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Anti-inflammatory effects of noradrenaline on LPS-treated microglial cells: Suppression of NFκB nuclear translocation and subsequent STAT1 phosphorylation

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ABSTRACT

Noradrenaline (NA) has marked anti-inflammatory effects on activated microglial cells. The present study was conducted to elucidate the mechanisms underlying the NA effects using rat primary cultured microglial cells. NA, an α1 agonist, phenylephrine (Phe) and a β2 agonist, terbutaline (Ter) suppressed lipopolysaccharide (LPS)-induced nitric oxide (NO) release by microglia and prevented neuronal degeneration in LPS-treated neuron-microglia coculture. The agents suppressed expression of mRNA encoding proinflammatory mediators. Both an α1-selective blocker terazocine and a β2-selective blocker butoxamine overcame the suppressive effects of NA. cAMP-dependent kinase (PKA) inhibitors did not abolish the suppressive NA effects. LPS decreased IκB leading to NFκB translocation into nuclei, then induced phosphorylation of signal transducer and activator of transcription 1 (STAT1) and expression of interferon regulatory factor 1 (IRF1). NA inhibited LPS-induced these changes. When NFκB expression was knocked down with siRNA, LPS-induced STAT1 phosphorylation and IRF1 expression was abolished. NA did not suppress IL-6 induced STAT1 phosphorylation and IRF1 expression. These results suggest that one of the critical mechanisms underlying the anti-inflammatory effects of NA is the inhibition of NFκB translocation. Although inhibitory effects of NA on STAT1 phosphorylation and IRF1 expression may contribute to the overall suppressive effects of NA, these may be the downstream events of inhibitory effects on NFκB. Since NA, Phe and Ter exerted almost the same effects and PKA inhibitors did not show significant antagonistic effects, the suppression by NA might not be dependent on specific adrenergic receptors and cAMP-dependent signaling pathway.

1. Introduction

Noradrenaline (NA) has been extensively documented for its immunosuppressive actions on microglial cells (Chang and Liu, 2000; Heneka et al., 2010; Kong et al., 2010; Madrigal et al., 2005; Markus et al., 2010; Mori et al., 2002; Qian et al., 2011). Microglial cells express adrenergic receptors (ARs) (Mori et al., 2002; Tanaka et al., 2002), and NA or other adrenergic agonists can suppress expression of proinflammatory cytokines and inducible nitric oxide synthase (iNOS) by lipopolysaccharide (LPS)-treated microglial cells. In Alzheimer's disease (AD), neurons in the locus ceruleus (LC), a major source of NA in the brain, degenerate during the course of AD pathogenesis. Degeneration of LC neurons causes the marked reduction of NA levels in the brain, which could be linked to microglial proinflammatory activities, which is thought to be one of the critical causes of progressive neuronal degeneration in AD (Heneka et al., 2010). Furthermore, reduced NA levels has been implicated in the impaired clearance of amyloid β (Aβ) by microglia, followed by elevated Aβ deposition in the cerebrum and hippocampus in AD brains (Heneka et al., 2010; Kong et al., 2010). Similar observations have been made in brains of Parkinson's disease patients (Zarow et al., 2003).

These effects of NA on microglial cells have been attributed to signaling through β-ARs. More specifically, β2-AR is thought to play a critical role in the suppression of microglial proinflammatory

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activities (Farmer and Pugin, 2000; Fujita et al., 1998; Markus et al., 2010; McNamee et al., 2010; Qian et al., 2009, 2011). These proinflammatory activities include the synthesis and release of proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and oxidative molecules, such as NO and superoxide anion (Choudhury et al., 2011; Qian et al., 2009; Tanaka et al., 1998). These molecules are potentially harmful to neurons. β-ARs increase intracellular cAMP levels, resulting in the suppression of microglial proinflammatory responses (Farmer and Pugin, 2000). Indeed, a cAMP analogue, 8Br-cAMP, or cAMP elevating agents, such as forskolin or phosphodiesterase inhibitors, down-regulate the expression of proinflammatory cytokines and iNOS (Woo et al., 2004; Zhang et al., 2002).

However, NA is a much weaker agonist for β2-AR than isoproterenol or adrenaline (Poyet et al., 1986). NA much less potently elevates cAMP levels compared to adrenaline. Additionally, NA preferentially binds to α1-AR, which has an affinity of a comparable level as an α1-adrenergic agonist, phenylephrine (Phe) (Minneman, 1983). Previously, we showed that Phe suppressed LPS-induced production of NO and TNF-α by microglial cells, without significant effects on intracellular cAMP levels (Mori et al., 2002). A few studies have mentioned the suppressive effects of Phe on LPS-induced proinflammatory actions *in vitro* (Chang and Liu, 2000) and *in vivo* (Markus et al., 2010). Based on the affinity of NA to ARs, it might be possible that NA exerts its anti-inflammatory effects through α1-AR rather than β2-AR. Furthermore, the molecular mechanisms underlying the suppressive effects of NA and other adrenergic agonists on microglial cells remain to be elucidated. Although expression of α2-AR by microglial cells has been demonstrated, an α2-adrenergic agonist clonidine does not mediate immunosuppressive actions (Mori et al., 2002).

In this study, we found that NA, Phe and a β2-adrenergic agonist terbutaline (Ter) similarly exert neuroprotective effects in LPS-treated neuron–microglia cocultures by suppressing iNOS expression. These agents suppressed LPS-induced NFκB nuclear translocation in microglial cells and the subsequent phosphorylation of signal transducer and activator of transcription 1 (STAT1) and up-regulation of interferon regulatory factor 1 (IRF1) expression. These activities of AR agonists are thought to be critical for their suppressive effects on microglial cells.

2. Materials and methods

2.1. Chemicals

Adrenergic agonists, NA and Phe, Clo and Ter, and an α1-AR blocker, terazocine (Trz), an α2-AR blocker, Yohimbine (Yoh), a β2-AR blocker, butoxamine (But), and a cAMP-dependent protein kinase (PKA) inhibitor, H89 were purchased from Sigma–Aldrich (St Louis, MO, USA). Another PKA inhibitor KT5720 was from Tocris Bioscience (Bristol, UK). Diethylenetriaamine NONOate (DETA NONOate) was obtained from Cayman Chemicals (Ann Arbor, MI, USA).

2.2. Cell culture

All experiments involving animals were conducted in accordance with the Guide for Animal Experimentation at Ehime University School of Medicine. Rat primary microglial cultures were prepared as reported elsewhere (Tanaka et al., 1998). Briefly, whole forebrains from neonatal rats were dissociated into individual cells that were cultured for 11 or 14 days as mixed glial cultures with 10% fetal calf serum (FCS)-supplemented Dulbecco's Modified Eagle's Medium (DMEM; Wako, Osaka, Japan). Microglial cells were purified from the culture to a purity of >99% (Yokoyama et al., 2004). In most experiments, purified microglial cells were cultured in serum-free E2 medium [DMEM containing 10 mM HEPES (pH 7.3; Gibco, Grand Island, NY), 4.5 mg/ml glucose, 5 μg/ml insulin, 5 nM sodium selenite, 5 μg/ml transferrin (Gibco) and 0.2 mg/ml bovine serum albumin (Sigma)]. For experiments to determine the expression levels of mRNA encoding ARs, some microglial cells were cultured in Eagle's Minimum Essential Medium (EMEM) containing either no additions, L-serine (L-Ser) or 1% FCS, or were cultured in DMEM with 3% FCS.

Cortical neurons dissociated from the cerebral cortices of 17-day-old rat embryos were seeded on poly-L-lysine (PLL)-coated 4-well culture plates at a density of 250,000 cells/cm^2 (Tanaka et al., 1999); these neurons were cultured in DMEM with 10% FCS for the first day. On the second day, the medium was exchanged with E2 medium. When neuron–microglia cocultures were prepared, microglial cells (100,000 cells/cm^2) were seeded on neurons on the second day.

LPS (from *Escherichia coli* serotype 055:B5; Sigma) was added at 1 μg/ml to the culture medium to activate microglial cells (Tanaka et al., 1998). In some experiments, adrenergic agonists and/or antagonists were added along with LPS. The effects of NA on IL-6-induced phosphorylation of STAT1 were evaluated 45 min after the addition of IL-6 (10 ng/ml; PeproTech, Rocky Hill, NJ, USA) into the microglia cultures. In this experiment, NA was added 30 min before the addition of IL-6.

2.3. NONOate-induced neuronal cell death

A NO donor, DETA NONOate, liberates 2 mol of NO per 1 mol into a culture medium with a half-life of 20 h. Primary neuronal cultures were incubated with E2 containing DETA NONOate (0–300 μM) for 48 h, followed by immunoblotting to detect synapsin I, which was performed to evaluate neuron viability.

2.4. Conventional and quantitative real-time RT-PCR (qPCR)

Cells from pure microglial cultures or neuron–microglia cocultures were lysed with ISOGEN.

(Nippon gene, Tokyo, Japan). Total RNA was collected. Then, cDNA was generated from DNase-I-treated RNA by reverse transcription using an oligo-(dT) 15 primer, as previously described (Takahashi et al., 2008). GoTaq DNA polymerase (Promega, Madison, WI, USA) was used to amplify each gene cDNA fragment from cDNA isolated from cultured macrophages. Amplified DNA fragments were visualized by agarose gel electrophoresis as previously described (Mori et al., 2002). Quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis was performed in triplicate using a MJ mini instrument (Bio-Rad, Hercules, CA, USA) using Fast Start Universal SYBR Green (Roche Diagnostic Japan, Tokyo, Japan) as described elsewhere (Sugimoto et al., 2014). All gene-specific mRNA expression values were normalized against those of *GAPDH* mRNA transcripts. Primer sequences for each gene are listed in Table 1.

2.5. Determination of nitrite release

Conditioned media of pure microglial cultures or neuron–microglia cocultures that were maintained in 24-well PLL-coated plates (Corning) for 48 h in E2 containing 1 μg/ml LPS were subjected to measurement of nitrite to evaluate LPS-induced NO release. The nitrite assay was carried out based on the Griess reaction, as described elsewhere (Zhang et al., 2002).

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