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Gemfibrozil, a lipid lowering drug, inhibits the activation of primary human microglia via peroxisome proliferator-activated receptor β

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Abstract

Microglial activation participates in the pathogenesis of various neuroinflammatory and neurodegenerative diseases. However, mechanisms by which microglial activation could be controlled are poorly understood. Peroxisome proliferator-activated receptors (PPAR) are transcription factors belonging to the nuclear receptor super family with diverse effect. This study underlines the importance of PPAR β/δ in mediating the anti-inflammatory effect of gemfibrozil, an FDA-approved lipid-lowering drug, in primary human microglia. Bacterial lipopolysaccharides (LPS) induced the expression of various proinflammatory molecules and upregulated the expression of microglial surface marker CD11b in human microglia. However, gemfibrozil markedly suppressed proinflammatory molecules and CD11b in LPS-stimulated microglia. Human microglia expressed PPAR- β and PPAR- γ , but not PPAR- α . Interestingly, either antisense knockdown of PPAR- β or antagonism of PPAR- β by a specific chemical antagonist abrogated gemfibrozil-mediated inhibition of microglial activation. On the other hand, blocking of PPAR- α and PPAR- γ had no effect on gemfibrozil-mediated anti-inflammatory effect in microglia. These results highlight the fact that gemfibrozil regulates microglial activation by inhibiting inflammatory gene expression in a PPAR- β dependent pathway and further reinforce its therapeutic application in several neuroinflammatory and neurodegenerative diseases.

Keywords

Gemfibrozil; Human microglia; Proinflammatory molecules; CD11b; PPAR- $\alpha/\beta/\gamma$

Introduction

Microglia, a resident macrophages and sensor cells of the CNS plays a key role in CNS remodeling and regeneration, and also act as a scavenger cells in the events of infection, inflammation, trauma, ischemia, and neurodegeneration in the CNS (Thomas 1992, El Khoury *et al.* 1998). Like macrophages, microglia respond to various stimuli by acquisition of a reactive phenotype as evidenced by the elevated expression of a number of cell surface molecules, including major histocompatibility complex class II antigens, CD45, complement receptors CR3 and CR4, immunoglobulin receptors FcGR1 and FcGR11, and intercellular adhesion molecule-1 (McGeer *et al.* 1993, Walker *et al.* 1995, Walsh *et al.* 2005). Importantly, activated microglia also produce different type of soluble factors including cytokines (IL-1 β , IL-6, TNF- α , and IL-12), NO, prostaglandins, and free oxygen radicals, which play an important role in host defense against CNS infections (Walsh *et al.* 2005,

Banati *et al.* 1993, Lee *et al.* 2002). However, excessive production of these substances may accelerate the neuronal damage caused by several pathological conditions such as ischemic brain damage, trauma and neurodegenerative diseases. Several reports demonstrated the presence of activated microglia in pathological lesions in several neurological diseases including multiple sclerosis (MS); (Lee *et al.* 2002), Alzheimer disease (AD) and Parkinson's disease (PD); (McGeer *et al.* 1992), and HIV-associated dementia (HAD) (Gelman 1993).

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcriptional factors belonging to the so-called nuclear receptor family (Desvergne & Wahli 1999, Straus & Glass 2001). To date, three different PPARs subtypes PPAR- α , PPAR- γ and PPAR- β have been identified in vertebrates (Straus & Glass 2001, Buchan & Hassall 2000, Bishop-Bailey 2000, Desvergne & Wahli 1999). PPARs can inhibit inflammatory gene expression by several mechanisms, including, competition for a limited pool of coactivators, direct interaction with p65 and p50 subunits and c-Jun, and modulation of p38 mitogen-activated protein kinase (MAPK) activity (Lee *et al.* 2003, Delerive *et al.* 1999, Li *et al.* 2000, Kelly *et al.* 2004, Ricote & Glass 2007). PPAR- β is ubiquitously expressed in different brain areas (Saluja *et al.* 2001, Polak *et al.* 2005, Moreno *et al.* 2004). Although the exact function of PPAR- β is poorly defined, it is likely to play a critical role in cell proliferation, differentiation, survival, lipid metabolism, and development (Cimini *et al.* 2003, Di Loreto *et al.* 2007, Hall *et al.* 2008). Even if not fully understood, the neuroprotective effect of PPAR- β agonists highlights their potential benefit to treat various acute or chronic neurological disorders.

Gemfibrozil (gem), an activator of PPAR- α , has been often prescribed in patients to lower the level of triglycerides (Woods *et al.* 2003, Rubins *et al.* 1999). This drug decreases the risk of coronary heart disease by increasing the level of high density lipoprotein (HDL) cholesterol and decreasing the level of low density lipoprotein (LDL) cholesterol (Rubins *et al.* 1999, Hsu *et al.* 2001). Earlier we have demonstrated that gem inhibits the expression of inducible nitric oxide synthase in human astroglia independent of PPAR- α (Pahan *et al.* 2002). Accordingly, we have shown that gem suppresses the activation of mouse microglia independent of PPAR- α (Jana *et al.* 2007a) and also inhibits the disease process of adoptively-transferred experimental allergic encephalomyelitis (EAE) independent of PPAR- α (Dasgupta *et al.* 2007). Others have also reported the anti-inflammatory effect of gem in other cells (Zhao *et al.* 2003, Xu *et al.* 2005, Xu *et al.* 2006).

While gem does not require PPAR- α for its anti-inflammatory activity in glial cells, the receptor that could be involved for this remained elusive. Here we demonstrate that PPAR- β , but not PPAR- α and PPAR- γ , is actually involved in anti-inflammatory activity of gem in primary human microglia.

Materials and Methods

Reagents: FBS and DMEM/F-12 were obtained from Invitrogen Life Technologies. LPS (*Escherichia coli*), gemfibrozil, and fenofibrate were obtained from Sigma-Aldrich. GW501516 (PPAR- β agonist) and GW1929 (PPAR- γ agonist) were purchased from Alexis Biochemical's. GSK0660 (PPAR- β antagonist), GW6471 (PPAR- α antagonist) and GW9662 (PPAR- γ antagonist) were purchased from Sigma-Aldrich. PPAR- β antibody was purchased from Santa Cruz Biotechnol. Phosphorothioate-labeled antisense and scrambled oligodeoxynucleotides were synthesized in the DNA-synthesizing facility of Invitrogen. Following antisense (ASO) and scrambled (ScO) oligonucleotides for different PPAR genes were used.

PPAR- β : Antisense (ASO): 5'-GCC TTG TCC CCG CAC ACC CG-3'

Scramble: (ScO): 5'- GCCTCCGCACACCGTTCCCG-3'

PPAR- γ : Antisense: 5'- TAC GGA GAG ATC CAC GGA G - 3'

Scramble: 5'- GGA GAT CCA CGG AGT ACA G - 3'

PPAR- α : Antisense: 5'-GGC CTC GAG TGG GGA GAG GGG - 3'

Scramble: 5'- TGC GCA CAC CCG CCC TGG CCT - 3'

Isolation of primary human microglia and astrocytes

Primary human microglia and astrocytes were prepared as described previously (Jana *et al.* 2007b). All of the experimental protocols were reviewed and approved by the Institutional Review Board of the Rush University Medical Center. Briefly, 11- to 17-week-old fetal brains obtained from the Human Embryology Laboratory (University of Washington, Seattle, WA) were dissociated by trituration and trypsinization (0.25% trypsin in PBS at 37°C for 15 min). The trypsin was inactivated with 10% heat-inactivated FBS (Mediatech, Washington, DC). The dissociated cells were filtered successively through 380 and 140 μ m meshes (Sigma, St. Louis, MO) and pelleted by centrifugation. The cell pellet was washed once with PBS and once with Neurobasal media containing 2% B27 and 1% antibiotic-antimycotic mixture (Sigma). In the first step, neurons were allowed to adhere to poly-D-lysine-coated plates for 5 min. Non-adherent cells were cultured on poly-D-lysine precoated 75 cm² flasks and incubated at 37°C with 5% CO₂ in air. Culture medium was changed after 3 days and every 3 days thereafter. On 9th day, flasks were placed on a rotary shaker at 240 rpm at 37°C for 2 h to remove loosely attached microglia. On 11th day, flasks were shaken again at 180 rpm for 18 h to remove oligodendroglia. Remaining adherent cells were astrocytes.

Immunostaining

Immunostaining was performed as described earlier (Jana *et al.* 2007b, Jana & Pahan 2009b, Jana & Pahan 2009a). Briefly, coverslips containing 200–300 cells/mm² were fixed with 4% paraformaldehyde for 15 min, followed by treatment with cold ethanol (–20 °C) for 5 min and two rinses in PBS. Samples were blocked with 3% BSA in PBS containing Tween 20 (PBST) for 30 min and incubated in PBST containing 1% BSA and primary antibody (1:50). After three washes in PBST (15 min each), slides were further incubated with Cy5 and Cy2 (Jackson ImmunoResearch, West Grove, PA, USA). For negative controls, a set of culture slides was incubated under similar conditions without the primary antibodies. The samples were mounted and observed under a Bio-Rad (Hercules, CA, USA) MRC1024ES confocal laser-scanning microscope.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from human primary microglia and astrocytes using RNA-Easy Qiagen kit following the manufacturer's protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase. Semiquantitative reverse transcriptase-coupled (RT)-PCR was performed as described previously (Jana *et al.* 2007a) using oligo (dT)_{12–18} as primer and moloney murine leukemia virus reverse transcriptase (Clontech) in a 20 μ l reaction mixture. The resulting cDNA was appropriately diluted, and diluted cDNA was amplified using Titanium Taq polymerase and the following primers for human proinflammatory genes: tumor necrosis factor- α (TNF- α), sense, 5'-CTG AGT CGG TCA CCC TTC TCC AGC T-3'; antisense, 5'-CCC GAG TGA CAA GCC TGT AGC CCA T-3'; IL-1 β , sense, 5'-GGA TAT GGA GCA AC A AGT GG-3'; antisense, 5'-ATG TAC CAG TTG GGG AAC T-3'; IL-6, sense, 5'-TTT TGG AGT TTG AGG TAT ACC TAG-3'; antisense, 5'-GCT GCG CAG AAT GAG ATG AGT TGT-3'; iNOS, sense, 5'-CTG CAG ACA CGT GCG TTA CTC CAC C-3'; antisense, 5'-GCA GGG CGT ACC ACT TTA GCT

CCA G-3'; IL-1A, sense, 5'-AGG AGA GCA TGG TGG TAG TAG C-3'; antisense, 5'-GTA ATG CAG CAG CCG TGA GGT A-3'; IL-18, Sense, 5'-TGG CTG CTG AAC CAG TAG AAG AC-3'; antisense, 5'-AGA GGC CGA TTT CCT TGG TCA AT-3'; LT- α , Sense: 5'-ACC ACG CTC TTC TGC CTG CTG CAC T-3'; antisense, 5'-GCC CTT GAA GAG GAC CTG GGA GTA-G-3'; IL-15, sense, 5'-AAA CCC CTT GCC ATA GCC AGC TCT T-3'; antisense, 5'-CTT CTG TTT TAG GAA GCC CTG CAC T-3'; CD11b, sense, 5'-CAA CCA AAG GGG CAG CCT CTA CCA G -3'; antisense, 5'-CTG GGA TGA TGC TAC CAG ACC ATC -3'; PPAR- α , sense, 5'-CCA GTA TTT AGG ACG CTG TCC -3'; antisense, 5'-AAG TTC TTC AAG TAG GCC TCG -3'; PPAR- γ , sense, 5'-TCT CTC CGT AAT GGA AGA CC -3'; antisense, 5'-GCA TTA TGA GAC ATC CCC AC -3'; PPAR- β , sense, 5'-CTG AGG TCC GGG AAG AGG AGG AGA A -3'; antisense, 5'-GCG CTC ACA CTT CTC GTA CTC CAG C -3'; GAPDH, sense, 5'-GGT GAA GGT CGG AGT CAA CG-3'; antisense, 5'-GTG AAG ACG CCA GTG GAC TC-3'.

Amplified products were electrophoresed on a 1.8% agarose gels and visualized by ethidium bromide staining. Message for the GAPDH gene was used to ascertain that an equivalent amount of cDNA was synthesized from different samples.

Real-time PCR analysis

It was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems, Foster City, CA) as described earlier (Jana & Pahan 2004, Jana & Pahan 2005, Dasgupta *et al.* 2004). Briefly, it was performed in a 96-well optical reaction plate (Applied Biosystem) on cDNA equivalent to 50 ng DNase-digested RNA in a volume of 25 μ l, containing 12.5 μ l TaqMan Universal Master Mix and optimized concentrations of FAM-labeled probe, forward and reverse primers following the manufacturer's protocol. All primers and FAM-labeled probes for human CD11b, IL-6, iNOS, LT- α , and GAPDH were obtained from Applied Biosystems. The mRNA expression of pro-inflammatory genes was normalized to the label of GAPDH mRNA. Data were processed by the ABI Sequence Detection System 1.6 software and analyzed by ANOVA.

Statistical analysis

All values are expressed as the mean $\pm$ SD of three independent experiments. Statistical differences between means were calculated by Student's *t* test. A *p* value of <0.05 ($p < 0.05$) was considered statistically significant.

Results

Gemfibrozil (gem) inhibits LPS-induced expression of iNOS and proinflammatory cytokines in primary human microglia

Microglia isolated from human fetal brain tissues were highly pure and contained 1–2% astroglia (Fig. 1A), but no oligodendroglia (Fig. 1B) and neurons (Fig. 1C). To determine the effect of gemfibrozil on the activation of human microglia, microglia were exposed to different concentrations of gemfibrozil (50–200 μ m) for 2 h, followed by treatment with LPS for 6 h and RNA was harvested for semi-quantitative RT-PCR. As expected, LPS markedly induced mRNA expression of iNOS and different proinflammatory cytokines (TNF- α , IL-1 β , IL-1 α , IL-6, Lt- α , IL-15, and IL-18) in human microglia (Fig. 1D). Although gemfibrozil itself was neither stimulatory nor much inhibitory to the expression of proinflammatory molecules in control microglia (data not shown), this drug dose-dependently reduced LPS-induced expression of iNOS and proinflammatory cytokines in microglia (Fig. 1D).

To investigate whether other fibrate drugs are also capable of suppressing the expression of proinflammatory molecules in human microglia, we examined the effect of clofibrate. Clofibrate is also a hypolipidemic drug that activates PPAR- α and induces the proliferation of peroxisomes in rats and mice (Lemberger *et al.* 1996). Similar to gemfibrozil, clofibrate also inhibited the expression of different proinflammatory molecules (TNF- α , IL-1 β , IL-1 α , IL-6, Lt- α , IL-15, and IL-18) in LPS-stimulated primary human microglia (Fig. 1E). However, clofibrate was less potent than gemfibrozil in suppressing the mRNA expression of proinflammatory molecules (Fig. 1D & E). These results suggest that fibrate drugs, in general, are inhibitory to LPS-induced expression of different proinflammatory molecules in primary human microglia.

Gemfibrozil suppresses CD11b expression in human microglia

Microglia are derived from a monocytic lineage and act functionally as macrophages in the brain (Banati *et al.* 1993). Microglia can be activated by secretory substances or signals associated with disease or injury, and become a phagocytic cell which also produces its own injurious molecules. In the activating process, its morphology is changed from a resting process-bearing cell (ramified form) into a rounded amoebic form displaying new or increased amounts of functional markers, such as cell surface receptors and class I and class II MHC molecules. Increased expression of the β 2-integrin CD11b/CD18 (MAC-1) has been also reported in either plaque-associated microglia in the AD brain or lesion-associated microglia in MS brain (McGeer & McGeer 1995). It has been also shown that LPS treatment of primary microglia results in morphological changes as well as increased expression of CD11b immunoreactivity (Horvath *et al.* 2008, Roy *et al.* 2006). We investigated if gemfibrozil was capable of down-regulating CD11b in LPS-stimulated primary human microglia. As expected, LPS markedly increased the mRNA expression of CD11b in microglia (Fig. 2A&B). However, gemfibrozil pretreatment markedly suppressed LPS-induced mRNA expression of CD11b (Fig. 2A&B). Immunofluorescence analysis of CD11b in primary microglia also shows that LPS stimulation increased the expression of CD11b and that gemfibrozil attenuated LPS-mediated CD11b expression (Fig. 2C). Similarly, clofibrate also inhibited LPS induced CD11b mRNA expression in primary human microglia (Fig. 2D&E). These data demonstrate that gemfibrozil and clofibrate are capable of inhibiting cellular activities responsible for activation and phenotypic conversion of human microglia.

Primary human microglia express PPAR- γ and PPAR- β , but not PPAR- α

Next we investigated mechanisms by which gem inhibited the proinflammatory gene expression in human microglia. Gemfibrozil, a known agonist of PPAR- α , stimulates the β -oxidation of fatty acids in peroxisomes and mitochondria via the activation of PPAR- α (Asayama *et al.* 1999). At first, we analyzed the level of different PPARs in cultured microglia and astrocytes. However, despite several attempts, we remained unable to detect the expression of PPAR- α mRNA in primary human microglia (Fig. 3A) and astroglia (Fig. 3C) by semi-quantitative RT-PCR. Interestingly, LPS decreased the mRNA expression of PPAR- β compared to control and gemfibrozil treatment markedly increased the level of PPAR- β in LPS-stimulated microglia (Fig. 3A&B) and astroglia (Fig. 3C&D). Consistent with the mRNA analysis, evaluation of PPAR- β protein expression in microglia also revealed an increase in PPAR- β expression in LPS-stimulated microglia by gemfibrozil treatment (Fig. 3E). In contrast to the decrease in PPAR- β expression, LPS stimulation markedly increased the mRNA expression of PPAR- γ in microglia (Fig. 3A) and astroglia (Fig. 3C). Interestingly, gemfibrozil decreased the mRNA expression of PPAR- γ in microglia and astroglia, which is also in contrast to the effect of this drug on PPAR- β (Fig. 3A–D). Immunofluorescence analysis also show that LPS decreased the level of PPAR- β (Fig. 3E), while increasing the level of PPAR- γ (Fig. 3F) in human microglia.

Gem inhibits the expression of proinflammatory molecules (IL-6 and iNOS) in primary human microglia via PPAR- α -independent mechanism

By overexpressing wild-type and dominant-negative constructs of PPAR- α in microglial cells and isolating primary microglia from PPAR- α ($-/-$) mice, we have recently demonstrated that gemfibrozil inhibits the activation of microglia independent of PPAR- α (Jana et al. 2007a, Pahan *et al.* 2002). Although we could not detect PPAR- α in human microglia by RT-PCR (Fig. 3), these cells may contain low level of PPAR- α that could be sufficient for mediating the anti-inflammatory effect of gem. Therefore, here we decided to investigate if gem required PPAR- α to suppress proinflammatory molecules in human microglia. Phosphorothioate-labeled antisense oligonucleotides provide an important option to knockdown specific gene in primary non-dividing cells without causing any cell death, which is always observed in transfection experiments. Therefore, we employed antisense oligonucleotides to knockdown PPAR- α in primary human microglia. Antisense knockdown of PPAR- α failed to abrogate the inhibitory effect of gem on the expression of iNOS and IL-6 in LPS-stimulated human microglia (Fig. 4A). To further confirm this result, we also treated microglia with PPAR- α antagonist (GW6471). As evident from figure 4B, GW6471 remained unable to block the inhibitory effect of gem on LPS-induced expression of IL-6 and iNOS in human microglia. These results clearly suggest that gem does not require PPAR- α to inhibit the expression of proinflammatory genes in primary human microglia.

Gem inhibits the expression of proinflammatory molecules in primary human microglia via PPAR- β , but not PPAR- γ

Next, we investigated the role of PPAR- β and PPAR- γ in gem-mediated inhibition of pro-inflammatory gene expression. Therefore, we employed antisense oligonucleotides to knockdown PPAR- β , and PPAR- γ in primary human microglia. Interestingly, antisense knockdown of PPAR- β markedly suppressed the inhibitory effect of gem on the expression of pro-inflammatory molecules in LPS-stimulated human microglia (Figs. 5A and 5B). These results were specific as scrambled oligonucleotides (ScO) remained unable to influence the anti-inflammatory effect of gem. Immunofluorescence analysis also shows that ASO, but not ScO, abrogated the inhibitory effect of gem on LPS-induced expression of iNOS protein (Fig. 5C). In contrast to antisense knockdown of PPAR- β , ASO against PPAR- γ had no effect on gem-mediated inhibition of iNOS in microglia (Fig. 5D). These results suggest that gem exhibits anti-inflammatory effect in human microglia via PPAR- β .

Effect of PPAR- β and PPAR- γ antagonist on LPS induced iNOS and pro-inflammatory gene expression in primary human microglia

To further bolster these results that gemfibrozil suppresses the expression of pro-inflammatory molecules via PPAR- β , but not PPAR- γ , in human microglia, cells were treated with either PPAR- β or PPAR- γ antagonist in the presence or absence of gem. As evident from semi-quantitative RT-PCR (Fig. 6A) and quantitative real-time PCR (Fig. 6B), GSK3787, a specific antagonist of PPAR- β , markedly abrogated gem-mediated inhibition of Lt- α , iNOS and IL-6 in human microglia, confirming the involvement of PPAR- β in anti-inflammatory effect of gem in human microglia. Furthermore, we observed that gem and GW501519, a specific PPAR- β agonist, remained unable to inhibit the expression of pro-inflammatory molecules (Lt- α , iNOS and IL-6) in the presence of GSK3787 (Fig. 6C & D). On the other hand, PPAR- γ agonist GW1929 inhibited the LPS-induced the expression of proinflammatory molecules in presence of PPAR- β antagonist (GSK3787) in human primary microglia (Fig. 6B & D), demonstrating the specificity of the effect. Moreover, we observed that gem inhibited the expression of pro-inflammatory molecules (Lt- α , iNOS and IL-6) in the presence of GW9662, a PPAR- γ antagonist, in human microglia (Fig. 6E & F). These results demonstrate that gem inhibits the LPS-induced proinflammatory molecule expression via PPAR- β , but not PPAR- γ , in human microglia.

Discussion

Common pathological hallmarks of several neurodegenerative diseases include the loss of invaluable neurons associated with or followed by massive activation of microglia. Although microglial activation has an important repairing function, once microglia are activated in the neurodegenerating microenvironment, it always goes beyond control and eventually detrimental effects override beneficial effects. Therefore, understanding mechanisms that regulate microglial activation is an important area of investigation that may enhance the possibility of finding a primary or an adjunct therapeutic approach against incurable neurodegenerative disorders.

The studies reported in this manuscript clearly demonstrate that gemfibrozil, a commonly used lipid-lowering drug and an activator of PPAR- α , suppresses the expression of proinflammatory molecules (iNOS, TNF- α , IL-1 β , and IL-6) in human primary microglia. Because these proinflammatory molecules have been implicated in the pathogenesis of demyelinating and neurodegenerative diseases, our results provide a potentially important mechanism whereby activators of PPAR- α may ameliorate neural injury. Although gemfibrozil exerted anti-inflammatory effect in LPS-stimulated human microglia upon 2 h preincubation, it should not be a problem in chronic neuroinflammatory and neurodegenerative disorders, which are associated with consistent generation of proinflammatory molecules. Therefore, in such conditions, gemfibrozil treatment is expected to suppress subsequent cycles of proinflammatory signaling pathways. Accordingly, we (Dasgupta et al. 2007) and others (Lovett-Racke et al. 2004, Gocke et al. 2009) have shown that gemfibrozil is capable of suppressing the progression of relapsing-remitting and chronic EAE in mice. Earlier we have demonstrated that Δ hPPAR- α (a dominant-negative mutant of human PPAR- α) is unable to block the inhibitory effect of gemfibrozil on the induction of iNOS in human astroglia, suggesting that gemfibrozil does not require PPAR- α to inhibit the induction of iNOS in human astroglia (Pahan et al. 2002). Although one study by Xu et al (Xu et al. 2005) has indicated the possible involvement of PPAR- α in gemfibrozil-mediated inhibition of microglial iNOS, this study does not attempt to examine the effect of gemfibrozil in PPAR- α (–/–) microglia. Recently, by using microglia from wild type and PPAR- α (–/–) mice, we have demonstrated that gemfibrozil inhibits the activation of mouse microglia without involving PPAR- α (Jana et al. 2007a). However, the nuclear receptor that was involved in anti-inflammatory efficacy of gemfibrozil in microglia was not known.

Here we demonstrate that gemfibrozil inhibits the induction of proinflammatory gene expression in human microglia via PPAR- β , but not PPAR- α and PPAR- γ . This conclusion is based on the following observations. *First*, PPAR- α was present in primary human microglia at an undetectable level. Gemfibrozil was also unable to stimulate the level of PPAR- α in microglia. *Second*, although gemfibrozil is a known activator of PPAR- α , either antisense knockdown of PPAR- α or antagonism PPAR- α by pharmacological drug did not block the anti-inflammatory effect of gemfibrozil. *Third*, fibrate drugs like clofibrate is more selective for PPAR- α than either PPAR- β or PPAR- γ (Forman et al. 1997, Berger et al. 1999). Accordingly, clofibrate was less potent in suppressing the mRNA expression of various proinflammatory molecules in human microglia. *Fourth*, human microglia expressed PPAR- γ and interestingly, gemfibrozil inhibited the expression of PPAR- γ , in microglia. Although activation of PPAR- γ leads to anti-inflammation, LPS stimulated the expression of PPAR- γ in human microglia. However, gemfibrozil inhibited the LPS-induced expression of PPAR- γ in human microglia. Consistently, either antisense knockdown of PPAR- γ or antagonism of PPAR- γ by chemical compound had no effect on gemfibrozil-mediated inhibition of proinflammatory molecules, suggesting that gemfibrozil does not need PPAR- γ for exhibiting its anti-inflammatory effect in human microglia. *Fifth*, human

microglia expressed PPAR- β and gemfibrozil stimulated the expression of PPAR- β in microglia. Antisense knockdown of PPAR- β abrogated the inhibitory effect of gemfibrozil on proinflammatory molecules. Similarly, GSK3787, an antagonist of PPAR- β , also blocked the anti-inflammatory effect of gemfibrozil in human microglia. Taken together, these results demonstrate that gemfibrozil exhibits anti-inflammatory effect via PPAR- β , but neither PPAR- α nor PPAR- γ .

Although activation of PPAR- γ leads to anti-inflammation and human microglia also express PPAR- γ , gemfibrozil does not require PPAR- γ for its anti-inflammatory effect. Interestingly, we observed a reciprocal relationship between PPAR- β and PPAR- γ in primary human microglia. LPS stimulation increased the expression of PPAR- γ , while decreasing the expression of PPAR- β . On the other hand, gemfibrozil increased the level of PPAR- β , while decreasing the level of PPAR- γ . It has been shown that PPAR- β inhibits the activities of PPAR- γ and PPAR- α in experimental models of PPAR- β over-expression in nontransformed normal monkey kidney CV-1 cells and mouse NIH 3T3 fibroblasts (Zuo *et al.* 2006, Shi *et al.* 2002). Similarly, siRNA knockdown of PPAR- β leads to an increase in PPAR- γ activation in colon cancer cells (Zuo *et al.* 2006). Therefore, these findings are consistent with our result that upregulation of PPAR- β by gemfibrozil leads to the down-regulation of PPAR- γ in primary human microglia. However, the precise mechanism by which PPAR- β inhibits the expression of PPAR- γ in human microglia requires further investigation.

Mechanisms by which PPAR- β could be coupled to proinflammatory gene expression are poorly understood. Among all the known proinflammatory transcription factors working in concert to transactivate promoters of proinflammatory genes, NF- κ B p50:p65 is literally the most important one (Ghosh 1999). The presence of multiple consensus sequences (κ B elements) in the promoter region of proinflammatory molecules for the binding of NF- κ B and the inhibition of proinflammatory gene expression in human, rat, and mouse glial cells with the inhibition of NF- κ B activation (Jana *et al.* 2001, Liu *et al.* 2002, Dasgupta *et al.* 2003, Ghosh 1999, Brahmachari *et al.* 2009, Ghosh & Hayden 2008, Saha *et al.* 2007, Xie *et al.* 1994) establishes an essential role of NF- κ B activation in the induction of proinflammatory molecules. Previous studies have provided evidence that PPAR can inhibit inflammatory gene expression by several mechanisms, including competition for a limiting pool of coactivators, direct interaction with NF- κ B p65 and p50 subunits, modulation of p38 mitogen-activated protein kinase (MAPK) activity, and partitioning the corepressor B-cells lymphoma 6 (BCL-6) (Li *et al.* 2005, Lee *et al.* 2003, Delerive *et al.* 1999, Kelly *et al.* 2004, Ricote & Glass 2007). Compared to PPAR- α and PPAR- γ , relatively little is known on the role of PPAR- β in the regulation of inflammatory responses (Ricote & Glass 2007). Gemfibrozil induced-PPAR- β may suppress the activation of NF- κ B by different models. In the direct interaction model, PPAR- β may be actively exported from the nucleus into the cytosol by interaction with NF- κ B, leading to an alteration in the expression of proinflammatory genes such as IL-6, iNOS and IL-1 β in human microglia. In the second model, in the absence of a ligand, PPAR- β may sequester BCL-6 from inflammatory response genes, leading to an increased expression of proinflammatory molecules. In contrast, in the presence of a ligand (e.g. gemfibrozil), PPAR- β releases the repressor; which distributes to NF- κ B-dependent promoters and exerts anti-inflammatory effects by repressing the transcription of proinflammatory genes. The release of BCL-6 is known to contribute to many of the anti-inflammatory actions of PPAR- β in macrophages both *in vitro* (Lee *et al.* 2003, Takata *et al.* 2008) and *in vivo* (Takata *et al.* 2008). Although the role of BCL-6 in other tissues is not clear and we have not been able to analyze the role of BCL-6 in human microglia due to scarcity of primary human microglia, it is possible that gemfibrozil employs the PPAR- β – BCL-6 pathway in order to suppress the expression of proinflammatory molecules in human microglia.

In this study, we have used LPS as a prototype inducer of proinflammatory molecules, which may not be directly relevant to neurodegenerative disorders like AD, PD etc. However, similar to LPS, other inducers like IL-1 β , amyloid- β peptides (an etiological reagent for AD) or MPP⁺ (an etiological reagent for PD) also activate glial cells via activation of NF- κ B (Jana et al. 2007a). Because gem is capable of inhibiting the activation of NF- κ B in mouse and human glial cells, this drug is expected to inhibit the activation of human microglia in response to disease-specific stimuli. In summary, we have demonstrated that gemfibrozil, an FDA-approved drug for hyperlipidemia, suppresses the activation of primary human microglia via PPAR- β . Although the *in vitro* situation of human fetal microglia in culture does not truly resemble the *in vivo* situation of microglia in the brain of patients with neurodegenerative disorders, our results suggest that specific modulation of PPAR- β by gemfibrozil may be an important therapeutic avenue to halt microglial activation in different neurodegenerative disorders

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Fig. 1. Effects of gemfibrozil on LPS induced proinflammatory gene expression

To examine the purity of microglia, cells were double-immunolabeled with CD11b and either GFAP, GalC, or MAP-2 and observed under a confocal laser-scanning microscope (A, B & C). Human primary microglia was pretreated with gemfibrozil 2 h before LPS (1 μ g/ml) treatment. At 6 h after LPS treatment, total RNA was isolated and analyzed by semi quantitative RT-PCR (D). Human primary microglia was pretreated with clofibrate (100 μ M) 2 h before LPS (1 μ g/ml) treatment. After 6 h of treatment, total RNA was isolated and analyzed by semi quantitative RT-PCR (E). The gel shown was one representative of results from three separate experiments.

Fig. 2. Effects of gemfibrozil on LPS induced-CD11b gene expression

Human primary microglia was pretreated with gemfibrozil 2 h before LPS (1 $\mu\text{g/ml}$) treatment. After 6 h of LPS treatment, total RNA was isolated and analyzed by semiquantitative RTPCR (A) and real-time PCR (B). Results represent mean $\pm$ SD of three separate experiments. ^a $p < 0.001$ vs control; ^b $p < 0.001$ vs LPS. The gel shown was one representative of results from three separate experiments. CD11B immunohistochemistry, cell type specific marker for microglia-macrophage lineage cells (C). Human primary microglia was pretreated with clofibrate 2 h before LPS (1 $\mu\text{g/ml}$) treatment. At 6 h after LPS treatment, total RNA was isolated and analyzed by semi-quantitative RT-PCR (D) and real-time PCR (E). Results represent mean $\pm$ SD of three separate experiments. ^a $p < 0.001$ vs control; ^b $p < 0.001$ vs LPS.

Fig. 3. Effect of gemfibrozil on the expression of PPAR-α, PPAR-β and PPAR-γ in human primary microglia and astrocytes

Primary microglia (A, B, E & F) and astrocytes (C & D) were pretreated with different concentrations of gem 2 h prior to the addition of 1 μg/ml of LPS under serum-free condition. After 6 h of stimulation, the mRNA expression of PPAR-α, PPAR-β and PPAR-γ was monitored by semi-quantitative RT-PCR (A & C). Bands were scanned and expressed (B & D) as relative expression (PPAR/GAPDH). Results represent mean ± SD of three separate experiments. ^a $p < 0.001$ vs control-PPAR-β; ^b $p < 0.001$ vs LPS-PPAR-β; ^c $p < 0.001$ vs control-PPAR-γ; ^d $p < 0.001$ vs LPS-PPAR-γ. After 18 h of stimulation, the expression of PPAR-β, PPAR-γ and CD11b protein was monitored by immunofluorescence (E & F). DAPI was used to visualize nucleus. Results represent three independent experiments.

Fig. 4. Effect of PPAR- α antisense knockdown on LPS induced- iNOS production in human primary microglia

Human primary microglia received either ASO or ScO at 1 μ M against PPAR- α , after 40 h of incubation, the cells were pretreated with gemfibrozil 2 h before LPS (1 μ g/ml) treatment. At 6 h after LPS treatment, total RNA was isolated and analyzed by semi quantitative RT-PCR (A). Cells were simultaneously pretreated with gemfibrozil and GW6471 (PPAR- α antagonist) 2 h before LPS (1 μ g/ml) treatment. At 6 h after LPS treatment, total RNA was isolated and assessed by semi quantitative RTPCR (B). Results represent three independent experiments.

Fig. 5. Effect of PPAR-β and PPAR-γ antisense knockdown on LPS induced- iNOS production in human primary microglia

Human primary microglia received either ASO or ScO at 1 μM against PPAR-β and PPAR-γ, after 40 h of incubation, the cells were pretreated with gemfibrozil 2 h before LPS (1 μg/ml) treatment. At 6 h after LPS treatment, total RNA was isolated and analyzed by semi quantitative RT-PCR (A and D) and real-time PCR (B). Results represent mean ± SD of three separate experiments. ^a*p* < 0.001 vs LPS; ^b*p* < 0.001 vs (LPS+Gem). After 18 h of stimulation, the expression of iNOS and CD11B protein was monitored by immunofluorescence (C). DAPI was used to visualize nucleus. Results represent three independent experiments.

Fig. 6. Effect of PPAR-β and PPAR-γ antagonist on the expression of pro-inflammatory genes expression

Human primary microglia were simultaneously pretreated either with gemfibrozil, or PPAR-γ agonist WY1929 and PPAR-β antagonist GSK3787 2 h before LPS (1 μg/ml) treatment. At 6 h after LPS treatment, total RNA was isolated and analyzed by semi quantitative RT-PCR (A and C) and real time- PCR (B and D). Results represent mean ± SD of three separate experiments. ^a $p < 0.001$ vs LPS; ^b $p < 0.001$ vs (LPS+Gem) for (A). ^a $p < 0.001$ vs LPS; ^b $p < 0.001$ vs (LPS+GW501519) for (B). Cells were simultaneously pretreated with gemfibrozil and GW9662 (PPAR-γ antagonist) 2 h before LPS (1 μg/ml) treatment. At 6 h after LPS treatment, total RNA was isolated and analyzed by semi quantitative RT-PCR and real time PCR (E and F). Results represent mean ± SD of three separate experiments. ^a $p < 0.001$ vs control; ^b $p < 0.001$ vs LPS.