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Lipoteichoic acid-induced nitric oxide synthase expression in RAW 264.7 macrophages is mediated by cyclooxygenase-2, prostaglandin E_2, protein kinase A, p38 MAPK, and nuclear factor-κB pathways

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Abstract

We recently reported that lipoteichoic acid (LTA), a cell wall component of the gram-positive bacterium *Staphylococcus aureus*, stimulated inducible nitric oxide synthase (iNOS) expression, nitric oxide (NO) release, and cyclooxygenase-2 (COX-2) expression in RAW 264.7 macrophages. This study was carried out to further investigate the roles of COX-2 and prostaglandin E_2 (PGE_2) in LTA-induced iNOS expression and NO release in RAW 264.7 macrophages. Treatment of RAW 264.7 macrophages with LTA caused a time-dependent increase in PGE_2 release. LTA-induced iNOS expression and NO release were inhibited by a non-selective COX inhibitor (indomethacin), a selective COX-2 inhibitor (NS-398), an adenylyl cyclase (AC) inhibitor (dideoxyadenosine, DDA), and a protein kinase A (PKA) inhibitor (KT-5720). Furthermore, both PGE_2 and the direct PKA activator, dibutyryl-cAMP, also induced iNOS expression in a concentration-dependent manner. Stimulation of RAW 264.7 macrophages with LTA, PGE_2, and dibutyryl-cAMP all caused p38 MAPK activation in a time-dependent manner. LTA-mediated p38 MAPK activation was inhibited by indomethacin, NS-398, and SB 203580, but not by PD 98059. The PGE_2-mediated p38 MAPK activation was inhibited by DDA, KT-5720, and SB 203580, but not by PD 98059. LTA caused time-dependent activation of the nuclear factor-κB (NF-κB)-specific DNA–protein complex formation. The LTA-induced increase in κB-luciferase activity was inhibited by indomethacin, NS-398, KT-5720, and a dominant negative mutant of p38 αMAPK (p38 αMAPK DN). These results suggest that LTA-induced iNOS expression and NO release involve COX-2-generated PGE_2 production, and AC, PKA, p38 MAPK, and NF-κB activation in RAW 264.7 macrophages.
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1. Introduction

Nitric oxide (NO) has been proven to be an important signaling molecule involved in regulating a wide range of biological activities in vascular, neural, and immune systems [1]. NO, formed from L-arginine by NO synthase (NOS), is generated by many cell types [2,3]. Several isoforms of NOS have been isolated, purified, cloned, and expressed [2,4]. Endotoxin and cytokines induce macrophages to express an

Abbreviations: AC, adenylyl cyclase; COX-2, cyclooxygenase-2; DDA, dideoxyadenosine; DMEM/Ham's F-12, Dulbecco's modified Eagle's medium/Ham's F-12; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NO, nitric oxide; NF-κB, nuclear factor-κB; PGE_2, prostaglandin E_2; PKA, protein kinase A.

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isoform of NOS [inducible NOS (iNOS), which is calcium independent] which through the generation of NO, plays an important role in the cytotoxicity of activated macrophages against microbes and tumor cells [5] and contributes to the circulatory failure associated with shock due to sepsis [6] and hemorrhage [7]. The iNOS protein has been identified in a wide variety of cell types including macrophages, mesangial cells, vascular smooth muscle cells, keratinocytes, chondrocytes, osteoclasts, and hepatocytes and can be induced by many immune stimuli [1,8]. Changes in NO formation in iNOS-expressing cells usually correlate with similar changes in iNOS mRNA levels, indicating that a major part of iNOS regulation occurs at the transcriptional level. The promoter region of the iNOS gene contains several binding sites for transcriptional factors, such as nuclear factor-κB (NF-κB) and activator protein-1, as well as for various members of the CCAAT/enhancer-binding protein, activating transcription factor/cAMP response element-binding protein, and Stat family of transcriptional factors [9]. Our previous reports showed that NF-κB appeared to be essential for iNOS expression seen in RAW 264.7 macrophages exposed to lipoteichoic acid (LTA) [10,11].

Endotoxin, a component of the outer membrane of gram-negative bacteria, has been identified as the prime initiator of gram-negative bacterial septic shock. In contrast to endotoxic shock, we know relatively little about the mechanisms of gram-positive bacteria-induced septic shock. LTA, a component of the membrane of gram-positive bacteria, induces iNOS expression in vascular smooth muscle cells and macrophages [12–14]. LTA also diminishes the contractile response to vasoconstrictor agents in rings from human arteries [15] and produces circulatory failure such as hypotension and vascular hyporeactivity to vasoconstrictor agents in rats by inducing iNOS protein and activity [16].

The intracellular signaling pathways through which LTA causes iNOS expression in macrophages involve a series of events resulting in the transmission of the signal from the plasma membrane through the cytoplasm to the nucleus, where iNOS gene expression is upregulated. Our previous studies showed that treatment of RAW 264.7 macrophages with LTA causes iNOS expression and NO release through two separate pathways: the phosphatidylcholine-phospholipase C (PC-PLC), phosphatidylinositol-phospholipase C (PI-PLC)/protein kinase C (PKC)/NF-κB cascade and the tyrosine kinase (TK)/phosphatidylinositiol 3-kinase (PI3K)/Akt/p38 mitogen-activated protein kinase (MAPK)/NF-κB cascade [10,11]. Previous studies have shown a positive association between endogenous PGE_2 production and iNOS expression both in vitro [17,18] and in vivo [19]. However, the signaling pathway between COX-2 induction and iNOS expression caused by LTA is still unknown. An increase in intracellular cAMP levels is an important intracellular signaling mechanism involved in the regulation of gene expression. Certain in vitro studies have shown that an increase in cAMP levels causes iNOS induction [18,20,21]. In the present study, we further explored the intracellular signaling pathway for activation of the LTA-induced adenylyl cyclase (AC)/cAMP/PKA pathway and its involvement in LTA-stimulated iNOS expression and NO production in RAW 264.7 macrophages. The results showed that LTA can cause activation of the AC/cAMP/PKA pathway by induction of COX-2 protein and formation of PGE_2, resulting in the activations of p38 MAPK and NF-κB, which in turn induce iNOS expression and NO production. The signaling pathway explored in this study had a delayed onset, whereas two separate pathways which induced NF-κB activation in RAW 264.7 macrophages we previously reported have a rapid onset [10,11].

2. Materials and methods

2.1. Materials

Lipoteichoic acid (LTA derived from *Staphylococcus aureus*), sulfanilamide, *N*-(1-naphthyl)-ethylenediamine, Trizma base, dithiothreitol (DTT), glycerol, phenylmethylsulphonyl fluoride (PMSF), pepstatin A, leupeptin, and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO). Dibutyryl-cAMP, indomethacin, NS-398, dideoxyadenosine (DDA), KT-5720, PD 98059, and SB 203580 were purchased from Calbiochem (San Diego, CA). Penicillin/streptomycin, fetal calf serum (FCS), T4 polynucleotide kinase, and Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/Ham's F-12) were purchased from Life Technologies (Gaithersburg, MD). A PGE_2 enzyme immunoassay kit was obtained from Cayman (Ann Arbor, MI). Rabbit polyclonal antibodies specific for iNOS, p38 MAPK, as well as horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody specific for COX-2 was purchased from BD Transduction Laboratories (Lexington, KY). An antibody specific for phospho-p38 MAPK was purchased from Cell Signaling Biotechnology (Beverly, MA). An antibody specific for α-tubulin was purchased from Oncogene Science (Cambridge, UK). Anti-mouse-IgG-conjugated alkaline phosphatase was purchased from Jackson Immuno Research Laboratories (West Grove, PA). The NF-κB probe was purchased from Promega (Madison, WI). 4-Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Boehringer Mannheim (Mannheim, Germany). The enhanced chemiluminescence detection agent was purchased from PerkinElmer Life Sciences (Boston, MA). pGL2-ELAM-Luc (which is under the control of one NF-κB binding site) and pBK-CMV-Lac Z were kindly provided by Dr. W.-W. Lin (National Taiwan University, Taipei, Taiwan). The dominant negative mutant of p38 αMAPK (p38 αMAPK DN) was kindly provided by Dr. C. M. Teng (National Taiwan University, Taipei, Taiwan). [γ- ^{32}P]ATP (6,000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). GenePORTER™ 2 was purchased from Gene Therapy System (San Diego, CA). All materials for SDS-PAGE and the protein assay reagents were purchased from Bio-Rad (Hercules, CA).

2.2. Cell culture

RAW 264.7, a mouse macrophage cell line, was obtained from American Type Culture Collection (Livingstone, MT), and cells were maintained in DMEM/Ham's F-12 nutrient mixture containing 10% FCS, 100 U/ml of penicillin G, and 100 μg/ml streptomycin in a humidified 37 °C incubator. After reaching confluence, cells were seeded onto 10-cm dishes for the electrophoretic mobility shift assay (EMSA), 6-cm dishes for the immunoblotting assays, 24-well plates for the nitrite assays, or 12-well plates for the PGE_2 enzyme immunoassay and κB-luciferase assays.

2.3. Measurement of PGE_2 release

RAW 264.7 macrophages were cultured in 12-well culture plates. After reaching confluence, cells were treated with LTA (10 μg/ml) for the indicated time intervals at 37 °C. After treatment, the medium were collected and stored at

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