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Cytochrome P4504f, a potential therapeutic target limiting neuroinflammation

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ABSTRACT

Inflammatory processes are involved in the pathogenesis and/or progression of acute central nervous system (CNS) infection, traumatic brain injury and neurodegenerative disorders among others indicating the need for novel strategies to limit neuroinflammation. Eicosanoids including leukotrienes, particularly leukotriene B_4 (LTB_4) are principle mediator(s) of inflammatory response, initiating and amplifying the generation of cytokines and chemokines. Cytochrome P450 (Cyp), a family of heme proteins mediate metabolism of xenobiotics and endogenous compounds, such as eicosanoids and leukotrienes. Cytochrome P4504F (Cyp4f) subfamily includes five functional enzymes in mouse. We cloned and expressed the mouse Cyp4f enzymes, assayed their relative expression in brain and examined their ability to hydroxylate the inflammatory cascade prompt LTB_4 to its inactive 20-hydroxylated product. We then examined the role of Cyp4fs in regulating inflammatory response in vitro, in microglial cells and in vivo, in mouse brain using lipopolysacharide (LPS), as a model compound to generate inflammatory response. We demonstrate that mouse brain Cyp4fs are expressed ubiquitously in several cell types in the brain, including neurons and microglia, and modulate inflammatory response triggered by LPS, in vivo and in microglial cells, in vitro through metabolism of LTB_4 to the inactive 20-hydroxy LTB_4. Chemical inhibitor or shRNA to Cyp4fs enhance and inducer of Cyp4fs attenuates inflammatory response. Further, induction of Cyp4f expression lowers LTB_4 levels and affords neuroprotection in microglial cells or mice exposed to LPS. Thus, catalytic activity of Cyp4fs is a novel target for modulating neuroinflammation through hydroxylation of LTB_4.

1. Introduction

Inflammatory processes are seen as acute response following infections of CNS or traumatic brain injury [1] and in chronic neurodegenerative disorders, such as Alzheimer's and Parkinson's disease [2]. Such inflammation can occur either through entry of monocytes from the periphery into the brain or can be mediated *in situ* in the brain through microglia [3]. In response to inflammatory stimuli microglia are activated and release excessive amounts of pro-inflammatory mediators including cytokines (TNF-α, IL-6, IL-1β, etc.), chemokines (MCP-1, MIP-α, etc.) and free radicals. Another class of key players in the inflammatory cascade, are the eicosanoids including leukotrienes particularly leukotriene B_4 (LTB_4) and prostaglandins which are also secreted by microglia. Microglia express pattern recognition receptors including toll-like receptors (TLRs) and scavenger receptors on their surface [3]. Binding of ligand(s), such as lipopolysaccharide (LPS) to these receptors activates microglia releasing inflammatory mediators, such as cytokines, chemokines and leukotrienes [4] which propagate neuroinflammation in many ways, including activation of phospholipase A2 (PLA_2), cyclo-oxgenases and lipo-oxygenses [5]. This causes release of arachidonic acid from neural membrane and generates pro-inflammatory leukotrienes and prostaglandins thus initiating a cyclical cascade. LTB_4 formed initiates and amplifies production of cytokines and chemokines, and is a key component of inflammatory response [6]. A close interdependent relationship exists between cytokines and chemokines on one hand and leukotrienes and prostaglandins on the other [7,8]. Among others, resolution of inflammation should therefore involve methods that inactivate leukotrienes thereby reducing the inflammatory response seen as increased cytokine and chemokine levels.

Cytochrome P450 (Cyp), a superfamily of heme proteins is involved in metabolism of xenobiotics and endogenous compounds [9]. While liver is the major organ involved in P450-mediated biotransformation, functional P450 enzymes are also present in brain wherein they metabolize a variety of compounds [10,11]. Cytochrome P4504F (Cyp4f) subfamily includes 7 functional enzymes in humans, 4 in rat and 5 in mice and some

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members of the Cyp4f family are known to biotransform leukotrienes [12,13].

In the present study we examined the involvement of P4504F family in the resolution of inflammation through the metabolism of LTB_4 to 20-hydroxy LTB_4 using an acute model of inflammation. Administration of single dose of LPS has been shown to result in acute and persistent inflammatory response in the brain, but no neurodegeneration in the time scale examined by us [14] and therefore provides an excellent model for studying the impact of inflammation in the brain. LPS exposure is known to not only increase the cytokines (TNF-α, IL-6 and IL-1β) and chemokines (MCP-1) in the brain and primary astroglial cultures [15] but also results in increased levels of leukotrienes, such as LTB_4 [16]. Our goal was to determine if Cyp4fs play a role in the resolution of neuroinflammation using this model.

2. Materials and methods

2.1. Materials

Antibody to Iba-1 was procured from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Alexa Fluor 488 and Alexa Fluor 594 were purchased from Molecular Probes (Eugene,Oregon). Antigen unmasking solution, all the other conjugated secondary antibodies, Vectastain-ABC Elite kit, VECTASHIELD mounting media and Nova Red substrate kit were purchased from Vector laboratories (Burlingame, CA). Cell culture products and Lipofectamine™ were obtained from Gibco BRL (Invitrogen, Carlsbad, CA). TRI reagent and bromochloropropane (BCP) were purchased from Molecular Research Centre (Cincinnati, OH, USA). Single-stranded cDNA synthesis kit and SYBR Green supermix were purchased from Applied Biosystems (Foster City, CA 94404, USA). Fenofibrate, lipopolysaccaride (LPS from *Escherichia coli* 055:B5) and 17-octadecynoic acid (17-ODYA) were obtained from Sigma Chemical Company (St. Louis, MO). DIG-RNA labeling and detection kit, antidigoxigenin Fab fragments linked to peroxidase and PCR reagents were purchased from Roche Diagnostics GmbH, Germany. Cytokine bead array (CBA) mouse inflammation kit was obtained from BD Biosciences (USA). The Assay designs™ leukotriene B_4 enzyme immunoassay kit was procured from Stressgen (Michigan; USA). Microslides (superfrost plus) were obtained from VWR International (USA). All other chemicals or reagents were of analytical grade and were obtained from Sigma–Aldrich, Merck or Qualigens (India).

2.2. Animals and LPS treatment

Male C57BL6J mice (2–3 months old, 25–30 g) were obtained from National Brain Research Centre (NBRC). Animals had access to pelleted diet and water *ad libitum*. All animal experiments were carried out according to institutional guidelines for the use and care of animals. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques where available. Animals were injected with LPS (3 mg/kg body weight in saline; subcutaneous). The group-matched controls received vehicle. Animals were sacrificed 12 or 24 h after LPS treatment and cortex was dissected out. In some experiments animals were perfused transcardially with buffered paraformaldehyde (4%, w/v) and brains were dissected out and processed for fluorescence *in situ* hybridization (FISH) or immunohistochemistry (IHC). For examining the effect of the ω-hydroxylase inhibitor, 17-octadecynoic acid (17-ODYA), mice were divided into four groups. Two groups received vehicle (0.5% DMSO in absolute ethanol, v/v; intrathecal) followed by normal saline or LPS 1 h later. The remaining two groups received 17-ODYA (0.2 mg/kg body weight; intrathecal) prior to LPS or saline treatment. Fenofibrate (150 mg/kg body weight; subcutaneous), an inducer of Cyp4fs was administered to two groups of mice for 4 days followed by LPS or saline 1 h later. An additional 2 groups received the vehicle (absolute ethanol, v/v; subcutaneous) followed by normal saline or LPS.

2.3. Cell-culture experiments

Murine microglial cells, BV-2 (American type culture collection, Manassas, VA, USA) which exhibit both phenotypic and functional properties of reactive microglial cells were grown in DMEM supplemented with 10% FBS, streptomycin and penicillin. Cells were treated with LTB_4 (0–100 nM) or LPS (25 ng/ml of media) for 24 or 12 h respectively. For inhibitor or inducer studies, cells were treated with 17-ODYA (100 μM) or fenofibrate (40 μg/ml) respectively 1 h prior and 1 h after exposure to LPS (25 ng/ml of media) and collected 12 h after treatment. Cell viability was assessed by MTT assay [17]. BV-2 cells exposed to LPS secrete cytokines and chemokines into the media. In order to assess the cytotoxicity of the medium in which BV-2 cells were grown, we exposed Neuro-2a cells (derived from mouse neuroblastoma) to this media as described earlier [18].

2.4. Amplification, cloning, and expression of Cyp4fs

Total RNA was isolated from mouse brain cortex using TRI reagent method [19]. The complete open reading frames (ORF) of Cyp4f13, Cyp4f14, Cyp4f15, Cyp4f16 and Cyp4f18 were amplified using specific primers shown in Table 1. The derivative PCR products were cloned into pcDNA3.1 mammalian expression vector, verified by DNA sequencing and transiently transfected to COS-1 cells.

2.5. Assay of LTB_4 ω-hydroxylase activity

The reaction mixture containing cell lysate (1.5 mg protein) suspended in HEPES buffer (20 mM; pH 7.5) containing sucrose (340 mM), EDTA (1 mM), NADPH (1 mM) and LTB_4 (60 μM) in a total volume of 0.1 ml was incubated at 37 °C for 15 min. The reaction product was then extracted with 3 ml of ethyl acetate, dried gently under nitrogen and analyzed using HPLC as described [20,21]. Blank reactions did not contain NADPH. The activities were obtained after normalization of the expression of the recombinant Cyp4f using qRT-PCR.

2.6. RNA isolation and quantitative real time-PCR

Total RNA was isolated from cortex of mice and cDNA was synthesized using random hexamers. Real-time PCR was performed on ABI 7500 sequence detection system using Power SYBR Green PCR Master Mix from Applied Biosystems according to the manufacturer's instructions. The sequence of the primers used for quantitative real time-PCR is listed in Table 1. All reactions were carried out in triplicate and negative control without the template was also run. 18S rRNA was used as an internal control for normalization. Data was analyzed using the comparative threshold cycle (ΔΔCt) method. The amplicon generated for qRT-PCR of Cyp4f15 was cloned into pCR II and used to generate sense and antisense riboprobes for fluorescence *in situ* hybridization (FISH) experiments using T7 and T3 RNA polymerases, respectively.

2.7. Fluorescence in situ hybridization

Coronal sections of the brains (10 μm thick) were cut on a cryostat and used for *in situ* hybridization [22]. Sense probes served as controls for FISH. In some experiments Cyp4f15 was localized using FISH and then the sections were immunostained for Iba-1.

Table 1
List of primers used for cloning and qRT-PCR.

Gene accession number	Primer sequence	Expected amplicon
Cyp4f13 full-length (NM_130882)	Forward 5′ AGAGAAGGTGCCGGGGTGGAAG 3′ Reverse 5′ CTGGTTCCAAGGCAGCTGTGAGG 3′	1622 bp
Cyp4f14 full-length (NM_022434)	Forward 5′ TCACTGCACATTTATTCACCCAGC 3′ Reverse 5′ TAGGGTGGCTCTAGACCAGGT 3′	1574 bp
Cyp4f15 full-length (NM_134127)	Forward 5′ GTCAGAGTGATCCTGTTGGCTTCA 3′ Reverse 5′ ACCCGTCCAGGTGCAGGGAG 3′	1604 bp
Cyp4f16 full-length (NM_024442)	Forward 5′ ATAAGGATGCTGCGGCTAAGTGTGTC 3′ Reverse 5′ TAGACCTGGTCACTGAGCGCC 3′	1594 bp
Cyp4f18 full-length (NM_024444)	Forward 5′ AAACACAGTGCTCCTGAGAGAAGG 3′ Reverse 5′ GAGGGTCCCACGCCAGCTCAG 3′	1574 bp
Cyp4f13 qRT	Forward 5′ CATCTTGGATTCTAGCCCGAA 3′ Reverse 5′ GAAGCAACGAAGGCGACTG 3′	74 bp
Cyp4f14 qRT	Forward 5′ ACTGGCTTATGGGTCACGTG 3′ Reverse 5′ ACCCACCAAACGAGTCAATTC 3′	74 bp
Cyp4f15 qRT	Forward 5′ CATGACATGGCTGGGTCCTA 3′ Reverse 5′ GAGGCATTGAGAACAGATCGA 3′	78 bp
Cyp4f16 qRT	Forward 5′ CCTTGCCTGGACTTACTCATT 3′ Reverse 5′ GTAACCAGCTGCATGCCTTC 3′	132 bp
Cyp4f18 qRT	Forward 5′ AGAGCCTGGTGCGAACCTT 3′ Reverse 5′ TGGAATATGCGGATGACTGG 3′	73 bp
TNF-α qRT (NM_013693)	Forward 5′ ATGGCCCAGACCCTCACACTCA 3′ Reverse 5′ ACAACCCATCGGCTGGCACCA 3′	184 bp
IL-6 qRT (NM_031168)	Forward 5′ ACCTGCTGGTGTGTGACGTTC 3′ Reverse 5′ GTCGTTGCTTGGTTCTCCTTGTAC 3′	179 bp
MCP-1 qRT (NM_011333)	Forward 5′ GCTGTAGTTTTTGTCACCAAGCTCAA 3′ Reverse 5′ TGAAGACCTTAGGGCAGATGCAG 3′	150 bp
IL-1β qRT (NM_008361)	Forward 5′ ACCTGCTGGTGTGTGACGTTC 3′ Reverse 5′ GTCGTTGCTTGGTTCTCCTTGTAC 3′	179 bp

2.8. Immunohistochemistry

Brains were sectioned on a cryostat and immunohistochemistry was done as described in detail [23]. Briefly, coronal sections (30 μm thick) were cut throughout the entire cortex and hippocampus. Cryosections were mounted on VWR microslides (USA) and sections were air dried. Quenching was done with 3% hydrogen peroxide for 20 min. Sections were washed with Tris-buffered saline (TBS) and subjected to heat induced epitope retrieval using antigen unmasking solution. Sections were washed and blocking was done with 3% (v/v) serum in which secondary was raised with 2% BSA (w/v) and 0.1% Triton X (v/v) for 2 h at room temperature. Cryosections were then incubated with the primary antibody to Iba-1 at 4–8 °C overnight. Immunostaining was visualized using following secondary antibodies, FITC conjugated, Alexa Fluor 488, Alexa Fluor 594 or HRP conjugated which was developed with Nova Red. Immunofluorescence was visualized under an upright Zeiss Axioplan/ApoTome using 20×, 40× and 100× objective lens and bright field images were captured using Leica DMRXA2 with 40× objective lens. Non-immune IgG was used as control for immunohistochemistry.

2.9. Quantitation of microglia

Immunohistochemistry for microglia was performed using Iba-1 antibody. Every 5th section was used for stereological assessment. Stereological analysis was done by determining number of Iba1-activated microglia per square millimeter using IM 50 software (Leica).

2.10. Down-regulation of Cyp4fs

Two sets of primers were designed to generate shRNA, which were cloned into mU6 vector (Table 2). Scrambled shRNA to Cyp4f15 was also designed and cloned into mU6, which served as a negative control. One set down-regulated all the Cyp4f genes while the other was specific to Cyp4f15. The shRNA containing plasmids were transfected into BV-2 cells, harvested after 72 h and RNA was isolated for qRT-PCR. The shRNA was also transfected into BV-2 cells for 72 h and the cells were treated with either LPS alone (25 ng/ml of media) or with fenofibrate (40 μg/ml) for 12 or 36 h respectively and the media was analyzed for cytokine levels.

2.11. Processing of tissue

Tissue (cortex) was homogenized in potassium phosphate buffer (0.1 M; pH-7.4) containing 0.25 M sucrose and was centrifuged at 100,000 × *g* for 1 h for cytosol preparation. Protein concentration was estimated by dye binding method [24]. The cytosol was used to quantitate the levels of cytokines and chemokines using Cytometric Bead array (CBA).

2.12. Estimation of cytokines, chemokines and LTB_4

Cytokine bead array was used to quantitatively measure cytokine and chemokine levels in the cytosol from cortex as also from the cell culture media. The assay was performed according to the manufacturer's instructions and analyzed on the FACS Calibur (Becton Dickinson). Following acquisition of sample data, analysis was done using the BD™ CBA Analysis Software that allows the

Table 2
Primer sequences used for generating shRNA.

shRNA-4f	Oligo1 – 5′ TTTGGCTGACACCTTCATGTTTAAGTTC TCTAAACATGAAGGTGTCAGCCTTTTT 3′ Oligo2 – 5′ CTAGAAAAAGGCTGACACCTTCATGTTT AGAGAACTTAAACATGAAGGTGTCAGC 3′
shRNA-4f15	Oligo1 – 5′ TTTGGAGAATCCCAGTGAGTATAAGTTC TCTATACTCACTGGGATTCTCCTTTTT 3′ Oligo2 – 5′ CTAGAAAAAGGAGAATCCCAGTGAGTATA GAGAACTTATACTCACTGGGATTCTC 3′
Scrambled shRNA-4f15	Oligo1– 5′ TTTGTGCCACCCTGACATCATTAAGTT CTCTAATGATGTCAGGGTGGCACTTTTT 3′ Oligo2 – 5′ CTAGAAAAAGTGCCACCCTGACATCA TTAGAGAACTTAATGATGTCAGGGTGGCA 3′

calculation of cytokine concentrations in unknown samples [25]. LTB_4 levels were measured using the Assay designs™ Leukotriene B_4 enzyme immunoassay kit according to manufacturer's instructions.

2.13. Statistical analysis

Sigma Stat 3.5 software was used for analysis of the data and Sigma Plot 10.0 for generating graphs. Significance was assessed using One-Way Analysis of Variance followed by post hoc tests (Student–Newmann–Keul's or Bonferroni) for multiple comparisons and Student's *t*-test for comparison between two groups. Values of $p < 0.05$ were taken to be statistically significant. Data is represented as mean ± standard deviation.

3. Results

3.1. Differential expression and catalytic activity of Cyp4fs towards the proinflammatory mediator LTB_4

We cloned, expressed the mouse Cyp4fs (4f13, 14, 15, 16, and 18) and examined their capability to hydroxylate LTB_4. Recombinant Cyp4f14, Cyp4f15 and Cyp4f18 are the most effective members of Cyp4f sub-family for LTB_4 metabolism, while Cyp4f13 and 16 are poor metabolizers (Fig. 1A). Cyp4f13, Cyp4f14 and Cyp4f15 are expressed at markedly higher levels than Cyp4f16 or Cyp4f18 in naive mouse brain cortex (Fig. 1B). Cyp4f15 is expressed ubiquitously in the mouse brain and can be visualized in the pyramidal neurons of CA1, CA2 and CA3 in the hippocampus and granule cells of the dentate gyrus. Further Cyp4f15 is also expressed in the Purkinje cells and the granule cell layer of the cerebellum. Diffuse staining of the cells including neurons was seen in the cortex and thalamus (Fig. 1C).

3.2. LPS prompts increase in inflammatory mediators and expression levels of Cyp4fs in mouse brain

Exposure to a single dose of LPS (3 mg/kg b.w.) for 12 h results in activation of microglia in cortex (Fig. 2A) and elevated levels of LTB_4 (Fig. 2B). The mRNA expression and protein levels of cytokines (TNF-α, IL-6, IL-1β; Fig. 2C and D) and chemokine (MCP-1; Fig. 2C and D) were markedly elevated, while the protective cytokine IL-10 levels were decreased (Fig. 2D) at 12 and 24 h after a single exposure to LPS. Cyp4f15, Cyp4f16 and Cyp4f18 mRNA expression increased significantly with no change in Cyp4f13 and Cyp4f14 after LPS treatment (Fig. 3A and B) indicating the regulation of LTB_4 metabolism during inflammatory process. Since Cyp4f15, the efficient metabolizer of LTB_4 is expressed in high amounts and is induced by inflammatory prompts unlike Cyp4f14 we focused our studies on Cyp4f15. Microglia are the site of LTB_4 production, and if indeed Cyp4fs are the key mediators of LTB_4 hydroxylation, the expression of Cyp4f in microglia could be potentially modulated by LPS. We therefore, examined the co-expression of Cyp4f15 and Iba-1, a marker for microglia in vehicle and LPS treated mouse brain. Both in cortex and hippocampus (Fig. 3C and D), Cyp4f15 was expressed in Iba-1 positive microglia and this was substantially enhanced in LPS treated mice indicating that LTB_4 hydroxylation could potentially occur within microglia.

3.3. Inhibition of Cyp4f potentiates LPS mediated increase in cytokine levels

Co-administration of 17-ODYA (17-octadecynoic acid, an inhibitor of Cyp4f mediated ω-hydroxylation) with LPS leads to significant increase in LTB_4 (Fig. 4A), mRNA (Fig. 4B and C) and protein levels (Fig. 4D and E) of TNF-α, IL-6, MCP-1 and IL-β in mouse brain cortex while IL-10 decreased (Fig. 4E) over that seen with LPS alone. This is consistent with the postulate that Cyp4fs inactivate LTB_4 thus decreasing inflammation and, when Cyp4fs are inhibited inflammatory response is exacerbated. Cyp4fs regulate constitutive levels of LTB_4 (Fig. 4A) and cytokines/chemokine (TNF-α, IL-6, MCP-1 and IL-1β; Fig. 4B–F) as shown by significant increase in the levels following 17-ODYA treatment alone. The exacerbation in inflammation resulted in increased microglial activation as quantitated using stereology (Fig. 4G).

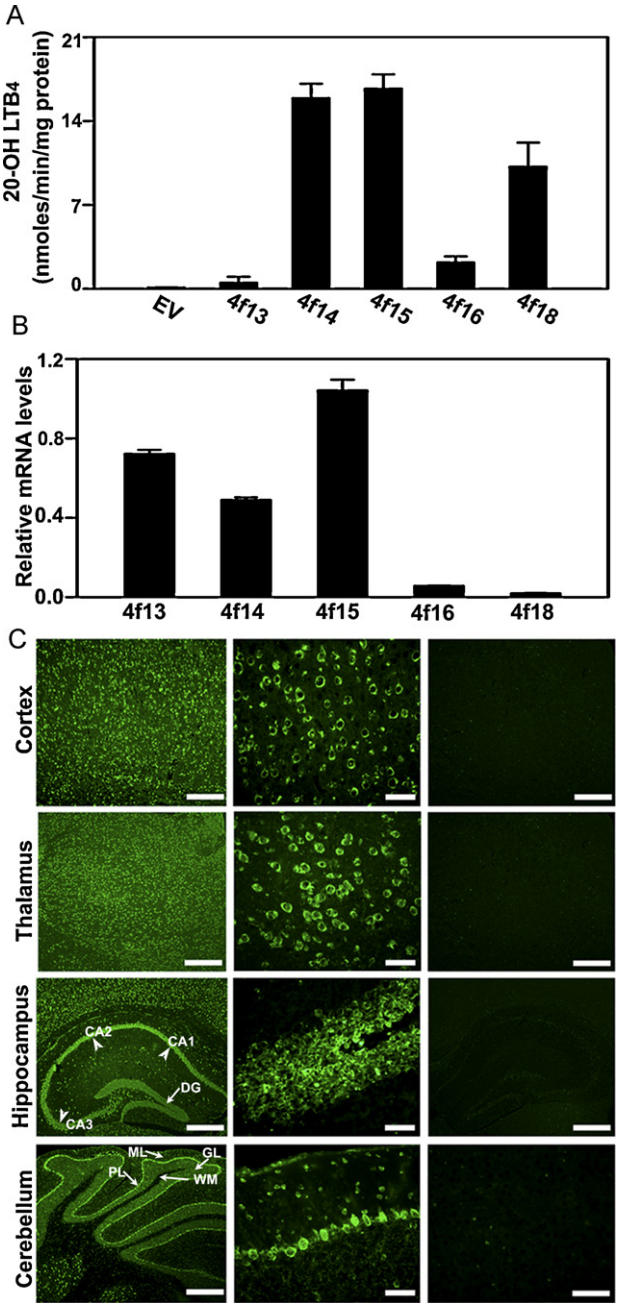


Fig. 1. Metabolism of LTB_4 by recombinant mouse Cyp4fs and relative expression of Cyp4fs in mouse brain cortex. (A) Cells expressing recombinant Cyp4fs metabolize LTB_4 to 20-OH LTB_4 while the cells transfected with empty vector do not metabolize LTB_4. EV represents cells transfected with empty vector ($n = 3$ per group). (B) Constitutive expression of Cyp4fs in mouse brain cortex was assessed by qRT-PCR. 18S rRNA was used for normalization ($n = 4$ per group). (C) Fluorescence *in situ* hybridization for Cyp4f15 mRNA expression was performed on coronal sections of mouse brain. Increased expression of Cyp4f15 mRNA was observed in the neurons of cerebral cortex, thalamus, hippocampus and cerebellum as seen in low (bar = 100 μm) and high magnification (bar = 25 μm). Corresponding control sections hybridized with sense probe are also shown (bar = 100 μm). DG = dentate gyrus, ML = molecular layer, GL = granular layer, PL = Purkinje layer, and WM = white matter.

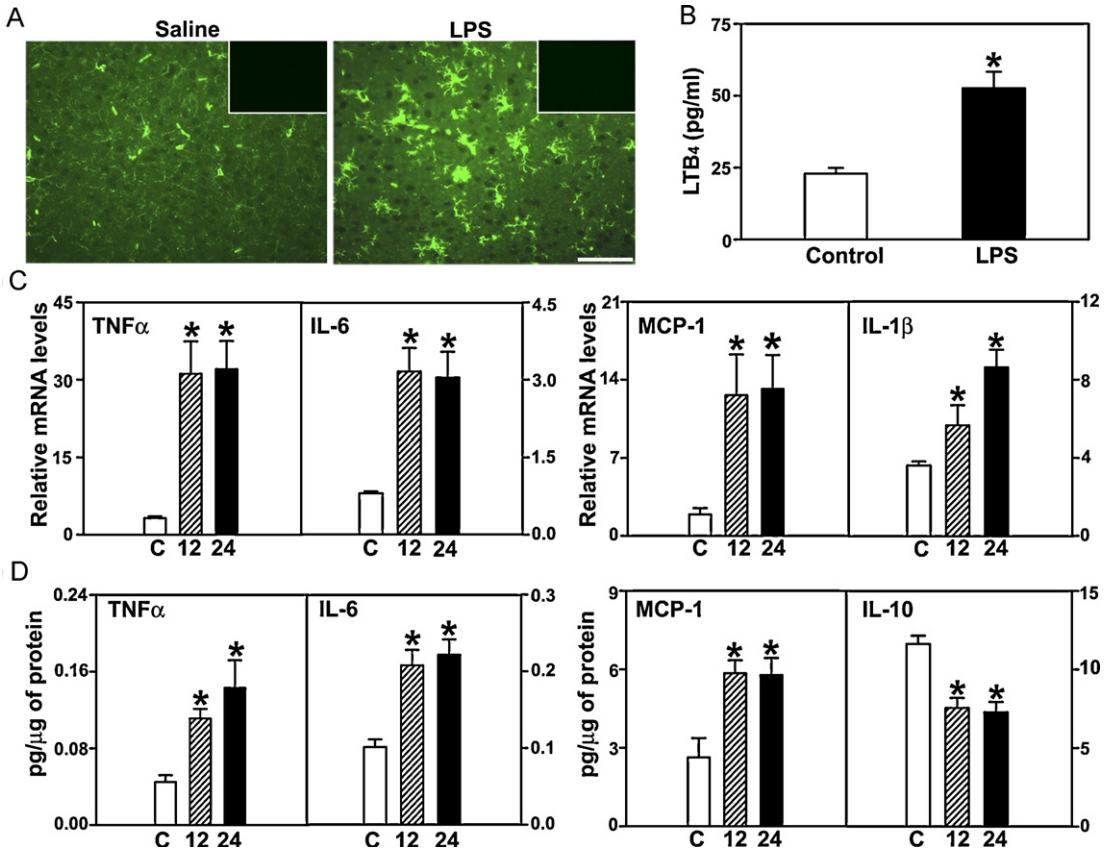


Fig. 2. Effect of LPS on microglial activation and relative expression of cytokines/chemokines in mouse brain cortex. Administration of LPS to mice resulted in a significant increase in number of Iba-1 positive cells (A) and LTB_4 levels (B) 12 h later in the cortex, bar = 100 μm. Inset depicts negative control for immunohistochemical localization of Iba-1, which was performed using normal rabbit IgG in place of Iba-1 antibody. There was a significant increase in mRNA expression (C) and protein levels (D) of cytokines (TNF-α, IL-6, IL-1β) and chemokine (MCP-1) while IL-10 (D) decreased in the cortex 12 and 24 h after LPS administration. 18S rRNA was used for normalization ($n = 6$ per group, $^*p < 0.05$).

3.4. Induction of Cyp4f attenuates the LPS mediated increase in cytokine levels

Administration of fenofibrate for 4 days induced the expression of Cyp4f15 and 4f18 in mouse brain cortex (Fig. 5A and B) while other Cyp4f enzymes were unchanged. It is interesting to note that while LPS alone induced certain Cyp4fs this induction was significantly greater when mice were pretreated with fenofibrate and then exposed to LPS (Fig. 5C). Concurrently, LTB_4 and cytokine/chemokine levels were lowered significantly. Thus, pretreatment with fenofibrate substantially reduced the LPS-mediated inflammatory response measured as LTB_4 (Fig. 5D), cytokine/chemokine mRNA (Fig. 5E) and protein (Fig. 5F) levels in the cortex while it significantly increased the protective cytokine, IL-10 levels (Fig. 5F). Fenofibrate treatment alone significantly decreased levels of LTB_4 (Fig. 5D) and cytokines/chemokine (TNF-α, IL-6, MCP-1 and IL-1β; Fig. 5E–G) indicating role of Cyp4s in inflammation. The biochemical measurements were validated with immunohistochemical quantitation of activated microglia using Iba-1, wherein activated microglia were decreased with fenofibrate in LPS treated mice (Fig. 5H).

3.5. Down-regulation of Cyp4f in vitro results in increased inflammatory response to LPS challenge

To pursue the mechanism involved, we turned to a cell culture approach. Since microglia are the major cell type involved in propagation and regulation of inflammatory response in brain, we used the mouse derived microglial cell line BV-2, which constitutively expresses Cyp4f13, 4f14, 4f15, 4f16 and 4f18 (Fig. 8A and B). Addition of LTB_4 to BV-2 cells increased the expression of TNF-α, IL-6 and IL-1β (Fig. 6A). Exposure of BV-2 cells to LPS increased the levels of LTB_4 (Fig. 6B). Treatment with 17-ODYA resulted in exacerbated LPS mediated release of TNF-α and IL-6 into the medium (Fig. 6C). Addition of this medium containing the cytokines/chemokine secreted by BV-2 cells treated with both LPS and 17-ODYA resulted in decreased cell viability of Neuro-2a cells compared to medium from cells treated with LPS alone. We chose the well characterized Neuro-2a cells to test the cytotoxicity of the medium in which BV-2 cells were grown since it is mouse neuroblastoma derived cell line and can be differentiated to neurons [26] indicating that inhibition of Cyp4fs enhanced the levels of toxic cytokines released into the medium which exacerbated the LPS mediated toxicity (Fig. 6D).

We next developed a single shRNA that accomplished down-regulation of all mouse Cyp4fs by developing shRNA sequence against a homologous element within Cyp4f family. Further, we also developed an shRNA with a sequence unique to Cyp4f15. A scrambled shRNA sequence was also cloned to serve as negative control. Both the active shRNAs markedly reduced the expression of 4f15 in BV-2 cells whereas the scrambled sequence had no effect (Fig. 7A). The shRNA designed commonly for all Cyp4fs also significantly reduced the expression of Cyp4f13 and 4f18 however, the expression of Cyp4f14 and 4f16 were unaltered.

It may be noted that Cyp4f14 is expressed at very low levels in BV-2 cells and although Cyp4f16 is expressed at high levels, its ability to hydroxylate LTB_4 is very low (Fig. 1A). Thus, this shRNA down-regulated the relevant Cyp4fs. Moreover, both shRNA

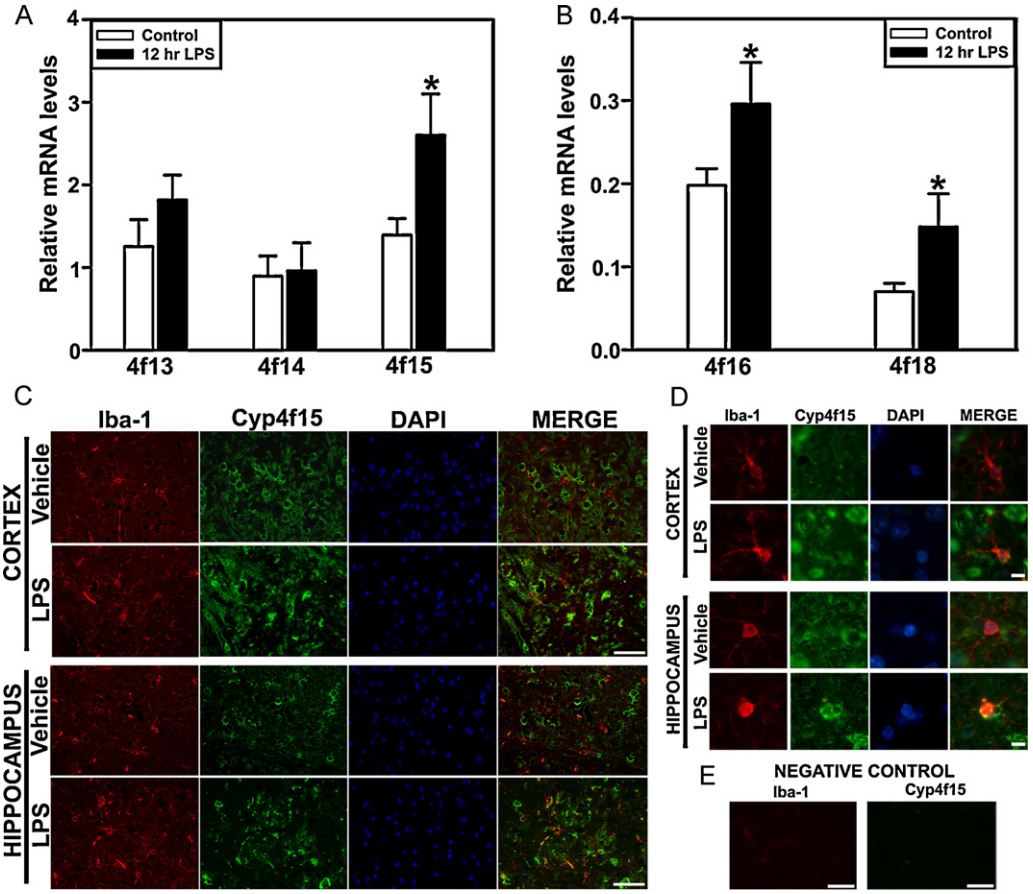


Fig. 3. Effects of LPS treatment on expression of Cyp4fs expression in mouse brain. (A and B) Exposure of LPS for 12 h selectively induced the expression of Cyp4f15, 4f16 and 4f18 mRNA in the cortex. 18S rRNA was used for normalization ($n = 6$ animals per group, $^*p < 0.05$). (C) Co-localization of Cyp4f15 mRNA (green) and Iba-1 (red) was performed in vehicle and LPS treated mouse brain cortex and hippocampus. LPS treatment for 12 h increased the number of Iba-1 positive microglia, which also co-expressed Cyp4f15 mRNA as seen in the magnified images depicted in (D), bar = 100 μm (C) and bar = 25 μm (D). (E) Control section of mouse brain cortex that was hybridized with sense probe, bar = 100 μm. Negative control for immunohistochemical localization of Iba-1, which was performed using normal rabbit IgG in place of Iba-1 antibody is also depicted, bar = 100 μm.

sequences significantly enhanced the release of inflammatory cytokines, TNF-α (Fig. 7B) and IL-6 (Fig. 7C) into the culture medium. Addition of medium from LPS treated BV-2 cells wherein Cyp4fs were down-regulated resulted in decreased viability of Neuro-2a cells compared to medium from cells treated with LPS alone (Fig. 7D) in a manner similar to that seen with 17-ODYA. These data together demonstrate the dependence of inflammatory response measured as LTB_4 levels and cytokine release on Cyp4f expression.

3.6. Effect of induction of Cyp4f by fenofibrate on LPS induced inflammatory response

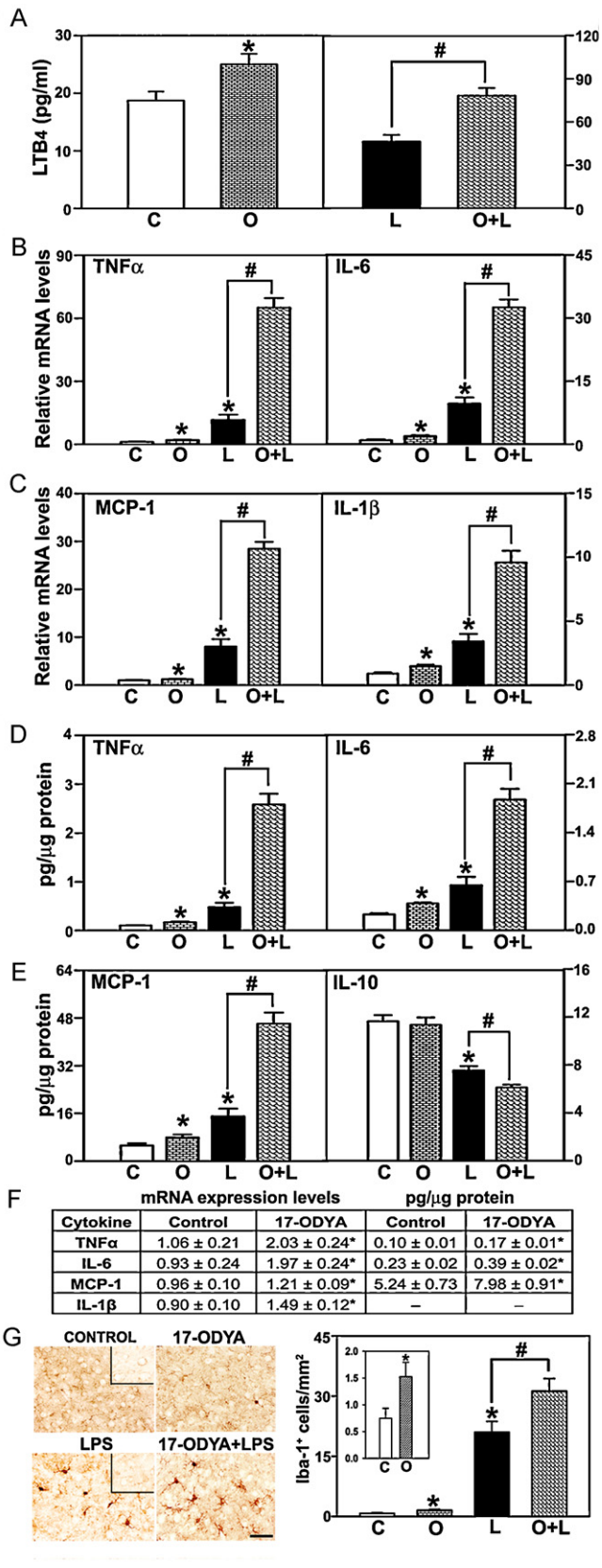
Treatment of BV-2 cells with fenofibrate for 24 and 48 h resulted in substantial induction of Cyp4f13, Cyp4f14, Cyp4f15 and Cyp4f18 in a time dependent manner while Cyp4f16 was unchanged (Fig. 8A and B). This pattern of induction was different from that observed in vivo, indicating that multiple subtypes of cells contribute to the differential induction pattern of Cyp4fs in mouse brain. Pretreatment with fenofibrate attenuated LPS-mediated release of cytokines, such as TNF-α and IL-6 (Fig. 8C). Interestingly, fenofibrate alone inhibited the release of TNF-α and IL-6 indicating that induction of Cyp4fs by fenofibrate probably down-regulates the constitutive levels of cytokines (Fig. 8C, inset).

In order to ascertain whether fenofibrate acts through the induction of Cyp4fs and not through other mechanism(s), we transfected BV-2 cells with the shRNAs to Cyp4fs and Cyp4f15 prior to treatment with LPS or LPS and fenofibrate. Transfection of shRNA abolished the fenofibrate-mediated induction of the mRNA expression of Cyp4fs in general and Cyp4f15 in particular (Fig. 8D). A point of interest is that while LPS treatment per se induced Cyp4fs in the mouse brain, this was not replicated in vitro, in cultured BV-2 cells, indicating the probable contribution of multiple cell types in the induction of Cyp4fs in the mouse brain, in addition to other factors.

Fenofibrate failed to attenuate LPS induced increase in the levels of TNF-α and IL-6 in cells transfected with shRNA-4f or shRNA-4f15 (Fig. 8E and F). These experiments indicate that fenofibrate provides neuroprotection against LPS-mediated release of inflammatory cytokines by inducing the expression of Cyp4fs. The above observations depict the importance of Cyp4fs in alleviating the inflammatory response, in vitro.

4. Discussion

We demonstrate that Cyp4fs play an important role in the resolution of inflammation in the brain through metabolism of LTB_4 to 20-hydroxy LTB_4. Inhibition of Cyp4f activity by down-regulation of gene expression using shRNA or chemical inhibitors results in increased LTB_4 levels and escalation of inflammatory response while elevated expression of Cyp4fs decreases the LTB_4 levels and affords neuroprotection in a LPS model of neuroinflammation both in vitro and in vivo, in mice.



Cytokine	Control	17-ODYA	Control	17-ODYA
TNFα	1.06 ± 0.21	2.03 ± 0.24*	0.10 ± 0.01	0.17 ± 0.01*
IL-6	0.93 ± 0.24	1.97 ± 0.24*	0.23 ± 0.02	0.39 ± 0.02*
MCP-1	0.96 ± 0.10	1.21 ± 0.09*	5.24 ± 0.73	7.98 ± 0.91*
IL-1β	0.90 ± 0.10	1.49 ± 0.12*	–	–

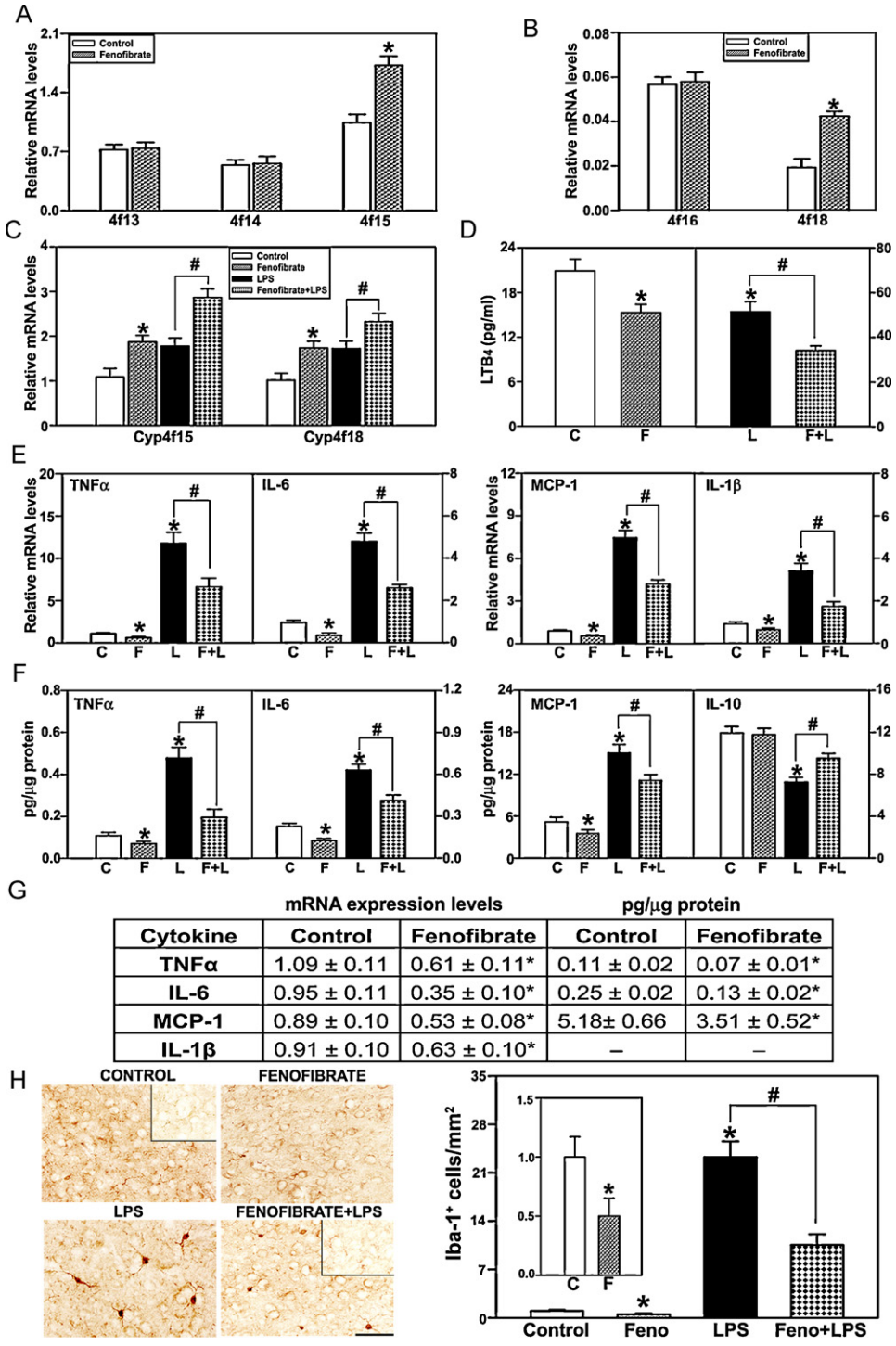
Fig. 4. Effect of inhibitor of Cyp4fs on LPS mediated inflammatory response in mouse brain cortex. (A) Pretreatment with 17-ODYA, an inhibitor of Cyp4f catalytic activity enhanced the LPS mediated increase in LTB_4 levels in the cortex. This effect correlated with the increased mRNA expression (B and C) and protein levels (D and E) of cytokines (TNF-α, IL-6, IL-1β) and chemokine (MCP-1) in 17-ODYA pretreated mice compared to LPS alone. Conversely, protective cytokine IL-10 production was decreased in 17-ODYA (E) as compared to LPS alone. Treatment with 17-ODYA alone results in increased levels of LTB_4 (A) and cytokines/chemokine (F). Data is represented as (C) vehicle control, (O) 17-ODYA, (L) LPS, (O + L) 17-ODYA + LPS treated mouse brain cortex. 18S rRNA was used for normalization ($n = 15$ per group, * and #$p < 0.05$). (G) Stereological assessment of microglia following immunolabeling with antibody to Iba-1 showed significant increase in number of activated microglia following 17-ODYA pretreatment compared to LPS alone (bar = 50 μm). Inset depicts the negative control for immunohistochemical localization of Iba-1, which was performed using normal rabbit IgG.

Fenofibrate, a PPARα agonist, which is used clinically as a statin has been shown to be neuroprotective in several models of acute and chronic neurodegenerative disorders, such as Parkinson's disease [27]. The neuroprotective role of fenofibrate has been attributed to its action as a PPARα effector [28]. PPARα expression is also regulated by LTB_4 [29] thus indicating its role in auto-regulation of inflammatory response. Here, we show that fenofibrate also induces a different class of genes, namely the Cyp4fs in brain, which are then able to enhance the hydroxylation of LTB_4, thereby reducing the levels of cytokines and chemokines, the key mediators of inflammation. Thus, we demonstrate a new mechanism of action for fenofibrate and provide evidence for its action through the induction of Cyp4fs by demonstrating that exposure of cells to fenofibrate wherein Cyp4fs are down-regulated by shRNA does not lead to resolution of inflammation (Fig. 8).

Cyp4fs can be induced potentially by a variety of small molecules. Promoter analysis reveals RXR heterodimer binding sites at 468 bp, 326 bp and 290 bp upstream of the Cyp4f15 start site. RXR and PPAR form heterodimers [30] to promote transcription and thus, may act cooperatively to enhance gene expression of Cyp4fs. Thus, we present a new drug target that could be exploited to generate a novel class of anti-inflammatory agents for use in brain disorders.

All the Cyp4f genes, namely Cyp4f13, Cyp4f14, Cyp4f15, Cyp4f16 and Cyp4f18 are expressed in the mouse brain cortex and possess differential abilities to hydroxylate LTB_4 (Fig. 1A and B). Cyp4f15 is expressed in significantly higher amounts, efficiently metabolizes LTB_4 to 20-hydroxy LTB_4 and is induced by exposure to LPS or fenofibrate, unlike Cyp4f14, which is expressed in high amounts and efficiently hydroxylates LTB_4, but is not induced by LPS (Figs. 1A, B and 3A) or fenofibrate (Fig. 5A). Since fenofibrate mediated anti-inflammatory response occurs through induction of Cyp4fs (Fig. 8E and F) it is less likely that Cyp4f14 contributes significantly to the anti-inflammatory action. Further, Cyp4f18 is induced by LPS (Fig. 3B) and fenofibrate (Fig. 5B) and efficiently hydroxylates LTB_4 but is expressed in very low amounts in the cortex (Fig. 1A and B). Therefore, Cyp4f15 may play a prominent role in the regulation of neuroinflammation through inactivation of LTB_4 in C57BL6J mice. Though Cyp4f15 is induced on exposure of LPS in vivo (Fig. 3A), there seems to be no effect on its levels in vitro (Fig. 8D). The differential response seen could be contributed by the brain milieu, which is composed of variety cell types besides microglia. The induction of certain Cyp4fs following LPS treatment indicates an endogenous mechanism to reduce inflammatory response. However, augmentation of this response by Cyp4f inducer, such as fenofibrate attenuated the inflammatory response further.

LTB_4 is a product of action of 5-lipoxygenase on arachidonic acid, while another class of inflammatory prompts, the prostaglandins, is formed from arachidonic acid by cyclo-oxygenases. The Cyp4f subfamily can also metabolize hydroxyeicosatetraenic acid (HETE) and hydroperoxyeicosatetraenoic acid (HPETE), which are metabolic signals for vasoconstriction/dilation and other functions [31]. Thus Cyp4fs could modulate the inflammatory cascade by hydroxylating and inactivating both leukotrienes and prostaglandins [12].



Cytokine	mRNA expression levels: Control	mRNA expression levels: Fenofibrate	pg/μg protein: Control	pg/μg protein: Fenofibrate
TNFα	1.09 ± 0.11	0.61 ± 0.11*	0.11 ± 0.02	0.07 ± 0.01*
IL-6	0.95 ± 0.11	0.35 ± 0.10*	0.25 ± 0.02	0.13 ± 0.02*
MCP-1	0.89 ± 0.10	0.53 ± 0.08*	5.18± 0.66	3.51 ± 0.52*
IL-1β	0.91 ± 0.10	0.63 ± 0.10*	–	–

Fig. 5. Effect of inducer of Cyp4fs on LPS mediated inflammatory response in mouse brain cortex. (A and B) Treatment with fenofibrate significantly induced the expression of Cyp4f15 and 18 in mouse cortex however the levels of Cyp4f13, 14 and 16 were unaltered. (C) Pretreatment with fenofibrate enhanced the LPS mediated induction of Cyp4f15 and 18 compared to LPS alone. (D) The levels of LTB_4 decreased significantly after fenofibrate treatment compared to LPS alone. This effect correlated with the decreased mRNA expression (E) and protein levels (F) of cytokines (TNF-α, IL-6, IL-1β) and chemokine (MCP-1) in fenofibrate pretreated mice as compared to LPS alone. Conversely, protective cytokine IL-10 production was increased in fenofibrate (F) pretreated mice compared to LPS alone. Treatment with fenofibrate alone results in decreased levels of LTB_4 (D) and cytokines/chemokine (G). Data is represented as (C) vehicle control, (F) fenofibrate, (L) LPS, (F + L) fenofibrate + LPS treated mouse brain cortex. 18S rRNA was used for normalization ($n = 15$ per group, * and #$p < 0.05$). (H) There was a significant decrease in Iba-1 positive cells after treatment with both fenofibrate (Feno) and LPS (bar = 50 μm). Inset depicts the negative control.

Recombinant mouse brain Cyp4f15 and 18 metabolize LTB_4 to 20-hydroxy LTB_4 efficiently. LTB_4 has been shown to play an important role in inflammatory response in CNS including spinal cord injury [32]. While LTB_4 is generated in the brain following LPS exposure [16] and plays an important role in propagating inflammatory processes as shown in the present study and elsewhere [32], it is also possible that Cyp4fs mediate metabolism of other molecules in the arachidonic acid cascade, which must further be addressed. In addition enzymes of Cyp4A family, may also metabolize LTB_4 [33], however, the shRNA experiments detailed herein suggest that in the experimental paradigm used by us, Cyp4fs are important for metabolism of LTB_4.

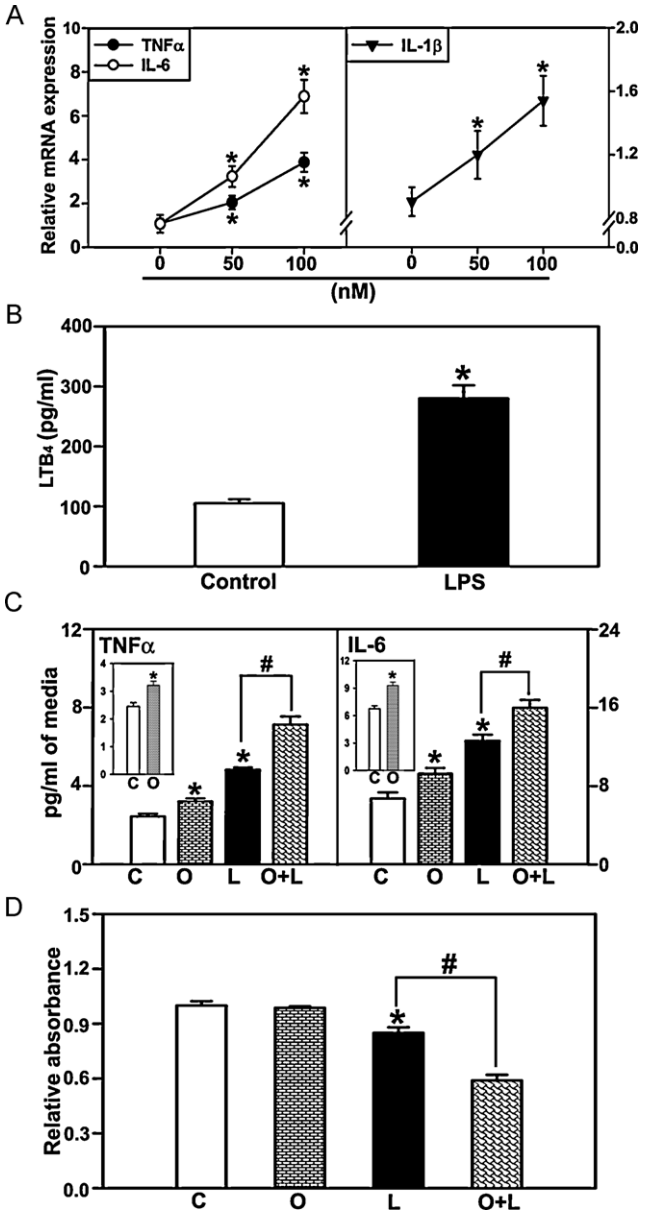


Fig. 6. Microglial BV-2 cells show inflammatory response, in vitro, to LTB_4 and LPS which is exacerbated following inhibition of Cyp4f. (A) Addition of LTB_4 to BV-2 cells resulted in dose dependent increase in TNF-α, IL-6 and IL-1β. (B) LTB_4 levels increased significantly in BV-2 cells following exposure to LPS (25 ng/ml) for 12 h. Cells were treated with vehicle (C), 17-ODYA (O), LPS (L) or both 17-ODYA and LPS (O + L). Exposure to 17-ODYA enhanced LPS mediated increased release of cytokines into the medium (C) and decreased viability of Neuro-2a cells (D) upon addition of the medium from BV-2 cells ($n = 12$ per group, * and $\#p < 0.05$).

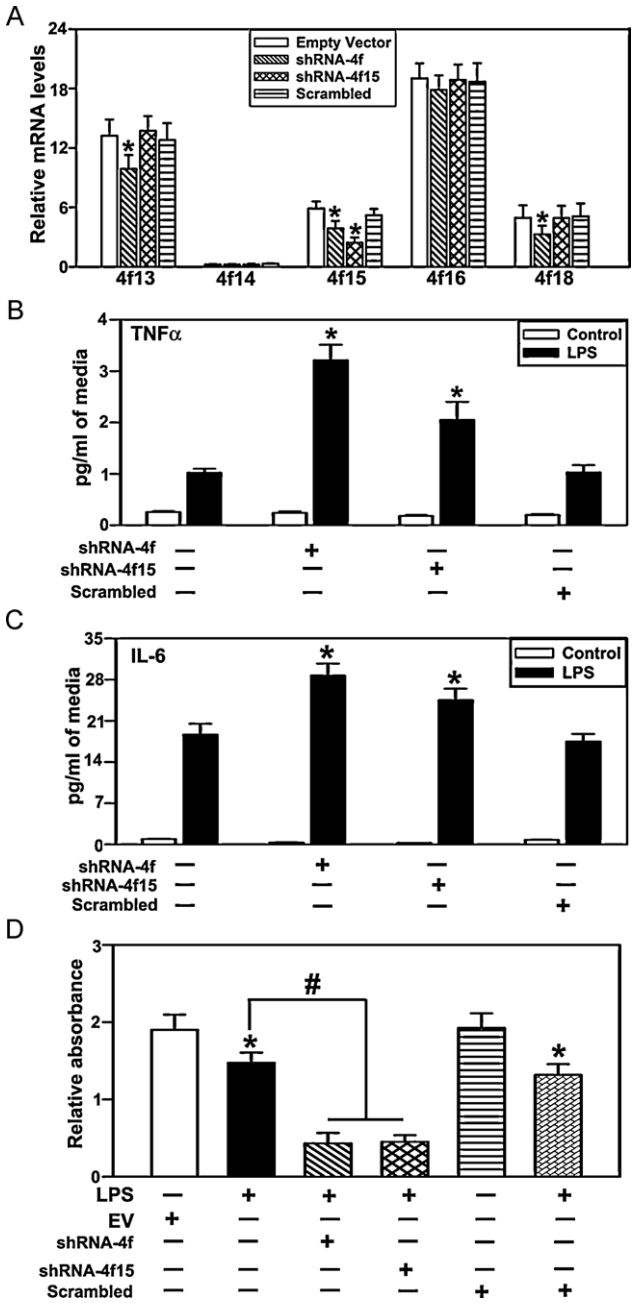


Fig. 7. Inflammatory response, in vitro, to LPS is enhanced following knock-down of Cyp4f. (A) BV-2 cells were transfected with empty vector (EV), shRNA to Cyp4fs (shRNA-4f), shRNA specific to Cyp4f15 (shRNA-4f15) or scrambled shRNA and the knock-down of Cyp4fs expression was assessed quantitatively. TNF-α (B) and IL-6 (C) released into the medium from cells transfected with shRNA-4f, shRNA-4f15 or scrambled shRNA in the presence and absence of LPS. (D) Addition of medium from cells transfected with the above shRNA and exposed to LPS resulted in decreased viability of Neuro-2a cells indicating that down-regulation of Cyp4fs enhanced LPS induced toxicity. 18S rRNA was used for normalization ($n = 10$ per group, * and $\#p < 0.05$).

The presence of Cyp4fs in microglia cells in mouse brain cortex indicates that LTB_4 can be metabolized at the site of production before being extruded out of the cell, where they interact with the cell surface receptors BLT1 and BLT2 [34] further propagating the inflammatory cascade. These receptors are present in the CNS neurons [35–37] and may play a role not only in modulating inflammatory response but also the function of ion channels [38].

It may be argued that Cyps are essentially localized in the endoplasmic reticulum (ER) while LTB_4 is generated in cytoplasm and therefore Cyp4fs may not be able to metabolize LTB_4. Cyps are anchored to ER membrane through N-terminal hydrophobic amino acids thus allowing the bulk of the protein including the catalytic subunit to be exposed to the cytosol, which would be accessible to LTB_4 [39]. Further, Cyps are also known to be present in the plasma membrane, where LTB_4 hydroxylation could presumably take place. This is further confirmed by the fact that inhibition of Cyp4fs either through the use of general inhibitor, 17-ODYA or through the use of shRNAs results in increased inflammatory response following LPS exposure. This study clearly demonstrates that Cyp4fs are indeed involved in detoxifying the mediators of inflammation and therefore may serve as potential drug targets which is relevant considering that CYP4F family of enzymes are expressed in human brain and have the capability to metabolize LTB_4 [40].

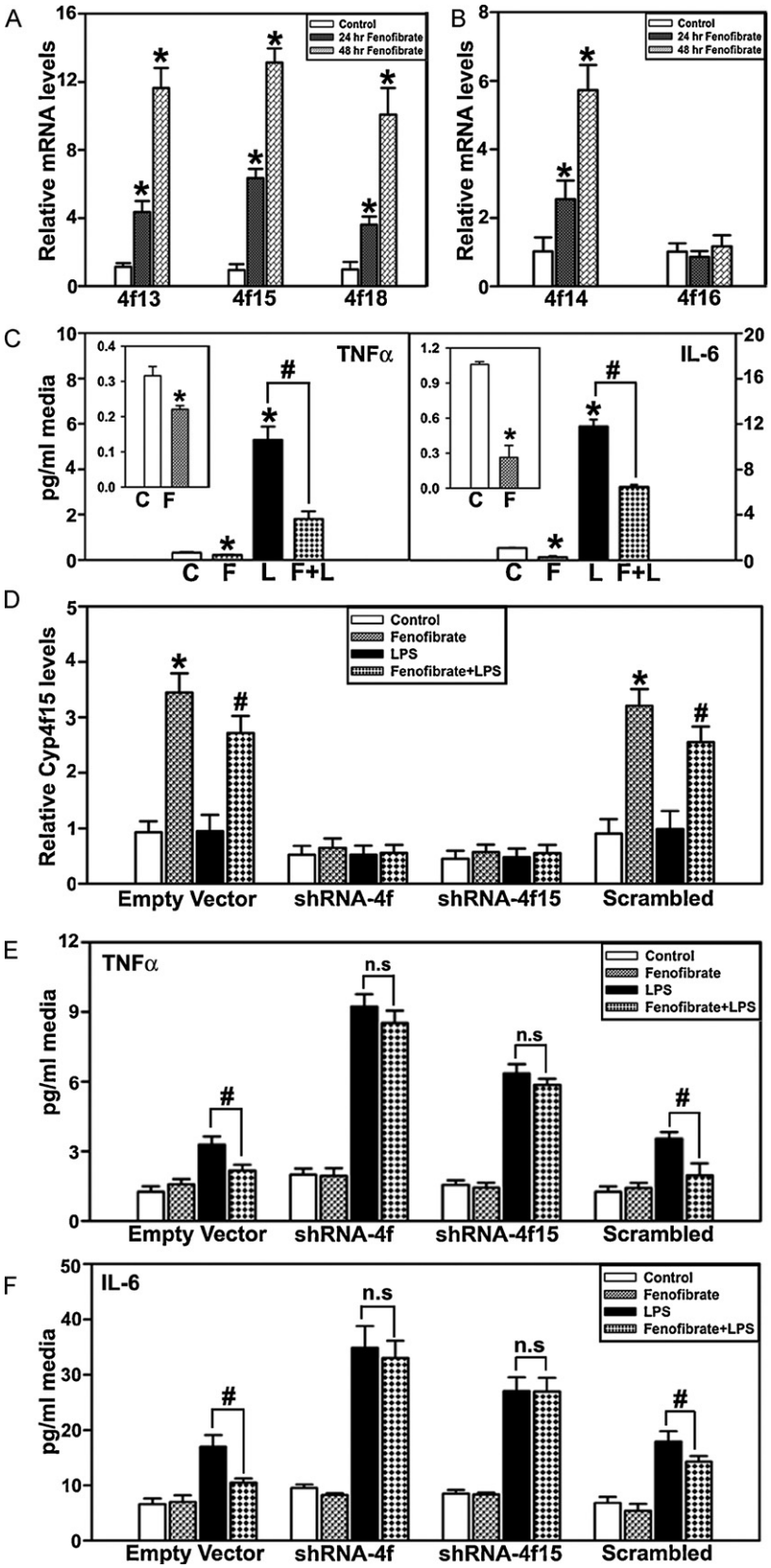


Fig. 8. Fenofibrate attenuates LPS mediated inflammatory response in BV-2 cells which is abolished by knock-down of Cyp4f. (A and B) Fenofibrate exposure induced the levels of Cyp4fs with the exception of Cyp4f16 in BV-2 cells. Cells were treated with vehicle (C), fenofibrate (F), LPS (L) or both fenofibrate and LPS (F + L). (C) Treatment with fenofibrate significantly decreased the levels of cytokines released into the medium after LPS treatment. Inset shows the decrease in cytokines after fenofibrate treatment alone in higher magnification. (D) BV-2 cells were transfected with empty vector, shRNA-4f, shRNA-4f15 or scrambled shRNA and expression of Cyp4f15 was quantitated from transfected cells treated with vehicle, fenofibrate, LPS, both LPS and fenofibrate. TNF-α (E) and IL-6 (F) released in the medium of the experimental groups described above demonstrates that the protective effect of fenofibrate against LPS mediated inflammatory response is lost when Cyp4f is knocked down. 18S rRNA was used for normalization ($n = 10$ per group, * and #$p \leq 0.05$); n.s. – not significant.

In NCBI, UniGene and HomoloGene databases, mouse Cyp4f18 is grouped with human CYP4F3A, Exhibit 81% homology and hydroxylate LTB_4. Mouse Cyp4f14 shares 81% homology with human CYP4F2 and is listed as orthologue, while mouse Cyp4f15 shares 78% amino acid sequence identity with human Cyp4F11 [33]. The human brain CYP4F enzymes are yet to be characterized. Further, considering the existence of unique splice variants of P450 enzymes in human brain that contribute differentially to biotransformation, it is difficult to extrapolate the data from mouse to humans. Nevertheless, the present studies demonstrate the potential implication of Cyp4f enzymes in resolution of inflammation and this remains to be characterized in human brain.

It has been debated whether the inflammatory response seen in a variety of brain disorders, such as CNS infections, brain/spinal cord injury or neurodegenerative disorders are the cause or the consequence of the pathogenic insult [41–43]. Nevertheless, it is well acknowledged that reduction of neuroinflammation results in better prognosis and slower progression of the disease [44,45]. Therefore, discovery of drug targets such as Cyp4fs offers a novel method of controlling inflammatory response both under acute conditions, such as infections of the nervous systems and traumatic brain injury as well as in chronic degenerative conditions. If indeed such agents could be developed they would potentially be useful as disease modifying therapies for brain disorders where neuroinflammation plays an important role in disease progression.

Contributors

N.S, V.A. performed experiments and analyzed the data. R.K performed significant proportion of the quantitative RT-PCR experiments. S.D.J. performed immunohistochemistry and *in situ* hybridization. L.A. did the cloning of Cyp4f18. V.R. designed the study and interpreted the data. V.R., H.W.S., and N.S. drafted the manuscript. All authors read and approved the final manuscript.

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