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Neutrophil elastase enhances IL-12p40 production by lipopolysaccharide-stimulated macrophages via transactivation of the PAR-2/EGFR/TLR4 signaling pathway

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ABSTRACT

Proteinase-activated receptor 2 (PAR-2) and toll-like receptor 4 (TLR4) are involved in innate immune responses and signaling cross-talk between these receptor molecules has the potential to augment an ongoing inflammatory response. The aim of this study was to evaluate the possible cooperative influence of PAR-2 and TLR4 on IL-12p40 production by macrophages after stimulation with lipopolysaccharide (LPS). During culture, GM-CSF upregulated PAR-2 expression by macrophages in a time-dependent manner. Stimulation with LPS enhanced IL-12p40 production by macrophages in a concentration-dependent manner. While human neutrophil elastase (HNE) did not induce IL-12p40 production, pretreatment of macrophages with HNE synergistically increased the IL-12p40 protein level after LPS exposure. Silencing of TLR4 with small interfering RNA blunted the synergistic enhancement of IL-12p40 by HNE combined with LPS. Silencing of β-arrestin 2, p22phox, or ERK1/2 also inhibited an increase of IL-12p40. Interestingly, transfection of macrophages with small interfering RNA duplexes for DUOX-2, EGFR, TLR4, or TRAF6 significantly blunted the increase of IL-12p40 in response to treatment with HNE plus LPS. U73122 and Rottlerin also inhibited the increased production of IL-12p40. In conclusion, HNE is involved in transactivation of TLR4 through activation of DUOX-2/EGFR and synergistically enhances IL-12p40 production by macrophages stimulated with LPS.

1. Introduction

Interleukin (IL)-12p40 is a component of the bioactive cytokines IL-12 and IL-23. Toll-like receptor 4 (TLR4) is a key member of the TLR family, and its role in inducing the production of inflammatory substances involved in innate immunity has been well characterized. A TLR4 agonist was reported to specifically promote production of the Th1-inducing cytokine IL-12 [1]. Protease-activated receptor 2 (PAR-2) is a 7-transmembrane G-protein-coupled receptor (GCPR) that has been reported to interact with TLR4 and enhance TLR4-dependent signaling [2–4], although the mechanisms involved have not been clarified.

PARs are characterized by a unique mechanism of activation that involves receptor cleavage by various proteinases at specific sites within the extracellular amino-terminus, leading to the exposure of amino-terminal "tethered ligand" domains that bind and activate the cleaved receptors. Neutrophil elastase has been reported to activate PAR-2 [5], which suggests that neutrophil elastase may interact cooperatively with TLR4 to enhance IL-12p40 production. Indeed, it has been reported that HNE upregulates IL-8 production via TLR4 [6].

Epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases. These transmembrane proteins are activated by binding with peptide growth factors from the EGF family of proteins. EGFR kinase activity is required for TLR4 signaling and for the septic shock response [7]. Activation of nuclear factor-kappaB (NF-κB) by TLR4 in response to stimulation by LPS was reported to be impaired by downregulation of EGFR expression, suggesting that this receptor is essential for LPS-induced signaling via TLR4 [8]. Neutrophils release soluble EGFR ligands and initiate EGFR/MEK/ERK signaling [9,10]. Cross talk between unrelated cell surface receptors, such as GPCRs and EGFR, is a crucial mechanism for expanding the cellular communication network. NADPH oxidase-dependent production of reactive oxygen species (ROS) mediates EGFR activation. The dual oxidases DUOX-1 and DUOX-2 are H_2O_2-producing isoforms of the NADPH oxidase family.

Abbreviations: EGFR, epidermal growth factor receptor; DUOX, dual oxidase; GCPR, G-protein-coupled receptor; GM-CSF, granulocyte–macrophage colony-stimulating factor; HNE, human neutrophil elastase; IL-12p40, p40 subunit of interleukin-12; LPS, lipopolysaccharide; PAR-2, protease-activated receptor 2; siRNA, small interfering RNA; TLR, toll-like receptor; TRAF6, tumor necrosis factor receptor-associated factor 6.

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Activation of TLR4 is required for induction of DUOX-2 [11], while activation of PAR-2 also leads to upregulation of the DUOX-2/ROS pathway [12].

Ligands of EGFR are derived from glycoprotein precursors consisting of an extracellular region, a transmembrane domain, and a cytoplasmic domain. Accordingly, proteolytic cleavage can result in the release of soluble growth factors [13]. Such cleavage occurs between the first and second motifs of the immature ligand precursor, leading to release of the subunit adjacent to the plasma membrane as a mature ligand [14]. A role of metalloproteinases (MMP)/a disintegrin and metalloproteinase (ADAM) in EGF ligand shedding mediated by GPCRs has been reported and is termed EGFR transactivation [15,16], while NOX/DUOX may be crucially involved in redox-dependent regulation of EGFR signaling [17].

We previously reported that neutrophil elastase activates phospholipase C (PLC)/protein kinase C (PKC) signaling that leads to cytokine production [18,19]. In addition, it was reported that PLC/PKC signaling modulates DUOX activity [20]. Furthermore, granulocyte–macrophage colony-stimulating factor (GM-CSF) enhances the expression of PAR-2 [21,22] and upregulates TLR4 [23,24]. These reports led us to the hypothesis that neutrophil elastase may be involved in transactivation of TLR4 via DUOX-2/EGFR signaling and promote synergistic enhancement of IL-12p40 expression by GM-CSF-dependent macrophages after stimulation with LPS.

2. Materials and methods

2.1. Ethics statement

Human peripheral blood samples were obtained from healthy volunteers and this study was approved by the Institutional Review Board of Kumamoto Health Science University. Written informed consent was obtained from all of the volunteers.

2.2. Chemicals and reagents

Human neutrophil elastase (HNE) with an activity of 200 U/L was purchased from SERVA Electrophoresis (Heidelberg, Germany). Recombinant human GM-CSF was obtained from Tocris Bioscience (Bristol, UK). U73122 (Tocris Bioscience) and Rottlerin (Tocris Bioscience) were employed to investigate the intracellular signaling pathways involved in IL-12p40 production.

2.3. Isolation of adherent monocytes from peripheral blood mononuclear cells

Lymphocyte medium for thawing (BBLYMPH1) was obtained from Zen-Bio, Inc. (Research Triangle Park, NC). Peripheral blood mononuclear cells (PBMCs) were isolated as described previously [25]. Briefly, heparinized blood samples were obtained from nonsmoking healthy volunteers and were diluted 1:1 with pyrogen-free saline. Further, PBMCs were isolated immediately after collection using Lymphoprep gradients (Axis-Shield PoC As, Norway). Then, cells were suspended with BBLYMPH1 incubated for 3 h. For monocyte isolation by plastic adherence, 1×10^6 cells per well were distributed into 12-well plates (Corning Inc. Costar, NY) and allowed to adhere a 5% CO_2 incubator at 37 °C for 2 h and washed 3 times with warm phosphate-buffered saline (PBS) to remove nonadherent cells. Then, monocytes were cultured in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) and 10×10^3 μg/L gentamicin at 37 °C in 5% CO_2 humidified air. The adherent monocytes were recovered with a cell scraper. The purity of monocytes was evaluated by fluorescent staining with CD14-phycoerythrin (PE) mouse anti-human monoclonal antibody (Life technologies, Staley Road Grand Island, NY) and Fluorescence Activated Cell Sorting (FACS) analysis. The recovery of monocytes was also evaluated by trypan blue staining and counted using a Zeiss microscope (Jena, Germany). CD^{14+} monocytes had a purity of 85.57 ± 0.086% (mean ± SE, n = 48, 85.0–87.3). Consequently, CD^{14+} adherent macrophages expressing one of a broad range of plasma membrane receptors, such as mannose receptor (CD206), could be obtained from these cells [26]. After monocytes were resuspended in RPMI-1640 medium (Sigma-Aldrich, Oakville, Ontario, Canada) supplemented with 25 mM HEPES (Sigma-Aldrich), 100 mM/L L-glutamine (Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich).

2.4. Induction of macrophages from adherent monocytes

GM-CSF is known to induce multiple populations of macrophages. Adherent monocytes (1×10^6 cells/mL) were seeded into 12-well tissue culture plates containing RPMI-1640 medium with 10% FCS and 4 mM L-glutamine, and were incubated in the presence of 10×10^3 ng/L recombinant human GM-CSF [27]. On days 3 and 6 of culture, the cells were washed and fresh medium containing GM-CSF was added. Day 9 cells were utilized as GM-CSF-dependent macrophages in this study.

2.5. ELISA for PAR-2 and IL-12p40

Monocytes (1×10^6 cells) were cultured with GM-CSF (10×10^3 ng/L) to obtain GM-CSF-dependent macrophages. On days 0, 3, 5, 7, and 9 of culture, the PAR-2 protein level in whole-cell lysates was determined by ELISA with an anti-PAR-2 antibody (Biocompare, South San Francisco, CA).

Escherichia coli 0111:B4 lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO). After GM-CSF-dependent macrophages were stimulated with LPS (0, 5, 10, 20, 30, or 50 ng) for 6 h, the IL-12p40 protein level in whole-cell lysates was measured by ELISA with an anti-IL-12p40 antibody (Abcam, Cambridge, UK).

In addition, GM-CSF-dependent macrophages were pretreated by incubation with or without HNE (50 μM) for 6 h and then were stimulated with LPS (0, 5, 10, 20, or 50 ng) for 6 h, after which the IL-12p40 protein level in whole-cell lysates was measured by the above-mentioned ELISA. Effect of small interfering (si) RNA for PAR-2, β arrestin 2, p22phox, ERK1/2, DUOX-2, EGFR, or TRAF6 on IL-12p40 production by macrophages stimulated with HNE and LPS.

Small interfering RNA duplexes (siRNAs) for PAR-2, β-arrestin 2, p22phox, ERK1/2, DUOX-2, EGFR, and TRAF6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Transfection of GM-CSF-dependent macrophages with siRNAs for β-arrestin 2 (50 nM), p22phox (50 nM), ERK1/2 (50 nM), DUOX-2 (50 nM), EGFR (50 nM), or TRAF6 (50 nM) was performed on days 7 to 8 of culture using Lipofectamine™ RNAiMAX (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Then the transfected macrophages (day 9, 1×10^6 cells) were pretreated with HNE (50 μM) for 6 h and stimulated with LPS (10 ng) for 6 h, after which the IL-12p40 protein level in whole-cell lysates was determined by ELISA with an anti-IL-12p40 monoclonal antibody (Abcam). Control siRNA-A (Santa Cruz Biotechnology) was utilized as a negative control for these experiments.

2.6. Effect of U73122 or Rottlerin on IL-12p40 production by macrophages

GM-CSF-dependent macrophages (9 day of culture) were pretreated with U73122 (2 μM) or Rottlerin (5 μM) and then were stimulated by LPS (10 ng) for 6 h with or without HNE (50 μM). Subsequently, the IL-12p40 protein level in whole-cell lysates was determined by ELISA with an anti-IL-12p40 monoclonal antibody (Abcam).

2.7. Statistical analysis

Results are expressed as the mean ± SE. Analysis of variance and the *t*-test for independent means were used to assess differences between multiple groups and differences between two groups,