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ScienceDirect
Free Radical Biology & Medicine 42 (2007) 686–697
www.elsevier.com/locate/freeradbiomed

Original Contribution

Nitric oxide inhibits interleukin-12 p40 through p38 MAPK-mediated regulation of calmodulin and c-rel

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Received 13 July 2006; revised 6 December 2006; accepted 11 December 2006
Available online 16 December 2006

Abstract

In activated macrophages, the rel/NF-κB transcription factors are known to play important roles in interleukin-12 (IL-12) p40 regulation by nitric oxide (NO). However, the relative contributions of these factors are not well understood. Here, we describe a dominant role for c-rel involving p38 mitogen-activated protein kinase (p38 MAPK) and calmodulin (CaM) protein in NO-mediated IL-12 p40 inhibition in activated macrophages. Inhibition of NO production by aminoguanidine increased, whereas sodium nitroprusside (SNP; an exogenous NO generator) reduced, nuclear c-rel levels in LPS + IFN-γ-activated RAW 264.7 macrophages. Overexpression of c-rel but not p65 NF-κB increased IL-12 p40 during NO treatment. The p38 MAPK phosphorylation is increased by NO, and inhibition of p38 MAPK in SNP-treated macrophages by SB203580 or transient expression of a dominant-negative mutant of p38 MAPK upregulated both nuclear c-rel and IL-12 p40 levels, indicating that NO targeted the p38 MAPK pathway to inhibit c-rel and IL-12 p40. Cytoplasmic CaM level was increased by NO, and SB203580 decreased the CaM level in NO-exposed macrophages. Inhibition of CaM activity by trifluoperazine rescued the inhibitory effect of NO on c-rel and IL-12 p40. Our findings indicate that c-rel plays an important role in NO-mediated inhibition of IL-12 p40 and is regulated by p38 MAPK through CaM protein.

Keywords: Interleukin-12 p40; Nitric oxide; p38 mitogen-activated protein kinase; c-rel; Calmodulin; Free radicals

The inducible effector responses of macrophages prominently include production of both nitric oxide (NO) [1,2] and interleukin-12 (IL-12) [3,4]. IL-12 strongly regulates T helper 1 responses [5–8] and induces cytotoxicity against intracellular pathogens [9–11] by activating cell-mediated immune responses [12,13]. Therefore, inhibition of IL-12 production is likely to be an important strategy of intracellular pathogens for establishing successful infection [14–16]. Recently, it has been observed that NO can inhibit IL-12 induction in activated macrophages [17,18]. Because NO production is increased during infection with intracellular pathogens [19–21], it is possible that the pathogens use the "NO machinery" to downregulate IL-12 induction and thereby evade the protective immune responses of the host. Understanding the detailed signaling mechanisms of IL-12 regulation by NO would be helpful in designing pharmacological modulators of immune responses to control these pathogens more effectively.

IL-12 is a heterodimeric protein of 70 kDa, composed of two subunits, IL-12 p35 and IL-12 p40 [12]. Regulation of biologically active IL-12 p70 depends upon transcriptional regulation of the gene encoding the IL-12 p40 subunit [22]. The Rel/NF-κB family of transcription factors is known to play a

Abbreviations: IL-12, interleukin-12; NF-κB, nuclear factor-κB; p38 MAPK, p38 mitogen-activated protein kinase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; IFN-γ, interferon-γ; AG, aminoguanidine; SNP, sodium nitroprusside; EIA, enzyme immunoassay; CaM, calmodulin; TFP, trifluoperazine; IL-10, interleukin-10; IRAK, IL-1 receptor-associated kinase.

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doi:10.1016/j.freeradbiomed.2006.12.014

critical role in IL-12 p40 transactivation [23,24]. Recently it has been shown that NO inhibits IL-12 p40 production in activated RAW 264.7 macrophages by targeting the Rel/NF-κB transcription factors [25]. The DNA-binding activities of p50 NF-κB, p65 NF-κB, and c-rel factor are found to be inhibited by NO [25]. However, it is not clear which particular factor actually plays the dominant role in the NO-mediated inhibition of IL-12 p40. Earlier we reported that macrophages from Bruton's tyrosin kinase-deficient mice can produce higher amounts of IL-12 [18] despite having decreased p50 and p65 expression and NO production compared to the wild-type mice [26], indicating that the NO-mediated IL-12 p40 inhibition may not be regulated solely by p50 and p65 NF-κB. This is further supported by observations that when BALB/c macrophages are stimulated with filarial antigens, they produce decreased levels of NO due to lower activation of p50 and p65; however, they can still produce higher levels of IL-12 p40 [27]. Therefore, it is possible that the c-rel is more important in IL-12 p40 downregulation by NO. In this study, we demonstrate that it is the c-rel transcription factor that plays an important role in NO-mediated inhibition of IL-12 p40 and NO targets the upstream p38 mitogen-activated protein kinase (MAPK) to regulate c-rel through enhancing calmodulin (CaM) protein.

Materials and methods

Mice

BALB/c mice were obtained from the National Institute of Nutrition (NIN), Hyderabad. All mice were 6–12 weeks of age. Experimental procedures were approved by the Institutional Review Committee for care and usage of animals of the NIN.

Macrophage stimulation assay

Peritoneal exudate cells (PECs) were harvested by injecting 4% thioglycolate broth as described by us earlier [18]. The RAW 264.7 macrophages were obtained from the National Centre for Cell Science, Pune, India, and maintained in Dulbecco's modified eagle medium (Invitrogen, Grand Island, NY, USA) containing 10% fetal calf serum and antibiotics. Macrophages (3×10^6/ml) were stimulated with a combination of 1 μg/ml LPS (Sigma–Aldrich, St. Louis, MO, USA) and 1 ng/ml IFN-γ (R&D Systems, Minneapolis, MN, USA). Wherever needed, aminoguanidine (AG; Sigma–Aldrich), sodium nitroprusside (SNP; Sigma–Aldrich), SB203580 (Sigma–Aldrich), trifluoperazine (TFP; Sigma–Aldrich) were all added 1 h before stimulation with LPS + IFN-γ. IL-12 p40 and NO levels were measured after 48 h of stimulation with LPS + IFN-γ.

Cytokine assay

IL-12 p40 cytokine (BD Biosciences Pharmingen, San Diego, CA, USA) was quantified by two-site sandwich enzyme immunoassay (EIA) as described earlier [18,26]. Standard curve for the cytokine was obtained using the recombinant standard protein provided by the manufacturer.

Nitrite estimation

The accumulated nitrite resulting from NO production by the stimulated macrophages in culture was measured using the Griess reaction [18]. The assay was performed in 96-well plates using equal volumes of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine (1:1) in 2.5% orthophosphoric acid) and sample. The plates were read at 550 nm absorbance. Nitrite concentrations were calculated based on a standard curve read from a prepared standard solution of sodium nitrite.

Western blot analysis

For p38 MAPK detection, cells were lysed using Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For detecting p65/c-rel/β-actin, nuclear extracts were prepared from NP-40-lysed cells as described elsewhere [28]. After electrophoretic transfer, the nitrocellulose membranes were incubated with rabbit antibody to either phosphorylated or total p38 MAPK (Cell Signaling Technology, Beverly, MA, USA) or rabbit antibody to p65 or c-rel or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) protein. The membrane was next incubated with anti-rabbit or anti-mouse immunoglobulin–horseradish peroxidase (Ig–HRP) conjugate (Sigma–Aldrich). Bound enzyme was detected by chemiluminescence following the manufacturer's protocol (ECL; Amersham Biosciences, Little Chalfont, UK) as described earlier [29]. Equal loading of protein was confirmed by measuring the β-actin level by Western blotting.

Transient transfection assay

The p65 and the c-rel overexpression plasmid constructs were kind gifts from Dr. Klaus Ruckdeschel (Max von Pettenkofer-Institute for Hygiene and Microbiology, Munich, Germany) and the dominant-negative mutant of p38 (DNp38) was kind gift from Dr. Jiahuai Han (The Scripps Research Institute, La Jolla, CA, USA). Cells were transfected with 10 μg of respective plasmid construct using Lipofectamine 2000 (Invitrogen) as described earlier [29]. In control experiments, cells were transfected in parallel with the respective backbone vectors. At 24 h posttransfection, cells were stimulated with LPS + IFN-γ in the absence or presence of SNP for 1 h to measure p65/c-rel by Western blotting or for 48 h to estimate IL-12 p40 level by EIA.

Generation and transfection of siRNA construct for c-rel

A vector-based system was used to knock down c-rel by RNA interference (RNAi) using the RNAi-Ready pSIREN-shuttle vector (Becton–Dickinson, San Jose, CA, USA). The target sequence for murine c-rel used was 5′-AACAACCGGACATACCCGTGT-3′ [30], which is located 129 bases downstream of the start codon [31] (National Center for Biotechnology Information Accession Nos. NM009044, X15842, and