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β_2-Adrenergic receptor regulate Toll-like receptor 4-induced late-phase NF-κB activation

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ABSTRACT

Stimulation of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) triggers myeloid differentiation factor 88 (MyD88)-dependent early-phase NF-κB activation and Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF)-dependent late-phase NF-κB activation. In a previous study, we have shown that β_2-adrenergic receptor (β_2AR) functions as a negative regulator of NF-κB activation through β-arrestin 2 in the macrophage cell line RAW264 and that down-regulation of β_2AR expression in response to LPS is essential for NF-κB activation and expression of its target gene, inducible nitric oxide synthase (NOS II). Here, we demonstrate that β_2AR plays an important role in TRIF-dependent late-phase NF-κB activation. LPS-stimulated down-regulation was induced in MyD88-knockdown cells, but not in TRIF-knockdown cells, suggesting that β_2AR expression was down-regulated by the TRIF-dependent pathway. On the other hand, depletion of β_2AR or β-arrestin 2 expression by siRNA decreased cytoplasmic IκBα and abrogated late-phase IκBα degradation and NF-κB activation in response to LPS. Inducible nitric oxide synthase (NOS II) expression was increased continuously during 24 h of LPS stimulation in control cells, but decreased in β_2AR or β-arrestin 2-knockdown cells after 6 h of LPS stimulation. These findings suggest that β_2AR functions not only as a negative regulator of NF-κB activation, but also as a stabilizing factor of the NF-κB/IκBα complex through cytoplasmic β-arrestin 2, and that TRIF-dependent down-regulation of β_2AR expression increases the level of cytoplasmic NF-κB/IκBα complex free from β-arrestin 2, leading to continuous late-phase NF-κB activation.

1. Introduction

The mammalian immune system is notable for its ability to sense and respond to a wide range of infectious microorganisms. Detection of microbes is mediated by several families of pattern-recognition receptors (PRRs), and this recognition leads to the activation of the innate and adaptive immune responses. Toll-like receptors (TLRs) are the best characterized PRRs and are linked to control of bacterial and viral infection (Takeda et al., 2003; Janeway and Medzhitov, 2002). Unique among the large family of TLRs, Toll-like receptor 4 (TLR4) engages two distinct adaptor proteins: myeloid differentiation factor 88 (MyD88), which elicits production of proinflammatory cytokines; and Toll/interleukin-1 receptor domain-containing adaptor-inducing IFN-β (TRIF), which activates the production of type I interferons and proinflammatory cytokines. The MyD88-dependent pathway is common to all TLRs except TLR3, whereas the TRIF-dependent pathway is unique to TLR3 and TLR4 (Adachi et al., 1998; Kawai et al., 1999, 2001; Kaisho et al., 2001; Akira and Takeda, 2004). The MyD88-dependent pathway recruits interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK4, which phosphorylate TNF receptor-associated factor 6 (TRAF6), leading to activation of NF-κB. Signalling pathways downstream of TRIF also lead to activation of NF-κB as well as interferon regulatory factor (IRF)-3. TLR4 induces activation of both the MyD88-dependent and TRIF-dependent pathways, which lead to early-phase and late-phase activation of NF-κB, respectively (Kawai et al., 1999; Yamamoto et al., 2003). However, it remains unclear why TLR4 signalling requires activation of both pathways, and the regulatory mechanisms of the TRIF-dependent late-phase pathway leading to NF-κB activation are not fully understood.

TLR4 is the signalling receptor of the bacterial component, lipopolysaccharide (LPS), and TLR4-initiated activation of NF-κB

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is essential for regulation of inducible nitric oxide synthase (NOS II) and several proinflammatory cytokines in response to invading pathogens. NO produced by NOS II has a number of important biological functions, including roles in tumor cell killing and host defense against intracellular pathogens. Although this basic definition is still accepted, over the past decade NO has been shown to play a much more diverse role in the immune system and in other organ systems, including both beneficial and detrimental effects. For example, NO may have a role in systemic inflammatory response syndrome, which includes severe septic shock and multiple organ system failure, and remains a leading cause of death in critically ill patients (Hierholzer et al., 1998; Bogdan, 2001). Therefore, the molecular mechanisms of TLR4 signalling that leads to NO production in response to LPS require clarification.

NF-κB is found predominantly in the cytoplasm, complexed with members of the IκB family. Release of NF-κB from IκB proteins is an essential step in the generation of transcriptionally competent NF-κB. The consensus is that IκB proteins mask the nuclear localization signals of NF-κB proteins, thereby regulating NF-κB activity, primarily by limiting their nuclear translocation. However, recent studies have indicated that IκBα is expressed in the nucleus as well as in the cytoplasm and that NF-κB/IκBα complexes shuttle between the nucleus and the cytoplasm under all conditions (Lain de Lera et al., 1999; Tergaonkar et al., 2005; Rodriguez et al., 1999). Nuclear NF-κB is unable to bind to DNA due to association with proteins of the IκB family, because nuclear IκBα is not sensitive to signal-induced degradation. Therefore, NF-κB activities following stimulation are dependent on the level of cytoplasmic NF-κB/IκBα complexes.

Recently, we demonstrated that the level of β_2 adrenergic receptor (β_2AR) expression influenced TLR4 signalling (Itoh et al., 2004; Kizaki et al., 2008a,b). β_2AR is a family of G protein-coupled receptors (GPCRs) that provide the key linkages for the sympathetic nervous system to regulate the immune system (Downing and Miyan, 2000; Elenkov et al., 2000). Primary and secondary lymphoid organs, such as the thymus, spleen and lymph nodes, receive extensive sympathetic/noradrenergic innervation, and lymphocytes, macrophages, and many other immune cells bear functional β_2AR. Therefore, stimulation of β_2AR regulates proinflammatory cytokine production, lymphocyte trafficking and proliferation, and antibody secretion through generation of cAMP and activation of protein kinase A (PKA) (Elenkov et al., 2000; Kohm and Sanders, 2001). However, the role of β_2AR in the TLR signalling pathway in macrophages is unclear.

Arrestins are cytoplasmic proteins that play a critical role in the regulation of GPCR signalling (Luttrell and Lefkowitz, 2002) and recent studies have indicated that β-arrestins also regulate NF-κB signalling by interacting with IκBα in HEK293, HeLa, and COS-7 cells (Gao et al., 2004; Witherow et al., 2004). Quite recently, we demonstrated that β-arrestin 2 interacts with cytoplasmic IκBα in the macrophage RAW264 cell line (Kizaki et al., 2008a,b). In addition, we showed that LPS-stimulated signals suppress β_2AR expression, which is accompanied by down-regulation of β-arrestin 2, and that abrogation of the down-regulation inhibits NF-κB activation in response to LPS, suggesting that β_2AR functions as a negative regulator of TLR4 signalling through β-arrestin 2 in macrophages. In the present study, we investigated the physiological consequence of the expression of β_2AR and β-arrestin 2 in macrophages and analyzed the cross-talk between signalling of β_2AR and TLRs. Our studies reveal that expression of β_2AR plays an important role in cytoplasmic retention of NF-κB/IκBα complexes. Furthermore, we show that β_2AR expression is down-regulated in a TRIF-dependent manner in macrophages and that this down-regulation is essential not only for TRIF-dependent late-phase NF-κB activation in response to LPS but also for "escaping" from anti-proinflammatory signals from β_2AR. We also found that β_2AR expression was down-regulated by a region in the adjacent gene, *LOC225609*, expression of which was down-regulated in a TRIF-dependent manner by LPS stimulation.

2. Materials and methods

2.1. Cell culture

The murine macrophage cell line RAW264 (RCB0535) was purchased from RIKEN Cell Bank (Ibaraki, Japan) and cultured as described previously (Kizaki et al., 2002). Cells were stimulated with 1 μg/ml LPS (Sigma–Aldrich, St. Louis, MO), 50 μg/ml poly(I:C) (Sigma–Aldrich), 1 μM CpG (ODN1826) oligonucleotides, or negative control of CpG (ODN1826 control) (Invivogen, San Diego, CA).

2.2. RNA interference

The siGENOME SMARTpoolTM reagent and siCONTROLTM non-targeting siRNA were obtained from Dharmacon (Chicago, IL). Transfection of siRNAs was carried out using HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) with a final siRNA concentration of 50 nM, in accordance with the manufacturer's instructions.

2.3. EMSA

Nuclear extracts were prepared as described (Kizaki et al., 2000). The NF-κB oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG-3′) was purchased from Promega (Madison, WI) and labeled with biotin at the 3′-end. The nuclear protein and labeled oligonucleotide probe were incubated in 20 μl of EMSA buffer [20 mM HEPES, pH 7.6, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 1 mM EDTA, 0.2% Tween, 30 mM KCl, 1 μg poly(dI-dC), 1 μg poly L-lysine] at room temperature for 15 min, electrophoresed in 7% polyacrylamide gels, transferred onto Nylon membranes, and UV cross-linked. To detect the signals, blots were incubated for 15 min with streptavidin-horseradish peroxidase conjugate in blocking reagent, and for 5 min with a chemiluminescence reagent. Blots were then exposed to Kodak X Omat AR film (GE Healthcare Bio-Science, Piscataway, NJ).

2.4. Western blotting analysis

Cell membrane proteins were prepared using a Plasma Membrane Protein Extraction Kit (Bio Vision, Mountain View, CA). Cytoplasmic and nuclear protein extracts were prepared as described previously (Kizaki et al., 2000). Protein concentration was determined using Bradford assay (Bio-Rad, Hercules, CA). The same amounts of samples were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Applied Biosystems, Foster City, CA). Membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) and incubated with goat polyclonal antibodies (Abs) against β_2AR or rabbit polyclonal Abs against IκBα or NOS II (Santa Cruz Biotechnology, Santa Cruz, CA), followed by an appropriate secondary Ab (horseradish peroxidase-conjugated rabbit anti-goat or goat anti-rabbit IgG; DAKO, Kyoto, Japan). In some experiment, to ensure equal protein loading, the membranes were incubated with rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz) after stripping. Immunoreactivity was visualized with an enhanced chemiluminescence reagent (ECL; GE Healthcare Bio-Science).

2.5. Determination of nitrite concentration

Nitrite in cell culture supernatants was measured using the assay system of Ding et al. (1988). The concentration of nitrite was calculated by comparison with sodium nitrite as a standard.