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G-protein coupled receptor agonists mediate Neu1 sialidase and matrix metalloproteinase-9 cross-talk to induce transactivation of TOLL-like receptors and cellular signaling

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ABSTRACT

The mechanism(s) behind GPCR transactivation of TLR receptors independent of TLR ligands is unknown. Here, GPCR agonists bombesin, bradykinin, lysophosphatidic acid (LPA), cholesterol, angiotensin-1 and -2, but not thrombin induce Neu1 activity in live macrophage cell lines and primary bone marrow macrophage cells from wild-type (WT) mice but not from Neu1-deficient mice. Using immunocytochemistry and NFκB-dependent secretory alkaline phosphatase (SEAP) analyses, bombesin induced NFκB activation in BMC-2 and RAW-blue macrophage cells, which was inhibited by MyD88 homodimerization inhibitor, Tamiflu, galardin, piperazine and anti-MMP-9 antibody. Bombesin receptor, neuromedin B (NMBR), forms a complex with TLR4 and MMP9. Silencing MMP9 mRNA using siRNA transfection of RAW-blue macrophage cells markedly reduced Neu1 activity associated with bombesin-, bradykinin- and LPA-treated cells to the untreated controls. These findings uncover a molecular organizational GPCR signaling platform to potentiate Neu1 and MMP-9 cross-talk on the cell surface that is essential for the transactivation of TLR receptors and subsequent cellular signaling.

1. Introduction

G-protein coupled receptors (GPCRs) exhibit key regulatory functions in innate and acquired immunity. In macrophage and dendritic cells, GPCRs have been shown to regulate diverse cell functions including cell–cell interactions, survival, chemotaxis and activation [1–3]. There is now evidence for a cross-talk between GPCR and TOLL-like receptor (TLR) signaling pathways [2,3], but the mechanism(s) behind this cross-talk has not been fully defined.

An insight into the mechanism(s) of GPCR agonist-induced transactivation of TLRs in the absence of TLR ligands came from our recent reports on the role of Neu1 sialidase and matrix metalloproteinase-9 (MMP-9) cross-talk in regulating TLR [4] and Trk [5] receptors. The findings disclosed a receptor signaling paradigm involving a process of receptor ligand-induced GPCR-signaling via Gαi-proteins, MMP-9 activation, and the induction of Neu1 activation. Central to this process was that Neu1–MMP-9 complex is tethered to TLR-4 receptors on the cell surface of naïve primary macrophages and TLR-expressing cell lines. This signaling paradigm proposes that ligand binding to the receptor on the cell surface induces a conformational change of the receptor to initiate GPCR-signaling via GPCR Gα subunit proteins and MMP-9 activation to induce Neu1. Activated Neu1 tethered to TLR on the cell surface targets and hydrolyzes sialyl α-2-3-linked β-galactosyl residues at the ectodomain of TLR receptors [4]. Taken together, these findings predict a prerequisite desialyation of TLR receptors caused by activated Neu1 enabling the removal of a steric hindrance to receptor association with subsequent activation of TLR receptors and cellular signaling.

This report describes the key players involved in the GPCR agonist-induced transactivation of TLR receptors in the absence of TLR ligands. They radically redefine the current dogma(s) governing the mechanism of the cross-talk between GPCR and TLR activation and cellular signaling, which may provide important pioneering approaches to disease intervention strategies. Here, GPCR agonists

Abbreviations: GPCR, G-protein coupled receptor; TLR, TOLL-like receptors; LPS, endotoxin lipopolysaccharide; pNFκB, NFκB p65 (Rel A) phosphospecific pS529; 4MUNANA, 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid; poly-IC, polyinosinic–polycytidylic acid; Tamiflu, oseltamivir phosphate; MMP, matrix metalloproteinase; GPCR, G-protein coupled receptor; SEAP, secreted embryonic alkaline phosphatase; PTX, pertussis toxin; PIPZ, piperazine; IC_{50}, 50% inhibitory concentration.

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mediate GPCR-signaling via membrane Gα subunit proteins to induce Neu1 and MMP-9 cross-talk at the TLR's ectodomain on the cell surface. This molecular organizational GPCR signaling platform is proposed to be an initial processing stage for GPCR agonist-induced transactivation of TLRs and subsequent downstream NFκB signaling.

2. Materials and methods

2.1. Reagents

TLR4 ligand, lipopolysaccharide (LPS from *Serratia marcescens* was purified by phenol extraction, Sigma or LPS-EK from *E. coli* K12 (Invivogen, San Diego, CA) was used at indicated optimal dosage. TLR3 ligand polyinosinic–polycytidylic acid (polyI:C, 20 μg/ml; Sigma) and TLR2 ligand, killed *Mycobacterium butyricum* (MYCO, 5 μg/ml, DIFCO) were used at the indicated optimal dosage. Bombesin acetate salt hydrate, bradykinin acetate salt, angiotensin I and II human acetate salt, oleoyl-L-α-lysophosphatidic acid sodium salt (LPA), and cholesterol (all purchased from Sigma-Aldrich) in Tris buffered saline were used at predetermined optimal dosage.

The sialidase substrate, 2′-(4-methylumbelliferyl)-α-D-N-acetyl neuraminic acid (98% pure, 4-MUNANA, Biosynth International Inc., Itasca, IL, USA) was used at the optimal concentration of 0.318 mM for the live cell sialidase assay as previously described [6–8].

2.2. Inhibitors

Tamiflu (99% pure oseltamivir phosphate, Hoffmann-La Roche Ltd., Mississauga, Ontario, Lot # BS00060168) and DANA (2-deoxy-2,3-dehydro-D-N-acetylneuraminic acid) (Sigma-Aldrich) were used at indicated concentrations. Pertussis toxin (PTX, from *Bordetella pertussis*, in buffered aqueous glycerol solution, Sigma-Aldrich) catalyzes the ADP-ribosylation of the α subunits of the heterotrimeric G proteins G_i, G_o, and G_t. This prevents the G protein heterotrimers from interacting with receptors, thus blocking their coupling and activation. Galardin (GM6001; N-[(2R)-2-(Hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide; Calbiochem-EMD Chemicals Inc., Darmstadt, Germany) is a potent, cell-permeable, broad-spectrum hydroxamic acid inhibitor of matrix metalloproteinases (MMPs). Piperazine (PIPZ, a MMPII inhibitor, N-Hydroxy-1,3-di-(4-methoxybenzenesulphonyl)-5,5-dimethyl-[1,3]-piperazine-2-carboxamide; Calbiochem-EMD Chemicals Inc.) is a potent, reversible, broad-range inhibitor of MMP-1 (IC_{50} = 24 nM), MMP-3 (IC_{50} = 18.4 nM), MMP-7 (IC_{50} = 30 nM), and MMP-9 (IC_{50} = 2.7 nM). MMP-3 inhibitor (MMP-3i, Stromelysin-1 Inhibitor, Calbiochem-EMD Chemicals Inc.) inhibits MMP-3 (IC_{50} = 5 nM). MMP-9 inhibitor (MMP-9i, Calbiochem-EMD Chemicals Inc.) is a cell-permeable, potent, selective, and reversible inhibitor (IC_{50} = 5 nM). It also inhibits MMP-1 (IC_{50} = 1.05 μM) and MMP-13 (IC_{50} = 113 nM) only at much higher concentrations.

MyD88 homo-dimerization inhibitor peptide (Imgenex, San Diego, CA; IMG-2005-1) contains a sequence from the MyD88 TIR homo-dimerization domain to which MyD88 monomers bind to the inhibitor peptide and block MyD88 homo-dimerization. The corresponding control peptide does not contain the MyD88 homo-dimerization sequence. The compounds were used according to Imgenex protocol.

2.3. Antibodies

Rabbit anti-human Neu1 IgG and rabbit polyclonal anti-MMP9, were all acquired from Santa Cruz Biotechnology, Santa Cruz, CA, USA. AlexaFluor-labeled secondary antibodies included F(ab′)2 goat anti-rabbit AlexaFluor 488 (Molecular Probes, Eugene, OR, USA), F(ab′)2 goat anti-rabbit AlexaFluor 594 (Molecular Probes), goat anti-mouse AlexaFluor 594 (Invitrogen, Corp.) were used at predetermined optimal concentrations in these studies. Horse radish peroxidase-labeled goat anti-rabbit antibody was obtained from Santa Cruz Biotechnology.

2.4. Cell lines

BMC-2 and BMA macrophage cells [9,10] were obtained from Dr. Ken L. Rock, University of Massachusetts Medical School, Worcester, MA. All cell lines were grown at 37 °C in 5% CO_2 in culture media containing 1× Dulbecco's Modified Eagle Medium (DMEM, Gibco, Rockville, MD) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, Utah, USA).

RAW-Blue™ cells (mouse macrophage reporter cell line, Invivogen, San Diego, CA) derived from RAW 264.7 macrophages were grown in culture medium under the selection of zeocin. The cells stably express a secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-kB and AP-1 transcription factors. Upon stimulation, RAW-Blue™ cells activate NF-kB and/or AP-1 leading to the secretion of SEAP which is detectable and measurable when using QUANTI-Blue™, a SEAP detection medium (Invivogen). RAW-Blue™ cells are resistant to Zeocin™ and G418.

2.5. NFκB-dependent secretory alkaline phosphatase (SEAP) assay

Briefly, a cell suspension of 550,000 RAW-blue cells/mL in fresh growth medium was prepared, and 180 μL of cell suspension (~100,000 cells) was added to each well of a Falcon flat-bottom 96-well plate (Becton Dickinson). After different incubation times, 1.5 μg/mL of LPS was added to each well together with either 250 μM Tamiflu or 100 μg/mL piperazine. The plates were incubated at 37 °C in a 5% CO_2 incubator for 18–24 h. A QUANTI-Blue™ solution which is a detection medium developed to determine the activity of any alkaline phosphatase present in a biological sample was prepared following the manufacturer's instructions. Briefly, 180 μL of QUANTI-Blue solution was added to each well of a flat-bottom 96-well plate, followed by 20 μL of supernatant from stimulated RAW-blue cells. The plate was incubated for 30 min to 3 h at 37 °C and the SEAP levels were determined using a spectrophotometer at 620–655 nm. Each experiment was performed in triplicate.

2.6. Silencing MMP9 mRNA using MMP9 siRNA

MMP9 siRNA (mouse) duplex components were obtained from Santa Cruz Biotechnology, Inc., containing a pool of three target-specific 20–25-nt siRNAs designed to knock down gene expression. RAW-blue or BMA cells were plated in a 6-well plate at 5×10^5 cells/well and incubated at 37 °C for 24 h. In serum-free medium, 10 μL of 40 μM siRNA duplex in a total volume of 250 μL of medium was mixed with 10 μL of Lipofectamine 2000 (Invitrogen) for 20 min at room temperature and further described in detail elsewhere [4].

2.7. Mouse models

Wild-type (WT), Neu1–CathA KD (Neu1 deficient and cathepsin A deficient) and CathA KD (normal Neu1 sialidase bound to inactive cathepsin A Ser190Ala mutant) mice were obtained from Dr. Alexey Pshezhetsky's laboratory. Neu1–CathA KD mice have a hypomorphic cathepsin A phenotype with a secondary ~90% reduction of the Neu1 activity [11]. CathA KD mice have an inactive cathepsin A with a S190A point mutation [11].

2.8. Primary mouse bone marrow macrophage cells

Bone marrow (BM) cells were flushed from femurs and tibias of mice with sterile Tris-buffered saline (TBS) solution. The cell suspension was centrifuged for 3 min at 900 rpm, and the cell pellet