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Original Contribution

Activation of c-Src: A hub for exogenous pro-oxidant-mediated activation of Toll-like receptor 4 signaling

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ABSTRACT

To study the role of c-Src kinase in pro-oxidant-induced stimulation of Toll-like receptor 4 (TLR4), we used lipopolysaccharide from *Escherichia coli* K12 (LPS-EK) and monophosphoryl lipid A, as TLR4-specific agonists and positive controls, and SIN-1 and potassium peroxychromate as pro-oxidant sources. We used the HEK-Blue mTLR4 cell line, which is stably transfected with mouse TLR4 and expresses optimized SEAP reporter under the control of a promoter inducible by NF-κB transcription factor. The level of SEAP released due to TLR4 stimulation was a measure of NF-κB activation. Treatment with either the pro-oxidants or LPS-EK increased SEAP release and TNF-α production in these cells. These treatments also increased intracellular reactive oxygen species accumulation, with an enhanced production of nitric oxide and TBARS to confirm oxidant stress in these cells. Pretreatment with c-Src kinase inhibitors, PP2 and Ca-pY, which act by different mechanisms, decreased these parameters. Pretreatment with SSG, a c-Src activator, enhanced the effects promoted by LPS-EK and pro-oxidants and rescued cells from the PP2- and Ca-pY-induced effects. Curiously, pro-oxidants, but not TLR4 agonist, increased the ratio of TNF-α to IL-10 released, suggesting that pro-oxidants can initiate and maintain an imbalance of TNF-α production over IL-10. To different degrees, both pro-oxidants and TLR4 agonist increased formation of c-Src complexes with TLR4 and IκB-α as coimmunoprecipitates. Both pro-oxidants and TLR4 agonist increased c-Src phosphorylation of the Tyr42 residue in IκB-α, but the pro-oxidant-induced effect was more robust and much longer lasting. Taken together, these studies provide a mechanism whereby c-Src assumes a central role in pro-oxidant-induced NF-κB activation in TLR4 signaling. Pro-oxidant-induced activation of TLR4 through c-Src/NF-κB/IκB-α coupling provides a basis for a molecular dissection of the initiation and maintenance of sterile inflammation that may serve as a "pathophysiologic primer" for many diseases.

Oxidative/nitrosative stress (ONS)[1], a pervasive condition of increased levels of reactive oxygen/nitrogen species (ROS/RNS) in cells or tissues, is now acknowledged to be a prominent feature of many acute and chronic diseases and even of the normal aging process [1]. As a "privilege" of aerobic organisms, ONS can be induced by exogenous oxidants as well as ROS/RNS generated during normal cellular processes [2]. Within cells, ROS can act as secondary messengers in intracellular signaling cascades to induce a dysregulated phenotype, but the mechanism(s) is not clear. ONS can activate many signaling pathways in various cell types, but the changes in cell signaling that may result in decreased or enhanced responsiveness after exposure to oxidants are not fully understood.

A number of tyrosine protein kinases and phosphatases play roles in oxidant-induced cell signaling [3,4]. c-Src is the leading member of the Src family of nonreceptor tyrosine kinases (SFKs) that are expressed in many cells and tissues [5,6] and are involved in diverse signal transduction pathways [7,8]. All members of the SFKs possess similar domain arrangements consisting of *src* homology 3 (SH3), SH2, and kinase (SH1) domains with a common myristoylated and/or palmitoylated membrane-anchoring N-terminal region known as the SH4 domain [9,10] and a unique domain [11]. Regulation of c-Src activity is crucial for its biological functions. Under basal conditions, 90–95% of c-Src is in a dormant state in the cell [12], but growth factors, including inflammatory cytokines and bacterial lipopolysaccharide [13] can rapidly activate it by phosphorylation. An important mechanism for the inactivation of c-Src is dephosphorylation of pTyr416 on c-Src by a

Abbreviations: ONS, oxidative/nitrosative stress; TLR, Toll-like receptor; HEK, human embryonic kidney; MD, myeloid of differentiation; Ca-pY, caffeic acid–pYEEIE; CD, cluster of differentiation; Eb, ebselen; MPLA, monophosphoryl lipid A; LPS–EK, lipopolysaccharide from *Escherichia coli* K12; PPC, potassium peroxychromate; SEAP, secreted embryonic alkaline phosphatase; TIRAP, Toll-interleukin 1 receptor (TIR) adaptor protein; TBARS, thiobarbituric acid-reacting substances; MDA, malondialdehyde; NF-κB, nuclear factor κB; ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF, tumor necrosis factor

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member of the nonreceptor tyrosine phosphatases (PTPases). The potential candidates for PTPase implicated in dephosphorylation of pTyr416 on c-Src include cytoplasmic PTP1B, SHP1 (Src homology 2 domain-containing tyrosine phosphatase 1), and SHP2 [14,15]. c-Src is sensitive to cellular redox stress [16,17], but its role in pro-oxidant-induced inflammatory processes is not known.

Stimulation of Toll-like receptors (TLRs) plays a critical role in innate immune responses [18] and subsequent development of adaptive immunity [19,20]. All mammalian TLRs have similar structural organization consisting of an ectodomain, a transmembrane domain, and a cytoplasmic domain with an intracellular Toll/interleukin 1 receptor (TIR) domain that is critical for signal transduction [19]. Toll-like receptor 4 (TLR4), a member of the TLR superfamily, is a pattern recognition receptor that is expressed mainly on immune cells and is involved in sterile inflammatory responses. TLR4, with an extracellular protein MD-2, is a native signaling receptor for lipopolysaccharide (LPS) [21], but also serves as an important sensor for oxidant stress [22]. The receptor comprises a trimolecular signaling complex of CD14 (as a TLR4 coreceptor), the TIR domain, and TLR4 itself [23–25]. The TLR4 signaling cascade is initiated by the coreceptor CD14, after interaction of LPS with LPS-binding protein. The receptor signaling is enhanced by its monodimerization followed by recruitment of adaptor proteins and kinases to the intracellular TIR domain of the receptor [26,27]. The cytosolic adapter proteins including myeloid differentiation primary response protein 88 (MyD88), TIR adaptor protein (TIRAP), and tumor necrotic factor receptor-associated factor 6 (TRAF6) [28] initiate the proximal events of TLR4-mediated intracellular signaling. Association of TLR4 with MyD88 [29] can recruit other adapter proteins, which leads to the activation of transforming growth factor-β-activated protein kinase 1 (TAK-1), which in turn results in NF-κB and activator protein-1 (AP-1) activation [30,31].

Recently, we have shown that exogenous pro-oxidants act through TLR4 to activate NF-κB [32]. NF-κB is activated by diverse signals and its activation regulates the promoter regions of a variety of genes. In unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form by interacting with inhibitory NF-κB (IκB) proteins. The key pathway in the regulation of NF-κB activation is its nuclear translocation after release from the inhibitory κB-α (IκB-α) subunit to which it is bound in the cytosol [33]. Regulation of NF-κB activation is usually achieved by phosphorylation of IκB-α on serine 32 and serine 36 residues ([pSer32/pSer36]) mediated by IκB-α kinase. NF-κB activation is a primary regulator of stress response in vivo [34]. Under ONS, we propose a novel pathway that involves tyrosine phosphorylation ([pTyr]) of IκB-α at the Tyr42 residue [17,35], a site that is present only in IκB-α, and that favors enhanced formation of [pTyr42]-IκB-α by c-Src over [pSer32/pSer36]-IκB-α. Stimulation of TLR4 appears to mediate both rapid and delayed activation of NF-κB. Phosphorylation of IκB-α at Tyr42 would activate NF-κB for a long time, which delays its ubiquitin-dependent degradation [36,37].

The role of c-Src in the molecular mechanisms that may underlie pro-oxidant-induced NF-κB activation through TLR4 signaling is still poorly understood. In this study, we sought to establish a primary role for c-Src in pro-oxidant-induced TLR4 signaling through the NF-κB activation pathway. With transfection of c-Src siRNA that blocked LPS-induced effects with high efficiency [38,39], we have hypothesized that c-Src among the SFKs plays a dominant role in NF-κB activation induced by exogenous pro-oxidants through TLR4 signaling. Here we present evidence that integrates exposure to pro-oxidants with c-Src/TLR4-coupled signaling to increased c-Src/IκB-α-induced effects in the NF-κB activation pathway (compared with that induced by LPS-EK, a specific agonist for TLR4). We hypothesize that the product of the oxidant-induced c-Src/TLR4/NF-κB-coupled pathway has the potential to initiate and maintain long-lasting "sterile" inflammatory processes that may serve as a primer for various disease states.

Materials and methods

Chemicals and materials

HEK-Blue selection medium, selection antibiotic zeocin, Quanti-Blue detection reagent (alkaline phosphatase detection medium), synthetic monophosphoryl lipid A (MPLA), LPS-EK from *E. coli* K12 (LPS-EK Ultrapure), and polyclonal rat IgG used as pAb neutralization control were obtained from InvivoGen (San Diego, CA, USA). Linsidomine chloride (SIN-1), an effective nitric oxide (NO) donor, was obtained from Cedarlane (Burlington, NC, USA). Ebselen was from Enzo Life Sciences (Farmingdale, NY, USA). Src family inhibitor (PP2) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Low endotoxin, azide-free affinity-purified rat IgG2a, κ-isotype anti-mouse TLR4 (CD284)/MD-2 complex pAb for neutralization of TLR4, and enzyme-linked immunosorbent assay (ELISA) kits for human-specific TNF-α and interleukin-10 (IL-10) were purchased from BioLegend (San Diego, CA, USA). CellROX deep red reagent and NucBlue Live ReadyProbes reagent were obtained from Invitrogen. (5*Z*)-7-Oxozeaenol (a potent and selective TAK-1 mitogen-activated protein (MAP) kinase kinase kinase inhibitor), caffeic acid-pYEEEIE (Ca-pY; phosphopeptide ligand inhibitor for Src SH2 domain), UO126 (a potent inhibitor of MAP kinase kinase family members MEK1 and MEK2), UO124 (an inactive analog of U0126), PP3 (a negative control for PP2), Parameter total nitric oxide and nitrate/nitrite, and Parameter thiobarbituric acid-reactive substances (TBARS) kits were purchased from R&D Systems (Minneapolis, MN, USA). Sodium stibogluconate (a protein tyrosine phosphatase inhibitor, in effect, a c-Src activator) was obtained from EMD Millipore (Billerica, MA, USA). A FACE c-Src kit was obtained from Active Motif (Carlsbad, CA, USA).

Preparation of potassium peroxychromate (PPC)

PPC, used in the study as a source of ROS [40], is not available for purchase from any commercial vendor. We prepared it in the laboratory according to a published protocol [41]. We characterized the product by elemental and infrared analyses, and the purity was determined to be $\geq 98\%$.

Cell lines and culture

Cells were derived from the human embryonic kidney-293 (HEK-293) cell line. HEK-Blue mTLR4 and HEK-Blue Null1-v cells were purchased from InvivoGen. HEK-Blue null-1v-1v1-v is the parental cell line of HEK-Blue mTLR4, but does not express mTLR4. HEK-Blue mTLR4 cells are stably transfected to express mTLR4 at high levels with MD-2 and CD14 coreceptor genes involved in TLR4 recognition and presentation. In addition, the cells stably express an optimized secreted alkaline phosphatase (SEAP) reporter gene under the control of a promoter inducible by NF-κB and AP-1 transcription factors. HEK-Blue Null1-v cells also express the SEAP reporter gene under the control of the interferon-β minimal promoter fused to NF-κB and AP-1 binding sites. We used the level of SEAP protein released into the culture medium to quantify the extent of TLR4 stimulation, which also represents the level of NF-κB activation.

Cells were grown in a 37 °C, 100% humidified incubator in Dulbecco's modified Eagle's medium (4.5 g of glucose/L) without pyruvate but supplemented with 2 mM L-glutamine, 10% (v/v) fetal

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