Involvement of Phosphatidylinositol 3-Kinase-Mediated Up-Regulation of IκBα in Anti-Inflammatory Effect of Gemfibrozil in Microglia

Malabendu Jana, Arundhati Jana, Xiaojuan Liu, Sankar Ghosh and Kalipada Pahan

*J Immunol* 2007; 179:4142-4152; doi: 10.4049/jimmunol.179.6.4142
http://www.jimmunol.org/content/179/6/4142

References

This article cites 42 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/179/6/4142.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Involvement of Phosphatidylinositol 3-Kinase-Mediated Up-Regulation of IκBα in Anti-Inflammatory Effect of Gemfibrozil in Microglia

Malabendu Jana,* Arundhati Jana,* Xiaojuan Liu,† Sankar Ghosh,‡ and Kalipada Pahan2*†

The present study underlines the importance of PI3K in mediating the anti-inflammatory effect of gemfibrozil, a prescribed lipid-lowering drug for humans, in mouse microglia. Gemfibrozil inhibited LPS-induced expression of inducible NO synthase (iNOS) and proinflammatory cytokines in mouse BV-2 microglial cells and primary microglia. By overexpressing wild-type and dominant-negative constructs of peroxisome proliferator-activated receptor-α (PPAR-α) in microglial cells and isolating primary microglia from PPAR-α−/− mice, we have demonstrated that gemfibrozil inhibits the activation of microglia independent of PPAR-α. Interestingly, gemfibrozil induced the activation of p85α-associated PI3K (p110β but not p110α) and inhibition of that PI3K by either chemical inhibitors or dominant-negative mutants abrogated the inhibitory effect of gemfibrozil. Conversely, overexpression of the constitutively active mutant of p110 enhanced the inhibitory effect of gemfibrozil on LPS-induced expression of proinflammatory molecules. Similarly, gemfibrozil also inhibited fibrillar amyloid β (Aβ),, prion peptide (PrP)-, dsRNA (poly IC)-, HIV-1 Tat-, and 1-methyl-4-phenylpyridinium (MPP⁺)-, but not IFN-γ-, induced microglial expression of iNOS. Inhibition of PI3K also abolished the inhibitory effect of gemfibrozil on Aβ-, PrP-, poly IC-, Tat-, and MPP⁺-induced microglial expression of iNOS. Involvement of NF-κB activation in LPS-, Aβ-, PrP-, poly IC-, Tat-, and MPP⁺-, but not IFN-γ-, induced microglial expression of iNOS and stimulation of IκBα expression and inhibition of NF-κB activation by gemfibrozil via the PI3K pathway suggests that gemfibrozil inhibits the activation of NF-κB and the expression of proinflammatory molecules in microglia via PI3K-mediated up-regulation of IκBα. The Journal of Immunology, 2007, 179: 4142–4152.

Microglia are considered as CNS-resident professional macrophages and sensor cells that function as the principal immune effector cells of the CNS responding to any pathological event. Activation of microglia has been implicated in the pathogenesis of a variety of neurodegenerative diseases, including Alzheimer’s disease (AD),1 Parkinson’s disease, Creutzfeld-Jacob disease, HIV-associated dementia (HAD), stroke, and multiple sclerosis (MS) (1). It has been found that activated microglia accumulate at sites of injury or plaques in neurodegenerative CNS (1–5). Although activated microglia scavenge dead cells from the CNS and secrete different neurotrophic factors for neuronal survival, it is believed that severe activation causes various autoimmune responses leading to neuronal death and brain injury (1–5). During activation, microglia express various genes related to inflammation, such as proinflammatory cytokines, proinflammatory enzymes, and proinflammatory adhesion molecules (1). Excessive production of these neurotoxic proinflammatory molecules plays an important role in enhancing the degenerative process in the inflamed CNS.

Peroxisome proliferator-activated receptors (PPARs), members of the nuclear hormone receptor superfamily, have been implicated in a variety of human diseases (6). Activation of PPAR-α mainly leads to the induction of a variety of genes such as those coding for the enzymes for β- and ω-oxidation of fatty acids (7). Gemfibrozil, an activator of PPAR-α, has been often prescribed to patients to lower the level of triglycerides (8, 9). This drug decreases the risk of coronary heart disease by increasing the level of high-density lipoprotein cholesterol and decreasing the level of low-density lipoprotein cholesterol (8, 9). In our previous studies (10), we have shown that gemfibrozil markedly inhibits the expression of inducible NO synthase (iNOS) and the production of NO in human astrocytes independent of PPAR-α. The aim of this study was to determine the effect of gemfibrozil on the expression of iNOS and proinflammatory cytokines in microglia and to find out its mode of action. In this study, we demonstrate that gemfibrozil also inhibited the expression of proinflammatory molecules in mouse microglia independent of PPAR-α. Interestingly, gemfibrozil induced the activation of PI3K in microglia and this is the first demonstration of activation of PI3K by any PPAR agonist in any cell type. Interestingly, inhibition of PI3K deleted the anti-inflammatory response.

1Department of Neurological Sciences, Rush University Medical Center, Chicago, IL 60612; 2Section of Neuroscience, Department of Oral Biology, College of Dentistry, University of Nebraska Medical Center, Lincoln, NE 68583; and 3Section of Immunobiology and Department of Molecular Biophysics and Biochemistry, School of Medicine, Yale University, New Haven, CT 06536

Received for publication August 25, 2006. Accepted for publication July 5, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grants from the National Multiple Sclerosis Society (RG3422A1/1) and the National Institutes of Health (NS39940 and NS48923).
2 Address correspondence and reprint requests to Dr. Kalipada Pahan, Department of Neurological Sciences, Rush University Medical Center, 1735 West Harrison Street, Suite 320, Chicago, IL 60612. E-mail address: Kalipada.Pahan@rush.edu
3 Abbreviations used in this paper: AD, Alzheimer’s disease; HAD, HIV-associated dementia; MS, multiple sclerosis; PPAR, peroxisome proliferator-activated receptor; iNOS, inducible NO synthase; ChIP, chromatin immunoprecipitation; SH2, Src homology 2; Aβ, amyloid β; PrP, prion peptide; MPP, 1-methyl-4-phenylpyridinium; SRC, superrepressor.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00

www.jimmunol.org
effect of gemfibrozil while stimulation of PI3K enhanced the same effect establishing a novel role of PI3K in mediating the anti-inflammatory function of gemfibrozil. We also demonstrate that gemfibrozil induced the expression of anti-inflammatory molecule IxBo in microglia through the PI3K pathway.

Materials and Methods

Reagents

FBS and DMEM/F-12 were obtained from Invitrogen Life Technologies. LPS (Escherichia coli), gemfibrozil, and fenobrate were obtained from Sigma-Aldrich. WY-14643 was purchased from Biomol. Wortmannin, LY294002, and Abs against the regulatory subunit of PI3K (p110α and p110β) and phosphatidylserine dispersed by sonication, and 0.1 mg/ml phosphatidylserine were obtained from Calbiochem. Abs against catalytic subunits of PI3K (p110α and p110β) and chromatin immunoprecipitation (ChIP) grade anti-p65 Abs were obtained from Santa Cruz Biotechnology. (γ-32P)ATP (3000 Ci/mM) was obtained from PerkinElmer. The dominant-negative mutant of p85α (Δp85α), the constitutively active mutant of p110α/p110β (p110α* and p110β*), and the kinase-dead mutant of p110α/p110β (p110-κd) were provided by Dr. J. R. Raymond (Medical University of South Carolina, Charleston, SC). The expression construct of PPARG-α and the dominant-negative mutant of PPARG-α (ΔPPARG-α) were provided by Dr. S. M. Fischer (University of Texas MD Anderson Cancer Institute, Houston, TX). The superrepressor (SR) construct of IxBo was provided by S. Ghosh (Yale University, New Haven, CT). PPARG-α−/− mice and littermate controls were purchased from The Jackson Laboratory.

Isolation of mouse primary microglia

Microglial cells were isolated from mixed glial cultures according to the procedure of Guilain and Baker (11). Animal maintenance and experimental protocols were approved by the Rush University Animal Care Committee. Briefly, mixed glial cells were prepared from 7- to 9-day-old mouse pups. On day 9, the mixed glial cultures were washed three times with DMEM/F-12 and subjected to a shake at 240 rpm for 2 h at 37°C on a rotary shaker. The floating cells were washed and seeded onto tissue culture flasks and incubated at 37°C for 1 h. The attached cells were removed by trypsinization and seeded on to new plates for further studies. To monitor purity, cells were immunostained with Abs (BD Pharmingen) against Mac-1 surface Ag, a marker for microglia/macrophages. Ninety to 95% of this preparation was found to be positive for Mac-1. For the induction of proinflammatory molecule production, cells were stimulated with LPS (Escherichia coli) oncDNA equivalent to 50 ng of DNase-digested RNA in a 96-well plate, containing 12.5 l of TaqMan Universal Master mix, 1 l of primer and optimized concentrations of FAM-labeled probe, forward and reverse primers following manufacturer's protocol. All primers and FAM-labeled probes for mouse iNOS, cytokines, and GAPDH were synthesized by guest on October 23, 2017 http://www.jimmunol.org/ Downloaded from

Assay of p85- and p110-associated PI3K

After stimulation, cells were lysed with ice-cold lysis buffer containing 1% v/v Nonidet P-40, 100 mM NaCl, 20 mM Tris (pH 7.4), 10 mM sodium-4-ethylamidine, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A. Lysates were incubated at 4°C for 15 min followed by centrifugation at 13,000 × g for 15 min. The supernatant was precipitated with protein G-Sepharose beads (Bio-Rad) for 1 h at 4°C followed by the addition of 1 μg/ml ps50 mAb. After a 2-h incubation at 4°C, protein G-Sepharose beads were added, and the reaction mixture was further incubated for 1 h at 4°C. The immunoprecipitates were washed twice with lysis buffer, once with PBS, once with 0.5 M LiCl and 100 mM Tris (pH 7.6), once in water, and once in kinase buffer (5 mM MgCl2, 0.25 mM EDTA, and 20 mM HEPES (pH 7.4)). PI3K activity was determined as described earlier (18, 19) using a lipid mixture of 100 μM of 0.1 mg/ml phosphatidylserine and 0.1 mg/ml phosphatidylserine dispersed by sonication in 20 mM HEPES (pH 7.0) and 1 mM EDTA. The reaction was initiated by the addition of 20 μCi of [γ-32P]ATP (3,000 Ci/mM; NEN) and 100 μM ATP and terminated after 15 min by the addition of 80 μl of 1 N HCl and 200 μl of chloroform:methanol (1:1). Phospholipids were separated by thin-layer chromatography and visualized by exposure to iodine vapor and autoradiography (18, 19). Similarly to monitor p110α- and p110β-associated PI3K activity, supernatants were immunoprecipitated with Abs against p110α and p110β followed by the immunocomplex lipid kinase assay as described above.

Expression of different mutant constructs of PI3K

Class IA PI3K consists of a catalytic subunit (p110) of 110 kDa and a regulatory subunit (p85) of 85 kDa. In the dominant-negative form of p85α, 35 aa in the inter-Src homology 2 (SH2) region from residues 479–513 of wild-type p85α, important for binding the p110α/p110β subunit of PI3K, are deleted, and two other amino acids (Ser-Arg) are inserted in this deleted position. The engineering of the construct and description of the vector driving the expression of the proteins have been published previously (20). In contrast, in the constitutively active mutant of p110α/p110β (p110α*), the inter-SH2 domain of p85 is ligated to the NH2 terminus of p110 whereas in the kinase-deficient mutant of p110α/p110β (p110-κd), the ATP-binding site is mutated (21). Cells plated in 12-well plates were transfected with 0.2–0.25 μg of different plasmids using Lipofectamine Plus (Invitrogen Life Technologies) using the manufacturer’s protocol as described previously (12, 13).

Semiquantitative RT-PCR analysis

The expression of different proinflammatory molecules was analyzed by semiquantitative RT-PCR using a RT-PCR kit from BD Clontech as described earlier (16, 22). Briefly, total RNA was isolated from stimulated or unstimulated cells by using the Qiagen mini kit followed by digestion with DNase to remove contaminating genomic DNA. Briefly, 1 μg of DNase-digested RNA was reverse transcribed using oligo(dT)12–18 as primer and MMLV reverse transcriptase (BD Clontech) in a 20-μl reaction mixture. The resulting cDNA was appropriately diluted, and diluted cDNA was amplified using Titanium TaqDNA polymerase and the following primers. The following primers were used to amplify mouse proinflammatory molecule IκBα (497 bp): sense: 5′-GCC TTC CGA AGG TTT GAC CAG CAC C-3′, antisense: 5′-GGC TGT CAG AGC ATG GCC GAG TAC TAC CAC C-3′; IL-1β (563 bp): sense: 5′-ATG GCA ACT GCT CCT GAA ACT AAC T-3′, antisense: 5′-CAG CAG GAC AGG TAT AGA TTA TCT CCT TTG-3′; p85α (354 bp): sense: 5′-TTC TGT CCT CTA CTG AAC TCG GGG GTG ATC GGT CCC-3′, antisense: 5′-GTA GAT GAC AGA ACC TCG GAC GGT GTG GG-3′; IL-6 (155 bp): sense: 5′-TGG AGG CAG AGG AGG TGC TTA GAC GAC GCC-3′. All primers were synthesized by guest on October 23, 2017 http://www.jimmunol.org/ Downloaded from

Real-time PCR analysis

Real-time PCR analysis was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems) as described earlier (16, 22). Briefly, it was performed in a 96-well optical reaction plate (Applied Biosystems) on cDNA equivalent to 50 ng of DNase-digested RNA in a volume of 25 μl, containing 12.5 μl of TaqMan Universal Master mix and optimized concentrations of FAM-labeled probe, forward and reverse primers following manufacturer’s protocol. All primers and FAM-labeled probes for mouse iNOS, cytokines, and GAPDH were measured after scanning the bands with a Fluor Chem 8800 Imaging System (Alpha Innotech).

ChIP assay

ChIP assays were performed using a kit (Upstate Biotechnology) according to the manufacturer’s protocol. Briefly, 2 × 106 microglial cells preincubated with 5% FBS, 10 μg/ml gemfibrozil or 50 μg/ml LPS were stimulated with LPS. After 3 h of stimulation, cells were fixed by formaldehyde (1% final concentration), and cross-linked adducts were resuspended and sonicated, resulting in an average chromatin fragment size of 400 bp. ChIP was
performed on the cell lysate by overnight incubation at 4°C with 2 μg of Abs against p65 followed by incubation with protein G-agarose (Santa Cruz Biotechnology) for 2 h. The beads were washed and incubated with elution buffer. To reverse the cross-linking and purify the DNA, precipitates were incubated in a 65°C incubator overnight and digested with proteinase K. DNA samples were then purified, precipitated, and precipitates were washed with 75% ethanol, air-dried, and resuspended in Tris-EDTA buffer. The following primers were used to amplify fragments flanking proximal NF-κB elements in the mouse iNOS promoter; sense: 5'-CAT GAG GAT ACA CCA CAG AG-3', antisense: 5'-AAG ACC TGA GGG TTT TC-3'.

The following primers were used to amplify fragments flanking distal NF-κB elements in the mouseiNOS promoter; sense: 5'-'ACC CGG CAT TTT CCC TCT CTC-3'); antisense: 5'-ACC CGG TAT TTT TCT TAT-3'; sense: 5'-CAT GCT CCA GAG GAT ACA A-3'; antisense: 5'-CGA GGG TCA GAT CTA GGG TTT TCT C-3'; sense: 5'-CAT GAG GAT ACA CCA CAG AG-3'; antisense: 5'-AAG ACC TGA GGG TTT TC-3'.

The PCRs were repeated by using varying cycle numbers and different amounts of templates to ensure that results were in the linear range of PCR.

**Assay of transcriptional activity of NF-κB**

Cells plated at 50–60% confluence in 12-well plates were cotransfected with 0.25 μg of pNF-κB-Luc (NF-κB-dependent reporter construct) and 12.5 ng of pRL-TK (a plasmid encoding Renilla luciferase, used as transfection efficiency control; Promega) using Lipofectamine Plus (Invitrogen Life Technologies). After 24 h of transfection, cells were stimulated with different stimuli for 6 h. Firefly and Renilla luciferase activities were analyzed in cell extracts using the Dual Luciferase kit (Promega) in a TD-20/20 Luminometer (Turner Designs) as described earlier (12, 13). Relative luciferase activity of cell extracts was typically represented as the ratio of firefly luciferase value:Renilla luciferase value × 10^{-3}.

**Cell viability measurement**

Mitochondrial activity was measured with the MTT assay (Sigma-Aldrich).

**Statistics**

Statistical comparisons were made using one-way ANOVA followed by the Student t test.

**Results**

**Gemfibrozil inhibits the expression of iNOS and proinflammatory cytokines in LPS-stimulated mouse BV-2 microglial cells**

Cells were cultured in serum-free medium in the presence LPS. It is evident from Table I that LPS alone markedly induced the production of NO and proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in BV-2 microglial cells. Next, we examined the effect of gemfibrozil, an activator of PPAR-α (23), on LPS-induced production of proinflammatory molecules. Gemfibrozil itself was neither stimulatory nor much inhibitory to NO and cytokine production in control cells. However, gemfibrozil, when added 2 h before the addition of LPS markedly inhibited LPS-induced production of NO, TNF-α, IL-1β, and IL-6 (Table I).

**Table I. Gemfibrozil inhibits the induction of NO, TNF-α, IL-1β, and IL-6 production in mouse BV-2 microglial cells**

<table>
<thead>
<tr>
<th>Proinflammatory Molecules</th>
<th>Control</th>
<th>LPS</th>
<th>LPS plus Gem (200 μM)</th>
<th>LPS plus Gem (300 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite (nM/mg/24 h)</td>
<td>3.7 ± 0.4</td>
<td>91.3 ± 10.7</td>
<td>51.7 ± 6.5^{a} (44)</td>
<td>38.5 ± 3.6^{a} (58)</td>
</tr>
<tr>
<td>TNF-α (ng/mg/24 h)</td>
<td>0</td>
<td>32 ± 4.6</td>
<td>15.5 ± 1.8^{a} (52)</td>
<td>11.6 ± 1.5^{a} (64)</td>
</tr>
<tr>
<td>IL-1β (ng/mg/24 h)</td>
<td>0</td>
<td>25.6 ± 3.2</td>
<td>12.2 ± 1.9^{a} (53)</td>
<td>8.2 ± 1.3^{a} (68)</td>
</tr>
<tr>
<td>IL-6 (ng/mg/24 h)</td>
<td>0</td>
<td>87.3 ± 11.2</td>
<td>50.7 ± 6.9^{a} (42)</td>
<td>39.8 ± 5.3^{a} (55)</td>
</tr>
</tbody>
</table>

^{a} Cells preincubated with different concentrations of gemfibrozil (Gem) for 2 h were stimulated with LPS (1 μg/ml) in serum-free DMEM/F-12. After 24 h of incubation, concentrations of nitrite, TNF-α, IL-1β, and IL-6 were assayed in supernatants as described in Materials and Methods. Data are expressed as the mean ± SD of three different experiments.

**Table II. Wy-14643 and fenofibrate inhibit the induction of NO production in mouse BV-2 microglial cells**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Nitrite (nM/mg Protein/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>LPS</td>
<td>91.3 ± 10.7</td>
</tr>
<tr>
<td>LPS plus Wy-14643 (200 μM)</td>
<td>40.5 ± 5.2^{a} (56)</td>
</tr>
<tr>
<td>LPS plus Wy-14643 (300 μM)</td>
<td>18.2 ± 1.6^{a} (80)</td>
</tr>
<tr>
<td>LPS plus fenofibrate (200 μM)</td>
<td>49.8 ± 4.6^{a} (44)</td>
</tr>
<tr>
<td>LPS plus fenofibrate (300 μM)</td>
<td>33.2 ± 4.5^{a} (64)</td>
</tr>
</tbody>
</table>

^{a} Cells preincubated with different concentrations of WY-14643 and fenofibrate for 2 h were stimulated with LPS (1 μg/ml) in serum-free DMEM/F-12. After 24 h of incubation, concentrations of nitrite were assayed in supernatants. Data are expressed as the mean ± SD of three different experiments.

**Conclusion**

Despite the inhibition of the expression of proinflammatory molecules (data not shown), significant inhibition was observed at higher concentrations (200 and 300 μM) of gemfibrozil (Table I). Similar to gemfibrozil, other PPAR-α agonists, such as WY-14643 and fenofibrate, also suppressed the production of nitrite in LPS-stimulated cells at 200 and 300 μM concentrations (Table II). To understand the mechanism of inhibition, we examined the effect of gemfibrozil on mRNA levels of iNOS and proinflammatory cytokines in LPS-stimulated cells. Gemfibrozil dose-dependently inhibited LPS-induced expression of iNOS (49% inhibition at 200 μM gemfibrozil), TNF-α (52% inhibition at 200 μM gemfibrozil), IL-1β (92% inhibition at 200 μM gemfibrozil), and IL-6 (90% inhibition at 200 μM gemfibrozil) mRNAs in BV-2 microglial cells (data not shown). Similarly, at 200 μM concentration, Wy-14643 and fenofibrate also attenuated the expression of iNOS and cytokines in LPS-stimulated microglial cells (see Fig. 3, C and D). To examine whether gemfibrozil affected cell viability, microglial cells were incubated with different concentrations (100, 200, and 300 μM) of gemfibrozil under serum-free condition as mentioned above and their viability was determined by the MTT assay. Gemfibrozil at different concentrations used did not decrease the viability of the cells (data not shown). Therefore, inhibition of the expression of proinflammatory molecules by gemfibrozil was not due to any change in viability of the cells.
Role of PPAR-α in gemfibrozil-mediated inhibition of proinflammatory molecules in LPS-stimulated primary microglia

Because gemfibrozil is a known activator of PPAR-α (6, 23, 24), a member of the nuclear hormone receptor superfamily, we examined whether gemfibrozil inhibited the expression of proinflammatory molecules through the activation of PPAR-α. Therefore, primary microglia were isolated from wild-type and PPAR-α−/− mice. As evidenced in Fig. 1, LPS induced the mRNA expression of iNOS, TNF-α, IL-1β, and IL-6 in both PPAR-α−/− and PPAR-α+/+ microglia. However, gemfibrozil, when added 2 h before the addition of LPS, markedly inhibited LPS-induced expression of proinflammatory molecules in both PPAR-α+/+ and PPAR-α−/− microglia (Fig. 1). Similarly, overexpression of either wild-type PPAR-α or dominant-negative PPAR-α (ΔPPAR-α) did not alter the inhibitory effect of gemfibrozil on LPS-induced expression of proinflammatory molecules (iNOS, TNF-α, IL-1β, and IL-6) in mouse BV-2 microglial cells (data not shown). Taken together, these results suggest that PPAR-α is not involved in gemfibrozil-mediated anti-inflammatory effect in microglial cells.

Activation of PI3K by gemfibrozil and abrogation of the anti-inflammatory effect of gemfibrozil by wortmannin

Next, we investigated mechanisms by which gemfibrozil may transduce inhibitory signals for the expression of proinflammatory molecules in microglia. PI3K, a dual protein and lipid kinase, transduces signals for multiple biological processes (25, 26). Class IA PI3K, which is regulated by receptor tyrosine kinases, consists of a heterodimer of a regulatory 85-kDa subunit and a catalytic 110-kDa subunit (p85α/p110). In contrast, class IB PI3K
FIGURE 3. Inhibitors of PI3K abrogate the suppressive effect of gemfibrozil and other PPAR-α agonists on the expression of proinflammatory molecules in LPS-stimulated BV-2 microglial cells. Cells preincubated with either 200 nM wortmannin (A) or different concentrations of LY294002 (LY) (B) for 30 min were treated with 200 μM gemfibrozil under serum-free condition. After 2 h of treatment, cells were stimulated with 1 μg/ml LPS. After 6 h of stimulation, total RNA was isolated and semiquantitative RT-PCR analysis was performed. Cells preincubated with different concentrations of wortmannin for 30 min were treated with either 200 μM WY-14643 (C) or 200 μM fenofibrate (D) under serum-free condition. After 2 h of treatment, cells were stimulated with 1 μg/ml LPS. After 6 h of stimulation, semiquantitative RT-PCR analysis was performed. The relative expression of cytokines or iNOS was measured after scanning the bands (lower panels). Results represent mean ± SD of three separate experiments. Values of p: a, p < 0.001 vs LPS + Gem for A; b, p < 0.001 vs LPS + Gem for B; c, p < 0.001 vs LPS + WY14643 for C; d, p < 0.001 vs LPS + Fenofibrate for D.
exert their anti-inflammatory effect in microglial cells via the PI3K pathway.

Next, we investigated whether gemfibrozil required PI3K to inhibit the expression of proinflammatory molecules in primary microglia as well. Similar to BV-2 microglial cells, wortmannin was also capable of abrogating the inhibitory effect of gemfibrozil on the expression of iNOS, TNF-α, and IL-6 mRNAs in LPS-stimulated mouse primary microglia (Fig. 4), suggesting the requirement of PI3K in the anti-inflammatory effect of gemfibrozil in primary microglia.

Inhibition of fibrillar amyloid β (Aβ), prion peptide (PrP), dsRNA (poly IC), HIV-1 Tat, and 1-methyl-4-phenylpyridinium (MPP⁺)-induced expression of iNOS in microglial cells by gemfibrozil via the PI3K pathway

Activated microglia are considered to play an important role in various pathological conditions associated with viral encephalopathy, AD, Parkinson’s disease, HAD, Creutzfeldt-Jakob disease, etc. Because gemfibrozil inhibited LPS-induced expression of proinflammatory molecules in microglia, we were prompted to investigate whether gemfibrozil was also capable of negating the expression of iNOS in microglial cells stimulated with etiological reagents of various neurological disorders. BV-2 microglial cells challenged with fibrillar Aβ peptides (etiological reagent for AD), fibrillar PrP peptides (etiological reagent for prion diseases),
Expression of Δp85α (a dominant-negative mutant of p85α) blocks gemfibrozil-mediated inhibitory effect on the expression of proinflammatory molecules in LPS-stimulated mouse BV-2 microglial cells. Cells were transfected with 0.25 μg of either Δp85α or an empty vector. Twenty-four hours after transfection, cells were incubated with gemfibrozil for 2 h followed by stimulation with LPS. After 6 h of stimulation, semiquantitative RT-PCR (A) was performed. The relative expression of cytokines or iNOS was measured after scanning the bands (B). Results represent mean ± SD of three separate experiments. Values of p: a, p < 0.001 vs vector-LPS-gemfibrozil.

FIGURE 6. Expression of Δp85α (a dominant-negative mutant of p85α) blocks gemfibrozil-mediated inhibitory effect on the expression of proinflammatory molecules in LPS-stimulated mouse BV-2 microglial cells. Cells were transfected with 0.25 μg of either Δp85α or an empty vector. Twenty-four hours after transfection, cells were incubated with gemfibrozil for 2 h followed by stimulation with LPS. After 6 h of stimulation, semiquantitative RT-PCR (A) was performed. The relative expression of cytokines or iNOS was measured after scanning the bands (B). Results represent mean ± SD of three separate experiments. Values of p: a, p < 0.001 vs vector-LPS-gemfibrozil.

Effect of p110α (a catalytically active mutant of p110α) and p110-kd (a kinase-dead mutant of p110α) on the anti-inflammatory effect of gemfibrozil in microglial cells

Because the expression of Δp85α blocked the anti-inflammatory effect of gemfibrozil (Fig. 6) and p85α dimerizes with either p110α or p110β, to confirm this finding further by a different approach, we examined the effect of p110α or p110β on the anti-inflammatory effect of gemfibrozil in microglial cells. It has been reported that expression of p110α but not that of p110-kd is sufficient to promote Glut 4 translocation in adipocytes (21). Therefore, to increase the activity of p110α or p110β, mouse microglial cells were transfected with p110α. Earlier, we have demonstrated that expression of p85α-associated lipid kinase activity of PI3K in C6 glial cells by the same dominant-negative mutant of p85α indicating that the overexpressed dominant-negative mutant protein of p85α did not associate with the catalytic subunit of PI3K. As evidenced by semiquantitative RT-PCR analysis in Fig. 6, LPS induced the expression of proinflammatory molecules (iNOS, TNF-α, and IL-6) in empty vector- as well as Δp85α-transfected BV-2 microglial cells. However, gemfibrozil inhibited the mRNA expression of these proinflammatory molecules in empty vector- but not Δp85α-transfected microglial cells (Fig. 6) suggesting again that gemfibrozil exhibits its anti-inflammatory effect in microglia via p85α-associated PI3K.

Effect of p110α (a catalytically active mutant of p110α) and p110-kd (a kinase-dead mutant of p110α) on the anti-inflammatory effect of gemfibrozil in microglial cells

Because the expression of Δp85α blocked the anti-inflammatory effect of gemfibrozil (Fig. 6) and p85α dimerizes with either p110α or p110β, to confirm this finding further by a different approach, we examined the effect of p110α or p110β on the anti-inflammatory effect of gemfibrozil in microglial cells. It has been reported that expression of p110α but not that of p110-kd is sufficient to promote Glut 4 translocation in adipocytes (21). Therefore, to increase the activity of p110α or p110β, mouse microglial cells were transfected with p110α. Earlier, we have demonstrated that expression of p85α-associated lipid kinase activity of PI3K in C6 glial cells by the same dominant-negative mutant of p85α indicating that the overexpressed dominant-negative mutant protein of p85α did not associate with the catalytic subunit of PI3K. As evidenced by semiquantitative RT-PCR analysis in Fig. 6, LPS induced the expression of proinflammatory molecules (iNOS, TNF-α, and IL-6) in empty vector- as well as Δp85α-transfected BV-2 microglial cells. However, gemfibrozil inhibited the mRNA expression of these proinflammatory molecules in empty vector- but not Δp85α-transfected microglial cells (Fig. 6) suggesting again that gemfibrozil exhibits its anti-inflammatory effect in microglia via p85α-associated PI3K.

Effect of p110α (a catalytically active mutant of p110α) and p110-kd (a kinase-dead mutant of p110α) on the anti-inflammatory effect of gemfibrozil in microglial cells

Because the expression of Δp85α blocked the anti-inflammatory effect of gemfibrozil (Fig. 6) and p85α dimerizes with either p110α or p110β, to confirm this finding further by a different approach, we examined the effect of p110α or p110β on the anti-inflammatory effect of gemfibrozil in microglial cells. It has been reported that expression of p110α but not that of p110-kd is sufficient to promote Glut 4 translocation in adipocytes (21). Therefore, to increase the activity of p110α or p110β, mouse microglial cells were transfected with p110α. Earlier, we have demonstrated that expression of p85α-associated lipid kinase activity of PI3K in C6 glial cells by the same dominant-negative mutant of p85α indicating that the overexpressed dominant-negative mutant protein of p85α did not associate with the catalytic subunit of PI3K. As evidenced by semiquantitative RT-PCR analysis in Fig. 6, LPS induced the expression of proinflammatory molecules (iNOS, TNF-α, and IL-6) in empty vector- as well as Δp85α-transfected BV-2 microglial cells. However, gemfibrozil inhibited the mRNA expression of these proinflammatory molecules in empty vector- but not Δp85α-transfected microglial cells (Fig. 6) suggesting again that gemfibrozil exhibits its anti-inflammatory effect in microglia via p85α-associated PI3K.

Effect of p110α (a catalytically active mutant of p110α) and p110-kd (a kinase-dead mutant of p110α) on the anti-inflammatory effect of gemfibrozil in microglial cells

Because the expression of Δp85α blocked the anti-inflammatory effect of gemfibrozil (Fig. 6) and p85α dimerizes with either p110α or p110β, to confirm this finding further by a different approach, we examined the effect of p110α or p110β on the anti-inflammatory effect of gemfibrozil in microglial cells. It has been reported that expression of p110α but not that of p110-kd is sufficient to promote Glut 4 translocation in adipocytes (21). Therefore, to increase the activity of p110α or p110β, mouse microglial cells were transfected with p110α. Earlier, we have demonstrated that expression of p85α-associated lipid kinase activity of PI3K in C6 glial cells by the same dominant-negative mutant of p85α indicating that the overexpressed dominant-negative mutant protein of p85α did not associate with the catalytic subunit of PI3K. As evidenced by semiquantitative RT-PCR analysis in Fig. 6, LPS induced the expression of proinflammatory molecules (iNOS, TNF-α, and IL-6) in empty vector- as well as Δp85α-transfected BV-2 microglial cells. However, gemfibrozil inhibited the mRNA expression of these proinflammatory molecules in empty vector- but not Δp85α-transfected microglial cells (Fig. 6) suggesting again that gemfibrozil exhibits its anti-inflammatory effect in microglia via p85α-associated PI3K.

Effect of p110α (a catalytically active mutant of p110α) and p110-kd (a kinase-dead mutant of p110α) on the anti-inflammatory effect of gemfibrozil in microglial cells

Because the expression of Δp85α blocked the anti-inflammatory effect of gemfibrozil (Fig. 6) and p85α dimerizes with either p110α or p110β, to confirm this finding further by a different approach, we examined the effect of p110α or p110β on the anti-inflammatory effect of gemfibrozil in microglial cells. It has been reported that expression of p110α but not that of p110-kd is sufficient to promote Glut 4 translocation in adipocytes (21). Therefore, to increase the activity of p110α or p110β, mouse microglial cells were transfected with p110α. Earlier, we have demonstrated that expression of p85α-associated lipid kinase activity of PI3K in C6 glial cells by the same dominant-negative mutant of p85α indicating that the overexpressed dominant-negative mutant protein of p85α did not associate with the catalytic subunit of PI3K. As evidenced by semiquantitative RT-PCR analysis in Fig. 6, LPS induced the expression of proinflammatory molecules (iNOS, TNF-α, and IL-6) in empty vector- as well as Δp85α-transfected BV-2 microglial cells. However, gemfibrozil inhibited the mRNA expression of these proinflammatory molecules in empty vector- but not Δp85α-transfected microglial cells (Fig. 6) suggesting again that gemfibrozil exhibits its anti-inflammatory effect in microglia via p85α-associated PI3K.

Effect of p110α (a catalytically active mutant of p110α) and p110-kd (a kinase-dead mutant of p110α) on the anti-inflammatory effect of gemfibrozil in microglial cells

Because the expression of Δp85α blocked the anti-inflammatory effect of gemfibrozil (Fig. 6) and p85α dimerizes with either p110α or p110β, to confirm this finding further by a different approach, we examined the effect of p110α or p110β on the anti-inflammatory effect of gemfibrozil in microglial cells. It has been reported that expression of p110α but not that of p110-kd is sufficient to promote Glut 4 translocation in adipocytes (21). Therefore, to increase the activity of p110α or p110β, mouse microglial cells were transfected with p110α. Earlier, we have demonstrated that expression of p85α-associated lipid kinase activity of PI3K in C6 glial cells by the same dominant-negative mutant of p85α indicating that the overexpressed dominant-negative mutant protein of p85α did not associate with the catalytic subunit of PI3K. As evidenced by semiquantitative RT-PCR analysis in Fig. 6, LPS induced the expression of proinflammatory molecules (iNOS, TNF-α, and IL-6) in empty vector- as well as Δp85α-transfected BV-2 microglial cells. However, gemfibrozil inhibited the mRNA expression of these proinflammatory molecules in empty vector- but not Δp85α-transfected microglial cells (Fig. 6) suggesting again that gemfibrozil exhibits its anti-inflammatory effect in microglia via p85α-associated PI3K.
with empty vector-transfected cells. In contrast, expression of p110-kd blocked this anti-inflammatory effect of gemfibrozil in LPS-stimulated microglial cells (Fig. 7), clearly delineating an essential role of p110B in gemfibrozil-mediated inhibition on proinflammatory molecules.

Inhibition of NF-κB activity in microglial cells by gemfibrozil

Next, we investigated mechanisms by which activation of p110β PI3K may limit the expression of proinflammatory molecules in microglia. Because the activation of NF-κB is necessary for the transcription of proinflammatory molecules (12, 13, 30–32), we investigated the role of NF-κB activation in the expression of iNOS. Mouse iNOS promoter harbors two NF-κB-binding sites: distal (nucleotides −971 to −962) and proximal (nucleotides −85 to −76) (32). At first, we used ChIP analysis to study the recruitment of RelA p65 to each of these two NF-κB-binding sites. After immunoprecipitation of LPS-stimulated microglial chromatin fragments by Abs against p65, we were able to amplify 307-bp fragments flanking the proximal NF-κB element (Fig. 8A). However, after several attempts, we failed to detect any amplification product spanning the distal NF-κB-binding site (data not shown). These results suggest that LPS induces the recruitment of p65 to the proximal NF-κB-binding site of the mouse iNOS promoter. Therefore, next we examined the effect of gemfibrozil on the recruitment of p65 to the proximal NF-κB-binding site of the iNOS promoter. Consistent to the inhibition of iNOS mRNA expression, gemfibrozil inhibited the recruitment of p65 to the iNOS promoter in LPS-stimulated microglia (Fig. 8A). In contrast, no amplification product was observed in any of the immunoprecipitates obtained with control IgG (left three lanes of Fig. 8A) suggesting the specificity of these interactions. These results also suggest that gemfibrozil interferes with the recruitment of NF-κB to the iNOS promoter.

Next, we examined whether NF-κB was also involved in Aβ, PrP, poly IC-, Tat-, MPP⁺, IL-1β, and IFN-γ-induced microglial iNOS mRNA expression. Similar to LPS, other stimuli (Aβ, PrP, poly IC, Tat, IL-1β, and MPP⁺) markedly induced the transcriptional activity of NF-κB as evident from reporter luciferase activity (Fig. 8C). In contrast, IFN-γ was unable to induce the activation of NF-κB (Fig. 8C). We used the SR construct of 1xBα to inhibit the function of NF-κB. Expectedly, the SR 1xBα markedly blocked LPS-, Aβ-, PrP-, poly IC-, Tat-, IL-1β-, and MPP⁺-induced activation of NF-κB (data not shown). Therefore, by using SR 1xBα, we investigated whether NF-κB activation was required for Aβ-, PrP-, poly IC-, Tat-, IL-1β-, and MPP⁺-induced microglial expression of iNOS. It is clear from Fig. 8B that SR 1xBα but not an empty vector was capable of suppressing LPS-, Aβ-, PrP-, poly IC-, Tat-, IL-1β-, and MPP⁺-induced expression of iNOS mRNA. In contrast, SR 1xBα had no effect on IFN-γ-induced expression of iNOS mRNA (Fig. 8B).

Because gemfibrozil inhibited the expression of iNOS and the recruitment of NF-κB to the iNOS promoter in activated microglia, we investigated whether gemfibrozil was capable of attenuating the activation of NF-κB in microglia as well. As evident from Fig. 8C, gemfibrozil attenuated LPS-, Aβ-, PrP-, poly IC-, Tat-, IL-1β-, and MPP⁺-induced NF-κB-dependent luciferase activity suggesting an inhibitory effect of gemfibrozil on the activation of NF-κB.
FIGURE 9. Role of PI3K in gemfibrozil-mediated increased expression of IκBα in BV-2 microglial cells. A. Cells were treated with 200 μM gemfibrozil under serum-free condition. At different minutes of treatment, the mRNA expression of IκBα was analyzed by semiquantitative RT-PCR. B. Cytoplasmic extracts were immunoblotted with Abs against IκBα and actin. C. Cells were treated with different concentrations of gemfibrozil under serum-free condition. After 30 min of treatment, total RNA was isolated and the mRNA expression of IκBα was analyzed by semiquantitative RT-PCR. Cells preincubated with different concentrations of either wortmannin (D) or LY294002 (E) for 30 min were treated with 200 μM gemfibrozil under serum-free condition. After 30 min of treatment, semiquantitative RT-PCR analysis was performed. F. Cells were transfected with 0.25 μg of either p110* or p110-kd, or an empty vector. Twenty-four hours after transfection, cells were incubated under serum-free condition for 2 h followed by semiquantitative RT-PCR analysis for IκBα. The relative expression of IκBα was measured after scanning the bands (lower panel). Results represent mean ± SD of three separate experiments. Values of p: a, p < 0.001 vs 0 min; b, p < 0.001 vs 0 min; c, p < 0.001 vs control (no gem); d, p < 0.001 vs gem only for D; e, p < 0.001 vs gem only for E; f, p < 0.001 vs empty vector for F.

However, this inhibitory effect of gemfibrozol on neurotoxin-induced NF-κB activation was abrogated by Ap110, a kinase-dead mutant of p110<sub>α</sub>/p110β, and augmented by p110*, a constitutively active mutant of p110<sub>α</sub>/p110β, as compared with empty vector-transfected cells (Fig. 8D). Taken together, these results suggest that all different stimuli of microglial iNOS except IFN-γ induce the expression of iNOS via NF-κB, that gemfibrozil inhibits neurotoxin-induced activation of NF-κB via the PI3K pathway in microglia, and that gemfibrozil is probably unable to interfere with IFN-γ-induced microglial expression of iNOS due to its independency on NF-κB activation.

Gemfibrozol induces the expression of IκBα in microglial cells via a PI3K-sensitive pathway

Because fibrates induce the expression of IκBα capable of arresting the classical NF-κB heterodimer (p65:p50) in the cytoplasm (31), we investigated whether gemfibrozil stimulates the expression of IκBα in microglial cells via a PI3K pathway. As expected, gemfibrozil alone time-dependently induced the expression of IκBα mRNA in BV-2 microglial cells (Fig. 9A). The increase in IκBα mRNA was visible as early as 15 min with the maximum increase found at 30 min of treatment (Fig. 9A). Western blot analysis also shows that gemfibrozil induced the expression of IκBα protein at different minutes of stimulation exhibiting the maximum increase at 90 min (Fig. 9B). Dose-dependent experiment shows that gemfibrozil exhibited maximum increase in IκBα mRNA at a dose of 200 μM or higher (Fig. 9C). However, interestingly, both wortmannin and LY294002 (inhibitors of PI3K) abrogated gemfibrozil-mediated stimulation of IκBα mRNA (Fig. 9D and E) suggesting that gemfibrozil induces/stimulates the expression of IκBα via the PI3K pathway. In addition to activating PI3K, gemfibrozol may activate other signaling pathways as well. Therefore, next we investigated whether the activation of PI3K alone was sufficient to induce the expression of IκBα in microglia. It is evident from Fig. 9F that overexpression of p110<sup>α</sup> (the constitutively active mutant of p110α/p110β), but not the empty vector, was capable of increasing the mRNA expression of IκBα. In contrast, p110-kd (the kinase-deficient mutant) had no effect on the mRNA expression of IκBα. These results clearly suggest that activation of PI3K is sufficient to induce microglial expression of IκBα.

Discussion

Common pathological hallmarks of several neurodegenerative diseases include the loss of invaluable neurons associated with or followed by massive activation of microglia (1, 33, 34). 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-1β, and IL-6) in mouse microglia. Because these proinflammatory molecules have been implicated in the pathogenesis of demyelinating and neurodegenerative diseases (3, 5, 34), our results provide a potentially important mechanism whereby activators of PPAR-α may ameliorate neural injury. However, gemfibrozil inhibited the expression of proinflammatory molecules in primary microglia isolated from wild-type as well as PPAR-α−/− mice. Furthermore, ΔPPAR-α was unable to block the inhibitory effect of gemfibrozil on the induction of proinflammatory molecules in microglial cells. These results suggest that gemfibrozil does not require PPAR-α to display its anti-inflammatory effect in microglia. A recent study by Xu et al. (35) has indicated the possible involvement of PPAR-α in gemfibrozil-mediated inhibition of microglial iNOS. However, this study did not attempt to examine the effect of gemfibrozil in PPAR-α knockout microglia.

Characterization of intracellular pathways that may negatively and positively regulate the expression of proinflammatory molecules in glial cells is an active area of investigation. PI3K is a key signaling molecule implicated in the regulation of a broad array of biological responses including receptor-stimulated mitogenesis, oxidative burst, and cell survival (25, 26). For class IA PI3K, the p85 regulatory subunit acts as an interface by interacting with the insulin receptor substrate-1 through its SH2 domain and thus recruits the p110 catalytic subunit to the cell membrane through its SH2 domain (25, 26). In contrast, for class IB PI3K, the p110 protein is activated by the engagement of G protein-coupled receptors. The p110 then catalyzes the reaction to release phosphatidylinositol (3,4,5)-triphosphate as the second messenger upon activation of the substrate and activates downstream signaling molecules like Akt/protein kinase B and p70 ribosomal S6 kinase (25, 26). Earlier Pahan et al. (18) have shown that inhibition of PI3K by either chemical inhibitors, such as wortmannin and LY294002, or a dominant-negative mutant of the regulatory subunit p85α induces/stimulates the expression of iNOS in LPS- or cytokine-stimulated C6 glial cells and rat primary astrocytes, suggesting that activation of p85α-associated PI3K may transduce an inhibitory signal for the expression of iNOS. Subsequently, we have demonstrated that expression of the catalytically active p110α/p110β subunit inhibits the production of NO and the expression of iNOS in human U373MG astrocytoma cells and primary astrocytes (19). Because these results support the conclusion that p85α-associated PI3K (p110α/p110β) signal transduction pathway is a negative regulator of the expression of iNOS, we were prompted to investigate whether p85α-associated PI3K plays a possible role in anti-inflammatory activity of gemfibrozil in microglia. Several lines of evidence presented in this study clearly support the conclusion that gemfibrozil attenuates the induction of proinflammatory molecules in microglia via PI3K.

Our conclusion is based on the following observations. First, gemfibrozil alone induced the activation of p85α-associated PI3K p110β but not p110α in microglia. Second, wortmannin, an inhibitor of PI3K, abrogated the inhibitory effect of gemfibrozil on the expression of proinflammatory molecules in BV-2 microglial cells and primary microglia. Third, overexpression of Δp85α, a dominant-negative mutant of p85α, also blocked the anti-inflammatory function of gemfibrozil. Fourth, overexpression of p110α, a catalytically active mutant of p110α/p110β, enhanced the inhibitory effect of gemfibrozil on the expression of proinflammatory molecules. In contrast, overexpression of p110-kd, a kinase-dead mutant of p110α/p110β, removed this anti-inflammatory effect of gemfibrozil in microglia. Taken together, these studies delineate an absolute requirement of p85α-associated PI3K for the inhibitory effect of gemfibrozil on microglial expression of proinflammatory molecules. However, we do not know mechanisms by which gemfibrozil induces the activation of p85α-associated p110β PI3K in microglia. In general, p85α-associated PI3K is activated via growth factor receptors. Tyrosine phosphorylation of growth factor receptors creates docking sites for binding of p85α through its SH2 domains. Because gemfibrozil is inducing the activation of PI3K within a minute interval, it may not be surprising if gemfibrozil and other fibrate drugs use any of these growth factor receptors to activate PI3K.

Among all the known proinflammatory transcription factors working in concert to transactivate promoters of proinflammatory genes, NF-κB p50:p65 is literally the most important one. The presence of multiple consensus sequences (κB elements) in the promoter region of proinflammatory molecules for the binding of NF-κB and the inhibition of proinflammatory gene expression in human, rat, and mouse glial cells with the inhibition of NF-κB activation (12, 13, 30, 31) establishes an essential role of NF-κB induction in the activation of proinflammatory molecules. In resting cells, the classical p65:p50 heterodimer is arrested in the cytoplasm as an inactive complex by IκBα (31). It has been demonstrated that newly synthesized IκBα protein accumulates in cytoplasm as well as in nucleus where it reduces NF-κB binding (36). Several studies have reported that ligands of PPAR inhibit the activation of NF-κB by up-regulating the expression of IκBα (37, 38). We have also observed induction of IκBα mRNA and protein in microglial cells by gemfibrozil. In addition, our results demonstrate a novel mechanism by which gemfibrozil induces/stimulates the expression of IκBα in microglia. Abrogation of gemfibrozil-mediated stimulation of IκBα by inhibitors of PI3K (LY294002 and wortmannin) and up-regulation of IκBα by overexpression of a constitutively active mutant of PI3K alone suggest an important role of PI3K in gemfibrozil-mediated increase in IκBα expression. Therefore, it appears that gemfibrozil-mediated activation p110β PI3K is capable of limiting the expression of proinflammatory molecules in microglia via up-regulation of IκBα. This is interesting because activation of p110α/p110β usually does the opposite. It induces/stimulates the expression of proinflammatory molecules through enhanced activation of NF-κB, i.e., through enhanced phosphorylation and rapid degradation of IκBα (28).

We extended the study beyond LPS and studied whether gemfibrozil suppressed microglial activation induced by other neurotoxins and etiological reagents of various neurodegenerative disorders via a PI3K pathway. It is important to know that gemfibrozil inhibited microglial expression of iNOS mRNA induced by various neurotoxins, such as Aβ, PrP, poly IC, Tat, IL-1β, and MPP⁺ via a PI3K pathway. Surprisingly, gemfibrozil was unable to suppress IFN-γ-induced expression of iNOS mRNA in microglia. Activation of NF-κB by LPS, Aβ, PrP, poly IC, Tat, IL-1β, and MPP⁺, but not by IFN-γ, and inhibition of LPS-, Aβ-, PrP-, poly IC-, Tat-, IL-1β-, and MPP⁺-, but not IFN-γ-, induced expression of iNOS mRNA by SR IκBα suggests that NF-κB is required for LPS-, Aβ-, PrP-, poly IC-, Tat-, IL-1β-, and MPP⁺-, but not IFN-γ-, induced expression of iNOS. It is consistent with a finding by Kleinert et al. (39) that delineates an important role of STAT1α in IFN-γ-induced expression of iNOS. Because gemfibrozil inhibits the activation of NF-κB via PI3K p110β-mediated up-regulation of IκBα, this drug is capable of attenuating the expression of only those proinflammatory molecules whose expression depends on the activation of NF-κB. However, IFN-γ induces the expression of iNOS independent of NF-κB (Fig. 8B), therefore, gemfibrozil is unable to inhibit IFN-γ-induced microglial expression of iNOS via activation of p110β PI3K.

At present, we do not know the mechanism by which p110β PI3K may transduce signals for enhanced expression of IκBα in microglia. It is known that the promoter of IκBα contains the
consensus NF-κB-binding site (40) and once NF-κB is activated, as part of its autoregulation, the transcription of the inhibitory subunit (IκBα) is turned on to suppress its activation. However, gemfibrozil alone does not induce the activation of NF-κB (data not shown) ruling out the possible involvement of NF-κB in transcriptional up-regulation of IκBα. In addition to having NF-κB-binding sites, the promoter of IκBα also houses several consensus sequences for CREB binding (41). Therefore, there is a possibility that gemfibrozil-mediated activated p110β transduces signals for phosphorylation and activation of CREB which in turn is involved in the transcription of the IκBα gene. Experiments are underway in our laboratory to reveal the role of p110β P3K in gemfibrozil-mediated up-regulation of IκBα in microglia.

Microglia are the sensor cells in the CNS that express proinflammatory molecules in response to any neurotoxic and degenerative insult. Recently, gemfibrozil has been shown to suppress the disease process of experimental allergic encephalomyelitis, an animal model of MS (42), suggesting that gemfibrozil may have a therapeutic effect in this neuroinflammatory disease. Although the in situ situation of mouse microglia in culture and its treatment with Parkinsonian neurotoxin and etiological reagents of AD, HAD, prion diseases, and viral encephalopathy may not truly resemble the in vivo situation of microglia in the brain of patients with these neurodegenerative disorders, our results identify gemfibrozil as a possible therapeutic agent to suppress microglial activation in neuroinflammatory and neurodegenerative disorders via P110β-mediated regulation of IκBα.

Disclosures

The authors have no financial conflict of interest.

References


Downloaded from http://www.jimmunol.org/ by guest on October 23, 2017