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# Synergistic activation of JNK/SAPK induced by TNF-α and IFN-γ: Apoptosis of pancreatic β-cells via the p53 and ROS pathway

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**Abstract**

IFN-γ and TNF-α are major proinflammatory cytokines implicated in islet β-cell destruction, which results in type-1 diabetes; however, the underlying mechanism is not clear. Using pancreatic β-cell line MIN6N8 cells, co-treatment with TNF-α and IFN-γ, but neither cytokine alone, synergistically induced apoptosis, correlated with the activation of the JNK/SAPK, which resulted in the production of reactive oxidative species (ROS) and loss of mitochondrial transmembrane potential (ΔΨm). Additionally, cells transfected with wild-type JNK1 became more susceptible to apoptosis induced by TNF-α/IFN-γ through ROS production and loss of Δψm, while cascading apoptotic events were prevented in dominant-negative JNK1-transfected or JNK inhibitor SP600125-treated cells. As the antioxidant, *N*-acetyl-cysteine, failed to completely suppress apoptosis induced by TNF-α/IFN-γ, an additional pathway was considered to be involved. The level of p53 was significantly increased through synergistic activation of JNK by TNF-α/IFN-γ. Furthermore, the synergistic effect of TNF-α/IFN-γ on apoptosis and ROS production was further potentiated by the overexpression of wild-type p53, but not with mutant p53. This synergistic activation of JNK/SAPK by TNF-α/IFN-γ was also induced in insulin-expressing pancreatic islet cells, and increased ROS production and p53 level, which was significantly inhibited by SP600125. Collectively, these data demonstrate that TNF-α/IFN-γ synergistically activates JNK/SAPK, playing an important role in promoting apoptosis of pancreatic β-cell via activation of p53 pathway together with ROS.


*Keywords:* Islet cells; Apoptosis; Cytokines; JNK/SAPK; Reactive oxygen species

## 1. Introduction

Autoreactive T-lymphocytes and macrophages are the most important effector cells in type-1 diabetes, ultimately inducing apoptosis of insulin-producing pancreatic islet cells through the production and activation of several cytokines from circulating inflammatory cells [1,2]. Although these cytokines, including interleukin (IL)-1β, interferon (IFN)-γ and tumor necrosis factor (TNF)-α [3], have been widely studied, the exact mechanisms by which they induce β-cell apoptosis are not clear. These cytokines may directly control β-cell gene expression and/or indirectly activate immune and inflammatory cells present within islet cells. Of the known inflammatory cytokines, TNF-α and its role in β-cell apoptosis has been extremely controversial and dependent upon the species and cell type examined [4]. Whereas TNF-α alone induces cell death in both the murine pancreatic cell line NIT-1 and the rat pancreatic cell lines, combination TNF-α and IFN-γ or IL-1β is required for apoptosis of INS-1 and RINm5F, MIN6N8 and primary murine β-cells [5,6]. The cytokine IFN-γ also plays a critical role in the

*Abbreviations:* JNK/SAPK, c-jun NH2-terminal kinase/stress-activated protein kinase; ROS, reactive oxygen species; ΔΨm, mitochondrial transmembrane potential; NAC, *N*-acetyl-L-cysteine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; DCFH-DA, dichlorodihydrofluorescein diacetate; PMSF, phenylmethylsulfonyl fluoride; FACS, fluorescent-activated cell sorter; VDAC, a voltage-dependent anion channel; PARP, poly(ADP-ribose)polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; BMH, bismaleimidohexane.

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pathogenesis of type-1 diabetes in NOD mice and streptozotosin-sensitizing (STZ) mice [7]. Mice deficient for the IFN-γ receptor (IFN-γR) were significantly less sensitive to STZ-mediated induction of diabetes, and the absence of the cytokine receptor has been shown to prevent insulitis and autoimmune diabetes in NOD mice [8]. Recently, it was reported that IFN-γ-induced STAT1/IRF-1 activation plays an essential role in β-cell apoptosis induced by TNF-α/IFN-γ [9], and additional evidence has implicated calcium channel activation in pancreatic β-cell apoptosis induced by TNF-α and IFN-γ together [10], suggesting that IFN-γ may act to increase the sensitivity of target cells to TNF-α. At present, however, the exact molecular mechanism(s) involved in apoptosis by these two cytokines in pancreatic β-cells has not been clearly elucidated.

Induced by TNF-α, JNK/SAPK and the p38 MAPK are mediators of pro-apoptotic processes associated with stress responses and cellular functions including growth and differentiation [11]. The pro-apoptotic targets of JNK include mitochondrial release of cytochrome *C* through the phosphorylation of Bcl-2 and Bcl-xL, as well as activation of FasL, TNF-α, c-myc and p53 promoters [12]. Although JNK/SAPK and p38 MAPK activation has been shown to induce apoptosis through several-related mechanisms in adipocytes and rat pancreatic β-cell lines [13], the relationships between these activators and events such as release of mitochondrial proteins, caspase activation, ROS production and loss of $\Delta\psi m$ remain controversial. Furthermore, JNK activation has been associated with insulin resistance and decreased insulin gene expression in pancreatic β-cells [14], but the exact nature with which JNK/SAPK is involved in the regulation of apoptosis induced by cytokines in pancreatic β-cells are still not clear.

The generation of ROS, such as superoxide anion and hydrogen peroxide, has been implicated in the pathogenesis of several abnormal conditions and diseases including ischemia, cancer and diabetes mellitus as well as maintenance of pathways for cell growth, which can incorporate proliferation, apoptosis and senescence [15]. As one of the major sources of ROS, mitochondria are highly susceptible to oxidative damage, causing mutations in mitochondrial DNA and changing the mitochondrial transmembrane potential, thereby affecting mitochondrial membrane integrity and preceding cell death induced by toxic compounds and cytokines [16]. Recently, various studies have demonstrated that elevated levels of mitochondrial ROS are sufficient to trigger β-cell death since *N*-acetyl-L-cysteine elevated intracellular glutathione levels and delayed ROS-induced cell death of pancreatic β-cells as well as adipocytes [17].

The p53 protein is a critical initiator of the intrinsic apoptosis pathway in response to stress from oncoproteins, DNA damage, hypoxia and survival factor deprivation [18]. p53 can also regulate apoptosis by activating mitochondrial genes to enhance ROS generation [19] or bind to anti-apoptotic proteins Bcl-2 and Bcl-xL to directly interact with mitochondria to promote mitochondrial membrane permeability [20]. Additionally, the p53 protein can also induce a conformational change in Bax, which triggers oligomerization and increases mitochondrial permeabilization [21].

In this investigation, we examined the role of JNK/SAPK stress-activated pathway in mediating signals contributing to TNF-α/IFN-γ-induced apoptosis. These results collectively indicate that JNK/SAPK activation could induce apoptosis of pancreatic β-cells through activation of the p53 pathway and through intracellular ROS. These studies provide a rationale for new clinical perspectives on the development of future therapy for type-1 diabetes.

## 2. Materials and methods

### 2.1. Cell lines and reagents

MIN6N8 cells, SV40 T-transformed insulinoma cells derived from NOD mice, were kindly provided by Dr. M.S. Lee (Sungkyunkwan University School of medicine, Seoul, Korea). Cells were grown in DMEM containing 15% fetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD). Anti-caspase-3, -7, -9 anti-PARP, anti-phospho-JNK1 ($Tyr^{701}$), anti-phospho-SEK1, anti-phospho-p38, anti-phospho-p42/44, anti-JNK1, anti-p38, anti-p42/44 and anti-SEK were obtained from Cell Signaling Technology (Beverly, MA). Anti-Bax, anti-Bad, anti-phospho-p53, anti-p53, anti-p21, anti-VDAC, anti-Bcl-xL, anti-insulin, anti-cytochrome *C*, anti-Bcl-2 and anti-β actin antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Murine IFN-γ and TNF-α were purchased from Biosource International (Camarillo, CA). Dichlorodihydrofluorescein diacetate (DCFH-DA) and DiOC6 were purchased from Molecular Probes (Eugene, OR). The specific JNK inhibitor, SP600125, was purchased from Calbiochem (San Diego, CA). *N*-Acetyl-cysteine (NAC), phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, penicillin, streptomycin, rhodamine 123 (Rh123), propidium iodide (PI) and other common chemicals came from Sigma (St. Louis, MO). Bismaleimidohexane (BMH) was obtained from Pierce Biotechnology (Rockford, IL). The plasmids pcDNA3-HA-JNK1 (wt JNK1), pEBG-SEK1 (wt SEK1) and their respective dominant negative mutants, pcDNA-HA-JNK1-KR (Lys-Arg, mt JNK1) or pEBG-SEK1-KR (mt JNK1) were generously provided by Dr. B.J. Song (National Institute of Alcohol Abuse and Alcoholism, Bethesda, MD), which have been described previously [22].

### 2.2. Isolation of mouse pancreatic islets

Islet cells were isolated from overnight-fasted ICR mice (weighing 20–25 g) by the collagenase digestion technique