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Role of CREB in modulation of TNFα and IL-10 expression in LPS-stimulated RAW264.7 macrophages

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ABSTRACT

The role of CREB in LPS signaling is controversial. The objective of this study was to evaluate the effect of LPS on phosphorylation and transcriptional activation of CREB, in comparison to isoproterenol, a β-adrenergic receptor agonist. We show here that LPS elevates intra-cellular cAMP level in RAW264.7 macrophages, with slower kinetics and lower magnitude than isoproterenol. The two agents stimulated CREB phosphorylation on Ser-133 to a similar extent, but with a different mechanism; rapid and mostly PKA-mediated for isoproterenol; slow and MSK1-mediated for LPS. Interestingly, LPS-stimulated phosphorylation of CREB did not result in transcriptional activation of a CRE-regulated luciferase reporter, in contrast to stimulation by isoproterenol. Furthermore, inhibitors of p38 and MSK1, but not PKA, completely blocked the production of IL-10 and TNFα in LPS-stimulated macrophages. Distinctively, the PKA inhibitor H89 blocked the suppressive effect of isoproterenol on TNFα production, as well as its stimulatory effect on IL-10 induction, in LPS-stimulated macrophages. Likewise, while over-expression of dominant negative CREB had no effect on LPS-stimulated TNFα production, it blocked the suppressive effect of isoproterenol on TNFα production in the LPS-stimulated macrophages. Our results thus indicate that PKA-mediated phosphorylation of CREB promotes TNFα suppression and IL-10 induction, whereas the same phosphorylation event initiated by LPS and mediated by MSK1 is non-functional for transcriptional modulation.

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1. Introduction

The pro-inflammatory cytokine tumor necrosis factor (TNF)α and the anti-inflammatory cytokine interleukin (IL)-10 are key players in inflammation, initiated, among other stimuli, upon toll-like receptor 4 (TLR4)-mediated detection of lipopolysaccharide (LPS), a molecular component of Gram-negative bacteria. Tight regulation of cytokine production is essential for the balance between proper immune reaction against the pathogen and prevention of excessive damage to the host (Beutler, 2004).

Activation of the cAMP pathway in macrophages by a multitude of G protein-coupled receptor (GPCR) ligands, such as agonists of the β-adrenergic receptor (β-AR), serves to down-regulate production of TNFα, as well as to up-regulate production of IL-10 (Kast, 2000; Szelenyi et al., 2000; Zidek, 1999). The canonical cAMP pathway propagates via protein kinase A (PKA)-mediated phosphorylation of CREB (Gonzalez and Montminy, 1989), a transcription factor which binds to the CRE sites, present at both TNFα (Kuprash et al., 1999) and IL-10 (Platzer et al., 1999) promoters.

Phosphorylation and activation of CREB following treatment of LPS-stimulated RAW264.7 macrophages with a cAMP inducer results in transcriptional activation at the IL-10 promoter (Avni et al., 2010; Goldsmith et al., 2009a). Consistently, mutations at CRE sites in the human IL-10 promoter result in loss of transcriptional activity (Platzer et al., 1999). These observations thus indicate that cAMP-activated CREB can positively regulate IL-10 transcription in LPS-stimulated macrophages.

The role of CREB in TNFα transcription is far less understood. On the one hand, cAMP inducers negatively regulate TNFα expression (Goldsmith et al., 2009b; Kast, 2000). On the other hand, mutations at the CRE site in the mouse TNFα promoter result in loss of transcriptional activity in LPS-stimulated (O'Donnell and Taffet, 2002) or mycobacteria-stimulated (Roach et al., 2005) mouse RAW264.7 macrophages. Thus, while cAMP is considered a negative regulator of TNFα transcription, the latter studies suggested that CREB may be a positive regulator of TNFα transcription.

In addition to the controversy regarding the role of CREB in TNFα transcription, it is unclear whether LPS itself activates CREB. First, some groups (Chen et al., 1999; Osawa et al., 2006), but not others

Abbreviations: PKA, protein kinase A; β-AR, β-adrenergic receptor; GPCR, G protein-coupled receptor; AC, adenylyl cyclase; PVDF, Immobilon-FL polyvinylidene fluoride; TLR, toll-like receptor; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin.

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(Avni et al., 2010; Greten et al., 1996), have been able to observe a cAMP increase in LPS-stimulated RAW264.7 macrophages. The apparent contradictory may be explained by the different incubation times; long and short, respectively. Second, LPS has been shown to stimulate phosphorylation of CREB on Ser-133 via MSK1, a kinase which is downstream to p38 (Caivano and Cohen, 2000; Eliopoulos et al., 2002). Yet, in these studies it was not directly demonstrated whether CREB was activated by this phosphorylation event.

Therefore, the goal of our research was to evaluate the role of CREB in transcriptional regulation of TNFα and IL-10, in LPS-stimulated macrophages. We show here that isoproterenol induced PKA-mediated phosphorylation of CREB as well as transcriptional activation of a cAMP-dependent luciferase reporter, whereas LPS induced MSK1-mediated phosphorylation of CREB, but failed to activate it. Furthermore, PKA and CREB activation was found to be required for transcriptional regulation of TNFα and IL-10 by isoproterenol, while it was found to be irrelevant for LPS stimulation of cytokine expression.

2. Materials and methods

2.1. Reagents, plasmids, and cell culture

Lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5), H89, rolipram, isoproterenol, propranolol, PMSF and DMSO, were purchased from Sigma–Aldrich (St. Louis, MO). Ro318220 was purchased from Calbiochem (Darmstadt, Germany). SB203580 was purchased from A.G. Scientific (San Diego, CA). L-Glutamine, penicillin and streptomycin were purchased from Biological Industries (Beit Haemek, Israel). DMEM and FBS were purchased from Gibco (Carlsbad, CA). BSA was purchased from Amresco (Solon, OH). ELISA reagents sets for TNFα and IL-10 were purchased from R&D Systems (Minneapolis, MN). The LANCE-cAMP kit was purchased from Perkin-Elmer (Waltham, MA). The antibodies against α-tubulin and general p38 were from Santa Cruz Biotechnology (Santa Cruz, CA). A CRE-containing EVX-1 promoter-luciferase reporter gene construct (hereafter CRE-luciferase) (Conkright et al., 2003), an antibody against phospho-Ser-133 CREB, and a dominant negative S133A CREB (Gonzalez and Montminy, 1989), were a kind gift from Dr. Marc Montminy (Salk Institute, La-Jolla, CA). The RSV control vector (for dominant negative CREB) was created by excision of the CREB cDNA fragment out of the construct with the restriction enzymes KpnI and BamHI (NEB, Ipswich, MA), blunting of the vector backbone overhang ends using Klenow DNA polymerase (NEB, Ipswich, MA), and ligation using T4 DNA ligase (Fermentas, Burlington, Canada). A luciferase reporter gene downstream of the full (−1167/+155) TNFα promoter (Chen et al., 2008) was a kind gift from Dr. Chundong Yu (Xiamen University, Xiamen, Fujian, China). The antibodies against phospho-Thr-581 MSK1 and doubly phosphorylated p38 were from Cell Signaling Technology (Danvers, MA). Infrared dye-labeled secondary antibodies and blocking buffer were from Li-Cor Biosciences (Lincoln, NE). Immobilon-FL polyvinylidene fluoride (PVDF) membranes were from Millipore (Billerica, MA). Complete protease inhibitors mixture and HD-fugene transfection reagent were purchased from Roche (Mannheim, Germany). Endofree Plasmid Maxi Kit was from Qiagen (Hilden, Germany). Dual-luciferase reporter assay kit was from Promega (Madison, WI). DH10B bacteria were from Invitrogen (Carlsbad, CA). Mouse RAW264.7 macrophage cells, obtained from American Type Culture Collection (ATCC, Rockville, MD), were grown to 80–90% confluence in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (hereafter culture medium) and with 10% FBS. The cells were grown and maintained at 37 °C in a humidified incubator with 5% CO_2.

2.2. Macrophages activation assay and cytokine measurement

RAW264.7 macrophages were maintained for 48 h prior to the experiment in 96-well plates, at 1.5×10^5 cells per well, in culture medium supplemented with 5% FBS. The culture medium was replaced 2 h before treatment in order to avoid the artifact of medium replacement on signaling (Smith et al., 1997). The cells were stimulated with LPS (100 ng/ml) in the presence or absence of isoproterenol (1 μM) at 37 °C for 2 h. TNF-α and IL-10 secretion to the medium was measured with a commercially available ELISA reagents set, according to the manufacturer's instructions, using a microplate reader (BioTek, Winooski, Vermont). The samples were stored at −80 °C until used.

2.3. Whole cell cAMP measurements

RAW264.7 macrophages were maintained for 48 h prior to the experiment in 96-well plates, at 1.5×10^5 cells per well, in culture medium supplemented with 5% FBS. The cells were incubated at 37 °C for 0–60 min with isoproterenol (1 μM) or LPS (100 ng/ml). The β-AR inverse agonist propranolol (1 μM) was added to LPS-treated cells and to control cells in order to prevent possible constitutive and basal β-AR activity. Lysis was then accomplished by incubation with HCl (0.1 M) in the presence of the phosphodiesterase 4 inhibitor rolipram (20 μM), for 20 min at 4 °C. The samples were diluted 2-fold with PBS/0.1% BSA and the intracellular cAMP level was measured by the LANCE-cAMP kit, in a 384-well plate suitable for time-resolved fluorescence (TRF) measurements, using a Synergy 2 multi-mode microplate reader (BioTek, Winooski, Vermont). The assay was performed according to the manufacturer's instructions, with the exception of antibody addition to the lysate rather than to the cells.

2.4. CREB, p38 and MSK1 phosphorylation assay

RAW264.7 macrophages were maintained for 24 h prior to the experiment in 12-well plates, at 5×10^5 cells per well, in culture medium supplemented with 0.1% FBS. The cells were stimulated with LPS (100 ng/ml) or isoproterenol (1 μM) at 37 °C for 0–60 min. The cells were then washed twice with cold PBS and lysed for 15 min at 4 °C with buffer containing Triton X-100 (1%), Tris buffer pH 8.0 (50 mM), NaCl (100 mM), β-glycerophosphate (50 mM), sodium orthovanadate (1 mM), EDTA (1 mM), EGTA (1 mM), glycerol (30%), PMSF (1 mM) and a complete protease inhibitor mixture diluted according to the manufacturer instructions. Cell extracts were centrifuged (14,000 × *g*, 15 min at 4 °C) and the supernatants were stored at −80 °C.

2.5. Western blotting

Cell extracts (30 μg protein) were boiled for 5 min in SDS-PAGE buffer, subjected to 10% SDS-PAGE, and proteins were transferred to PVDF membrane. An antibody raised against phospho-Ser-133 CREB was used together with an antibody against α-tubulin, or separately from an antibody against CREB (total). Antibodies against doubly phosphorylated p38 and against phospho-Thr-581 MSK1 were used together with an antibody against p38 (total). Two-color imaging and quantitative analysis of western blots was performed using the Odyssey infrared imaging system (Li-Cor Biosciences), according to the manufacturer's instructions.

2.6. Protein determination

Protein was determined by a modification of the Bradford procedure, which yields linear and thus more accurate results,

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