Quantitative Bio-Science 33(1), 41~45(2014)

Attenuation of Translocator Protein 18 kDa (TSPO) Up-Regulation by Peroxisome Proliferator-Activated Receptor γ Ligand in Activated Microglia

Hyojin Cho, Hyun-Jung Shim, Seong-Woon Yu*

Department of Brain Science, Daegu Gyeongbuk Institute of Science and Technology (DGIST), Daegu 711-873, Korea

(Received May 13, 2014; Revised May 23, 2014; Accepted May 24, 2014)

ABSTRACT

Translocator protein (18 kDa) (TSPO) is a five transmembrane domain protein localized primarily in the outer mitochondrial membrane. Recently, we reported that TSPO is a negative regulator of neuroinflammation in microglia. Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-specific transcriptional factor belonging to the nuclear receptor superfamily and predicted as a putative TSPO transcriptional factor. A number of studies suggest that the activation of PPARγ has anti-inflammatory effects. In this study, we observed that treatment of rosiglitazone, a PPARγ ligand significantly decreased the NO production in lipopolysaccharide-stimulated BV2 microglia cell, indicating inhibition of microglial activation. The inhibitory effect of rosiglitazone extended to attenuated protein level of TSPO. TSPO up-regulation seems an adaptive anti-inflammatory response to overcome microglia activation, according to our previous report. Taken together, these results indicate that PPARγ activation by rosiglitazone attenuates neuroinflammation and leads to reduced expression of TSPO in the BV2 microglial cells.

Key words : Microglia, Neuroinflammation, PPARγ, Translocator protein 18 kDa (TSPO)

Introduction

Microglia are resident macrophages of the brain and spinal cord sensitive to brain injury and disease. Under normal conditions, microglia cells contribute to brain development and maintenance of tissue homeostasis. However, microglia also have a potential to cause neuronal damage when activated inflammatory responses process chronically [1-3].

Translocator protein (18 kDa) (TSPO) is a five transmembrane domain protein localized primarily in the outer mitochondrial membrane. TSPO is expressed in various tissue types including the central nervous system, especially in microglia and reactive astrocytes [4]. In the previous study, we demonstrated that TSPO is a negative regulator of neuroinflammation in microglia. Overexpression of TSPO diminished microglia activation and proinflammatory responses upon lipopolysaccharide (LPS) treatment while TSPO knockdown led to the opposite events [5].

A transcription factor (a sequence-specific DNA-binding factor) is a protein that controls the activity of a gene by binding to specific DNA sequences. Transcription factors exert its activity by promoting or blocking the recruitment of RNA polymerase to specific genes through complex with other proteins or alone [6]. Using the bioinformatics tools peroxisome proliferator-activated receptor γ (PPARγ) was predicted as a putative TSPO transcriptional factor. PPARs are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes [7,8]. Recently, there are reports showing inhibitory effects of PPARγ ligands on the production of microglia-derived proinflammatory molecules. It is known that thiadiazolidinones (TDZDs) and thiazolidinediones (TZDs), such as rosiglitazone, pioglitazone, and troglitazone, are ago-

* Correspondence should be addressed to Dr. Seong-Woon Yu, Department of Brain Science, Daegu Gyeongbuk Institute of Science and Technology (DGIST), Daegu 711-873, Korea. Tel: +82-53-785-6113, Fax: +82-53-785-6109, E-mail: yusw@dgist.ac.kr

nists of PPARγ. It has recently been shown that TDZDs inhibit inflammatory activation of cultured brain astrocytes and microglia [9-12]. These studies suggest the possibility that PPARγ is related to the regulation of TSPO gene expression, exhibiting anti-inflammatory activities in common. Here we report that administration of rosiglitazone, a specific ligand for PPARγ, attenuated neuroinflammation in BV2 microglia cells. The improved neuroinflammation upon rosiglitazone administration was associated with the attenuated expression level of TSPO.

Materials and Methods

1. Chemicals and reagents

Hyclone DME/F-12 was purchased from Thermo scientific. Penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). LPS was purchased from Calbiochem (La Jolla, CA). Rosiglitazone was purchased from Sigma-Aldrich.

2. Cell culture

BV2 microglial cells were maintained in DME/F-12 supplemented with 5% FBS and 1% penicillin-streptomycin. Cells were grown at 37°C in an atmosphere containing 5% CO_2. For experiments the cells were plated at a density of 1.0×10^5 cells per cm^2. For stock solutions, rosiglitazone was prepared in dimethyl sulfoxide (DMSO) and LPS was prepared in phosphate buffered saline (PBS), respectively.

3. Cell viability

Viability of BV2 cells was determined using The CellTiter-Blue® Cell Viability Assay (Promega, Madison, WI, USA). This assay provides a homogeneous, fluorescent method for monitoring cell viability. It is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). The 10 μL of reagent was directly added to the assay plate. The plate was incubated at 37°C for 2 h. After an incubation step, data were recorded at 490 nm with a SpectraMAX 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

4. Nitrite assay

The production of nitric oxide (NO) was determined by nitrite assay. This assay is to measure the accumulated level of the NO metabolite nitrite (NO_2^-) in the supernatant as a surrogate index of NO using a colorimetric reaction with Griess reagent (0.1% naphthylethylenediamine, 1% sulfanylamide and 2.5% H_3PO_4; Promega) 24 h after LPS treatment. Absorbance was measured at 540 nm with a microplate reader.

5. RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was purified using the QIAzol reagent (Qiagen, Valencia, CA, USA) and cDNA was prepared from total RNA using Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. cDNA generation and RT-PCR reaction were performed according to the previous report with slight modifications [5] using QuantiTechSYBR Green PCR kit (Qiagen) and the Rotor gene Q real-time amplification instrument (Qiagen). The PCR conditions and the sequences of the primers for TSPO and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as reported [5]. Each PCR reaction was performed in triplicates and for each sample, the levels of TSPO mRNA expression were normalized to GAPDH levels using the comparative C_T ($2^{-\Delta\Delta C}{}_T$) method and were expressed as fold induction.

6. Western blot analysis

BV2 cells were harvested at the indicated time point and lysed in cell lysis buffer (250 mM sucrose, 50 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF) in 20 mM Tris-HCL, pH 7.2) containing 1 × protease cocktail inhibitors (Roche Diagnostics, Indianapolis, IN, USA) and 1 × phosphatase cocktail inhibitors (Pierce, Rockford, IL, USA) for 30 min on ice. The cell lysates were cleared by centrifugation at 14,000 rpm for 15 min. The protein concentration of cell lysates was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Typically, 10-20 μg of proteins per well were loaded for Western blotting analysis. The proteins were electrotransferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA), and the membranes were blocked for 1 h at room temperature in a blocking solution of 5% nonfat dry milk in PBS-0.1% Tween 20 (PBST). The membranes were incubated overnight with primary antibody. After rinsing with blocking solution, the membranes were incubated for 1 h at room temperature in blocking solution contain-

ing horseradish peroxidase conjugated secondary antibodies. After washing, the membranes were processed for analysis using an enhanced chemiluminescence kit (Thermo Scientific) as described by the manufacturer.

7. Statistical methods

The difference between two groups was analyzed by the t-test. Multiple comparisons among groups were performed by one-way ANOVA. All statistical analyses were performed using GraphPad Prism (Graphpad software, Inc).

Results

1. Rosiglitazone reduces NO production

We first confirmed that a representative PPARγ ligand, rosiglitazone suppresses microglia activation and neuroinflammation. BV2 cells are an immortalized murine microglia cell line [13]. LPS was used as a microglia activator since LPS is the major component of the outer membrane of gram-negative bacteria and is commonly used as an inducer in immune cells [14]. To observe neuroinflammatory response of BV2 microglial cells, we carried out NO production assay that can detect nitrite (NO_2^-) as a surrogate marker of NO. Rosiglitazone was pretreated 1 h prior to LPS treatment in BV2 cell cultures. The levels of NO_2^- as a marker of BV2 cell activation were measured at 24 h after LPS treatment. The results showed that 100 ng/mL LPS treatments induced about 5 fold increase in NO production, compared with untreated control at 24 h after LPS treatment. However, pretreatment with rosiglitazone attenuated the levels of NO_2^- in a dose dependent manner (Fig. 1A). The doses of LPS and rosiglitazone (Fig. 1B) used in this study showed no toxicity on BV2 cells, as confirmed by CellTiter Blue Assay.

2. Rosigiltazone attenuated TSPO expression level

BV2 cells were treated with 100 ng/mL LPS and then the levels of TSPO expression were measured by RT-PCR and Western blot analyses. RT-PCR revealed a moderate reduction in TSPO mRNA level 24 h after rosiglitazone administration, but it did not reach a statistically significant level (Fig. 2). However, the levels of TSPO protein were significantly decreased by rosiglitazone treatment, as shown by Western blot analysis when measured 24 h after LPS treatment (a representative image of three experiments in Fig. 3A and quantification of protein band intensities in Fig. 3B). In our previous report, we suggested that TSPO level in activated microglia increases in response to inflammatory stimuli as an adaptive response to resolve sustained microglia activation. Therefore, if rosiglitazone suppresses inflammation, the increase of TSPO level is expected to be prevented. The current data are in accordance with this notion that rosiglitazone administration reduces neuroinflammation and thus, up-regulation of TSPO expression is suppressed.

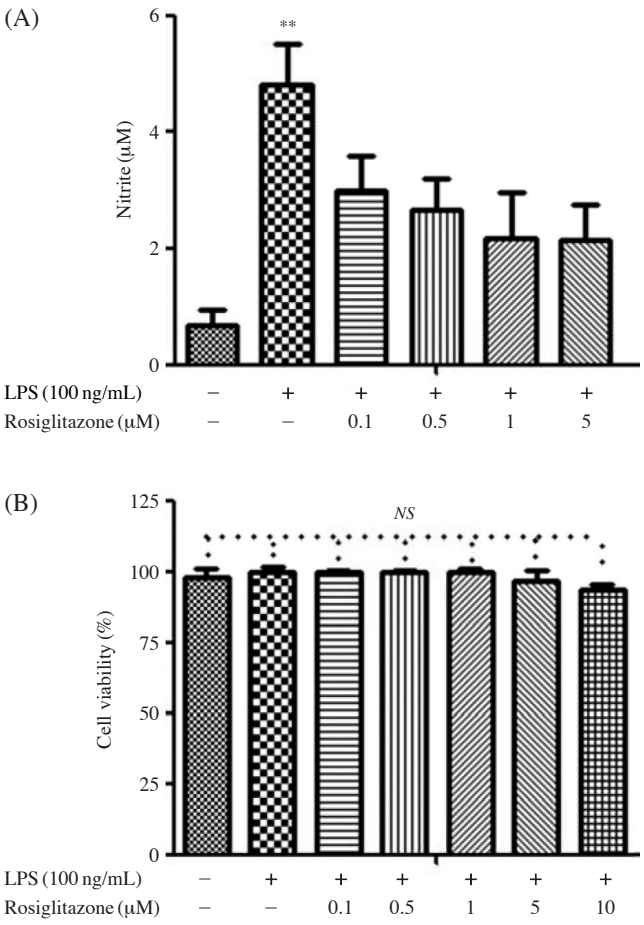

Fig. 1. (A) Rosiglitazone was pretreated 1 h prior to LPS treatment in BV2 cell cultures and then the level of NO_2^- was measured as markers on BV2 cell activation. The NO_2^- levels were determined at 24 h after LPS treatment. (B) The viability of BV2 cells was not affected by the doses of rosiglitazone used in this study, when measured by CellTiter Blue assay. Rosiglitazone was pretreated 1h prior to LPS treatment in BV2 cell cultures. The data are presented as mean±SD (n=3). $^{**}p<0.01$, compared with control. NS, not significant.

Discussion

Microglia are a resident macrophage population in the central nervous system (CNS). Activated microglia are a source of proinflammatory cytokines and chemokines, which act to

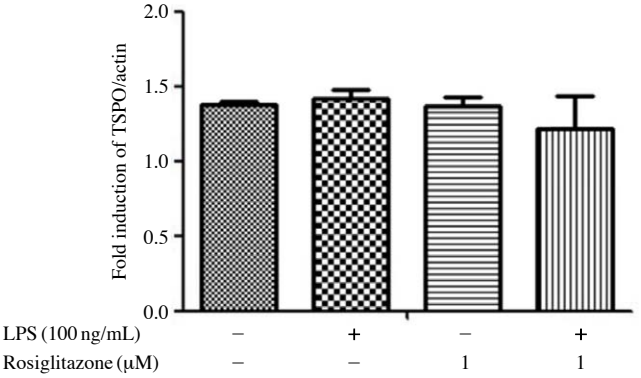

Fig. 2. mRNA expression level of TSPO following LPS (100 ng/mL) treatment was measured by RT-PCR in BV2 cells. mRNA expression levels were normalized to GAPDH.

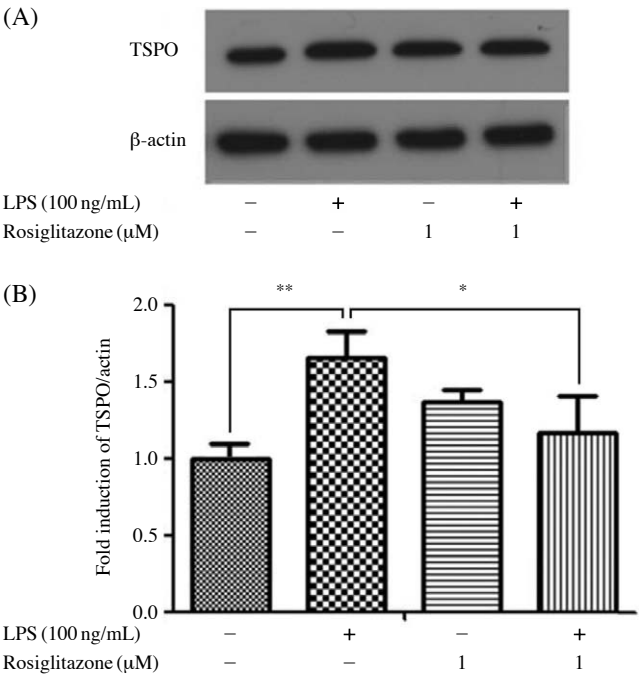

Fig. 3. Immunoblots with the indicated antibodies. (A) TSPO Protein expression levels were normalized to β-actin. (B) The lower panel shows quantification analysis of Western blotting data. Results are the mean ± S.D. (n=3), one-way ANOVA with Bonferroni correction: $*p<0.05$, $**p<0.01$.

initiate or promote inflammatory process in the CNS [1,2,5]. Because TSPO expression level is especially elevated in activated glial cells of the brain, TSPO can serve as a marker for brain gliosis [15]. In spite of its up-regulation in injured brain areas suffering neuroinflammation, the physiological role of TSPO and relevant signaling mechanisms in neuroinflammation remained unclear. Our previous work demonstrates at the molecular level that TSPO plays a negative role in neuroinflammation of murine microglial BV2 cell line [5].

The peroxisome proliferator-activated receptors (PPARs) are a member of the nuclear receptor superfamily of ligand-dependent transcription factors. It is known that PPARγ is especially detected in adipose tissue, intestine and macrophages [16]. A growing body of evidence suggests that PPARγ agonists exert the anti-inflammatory activities [17]. These studies suggest the possibility that PPARγ may be related to the regulation of TSPO gene expression, both of which have anti-inflammatory activities in common.

The present study demonstrates that treatment of rosiglitazone, a prototype PPARγ ligand, significantly decreased the levels of NO production in a dose dependent manner, indicating inhibition of microglial activation (Fig. 1A). The reduction of neuroinflammation induced the attenuated expression level of TSPO on rosiglitazone administration (Figs. 2, 3). Taken together with these data, it is suggested that PPARγ activation by rosiglitazone mitigated neuroinflammation and reduced levels of TSPO.

Acknowledgements

This work was supported by the DGIST Convergence Science Center Program of the Ministry of Science, ICT and Future Planning (14-BD-04).

References

1. Perry VH, Nicoll JA, Holmes C. Microglia in neurodegenerative disease. Nat Rev Neurol 2010;6:193-201.
2. Cardona AE, Pioro EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, et al. Control of microglial neurotoxicity by the fractalkine receptor. Nat Neurosci 2006;9:917-924.
3. Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 2007; 8:57-69.
4. Rupprecht R, Papadopoulos V, Rammes G, Baghai TC, Fan J, Akula N, et al. Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. Nat Rev Drug Discov 2010;9:971-988.
5. Bae KR, Shim HJ, Balu D, Kim SR, Yu SW. TSPO negatively regulates inflammation in microglia. J Neuroimmune Pharmacol 2014;9:424-437.
6. Latchmann DS. Transcription factors: an overview. Int J Biochem Cell Biol 1997;29:1305-1312.
7. Michalik L, Auwerx J, Berger JP, Chatterjee VK, Glass CK,

Gonzalez FJ, et al. International union of pharmacology. LXI. Peroxisome proliferator-activated receptors. Pharmacol Rev 2006;58:726-741.

8. Kielian T, Drew PD. Effects of peroxisome proliferator-activated receptorγ agonists on central nervous system inflammation. J Neurosci Res 2003;71:315-325.
9. Luna-Medina R, Cortes-Canteli M, Alonso M, Santos A, Martínez A, Perez-Castillo A. Regulation of inflammatory response in neural cells in vitro by thiadiazolidinones derivatives through peroxisome proliferator-activated receptor gamma activation. J Biol Chem 2005;280:21453-21462.
10. Cuzzocrea S, Pisano B, Dugo L, Ianaro A, Maffia P, Patel NS, et al. Rosiglitazone, a ligand of the peroxisome proliferator-activated receptor-gamma, reduces acute inflammation. Eur J Pharmacol 2004;483:79-93.
11. Gottfried-Blackmore A, Sierra A, Jellinck PH, McEwen BS, Bulloch K. Brain microglia express steroid-converting enzymes in the mouse. J Steroid Biochem Mol Biol 2008;109:96-107.
12. Rosario L-M, Marta C-C, Mercedes A, Angel S, Ana M, Ana P-C. Regulation of inflammatory response in neural cells *in vitro* by thiadiazolidinones derivatives through peroxisome proliferator activated receptor γ activation. J Biol Chem 2005; 280:21453-21462.
13. Bocchini V, Mazzolla R, Barluzzi R, Blasi E, Sick P, Kettenmann H. An immortalized cell line expresses properties of activated microglial cells. J Neurosci Res 1992;31:616-621.
14. Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE, et al. The Toll-Like Receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. J Neurosci 2002;22:2478-2486.
15. Karlstetter M, Nothdurfter C, Aslanidis A, Moeller K, Horn F, Scholz R, et al. Translocator protein (18 kDa) (TSPO) is expressed in reactive retinal microglia and modulates microglial inflammation and phagocytosis. J Neuroinflammation 2014;8:11-13.
16. Delerive P, Fruchart J-C, Staels B. Peroxisome proliferator-activated receptors in inflammation control. J Endocrinol 2001; 169:453-459.
17. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor is a negative regulator of macrophage activation. Nature 1998;391:79-81.