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MEKK3 is required for lysophosphatidic acid-induced NF-κB activation

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ABSTRACT

Lysophosphatidic acid (LPA) is a potent agonist that exerts various cellular functions on many cell types through binding to its cognate G protein-coupled receptors (GPCRs). Although LPA induces NF-κB activation by acting on its GPCR receptor, the molecular mechanism of LPA receptor-mediated NF-κB activation remains to be well defined. In the present study, by using MEKK3-, TAK1-, and IKKβ-deficient murine embryonic fibroblasts (MEFs), we found that MEKK3 but not TAK1 deficiency impairs LPA and protein kinase C (PKC)-induced IκB kinase (IKK)-NF-κB activation, and IKKβ is required for PKC-induced NF-κB activation. In addition, we demonstrate that LPA and PKC-induced IL-6 and MIP-2 production are abolished in the absence of MEKK3 but not TAK1. Together, our results provide the genetic evidence that MEKK3 but not TAK1 is required for LPA receptor-mediated IKK-NF-κB activation.

1. Introduction

The nuclear factor-κB (NF-κB) family of transcription factors plays an important role in regulating the expression of genes responsible for innate and adaptive immunity, stress responses, anti-apoptosis, cell proliferation, and differentiation [1–4]. In resting cells, NF-κB is retained in the cytosol in an inactive form through interaction with IκB inhibitory proteins. Release of NF-κB for translocation to the nucleus and activation of NF-κB dependent genes is accomplished through a signal-induced phosphorylation of IκB by IκB kinase (IKK) and subsequent IκBα degradation [5–8]. Upon binding to their agonist ligands, various cell surface receptors, including receptors for proinflammatory cytokines such as TNFα and IL-1, Toll-like receptors (TLRs), antigen receptors, and GPCRs, induce distinct signaling pathways that eventually converge on IKK complex to activate NF-κB [9]. A major challenge in the NF-κB field is to understand how these distinct receptor-mediated signaling effectors activate IKK/NF-κB in a signal-specific manner [10].

Lysophosphatidic acid (LPA) is a naturally occurring, water-soluble glycerophospholipid that exerts hormone- and growth factor-like activities on many cell types including fibroblasts, endothelial cells, and smooth muscle cells [11–12]. LPA is involved in the regulation of various cellular responses such as cell proliferation, chemotaxis and survival through binding to its cognate G protein-coupled receptors (GPCRs) and activating LPA receptor-mediated multiple effector molecules, including NF-κB [11]. Recently, the adaptor and scaffold proteins β-arrestin2, Bcl10, MALT1 and CARMA3 were identified as essential signal transducers to mediate LPA-induced NF-κB activation [13–17]. Two members of MAP3K serine/threonine kinase family, MEKK3 and TAK1 have been demonstrated to be involved in regulating NF-κB activation through IKK [18–22]. Surprisingly, it has been suggested that TAK1 is not essential in the LPA-mediated NF-κB activation [15]. We therefore tested whether MEKK3 is required for LPA-induced NF-κB activation. Using MEKK3- and TAK1-deficient MEF cell lines, we demonstrate that MEKK3 but not TAK1 is required for LPA receptor-mediated IKK-NF-κB activation. These results reveal that MEKK3, but not TAK1, preferentially mediates GPCR-induced NF-κB activation.

2. Materials and methods

2.1. Antibodies, plasmids and reagents

Antibodies against ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, IκBα, phospho-IκBα, IKKβ, phospho-IKKα/β, TAK1, and secondary antibodies conjugated to horseradish peroxidase were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against PCNA (PC-10), NF-κB-p65 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against MEKK3 was from BD Biosciences Pharmingen

Abbreviations: NF-κB, nuclear factor-κB; IKK, IκB kinase; LPA, lysophosphatidic acid; GPCR, G protein-coupled receptor; PKC, protein kinase C; TAK1, TGF-β activated kinase 1; MEKK3, mitogen-activated protein kinase kinase kinase 3.

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(San Diego, CA). Antibody against β-actin were from Sigma (St. Louis, MO). pBabe-vector and pBabe-HA-MEKK3 expression vector has been described previously [20]. The NF-κB-dependent *firefly* luciferase reporter plasmid and pCMV promoter-dependent *Renilla*-luciferase reporter were purchased from Clontech (Mountain View, California). LPA, phorbol-12-myristate-13-acetate (PMA) and ionomycin (Iono) were purchased from Sigma. Mouse IL-6 and MIP-2 ELISA kits were purchased from BD Biosciences and R & D Systems (Minneapolis, MN), respectively. The ECL-Plus Western blotting system was purchased from GE Healthcare Bio-sciences Corp.

2.2. Cell culture and transfection

MEKK3$^{-/-}$, TAK1$^{-/-}$ and *IKK*β$^{-/-}$ as well as the reconstituted MEF cell lines have been described previously [20,22,23]. These cells are maintained in DMEM containing 10% FCS at 37 °C with 5% CO_2, and transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

2.3. Luciferase reporter gene assay

Luciferase reporter gene assay was performed using a dual luciferase reporter assay system (Promega, Madison, WI) and a Monolight 3010 luminometer (BD Pharmingen) as described previously [20]. Briefly, targeted cells were transiently cotransfected with specific vectors and an NF-κB-dependent *firefly* luciferase reporter construct as well as a *Renilla*-luciferase control construct. Cellular extracts were prepared 36 h post-transfection and the luciferase activities were determined. Relative NF-κB luciferase activity was normalized to *Renilla*-luciferase activity. Changes in luciferase activity with respect to control were calculated. Each experiment was conducted in triplicate.

2.4. Preparation of nuclear and cytosolic fractions

Nuclear and cytosolic extracts were made as described [20]. In brief, cells were harvested in ice-cold PBS (pH 7.4) and were pelleted by centrifugation at 500 ×*g* for 3 min and then lysed for 30 min on ice in buffer B (10 mM HEPES buffer, pH 7.9, containing 0.1 mM EDTA, 10 mM KCl, 0.4% (v/v) IGEPAL, 0.5 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Lysates were centrifuged at 15,000 ×*g* for 10 min. The resulting supernatants constituted cytosolic fractions. The pellets were washed three times with buffer B and resuspended in buffer C (20 mM HEPES buffer, pH 7.9, containing 400 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF) and incubated for 30 min on ice and centrifuged at 15,000 ×*g* for 10 min. The supernatants were used as nuclear extracts.

2.5. Electrophoretic mobility shift assay (EMSA)

NF-κB oligonucleotide probes were labeled with [γ-^{32}P]ATP. MEF cells (1×10^6) were starved for 12 h and stimulated for the indicated

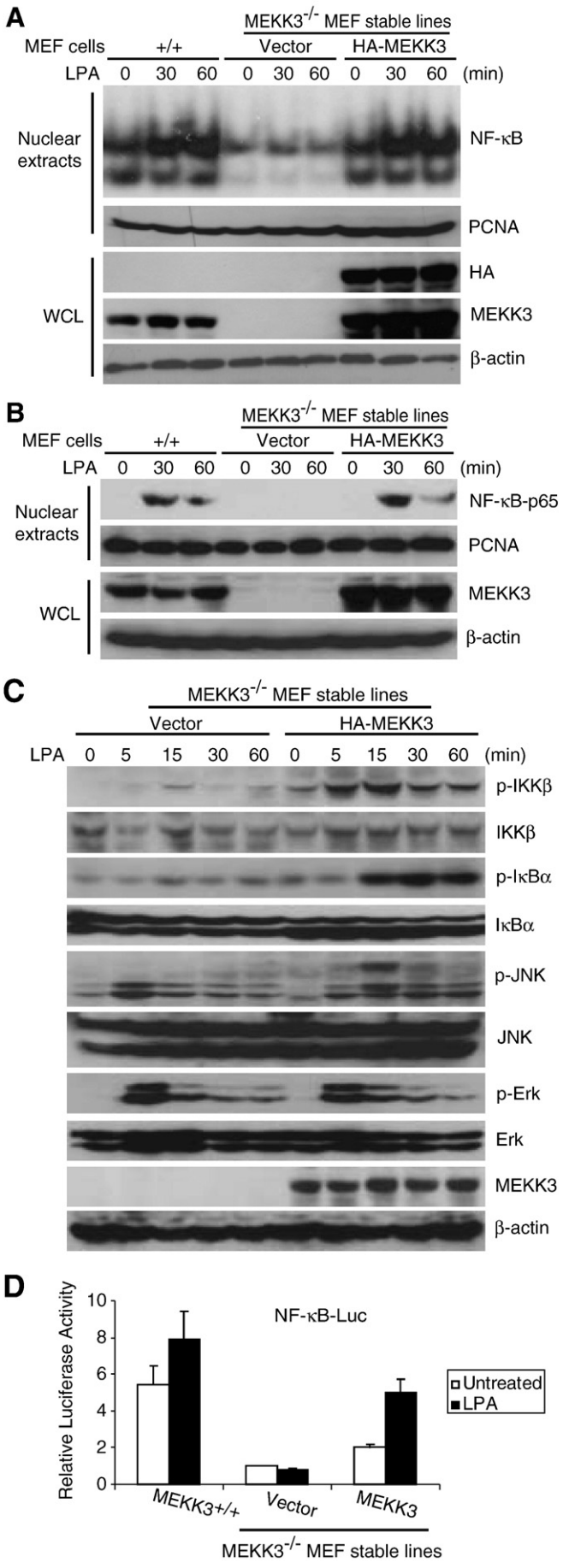

Fig. 1. MEKK3 is required for LPA-induced IKK-NF-κB activation. (A) MEKK3$^{+/+}$ and MEKK3$^{-/-}$ MEF cells reconstituted with empty vector or HA-MEKK3 were either untreated or treated with LPA (30 μM) for 0, 30, and 60 min, then harvested. Nuclear extracts were prepared and subjected to EMSA by using ^{32}P-labeled NF-κB probes. Whole cell lysates (WCL) were subjected to SDS-PAGE and immunoblotting with antibodies indicated. PCNA was used as a loading control for nuclear extracts and β-actin was detected as a loading control for WCL. (B) MEKK3$^{+/+}$ and MEKK3$^{-/-}$ MEF cells reconstituted with empty vector and MEKK3 were stimulated as in A. Then nuclear extracts were prepared and subjected to the immunoblotting analysis as indicated. (C) MEKK3$^{-/-}$ MEF cells reconstituted with empty vector and MEKK3 were either untreated or treated with LPA (30 μM) for 0, 5, 15, 30, and 60 min, then harvested. WCL were subjected to SDS-PAGE and immunoblotting analysis as indicated. (D) One microgram of NF-κB luciferase reporter and 20 ng of *Renilla*-Luc plasmids were cotransfected into MEF cells indicated. Twenty-four hours after transfection, cells were starved for 12 h followed by the addition of LPA (30 μM) for 8 h. The relative luciferase activity was measured and normalized with the *Renilla* activity. Error bars indicate ± standard deviation in triplicate experiments.