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Lipopolysaccharide (LPS)-mediated priming of toll-like receptor 4 enhances oxidant-induced prostaglandin E_2 biosynthesis in primary murine macrophages☆

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ABSTRACT

Agonists and pseudo-agonists for toll-like receptor 4 (TLR4) are common in our environment. Thus, human exposure to these agents may result in "priming or sensitization" of TLR4. A body of evidence suggests that LPS-mediated sensitization of TLR4 can increase the magnitude of responses to exogenous agents in multiple tissues. We have previously shown that reactive oxygen and nitrogen species (RONS) stimulate TLR4. There is no evidence that LPS-primed TLR4 can influence the magnitude of responses to oxidants from either endogenous or exogenous sources. In the present study, we directly tested the hypothesis that LPS-primed TLR4 will sensitize primary murine peritoneal macrophages (pM) to oxidant-mediated prostaglandin E2 (PGE_2) production. We used potassium peroxychromate (PPC) and potassium peroxynitrite (PPN) as direct *in vitro* sources of exogenous RONS. Our results showed that a direct treatment with PPC or PPN alone as sources of exogenous oxidants had a limited effect on PGE_2 biosynthesis. In contrast, pM sensitized by prior incubation with LPS-EK, a TLR4-specific agonist, followed by oxidant stimulation exhibited increased transcriptional and translational expression of cyclooxygenase-2 (COX-2) with enhanced PGE_2 biosynthesis/production only in pM derived from TLR4-WT mice but not in TLR4-KO mice. Thus, we have shown a critical role for LPS-primed TLR4 in oxidant-induced inflammatory phenotypes that have the potential to initiate, propagate and maintain many human diseases.

1. Introduction

A progressive change in receptor sensitivity with prior exposure to an activating ligand is referred to as "priming" or "sensitization" of the receptor [1]. A potential "priming" of TLR4 by pseudo-TLR4 ligands such as common drugs especially opioids [2], tricyclic antidepressants [3,4] and even ethanol [5,6] appears to be commonplace. Furthermore, human exposure to LPS, a native TLR4 ligand, is commonplace *via* multiple sources. Exposure may be through different sources such as bacterial infection, microbiome translocation of gut microflora, gut injury, dietary alteration and in a variety of occupational and environmental settings [7].

LPS is a complex glycolipid in the outer membrane of Gram-negative and select Gram-positive bacteria and plays a critical role in activating the innate immunity [8,9,10], which includes initiation of macrophage responses that may involve secretion of cytokines, nitric oxide and eicosanoids (prostaglandins). LPS is also a natural ligand for TLR4/MD-2/CD14 receptor complex in many cells especially dendritic, monocytes, macrophages and B-cells [11]. Besides, injured tissue such as dying tumor cells can persistently release endogenous danger signals collectively termed damage associated molecular patterns (DAMPs) such as certain heat shock protein (HSPs), high mobility group box 1(HMGB1) protein that serve as endogenous and exogenous ligands for TLR4 as well [12]. In addition, TLR4 interacts with molecules, *e.g.* LPS, released by bacteria, which are collectively called pathogen associated molecular patterns (PAMPs). Because TLR4/ CD14/MD-2 receptor complex shares a common signaling cascade with IL-1 receptor, we hypothesize that prior exposure to LPS might sensitize prostaglandin-

Abbreviations: ONS, oxidative/nitrosative stress; TLR, toll-like receptor; LPS-EK (Ultrapure), lipopolysaccharide from *E. coli* K12; TLR4-WT macrophages, macrophages derived from wild-type mice; TLR4-KO macrophages, macrophages derived from complete TLR4 knock-out mice; TAOC, total antioxidant capacity; pM, primary peritoneal macrophages; PPC, potassium peroxychromate; PPN, potassium peroxynitrite; $cPLA_2$, cytosolic phospholipase A 2; $sPLA_2$, secretory phospholipase A 2; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; mPGES-1, microsomal prostaglandin E synthase-1; mPGES-2, microsomal prostaglandin E synthase-2; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; ANOVA, analysis of variance; iROS, intracellular reactive oxygen species; ELISA, enzyme-linked immuno-sorbent assay

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synthesizing enzyme to oxidants in primary murine peritoneal macrophages (pM). LPS can induce the release of prostaglandins in macrophages [13].

Prostaglandin E_2 (PGE_2), a prostanoid synthesized from arachidonic acid (AA), can increase vascular permeability, induce fever, play a role in muscle regeneration and maintain hyperalgesic responses [14]. Because of multiple important physiological roles of PGE_2, many key patents describing inventions for utilization of PGE_2 for either diagnostic or therapeutic purposes have been filed ([15]; also US Pat No. 3,691,216 for PGE_2 methyl ester and PGE_2 methyl ester diacetate, filed 1972; US Pat No 3,795,697, filed 1974). PGE_2 is not stored but is synthesized *de novo* from membrane-released AA when cells are activated by stimuli such as mechanical trauma, cytokines, and growth factors. AA metabolites play critical roles in initiating and/or terminating inflammatory processes [16,17].

A family member of phospholipase A 2 (PLA_2) enzymes initiates PGE_2 synthesis. The PLA_2 enzyme family catalyzes the hydrolysis of membrane phospholipids at the sn-2 position to liberate AA (a 20-carbon unsaturated fatty acid) to initiate PGE_2 synthesis. Both cytosolic PLA_2 ($cPLA_2$) and secretory group V PLA_2 ($sPLA_2$-V) are involved in regulating AA mobilization in response to macrophage exposure to TLR4 activation [18]. The AA released in the membrane is rapidly oxidized into the unstable metabolite, prostaglandin G2 (PGG_2), which is subsequently presented to PGH_2 by the cyclooxygenase (COX) enzymes. COX-1, expressed constitutively in most cells, is the dominant source of prostanoids that serve housekeeping functions, whereas COX-2 is the more important source of prostanoids formed in inflammation [19]. The final step in the biosynthesis of PGE_2 is catalyzed by prostaglandin E synthases (PGESs), a family of oxido-reductases, which has generated increasing interest as a therapeutic target in the treatment of inflammatory-related diseases. mPGES-1 responds to inflammatory stimuli and is frequently induced concomitantly with COX-2 after stimulation by LPS, TNF-α, or IL-1β [20].

PGE_2 then exerts its action locally by binding to one or more of its four cognate receptors, termed EP1-EP4, which are all G-protein-coupled receptors (GPCRs). In the onset of the inflammatory response, PGE_2 acts as a vasodilator to facilitate tissue influx of neutrophil of immune cells from the blood stream resulting in swelling and edema at the site of infection or tissue injury [16]. Furthermore, PGE_2 can stimulate sensory nerves to increase pain response and act on neurons in the pre-optic area to promote pyrogenic effects [21]. In addition, recent studies underscore that PGE_2 exacerbates inflammation by promoting the activation of TH17 cells, a subset of CD4 + helper T cells. PGE_2-mediated production of IL-17 can exacerbate the development of multiple inflammatory diseases, such as inflammatory bowel disease (IBD) and collagen-induced arthritis in mice [22,23]. PGE_2 plays a key role in inflammation, a common and critical pathologic process with its classical acute symptoms of pain, heat, swelling and loss/gain of function.

An extensive body of evidence suggests that LPS priming of TLR4 can change the magnitude of responses to exogenous agents in the liver, kidney, respiratory tract and lymphoid tissue [7]. Furthermore, reactive oxygen and nitrogen species (RONS) appear to participate in the regulation of TLR4 gene expression [24]. Because prooxidants can regulate TLR4 gene expression [25,26], it is still not understood whether LPS-primed TLR4 can influence the magnitude of responses to oxidants from exogenous sources. Thus, the effect of LPS-primed TLR4 can potentially affect critical events in cells of macrophage lineage. Therefore, we will address two basic questions: i) would prior exposure to LPS sensitize TLR4 to responses to exogenous oxidants? and ii) if so, what mechanism (s) is involved with respect to the transcriptional and/or translational activation of PGE_2 biosynthetic enzymes to enhance PGE_2 biosynthesis and release? We sensitized pM by prior exposure to LPS-EK (Ultrapure) [a specific TLR4/MD-2/CD-14 receptor complex agonist at 100 ng/ml for 4 h], which we determined empirically as an optimal priming condition in this pM system. In the present study, we directly tested the hypothesis that LPS-mediated priming of TLR4 will sensitize pM to oxidant-induced PGE_2 biosynthesis.

2. Materials and methods

2.1. Antibodies

Anti-CD11 b antibody (M1/70), isotype control rat (IgG2b) and anti-mPGES-1 were purchased from Abcam (Cambridge, England CB4 0FL), whereas anti-TLR4 antibody was obtained from Novus Biologicals (Littleton, Colorado, USA). Anti-COX-1 (D2G6) rabbit mAb, anti-COX-2 (D5H5) rabbit mAb and anti-$cPLA_2$ antibody were purchased from Cell Signaling Technology (Cambridge, MA, USA). HRP-conjugated ACTB were purchased from Proteintech Group.

2.2. Oxidants and other chemicals

Potassium peroxychromate (PPC), used in the study as a primary exogenous source of ROS, is not available commercially, but was synthesized in the laboratory according to a previously published protocol [27]. It was characterized by elemental and infrared analyses with a purity of > 98%. PPC has been used as a source of ROS to examine their effects on biochemical and biological functions [28]. PPC decomposes readily in aqueous systems to release several oxygen-centered free radicals including H_2O_2, hydroxyl radical (·OH), singlet oxygen ($^1O_2$) and possibly superoxide anion ($O2\cdot^-$). Potassium peroxynitrite (PPN) (Millipore, Billerica, Mass, USA) was used as a direct donor of peroxynitrite anion ($-OONO$) under physiological conditions. Linsidomine chloride (SIN-1) was obtained from AdipoGen (San Diego, CA, USA) and produces cell permeable peroxynitrite anions that can react with lipids, DNA and proteins by direct oxidative reaction or by indirect radical-induced mechanisms [29]. The intracellular total antioxidant capacity assay kit and the ELISA kit for PGE_2 were purchased from Cayman Chemical (Ann Arbor, MI, USA). The ELISA kit for mouse-specific TNF-α was purchased from BioLegend (San Diego, CA, USA). LPS-EK from E. coli K12 (LPS-EK Ultrapure) was obtained from InvivoGen (San Diego, CA, USA). TRI Reagent for RNA extraction was obtained from Molecular Research Center (Cincinnati, OH, USA). High-capacity cDNA reverse transcription assay kit and Restore Western Blot Stripping Buffer were obtained from ThermoFisher Scientific (Grand Island, NY, USA). Thioglycollate brewer powder was purchased from BD Biosciences (San Jose, CA).

2.3. Isolation and characterization of thioglycollate-elicited peritoneal macrophages (pM)

We isolated primary pM from mouse strain B6·B10ScN-Tlr4 lps-del/JthJ (Jackson Labs) with complete deletion of TLR4 gene (knockout) [TLR4-KO] [30] with a corresponding wild-type (TLR4-WT) control strain C57BL6. These mice were cared for and maintained as approved by UMKC-IACUC in accordance with NIH guidelines. We isolated and characterized primary pM from both strains according to a standard published method [31]. pM were plated in tissue culture plates for 1 h, and media containing floating non-adherent cells were removed and replaced with DMEM/12 medium supplemented with 10% (v/v) FBS, 50 units/ml penicillin and 50 μg/ml streptomycin for 24 h before they were used in subsequent experiments. Incubation with DMEM/12 medium for 24 h further enriched the purity of pM [32].

2.4. Sensitization and treatment of pM

pM were sensitized by incubation with 100 ng/ml LPS-EK for 4 h in culture medium supplemented with 10% heat-inactivated (HI) FBS, rinsed once thoroughly with culture medium supplemented with 1% HI FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. Finally, cells were incubated overnight with fresh medium supplemented with 1% HI FBS without oxidants (control) or with PPC or PPN.