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Molecules and Cells

Suppression of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 by Cell-Permeable Superoxide Dismutase in Lipopolysaccharide-Stimulated BV-2 Microglial Cells

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Oxidative stress plays a pivotal role in uncontrolled neuroinflammation leading to many neurological diseases including Alzheimer's. One of the major antioxidant enzymes known to prevent deleterious effects due to oxidative stress is Cu,Zn-superoxide dismutase (SOD). In this study, we examined the regulatory function of SOD on the LPS-induced signaling pathways leading to NF-kappaB activation, expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), in BV-2 cells using cell-permeable SOD. Treatment of BV-2 cells with cell-permeable SOD led to a decrease in LPS-induced reactive oxygen species (ROS) generation and significantly inhibited protein and mRNA levels of iNOS and COX-2 up-regulated by LPS. Production of NO and PGE_2 in LPS stimulated BV-2 cells was significantly abrogated by pretreatment with a cell-permeable SOD fusion protein. Furthermore, cell-permeable SOD inhibited LPS-induced NF-kappaB DNA-binding activity and activation of MAP kinases including ERK, JNK, and p38 in BV-2 cells. These data indicate that SOD has a regulatory function for LPS-induced NF-kappaB activation leading to expression of iNOS and COX-2 in BV-2 cells and suggest that cell-permeable SOD is a feasible therapeutic agent for regulation of ROS-related neurological diseases.

INTRODUCTION

Uncontrolled neuroinflammation in the central nervous system (CNS) plays a crucial role in many neurological disorders such as Alzheimer's diseases, Parkinson's diseases and HIV-associated dementia (Gerard and Rollins, 2001; Minghetti, 2005). Microglia plays an important role in immune regulation and become activated in neuropathological processes. Deregulated production of inflammatory mediators such as cytokines/chemokines, nitric oxide (NO), and prostaglandin E_2 (PGE_2), from activated microglia would exacerbate the pathologic process of inflammatory CNS diseases (Minghetti, 2004; Phillis et al., 2006; Ransohoff, 2002). NO produced by inducible nitric oxide synthase (iNOS) and PGE_2, the major product of cyclooxygenase-2 (COX-2) have neurotoxic effects.

A variety of stimuli including LPS can increase levels of reactive oxygen species (ROS) which in turn activates redox-sensitive transcriptional factors such as NF-κB (Flohe et al., 1997; Schulze-Osthoff et al., 1997) leading to expression of many proinflammatory genes including iNOS and COX-2. Therefore, ROS plays a critical role in a variety of neurodegenerative and neuroinflammatory diseases such as Alzheimer's (Floyd, 1999). Antioxidant enzymes, such as SOD, play a major role in cellular defense against ROS (Mates, 2000). SOD catalyzes the decomposition of superoxide to generate hydrogen peroxide that is converted by catalase and glutathione peroxidases into water and oxygen (Halliwell and Gutteridge, 1990).

Attenuation of microglial activation by antioxidants is considered to be a therapeutic approach for treatment of various neurological diseases; however, antioxidants have broad effects on cellular functions. Antioxidant enzymes such as SOD have been considered to have a beneficial effect on various diseases mediated by ROS (Greenwald, 1990). Recently, we generated a cell permeable SOD, Tat-SOD, by use of HIV-1 Tat protein transduction domain (PTD), which is capable of delivering protein into cells (reviewed in Schwarze and Dowdy, 2000). Tat-SOD has been shown to have protective effects against oxidative stress (Eum et al., 2004; Kwon et al., 2000; Song et al., 2008). Even though cell-permeable SOD has been demonstrated to protect against oxidative stress in various cell types and *in vivo* disease models (Eum et al., 2004; Song et al., 2008; Zhang et al., 2009), the molecular mechanisms responsible for these biological activities are yet to be determined. In this study, we investigated the possible immuno-modulatory roles of cell-permeable SOD in

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LPS-stimulated microglia. Treatment of BV-2 cells with cell-permeable SOD blocked LPS-induced ROS production. Cell-permeable SOD not only suppressed expression of iNOS and COX-2 but also inhibited resulting production of NO and PGE_2 in LPS-stimulated BV-2 microglial cells. Cell-permeable SOD significantly inhibited activation of NF-κB binding activity and IkBα phosphorylation in LPS-stimulated BV-2 cells. Furthermore, cell-permeable SOD inhibited LPS-induced activation of MAP kinases including ERK, JNK, and p38 in BV-2 cells.

MATERIALS AND METHODS

Cell culture

The immortalized murine BV-2 microglial cell line was maintained in Dulbucco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified incubator under 5% CO_2.

Reagents

Lipopolysaccharide and 2′,7′-Dichlorofluorescin diacetate (DCF-DA) were purchased from Sigma (USA). Primary antibodies against iNOS (BD Biosciences PharMingen, USA), COX-2 and actin (Santa Cruz, USA) were obtained commercially. Phosphor-IκBα, phosphor-p38, phosphor-ERK, phosphor-JNK, total- IκBα, total-p38 MAPK, total-ERK, and total-JNK were purchased from Cell Signaling Technology (USA).

Expression and purification of SOD fusion proteins

Expression and purification of SOD fusion proteins were carried out as described previously (Eum et al., 2004; Song et al., 2008). The amounts of SOD fusion proteins taken up into the cells were analyzed by Immunoblot analysis with an anti-histidine polyclonal antibody (1:500, USA).

Immunoblot analysis

Cells were incubated in lysis buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 10% v/v glycerol) at 4°C for 30 min. Samples of 50 μg protein were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and the proteins electrotransferred to a nitrocellulose membrane, which was probed with the indicated antibodies. Immunoreactive bands were visualized by chemiluminescence (ECL; Amersham).

Measurement of intracellular ROS

Intracellular ROS were measured using 2′,7′-dichlorofluorescein diacetate (DCF-DA) (Sigma, USA), which is converted by ROS into fluorescent 2′,7′-dichlorofluorescein (DCF), as described previously (Song et al., 2008).

RT-PCR analysis

Total RNA was isolated from BV-2 cells using a Trizol reagent kit (Invitrogen, USA). The RNA (2 μg) was reversibly transcribed with 10,000 U of reverse transcriptase and 0.5 μg/μl oligo-$(dT)_{15}$ primer (Promega, USA). PCR amplification of cDNA aliquots was performed with the following sense and antisense primers (5′ → 3′): iNOS sense, CCC TTC CGA AGT TTC TGG CAG CAG C; iNOS antisense, GGC TGT CAG AGC CTC GTG GCT TTG G; COX-2 sense, ACT CAC TCA GTT TGT TGA GTC ATT C; COX-2 antisense, TTT GAT TAG TAC TGT AGG GTT AAT G; beta-actin sense, GCG GGA AAT CGT GCG TGA CAT T; and beta-actin antisense, GAT GGA GTT GAA GGT AGT TTC GTG. PCR products were resolved on a 1% agarose gel and visualized with UV light after ethidium bromide staining.

Measurement of nitrite and PGE_2

BV-2 cells were plated at a density of 5×10^5 cells in a 24-well cell culture plate with 500 μl of culture medium and incubated for 12 h. Cells were treated with Tat-SOD or SOD for 1 h and then stimulated with LPS (100 ng/ml) for 18 h. The amount of nitrite, the oxidized product of NO, was measured in cell culture media using the Griess reagent system (Promega). The amount of PGE_2 produced was measured using an ELISA kit (R&D, USA) according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)

BV-2 cells were treated with LPS (100 ng/ml) for 1 h, then nuclear extracts of cells were prepared and analyzed for NF-κB binding activity, as described previously (Park et al., 2003; 2004). Nuclear extracts (5 μg) were equilibrated for 15 min in binding buffer (10 mM Tris-HCl, pH 8.0, 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.25 mM dithiothreitol) and 1 μg of poly dI/dC. A ^{32}P-labeled oligonucleotide probe (20,000 cpm) was added and the reaction incubated on ice for an additional 20 min. Samples were then resolved by electrophoresis on a 6% native polyacrylamide gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA). The gel was then dried and exposed to X-ray films.

In vitro kinase assay

BV-2 cells were treated with Tat-SOD or control SOD for 1 h and stimulated with LPS (100 ng/ml) for 15 min. The cells were washed with ice-cold PBS and incubated in lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) for 15 min. Cell lysates were analyzed for IKK activity by *in vitro* kinase assay. Cell lysates were incubated with anti-IKKα polyclonal antibody (Cell Signaling Technology, USA) for 1 h at 4°C. IKK-antibody complex was precipitated with protein A/G-conjugated agarose beads at 4°C overnight. The immune complexes were washed with kinase reaction buffer (20 mM Tris-HCl, pH 7.5 and 4 mM $MgCl_2$), and incubated in 15 μl of kinase reaction buffer containing 1 μg of GST-IκBα (Santa Cruz, USA) and 0.66 mM ATP at 30°C for 30 min. Samples were analyzed by 12% SDS-PAGE gel electrophoresis and the levels of phosphorylated GST-IκBα determined by immunoblot analysis with anti-IκBα antibody. To verify that equal amounts of kinases were involved in the reaction, the same blot was striped and the level of total IKKα determined by immunoblot analysis with anti-IKKα antibody.

Statistical analysis

The results were expressed as the mean ± SEM from at least 3 independent experiments. The values were evaluated via one-way ANOVA, followed by Duncan's multiple range tests using GraphPad Prism 4.0 software (GraphPad Software, Inc., USA). Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Delivery of SOD fusion proteins into BV-2 cells and the effect on ROS generation by LPS

To evaluate the cellular uptake of SOD fusion proteins into the BV-2 cells, SOD fusion proteins were incubated with cells at varying concentrations for 1 h, and the amounts of protein taken up were analyzed by immunoblot analysis. As shown in Fig. 1A, Tat-SOD efficiently entered the cells in a dose-dependent manner whereas the control SOD did not. Next, we examined the effect of SOD on ROS generation induced by LPS in the BV-2 cells. Pretreatment with Tat-SOD inhibited the LPS-induced increase of intracellular ROS levels in BV-2 cells

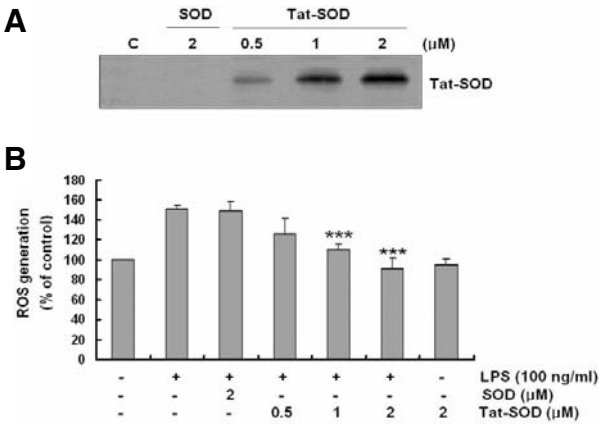

Fig. 1. Delivery of SOD fusion proteins into BV-2 cells and its effect on ROS generation by LPS. (A) Cellular uptake of SOD fusion proteins. BV-2 cells were incubated with various concentrations of Tat-SOD or SOD for 1 h. Cellular lysates were prepared for immunoblot analysis to determine the cellular uptake of SOD fusion proteins. C, untreated control cell lysates. (B) Effects of SOD fusion proteins on ROS generation in the LPS-stimulated BV-2 cells. BV-2 cells were treated with 0.5, 1 and 2 μM Tat-SOD or 2 μM SOD fusion proteins for 1 h, and then stimulated with LPS (100 ng/ml) for 15 min. Intracellular ROS levels were measured after staining with a fluorescent dye, DCF-DA. The data are the mean ± SEM of 3 separate experiments. In (B), $^{***}P < 0.001$ compared with BV-2 cells treated with LPS alone.

whereas the control SOD had a minimal effect (Fig. 1B). These results indicate that Tat-SOD can efficiently remove ROS generation by LPS stimulation in BV-2 cells.

Effects of SOD on LPS-induced expression of iNOS and COX-2 and NO/PGE_2 production

Previous studies reported that ROS was involved in LPS-induced increases of iNOS and COX-2 expression by microglial cells (Pawate et al., 2004; Wang et al., 2004). We used SOD fusion proteins to analyze the role of ROS on LPS-induced up-regulation of iNOS and COX-2 expression in BV-2 cells. Cells were incubated in the absence or presence of SOD fusion proteins for 1 h, treated with LPS (100 ng/ml), and expression of iNOS and COX-2 analyzed by RT-PCR and immunoblot analysis. Pretreatment with Tat-SOD inhibited iNOS and COX-2 mRNA (Fig. 2A) and protein (Fig. 2B) expression in a dose-dependent manner. Next, we evaluated the effect of SOD on the production of NO and PGE_2 in LPS-stimulated BV-2 cells, treated with Tat-SOD or SOD for 1 h, and stimulated with LPS for 18 h. Tat-SOD significantly inhibited LPS-induced NO and PGE_2 production in BV-2 cells (Fig. 2C). An experiment to determine the effect of Tat-SOD on LPS-induced NO and PGE_2 production in time-dependent manner was also performed. As shown in Figs. 2D and 2E, Tat-SOD fusion protein significantly inhibited LPS-induced production of NO and PGE_2 until 72 h. These results suggest that SOD is capable of modulating LPS-induced pro-inflammatory mediators in microglia.

The effects of SOD on LPS-induced activation of NF-κB, IκBα phosphorylation, and IKK activity in BV-2 cells

Since LPS has been reported to induce activation of NF-κB, a redox-sensitive transcription factor (Dimayuga et al., 2007; Pawate et al., 2004), known to regulate expression of various pro-inflammatory genes in microglia, we attempted to determine the effect of Tat-SOD on LPS-induced activation of NF-κB in BV-2 cells. BV-2 cells were treated with LPS (100 ng/ml) for 1 h, then nuclear extracts of BV-2 cells were prepared and analyzed for NF-κB binding activity by EMSA. Pre-treatment with Tat-SOD resulted in a decrease in LPS-induced DNA binding activity of NF-κB in a dose-dependent manner (Fig. 3A). Next, we examined the regulatory effect of Tat-SOD on the LPS-induced signal cascade of NF-κB activation, such as IκBα phosphorylation by immunoblot analysis using a phosphor antibody against IκBα. As shown in Fig. 3B, Tat-SOD significantly inhibited LPS-induced IκBα phosphorylation in BV-2 cells, while control SOD had a minimal effect. Next, we examined the effect of Tat-SOD on IKK activity induced by LPS. BV-2 cells were treated with Tat-SOD for 1 h, exposed to LPS for 15 min and the levels of IKK activity in cell lysates evaluated by an *in vitro* kinase assay. Tat-SOD inhibited LPS-induced increase of IKKκ activity whereas the control SOD did not (Fig. 3C). These data suggest that Tat-SOD inhibits LPS-induced NF-κB activation via suppression of IKK activation.

Inhibition of LPS-induced MAP kinase activation by Tat-SOD in BV-2 cells

The mitogen-activated protein kinase (MAPK) signaling cascades have been shown to be involved in activation of NF-κB upon LPS stimulation (Akundi et al., 2005; Fiebich et al., 2002). We further investigated the regulatory effect of Tat-SOD on the activity of MAP kinases such as p38, JNK, and ERK protein kinase. To examine the effect of Tat-SOD on LPS-induced MAPK activation, BV-2 cells were incubated in the absence or presence of Tat-SOD or SOD fusion protein for 1 h, followed by exposure to LPS (100 ng/ml) for 1 h, after which MAP kinase activation was analyzed by immunoblot analysis using phospho-specific antibodies against MAPK proteins. As shown in Fig. 4A, Tat-SOD decreased LPS-induced phosphorylation of ERK, p38 MAP kinase, and JNK in a dose-dependent manner. Next, we investigated the relative contribution of ERK, p38 MAP kinase and JNK in LPS-induced iNOS/COX-2 expression and resulting NO/PGE_2 production using MAPK inhibitors. As shown in Figs. 4B and 4C, pretreatment with ERK and JNK inhibitors (PD98059 and SP600125) significantly suppressed LPS-induced iNOS expression and NO production, while the p38 MAP kinase inhibitor (SB203580) did not. ERK, p38 MAP kinase, and JNK inhibitors suppressed LPS-induced COX-2 expression and PGE_2 production. These results suggest that different signaling cascades are involved in LPS-induced iNOS and COX-2 expression in BV-2 cells.

Our results are consistent with previous reports that SOD has a protective effect against proinflammatory responses. LPS-induced activation of NF-κB and release of cytokines such as TNF-α and IL-6 were significantly suppressed in microglial cells stably expressing SOD (Dimayuga et al., 2007). In addition, SOD overexpression mediated by transfection of SOD cDNA led to less production of NO and cytokines such as IL-1α and TNF-α in LPS activated microglial cells (Chang et al., 2001). Taken together, these studies suggest that SOD has anti-inflammatory activities, modulating ROS-dependent signaling pathways.

PTD derived from the basic domain of HIV-1 Tat has been shown to mediate uptake of exogenous proteins not only into living cells but also into various organs of mice, including the brain (Schwarze et al., 1999). The ability of PTDs such as Tat to deliver target proteins into the brain will facilitate the rational design of therapeutic proteins against various neurological diseases. We previously reported that intraperitoneal injection of cell-permeable SOD resulted in delivery of the SOD protein

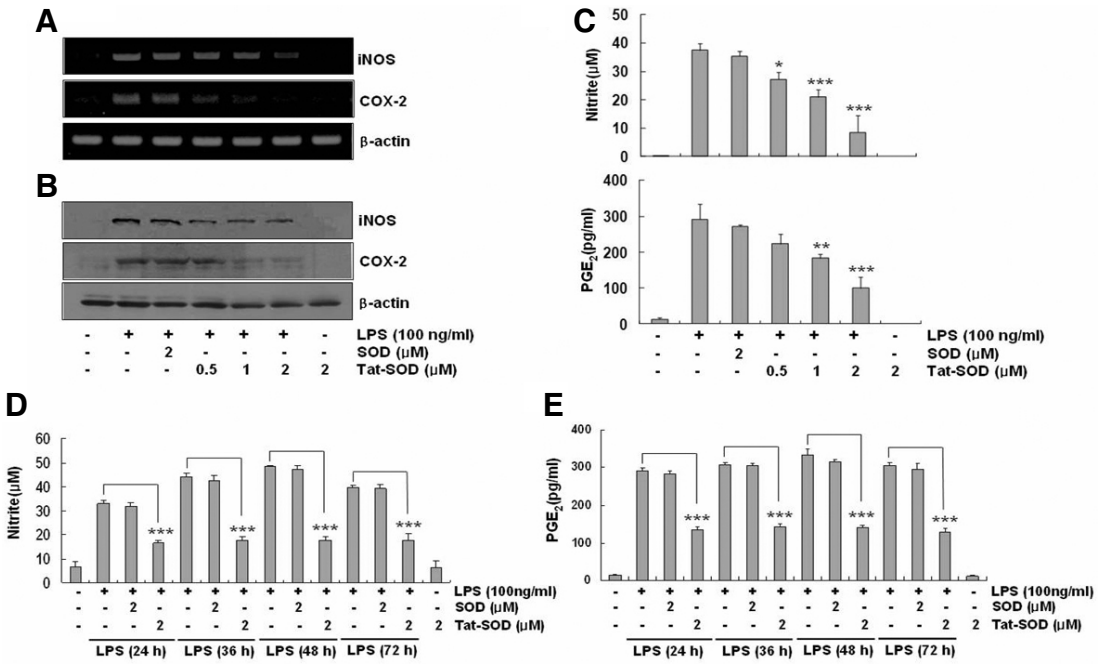

Fig. 2. Effects of SOD on LPS-induced expression of iNOS and COX-2 and NO/PGE_2 production. (A) Effects of SOD fusion proteins on LPS-induced expression of iNOS and COX-2 in BV-2 cells. BV-2 cells were treated with 0.5, 1 and 2 μM Tat-SOD or 2 μM SOD fusion proteins for 1 h, and exposed to LPS (100 ng/ml) for 8 h. Total RNA was extracted. iNOS, COX-2 and β-actin mRNA were analyzed by RT-PCR using specific primers. (B) BV-2 cells were treated with LPS (100 ng/ml) for 12 h with or without pretreatment with Tat-SOD or SOD fusion protein for 1 h. Cell extracts were prepared and analyzed by immunoblotting for iNOS and COX-2 protein expression. (C) Inhibitory effect of Tat-SOD on NO and PGE_2 production in LPS stimulated BV-2 cells. BV-2 cells were stimulated with LPS (100 ng/ml) for 18 h with or without pretreatment with Tat-SOD or SOD fusion proteins for 1 h. Culture medium was harvested and analyzed for NO and PGE_2 levels by a spectrophotometric method based on the Griess reactions and an ELISA kit, respectively. The data are the mean ± SEM of 3 separate experiments. To determine the effect of Tat-SOD on LPS-induced NO and PGE_2 production in a time-dependent manner, BV-2 cells pretreated with Tat-SOD or SOD fusion proteins for 1 h were stimulated with LPS (100 ng/ml) for 24 h, 36 h, 48 h and 72 h. Culture medium of BV-2 cells was harvested. The levels of NO (D) and PGE_2 (E) in the culture medium were determined. The data are the mean ± SEM of 3 separate experiments. In (C), (D) and (E), *P < 0.05, **P < 0.01, and ***P < 0.001 compared with cells treated with LPS alone.

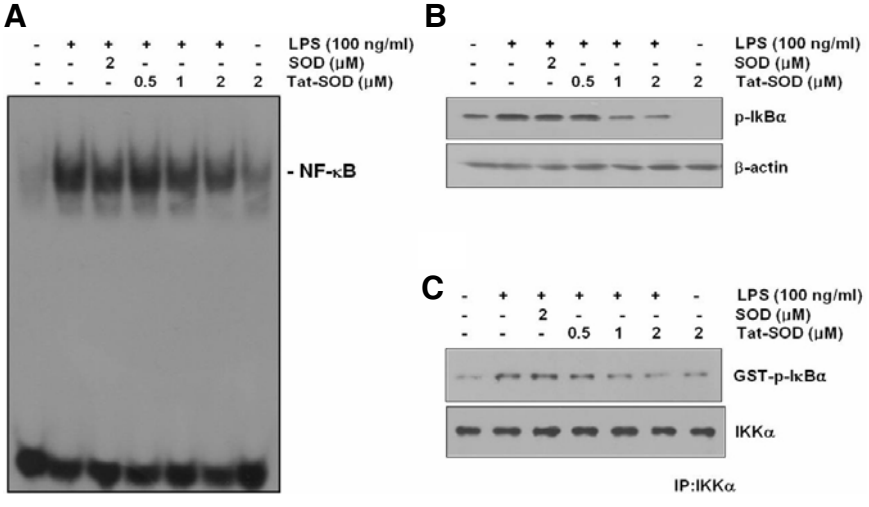

Fig. 3. Effects of SOD on LPS-induced activation of NF-κB, IκBα phosphorylation, and IKK activity in BV-2 cells. BV-2 cells were treated with Tat-SOD or SOD fusion proteins and stimulated with LPS (100 ng/ml) for 1 h. (A) DNA-binding activity of NF-κB in the nuclear extracts of the BV-2 cells was measured by EMSA. (B) Cell lysates were prepared from BV-2 cells treated with LPS and levels of phosphorylated IκBα determined by immunoblot analysis. (C) IKKα immunocomplex was prepared from LPS-stimulated BV-2 cells and IKK activity determined by *in vitro* kinase assay using GST-IκBα and ATP. As an internal control, the levels of IKKα were determined by Immunoblot analysis with anti-IKKα antibody (low panel).

to the brain in animals and exerted a protective effect against oxidative stress in *in-vivo* neurological disease models including transient forebrain ischemia and Parkinson's (Choi et al., 2006; Kim et al., 2005). These results suggested that Tat-SOD could be delivered systemically into the brain. Although Tat-SOD has a potential as a therapeutic tool against some neurologic diseases, great improvement in the delivery, bioavailability, and biosafety of these proteins into the brain will be required for further clinical trial. Advantages and limitations concerning protein transduction technology have been extensively discussed

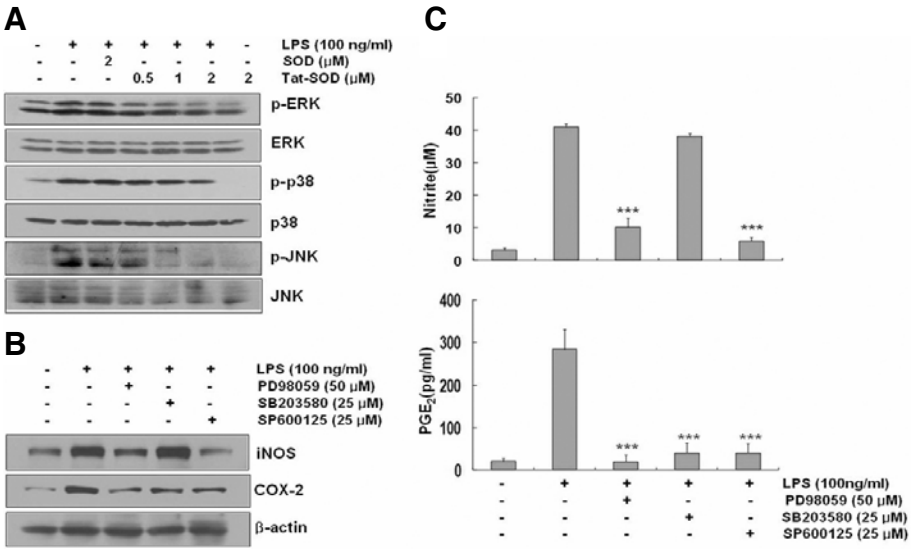

Fig. 4. Inhibition of LPS-induced MAP kinase activation by Tat-SOD in BV-2 cells. (A) Effect of SOD fusion proteins on the MAP Kinase activation in BV-2 cells stimulated with LPS (100 ng/ml) for 1 h with or without pretreatment with Tat-SOD or SOD fusion protein for 1 h. Cell extracts were prepared and analyzed for MAP kinase protein activation by immunoblot using phospho-specific antibodies against MAPK proteins. (B) BV-2 cells were pretreated with various MAPK inhibitors for 1 h and stimulated with LPS (100 ng/ml) for 12 h. Cell extracts were prepared and analyzed by immunoblotting for iNOS and COX-2 protein expression. (C) BV-2 cells were pretreated with various MAPK inhibitors for 1 h and stimulated with LPS (100 ng/ml) for 18 h. Culture medium was harvested and levels of NO and PGE_2 production in the culture medium determined as described in Materials and Methods. The data are the mean ± SEM of 3 separate experiments. In (C), ***P < 0.001 compared with cells treated with LPS alone.

(Chauhan et al., 2007; Dietz and Bähr, 2004).

In summary, we observed that treatment with cell-permeable SOD not only suppressed LPS-induced expression of iNOS and COX-2 but also inhibited LPS-induced production of NO and PGE_2 by BV-2 microglial cells. We demonstrated that SOD inhibited activation of NF-κB binding activity and IκBα phosphorylation in LPS-stimulated BV-2 cells. Furthermore, SOD inhibited LPS-induced activation of MAP kinases including ERK, JNK and p38 in BV-2 cells. These results collectively suggest that SOD may exert anti-inflammatory responses by inhibiting expression of pro-inflammatory mediators by microglial cells in the brain. Therefore, the cell-permeable Tat-SOD may be useful in the treatment of ROS-related neurological disorders.

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