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Activation of PPARα Attenuates IFNγ and IL-1β-induced Cell Proliferation in Astrocytes: Involvement of IL-6 Independent Pathway

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The present study demonstrates the effect of fibrates, agonists of PPARα on cytokines-induced proliferation in primary cultured astrocytes. Alone or combination treatment with cytokines, such as IL-1β (10 ng/ml), IFNγ (10 ng/ml), and TNF-α (10 ng/ml) cause a significant increase of cell proliferation in a time-dependent manner. Treatment of astrocytes with bezafibrate and fenofibrate (0, 5, and 10 μM) reduced the IFNγ and IL-1β-induced cell proliferation in a dose-dependent manner. To address the involvement of IL-6 on the IFNγ and IL-1β-induced cell proliferation, released IL-6 level was measured. IFNγ and IL-1β cause an increase of released IL-6 protein level in a time-dependent manner. Furthermore, pretreatment with IL-6 antibody (0, 0.1, 1, 2.5, and 5 ng/ml) dose-dependently inhibited the IFNγ and IL-1β-induced cell proliferation. However, bezafibrate and fenofibrate did not affect increased mRNA and protein levels of IL-6 in IFNγ and IL-1β-stimulated astrocytes. Taken together, these results clearly suggest that activation of PPARα attenuates the IFNγ and IL-1β-induced cell proliferation through IL-6 independent pathway.

Key Words: Astrocytes, Bezafibrate, Fenofibrate, IL-6, Proliferation

INTRODUCTION

Astrocytes, constituting the major glial cell population in the central nervous system (CNS), play important physiological, signaling, and immunological roles in CNS structure and function. Astrocytic proliferation, or astrogliosis, occurs in acute or chronically damaged areas of the CNS and is associated with regional neuronal loss. The reactivity and rapid proliferation of astrocytes is seen in pro-inflammatory cytokine-stressed astroglial cells, after acute, focal mechanical trauma and viral infection, and is associated with chronic neurodegenerative diseases including Alzheimer's disease (AD) [1,2].

The peroxisome proliferator-activated receptors (PPARs) represent a class of nuclear hormone receptors within a superfamily of proteins that also includes sex steroid receptors [3]. Three PPAR subtypes exist (α, δ and γ). Each of these receptors exhibits distinct tissue distribution and ligand specificities. PPARα, upon ligand activation, regulates transcription after dimerizing with the retinoid X receptor (RXR) and DNA-binding to PPAR-response elements (PPREs) within the regulatory regions of target genes [4]. The role of PPARs in glucose and lipid metabolism has been studied extensively [5]. In the CNS, a variety of studies indicate that PPARγ plays an important role for the pathogenesis of several neurological disorders, such as multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), experimental allergic encephalitis (EAE), AD, Parkinson's disease (PD), stroke and ischemic damage [6]. However, the exact molecular mechanism by which PPARα agonists contribute to cell proliferation remains to be understood.

In the present study, we have investigated whether bezafibrate and fenofibrate affect the IFNγ and IL-1β-induced cell proliferation and IL-6 expression in astrocytes. Our results indicate that activation of PPARα may play an important role in cell proliferation through IL-6 independent pathway in astrocytes.

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METHODS

Cell culture and reagents

Primary astrocyte-enriched cultures were prepared from the whole cortex of 1-d-old Sprague Dawley rats as described earlier [7]. Briefly, the cortex was dissected rapidly in ice-cold calcium/magnesium-free HBSS (Invitrogen, Carlsbad, CA, USA), pH 7.4, as described previously. Then the tissue was minced, incubated in HBSS containing trypsin (2 mg/ml) for 20 min, and washed twice in plating medium containing 10% FBS and 10 μg/ml gentamicin; next

ABBREVIATIONS: PPARα, peroxisome proliferator-activated receptor alpha; IFNγ, interferon gamma; IL-1β: interleukin-1 beta.

it was disrupted by triturating through a Pasteur pipette, after which the cells were seeded in 75 cm^2 culture flasks (Falcon, Franklin, NJ, USA). After incubation at 37°C in 5% CO_2 for 1 d, the medium was changed completely to the culture medium (DMEM containing 5% FBS and 10 μg/ml gentamicin). The cultures received half-exchanges with fresh medium twice a week. After 14~15 d the cells were shaken for at least 24 hr on an orbital shaker to remove the microglia and then seeded on multi-well tissue culture dishes. The cells were incubated with serum-free DMEM for 24 hr before the incubation with drugs. Recombinant IL-1β, IFN-γ and TNF-α were obtained from R&D systems (Minneapolis, MN). Bezafibrate and fenofibrate obtained from Sigma and Calbiochem, respectively.

Cell proliferation assay

Proliferation of primary cultured astrocytes was assayed by using the cell proliferation ELISA, BrdU colorimetric assay kit (Roche Applied Science) according to the manufacturer's protocol. Briefly, cells were seeded in 96-well plates, and following overnight serum starvation they were stimulated with cytokines and fibrates. Two hour before termination of assay, BrdU (10 μM) was added to each well, following which cells were fixed, and levels of incorporated BrdU were assayed using a conjugated anti-BrdU enzyme. Colorimetric analysis was done by measuring absorbance at 370 nm using a spectrophotometer.

IL-6 ELISA

Released IL-6 level was measured from media using the rat IL-6 ELISA kit (R& D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Total RNA isolation and reverse transcription

Cells were cultured in six-well culture plates. After drugs treatment, the cells were homogenized in TRIzol® reagent (Invitrogen). Total RNA was extracted from the cells according to the manufacturer's suggested protocol. Total RNA concentration was determined from spectrophotometric optical density measurement (260 and 280 nm). Total RNA (2 μg) was treated with 1 U DNase I (Promega, Madison, WI, USA) for 15 min at room temperature in 18 μl of volume containing 1× PCR buffer and 2 mM $MgCl_2$. Then it was inactivated by incubation with 2 μl of 25 mM EDTA at 65°C for 15 min. Reverse transcriptase reactions were carried out using MuLV Reverse Transcriptase (Promega) according to the manufacturer's protocol. Each reaction tube contained 2 μg of total in a volume of 25 μl containing 5 μl of MuLV 5× RT buffer, 1 μg of Oligod(T)15 (Promega), 2.5 μl of dNTP Mixture (Promega), 40 U of RNasin (Promega), 20 U of MuLV Reverse Transcriptase and nuclease-free water to volume. Reverse transcriptase reactions were carried out in a DNA Thermal Cycler 480 (Perkin Elmer, Branchburg, NJ, U.S.A.) at 25°C for 20 min, 42°C for 60 min and 95°C for 10 min. The cDNA was then stored at −20°C.

Quantitative real-time PCR

Real-time PCR for the analysis of IL-6 mRNA levels were performed in a Rotor-Gene Q (Qiagen). The primer sets for real-time PCR were designed using PrimerQuest (Integrated DNA Technologies) and synthesized from Bioneer (Daejeon, Korea). The primer sequences for IL-6 and GAPDH were as follows: rat IL-6 (forward, 5'-cgaaagtcaactccatctgcc-3', and reverse, 5'-ggcaactggctggaagtctct-3') and GAPDH (forward, 5'-cctacccccaatgtatccgttgtg-3', and reverse, 5'-ggaggaatgggagttgctgttgaa-3'). QuantiTect SYBR Green PCR kit was purchased from Qiagen. The reaction mixture consisted of 2 μl of cDNA template, 10 μl of SYBR Green PCR master mix and 10 pmol of primers in total volume of 20 μl. The cDNA was denatured at 95°C for 10 min followed by 45 cycles of PCR at 95°C for 10 sec, 58°C for 15 sec and 72°C for 20 sec. Data acquisition and analysis of real-time PCR were performed using the Rotor-Gene Q series software (version 1.7). Delta-delta Ct method was used to calculate the relative quantitation of each target gene normalized with GAPDH level in each individual sample.

Statistical analysis

All values shown in the figures are expressed as the mean± SD obtained from at least three independent experiments. Statistical analysis was carried out by one-way analysis of variance (ANOVA) with Tukey's post-hoc test using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA). A p values less than 0.05 were considered to indicate statistical significance.

RESULTS

Effects of PPARα agonists on IFNγ and IL-1β-induced proliferation of astrocytes

In the present study, we first examined the effect of PPARα agonists, such as bezafibrate and fenofibrate on cell proliferation, which was assayed by BrdU incorporation in cytokines-stimulated primary cultured astrocytes. The cells were treated with cytokines, such as IL-1β (10 ng/ml), TNF-α (10 ng/ml), and IFNγ (10 ng/ml) alone or co-treatment. As shown in Fig. 1A, cytokines significantly induced proliferation of astrocytes in a time-dependent manner. Compared to an untreated group, both the IFNγ and IL-1β-treated group and the TNF-α and IL-1β-treated group, but not the IFNγ-treated group, showed about a 2 to 2.5 fold increase of cell proliferation at 24 hr after treatment. To examine the effect of PPARα agonists on IFNγ and IL-1β-induced cell proliferation, cells were pre-treated for 0.5 hr with differing concentrations of bezafibrate and fenofibrate (0, 1, 5, and 10 μM) followed by stimulation with IFNγ and IL-1β for 24 hr. Both bezafibrate and fenofibrate dose-dependently inhibited cell proliferation (Fig. 1B), whereas one microM concentration of fibrates have no effect (Data not shown in graph). These data suggest that activation of PPARα may play a role in the regulation of IFNγ and IL-1β-mediated proliferation of astrocytes.

Involvement of IL-6 on IFNγ and IL-1β-induced proliferation of astrocytes

We next examined released level of IL-6 at 24 hr after cytokines stimulation. As shown in Fig. 2A, stimulation of IFNγ and IL-1β as well as TNF-α and IL-1β significantly induced release of IL-6 in a time dependent manner, whereas IFNγ alone has no effect. To address the involvement of IL-6 in the IFNγ and IL-1β-induced cell proliferation,

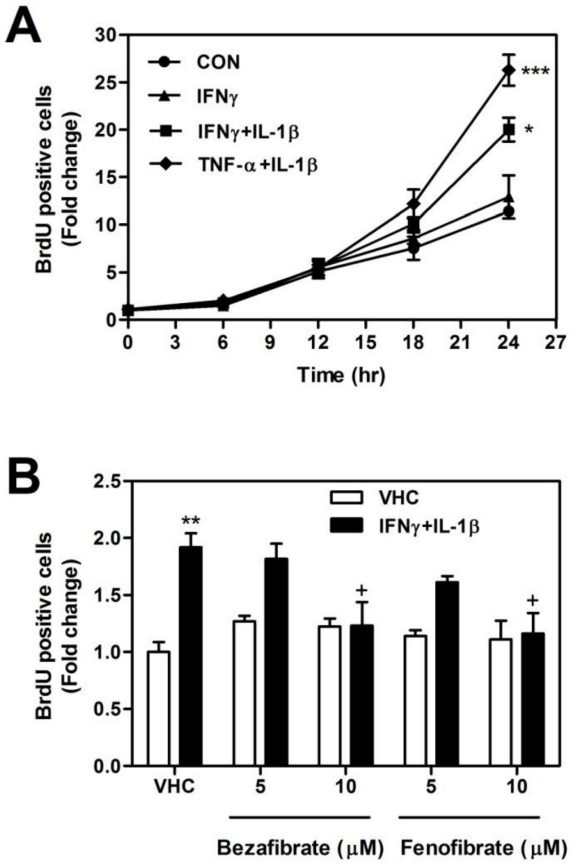

Fig. 1. Effect of bezafibrate and fenofibrate on the cell proliferation in IFNγ and IL-1β-treated astrocytes. Cells were treated with TNF-α (10 ng/ml), IFN-γ (10 ng/ml), and IL-1β (10 ng/ml). (A) The effect of cytokines on astrocytes proliferation, assayed by BrdU incorporation, was examined at each time point (0, 6, 12, 18, and 24 hr) following alone or co-stimulation with cytokines. (B) The effect of fibrates on cell proliferation was assayed. Cells were pre-treated with bezafibrate and fenofibrate (0, 5, and 10 μM) for 0.5 hr before stimulation with IFN-γ and IL-1β. Data are represented as mean±S.D. from three independent experiments (**$p<0.01$; vehicle *vs.* IFN-γ and IL-1β-treated group, $^{+}p<0.05$; IFN-γ and IL-1β *vs.* IFN-γ and IL-1β plus fibrates-treated group).

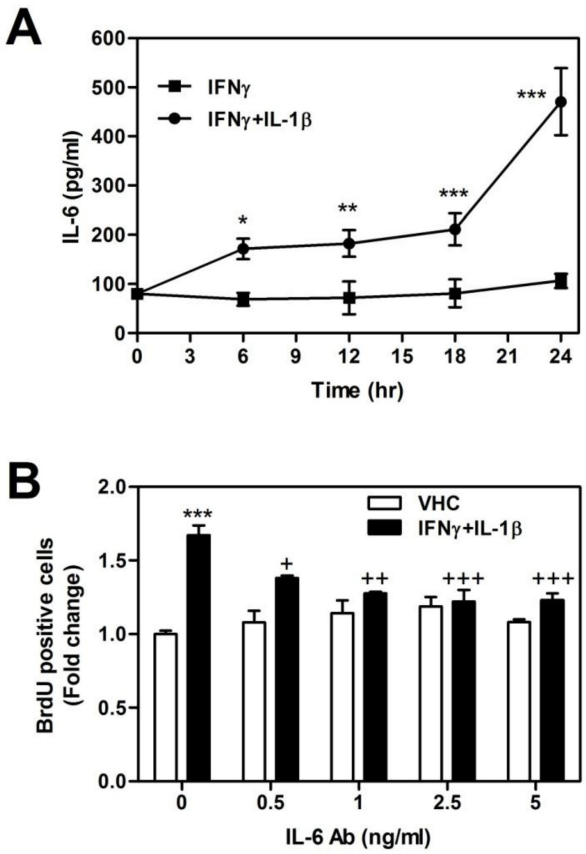

Fig. 2. Involvement of IL-6 on the cell proliferation in IFNγ and IL-1β-treated astrocytes. Cells were treated with IFN-γ (10 ng/ml) and IL-1β (10 ng/ml). (A) Released IL-6 Protein levels were measured using IL-6 ELISA kit from cultured media after stimulation. Values are mean±S.D. (*$p<0.05$, **$p<0.01$, ***$p<0.001$; IFN-γ *vs.* IFN-γ and IL-1β). (B) The effect of antagonizing of IL-6 using antibody on cell proliferation was assayed. Cells were pre-treated with IL-6 antibody (sodium azaide free) (0, 0.5, 1, 2.5, and 5 ng/ml) for 0.5 hr before stimulation with IFN-γ and IL-1β. Data are represented as mean±S.D. from three independent experiments (***$p<0.001$; vehicle *vs.* IFN-γ and IL-1β-treated group, $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$; IFN-$\gamma$and IL-1β *vs.* IFN-γ and IL-1β plus IL-6 Ab-treated group).

cells were pre-treated for 0.5 hr with varying concentrations of IL-6 antibody (0, 0.1, 1, 2.5, and 10 ng/ml) followed by stimulation with IFNγ and IL-1β for 24 hr. IL-6 antibody significantly inhibited cell proliferation in a dose dependent manner (Fig. 2B). These results suggest that IL-6 pathway can mediate IFNγ and IL-1β-induced cell proliferation.

Effects of PPARα agonists on IFNγ and IL-1β-induced IL-6 expression of astrocytes

Since cell proliferation was regulated by PPARα agonists and IL-6, lastly we examined whether bezafibrate and fenofibrate can regulate the IFNγ and IL-1β-induced IL-6 expression in astrocytes. Cells were pre-treated for 0.5 hr with different concentrations of bezafibrate and fenofibrate (0, 5, and 10 μM) followed by stimulation with IFNγ and IL-1β, and then the mRNA level of IL-6 was examined at 6 hr using the real-time RT-PCR and released protein level of IL-6 was determined using ELISA at 24 hr. Bezafibrate and fenofibrate had no affect on the increased mRNA and protein levels of IL-6 in the IFNγ and IL-1β-stimulated astrocytes (Fig. 3). These results clearly demonstrate that activation of PPARα has no effect on expression and release of IL-6 in the IFNγ and IL-1β-stimulated astrocytes.

DISCUSSION

In the present study, we have investigated the effect of PPARα agonists on cell proliferation in cytokines-stimulated astrocytes. Our results indicate that stimulation of IFNγ and IL-1β induces cell proliferation in astrocytes (Fig. 1A). Several lines of evidence suggest that glial cells can secrete pro-inflammatory cytokines, such as IL-1β, IL-6, INFγ, and TNF-α, and, in turn, can act on these cells in an autocrine manner. Cytokines can induce the prolifer-

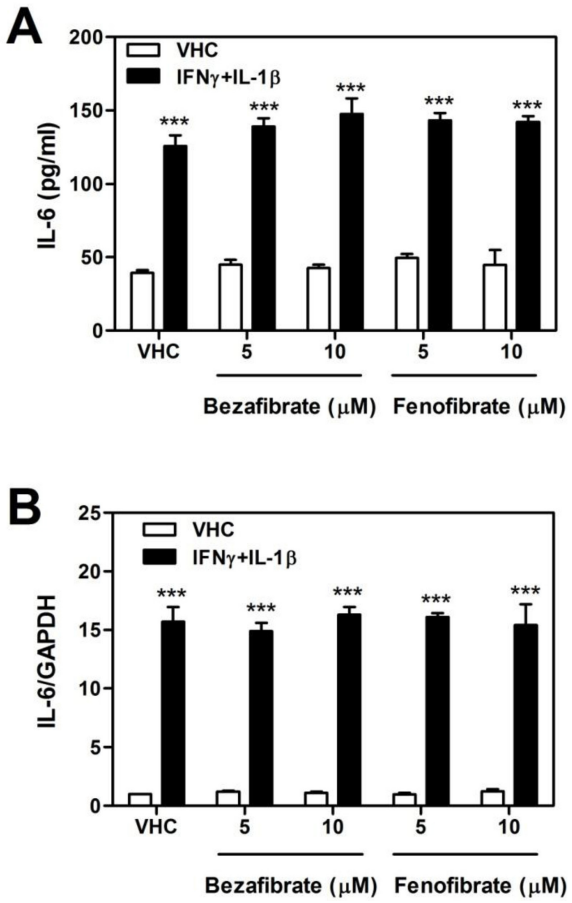

Fig. 3. Effect of bezafibrate and fenofibrate on the expression of IL-6 in IFNγ and IL-1β-treated astrocytes. Cells were treated with IFN-γ (10 ng/ml) and IL-1β (10 ng/ml). (A) Released IL-6 Protein levels were measured using IL-6 ELISA kit from cultured media after stimulation. (B) Total RNA was isolated from 6 hr in IFN-γ and IL-1β-treated astrocytes. Gene expression of IL-6 was measured by quantitative real-time PCR using Rotor-Gene Q (Qiagen). The expression of IL-6 gene was normalized with GAPDH gene expression. Data were obtained from triplicated IL-6 ELISA and PCR reactions with three different cultures, and values are mean±S.D. (***$p < 0.001$; vehicle *vs.* IFN-γ and IL-1β).

ation of astrocytes in neuroinflammatory conditions [1,7-9].

Fibrates are synthetic ligands for PPARα, which mediates the lipid-lowering activity [10]. In the CNS, several studies reported the beneficial effects of fibrates, such as neuroprotection and anti-neuroinflammation [6]. Based on these effects, we investigated the involvement of fibrates in proliferation of astrocytes. Bezafibrate and fenofibrate inhibited IFNγ and IL-1β-induced cell proliferation (Fig. 1B). Our results indicate that pathway of PPARα activation can regulate the proliferation of astrocytes. In line with our results, several previous studies demonstrated the anti-proliferative effect of PPARs agonists in several cell types [11-15]. Furthermore, the present work expands the list of beneficial effects by demonstrating inhibition of cell proliferation by fibrates.

To address the mediator of cell proliferation, we measured production of other cytokines from IFNγ and IL-1β-treated media. Stimulation of IFNγ and IL-1β causes a significant increase in production of IL-6 and treatment of its antibody dose-dependently attenuated the IFNγ and IL-1β-mediated cell proliferation (Fig. 2). Previous studies demonstrate IL-6-induced cell proliferation in several cell types [8,9,16,17]. In addition, brain injury-induced reactive gliosis and MPTP-induced microgliosis declined in IL-6 KO mice [18,19]. Accordingly, these results clearly indicate that cell proliferation is regulated by IL-6 dependent pathway.

Since cell proliferation was attenuated by activation of PPARα and inhibition of IL-6, we determined whether PPARα agonists can regulate the expression of IL-6 in IFNγ and IL-1β-treated astrocytes. Bezafibrate and fenofibrate did not affect the expression and production of IL-6 (Fig. 3). In contrast to our results, PPARα agonists inhibited the secretion of IL-6 as well as the pro-inflammatory cytokines such as TNF-α and IL-1β by LPS-stimulated astrocytes. Furthermore, fenofibrate inhibited NF-κB DNA binding activity, suggesting a mechanism by which PPARα agonists may regulate the expression of genes encoding these pro-inflammatory molecules [20]. A controversial effect of fibrate on IL-6 production may result from a difference of final concentration and intensity of stimulants. The present study suggests that the expression and release of IL-6 is not regulated by activation of PPARα. Taken together, our results indicate that IFNγ and IL-1β-induced cell proliferation is differently regulated by activation of PPARα and antagonization of IL-6 pathways.

In summary, our results clearly suggest that PPARα agonists attenuate the IFNγ and IL-1β-induced cell proliferation through IL-6 independent pathway. Although further studies will be necessary to reveal the exact molecular mechanism of anti-proliferation, this beneficial effect of PPARα agonists can be applied to reduce an astrogliosis as well as other inflammatory responses in CNS diseases.

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