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Interaction of inflammatory and anti-inflammatory responses in microglia by *Staphylococcus aureus*-derived lipoteichoic acid

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ABSTRACT

We investigated the interaction between proinflammatory and inflammatory responses caused by *Staphylococcus aureus*-derived lipoteichoic acid (LTA) in primary cultured microglial cells and BV-2 microglia. LTA induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein levels increase in a concentration- and time-dependent manner. Meanwhile, LTA also increased nitric oxide (NO) and PGE_2 production in microglia. Administration of TLR2 antagonist effectively inhibited LTA-induced NO, iNOS, and COX-2 expression. Moreover, treatment of cells with LTA caused a time-dependent activation of ERK, p38, JNK, as well as AKT. We also found that LTA-induced iNOS and COX-2 up-regulation were attenuated by p38, JNK, and PI3-kinase inhibitors. On the other hand, LTA-enhanced HO-1 expression was attenuated by p38 and PI3-kinase inhibitors. Treatment of cells with NF-κB and AP-1 inhibitors antagonized LTA-induced iNOS and COX-2 expression. However, only NF-κB inhibitors reduced LTA-induced HO-1 expression in microglia. Furthermore, stimulation of cells with LTA also activated IκBα phosphorylation, p65 phosphorylation at Ser^{536}, and c-Jun phosphorylation. Moreover, LTA-induced increases of κB-DNA and AP-1-DNA binding activity were inhibited by p38, JNK, and PI3-kinase inhibitors. HO-1 activator CoPP IX dramatically reversed LTA-induced iNOS expression. Our results provided mechanisms linking LTA and inflammation/anti-inflammation, and indicated that LTA plays a regulatory role in microglia activation.

Introduction

Bacteria stimulate the innate immune system of a host and the release of inflammatory molecules such as cytokines and chemokines as a response to infection (Medzhitov and Janeway, 1998). Lipopolysaccharide (LPS) from Gram negative bacteria is a well-known activator of the innate immune system. With regard to Gram-positive infection, when no endotoxin is present, lipoteichoic acid (LTA), the major component of the cell wall of Gram-positive bacteria, activates the innate immune system of the host and induces the release of chemokines and cytokines (Hsieh et al., 2010; Tang et al., 2010). These inflammatory molecules are the major causes of the various signs and symptoms that occurred during bacterial infection, including fever, inflammation, and acute phase responses (Dziarski et al., 2000; Ulevitch and Tobias, 1995). It has been reported that LTA exerts multiple effects on regulation of immune responses in astrocytes (Hsieh et al., 2010, 2012). Even though previous reports showed that LTA mediates TLR2 signaling, which induces glial inflammatory activation (Carpentier et al., 2008). Little is known about how LTA regulates induction of the inflammatory mediators in microglia.

Lots of evidence has revealed that microglia plays a most important role in host defense and tissue repair in the CNS (Graeber et al., 2011). Microglia has been implicated as the predominant cell type and un-controlled activation of microglia is capable of producing a variety of inflammatory mediators and potential neurotoxic compounds, which govern inflammation-mediated neuronal damage (Polazzi and Monti, 2010). The microglia-related immune responses must be tightly regulated to avoid over activation and disastrous

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neurotoxic consequences (Tian et al., 2012). Over activation of microglial cells may cause severe brain tissue damages in various neurodegenerative diseases (Czeh et al., 2011). Microglia activation involves morphologic changes and synthesis of new inflammatory proteins, such as iNOS and COX-2 (Gebicke-Haerter, 2001). The mechanism by which activated glial cells induce neuronal cell death has been shown to involve nitric oxide, reactive oxygen species and prostaglandins (Bal-Price and Brown, 2001). It has been reported that iNOS and COX-2 are induced in various types of CNS injuries and diseases (Hunot et al., 1996; Teismann et al., 2003). These two enzymes are often co-expressed in disease states associated with gliosis and in glial cells of substantia nigra of post-mortem Parkinson's patients (Knott et al., 2000).

Heme oxygenase (HO) is a rate-limiting enzyme that converses heme to CO, iron and biliverdin. There are two isoforms of HO that can be found in the human brain. HO-1 is an inducible form of heat shock protein found in large quantity in brain or other tissues, and it can be rapidly induced by various oxidative-inducing agents (Otterbein and Choi, 2000), while HO-2 expresses constitutively. Numerous reports have shown that HO-1 plays an important role in neuroprotective effect (Liu et al., 2012). Recently, we have reported that induction of HO-1 expression exerts anti-neuroinflammation and neuroprotection in microglia (Lu et al., 2010, 2013), astrocytes (Chen et al., 2012), and neurons (Lu et al., 2012). These findings suggest that HO-1 may act as an endogenous antioxidant protein to regulate inflammatory response and oxidative states.

Although the molecular mechanisms of downstream of LPS are well known in several cell types including macrophages and microglia, the signaling pathways related to LTA-induced inflammatory cytokine expression have not yet been investigated thoroughly in microglia. Therefore, in the present study, we showed that the regulation mechanisms of LTA in neuroinflammatory responses involve changes of iNOS, COX-2, and HO-1 expression in microglia.

Materials and methods

Materials. LTA (derived from *Staphylococcus aureus*), curcumin, PDTC, Tanshinone IIA and Bay 11-7082 were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), and OPTI-MEM were purchased from Invitrogen (Carlsbad, CA). Secondary antibodies, primary antibodies against β-actin, IκBα, p65, ERK2, phosphorylated ERK1/2, p38, JNK, TLR2 and TLR4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibody against HO-1 was purchased from StressGen Biotechnologies (Victoria, BC, Canada). Primary antibody against iNOS was purchased from BD Transduction Lab (Lexington, KY). Primary antibody against COX-2 was purchased from Cayman Chemicals (Ann Arbor, MI). Primary antibodies against phosphorylated p38, phosphorylated JNK, phosphorylated p65 at Ser^{536}, AMPK phosphorylated at Thr172 and Ser485, CaMKII phosphorylated at Thr286, and LKB1 phosphorylated at Ser428 were purchased from Cell Signaling and Neuroscience (Danvers, MA). CoPP IX was purchased from Calbiochem (San Diego, CA). The electrophoretic mobility shift assay (EMSA) gel shift kit was purchased from Panomics (Redwood City, CA).

Cell culture. All protocols for animal experiments were approved by the Animal Care Committee of China Medical University. Sprague–Dawley rats were obtained from the National Laboratory Animal Center of Taiwan. Rat primary microglia cultures were prepared according to our previous reports (Lin et al., 2011; Lu et al., 2010). Briefly, mixed glial cells were cultured in 75 cm^2 flasks for 12–14 days in DMEM/F12 (Gibco, Grand Island, NY) and microglia cells were separated by a rotary shaker. Cells were passed through a 70 μm nylon mesh filter (Falcon, Belgium) and seeded into 24-well plates at a density of 5×10^5 cells/well. The purity of microglia cultures was assessed by using Iba-1 antibody (Serotec, Oxford, UK), and cells were cultured in DMEM with 2% FBS for two days before drug treatment. The murine BV-2 cell line was cultured in DMEM with 10% FBS at 37 °C in a humidified incubator under 5% CO_2 and 95% air.

Assay of nitric oxide. Production of nitric oxide was assayed by measuring the nitrite levels of nitric oxide metabolite in culture medium which was determined by colorimetric assay with Griess reagent. Equal volume of culture supernatant reacted with Griess reagent (one part 0.1% naphthylethylenediamine and one part 1% sulfanilamide in 5% H_3PO_4). The absorbance was determined using a microplate reader (Bio-Tek, Winooski, VT) at 550 nm.

Measurement of PGE_2 production. Secreted PGE_2 in the medium was assayed with PGE_2 ELISA kit (Cayman Chemicals, Ann Arbor, MI), according to the manufacturer's procedure.

Western blot analysis. Microglia were treated with LTA for indicated time periods and lysed with radioimmunoprecipitation assay buffer. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk and probed with primary antibodies. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and a shareware Image J.

Preparation of nuclear extracts. The nuclear extract preparation was according to the previous report (Tsai et al., 2012) and has modified. Cells were suspended in buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice. The lysates were separated into cytosolic and nuclear fractions by centrifugation at 12,000 *g* for 10 min. The supernatants containing cytosolic proteins were collected. A pellet containing nuclear fraction was re-suspended in buffer C (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 0.4 M NaCl) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 13,000 *g* for 20 min and stored at −80 °C.

Electrophoretic mobility shift assay (EMSA). EMSA assay was performed according to the manufacturer's protocol. Briefly, nuclear extracts (2 μg) of cells were incubated with poly d (I-C) and then incubated with biotin-labeled probes. After electrophoresis on polyacrylamide gel, samples were transferred onto a presoaked Immobilon-Nyt membrane (Millipore, Billerica, MA). The membrane was cross-linked in an oven and developed with streptavidin–horseradish peroxidase conjugate before Western blot analysis.

Statistics. The values given are means ± S.E.M. The significance of difference between the experimental group and control was assessed by Student's *t* test. The difference was considered to be significant if the p value is less than 0.05.

Results

LTA induced the expressions of iNOS, COX-2 and HO-1 in microglia

Stimulation of LTA with various concentrations (5, 10 or 20 μg/ml for 24 h) or for indicated time periods (0, 4, 8, 16 or 24 h with a concentration of 10 μg/ml) increased iNOS, COX-2 and HO-1 expression in BV-2 microglial cells (Figs. 1A and B). Furthermore, LTA also increased iNOS, COX-2 and HO-1 up-regulation in rat primary cultured microglia (Fig. 1C). Moreover, stimulation of LTA with various concentrations (5, 10 or 20 μg/ml for 24 h) increased the secretion of nitric oxide (Suppl. Fig. 1) and PGE_2 (Fig. 1D) in BV-2 microglia as well. Additionally, LTA-induced iNOS expression is similar in BV-2 microglia and primary