p38 MAPK (SB203580) and JNK (SP600125) were used to access the effect of their inhibition on LPS-induced PGE_2 and nitric oxide production in primary microglia-enriched cultures. The role of PI3/Akt activation in the mediation of p38 MAPK / JNK activity was evaluated by its selective inhibitor wortmannin in primary microglia-enriched cultures. The effect of PPARγ activation on the activity of the COX-2 and iNOS, which are the rate-limiting enzymes for PGE_2 and NO, respectively, was measured by western blot. The inhibition of COX-2 and iNOS on LPS-induced dopaminergic neuronal loss was evaluated by immunocytochemistry.

CHAPTER 2: Dopaminergic neuronal loss induced by LPS is microglia-dependent and PPARγ activation by pioglitazone is neuroprotective by inhibiting LPS-induced microglial activation

(From published paper: B Xing., M Liu., GY Bing. (2007) J. Neuroimmunol. 192:89-98). M Liu took part in the experiment in the immunocytochemistry.

Introduction

It has been demonstrated that glial-related cytokines are increased either in PD postmortem study or in the assay of patients' serum / cerebrospinal fluid (CSF). The level of TNF-α from PD striatum and CSF is significantly higher than that in the control group (Mogi et al., 1994a), and other cytokines including IL-6 and IL-1β are also increased in the dopaminergic regions (Mogi et al., 1994b).

Although both activated glial cells (microglia / astrocytes) can release proinflammatory cytokines under certain cellular stress, and may take part in the detrimental process resulting to the death of dopaminergic neurons, increasing evidence indicates that activated microglia rather than astrocytes play a major role in the pathological process of PD. First, a large number of reactive microglia was found surrounding dopaminergic neurons in the SN of PD brains (McGeer et al., 1988b). Second, the SN area where dopaminergic neurons are degenerated has the highest density of microglia in the brain, whereas astrocytes have a higher density in the hippocampus and hypothalamus (Lawson et al., 1990; Savchenko et al., 2000). Third, a postmortem SN study found that glial fibrillary acidic protein (GFAP) immunoreactivity for astrocytes is often unobservable compared to the strong microglial immunostaining (Banati et al., 1998). Fourth, animal experimental studies demonstrated that neurotoxin-induced dopaminergic neuronal death is often preceded by microglia activation (Gao et al., 2002).

As a potent inducer of inflammation, the LPS PD model provides a very useful tool to study the mechanisms of microglia-mediated neuroinflammation in the pathological process of PD, since LPS selectively induces the death of dopaminergic neurons (Herrera et al., 2000) and microglia activation (Castano et al., 1998). The LPS *in vitro* model provides a useful chance to study the detrimental role of both astrocytes and microglia. However, the current *in vitro* model did not mechanically remove the role

of astrocytes since either primary mesencephalic cultures or primary mesencephalic-glia cultures were used in their studies. In this way, it is relatively difficult to explain the data. To investigate the activated microglia-mediated neuroinflammation and the dopaminergic neuronal degeneration, our LPS *in vitro* model systems were set up. From the reports that LPS neurotoxicity is glia-dependent (Bronstein et al., 1995), the first hypothesis is that LPS neurotoxicity is microglia-dependent and pioglitazone protects dopaminergic neurons by inhibiting microglia activation. The first specific aim is to administer LPS into both primary neuron-enriched cultures and primary neuron-microglia mixed cultures, then to test the survival of dopaminergic neurons, and to obtain the optimal (minimal) LPS toxicity concentration for the future study.

Several studies have suggested that PPARγ agonists inhibit macrophage / monocyte activation-derived cytokines such as TNF-α and NO (Jiang et al., 1998; Ricote et al., 1998). MPTP animal studies showed neuroprotection of a PPARγ agonist toward dopaminergic neurons (Breidert et al., 2002; Dehmer et al., 2004). The second hypothesis is that the PPARγ agonist pioglitazone protects dopaminergic neurons via inhibiting microglia activation in LPS *in vitro* model. The second specific aim is to test 1) if pioglitazone inhibits LPS-induced microglia activation, and 2) if pretreatment of pioglitazone into primary neuron-microglia mixed cultures shows a neuroprotective effect.

Materials and methods

Animals

Twenty one timed-pregnant Sprague Dawley rats obtained from Harlan (Indianapolis, IN, USA) and were maintained in a strict pathogen free environment. Animal use was performed in strict accordance with the National Institutes of Heath guidelines and was approved by the Institute's Animal Care and Use Committee at the University of Kentucky.

Reagents

Salmonella minnesota LPS and pioglitazone were obtained from Sigma-Aldrich (St Louis, MO, USA), Ara-C was obtained from Sigma-Aldrich (St Louis, MO, USA), antibodies used were as follows: polyclonal anti-tyrosine hydroxylase (TH) antibody from Pel-Freez

Biologicals (Rogers, AR, USA), monoclonal anti-rat CD11blc antibody for OX-42 from Pharmingen (San Jose, CA, USA), and monoclonal anti-β-actin from Sigma-Aldrich (St. Louis, MO, USA). The ABC kit and biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA, USA). All culture medium and products were obtained from Invitrogen (Carlsbad, CA, USA).

Mesencephalic neuron-microglia mixed cultures

Neuron-glia cultures were prepared from the SD ventral mesencephalic tissues of embryonic day 13-14. Briefly, midbrain tissues were dissected from day 14 SD rat embryos in Ca^{++}/Mg^{++} free medium (CMF). Cells were dissociated via gentle mechanical trituration in Hanks' Balanced Salt Solution (HBSS) containing newborn calf serum (3.5:1 v/v). The concentration of the cell suspension was ~ 1.2 x 10^7 cells / ml before seeding, and the cells were seeded in poly D-lysine (50μg/mL) pre-coated 24-well plates (1 x 10^5/well). Cells were fed with Dulbecco's Modified Eagle Medium (DMEM / F12) containing 10% horse serum and 10% fetal bovine serum. Three days after seeding, the cells were replenished with 500ml of fresh DMEM / F12 with 5% horse serum and fetal bovine serum. At DIV6 or DIV7, 2 x 10^5 microglia were added into primary mesencephalic neuron-enriched cultures containing 1 x 10^5 cells/well, and after 24h, the cultures were treated with either pioglitazone or LPS dissolved in DMSO, and the control groups were only treated with same amount of DMSO (10μl).

Primary mesencephalic neuron-enriched cultures

Twenty four hours after seeding, 5μM Ara-C was added into the culture medium for 48h, followed by replacement with fresh DMEM / F12 medium with 5% horse serum and fetal bovine serum. Cultures were exposed to treatment seven days after seeding.

Microglia-enriched cultures

Primary glial cell cultures were established from cerebral cortices of 2-3 day-old neonatal SD rats. Briefly, cerebral cortices were minced and gently dissociated by repeated pipetting in HBSS supplemented with fetal bovine serum (3.5:1 v/v). Cells were collected by centrifugation at 1000g for 6mins, resuspended in DMEM containing 10% fetal bovine serum, 100U/ml penicillin, and 100μg/ml streptomycin, and were cultured on 175cm^2 cell culture flasks in 5% CO_2 at 37°C. Floating microglia were harvested

between 2-8 weeks by shaking off at 200rpm, and the concentration of the cell suspension was ~1.4 x 10^6 microglial cells/ml and were re-seeded back into 24-well plates (2 x 10^5) for other assays. After 30mins, cultures were washed to remove non-adherent cells and fresh medium was added, and the cultures were treated 24h after seeding.

Immunocytochemistry

Culture medium was removed and the cells were rinsed in Tris buffer (pH = 7.3), fixed in 4% paraformaldehyde for 20mins, and rinsed again in Tris. Non-specific staining was blocked with 10% goat serum or horse serum for 60mins. Cells were incubated overnight at 4°C with primary anti-TH antibody (1:10,000) or anti-OX-42 primary antibody (1:1000). After several Tris rinses, cells were incubated with the following biotin-conjugated secondary antibodies for 1hr: goat anti-rabbit IgG (1:1000) for TH or horse anti-mouse IgG (1:1000) for OX-42. The cells were rinsed several times and incubated in an ABC-peroxidase reagent for 1hr. This was followed by several rinses and a color development with 3,3'-diaminobenzidine and 0.03% H_2O_2 in Tris buffer. Images were acquired using a Zeiss Axioplan 2 microscope connected to a digital Zeiss Axio camera operated with AxioVision software (Zeiss). To quantify OX-42 positive cells, nine representative areas per well, in the 24-well plate, were counted under the microscope at 100X magnification, and the microglia with an expanding transparent part were considered as activated. The TH-positive neurons were counted in each 24-well plate, and the averages are reported. TH-immunostained neurons were considered healthy, if the length of all the neurites was two times longer than the diameter of its cell body and if the cell had at least two neurites.

Statistical Analysis

The data are expressed as the mean ± SEM and statistical significance was assessed by ANOVA followed by a Tukey comparisons test using the SYSTAT 10 software (SPSS Inc., Chicago, Illinois). A value of $p < 0.05$ was considered statistically significant.

Results

LPS neurotoxicity is microglia-dependent and the PPARγ agonist pioglitazone protects dopaminergic neurons against LPS insult

It has been shown that LPS neurotoxicity is glia-dependent in mesencephalic cultures (Bronstein et al., 1995), but the effect of astrocytes were not excluded in their study. In order to test if LPS toxicity in our culture system is microglia-dependent, and to facilitate studying the cellular and molecular mechanisms between initial microglial activation and secondary dopaminergic neuronal death, the survival of TH-positive neurons upon LPS exposure, in the presence and absence of microglia, was investigated in primary neuron-enriched and neuron-microglia mixed cultures. We found that LPS failed to induce dopaminergic neuronal loss in neuron-enriched cultures (Figure 2.1). In contrast, in the presence of microglia, 1-40μg/ml LPS induced ~38-75% loss of the dopaminergic neurons (Figure 2.2) after a 72hr LPS treatment. Pretreatment with pioglitazone (10μM), 1hr prior to LPS (1μg/ml), significantly protected dopaminergic neurons by about 90% compared to the 60% cell loss upon LPS (1μg/ml) alone ($p<0.05$, Figure 2.2). Pioglitazone alone did not alter cell viability.

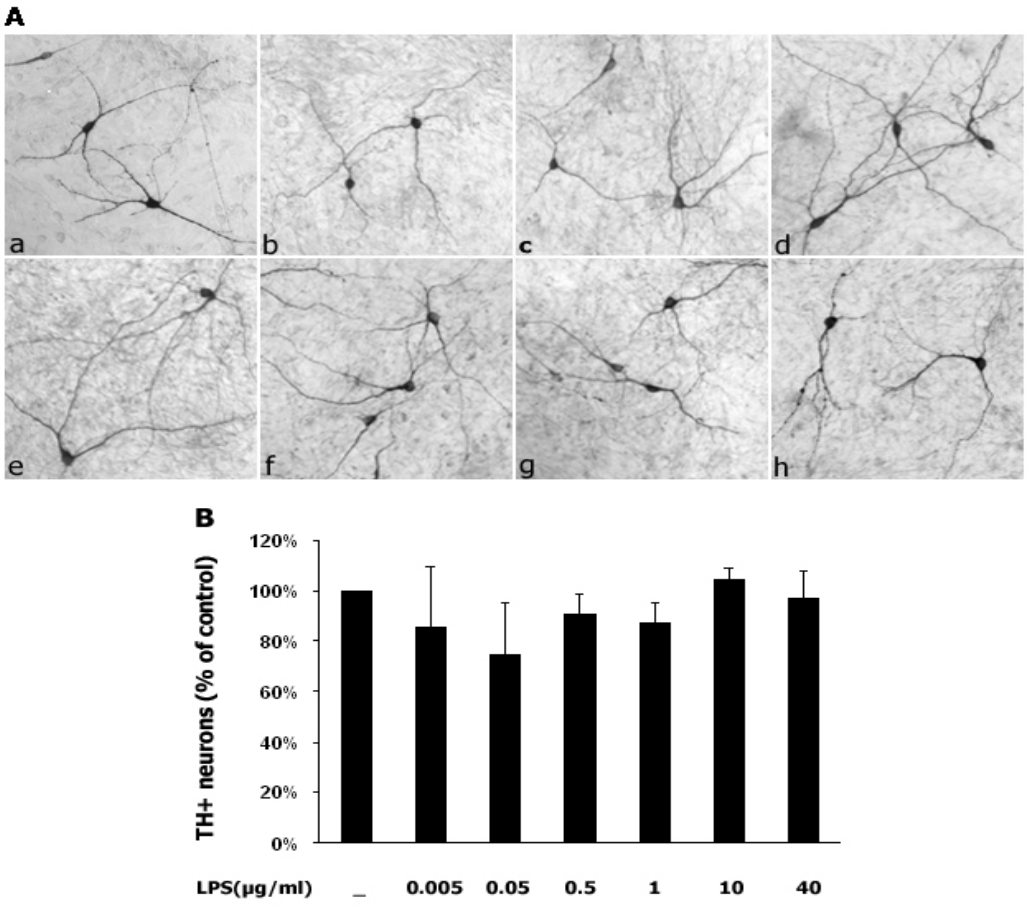
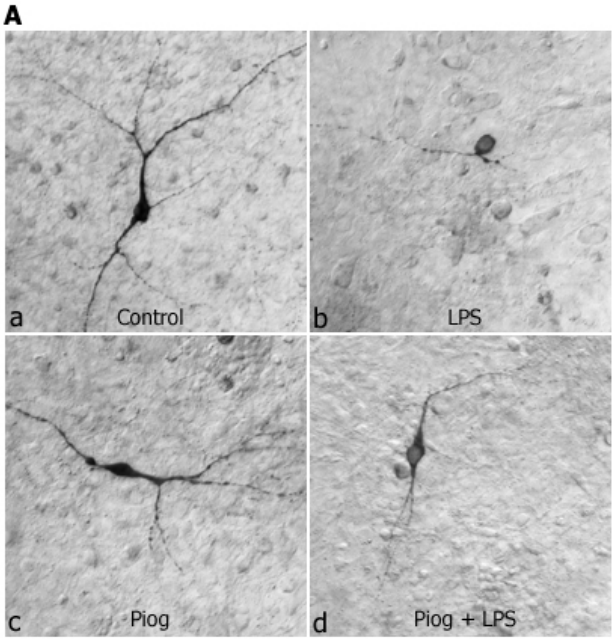
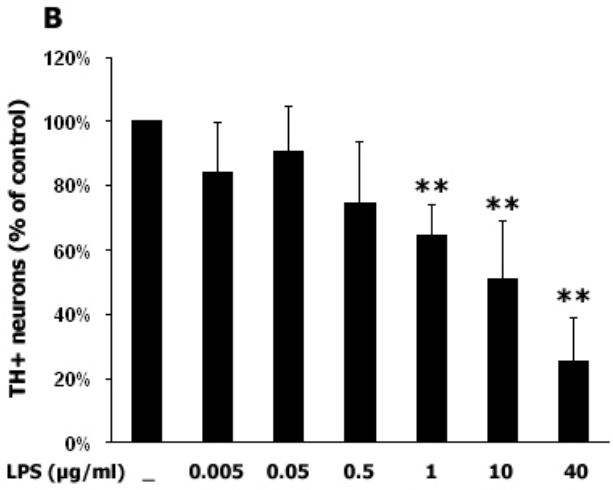


Figure 2. 1 LPS treatment in primary neuron-enriched cultures.
Rat mesencephalic neuron-enriched cultures (1×10^5 cells/well) were treated with LPS for 72 h, and examined with immunocytochemistry for TH-positive neurons. **A:** Increasing concentrations of LPS (a–h) did not decrease the number of TH-positive neurons. Both control and LPS treated groups had long smooth neuritis. **B:** No significance difference was found between control group and different doses of LPS groups (5ng/ml-40µg/ml). The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3). (a=control; b=LPS 5 ng/ml; c=LPS 10 ng/ml; d=LPS 50 ng/ml; e=LPS 100 ng/ml; f=LPS 1 µg/ml; g=LPS 10 µg/ml; h=LPS 40 µg/ml).


A
a
Control
b
LPS
c
Piog
d
Piog + LPS


B
TH+ neurons (% of control)
120%
100%
80%
60%
40%
20%
0%
**
**
**
LPS (µg/ml) _ 0.005 0.05 0.5 1 10 40

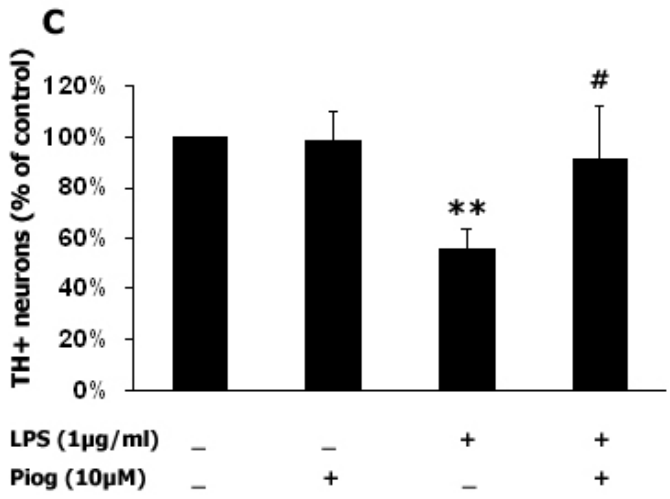
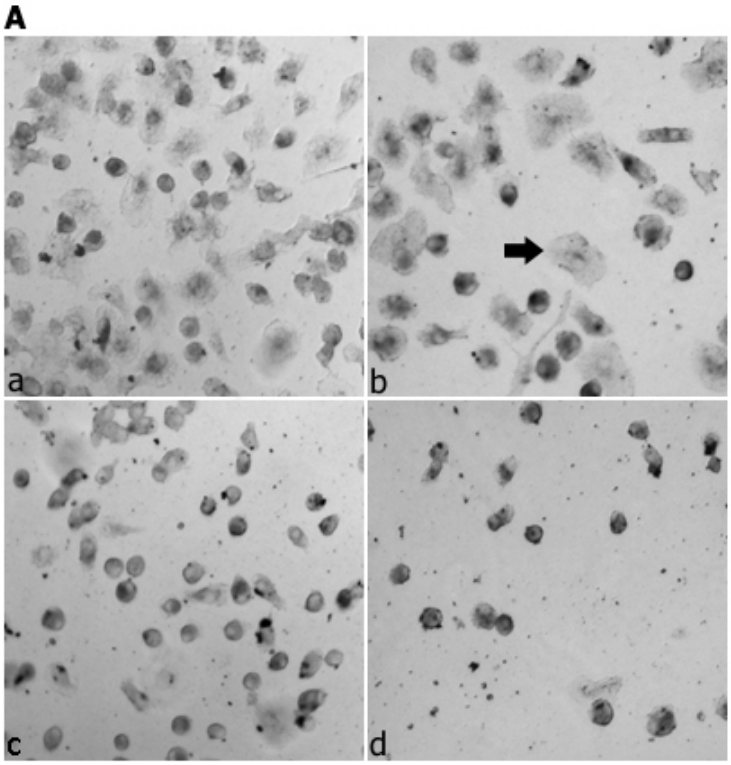


Figure 2. 2 LPS is neurotoxicity in neuron-microglia mixed cultures.
Rat neuron-microglia cultures (with 1×10^5 neurons and 2×10^5 microglia per well) were treated with various doses of LPS (5ng–40µg/ml) for 72 h, and the number of TH-positive neurons was examined with immunocytochemistry. **A:** control cultures have long and smooth neurites (a), LPS (1µg/ml) treated cultures have discontinuous neurites (b), and LPS (1µg/ml – 40µg/ml) induces a dose-dependent neurotoxicity as indicated by the discontinuous neurites (b). Pioglitazone (10µM) alone did not influence the general morphology and survival of the TH positive neurons (c). Pretreatment with pioglitazone (10µM) 1 h before LPS (1µg/ml) treatment represented in (d) showed normal morphology. **B:** LPS did not show neurotoxicity from 0.005µg/ml to 0.5µg/ml, however, 1µg/ml-40µg/ml LPS showed dose-dependent neurotoxicity. **C:** LPS (1µg/ml) decreased the number of TH positive neurons, and pretreatment of pioglitazone protected the TH-positive neurons against cell death, as determined by cell counts. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (**p<0.01 versus control, #p<0.05 versus LPS).

Pioglitazone suppresses LPS-induced microglia activation

LPS did not induce dopaminergic neuronal loss in the absence of microglia and pioglitazone protected dopaminergic neurons against LPS neurotoxicity in the presence of microglia, suggesting its neurotoxicity is dependent on microglia. In the next step we

tested the effect of pioglitazone on microglia activation. First, microglia-enriched cultures were treated with LPS (100ng-1μg/ml) in the presence or absence of pioglitazone (10μM) for 24 and 48h. Then, microglia activation was determined by the obvious swelling and morphological changes of the OX-42 positive cells. After 24h of 100ng/ml LPS exposure, 45% of the microglia were activated, and 60% microglia activation was observed with 1μg/ml LPS (Figure 2.3). In contrast, pretreatment with pioglitazone, 1hr before LPS, suppressed microglia activation to the control level (p<0.01). After 48h, LPS activated ~80% of the microglia (Figure 2.4), and pretreatment with pioglitazone (1μM and 10μM) significantly suppressed microglial activation within 50% and 30%, respectively. In addition, the suppressive effect of pioglitazone (10μM) on the LPS-induced microglia proliferation is observed in primary microglia-enriched cultures after 48h LPS exposure (Figure 2.5).



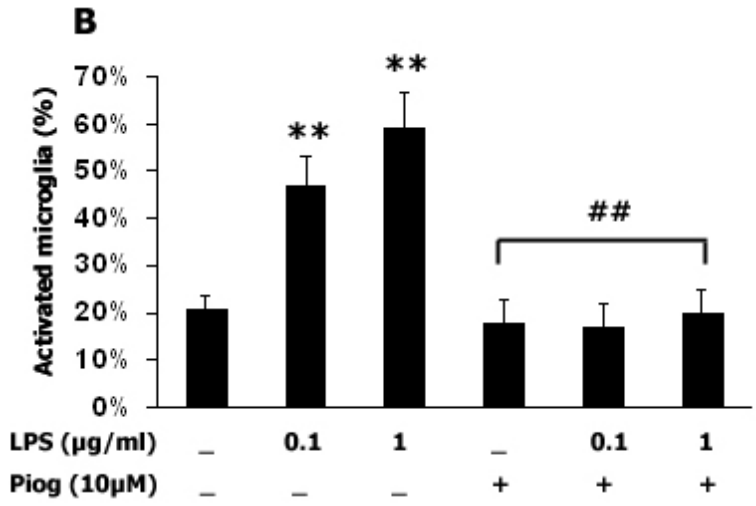


Figure 2. 3 Pioglitazone inhibits microglia activation in rat microglia-enriched cultures (24hr).

Twenty four hours after seeding (2×10^5 cells/well) into 24-well plates, cultures were treated with LPS (0.1 μg/ml–1 μg/ml) for 24h, and immunostaining for OX-42 was done. **A:** Microglia in control (a) are small than in the LPS-treated group (b) (1 μg/ml), in which the cells swelled, as represented by an arrow. The cell morphology did not change in the pioglitazone alone group (c), and pretreatment with pioglitazone 10μM had less swollen cells (d). **B:** The different doses of LPS activated 50%–60% of the microglia, and pretreatment with pioglitazone (10μM) significantly attenuated microglia activation. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (**p<0.01 versus control; ##p<0.01 versus LPS).

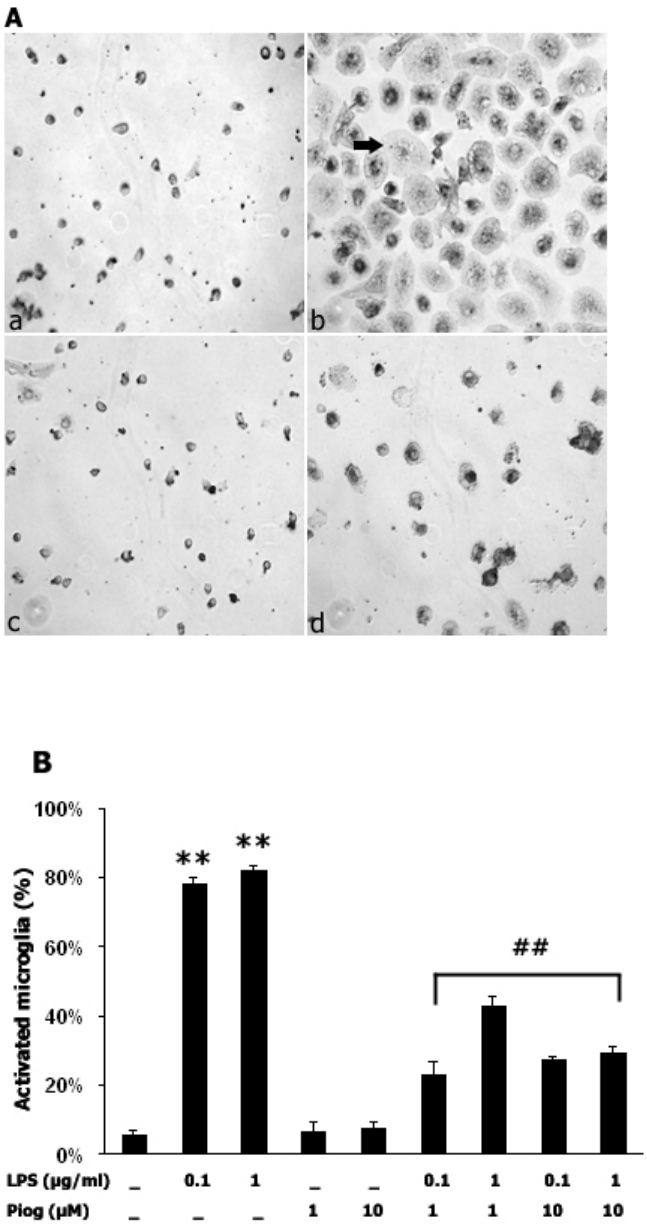


Figure 2. 4 Pioglitazone inhibits microglia activation in rat microglia-enriched cultures (48hr).

Twenty four hours after seeding (2×10^5 cells/well) into 24-well plates, cultures were treated with LPS (0.1μg/ml–1μg/ml) for 48h, and immunostaining with OX-42 was done. **A:** In LPS-treated group (b), the immunostained cells become much bigger than control group (a). An arrow in LPS-treated group indicates a typical activated microglia. Pioglitazone alone did not change morphology of the cells (c), and pretreatment with pioglitazone reduced the activated microglia (d), with some difference in their shape. **B:**

Forty-eight hours of LPS exposure activated about 80% of the microglia in LPS group (0.1μg/ml and 1μg/ml), and pretreatment with pioglitazone (1μM or 10 μM) significantly reduced microglia activation. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (**p<0.01 versus control; ##p<0.01 versus LPS).

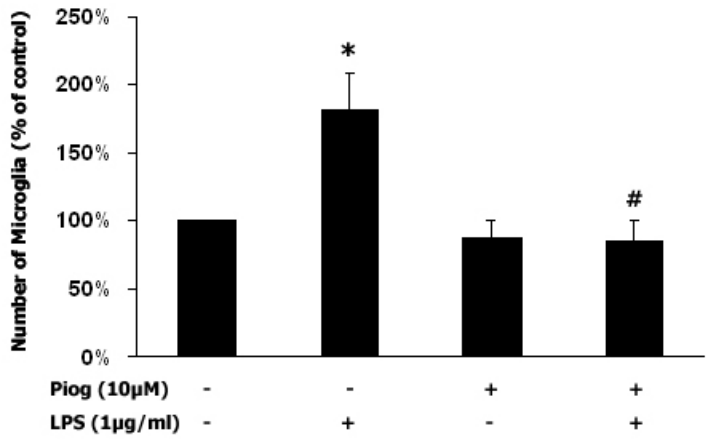


Figure 2. 5 Pioglitazone inhibits LPS-induced microglia proliferation (48hr).
Twenty four hours after seeding (2×10^5 microglia cells/well) into 24-well plates, cultures were treated with LPS (1μg/ml) for 48 h, and immunostained with OX-42. Forty-eight hours of LPS exposure increased the number of microglia, however, pretreatment with pioglitazone (10μM) reduced microglia proliferation. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (*p<0.05 versus control; #p<0.05 versus LPS).

Discussion

The results show that LPS neurotoxicity is microglia-dependent, and the PPARγ agonist pioglitazone protects dopaminergic neurons against LPS insult via inhibiting microglia activation. The current study shows that our *in vitro* cell culture system can be used as a reasonable tool to further study the molecular mechanisms of neuroinflammation-mediated dopaminergic neuronal death.

The brain has been considered as an immunological privileged site based on the fact that blood-brain-barrier protects the brain from immune reaction. However, increasing evidence suggested that this postulation may have exceptions; especially under the condition that microglia is excessively activated. LPS-induced systematic inflammation causing parkinsonism was reported from a case study (Niehaus et al., 2004), in which a laboratory worker was contaminated with LPS (*Salmonella minnesota, 10μg/ml*) by wound in an accident and developed parkinsonism after three weeks (Niehaus et al., 2004). Importantly, microglia activation but not reactive astrocytes has been previously demonstrated in the SN of PD brains (McGeer et al., 1988b; Mirza et al., 2000), and a growing body of evidence suggested that microglia activation could play a critical role in mediating the survival of dopaminergic neurons in the PD midbrain (Ouchi et al., 2005) as well as in animal models of Parkinson's disease (Czlonkowska et al., 1996; Lu et al., 2000; Le et al., 2001; Gao et al., 2002; Wu et al., 2002; Arai et al., 2004; Wang et al., 2006; Hunter et al., 2007; Purisai et al., 2007).

The present study clearly demonstrates that LPS treatment of neuron-enriched cultures fails to induce TH-positive cell loss, however, in the presence of microglia, a dose-dependent TH-positive cell loss is observed with LPS treatment. This suggests that the toxic effects of LPS on the mesencephalic dopaminergic neurons are dependent on microglia activation and the subsequent release of proinflammatory factors, which is consistent with the study from other labs (Liu et al., 2003; Qian et al., 2006; Mount et al., 2007; Zhao et al., 2007). While the causal relationship of microglial activation and dopaminergic neuron survival is still debatable, our finding that LPS failed to show dopaminergic neurotoxicity in the absence of activated microglia supports the idea that uncontrolled microglia activation can initiate dopaminergic neurodegeneration. Pioglitazone significantly reduced the LPS-induced dopaminergic neuronal loss,

suggesting its neuroprotective effect is related to its inhibiting microglia activation, which can initiate neuronal loss by increasing proinflammatory factors such as superoxide, NO, cytokines, and prostaglandins (Banati et al., 1993; Minghetti and Levi, 1998). The protective effects of pioglitazone are in line with several previous studies that demonstrated significant protection with pioglitazone in the MPTP PD model (Breidert et al., 2002; Dehmer et al., 2004), experimental autoimmune encephalomyelitis (EAE) model (Storer et al., 2005), transient cerebral ischemia model (Victor et al., 2006), and LPS PD model (Hunter et al., 2007).

Traditionally, three classic types of microglia in terms of their morphology (ameboid, ramified, and intermediate) was described according to observation from silver carbonate staining developed 70 years ago (del Rio Hortega, 1932; Kershman, 1939). With the gradual understanding of microglial function, the classification of microglia is switched according to their activation condition: resting microglia, activated microglia, and phagocytic microglia (Streit et al., 1989) . In our culture system, LPS increases the ratio of activated microglia and pioglitazone inhibits their activation. On the other hand, we observed that the total number of microglia, including activated and non-activated microglia, is significantly less in the pioglitazone treated group in microglia-enriched culture, suggesting pioglitazone suppresses LPS-induced microglia proliferation. Indeed, it showed that the proliferation rate of microglia is increased upon LPS stimuli (Shankaran, 2007), and the PPARγ agonist 15d-PGJ2 induces microglial death (Bernardo, 2003). However, two basic phenotypes of microglia were observed in the primary glial cultures (Floden and Combs, 2007), and recently subsets of microglia in the mouse brain were studied showing two cell groups with distinct ability to produce ROS (Nagatsu and Sawada, 2006). It will be very informative to know the potential differential role of PPARγ agonists in the microglia with different phenotypes, thus targeting the microglia specifically detrimental to the dopaminergic neurons can be an effective strategy for PD treatment. Another interesting question is: By what mechanisms does pioglitazone inhibit microglia proliferation? PGE_2 is known to induce microglia death, probably via its receptor EP2 and cAMP signaling, however, the present result showed that the decrease in PGE_2 level by pioglitazone is parallel with the decrease of microglia proliferation. Whether pioglitazone induces microglia death via modulating the

expression of its cell cycle protein is interesting to be explored. Our result suggested that the inhibitory effect of pioglitazone on microglia proliferation is LPS-dependent, since no significant difference of microglia proliferation was observed between the control and pioglitazone groups. The result is consistent with several studies, which demonstrated that the PPARγ agonist BRL49653 induces significant macrophage apoptosis upon cytokine TNF-α/IFNγ stimuli by DNA fragmentation assay (Chinetti et al., 1998) and ciglitazone induces the death of activated microglia (Yang et al., 2006). Based on the observation of LPS-dependent inhibition of proliferation in our experimental context, it is reasonable to speculate that certain active molecule(s) released from activated microglia take part in the PPARγ-mediated stress-dependent regulation on the microglia proliferation.

In summary, our present results demonstrate that LPS neurotoxicity is microglia-dependent, and pioglitazone protects dopaminergic neurons via suppressing microglia activation and / or microglia proliferation. In the next chapter (3), the underlying mechanisms of LPS-induced dopaminergic neuronal loss and its interference by pioglitazone were explored, focusing on the signaling transduction relevant to LPS-induced COX-2 / PGE_2.

CHAPTER 3: Neuroprotective effect of PPARγ activation with pioglitazone via inhibiting COX-2 activity is associated with its inhibition of NF-kB and JNK activation

(From published paper: B Xing., M Liu., GY Bing. (2007) J. Neuroimmunol. 192:89-98). M Liu took part in the experiment in the immunocytochemistry.

Introduction

In Chapter 2, microglia-dependent LPS neurotoxicity was demonstrated, and it also showed that the PPARγ agonist pioglitazone protects dopaminergic neurons against LPS insult in our culture systems. The questions addressed in this chapter are: 1) the potential inhibitive effect of pioglitazone on LPS-induced COX-2 / PGE_2, and 2) the potential interference of pioglitazone on the related signaling transduction pathways - NF-kB and JNK.

A few postmortem studies demonstrated that the activity of NF-kB and COX-2 are significantly increased in the SN and striatum of PD brain (Hunot et al., 1997; Teismann et al., 2003b). The percentage of immunoreactive nuclear NF-kB in PD midbrain is increased 70 fold compared to control samples (Hunot et al., 1997). The microglial-related iNOS immunoreactivity in the PD SN and COX-2 / PGE_2 level in ventral midbrain are increased 3 fold compared to the control group (Teismann et al., 2003b).

It is known that JNK and NF-kB are important mediators of inflammation associated with COX-2 activity and its production of PGE_2 (Uto et al., 2005; Won et al., 2005; Nieminen et al., 2006). One study reported c-Jun activation in MPTP-induced PD and in the dopaminergic neurons of PD patients, where deficiency of JNK prevented neurodegeneration accompanied by a decrease in COX-2 expression (Hunot et al., 2004).

We have showed PPARγ agonism with pioglitazone protects SN dopaminergic neurons against intrastriatal LPS insult, which was associated with COX-2 inhibition (Hunter et al., 2007). In addition, pioglitazone protects dopaminergic neurons against MPTP insult by inhibition of NF-kB (Breidert et al., 2002; Dehmer et al., 2004). However, the effect of pioglitazone on JNK-mediated COX-2 and PGE_2 generation upon LPS insult has not been studied. The hypotheses in this chapter are: 1) pioglitazone inhibits LPS-induced COX-2 expression and PGE_2 synthesis; 2) pioglitazone protects

dopaminergic neurons against LPS insult via inhibiting COX2 / PGE_2 level by interfering with NF-kB and JNK pathway. The specific aims are: 1) to test inhibitory effect of pioglitazone on LPS-induced COX-2 expression by western blot, and PGE_2 level by EIA assay; 2) to measure the role of COX-2 inhibition in the survival of TH positive neurons by using a selective COX-2 inhibitor CAY10404; 3) to test the inhibition of NF-kB / JNK by pioglitazone using western blot analysis, and to test the role of NF-kB and JNK inhibition in the LPS-induced PGE_2 synthesis by using selective inhibitors.

Materials and methods

Animals

Twenty one timed-pregnant Sprague Dawley rats were obtained from Harlan (Indianapolis, IN, USA) and were maintained in a strict pathogen free environment. Animal use was performed in strict accordance with the National Institutes of Heath guidelines and was approved by the Institute's Animal Care and Use Committee at the University of Kentucky.

Reagents

Salmonella minnesota LPS and pioglitazone were obtained from Sigma-Aldrich (St Louis, MO, USA). Selective inhibitors were as follows: T0070907-PPARγ inhibitor and CAY10404–COX-2 inhibitor from Cayman (Ann Arbor, MI, USA), SP600125-JNK inhibitor from A.G. Scientific (San Diego, CA, USA), and sulfasalazine-NF-kB inhibitor from Sigma-Aldrich (St. Louis, MO, USA). Antibodies used were as follows: polyclonal anti-tyrosine hydroxylase (TH) antibody from Pel-Freez Biologicals (Rogers, AR, USA), polyclonal anti-COX-2 and polyclonal anti-NF-kB p65 from Cell Signaling (Danvers, MA, USA), monoclonal anti-phospho-JNK antibody Santa Cruz (Santa Cruz, CA, USA), and monoclonal anti-β-actin from Sigma-Aldrich (St. Louis, MO, USA). The other reagents and culture medium were the same as those mentioned in chapter 2.

Mesencephalic neuron-microglia mixed cultures

The same methods were used as mentioned in chapter 2. Briefly, midbrain tissues were dissected from day 14 SD rat embryos, dissociated via gentle mechanical trituration, and the cells were seeded in poly D-lysine (50μg/mL) pre-coated 24-well plates ($1x10^5$/well)

or in pre-coated 6-well plates for western blot ($1x10^6$ or $2x10^6$ cells/well). Cells were fed with DMEM/F12 containing 10% horse serum and 10% fetal bovine serum. Three days after seeding, the cells were replenished with fresh medium. At DIV6 or DIV7, microglia were added into primary mesencephalic neuron-enriched cultures containing $1x10^5$ cells/well, and after 24h, the cultures were treated with either pioglitazone or LPS dissolved in DMSO, and the control groups were only treated with same amount of DMSO (10μl).

Primary mesencephalic neuron-enriched cultures
Cultures were exposed to treatment seven days after seeding.

Microglia-enriched cultures
Primary glial cell cultures were established from cerebral cortices of 2-3 day-old neonatal SD rats. Briefly, cerebral cortices were minced and gently dissociated. Cells were collected by centrifugation and were cultured in $175cm^2$ cell culture flasks in 5% CO_2 at 37°C. Floating microglia were harvested between 2-8 weeks, either seeded into 24-well plates for primary microglia culture, or re-seeded back into 24-well plates ($2x10^5$) for other assays. After 30mins, cultures were washed to remove non-adherent cells and fresh medium was added, and the cultures were treated 24h after seeding.

Immunocytochemistry
Same as that mentioned in chapter 2, except the following: In the double-labeling of OX-42 (1:1000) and NF-kB p65 (1:125), the color was developed with nickel-intensified 3,3'-diaminobenzidine in Tris buffer during the final step.

PGE_2 production
PGE_2 in the supernatant was measured with a PGE_2 EIA kit from Cayman (Ann Arbor, MI, USA) according to the manufacturer's instructions. The supernatant from primary microglia-enriched culture ($2x10^5$cells/well) was measured for total PGE_2 production. The culture medium was removed for extracellular PGE_2 measurement and 1ml PBS was added into each well plate followed by sonication 3 times X 6 seconds for intracellular PGE_2 measure. For the PPARγ inhibition study, T0070907 (1nM) was added into culture medium 1hr prior to either pioglitazone (10μM) and / or LPS (1μg/ml) treatment for 48hr.

Western blot

Cells were collected and lysed for western blot analysis of COX-2, NF-kB and phospho-JNK. Protein concentrations were determined with the bicinchoninic acid assay following the manufacturer's guide. Equal amounts of protein were separated by a SDS PAGE gel and were transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk and incubated with polyclonal anti-COX2 antibody (1:1000), polyclonal anti-NF-kB antibody (1:1000), and monoclonal anti-phospho-JNK (1:1000) either for 1hr at room temperature or overnight at 4°C. Peroxidase-linked anti-rabbit or anti-mouse IgG (1:4000; 1hr at 25°C) and ECL-Plus reagents (Amersham Biosciences Inc., Piscataway, NJ) were used as a detection system. The optical density was measured using the scion image^{TM} software (Frederick, MD, USA).

Statistical Analysis

The data are expressed as the mean ± SEM and statistical significance was assessed by ANOVA followed by a Tukey comparisons test using the SYSTAT 10 software (SPSS Inc., Chicago, Illinois). A value of $p < 0.05$ was considered statistically significant. Data presented represent three independent experiments.

Results

Pioglitazone inhibits LPS-induced COX-2 / PGE_2 expression in microglia-enriched cultures

PGE_2 generated by COX-2 is a major component of the innate immune response. Previous reports have shown that LPS strongly activates microglia, induces COX-2 expression, and enhances PGE_2 synthesis (Bauer et al., 1997; Fiebich et al., 2003). In this set of experiments, LPS-induced COX-2 expression was tested in the presence and absence of pioglitazone. Western blot analysis revealed that COX-2 was undetectable under basal conditions and with pioglitazone treatment, but COX-2 expression increased with LPS treatment. The LPS-induced increase was suppressed by pretreatment with pioglitazone (10μM) (Figure 3.1). In addition, a 24hr LPS challenge (1μg/ml and 10μg/ml) induced a 50 and 100-fold induction of PGE_2 ($p<0.001$), and pioglitazone significantly attenuated PGE_2 generation ($p<0.01$, Figure 3.2).

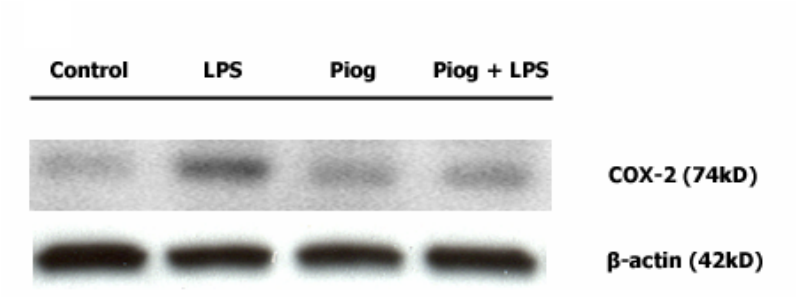


Figure 3. 1 Pioglitazone inhibits LPS-induced COX-2 expression.
Primary mesencephalic cultures (1×10^6 cells/well) were treated with 1μg/ml LPS for 6 h, and LPS treatment upregulated COX-2 expression. Pretreatment with pioglitazone (10μM) reduced COX-2 expression.

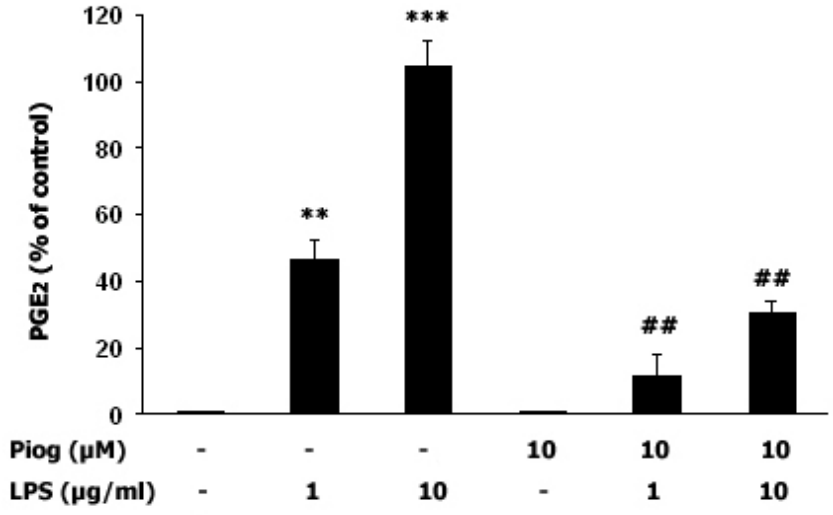


Figure 3. 2 Pioglitazone inhibits LPS-induced PGE_2 synthesis in microglia-enriched cultures.
In rat microglia-enriched cultures (2×10^5 cells/well), LPS (1μg/ml) increased PGE_2 about 50 fold in 24h, and pretreatment with pioglitazone 1h prior to LPS administration significantly decreased PGE_2 generation. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (**p<0.01 versus control; ***p<0.001 versus control; ##p<0.01 versus LPS).

Inhibition of COX-2 protects dopaminergic neurons against LPS challenge

In order to confirm whether inhibition of COX-2 activity protects dopaminergic neurons from LPS exposure, a selective COX-2 inhibitor (CAY10404, 1μM) was used in neuron-microglia mixed culture, 1hr before a 96h LPS exposure. As shown in Figure 3.3, inhibition of COX-2 protects dopaminergic neurons against LPS treatment (p<0.05).

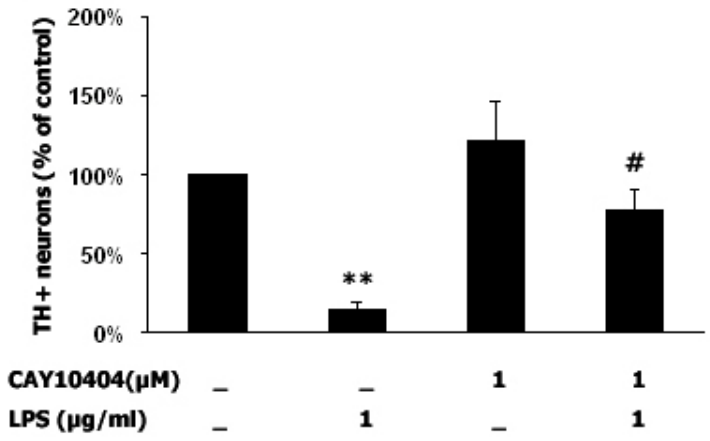


Figure 3. 3 Inhibition of COX-2 protects dopaminergic neurons against LPS insult in rat neuron-microglia mixed cultures.

Microglia (2×10^5 cells/well) were added back into neuron-enriched cultures (1×10^5 cells/well) at DIV6 along with 1 μM CAY10404 (a specific COX-2 inhibitor) 1 h before a 96 h LPS (1μg/ml) treatment. TH-positive immunostained neurons were counted and the results show that COX-2 inhibition protects the neurons. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (**p<0.01 versus control; #p<0.05 versus LPS).

Suppression of PGE_2 generation by pioglitazone is associated with interference with NF-kB and JNK activity

Since both the JNK and NF-kB pathways are involved in the proinflammatory response to LPS insult (Hambleton et al., 1996), we tested whether pioglitazone can suppress JNK and NF-kB phosphorylation. As shown in Figure 3.4, LPS (1μg/ml) increased the amount of phosphorylated JNK (Thr-183 and Tyr-185) and phosphorylated NF-kB (p65 at ser276). In contrast, pioglitazone treatment, 1hr before LPS, inhibited the phosphorylation of JNK and NF-kB ($p<0.05$). To investigate our hypothesis that pioglitazone inhibits NF-kB nuclear translocation in microglia, immunostaining of OX-42 (an indicator for microglia) and phosphorylated NF-kB p65 was performed in primary mesencephalic culture, and NF-kB nuclear translocation was observed in microglia upon LPS (1μg/ml) treatment (Figure 3.5 b). In contrast, pretreatment with pioglitazone suppressed NF-kB nuclear translocation (Figure 3.5 d).

To further determine if inhibition of JNK or NF-kB leads to the suppression of PGE_2 generation, sulfasalazine, a selective NF-kB inhibitor, and SP600125, a selective JNK inhibitor, were used in microglia-enriched cultures, 1hr before LPS treatment (1μg/ml and 10μg/ml). As shown in Figure 3.6, a 50 and 130-fold increase in PGE_2 generation were found, respectively ($p<0.01$ or $p<0.001$). Pretreatment with sulfasalazine (10μg/ml) or SP600125 (5μM) significantly suppressed PGE_2 generation after a 24h LPS exposure ($p<0.01$).

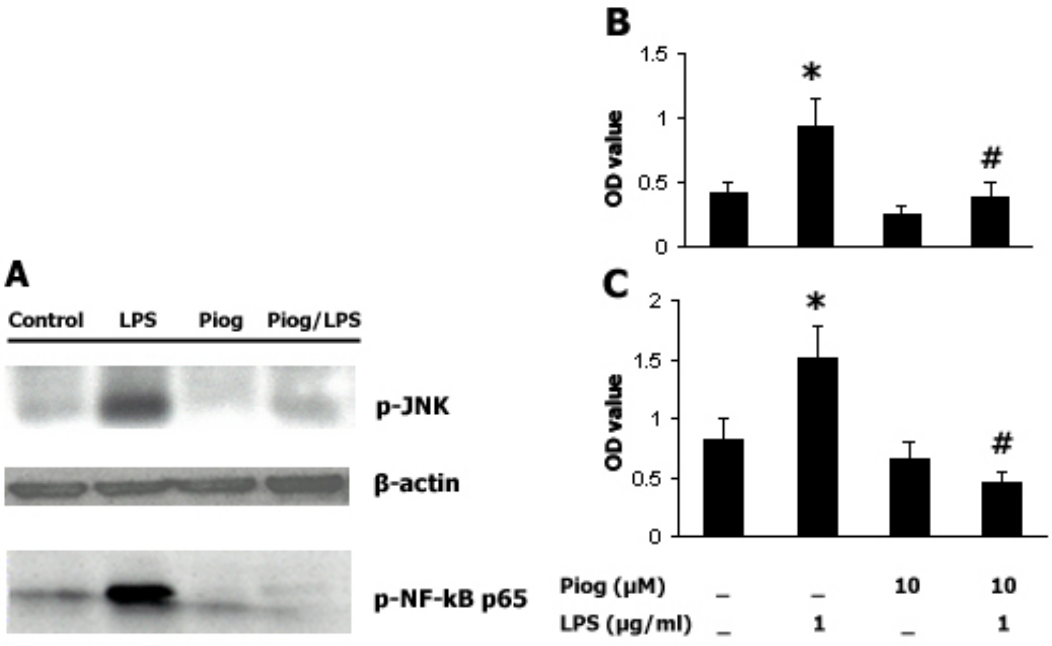


Figure 3. 4 Pioglitazone inhibits LPS-induced phosphorylation of JNK and NF-kB in neuron-microglia mixed cultures.

A: Microglia were added back into neuron-enriched cultures, and increased phosphorylated JNK and NF-κB were found upon 1μg/ml LPS exposure. Pretreatment with pioglitazone (10μM), for 1 h, inhibited JNK phosphorylation (Thr-183 and Tyr-185) and NF-κB p65 (Ser276) phosphorylation. **B and C:** Densitometric measurements showed LPS-induced increased JNK (B) and NF-kB (C) phosphorylation and inhibition by pioglitazone. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (*p<0.05 versus control; #p<0.05 versus LPS).

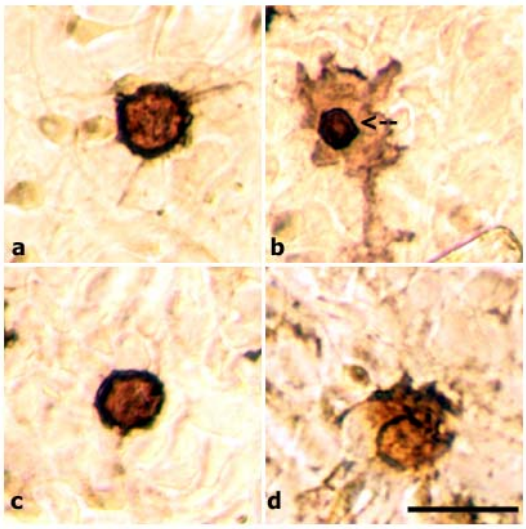


Figure 3. 5 Pioglitazone inhibits NF-kB nuclear translocation in microglia upon LPS stimuli.
Primary mesencephalic cultures (1×10^5 cells/well) were immunostained with anti-OX-42 antibody (1:1000), followed by anti-NF-κB p65 (1:125) staining. In the control group, NF-κB p65 staining (dark blue) was in the cytoplasm (a), however, sixty minutes of LPS (1μg/ml) exposure induced NF-κB p65 nuclear translocation, as shown by the arrow in (b). Both the pioglitazone group (c) and pioglitazone plus LPS group (d) showed that NF-κB p65 staining was in the cytoplasm, rather than the nucleus, suggesting that pretreatment with pioglitazone 1 h before LPS treatment suppressed NF-κB nuclear translocation.

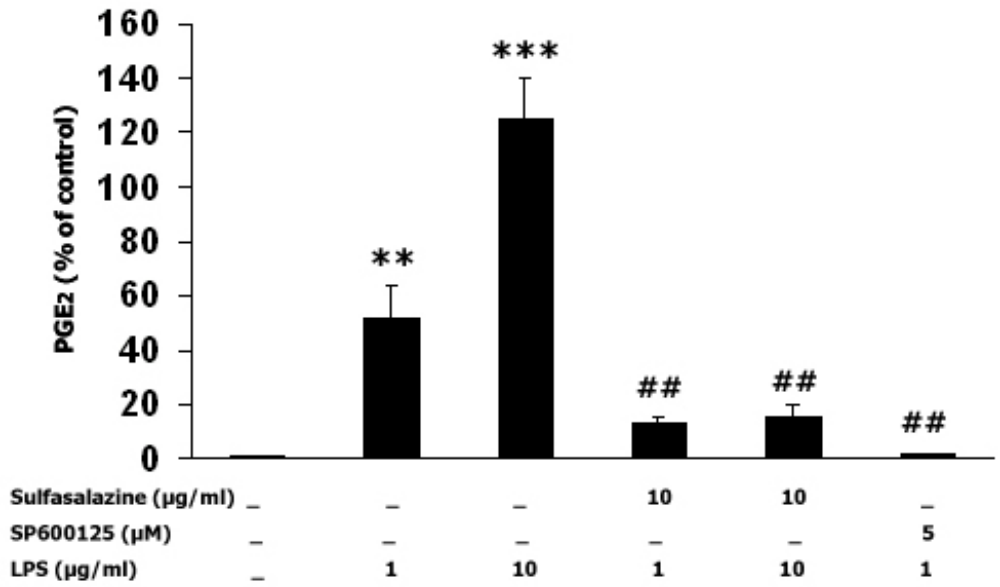
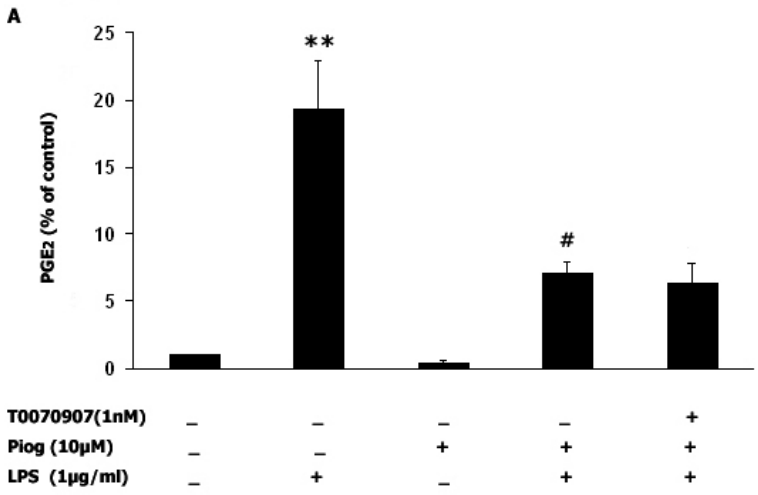


Figure 3. 6 Inhibition of NF-kB and JNK reduces PGE_2 generation in microglia-enriched cultures.

Microglia-enriched cultures (2×10^5 cells/well) were treated 24 h after seeding, and a 24h LPS (1μg/ml) treatment induced PGE_2 generation in a dose-dependent manner. The NF-kB inhibitor (Sulfasalazine) or JNK inhibitor (SP600125) was added 1 h before the LPS treatment and the LPS induced PGE_2 generation was significantly reduced. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (**p<0.01 versus control; ***p<0.001 versus control; ##p<0.01 versus LPS 1μg/ml).

A PPARγ antagonist failed to reverse pioglitazone inhibition of LPS-induced PGE_2 generation in primary microglia-enriched culture

Our preliminary study suggested that PGE_2 generation induced by LPS is PPARγ-independent. In order to investigate the possibility that pioglitazone inhibition of LPS-induced PGE_2 might be PPARγ-dependent in terms of intracellular and extracellular PGE_2, a highly selective antagonist T0070907 was used in the primary microglia-enriched culture. Forty-eight hour exposure to LPS (1μg/ml) stimuli induced a ~20 fold increase in the extracellular PGE_2 level, and pretreatment with pioglitazone significantly inhibited it (Figure 3.7 A, $p<0.05$), however, T0070907 (1nM) failed to show reversing effect on the inhibition of LPS-induced PGE_2 by pioglitazone. The similar response pattern was observed to measure intracellular PGE_2 level, and pioglitazone decreased its level, however, pretreatment with T0070907 (1nM) did not show its reversing effect on the PGE_2 inhibition by pioglitazone (Figure 3.7 B).



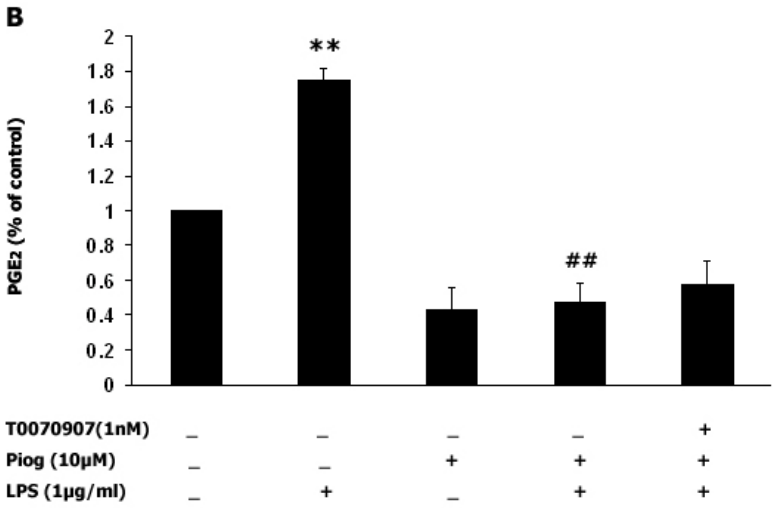


Figure 3. 7 Effect of T0070907 on the pioglitazone inhibition of LPS-induced PGE_2 generation.

Primary microglia-enriched culture ($2x10^5$/well) was treated with T0070907 (1nM) either alone or plus pioglitazone (10μM) and / or LPS (1μg/ml) for 48hr, then extracellular and intracellular PGE_2 levels were measured (Figure 3.7 A and B). **A:** (extracellular PGE_2) Pioglitazone inhibited LPS-induced PGE_2 levels, pretreatment with T0070907 did not block the inhibition of LPS-induced PGE_2 production by pioglitazone. **B:** (intracellular PGE_2) a similar pattern was found. Pretreatment with T0070907 (1nM) failed to reverse pioglitazone inhibition of LPS-induced PGE_2. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (**p<0.01 versus control; #p<0.05 versus LPS; ##p<0.01 versus LPS).

Discussion

In the previous chapter, our results showed that pioglitazone protects dopaminergic neurons against LPS neurotoxicity via inhibiting microglia activation.

In this chapter, LPS-induced COX-2 and PGE_2 was emphasized, and the potential interference of the relevant signaling pathways with pioglitazone was investigated. In addition, the potential inhibition of PGE_2 by pioglitazone was explored. The present *in vitro* study demonstrates that the neuroprotective effects of pioglitazone are associated with inhibiting JNK and NF-kB pathways, which inhibits the inflammatory reaction by attenuating the expression of COX-2 and PGE_2 generation, resulting in the survival of dopaminergic neurons. In addition, it appears that the inhibition of PGE_2 synthesis by pioglitazone might not be PPARγ-dependent.

COX-2 has been suggested as a key element in the pathological process of PD. COX-2 expression in the ventral midbrain of PD brain samples is increased (Teismann et al., 2003b) and COX-2 inhibition or deficiency protects dopaminergic neurons against MPTP and 6-OHDA toxicity (Teismann and Ferger, 2001; Sanchez-Pernaute et al., 2004; Vijitruth et al., 2006). Our LPS *in vitro* PD model demonstrated that pioglitazone inhibits COX-2 expression (Hunter et al., 2007). The present study reports that pioglitazone inhibits LPS-induced COX-2 in the primary mesencephalic-microglia mixed culture and PGE_2 synthesis in microglia. Combined with the observation that LPS neurotoxicity is microglia-dependent described in chapter 2 and inhibition of COX-2 rescues the neurons, the results suggest that microglia-derived PGE_2 may play an important role in the detrimental process of dopaminergic neuronal death, and pioglitazone provides beneficial effects via inhibiting its synthesis. Although the underlying mechanisms of cellular PGE_2–induced neurotoxicity in human are not known, the distribution of PGE_2 receptors (EP1, 2, 3, and 4) in rat midbrain and the potential PGE_2 receptor-mediated neurotoxicity in 6-OHDA *in vitro* model was investigated (Carrasco et al., 2007). Their studies identified EP1 and EP2 receptors on the dopaminergic neurons by immunohistochemistry for TH and PGE_2 receptors. Interestingly, only activation of EP1 receptor is toxic to dopaminergic neurons, in agreement with the similar toxicity induced by PGE_2 treatment. In contrast, selective

antagonist for EP1 receptor rescues dopaminergic neurons against 6-OHDA insult in primary mesencephalic neuronal cultures.

Based on the aforementioned reports, pioglitazone protects dopaminergic neurons probably via inhibiting LPS-induced PGE_2 synthesis. In addition, pioglitazone might also regulate EP1 receptor activity to attenuate PGE_2-mediated toxicity. Indeed, this could be possible since a PPARβ agonist increased EP4 mRNA and protein levels in human lung carcinoma cell line (Han et al., 2005).

Further study on the NF-kB and JNK activity in mixed cultures showed that pioglitazone suppresses their phosphorylation and NF-kB nuclear translocation induced by LPS. In addition, inhibition of NF-kB and JNK activity decreases PGE_2 level, suggesting not only NF-kB but also JNK mediate COX-2 activity, which is the first report demonstrating LPS-induced COX-2-mediated dopaminergic neuronal death *in vitro* model is associated with increased JNK activity.

Increased NF-kB immunoreactivity and expression were observed in the midbrain of PD patients (Hunot et al., 1997; Mogi et al., 2007). The current study showed that pioglitazone inhibits LPS-induced NF-kB phosphorylation and microglial nuclear translocation, in agreement with the study on the pioglitazone neuroprotection in MPTP animal model (Dehmer et al., 2004). The underlying mechanisms of the inhibitory effect of NF-kB activity by PPARγ agonists are not clearly known due to the wide spectrum of gene transcriptions regulated by both PPARγ and NF-kB. However, results from different cell culture systems suggested that PPARγ agonists including synthetic ligands inhibit NF-kB activation via PPARγ-independent mechanisms. For example, the PPARγ agonist 15d-PGJ2 inhibits the LPS-induced degradation of IkBα in RAW264.7 cells which only express very low PPARγ (Straus et al., 2000; Castrillo et al., 2001).

The direct implication that JNK activation may be related to the death of dopaminergic neurons came from the observation that the JNK inhibitor CEP-1347 protected neurons against MPTP toxicity (Saporito et al., 1999). Further results from an experimental PD model provided a link between the activation of JNK activation / COX-2 expression and survival of dopaminergic neurons. In their studies, MPTP increased c-JUN phosphorylation in the midbrain, in contrast, the JNK inhibitor CEP-11004 or JNK deficiency suppressed its expression accompanied with a decreased COX-2 expression

(Teismann et al., 2003b; Hunot et al., 2004). In the midbrain slice culture system, inhibition of JNK activity protects dopaminergic neurons against LPS/IFNγ insult (Shibata et al., 2006). It was suggested that MPTP activates the JNK signaling cascade, and JNK kinase (MKK4) / JNK / c-Jun / caspase-3 sequential activation is involved in dopaminergic neuronal death (Xia et al., 2001). Further study on the interference of JNK signaling-mediated neuronal loss by pioglitazone will be informative to understand its underlying protective mechanisms.

It is worth noting that T0070907, a highly selective PPARγ antagonist (Lee et al., 2002), has generated great interest recently as it is more potent and selective (with its IC_{50} 1nM for rosiglitazone) than other antagonists such as GW9662. We used T0070907 to test if the inhibition of LPS-induced PGE_2 generation by pioglitazone is PPARγ-dependent. Under current experimental condition, no significant blocking effect of T0070907 on the inhibition of PGE_2 by pioglitazone was observed in the measurement of both intracellular-PGE_2 and excellular-PGE_2 levels, suggesting pioglitazone might suppress LPS-induced PGE_2 in a PPARγ-independent manner. One study on the PGE_2 synthesis in lung cancer cell line revealed PPARγ-independent mechanisms since the PPARγ antagonist GW9662 did not block pioglitazone-mediated inhibition of PGE_2 generation (Hazra et al., 2007). On the other hand, another study on the TNFα-induced PGE_2 production in human smooth muscle cell culture demonstrated a PPARγ-dependent effect (Ringseis et al., 2006). Since pioglitazone inhibits PGE_2 synthesis partially via inhibiting NF-kB, which can be inhibited by PPARγ agonists via receptor-independent mechanisms (Straus et al., 2000; Castrillo et al., 2001), the PPARγ-independent inhibition of PGE_2 in our cell culture system may be related to the PPARγ-independent inhibition of NF-kB. PPARγ knockdown will be very helpful to address if its inhibitive effect on PGE_2 is receptor-dependent or independent.

The aforementioned studies indicate that the neuroprotective effects of pioglitazone are associated with its blocking multiple proinflammatory signaling pathways, including NF-kB and JNK pathways. However, its potential inhibitory effect on microsomol PGE synthase-1 (mPGES-1), which is the enzyme converting PGD_2 to PGE_2, cannot be ruled out, since LPS administration *in vivo* and *in vitro* causes persistent upregulation of mPGE-1 (Devaux et al., 2001; Ajmone-Cat et al., 2003). In addition,

activation of PPARγ blocked the up-regulation of COX-2, mPGES-1, and PGE_2 in mouse embryo fibroblasts (Kapoor et al., 2007). Therefore, in the future, it would be very interesting to determine if pioglitazone influences mPGES-1 activity in our cell culture systems.

In summary, the studies described in the current chapter suggest that the neuroprotective effects of pioglitazone are associated with the inhibition of JNK and NF-kB pathways as well as with the suppression of COX-2 activity and decreased PGE_2 generation, probably via PPARγ-independent mechanisms. This ultimately protects dopaminergic neurons via attenuating microglia-mediated and inflammation-induced neurodegeneration.

In the next chapter 4, LPS-induced iNOS / NO and its inhibition by pioglitazone via suppression of p38 MAPK signaling pathway will be emphasized.

CHAPTER 4: Pioglitazone inhibition of lipopolysaccharide-induced nitric oxide synthase is associated with altered activity of p38 MAP kinase and PI3K/Akt

(From published paper: B Xing, T Xin, RL Hunter, GY Bing. (2008) J. Neuroinflammation 5:4). T Xin took part in Western blot; RL Hunter took part in experimental design.

Introduction

Chapter 2 demonstrated that LPS neurotoxicity is microglia-dependent, and pioglitazone protects dopaminergic neurons via inhibiting microglia activation. In chapter 3, the study suggested that pioglitazone protects dopaminergic neurons against LPS insult by suppressing COX-2 expression and PGE_2 synthesis via interfering with NF-kB and JNK pathways. In this chapter, LPS-induced iNOS and NO generation are emphasized, and the following questions are discussed: 1) the inhibitory effects of pioglitazone on LPS-induced iNOS and NO production; 2) the potential differential role of p38 MAPK and JNK pathway in the LPS-induced NO generation; 3) the potential differential mediation of p38 MAPK and JNK by pioglitazone; 4) the potential mediation of PI3K / Akt activity by pioglitazone and its effect on NO generation.

One of the major effectors responsible for neurodegeneration is NO. Under normal condition, NO is a necessary component for synaptic transmission (Bredt, 1999). Under pathological condition, however, excessive NO can react with superoxide and form the lipid-permeable oxidant peroxynitrite which can directly oxidize proteins, lipids, and DNA. In addition, peroxynitrite can disrupt mitochondria function (Brown, 1999) and is related to mitochondria-driven reactive oxygen and reactive nitrogen species production (Poderoso et al., 1996), ultimately resulting in cell death.

Twenty years ago, postmortem analysis revealed that a great number of activated microglia surround dopaminergic neurons in the SN of PD brain samples (McGeer et al., 1988b). Recent postmortem studies provided evidence on the detrimental role of activated microglia-mediated iNOS / NO in dopaminergic neuron degeneration. In their studies upregulated microglial iNOS expression was observed in the SN of PD brain (Hunot et al., 1996; Knott et al., 2000). The experimental studies demonstrated that increased iNOS activation is associated with the death of dopaminergic neurons *in vitro* (Okuno et al., 2005; Lin et al., 2007) and *in vivo* (Liberatore et al., 1999; Teismann and

Ferger, 2001; Arimoto and Bing, 2003). In contrast, inhibition of iNOS showed neuroprotection toward dopaminergic neurons against toxic insults (Wang et al., 2002; Katsuki et al., 2006).

p38 MAPK and JNK pathways are two main signaling cascades modulating inflammatory response and stress (Kyriakis and Avruch, 2001). Experimental *in vitro* study suggested that activation of p38 MAPK is involved in LPS-induced insults in glial cells (Bhat et al., 1998). Activated microglia-induced cortical neuronal death has been attributed to p38 MAPK activation (Xie et al., 2004), and inhibition of p38 MAPK activity rescued dopaminergic neurons from a thrombin-activated microglia insult (Lee et al., 2005). Recent LPS *in vitro* and *in vivo* studies strongly implicated a role of p38 MAPK in the LPS-induced microglia-mediated dopaminergic neuronal loss. Inhibition of p38 MAPK pathway by its inhibitor protected neurons and NO production in the primary mesencephalic culture was decreased (Jeohn et al., 2002). The other study showed that intranigral co-injection of LPS with p38 MAPK inhibitor rescued dopaminergic neurons in the SN, accompanied with reduced iNOS expression (Ruano et al., 2006). In contrast, the role of the JNK pathway in the LPS model is less studied. The result from a midbrain slice culture study suggested that inhibition of JNK activity rescues LPS/IFNγ-induced dopaminergic neuronal loss without influencing iNOS expression (Shibata et al., 2006). However, in their studies the role of astrocytes was not ruled out, and the potential role of JNK pathway in the mediation of LPS-induced NO were not tested.

One study on mouse peritoneal macrophages demonstrated that the PPARγ agonist 15d-PGJ2 suppresses IFN-γ-induced iNOS protein and its mRNA (Ricote et al., 1998). An *in vivo* study demonstrated that the PPARγ agonist troglitazone suppresses cerebellular iNOS expression and protected cerebellular granule cells against LPS/IFN-γ insult (Heneka et al., 2000). A very recent study on the experimental PD model demonstrated that the PPARγ agonist pioglitazone protected dopaminergic neurons from MPTP insult, and iNOS-positive microglial cells were suppressed (Breidert et al., 2002); further study from the same research group suggested that pioglitazone protected TH positive neurons against MPTP insult probably via PPARγ activation and suppression of

iNOS expression (Dehmer et al., 2004). However, whether the inhibition of iNOS by pioglitazone is PPARγ-dependent or PPARγ-independent is still not defined.

The above studies strongly suggested that PPARγ agonists protect dopaminergic neurons via inhibiting microglial iNOS expression. However, several questions remain to be addressed. First, the role of pioglitazone in mediating LPS-induced NO production in microglia has not been studied; Second, the role of pioglitazone in the LPS-induced p38 MAPK pathway have not been investigated. So, the first hypothesis in this chapter is that pioglitazone inhibits LPS-induced NO generation probably via interference with p38 MAPK and JNK pathways. The first specific aim is to test 1) the inhibitory effect of pioglitazone on LPS-induced NO generation (by nitrite assay) in primary microglia cultures and iNOS expression (by western blot) in mixed cultures, 2) the inhibitory effect of pioglitazone on LPS-induced p38 MAPK in mixed cultures (by western blot), 3) the effect of p38 MAPK and JNK inhibition on NO production in microglia by using their selective inhibitors, and 4) if the inhibition of LPS-induced NO generation by pioglitazone is PPARγ-dependent or PPARγ-independent.

PI3K / Akt is a well-known cell survival signaling pathway (Brazil and Hemmings, 2001). Neurotrophic factors such as nerve growth factor promote neuronal survival via activating the PI3K/Akt cascade (Halvorsen et al., 2002; Nusser et al., 2002; Bonnet et al., 2004; Zhou et al., 2004; Yoshii and Constantine-Paton, 2007). On the other hand, PI3K/Akt mediates both the apoptosis and inflammatory responses (Cantley, 2002). Inhibition of PI3K by LY294002 promoted MPP^+-induced SH-SY5Y apoptosis, and activation of Akt can protect cells (King et al., 2001). Nerve growth factor treatment prevented MPP+-induced SH-SY5Y apoptosis possibly via increasing the phosphorylation of PI3K / Akt (Halvorsen et al., 2002). Recent studies demonstrated that the PI3K/Akt pathway inhibits the inflammatory response in monocytes via inhibiting the JNK and p38 MAPK pathways (Guha and Mackman, 2002). It is interesting to know if PI3K differentially mediates p38 MAPK and JNK activity inhibiting LPS-induced NO. Although the potential effects of PPARγ agonists on the PI3K activity have not been studied, one study on the LPS-induced iNOS in mucous acinar cells demonstrated that the PPARγ agonist ciglitazone inhibited its expression and inhibition of PI3K countered its inhibitive effect (Slomiany and Slomiany, 2003). Whether pioglitazone alters the activity

of PI3K/Akt pathway and thus inhibits LPS-induced NO is a very interesting question. The second hypothesis is that 1) the PI3K pathway takes part in LPS-induced p38 MAPK / JNK activation; 2) pioglitazone alters activation of the PI3K/Akt pathway. The second aim is to test 1) if inhibition of PI3K activity increases LPS-induced p38 MAPK and JNK phosphorylation (western blot), by using the PI3K selective inhibitor wortmannin; 2) if inhibition of PI3K increases LPS-induced p38 MAPK-mediated NO production in primary microglia culture (nitrite assay); and 3) if PI3K / Akt activity is altered by pioglitazone treatment (western blot).

Methods

Animals

Thirty two timed-pregnant Sprague Dawley rats were obtained from Harlan (Indianapolis, IN, USA), and maintained in a pathogen-free environment. Housing, breeding, and experimental use of the animals were performed in strict accordance with the National Institutes of Heath guidelines and were approved by the Institute's Animal Care and Use Committee at the University of Kentucky.

Reagents

Same as these mentioned in chapter 2 and 3 except the following: T0070907-PPARγ antagonist and 1400W-iNOS inhibitor from Cayman Chemical (Ann Arbor, MI, USA), SP600125-JNK inhibitor and SB203580-p38 inhibitor from A.G. Scientific (San Diego, CA, USA), and wortmannin-PI3K inhibitor from Sigma-Aldrich. Antibodies: polyclonal anti-iNOS from Millipore (Billerica, MA, USA), monoclonal anti- phospho p38 from Cell Signaling (Danvers, MA, USA), monoclonal anti-PPARγ (ser473) from Upstate (Billerica, MA, USA), polyclonal anti-PI3K p110 and polyclonal anti-Akt (Thr308) from Santa Cruz (Santa Cruz, CA, USA).

Mesencephalic neuron-microglia mixed cultures

Same as that mentioned in chapters 2 and 3.

Primary mesencephalic neuron-enriched cultures

Same as that mentioned in chapters 2 and 3.

Microglia-enriched cultures
Same as that mentioned in chapters 2 and 3.

Nitrite oxide assay
The production of NO was assessed by the accumulation of nitrite in culture supernatants by using the colorimetric reaction of the Griess reagent. Culture supernatants were collected at different time points following LPS stimulation and were mixed with Griess reagent (0.1% N-[1-naphthyl] ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4). The absorbance at 548nm was measured with a spectraMAX microplate reader from Molecular Devices (Sunnyvale, CA, USA). For the PPARγ inhibition study, T0070907 (1nM) was added into the culture medium 1hr prior to either pioglitazone (10μM) and / or LPS (1μg/ml) treatment for 48hr.

Western blot
Same as that described in chapters 2 and 3 except different antibodies were used. The antibodies and their concentrations used in western blot are: Polyclonal anti-iNOS antibody (1:1000), monoclonal anti-p38 (1:2000), monoclonal anti-PPARγ (1:250), polyclonal anti-PI3K p110 (1:250), and polyclonal anti-Akt (1:250).

Statistical analysis
The data are expressed as the mean ± SEM and statistical significance was assessed by ANOVA followed by a Tukey comparisons test using the SYSTAT 10 software (SPSS Inc., Chicago, Illinois). A value of $p<0.05$ was considered statistically significant.

Results

Pioglitazone inhibits LPS-induced nitric oxide generation in microglia-enriched cultures
To determine the effect of the PPARγ agonist pioglitazone on NO generation, two different doses of pioglitazone (1μM and 10μM) were administered to microglia-enriched cultures 1hr before LPS (1μg/ml) treatment. LPS induced a 4-fold increase in NO generation ($p<0.001$) after 48hr, and pretreatment with pioglitazone reduced NO production by about 40% to 60% ($p<0.001$), respectively (Figure 4.1). However, administration of pioglitazone concurrent with LPS, or 1hr after LPS, failed to inhibit the

LPS-induced NO increase (Figure 4.2). In addition, pioglitazone alone did not alter NO production.

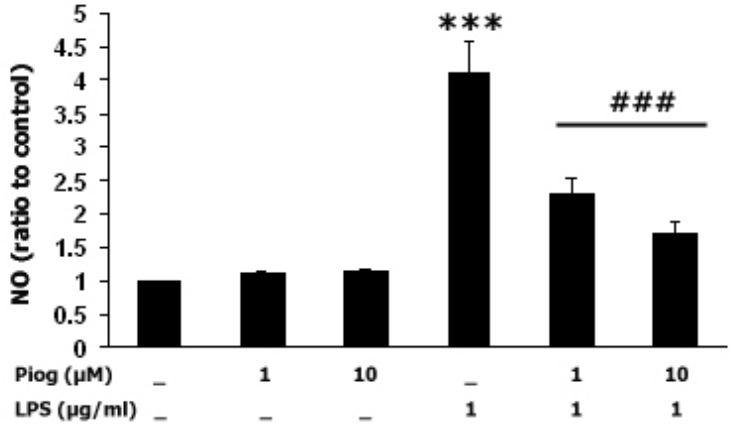


Figure 4. 1 Pioglitazone inhibits LPS-induced NO production in microglia-enriched cultures.
Microglia cultures ($2x10^5$ cells/well) were treated with pioglitazone (1 μM and 10μM) 1 hr before LPS treatment, and 48 h later NO levels were measured. LPS significantly induced NO generation, and pretreatment with pioglitazone inhibited this LPS-induced NO production in a dose-dependent manner. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (***$p <$ 0.001 versus. control; ###$p < 0.001$ versus. LPS)

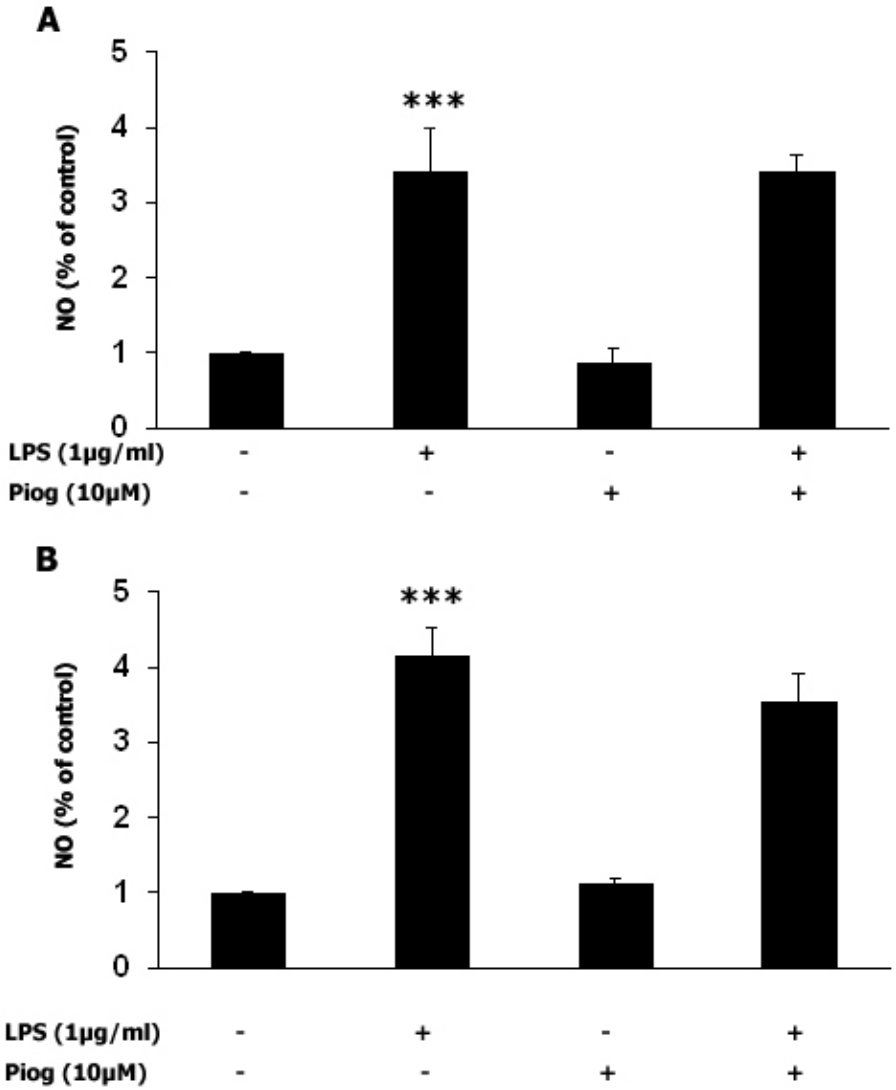


Figure 4. 2 Post-treatment of pioglitazone did not show inhibition of LPS-induced NO production in microglia-enriched cultures

Pioglitazone was added with LPS at the same time or 1 hr after LPS treatment in microglia-enriched cultures ($2x10^5$ cells/well), and 48 h later NO levels were measured. **A:** pioglitazone was added with LPS at the same time. **B:** pioglitazone was added 1h after LPS treatment. In both A and B, LPS significantly induced NO generation, however, no inhibitory effect of NO was observed. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (***$p <$ 0.001 versus. control)

Pioglitazone inhibits LPS-induced iNOS expression, and iNOS inhibition protects dopaminergic neurons from LPS insults in mesencephalic mixed cultures

In this set of experiments, iNOS expression was determined by western blot performed 48h after LPS (1μg/ml) treatment. As shown in Figure 4.3, basal iNOS expression was decreased by pioglitazone (p<0.001), LPS treatment produced significantly enhanced iNOS expression (p<0.01), and pretreatment with pioglitazone (10μM) significantly reduced this LPS-induced increase in iNOS expression (p<0.01). In addition, we used immunocytochemistry for TH-positive cells to assess the effect of a specific iNOS inhibitor, 1400W (1nM to 10μM), on the survival of dopaminergic neurons 72hr after LPS treatment. Figure 4.4 shows that LPS induces a significant loss (90%) of the TH-positive neurons when the iNOS inhibitor is administered 1hr before LPS (1μg/ml). Partial neuroprotection against the LPS insult was seen when using 1400W at 100nM (p<0.05) and 1μM (p<0.001).

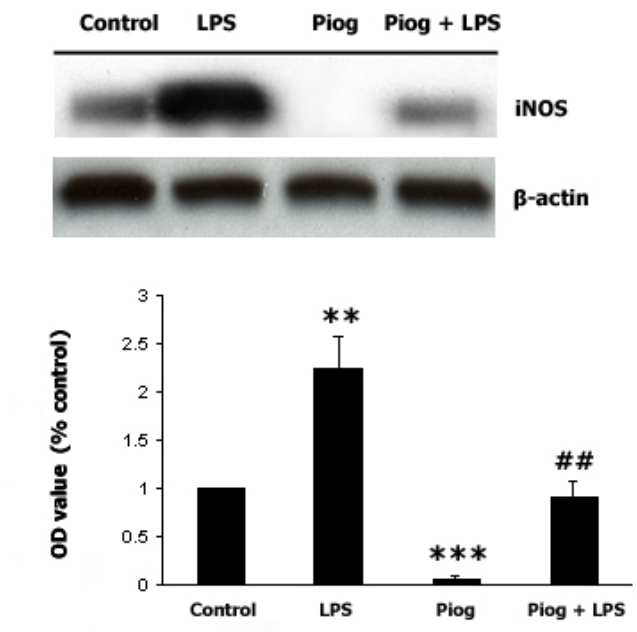


Figure 4. 3 Pioglitazone inhibits LPS-induced iNOS expression.
Rat mesencephalic mixed cultures ($2x10^6$cells/well) were treated with 1 μg/ml LPS for 48 hours. LPS treatment upregulated the expression of iNOS, and pretreatment with pioglitazone (10 μM), 1 hr before LPS, prevents its expression. The data were expressed as the percentage of control and were the mean ± SEM, from three independent

experiments. (n=3) (**p < 0.01 versus. control, ***p < 0.001 versus. control, ##p < 0.01 versus. LPS).

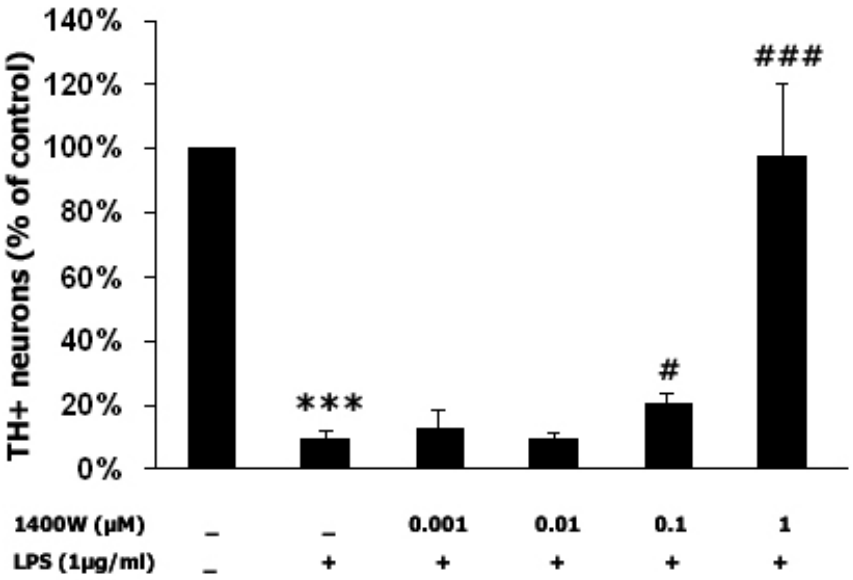


Figure 4. 4 iNOS inhibition protects dopaminergic neurons from LPS insults.
Rat mesencephalic mixed cultures ($2x10^5$ microglia added) were treated with the selective iNOS inhibitor 1400 W, with different doses from 1 nM to 1μM, 1 hr before a 72 hr LPS exposure. The number of TH-positive neurons was determined by immunocytochemistry. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (***p < 0.001 versus. control, #p < 0.05 versus. LPS, ###p < 0.001 versus. LPS).

Pioglitazone reduces NO levels by inhibition of p38 MAPK activity

In this set of experiments, pretreatment with pioglitazone (10μM) 1hr before LPS (1μg/ml) decreased phosphorylation of p38 MAPK (Figure 4.5), and pretreatment with wortmannin (1μM and 10μM) increased LPS-induced p38 MAPK phosphorylation in a dose-dependent manner (Figure 4.6 A-C. $p<0.05$). An increase in phosphorylation of p38 MAPK was not found when wortmannin was administered alone, without LPS stimuli (Figure 4.6 D). Wortmannin also did not change JNK expression (Figure 4.6 A and C). In addition, two proinflammatory pathways were examined, in order to demonstrate their involvement in the LPS-induced increase in NO production. Either SB203580 (a selective p38 MAPK inhibitor) or SP600125 (a selective JNK inhibitor) was administered to microglia-enriched cultures 1hr before LPS (1μg/ml) exposure. As shown in Figure 4.7, LPS significantly increased NO generation ($p<0.001$) and inhibition of p38 MAPK activity by pretreatment with SB203580 (5μM) decreased this NO production ($p<0.05$).

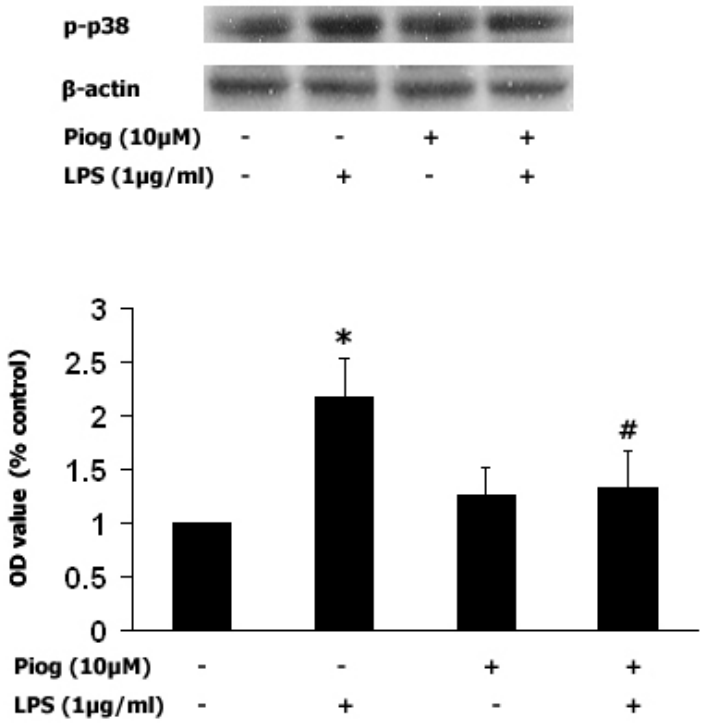


Figure 4. 5 Pioglitazone inhibits LPS-induced p38 MAPK activity.
Pretreatment with pioglitazone inhibited LPS-induced phosphorylation of p38 MAPK in mesencephalic neuronal-microglia mixed cultures. Pioglitazone was added 1 hr before LPS treatment (1 μg/ml) and p38 MAPK was immunobloted. LPS increased phosphorylation of p38 MAPK, and pretreatment with pioglitazone inhibited this expression. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (*$p < 0.05$ versus. control, #$p < 0.05$ versus. LPS).

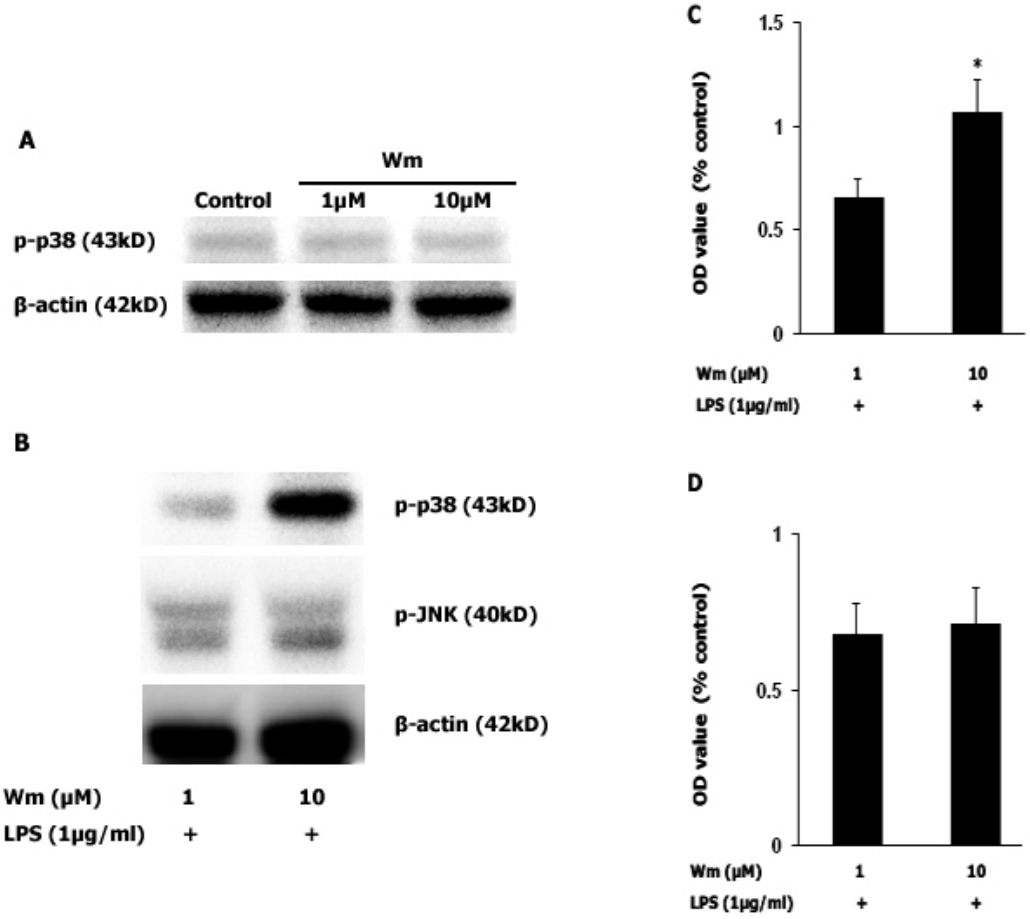


Figure 4. 6 Inhibition of PI3K activity increases LPS-induced p38 MAPK activity upon LPS stimuli.

Wortmannin (1 μM and 10 μM) was administered to mesencephalic neuron-microglia mixed cultures alone or before LPS (1 μg/ml) was added and, after 30 mins, p-JNK and / or p-p38 MAPK was immunobloted. **A:** In the absence of LPS stimuli, inhibition of PI3K did not increase p38 MAPK phosphorylation. **B:** Upon LPS stimuli, inhibition of PI3K increases p38 MAPK phosphorylation rather than JNK phosphorylation. **C:** Densitometric measurements showed that in the presence of LPS stimuli, inhibition of PI3K increased p38 MAPK phosphorylation. **D:** Densitometric measurements showed that in the presence of LPS stimuli, inhibition of PI3K did not increase JNK phosphorylation. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (*$p < 0.05$ versus. wortmannin 1μM plus LPS).

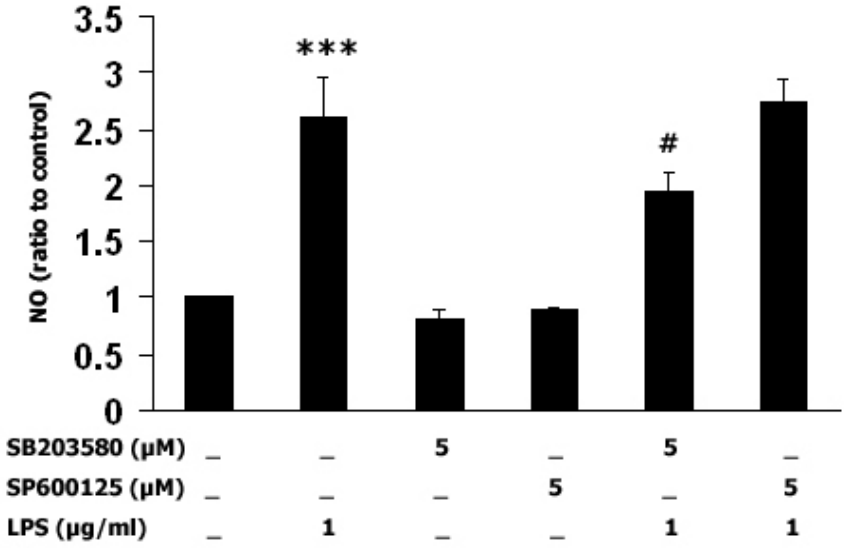


Figure 4. 7 Inhibition of p38 MAPK decreases LPS-induced NO generation in microglia-enriched cultures.
A selective p38 MAPK inhibitor (SB203580), or a selective JNK inhibitor (SP600125), was added to microglia-enriched cultures 1 hr before LPS (1 μg/ml) exposure and, after 24 h, NO levels were measured. The inhibition of LPS-induced NO was only found using SB203580. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (***p<0.001 versus control; #p<0.05 versus LPS)

The alteration of PPARγ activity is not shown in primary neuron-enriched cultures upon pioglitazone treatment and LPS insult

As shown in Figure 4.8, in primary neuron-enriched culture, activation of PPARγ by pioglitazone is not observed.

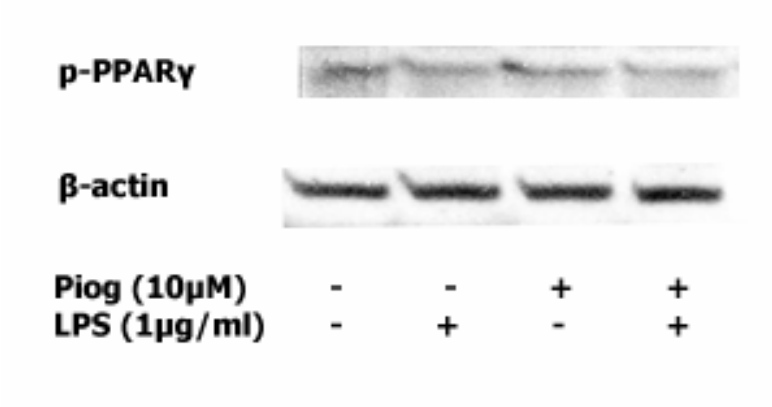


Figure 4. 8 Activation of PPARγ by pioglitazone is not observed in the primary neuron-enriched culture.

Primary neuron-enriched cultures (2×10^6 cells/well) were treated with pioglitazone (10 μM) 1 hr before LPS (1 μg/ml) exposure. PPARγ activation was assessed after 10 min. PPARγ activation was not observed in the both pioglitazone- and LPS-treated group. The data were from three independent experiments. (c=control, L1=LPS (1 μg/ml), P10 =pioglitazone (10 μM)).

Inhibition of PI3K activity prevents the inhibitory effect of pioglitazone on LPS-induced NO production and pharmacological inhibition of PPARγ activity increases LPS-induced NO level

To determine if pioglitazone enhances PI3K/Akt expression and if its inhibition enhances LPS-induced NO generation, the levels of PI3K and Akt were determined. PPARγ, PI3K, and Akt phosphorylation were measured after LPS (1μg/ml) exposure. As shown in Figure 4.9, PPARγ activation was observed in pioglitazone-treated cultures within 10min after DMSO or LPS. PI3K and phosphorylated Akt were increased 60min after LPS in the pioglitazone-treated cultures ($p<0.05$). Next, wortmannin (1μM) was added 30mins before pioglitazone (10μM) treatment and the NO level was measured 48h after LPS

(1μg/ml). The results showed that pretreatment with pioglitazone inhibited the LPS-induced NO increase (Figure 4.10, $p<0.01$). However, when wortmannin was given 30mins before pioglitazone, NO production was increased over LPS exposure ($p<0.05$). Interestingly, administration of wortmannin (1μM) 30min before pioglitazone followed by LPS 1hr later did not show the inhibitive effect of pioglitazone on NO level. Wortmannin alone, or together with pioglitazone, did not influence NO generation without LPS stimulation. Thus, pioglitazone prevents LPS-induced NO production, and pretreatment with wortmannin increases NO generation (Figure 4.10). As shown in Figure 4.11, pretreatment with T0070907 (1nM) reverses the inhibitive effect of pioglitazone on the LPS-induced NO production ($p<0.001$).

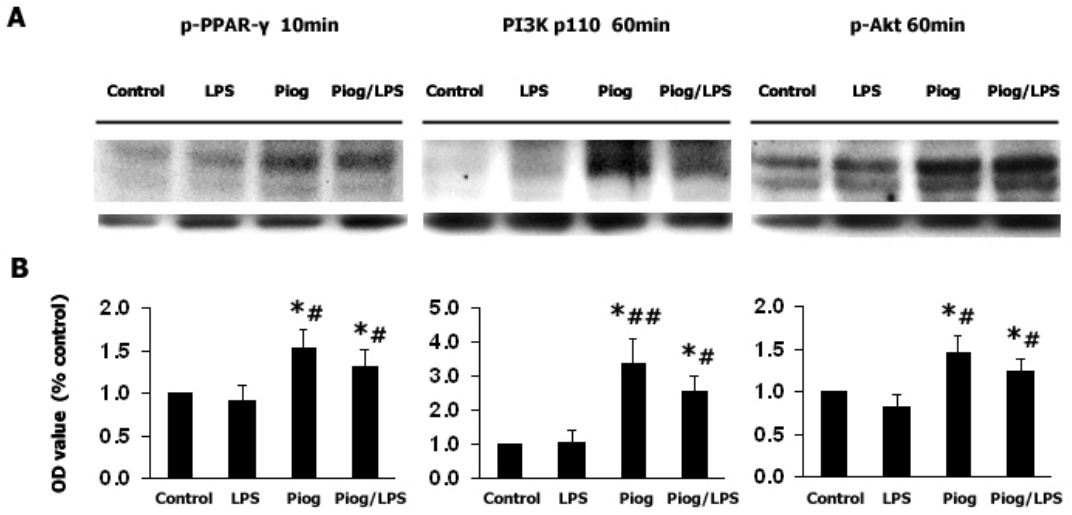


Figure 4. 9 Pioglitazone activates PPARγ and enhances PI3K/Akt activity.
Rat mesencephalic cultures (2×10^6 cells/well) were treated with pioglitazone (10 μM) only, or 1 hr before LPS (1 μg/ml) exposure. **A:** PPARγ activation was assessed after 10 min, and P13K and Akt were assessed after 60 min. PPARγ activation, PI3K and Akt expression were observed in the pioglitazone-treated cultures, compared to control and LPS alone groups. **B:** Densitometric measurements showed that the expression of phosphorylated PPARγ, PI3K, and Akt is higher than control group and LPS alone group. The data were expressed as the percentage of control and were the mean ± SEM, from

three independent experiments. (*$p < 0.05$ versus. control, #$p < 0.05$ versus. LPS, ##$p < 0.01$ versus. LPS).

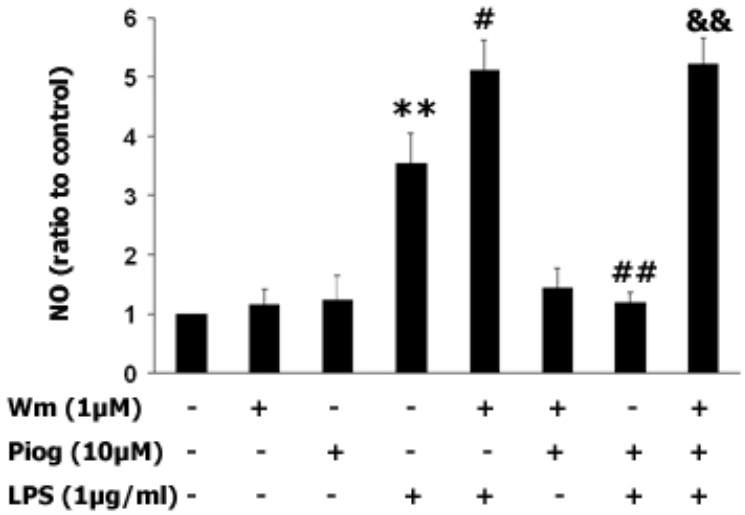


Figure 4. 10 PI3K negatively regulates the LPS-induced increase in NO production.
The specific PI3K inhibitor wortmannin (1 μM) was administered individually 90 mins before LPS treatment (1 μg/ml), or 30 mins before pioglitazone followed by LPS 60 mins later in microglia-enriched culture, and after 48 h NO levels were measured. The results show that the LPS-induced NO level was significantly higher than control ($p < 0.01$), and that pretreatment with pioglitazone inhibits LPS-induced NO ($p < 0.01$). In contrast, pretreatment with wortmannin enhanced the LPS-induced increase in NO generation ($p < 0.05$), and this pretreatment reversed the inhibitory effect of pioglitazone on LPS-induced NO generation. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (**$p < 0.01$ versus. control, #$p < 0.05$ versus. LPS, ##$p < 0.01$ versus. LPS, &&$p < 0.01$ versus. Piog plus LPS).

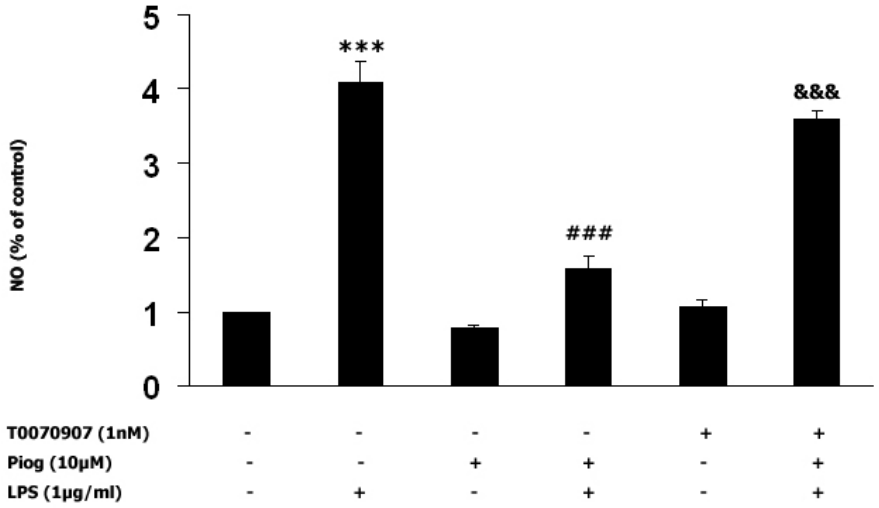


Figure 4. 11 Effect of PPARγ antagonist T0070907 on the LPS-induced NO production. Primary microglia-enriched culture ($2x10^5$/well) was treated with T0070907 (1nM) either alone or before pioglitazone treatment (10μM), followed by LPS administration (1μg/ml). After 48hr, NO production was measured. Pretreatment with T0070907 significantly reverses the inhibitive effect of pioglitazone on the LPS-induced NO production. The data were expressed as the percentage of control and were the mean ± SEM, from three independent experiments. (n=3) (***p<0.001 vs. control; ###p<0.001 vs. LPS; &&&p<0.001 vs. corresponding pioglitazone plus LPS group).

Discussion

In chapter 3, the results suggested that pioglitazone protects dopaminergic neurons by inhibiting LPS-induced COX-2 expression and PGE_2 synthesis via interference with NF-kB and JNK pathways. In this chapter, the results demonstrate that 1) pioglitazone protects dopaminergic neurons also via inhibiting LPS-induced iNOS and NO generation, 2) pioglitazone inhibits NO generation via inhibiting p38 MAPK activity, 3) the inhibition of p38 MAPK pathway by pioglitazone might be via the activation of PPARγ and PI3K pathway.

Pretreatment with microglia-enriched cultures with pioglitazone significantly inhibited the LPS-induced increase in NO production. Previous studies have shown that pretreatment with pioglitazone decreased iNOS-positive cells in the SN and striatum of MPTP-treated mice (Dehmer et al., 2004) as well as decreased iNOS expression in the intrastriatal LPS model (Hunter et al., 2007). These findings support our present results. In addition, we failed to observe any inhibitory effect of pioglitazone on LPS-induced NO production when pioglitazone was administered concurrent with LPS or 1hr after LPS treatment, which suggests that PPARγ-mediated anti-inflammatory pathways and LPS-mediated inflammatory pathways might target and interact with common active molecules. There are several potential candidates that can be competitively targeted within these two pathways. The first candidate is LPS-induced MAPK activation. As Camp's study demonstrated using 293T cells, PPARγ can be phosphorylated by p38 MAPK at its ser82 residue, and an increase in PPARγ phosphorylation may reduce its sensitivity to PPARγ ligands such as pioglitazone (Camp and Tafuri, 1997; Camp et al., 1999). The second candidate is CD14, where LPS-induced microglia activation is mediated by CD14. However, the PPARγ agonist 15d-PGJ2 and rosiglitazone negatively regulate CD14 mRNA transcription in primary mouse microglia cultures (Xu and Drew, 2007); although, a caveat to this finding is that 15d-PGJ2 was recently shown not to be a biologically relevant PPARγ agonist (Giri et al., 2004). A third candidate for competitive targeting by LPS and PPARγ is RXR. Recent studies have shown that rosiglitazone inhibits LPS-mediated RXR nuclear export, resulting in increased nuclear binding of RXR in hepatocytes of mice (Park et al., 2004), and that the RXR agonist, 9-cis retinoic acid, inhibits NO production by LPS-activated microglia (Xu et al., 2005). Our results

also demonstrate that inhibition of iNOS, with its specific inhibitor 1400W, protects dopaminergic neurons against LPS-induced neurotoxicity. This data is supported by a previous study using an iNOS inhibitor to attenuate dopaminergic neuron loss after intranigral LPS treatment (Arimoto and Bing, 2003). Therefore, we speculate that pioglitazone protects dopaminergic neurons at least via inhibition of iNOS expression and function, which is consistent with other studies (Dehmer et al., 2004; Hunter et al., 2007).

To further clarify which proinflammatory pathways might be involved in mediating the inhibition of LPS-induced NO by pioglitazone, selective inhibitors for p38 MAPK (SB203580) and for JNK (SP600125) were administered before LPS stimulation. It is interesting that inhibition of LPS-induced NO production was only observed with administration of the p38 MAPK inhibitor, but not with the JNK inhibitor, in microglia-enriched cultures. These results suggest that p38 MAPK might be associated with LPS-mediated iNOS regulation, but not with JNK. In addition, our study showed that pretreatment with pioglitazone before LPS reduces phosphorylation of p38 MAPK, which suggests that pioglitazone inhibits LPS-induced iNOS and NO production via suppression of p38 MAPK phosphorylation. Evidence has shown that inhibition of different MAPK pathways is associated with decreases in LPS-induced NO production (Bhat et al., 1998), where the inhibitory effect of p38 MAPK has been consistently observed (Chen and Wang, 1999). In addition, our results are also consistent with two recent *in vivo* studies which suggest a role for p38 MAPK, but not JNK, in LPS-induced activation of iNOS (Ruano et al., 2006; Shibata et al., 2006).

Inhibition of PI3K with wortmannin did not enhance JNK phosphorylation upon LPS stimulation. In contrast, wortmannin enhanced p38 MAPK phosphorylation upon LPS stimulation in a dose-dependent manner, suggesting that PI3K/Akt mediated LPS-induced p38 MAPK activity and pioglitazone might inhibit LPS-induced NO generation via regulation of PI3K/Akt activity.

Western blot analysis demonstrated that pioglitazone increases PPARγ activation followed by PI3K/Akt activation, and LPS treatment has no significant influence on their activation. On the other hand, PPARγ activation by western blot analysis was not found in the primary neuron-enriched culture. The result suggested that the increased PI3K/Akt

activity is mainly from microglia. Generally, these results suggest that activation of the PI3K/Akt pathway by pioglitazone might be via PPARγ activation. Whether the activation of PI3K/Akt by pioglitazone is PPARγ dependent or independent needs to be further clarified.

Our present study shows that inhibition of PI3K activity significantly enhances LPS-induced NO production. Furthermore, pretreatment with wortmannin reverses the inhibitory effect of pioglitazone on the LPS-induced increase in NO production, suggesting that inhibition of NO by pioglitazone may be PI3K-dependent. Indeed, it showed that inhibition of the P13K pathway enhances LPS-induced NO production in macrophages (Park et al., 1997). A more recent study demonstrated that human iNOS promoter induction by LPS/IFN-γ is suppressed by PI3K/Akt via inhibition of forkhead transcription factor FKHRL1 (Kristof et al., 2006).

In the current study, it suggests that pioglitazone inhibits LPS-induced NO synthesis via PPARγ-dependent mechanisms. The present results are supported by a recent study on the mechanisms of iNOS inhibition by PPARγ agonist Rosiglitazone. Their results demonstrated that ligand-binding causes SUMOylation on PPARγ, leading to inhibiting the removal of corepressor complex by 19s proteasome on the iNOS promoter, thus keeping iNOS transcription in an inactivated status (Pascual et al., 2005).

The current study shows that pioglitazone significantly inhibits LPS-induced microglia-mediated iNOS expression and NO production. This might be mediated by activation of the PI3K/Akt pathway, followed by inhibition of p38 MAPK activity, which may contribute to the inhibitory effects of pioglitazone on LPS-induced NO generation; thus, protecting dopaminergic neurons against LPS toxicity.

CHAPTER 5: Discussion and Conclusions

Summary of activated microglia-mediated neurodegeneration and its interference with PPARγ activation

Microglia-dependent LPS neurotoxicity to dopaminergic neurons

The survival study of the dopaminergic neurons upon LPS insult in our culture system showed that LPS-induced inflammation-mediated neurotoxicity is microglia-dependent. The loss of dopaminergic neurons is only observed in the presence of microglia. Since the finding that reactive microglia surround dopaminergic neurons in the SNpc of PD patients (McGeer et al., 1988b), an increasing body of evidence suggested unlimited microglial activation upon a variety of stresses may play a major role in the pathogenesis of PD. Although the role of activated microglia as an initial event leading to dopaminergic neuronal death has still been under debate, however, the highest density of microglia found in the SN of the brain provides the physical base of initial neuroinflammatory response. The *in vitro* model has advantage over *in vivo* animal model since the molecular mechanisms of activated microglia-mediated dopaminergic neuronal death can be investigated more easily via dissecting "initial" pathological events in microglia and "secondary" signaling transduction cascades occurring in the dopaminergic neurons. On the other hand, it would be very helpful to identify microglia subgroups with different phenotypic and physiological properties. In this way, the microglia subgroups which are more vulnerable to the stress signals and are mainly responsible for the release of proinflammatory molecules can be selected out. It will provide valuable tool to develop effective therapeutic strategy for Parkinson's disease and other neurodegenerative diseases. The number of microglia decreased in the pioglitazone plus LPS group. The cell counting in current study still can not tell whether it is due to microglia apoptosis or necrosis. The TUNEL analysis could provide more information.

PPARγ agonist pioglitazone protects dopaminergic neurons against activated microglia-mediated NO and PGE_2 via mediating MAPKs (p38 and JNK) and PI3K activity

PGE_2 and NO released from activated microglia are shown to be neurotoxic to dopaminergic neurons in primary neuron-microglia cultures. Pharmacological inhibition of JNK and p38 pathways suggests that PGE_2 and NO generation induced by LPS might

be differentially mediated by JNK and p38 pathways respectively, consistent with other studies (Pyo et al., 1998; Han et al., 2002). The different report from another study (Wang et al., 2004) might be due to different cell types in which BV-2 microglia was used. Interestingly, no change of p38 MAPK phosphorylation was observed with the inhibition of PI3K by wortmannin in the absence of LPS stimuli. In contrast, western blot analysis showed that inhibition of PI3K activity before LPS insult significantly increases the phosphorylation of p38 other than JNK, suggesting PI3K negatively mediate LPS-induced p38 activity. The results from the NO assay also support this finding, showing PI3K inhibition in the primary microglia-enriched culture reverses the inhibitive effect of pioglitazone on the LPS-induced NO generation, strongly suggesting PPARγ agonist pioglitazone suppresses induced-NO in a PI3K-dependent manner. This first report is further supported by the observation in western blotting analysis, showing that pioglitazone increases the phosphorylation of PPARγ, followed by PI3K / Akt activation in both pioglitazone treated group and LPS treated group. Due to that pioglitazone is administered before LPS treatment, and pioglitazone alone increase PI3K/Akt activity, its increases seen in LPS group are more likely originated from pioglitazone-induced PPARγ activation. Since no change of PPARγ activation was found in primary neuron-enriched culture upon pioglitazone and LPS treatment, it is more likely that the neuroprotective effect of pioglitazone is originated from its dynamic influence on the microglia but not on the dopaminergic neurons, which is supported by the evidence that PPARγ has only low expression in normal neurons and in the SN even upon MPTP insult (Dehmer et al., 2004; Cimini et al., 2005). Interestingly, a pharmacological PPARγ inhibition study using T0070907 suggested that pioglitazone inhibits LPS-induced NO production in a PPARγ-dependent, however, inhibition of LPS-induced PGE_2 synthesis by pioglitazone appears PPARγ-independent. It is interesting to know whether LPS-induced PGE_2 and NO can in turn influence the phosphorylation of PPARγ in a differential way.

In summary, pioglitazone protects dopaminergic neurons by inhibiting iNOS and COX-2 expression via interference with p38 MAPK and JNK / NF-kB in a differential mediating manner, either in a PPARγ-dependent or PPARγ-independent way.

Furthermore, the inhibition of iNOS / NO may be mediated by the activation of PPARγ and PI3K / Akt pathway.

Potential areas for the further inquiry

To investigate more effectively whether PPARγ agonist inhibition of LPS-induced NO and PGE_2 synthesis follows PPARγ-dependent mechanisms, knockdown of PPARγ in the primary microglia-enriched cultures could answer this question directly. The other question which is interesting to know is the potential feedback influence of PGE_2 and NO on the PPARγ activity either in microglia-enriched culture or in primary neuron-enriched cultures. Another question that was not directly addressed is the detailed signal transduction pathways related to LPS-induced inflammation and oxidative stress in the dopaminergic neurons, this could be explored via using conditioned medium from activated microglia culture.

Extrapolation to Parkinson's disease

From the extracellular signals to intracellular signaling transduction for either cellular survival or death, numerous activated molecules take part in this very complicated process. MAPKs and PI3K are the candidates to be preferred targeted and modified since 1) their signaling cascades are well understood; 2) they are associated with the death or survival signaling. PPARγ agonists have a broad spectrum of biological functions, from lipid metabolism in the peripheral tissue to the anti-inflammatory and antioxidant properties in the central nervous system, providing a very wide range that can be utilized to design effective and selective therapeutic strategies. By dissecting the molecular role of PPARγ activation in the neuroinflammation-mediated MAPKs and PI3K pathways, the downstream executive molecules can be identified. In this way, the undesirable and side effects of PPARγ activation on the survival of dopaminergic neurons can be removed by specific pharmacological inhibition or gene modification. For example, what is the consequence of long-term treatment of PPARγ agonists in Parkinson's disease? What is the long-term effect of PPARγ agonists on the astrocytes and microglia? The concern is not unnecessary since one study on the human astrocytic cells implicated its apoptotic property on these cells (Chattopadhyay et al., 2000). The current study suggested that

MAPKs pathways have differential function on the mediation of LPS-induced NO and PGE_2 level, and PPARγ activation inhibits their synthesis probably via PI3K activation and accordingly potential differential regulation of MAPKs activity. The further study on the physiological function of PPARγ and their roles in the proinflammatory process may be very useful to investigate the possibility of clinical therapy, and further understanding the molecular mechanisms of PPARγ agonists on their anti-inflammatory features in terms of receptor-dependent or receptor-independent will be very helpful to develop PPARγ synthetic drugs with efficacy and safety.

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Professional Publications:

Peer-reviewed Publications

1. **Xing, B.,** Zeng,Y.-S. (1998) The separation and bioactivity assay of the neurotrophic substances in the two molecular weight fractions from spinal dorsal horn of spared root rats treated by morphine. Chinese Journal of Anatomy, 1998, 21

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6. **Xing, B.,** Bing,GY., Gash,D.M. (2004) GDNF Protects Dopaminergic Neurons Against Lipopolysaccharide-induced Neuronal Loss in Organotypic Culture. (SFN meeting San Diego, CA. Oct, 2004)

7. **Xing, B.,** Chen, Y., Gash, D.M.**,** Bing, GY (2005) GDNF Protects Dopaminergic Neurons Against Lipopolysaccharide-induced Neuronal Loss in Organotypic Culture and 6-OHDA-Lesioned Model. (SFN meeting Washington, DC. 2005)

8. **Xing, B.,** Gash, D.M., Bing, GY (2006) PPARγ agonist Pioglitazone Protects Dopaminergic Neurons Against Lipopolysaccharide-induced Neuronal Loss by Microglial Activation. (SFN meeting Atlanta, GA. 2006)

9. **Xing B.,** Liu M., Bing, GY (2007) Pioglitazone Inhibits LPS-Induced COX-2 and iNOS function via interfering with the JNK, NF-kB, and p38 MAPK Pathways. (SFN meeting San Diego, CA. Nov, 2007)

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