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IRF3 and ERK MAP-kinases control nitric oxide production from macrophages in response to poly-I:C

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ABSTRACT

Understanding nitric oxide (NO) in innate anti-viral immunity and immune-mediated pathology is hampered by incomplete details of its transcriptional and signaling factors. We found in macrophages that IRF3, ERK MAP-kinases, and PKR are essential to NO production in response to RNA-virus mimic, poly I:C, a TLR3 agonist. ERK's role in NO induction may be through phosphorylation of serine-171 of IRF3 and expression of NO-inducing cytokines, IL-6 and IFN-β. However, these cytokines induced less NO in IRF3 knockout or knockdown macrophages. These findings show that ERK and IRF3 coordinate induction of NO by macrophages in response to stimulation of TLR3.

1. Introduction

Nitric oxide (NO) production by macrophages plays an important role in innate immunity and disease progression. NO can contribute to host defense by limiting viral infections [1,2] but also contribute to lung pathology during influenza virus infection [3]. Additionally, NO is an important component of central nervous system disorders such as Alzheimer's disease, multiple sclerosis, and amyotropic lateral sclerosis [4,5]. Therefore, an understanding of signaling pathways that contribute to NO production is important for both the study and treatment of numerous human diseases.

Toll-like receptors (TLRs) recognize pathogen associate molecular patterns and induce signal transduction pathways that lead to activation of immune responses [6]. In particular, TLR3 recognizes double stranded RNA (dsRNA) and the dsRNA analog, poly I:C, and induces expression of antiviral genes and secretion of reactive oxygen species such as NO [1,7]. Recognition of poly I:C by TLR3 recruits the adapter toll/IL-1R (TIR) domain-containing adaptor-inducing IFN-β (TRIF) and activates the kinases TBK1 and IKK$_\varepsilon$, which phosphorylate the C-terminus of interferon regulatory factor 3 (IRF3) [7,8], leading to its activation, and expression of cytokines such as IFN-β [9] or IL-6 [10]. However, poly I:C also leads to activation of non-canonical kinases such as mitogen activated protein kinases (MAPKs) [11,12]. Interestingly, early studies on IRF3 activation pointed to its phosphorylation by MAPKs such as JNK and p38 [13–15]. It remains unclear in which contexts, if any, MAPK phosphorylation could lead to IRF3 activation.

In this study, we found that extracellular signal related kinase (ERK) MAPK inhibitor, U0126, protein kinase R (PKR) inhibitor, 2-aminopurine, and phosphoinositide-3-kinase (PI3K) inhibitor, LY294002, all reduced NO induced by poly I:C in the mouse macrophage cell line, RAW264.7, and primary mouse macrophages. In addition, U0126 inhibited poly I:C-induced IFN-β, IL-6, and iNOS expression as well as induction of NO downstream of these cytokines. Furthermore, data showing ERK phosphorylation of IRF3 and decreased IL-6- or IFN-p-induced NO in IRF3 knockout or knockdown macrophages suggest that activation of IRF3 contributes to IL-6-induced NO. Therefore, both poly I:C-induced IFN-β

Abbreviations: IRF3, interferon response factor 3; NO, nitric oxide; TLR, toll like receptor; ERK, extracellular signal related kinase; PKR, protein kinase R; PI3K, phosphoinositide-3-kinase; TIR, toll/IL-1 receptor; TRIF, TIR domain-containing adaptor-inducing IFN-β; LPS, lipopolysaccharide; NF-κB, nuclear factor κ-B; IRF3KO, IRF3 deficient mice; BL/6, C57BL/6 mice; ODN, oligodeoxynucleotide

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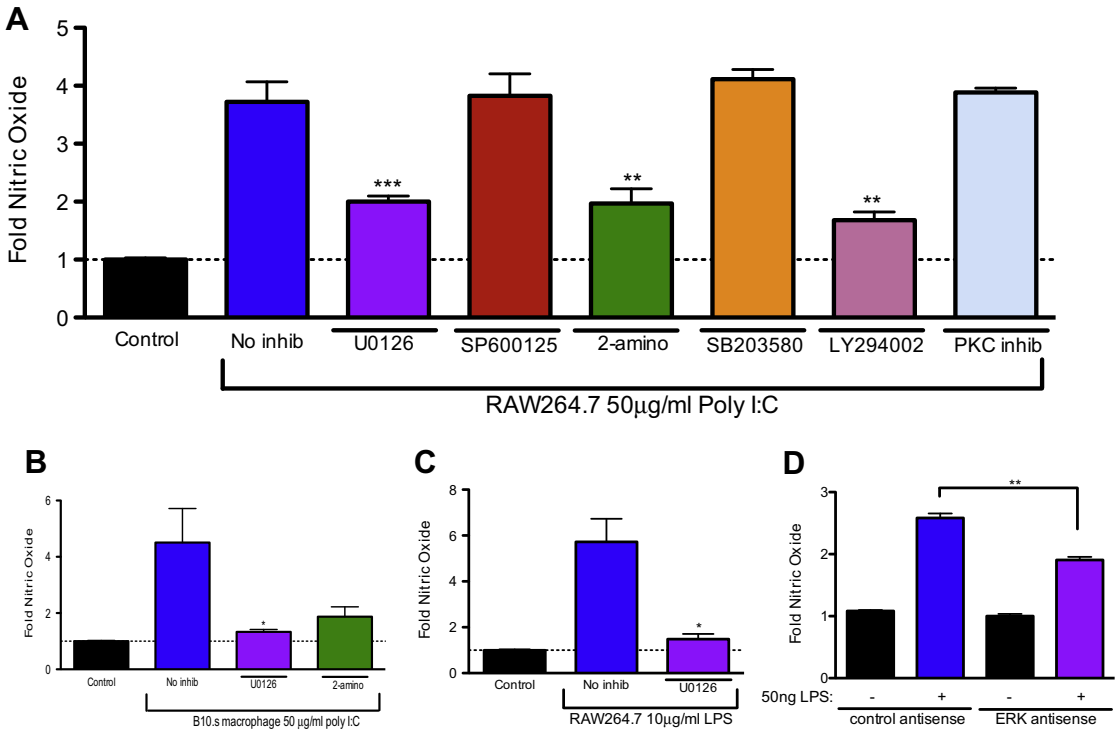

Fig. 1. ERK is involved in poly I:C-induced nitric oxide production in macrophages. (A) RAW264.7 cells were untreated (no inhib) or pretreated with U0126, SP600125, 2-aminopurine (2-amino), SB203580, LY294002, or PKC inhibitor for 30 min and then treated with 50 μg/ml poly I:C for 24 h or left untreated (control). (B, C) B10.s peritoneal macrophages (B) or RAW264.7 cells (C) were pre-treated with U0126 and then treated with 50 μg/ml poly I:C (B) or 10 μg/ml LPS (C), or transfected with ERK MAPK specific ODNs or scrambled control ODNs and then stimulated with 50 μg/ml LPS (D) for 24 h or left untreated (control). The supernatant was removed and nitric oxide was analyzed by Greiss assay. Data are means ± S.E.M. of 4–6 samples per group.

and IL-6 expression and induction of NO downstream of IL-6 and IFN-β could depend on ERK phosphorylation of IRF3.

2. Materials and methods

2.1. Mice, cell lines, and reagents

B10.s mice were offspring of breeder pairs generously provided by Dr. Michel Brahic (Stanford University). IRF3 deficient mice (IRF3KO) on the C57BL/6 (BL/6) background were offspring of breeder pairs obtained from Dr. Karen Mossman (McMaster University) [16]. OT-II mice on a BL/6 background were used as $IRF3^{+/+}$ mice and were obtained from Jackson Labs, (Bar Harbor, ME). RAW264.7 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in culture media (DMEM with 10% FBS with 50 μg/ml gentamycin). For stable selection of shRNAs, RAW264.7 cells were transfected with 2 μg psh-IRF3 or psh-Control (psiRNA vector, Invivogen) with the Amaxa nucleofector. Stable lines were selected with 100 μg/ml Zeocin. Knockdown was confirmed by Western blot and qRT-PCR (data not shown). For transient knockdown of ERK MAPKs, 2 μM phosphorothioate-oligonucleotides (ODNs) that target ERK 1 and 2 (5′-GCCGCCGCCGCCGCCAT-3′) or scrambled ODNs (5′-CGCGCG CTCGCGCACCC-3′) were transfected into RAW264.7 cells with the Amaxa nucleofector as previously described [17]. *Escherichia coli* lipopolysaccharide (LPS)O127:B8 was obtained from Sigma Chemical Co. (St. Louis, MO), and poly I:C from InvivoGen (San Diego, CA). The p38 MAPK inhibitor SB203580 (10 μM), Myristoylated PKC inhibitor (400 μM), PI3K inhibitor LY294002 (50 μM), and ERK MAPK inhibitor U0126 (40 μM) were from Promega Corporation (Madison, WI). The JNK MAPK inhibitor SP600126 (44 μM) was from Biosource (Camarillo, CA) and the PKR inhibitor 2-aminopurine (5 mM) from InvivoGen.

2.2. Macrophage preparations

Peritoneal macrophages were elicited by i.p. injection of 2 ml sterile thioglycollate broth into mice. Three days later, the peritoneal cavities were flushed with 2 ml DMEM and cells were incubated at 1×10^6 cells/2 ml culture media. After overnight incubation, non-adherent cells were removed and 1 ml of culture medium added. Adherent cells were greater than 90% Mac-1 + as determined by FACS analysis [18]. These macrophages were either untreated or treated as described in the figure legends.

2.3. ERK kinase assay

40 μg of IRF3-S171 peptide (DEGSSDLAIVSDPSQQLPSPNVN NFLNPAP) or IRF3-S135 peptide (GASPDTNGKSSLPHSQENLPKL FDGLILGP), or no peptide (synthesized from Thermo scientific/ Fisher) was added to kinase buffer (40 mM Tris, 20 mM $MgCl_2$, 0.1 mg/ml BSA) containing 770 μM ATP, and 250 ng active ERK1 (Signal Chem, Richmond, BC, Canada) or no ERK. The kinase reaction was allowed to proceed at room temperature for 40 min and phosphorylation was detected using the kinase detection kit from Promega.

2.4. RNA preparations and qRT-PCR

RNA was extracted from cells using the Purelink kit from Ambion/Invitrogen (Carlsbad, CA), according to the manufacturer's specifications. One hundred nanograms to 1 μg of RNA was reverse transcribed in .5 mM each of dATP, dGTP, dTTP, and dCTP, 20 U of RNAse inhibitor with Superscript II reverse transcriptase (Invitrogen) at 42 °C for 1.5 h followed by 95 °C for 5 min. The cDNA was diluted 1:2 and 1 μl was incubated with .4 mM of the following primer pairs (Invitrogen): IFN-β sense 5′-ATGAACAACAG