Nitric Oxide Induces Apoptosis via Ca\(^{2+}\)-Dependent Processes in the Pancreatic \(\beta\)-cell Line MIN6

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**Abstract.** An excessive production of nitric oxide (NO) in response to cytokines has been shown to be the major cause of the destruction of islet \(\beta\)-cells associated with type 1 (insulin-dependent) diabetes mellitus. The NO-induced \(\beta\)-cell death is the typical apoptosis. In the present study, we show evidence that supports a tight link between NO, Ca\(^{2+}\), protease and apoptosis in \(\beta\)-cells. Three different NO donors, SNAP, NOR3 and NOCT, induced apoptosis in a \(\beta\)-cell line, MIN6 cells, in a concentration-dependent manner. SNAP at 200 \(\mu\)M increased cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)])\(_i\) and induced apoptosis. The SNAP-induced apoptosis was blocked by a Ca\(^{2+}\) chelator, BAPTA-AM, and by an inhibitor of a Ca\(^{2+}\)-dependent protease, calpain. In conclusion, an excessive NO production induces apoptosis, wherein an increase in [Ca\(^{2+}\)], and resultant activation of calpain play a key role.

**Key words:** pancreatic \(\beta\)-cell/apoptosis/nitric oxide/cytosolic Ca\(^{2+}\)/protease/diabetes

Pancreatic \(\beta\)-cells possess constitutive and inducible nitric oxide synthases (cNOS and iNOS), the enzymes that synthesize NO from L-arginine (2, 8). Cytokines such as IL-1\(\beta\) stimulate NO production by activating iNOS mRNA expression in pancreatic islets (2). The consequent overproduction of NO by islet \(\beta\)-cells in response to cytokines was shown to be the major cause of the inhibition of insulin release and ultimate destruction of islet \(\beta\)-cells associated with type 1 diabetes (2, 4). The NO-induced \(\beta\)-cell death is the typical apoptosis (4). Furthermore, it has been shown that NO is linked to [Ca\(^{2+}\)]\(_i\) signaling in rat \(\beta\)-cells and INS-1 insulinoma cells, wherein a cGMP-mediated mechanism and Ca\(^{2+}\) release from mitochondria have been suggested (5, 11). However, the role of Ca\(^{2+}\) in the NO-induced apoptosis of \(\beta\)-cells remains to be ascertained. In this study, we examined the Ca\(^{2+}\) dependency of the NO-induced apoptosis in MIN6 cells, a \(\beta\)-cell line that retains responsiveness to glucose.

**Materials and Methods**

**Cell culture**  
A murine insulinoma cell-line, MIN6 cells (a gift from Dr. Y. Oka), were maintained in Dulbecco's Modified Eagle Medium (high glucose) (Gibco BRL, Co., USA) with 15% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin at 37\(^\circ\)C in 5% CO\(_2\).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cellular proliferation assay

MIN6 cells were incubated with SNAP, NOR3 or NOCT. In some experiments, cells were preincubated with BAPTA-AM for 30 min prior to the treatment with SNAP. After each individual time interval, 10 \(\mu\)l of sterile filtered MTT at a concentration of 5 mg/ml in PBS was added. Cells were incubated at 37\(^\circ\)C for 4 hours. One hundred \(\mu\)l of acidic isopropanol (0.04 M HCl in isopropanol) was added and the mixture was further incubated overnight. Solubilized formazan
was quantified at the absorbance ratio between 570 and 630 nm.

**Measurement of [Ca\(^{2+}\)]\(_i\)**

[Ca\(^{2+}\)]\(_i\) was measured by dual-wavelength fura-2 microfluorometry combined with digital imaging as previously reported (7, 12). Briefly, cells on coverslips were loaded with fura-2 by incubation with 2 \(\mu\)M fura-2 acetoxy-methyl ester for 30 min at 37°C in Krebs Ringer bicarbonate buffer (KRB) composed of (in mM) 121.7 NaCl, 4.4 KCl, 1.2 KH\(_2\)PO\(_4\), 2.0 CaCl\(_2\), 1.2 MgSO\(_4\), 5.0 NaHCO\(_3\), and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.4 with NaOH. The cells were then mounted in a chamber and superfused with KRB at a rate of 1 ml/min at 37°C. The cells were excited at 340 and 380 nm alternately every 2.5 sec, emission signals at 510 nm were detected with an intensified charge-coupled device (CCD) camera, and ratio images were produced by an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan).

**Flow cytometry**

Following each treatment, MIN6 cells were trypsinized and collected. The cells were permeabilized with 70% ethanol for 30 min and incubated with phosphate-buffered saline (PBS) containing 250 mg/ml propidium iodide (PI) for 15 min at room temperature in the dark. Cells from each group were analyzed by flow cytometry.

**Materials**

S-nitroso-N-acetyl-penicillamine (SNAP), (±)-(E)-Ethyl-2-[(E)-hydroxyiminol]-5-nitro-3-hexeneamide (NOR3), 1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC7), O,O′-Bis(2-aminophenyl)ethyleneglycol-N,N,N′,N′-tetracetic acid tetraacetoxymethyl ester (BAPTA-AM) and fura-2/acetoxymethyl ester were purchased from Dojin Chemical (Kumamoto, Japan). Z-Leu-Leu-Leu-H\(_2\), a calpain inhibitor, was purchased from Peptide Institute Inc. (Osaka, Japan).

**Statistical analysis**

The calculated values are expressed as mean ± SEM (n = number of observations). The statistical analysis was carried out by Student’s t-test.

**Results**

**NO donors decrease the viability of MIN6 cells**

SNAP, NOR3 and NOC7 were used as NO donors. Incubation of MIN6 cells with the NO donors for 24 hours markedly decreased the viability of the cells, as determined by the MTT assay, and these effects of NO donors were dose-dependent (Fig. 1). Since apoptosis is characterized by endonucleolytic degradation of DNA,
we used PI staining and flow cytometric analysis to specifically detect and quantitate apoptotic nuclei. SNAP dose-dependently increased the fraction of hypodiploid nuclei in MIN6 cells (Fig. 2).

**SNAP increases [Ca\(^{2+}\)]\(_i\) in MIN6 cells**

In MIN6 cells, an elevation of the glucose concentration from a basal level of 2.8 mM to 25 mM, a stimulatory concentration for insulin release, evoked an increase in [Ca\(^{2+}\)]\(_i\), the response typical of \(\beta\)-cells (12). The [Ca\(^{2+}\)]\(_i\) increase was characterized by a large sharp peak followed by a moderate elevation of [Ca\(^{2+}\)]\(_i\) (Fig. 3), which confirms previous reports (7, 12). In the presence of 16.7 mM glucose and on the moderate elevation of [Ca\(^{2+}\)]\(_i\), SNAP at 200 \(\mu\)M induced a large increase in [Ca\(^{2+}\)]\(_i\) in 7 of 7 cells (Fig. 3). SNAP (200 \(\mu\)M) also increased [Ca\(^{2+}\)]\(_i\) in rat pancreatic \(\beta\)-cells (data not shown).

**Intracellular Ca\(^{2+}\) chelator, BAPTA-AM, counteracts the effect of SNAP on the cell viability of MIN6 cells**

The results that SNAP increased [Ca\(^{2+}\)]\(_i\) and induced

![Graph](image1)

![Graph](image2)

**Fig. 3.** An NO donor increases [Ca\(^{2+}\)]\(_i\). In the presence of a stimulatory glucose concentration of 25 mM an NO donor, SNAP, at 200 \(\mu\)M induced a large increase in [Ca\(^{2+}\)]\(_i\) in MIN6 cells. The cell also responded to a rise in glucose concentration from 2.8 (G2.8) to 25 mM. The results shown are representative of 5 similar experiments.

**Fig. 4.** BAPTA-AM counteracts the induction by SNAP of cell death as assessed by MTT assay (A) and flow cytometry using propidium iodide (B). Following exposure of MIN6 cells to SNAP (200 \(\mu\)M) alone or with BAPTA-AM (10 \(\mu\)M) for 24 hrs, cell death was determined. Values are expressed as mean ± SEM for 6 experiments for each data in (A). Significant difference (p < 0.005) between control and SNAP, and between SNAP and SNAP + BAPTA-AM.
apoptosis in MIN6 cells suggested that the [Ca\(^{2+}\)] increase could mediate apoptosis. We then examined the effect of an intracellular Ca\(^{2+}\) chelator, BAPTA-AM, on the SNAP-induced apoptosis. Following incubation of MIN6 cells with SNAP (200 \(\mu\)M) in the absence and presence of BAPTA-AM (10 \(\mu\)M), the cell viability was measured by MTT assay. The SNAP-induced decrease in the cell viability was blocked by BAPTA-AM (Fig. 4A). BAPTA-AM also counteracted the action of SNAP to induce hypodiploid nuclei, as determined by PI staining and flow cytometric analysis (Fig. 4B).

**Calpain inhibitor, Z-Leu-Leu-H, counteracts the effect of SNAP on the cell viability of MIN6 cells**

Proteases are suggested to be implicated in apoptosis (6). In the present study, we examined the effect of calpain, a Ca\(^{2+}\)-dependent protease, on the apoptosis induced by SNAP. SNAP-induced apoptosis was blocked when Z-Leu-Leu-H (10 \(\mu\)M) was preloaded prior to incubation with SNAP (Fig. 5). The data suggest that calpain is activated by the NO-induced [Ca\(^{2+}\)] increase and plays a key role in apoptosis.

**Discussion**

Cytokines such as IL-1\(\beta\) stimulate NO production by activating iNOS mRNA expression in pancreatic islets. The cytokine-induced NO production has been implicated in the inhibition of insulin secretion and destruction of \(\beta\)-cells in type 1 diabetes. In NOD mouse, an animal model for type 1 diabetes, an elevation of the basal [Ca\(^{2+}\)] in islet cells has previously been shown (10). Elevation of [Ca\(^{2+}\)], often plays a key role in the stimulation of apoptotic process (9). Sustained increases in [Ca\(^{2+}\)] can activate endonuclease and promote apoptotic cell death (9). It was previously suggested that NO increases [Ca\(^{2+}\)], via a cGMP-mediated mechanism in rat \(\beta\)-cells (11) and that NO induces Ca\(^{2+}\) release from mitochondria in INS-1 insulinoma cells (5). However, the role of the [Ca\(^{2+}\)] increase in the NO-induced apoptosis of \(\beta\)-cells remains to be further ascertainment. The present study revealed a tight link between NO, [Ca\(^{2+}\)], protease and apoptosis in \(\beta\)-cells: 1) production of NO by SNAP increases [Ca\(^{2+}\)], and induces apoptosis in a \(\beta\)-cell line, MIN6 cells, 2) a Ca\(^{2+}\) chelator, BAPTA-AM, inhibits the NO-induced apoptosis, and 3) an inhibitor of calpain, a Ca\(^{2+}\)-dependent protease, prevents the NO-induced apoptosis. These results indicate that a large amount of NO introduced by a NO donor SNAP increases [Ca\(^{2+}\)], in an excessive and unregulated manner, which leads to the activation of Ca\(^{2+}\)-dependent protease and resultant apoptosis.

It has recently been shown that exposure of \(\beta\)-cells to high glucose and sulfonylurea induces apoptosis, at least partly, by Ca\(^{2+}\)-dependent mechanisms (1). Serum from patients with type I diabetes was shown to increase Ca\(^{2+}\) influx via L-type calcium channels and induce apoptosis in \(\beta\)-cells (3). Accordingly, an excessive [Ca\(^{2+}\)] increase or Ca\(^{2+}\) overload appears to play a crucial role in the process of apoptosis in \(\beta\)-cells. When the NO producing pathway, which is linked to Ca\(^{2+}\) signaling (5, 7, 11), is upregulated by cytokines, an excessive [Ca\(^{2+}\)] increase may take place and trigger Ca\(^{2+}\)-dependent apoptosis of \(\beta\)-cells.

**References**

NO-Induced Ca\textsuperscript{2+}-Dependent Apoptosis in β-cells


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