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Rac1 regulates peptidoglycan-induced nuclear factor-κB activation and cyclooxygenase-2 expression in RAW 264.7 macrophages by activating the phosphatidylinositol 3-kinase/Akt pathway

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ABSTRACT

Previously, we found that peptidoglycan (PGN), a cell wall component of the gram-positive bacterium *Staphylococcus aureus*, may activate the Ras/Raf-1/extracellular signal-regulated kinase (ERK) pathway, which in turn initiates IκB kinases α/β (IKKα/β) and nuclear factor-κB (NF-κB) activation, and ultimately induces cyclooxygenase-2 (COX-2) expression in RAW 264.7 macrophages. In this study, we further investigated the roles of Rac1, phosphatidylinositol 3-kinase (PI3K), and Akt in PGN-induced NF-κB activation and COX-2 expression in RAW 264.7 macrophages. PGN-induced COX-2 expression was attenuated by a Rac1 dominant negative mutant (RacN17), PI3K inhibitors (wortmannin and LY 294002), and an Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol2-[(R)-2-O-methyl-3-O-octadecylcarbonate]). PGN-induced PGE_2 release was also inhibited by RacN17. Treatment of RAW 264.7 macrophages with PGN caused the activation of Rac and Akt. PGN-induced Akt activation was inhibited by RacN17, LY 294002, and the Akt inhibitor. Stimulation of RAW 264.7 macrophages with PGN resulted in an increase in IKKα/β phosphorylation and p65 Ser536 phosphorylation; these effects were inhibited by RacN17, LY 294002, an Akt inhibitor, and an Akt dominant negative mutant (AktDN). The PGN-induced increases in κB-luciferase activity were also inhibited by RacN17, wortmannin, LY 294002, an Akt inhibitor, and AktDN. Treatment of macrophages with PGN induced the recruitment of p85α and Rac1 to Toll-like receptor 2 (TLR2) in a time-dependent manner. These results indicate that PGN may activate the Rac1/PI3K/Akt pathway through the recruitment of p85α and Rac1 to TLR2 to mediate IKKα/β activation and p65 phosphorylation, which in turn induces NF-κB transactivation, and ultimately causes COX-2 expression in RAW 264.7 macrophages.

1. Introduction

Prostaglandins (PGs), lipid mediators, play important roles in many biological processes, including cell division, blood pressure regulation, immune responses, ovulation, bone development, wound healing, and water balance. Altered prostanoid production is associated with a variety of illnesses, including acute and chronic inflammation, cardiovascular disease, colon cancer, and allergic diseases (DeWitt, 1991; Smith et al., 1991). Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to PGH_2, which is then further metabolized to various PGs, prostacyclin, and thromboxane A_2 (Vane et al., 1998). Two COX isoforms, COX-1 and COX-2, have been identified in humans (Mitchell et al., 1995). COX-1 is generally thought to produce prostaglandins which serve to maintain cellular homeostasis and is known to be constitutively expressed in many

Abbreviations: COX, cyclooxygenase; DMEM/F-12, Dulbecco's modified Eagle's medium/Ham's F-12; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GFP, green fluorescence protein; IKK, IκB kinase; IL-1, interleukin-1; ILR, IL-1 receptor; IRAK, IL-1 receptor-associated kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MLB, magnesium lysis buffer; MyD88, myeloid differentiation protein; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; PBS, phosphate-buffered saline; PGs, prostaglandins; PGN, peptidoglycan; PI3K, phosphatidylinositol 3-kinase; TIR, Toll/IL-1 receptor; TLR2, Toll-like receptor 2; TIRAP, TIR domain-containing adaptor protein; TRAF6, tumor necrosis factor receptor-associated factor 6.

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cell types, including endothelial cells, platelets, and gastric mucosa (Vane et al., 1994), whereas COX-2 is inducible expressed in most mammalian cells. COX-2 expression occurs rapidly by cytokines, growth factors, or bacterial endotoxin stimulation (Vane et al., 1998; Lin et al., 2001). COX-2 plays a major role in inflammatory processes, and its expression has been linked to several diseases associated with inflammation and colon cancer (Williams et al., 1999).

Peptidoglycan (PGN) is the major component of the cell wall of gram-positive bacteria. PGN is composed of alternating β-linked N-acetylmuramyl and N-acetylglucosaminyl glycan that are interlinked by peptide bridges resulting in a large, complex macromolecular structure (Bone, 1994; Ulevitch and Tobias, 1995). Like lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, PGN induces most of the clinical manifestations of bacterial infections, including inflammation, fever, and septic shock (Schleifer and Kandler, 1972). Of importance, PGN can also induce the production of proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8/CXCL8, and tumor necrosis factor (TNF)-α (Bhakdi et al., 1991; Mattsson et al., 1993; Wang et al., 2000, 2001; Xu et al., 2001). PGN binds CD14 and Toll-like receptor 2 (TLR2) to trigger several crucial intracellular signaling responses including activation of transcription factor nuclear factor-κB (NF-κB) and induction of proinflammatory cytokines (Dziarski et al., 1998; Wang et al., 2001). Previous work suggested that PGN-induced NF-κB activation is mediated through TLR2-dependent multiple signaling molecules including myeloid differentiation protein (MyD88), IL-1 receptor-associated kinase (IRAK), TNF receptor-associated factor 6 (TRAF6), NF-κB-inducing kinase (NIK), and the IκB kinase (IKK) signaling pathway (Wang et al., 2001; Xu et al., 2001). NF-κB is composed of Rel family homo- and heterodimers such as p50 and p65. This heterodimer is complexed to the inhibitory subunit, IκBα, which upon stimulation, is phosphorylated and subsequently degraded. This process releases active NF-κB, which is then translocated from the cytosol to the nucleus, to bind specific DNA enhancer sequences, and induce gene transcription (Baldwin, 1996; Barnes and Karin, 1997). However, recent results suggest that phosphorylation of the p65 subunit of NF-κB subunits positively controls NF-κB transcriptional activity by an IκB-independent pathway (Vanden Berghe et al., 1998; Zhong et al., 1998; Sizemore et al., 1999; Jefferies and O'Neill, 2000).

Rac1, a Rho family GTPase, participates in regulation of various cellular functions such as cytoskeletal reorganization, cellular growth, and apoptosis (Van Aelst and D'Souza-Schorey, 1997). Rac1 is involved in different aspects of host defense against bacteria, including leukocyte chemotaxis (Van Aelst and D'Souza-Schorey, 1997), pathogen phagocytosis (Chen et al., 1996; Lee et al., 2000), and the production of oxygen radicals (Knaus et al., 1991; Roberts et al., 1999). It was previously shown that Rac1 mediates a cytokine-stimulated, redox-dependent pathway necessary for NF-κB activation (Sulciner et al., 1996). Additionally, Rac1, Rho, and cdc42 induce transcriptional activity of NF-κB by phosphorylation of IκB (Perona et al., 1997), and activation of Rac1 induces NF-κB binding and activity and enhances expression of cyclin D1 (Joyce et al., 1999). More recently, Zampetaki et al. (2005) reported that biomechanical stress-induced NF-κB activation is mediated by Ras/Rac1.

Akt, a serine/threonine kinase, is a direct downstream effector of phosphatidylinositol 3-kinase (PI3K) (Franke et al., 1997). Akt can be modulated by multiple intracellular signaling pathways and acts as a transducer for many pathways initiated by growth factor receptor-activated PI3K. Akt can stimulate signaling pathways which upregulate the activity of NF-κB in Jurkat T-cells (Kane et al., 1999). In addition, activation of PI3K is involved in bradykinin-stimulated NF-κB activation in human pulmonary epithelial cells (A549) (Pan et al., 1999). The PI3K/Akt pathway plays a critical role in cGMP-mediated NF-κB activation and COX-2 expression (Chang et al., 2004). Whether Rac1, PI3K, and Akt participate in either pathway culminating in IKK activation or p65-mediated transactivation following PGN stimulation has not been investigated.

Recent studies from our laboratory showed that PGN induces TLR2, p85α, and Ras complex formation, and subsequently activates the Ras/Raf-1/extracellular signal-regulated kinase (ERK) pathway, which in turn initiates IKKα/β and NF-κB activation, and ultimately induces COX-2 expression in RAW 264.7 macrophages (Chen et al., 2004). However, little information is available about the roles of Rac1, PI3K, and Akt in regulating NF-κB activation and COX-2 expression following PGN stimulation. In this study, we attempted to identify the signaling pathway of PGN-induced PI3K/Akt activation and its roles in PGN-mediated NF-κB activation and COX-2 expression in RAW 264.7 macrophages. Our hypothesis was that PGN might activate the Rac1/PI3K/Akt pathway through recruiting Rac1 and p85α to TLR2 to mediate IKKα/β activation and p65 phosphorylation, which in turn induces NF-κB transactivation, ultimately causing COX-2 expression in RAW 264.7 macrophages.

2. Materials and methods

2.1. Materials

PGN (derived from *Staphylococcus aureus*) was purchased from Fluka (Buchs, Switzerland). Wortmannin and LY 294002 were obtained from Calbiochem (San Diego, CA). The Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol2-[(R)-2-O-methyl-3-O-octadecylcarbonate]) was purchased from Alexis (Lausen, Switzerland). The pure histone H2B from the calf thymus was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12), fetal calf serum (FCS), and penicillin/streptomycin were purchased from Life Technologies (Gaithersburg, MD). Antibodies specific for α-tubulin and COX-2 were purchased from Transduction Laboratories (Lexington, KY). Protein A/G beads, antibodies specific for IKKα/β, Akt, TLR2, Rac1, p85α, and isotype immunoglobulin G (IgG) as well as anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for phospho-Akt (Ser473) and phospho-IKKα (Ser180)/IKKβ (Ser181) were purchased from New England Biolabs (Beverly, MA). Anti-mouse and anti-rabbit IgG-conjugated alkaline phosphatases were purchased from Jackson Immuno Research Laboratories (West Grove, PA). The pcDNA was kindly provided by Dr. M.-C. Chen (Taipei Medical University, Taipei, Taiwan). 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). Rac1 expression construct sequences carrying the T17N (dominant negative, RacN17) and Q61L (constitutively active, RacL61) mutations and a Rac activity assay kit were purchased from Upstate (Lake Placid, NY). The PGE_2 enzyme immunoassay kit was obtained form Cayman (Ann Arbor, MI). [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). The myc-His tagged expression construct for the dominant negative Akt1-K179M mutant (AktDN) was a kind gift from Prof. C.-M. Teng (National Taiwan University, Taipei, Taiwan). GenePORTER™ 2 was purchased from Gene Therapy System (San Diego, CA). All materials for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Hercules, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Cell culture

The mouse macrophage cell line, RAW 264.7, was obtained from American Type Culture Collection (Livingstone, MT), and cells were