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TRAF2 mediates JNK and STAT3 activation in response to IL-1β and IFNγ and facilitates apoptotic death of insulin-producing β-cells

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ABSTRACT

Interleukin-1β (IL-1β) and interferon-γ (IFNγ) contribute to type 1 diabetes (T1D) by inducing β-cell death. Tumor necrosis factor (TNF) receptor-associated factor (TRAF) proteins are adaptors that transduce signaling from a variety of membrane receptors including cytokine receptors. We show here that IL-1β and IFNγ upregulate the expression of TRAF2 in insulin-producing INS-1E cells and isolated rat pancreatic islets. siRNA-mediated knockdown (KD) of TRAF2 in INS-1E cells reduced IL-1β-induced phosphorylation of JNK1/2, but not of p38 or ERK1/2 mitogen-activated protein kinases. TRAF2 KD did not modulate NFκB activation by cytokines, but reduced cytokine-induced inducible nitric oxide synthase (iNOS) promotor activity and expression. We further observed that IFNγ-stimulated phosphorylation of STAT3 required TRAF2. KD of TRAF2 or STAT3 reduced cytokine-induced caspase 3/7 activation, but, intriguingly, potentiated cytokine-mediated loss of plasma membrane integrity and augmented the number of propidium iodide-positive cells. Finally, we found that TRAF2 KD increased cytokine-induced production of reactive oxygen species (ROS). In summary, our data suggest that TRAF2 is an important mediator of IL-1β and IFNγ signaling in pancreatic β-cells.

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1. Introduction

Immune-mediated destruction of the pancreatic β-cells is a hallmark of type 1 diabetes (T1D). Pro-inflammatory cytokines including interleukin-1β (IL-1β) and interferon-γ (IFNγ) play a key role in this process by causing β-cell functional impairment and death (Mathis et al., 2001; Eizirik et al., 2009). The stress- and mitogen-activated protein kinase c-jun N-terminal kinase (JNK), and the transcription factors nuclear factor κB (NFκB) and signal transducer and activator of transcription (STAT)-1 are critical mediators of cytokine-induced β-cell cytotoxicity (Kim et al., 2007; Heimberg et al., 2001; Giannoukakis et al., 2000; Haefliger et al., 2003; Ammendrup et al., 2000; Bonny et al., 2001). JNK may cause β-cell apoptosis via activation of p53 and the pro-apoptotic BCL-2 family member BIM (Santin et al., 2011; Kim et al., 2005). NFκB and STAT1, activated by IL-1β and IFNγ, respectively, cause complex defensive and detrimental changes in gene transcription networks which collectively favors a pro-apoptotic response (Moore et al., 2011; Ortis et al., 2008; Cardozo et al., 2001). Inducible nitric oxide synthase (iNOS) is a major gene target of STAT1 and NFκB and studies have demonstrated that nitric oxide (NO) contributes to β-cell death (Heitmeier et al., 1999; Liu et al., 2000a). Nitric oxide may cause β-cell death via potentiation of JNK activity and inhibition of Akt, and by inhibiting the endoplasmic reticulum (ER) calcium pump SERCA2b leading to ER calcium depletion and stress (Storling et al., 2005; Oyadomari et al., 2001). While the importance of NFκB, STAT1, JNK, and iNOS in mediating cytokine-induced cell death has been elucidated, the upstream signaling mechanisms leading to activation of these pathways are not fully understood. Understanding of these mechanisms may lead to improved treatment strategies and design of novel drugs to prevent β-cell destruction and diabetes.

The tumor necrosis factor (TNF) receptor-associated factor

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(TRAF) family of proteins functions as intracellular adaptors that bind to and transduce signaling from a wide variety of membrane receptors including cytokine- and Toll-like receptors. There are six members of the TRAF family (TRAF1-6) all of which share a C-terminal TRAF domain. This domain mediates both protein–protein interactions with up- and downstream molecules but also facilitates oligomerization of TRAF proteins (Lee and Lee, 2002; Bradley and Pober, 2001). TRAFs regulate a plethora of cellular processes, including proliferation, apoptosis, autophagy, and survival. Several TRAF proteins have overlapping functions and signaling effects highlighting the complexity of TRAF biology (Xie, 2013). Only limited information about the involvement of TRAF proteins in mediating cytokine-induced β-cell signaling and death currently exists, but evidence supports that TRAF6, as in other cells, regulates IL-1β signaling to JNK and NFκB (Ortis et al., 2012; Frobose et al., 2006). Interestingly, however, exposure of insulin-producing rat INS-1E cells or primary rat β-cells to IL-1β + IFNγ causes upregulation of TRAF2 expression (Cardozo et al., 2001; Kutlu et al., 2003) suggesting that TRAF2 may participate in IL-1β/IFNγ signaling. Although TRAF2 foremost is known to regulate TNF receptor signaling (Rothe et al., 1994), TRAF2 is also involved in other signaling pathways such as those triggered by Toll-like and IL-17 receptors (Xie, 2013). In a cell type- and context-dependent manner, signaling via TRAF2 may lead to cell differentiation (Peng et al., 2012), survival (Liu et al., 1996; Yeh et al., 1997a), oxidative stress (Liu et al., 2000b; Shen et al., 2004; Noguchi et al., 2005), or apoptosis (Mauro et al., 2006). In cells exposed to TNFα or under ER stress conditions, TRAF2 mediates JNK and NFκB activation (Yeh et al., 1997a; Urano et al., 2000; Tada et al., 2001; Habelhah et al., 2004). Involvement of TRAF2 in type I IFN signaling has also been demonstrated (Yang et al., 2005). TRAF2 binds directly to the IFNAR1 subunit of the type I IFN receptor and is selectively required for IFNα-induced NFκB, but not STAT activation (Yang et al., 2008). Importantly, whether TRAF2 is involved in mediating IL-1β and/or IFNγ signaling in β-cells has not been established.

In the present study we examined the potential role of TRAF2 in IL-1β and IFNγ signal transduction in INS-1E cells. We show that TRAF2 is required for IL-1β activation of JNK and is essential for IL-1β + IFNγ-induced caspase 3/7 activation. Further, we demonstrate that TRAF2 is required for IFNγ-induced activation of STAT3, which contributes to caspase activation. Our study adds new knowledge to the molecular mechanisms and pathways activated by pro-inflammatory cytokines and leading to β-cell death.

2. Materials and methods

2.1. Cell culture and reagents

The clonal rat β-cell line INS-1E was cultured in culture flasks (Greiner) in complete medium (CM) consisting of RPMI-1640 with glutaMAX containing 11 mmol/l glucose supplemented with 50 μmol/l β-mercaptoethanol, 100 Units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) (Life Technologies). Cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C.

In experiments where cells were exposed to combinations of cytokines, the following concentrations were used: 1xCyt = 10 pg/ml IL-1β + 0.2 ng/ml IFNγ; 5xCyt = 50 pg/ml IL-1β + 1 ng/ml IFNγ; 15xCyt = 150 pg/ml IL-1β + 5 ng/ml IFNγ. Recombinant mouse IL-1β was obtained from BD Pharmingen, recombinant rat IFNγ and recombinant human TNFα were from R&D Systems.

2.2. Rat islet culture and treatment

Neonatal rat islets were isolated from 5 − 7-day old Wistar rats (Taconic) as previously described (Brunstedt, 1980). Rats were killed by decapitation and pancreases dissected and placed in ice-cold serum-free Hanks' balanced salt solution (HBSS) (Lonza). Pancreases were partially dissociated by 1.5 mg/ml collagenase (Roche). Islets were separated by Histopaque (1.077 g/ml density) (Sigma), centrifuged for 20 min at 1400×*g* without brake, washed with ice-cold HBSS containing 10% newborn calf serum (NCS) (Biological Industries) and handpicked under a dissection microscope. Islets were pre-cultured for 7–10 days in RPMI 1640 with ultraglutamine (Lonza) supplemented with 10% NCS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), in 5% CO_2 at 37 °C. Upon experimentation, rat islets were transferred to multi-well dishes with RPMI 1640 with ultraglutamine (Lonza) supplemented with 2% human serum (Biowest s4190-100), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Following experimental exposures, islets were lysed on ice for ~30 min in either RIPA buffer containing a Complete Mini protease inhibitor tablet (Roche), 10 mM β-glycerolphosphatate and 1 mM sodium orthovanadate or in M-PER mammalian protein extraction reagent with added phosphatase- and protease inhibitors (all from Thermo Scientific). Insoluble material was pelleted by centrifugation for 15 min at 10,000×*g*. Supernatants were used for Western blotting.

2.3. 2.3. cDNA and RT-qPCR

Total RNA was extracted by miRNeasy mini kit spin columns (Qiagen), and cDNA was synthesized by the iScript™ cDNA Synthesis Kit (Biorad). All transcripts were quantified by real-time PCR using the CFX384 C1000 Termalcycler (Biorad) or the Vii7 Real-time PCR system (Applied Biosystems). For evaluation of gene expression, the following TaqMan® Gene Expression Assays (Applied Biosystems) were used: *Traf2* (Rn01758426_m1), *Ppia* (Rn00690933_m1), *Irf-1* (Rn01456791_m1), *Socs1* (Rn01643811_s1), *Cxcl10* (Rn01413889_g1), *Nos2* (Rn00561646_m1), Hprt1 (Rn01527840_m1). Expression levels were calculated either by the delta–delta Ct method or by using standard curves and normalized against *Ppia* (cyclophilin) or the mean of *Ppia* and *Hprt1*. All TaqMan primer PCR reactions were performed using the TaqMan® Gene Expression Master Mix (Applied Biosystems).

2.4. Western blotting

Cells were lysed in M-PER mammalian protein extraction reagent with added phosphatase- and protease inhibitors on ice for ~30 min, and insoluble material was pelleted by centrifugation at 15,000×*g* for 10 min at 4 °C. Protein concentrations in lysates were measured by the Bradford method (Bio-Rad). Equal amounts of protein were mixed with 4 x LDS sample buffer (Invitrogen), denatured at 80 °C for 5 min and loaded on SDS NuPAGE or Bolt Bis-Tris gels (Invitrogen). Following transfer onto nitrocellulose filter membranes (Invitrogen) total protein was stained by Ponceau to assure equal transfer, and Western blotting was performed as described (Berchtold et al., 2011). Antibodies to TRAF2, P-JNK1/2, total JNK1/2, P-ERK1/2, total ERK1/2, P-p38, total p38, P-STAT1, total STAT1, P-STAT3, and total STAT3 were all from Cell Signaling. Anti-iNOS antibody was from BD Bioscience, anti-β-actin antibody was from Abcam, and anti-Tubulin was from Sigma. Immune complexes were detected by chemiluminescence and images were captured digitally by use of FUJI LAS4000 (Fujifilm) or Gbox Chemi XR5 (Syngene).

2.5. Electrophoretic mobility shift assay

Nuclear extracts from INS-1E cells were prepared and EMSA performed as previously described (Ronn et al., 2002). Briefly, after exposure to cytokines cells were washed twice with ice-cold PBS

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