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Apomorphine prevents LPS-induced IL-23 p19 mRNA expression via inhibition of JNK and ATF4 in HAPI cells

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ABSTRACT

Inflammation has been reported to be closely related to exaggeration of cerebral ischemia and neurodegenerative diseases. Microglia, resident immune cells in the central nervous system, can be activated in response to neuronal injury and produce proinflammatory cytokines, resulting in further aggravation of neuronal injury. Interleukin (IL)-23, which consists of p19 and IL-12 p40 subunits, has been shown to be involved in brain injury associated with neuroinflammation. Apomorphine (Apo), a nonselective dopamine receptor agonist, has been used for clinical therapy of Parkinson's disease. Besides the pharmacological effect, Apo is known to have pleiotropic biological functions. In this study, to elucidate the effect of Apo on lipopolysaccharide (LPS)-induced IL-23 p19 mRNA expression in microglial cell line HAPI cells, we pretreated cells with various concentrations of Apo (10 – 30 μM) for 8, 16, and 24 h, followed by exposure to LPS (100 ng/ml). Pretreatment with Apo dose- and time-dependently suppressed the induction of IL-23 p19 mRNA. However, this effect of Apo was exerted independently of dopamine receptors. JNK and ATF4, an endoplasmic reticulum (ER) stress-inducible transcription factor, were involved in expression of LPS-induced IL-23 p19 mRNA. Pretreatment with Apo (30 μM) for 24 h inhibited LPS-induced activation of JNK and the nuclear accumulation of ATF4. Thapsigargin (Tg), an ER stress inducer, stimulated IL-23 p19 mRNA expression via an ATF4 dependent mechanism. We also found that Apo inhibited Tg-induced ATF4 accumulation and IL-23 p19 mRNA expression. Taken together, our findings suggest that Apo exerts anti-inflammatory effects through inhibition of JNK and ATF4 signaling pathways.

1. Introduction

There is growing evidence that inflammation is closely associated with cerebral ischemia and neurodegenerative and neuroimmunological diseases. Microglia are resident immune cells in the central nervous system (CNS) and play a critical role in the maintenance of neuronal functions in the healthy brain. However, under pathological conditions, microglia are activated in response to neuronal injury (Weinstein et al., 2010). Activated microglia produce injurious molecules, such as nitric oxide (NO), superoxide, and proinflammatory cytokines, leading to further aggravation of neuronal injury (Bal-Price and Brown, 2001; Knoch et al., 2008). Several reports have demonstrated that post-ischemic inflammation participates in brain damage following cerebral ischemia (Hua et al., 2007; Shichita et al., 2009).

Interleukin (IL)-23 is a heterodimeric cytokine composed of the p19 subunit unique to IL-23 and the p40 subunit common to IL-12, and is produced by antigen-presenting cells such as macrophages, dendritic cells, and microglia. IL-23 stimulates differentiation into IL-17-producing T helper (Th17) cells (Aggarwal et al., 2003). The IL-23/Th17 axis is thought to play a critical role in neuroinflammation. Blockade of IL-23 or its downstream factor IL-17 ameliorates experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (Cua et al., 2003; Park et al., 2005). Li et al. (Li et al., 2007) reported that activated macrophages/microglia produce IL-23 p19 in active and chronic active multiple sclerosis lesions. In IL-23 p19 or IL-17 knockout mice, cerebral infarct volume resulting from cerebral ischemic-reperfusion is reduced (Shichita et al., 2009). These findings suggest that microglial activation and subsequent production of pro-inflammatory cytokines, such as IL-23, are closely related to the progression of inflammation in autoimmune disorders and ischemic brain injury.

Apomorphine (Apo), a nonselective dopamine receptor agonist, has been used for clinical therapy of Parkinson's disease (Lees, 1993). Besides the pharmacological effects, Apo has been reported to have pleiotropic biological functions. Indeed, Apo markedly increases the expression of neurotrophic factors such as nerve growth factor in mouse astrocytes (Ohta et al., 2000). It has been demonstrated that Apo prevents neuronal cell death associated with oxidative stress

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induced by hydrogen peroxide or the neurotoxin 6-hydroxydopamine (Gassen et al., 1998; Hara et al., 2006, 2003). Subcutaneous administration of Apo significantly attenuates dysfunction of motor neurons in the SOD1(G93A) transgenic mouse model of amyotrophic lateral sclerosis (Mead et al., 2013). These effects of Apo are thought to be exerted independently of dopamine receptors.

Targeting IL-23 may provide a therapeutic benefit for the aforementioned disorders. Although Apo can directly protect neuronal cells from oxidative stress, whether Apo suppresses microglia-mediated neuroinflammation has not been investigated to date. Lipopolysaccharide (LPS) has been reported to induce IL-23 p19 gene expression via Toll-like receptor 4 (TLR4) (Liu et al., 2009). Therefore, in this study, we examined the effect of Apo on LPS-induced IL-23 p19 mRNA expression in microglial cell line HAPI cells, which are well known to exhibit the properties of microglia/brain macrophages (Cheepsunthorn et al., 2001).

2. Materials and methods

2.1. Materials

Apo and thapsigargin (Tg) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dopamine was purchased from Wako Pure Chemical (Osaka, Japan). SCH23390 was purchased from Tocris Bioscience (Bristol, United Kingdom). Anti-activating transcription factor 4 (ATF4) and anti-NF-κB antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-JNK (Thr183/Thr185), anti-JNK, and anti-phospho-MKK4 (Ser257) antibodies and calyculin A (Cal A) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-actin antibody was purchased from Millipore (Billerica, MA, USA).

2.2. Cell culture

Rat immortalized microglia HAPI cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum, 4 mM glutamine, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin in a humidified 5% CO_2/95% air incubator at 37 °C.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

After the treatment, the cells were washed with phosphate-buffered saline (PBS) and total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 4 μg of total RNA. Target genes were amplified using primers specific for rat IL-23 p19 (forward primer, 5′-ACACTAGCCTGGAGTGCACA-3′; reverse primer, 5′-GAGGCATC TGTTGAGTCTCC-3′), ATF4 (forward primer, 5′-TCGGA TCCATGACCGAAATGAGCTTCCT-3′; reverse primer, 5′-CTT-CAGTGATATCCACTTCA-3′), dopamine D_1 receptor (forward primer, 5′-TCCTGATTAGCGTAGCATGG-3′; reverse primer, 5′-CTCCCTCTTGAAGGACATCT-3′), dopamine D_2 receptor (forward primer, 5′-GCCTACATAGCAACCCTGAC-3′; reverse primer, 5′-CCATGTGAAGGCGCTGTAGA-3′), dopamine D_4 receptor (forward primer, 5′-TGTCCGCTCATGCTACTGCT-3′; reverse primer, 5′-GCTCCCTTCCAGTGATCTTG-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer, 5′-ACCACAGTCCATG CCATCAC-3′; reverse primer, 5′-TCCACCACCCTGTTGCTGTA-3′). PCR amplification of IL-23 p19 and ATF4 was conducted using EX Taq polymerase (Takara Bio, Otsu, Japan) as follows: 2 min at 94 °C, 1 cycle; 40 s at 94 °C, 40 s at 58 °C, and 1 min at 72 °C, 30 cycles. PCR amplification of GAPDH and dopamine receptors were conducted using Taq DNA polymerase (Invitrogen) as follows: 2 min at 94 °C, 1 cycle; 40 s at 94 °C, 40 s at 58 °C, and 1 min at 72 °C, 18 and 30 cycles, respectively. We ascertained that there were linear correlations between the amounts of the PCR products and the template cDNAs. Aliquots of the PCR mixtures were separated on a 2% agarose gel and stained with ethidium bromide. Densitometric analyses were performed using the Multi Gauge software (Fuji Film, Tokyo, Japan). The mRNA levels were normalized relative to the GAPDH mRNA level of each sample.

2.4. Preparation of whole-cell lysates and nuclear extracts

After the treatment, cells were washed twice with ice-cold PBS. For the preparation of whole-cell lysates, the cells were collected using 150 μl of lysis buffer (20 mM Tris–HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT)), and lysed on ice for 30 min. The lysates were centrifuged at 18,000*g* for 10 min at 4 °C to remove cellular debris. For the preparation of nuclear extracts, the cells were collected using buffer A (20 mM HEPES–NaOH, pH 7.8, containing 15 mM KCl, 2 mM $MgCl_2$, 5 μg/ml leupeptin, 0.5 mM PMSF, and 2 mM DTT) and then centrifuged at 800*g* for 30 s at 4 °C. The cells were lysed in buffer B (buffer A containing 0.2% Nonidet P-40) for 5 min on ice, and then centrifuged at 10,000*g* for 30 s at 4 °C. Finally, the pellets were suspended in 20 mM HEPES–NaOH, pH 7.8, containing 0.4 M NaCl, 10% glycerol, 5 μg/ml leupeptin, 0.5 mM PMSF, and 2 mM DTT and stood on ice for 30 min. The nuclear extracts were centrifuged at 18,000*g* for 10 min at 4 °C to remove cellular debris. The protein concentration of the supernatants was determined using Bio-Rad protein assay reagent.

2.5. Western blotting

Whole-cell lysates (30 μg) or nuclear extracts (20 μg) were subjected to Western blot analysis. Western blotting was performed with SDS-PAGE as previously described (Doi et al., 2015). Proteins were detected using Super-signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA) or ImmunoStar LD (Wako Pure Chemical) and imaged using an LAS-3000 (Fuji Film).

2.6. siRNA transfection

HAPI cells were seeded in a 6-cm culture dish at a cell density of 6×10^5 cells/dish. The next day, cells were transfected with ATF4 (RSS340173, Thermo Fisher Scientific) or negative control (12935-112, Thermo Fisher Scientific) siRNAs using Lipofectamine RNAiMAX transfection reagent (Invitrogen). Twenty-four hours later, the culture medium was replaced with fresh medium and the cells were treated with LPS or Tg.

2.7. Statistical analysis

Data were analyzed using ANOVA followed by *post hoc* Bonferroni tests or Student's *t*-test. A P value of less than 0.05 was considered significant.

3. Results

3.1. Inhibitory effects of Apo on LPS-induced IL-23 p19 mRNA in HAPI cells

First, we examined the effect of LPS on IL-23 p19 mRNA expression in HAPI cells. As shown in Fig. 1A, LPS induced IL-23 p19 mRNA expression, which reached peak expression at 3 h. Therefore, in this study, we treated HAPI cells with LPS at 3 h. We have previously demonstrated that Apo has preconditioning effects against oxidative stress- and zinc-triggered toxicity (Hara et al., 2013, 2006). To determine the effects of pretreatment with Apo on LPS-induced IL-

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