

**The MAPK kinase kinase-1 is essential for cytokine-induced JNK and NF- $\kappa$ B activation in human pancreatic islet cells**

**Dariusz Mokhtari<sup>a</sup>, Jason W Myers<sup>b</sup>, Nils Welsh<sup>a</sup>**

<sup>a</sup>*Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden.*

<sup>b</sup>*Department of Biochemistry, Stanford University School of Medicine, Stanford California, USA.*

Running title: MEKK-1 and cytokine-induced JNK and NF- $\kappa$ B activation

Correspondence shall be addressed to: Nils Welsh, Dept. Of Medical Cell Biology, Uppsala University, Biomedicum, P.O. Box 571, S-751 23, Uppsala, Sweden.

Email: Nils.Welsh@mcb.uu.se

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*Objective:* The transcription factor NF- $\kappa$ B and the MAP kinases JNK1/2 are known to play decisive roles in cytokine-induced damage of rodent  $\beta$ -cells. The upstream events by which these factors are activated in response to cytokines are, however, uncharacterized. The aim of the present investigation was to elucidate a putative role of the MAP kinase kinase kinase-1 (MEKK-1) in cytokine-induced signaling.

*Research Design and Methods:* To establish a functional role of MEKK-1, the effects of transient MEKK-1 overexpression in  $\beta$ TC-6 cells, achieved by lipofection and cell sorting, and MEKK-1 downregulation in  $\beta$ TC-6 cells and human islet cells, achieved by dicer-siRNA treatment, were studied.

*Results:* We observed that overexpression of wild type MEKK-1, but not of a kinase dead MEKK-1 mutant, resulted in potentiation of cytokine-induced JNK activation, I $\kappa$ B degradation, NF- $\kappa$ B translocation and cell death. Downregulation of MEKK-1 in human islet cell provoked opposite effects, i.e. attenuation of cytokine-induced JNK and MKK4 activation, I $\kappa$ B stability and a less pronounced NF- $\kappa$ B translocation.  $\beta$ TC-6 cells with a downregulated MEKK-1 expression displayed also a weaker cytokine-induced iNOS expression and lower cell death rates. Also primary mouse islet cells with downregulated MEKK-1 expression were protected against cytokine-induced cell death.

*Conclusions:* MEKK-1 mediates cytokine-induced JNK- and NF- $\kappa$ B activation and this event is necessary for iNOS expression and cell death.

**T**ype-1 diabetes is an autoimmune disease that results in destruction of the insulin producing  $\beta$ -cells (1). Cytokines, such as interleukin- $1\beta$  (IL- $1\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ), induce  $\beta$ -cell death *in vitro*, and the local release of the same cytokines, by islet infiltrating lymphocytes and macrophages, has been proposed to mediate pancreatic  $\beta$ -cell destruction *in vivo* (2). Indeed, levels of pro-inflammatory cytokines have been correlated to insulinitis and  $\beta$ -cell destruction in both NOD mice (3) and human pancreatic biopsies from patients with recent onset Type 1 diabetes (4). Following receptor activation, cytokine-induced signaling involves the activation of the mitogen activated protein kinases (MAPKs) c-Jun NH<sub>2</sub>-terminal kinase (JNK), extracellular signal regulated kinase (ERK) and p38 (5,6). Interestingly, inhibition of JNK or p38 results in protection against cytokine-induced  $\beta$ -cell death (7,8), which points to a prominent role of these MAP kinases in cytokine-induced  $\beta$ -cell death. In addition to the MAP kinases, IL- $1\beta$  and TNF- $\alpha$ -induced signaling results in activation of the transcription factor Nuclear Factor-kappaB (NF- $\kappa$ B) (9,10). In rodent islets, cytokine-induced cell death is caused by increased nitric oxide (NO) production, which results from activation of NF- $\kappa$ B-mediated inducible nitric oxide synthase (iNOS) gene transcription (2).

The MAPKs JNK, ERK and p38 transduce a large variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, proliferation and apoptosis (11). MAPKs are activated by dual phosphorylation of threonine and tyrosine residues and are organized in signaling cascades consisting of a three-kinase set. Consequently, a MAPK kinase (MAP3K, MKKK or MEKK) phosphorylates a MAPK kinase (MAP2K,

MKK or MEK), which in turn activates a MAPK (11).

The 196-kDa serine/threonine protein kinase MEKK-1, which belongs to the MAP3K family, mediates death signals when persistently activated. Studies in various cell types have reported that MEKK-1 promotes apoptosis in response to genotoxic stimuli, such as UV irradiation, etoposide and cisplatin (12), as well as non-genotoxic stimuli, including Fas stimulation, anoikis and pro-inflammatory cytokines (13). In most cell types MEKK-1 activates all three major MAPK pathways (12,14) with the strongest effect on the JNK pathway through phosphorylation of MKK4 (15). Besides its serine/threonine kinase activity, MEKK-1 also acts as a ubiquitin E3 ligase (16).

The transcription factor NF- $\kappa$ B is activated by a wide range of stimuli including stress signals, and proinflammatory cytokines (17). The classical NF- $\kappa$ B pathway involves the release of the p50/p65 subunits from the inhibitory- $\kappa$ B (I $\kappa$ B) complex in the cytosol, a step induced by phosphorylation of I $\kappa$ B by I $\kappa$ B kinases (IKK). When released from I $\kappa$ B, the p50/p65 dimer translocates to the cell nucleus and regulates gene expression (17). Activation of NF- $\kappa$ B has been shown to be protective in most non-islet cells (17). However, in pancreatic islets, the role of NF- $\kappa$ B is far from clear and several recent reports propose a pro-apoptotic role for NF- $\kappa$ B in pancreatic  $\beta$ -cells (18,19).

The chain of events that promote cytokine-induced activation of JNK and NF- $\kappa$ B in  $\beta$ -cells is essentially unknown. Considering the prominent role of MEKK-1 as an activator of both JNK and NF- $\kappa$ B (15,20), the aim of the present study was to investigate the effect of genetic gain or loss of MEKK-1 function on MAPK and NF- $\kappa$ B activation in response to proinflammatory cytokines.

## RESEARCH DESIGN AND METHODS

**Cell culture.** Murine  $\beta$ TC-6 cells (American Tissue Culture Collection, Manassas, VA, USA) at passage numbers 20-30, were maintained in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10 % fetal calf serum (Sigma Chemicals St Louis, MO, USA), 2 mM L-glutamine, streptomycin (0.1 mg/ml) and benzylpenicillin (100 U/ml). Islets from NMRI mice (Naval Medical Research Institute-established, Mölle and Bomholt gård, Denmark) were isolated by collagenase digestion as described previously (8) and incubated free floating in WS. Isolated human pancreatic islets were kindly provided by Dr Olle Korsgren at the Dept. of Radiology, Oncology and Clinical Immunology at Uppsala University Hospital, Uppsala, Sweden. Human islets were maintained in RPMI 1640 (Sigma) medium supplemented as above and the glucose concentration of the media was lowered to 5.6 mM. All cells were grown at 37°C in a humidified air incubator with 5 % CO<sub>2</sub>.

**Plasmids.** pcDNA3 (Invitrogen, Carlsbad, CA, USA) plasmids containing either the full length mouse MEKK-1 or the kinase inactive MEKK-1 (MEKK-1 K(1253)→M) (mut MEKK-1) cDNA were kindly provided by Pär Gerwins at the Dept. of Genetics and Pathology at Uppsala University, Uppsala, Sweden. The plasmids were control sequenced at insertion sites and at the mutation site to verify the integrity of construct prior to experimentation. For GFP expression, the pd2EGFP-N1 (Clontech Laboratories Inc, Mountain View, CA, USA), vector was used. To overexpress the MEKK-1 constructs in  $\beta$ TC-6 cells, Lipofectamine 2000 was used as previously described (21).

**D-siRNA (diced-small interfering-RNA)-mediated down regulation of MEKK-1 in human islet cells.** Human islets, in groups of 100, were trypsinized (0.5%) for 5 min at 37°C and then treated with DNase I (Amersham Life Science, Piscataway, NJ,

USA) (30 units/ml) for 2 min. The resulting free islet cells were placed in non-attachment plates and transfected with 100 ng of d-siRNA directed against mouse MEKK-1 or GL3 (a firefly luciferase gene). D-siRNA directed against the 3'-end of coding region of the mouse MEKK-1 gene and 5'-end of Photinus Pyralis GL3 luciferase gene was synthesized as described previously (22). The in vitro transcription templates were amplified from cloned cDNAs using PCR and the following primers: MEKK-1 Forward-5'-**GCGTAATACGACTCACTATAGGGCT** G AAGTTCTAAGCAGCGCACG-3'; MEKK-1 Reverse-5'-**GCGTAATACGACTCACTA** TAGGGAGACAGGATATGCAACCGGGA G-3'; GL3-Forward-5'-**GCGTAATACGACTCACTATAGGAACAATTGCTTTTAC** AGATGC-3'; GL3-Reverse-5'-**GCGTAATACGACTCACTATAGGAGGCAGACCAGT** AGATCC-3'. The T7 polymerase promoter sequence is shown in bold. D-siRNA was introduced into islet cells during a 2 h incubation using Lipofectamine™ 2000 in 200  $\mu$ l of serum-free culture medium. The transfection medium was then replaced by full culture medium and the cells were cultured for 24-48 h.

**In vitro treatment of cells and evaluation of cell viability.** Transfected cells were either left untreated or treated with a mixture of cytokines (50 U/ml IL-1 $\beta$ , 1000 U/ml IFN- $\gamma$  and 1000 U/ml TNF- $\alpha$ ) for 48 hours. Viability of  $\beta$ TC-6 or mouse islet cells was determined by staining the cells with propidium iodide (Sigma) (20  $\mu$ g/ml) and bisbenzimidazole (Sigma) (5  $\mu$ g/ml) for 10 min at 37°C. After careful washing cells were trypsinized and analyzed by fluorescence microscopy using Openlab 3.0.4 software. Total number of cells, as well as propidium iodide positive cells, were counted using the NIH Image 1.63 software by investigators not aware of sample identity.

**Immunoprecipitation of MEKK-1.** Cells





































