A20 Inhibits Cytokine-induced Apoptosis and Nuclear Factor κB-dependent Gene Activation in Islets

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Summary

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease resulting from apoptotic destruction of β cells in the islets of Langerhans. Low expression of antioxidants and a predilection to produce nitric oxide (NO) have been shown to underscore β cell apoptosis. With this perspective in mind, we questioned whether β cells could mount an induced protective response to inflammation. Here we show that human and rat islets can be induced to rapidly express the antiapoptotic gene A20 after interleukin (IL)-1β activation. Overexpression of A20 by means of adenovirus-mediated gene transfer protects islets from IL-1β and interferon-γ-induced apoptosis. The cytoprotective effect of A20 against apoptosis correlates with and is dependent on the abrogation of cytokine-induced NO production. The inhibitory effect of A20 on cytokine-stimulated NO production is due to transcriptional blockade of inducible NO synthase (iNOS) induction; A20 inhibits the activation of the transcription factor nuclear factor κB at a level upstream of IκBα degradation. These data demonstrate a dual antiapoptotic and antiinflammatory function for A20 in β cells. This qualifies A20 as part of the physiological cytoprotective response of islets. We propose that A20 may have therapeutic potential as a gene therapy candidate to achieve successful islet transplantation and the cure of IDDM.

Key words: A20 • β cells • nuclear factor κB • nitric oxide • apoptosis

Type I insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease resulting from specific destruction of the insulin-producing β cell within the islet of Langerhans. Many studies have focused on the initiation phase of the disease, exploring the factors that permit or provoke the autoimmune attack (2–4). More recently, greater attention has been devoted to understanding the mechanisms of β cell susceptibility to death. Although multiple mechanisms are involved in the destruction of β cells, the common unifying theme remains that most of these trigger the apoptotic machinery of the β cell (5, 6).

β cell apoptosis can be induced by either specific T lymphocyte-mediated killing or proinflammatory cytokines. β cell–mediated β cell destruction occurs through direct cognate interactions using the granzyme/perforin or Fas/Fas ligand (FasL) systems (7, 8). Cytokine-mediated β cell apoptosis requires the active participation of the β cells. The intradermal release of IL-1β, TNF-α, and IFN-γ by activated mononuclear cells activates β cells to upregulate inducible nitric oxide synthase (iNOS) (9, 10). Generation of iNOS results in the production of high levels of nitric oxide (NO) and, to a lesser extent, superoxide (11, 12). NO and its reactive oxygen species derivatives, including peroxynitrite (ONOO−), are cytotoxic to β cells (13, 14). NO-mediated toxicity is the predominant mechanism responsible for β cell dysfunction and apoptosis induced by soluble mediators. In addition to its direct toxic potential, NO induces Fas expression on β cells, priming them to T lymphocyte-mediated killing (15). The central role played by NO in the pathophysiology of β cell loss during IDDM is directly demonstrated by the acceleration of IDDM in nonobese diabetic (NOD) mice (a well-studied experimental model of autoimmune diabetes) carrying the ins2 transgene under the control of the insulin promoter (16).

Since the early work of Reckard et al. (17) and Ballinger (18) showing that islet transplantation could cure diabetes in rodents, islet transplantation for humans has been regarded as a potential cure for diabetes (17–20). However, several obstacles still need to be overcome before successful islet transplantation becomes a reality, namely, (a) primary

Abbreviations used in this paper: β-gal, β-galactosidase; EM SA, electrophoretic mobility shift assay; GSN O, S-nitrosoglutathione; IDDM, insulin-dependent diabetes mellitus; IL-1β, inhibitor of NF-κB; iNOS, inducible NOS synthase; L-NIO, L-N-(1-iminoethyl) ornithine, dihydrochloride; MnSOD, manganese superoxide dismutase; MΩI, multiplicity of infection; NF-κB, nuclear factor κB; NO, nitric oxide; NOD, nonobese diabetic; NOS1, NOS2; N (2-hydroxy-2-nitrosohydrazino-1,2-ethylenediamine; rAd, recombinant adenovirus; RT, reverse transcription; TRAF, TNF receptor–associated factor.
nonfunction in the immediate posttransplantation period, (b) recurrence of autoimmune disease, and (c) allograft rejection (21–23). Whether related to hypoxia, loss of nutrients, induction of nonspecific inflammatory reactions, or immune effectors implicated in the development of autoimmune disease or allograft rejection, the final outcome of these processes is destruction of the transplanted islets by apoptosis.

One way to achieve successful islet transplantation for the treatment of IDDM would be to genetically engineer β cells to express antiapoptotic and antiinflammatory proteins (24). The zinc finger protein A20 represents one such candidate for genetic engineering of β cells. A20 was originally described as an antiapoptotic TNF-α-induced gene in endothelial cells (25, 26). Besides protection from apoptosis, we have demonstrated previously that A20 also inhibits proinflammatory responses in endothelial cells (27, 28). In this paper, we evaluate the efficacy of A20 to protect islets from apoptosis. We demonstrate that recombinant adenovirus (rAd)-mediated gene expression of A20 in rodent islets protects against cytokine-induced apoptosis and inhibits cytokine-induced NO generation. A20 suppresses cytokine-induced NO generation at the level of iNOS transcription through blockade of the transcription factor, nuclear factor κB (NF-κB). Furthermore, we report for the first time that A20 mRNA is rapidly induced in human and rat islets after cytokine stimulation. These latter data indicate that A20 is part of the physiological protective response of islets, further supporting its consideration for human gene therapy.

Materials and Methods

Islets. Male Sprague-Dawley rats were purchased from the Jackson Laboratory, and islets were isolated as described previously (23). Human islets were a gift from Dr. C. Ricordi (Diabetes Research Institute, University of Miami School of Medicine, Miami, FL). Both rodent and human islets were cultured in RPMI 1640, 10% FCS with 2 mM l-glutamine, 5 mM d-glucose, and 50 U/ml of penicillin and streptomycin, at 37°C with 5% CO2.

Analysis of A20 mRNA Expression in Islets. Total mRNA was isolated from human and rodent islets (RNAeasy Mini-Protocol; Qiagen) and cDNA was synthesized using random hexamers (SuperScript Preapcloniase System for First Strand C DNA Synthesis; Gibco BRL). PCR reactions were performed with the following primers: rodent β-actin: sense, 5′-CTTGACCGAGCTTGCTACGC-3′, and antisense 5′-AGCGCTAGGCAATCGGAAC-3′; A20: sense, 5′-TTTGGACACATATGGCGAAC-3′, and antisense 5′-AGTTGTCCATCCATGCTATTCC-3′; rat iNOS: sense, 5′-TGACCTGAAAGGAGAAGGAC-3′, and antisense 5′-CAGTTTTTATGGATCACG-3′. PCR was performed over a range of cycles (15–40) to ensure that amplification occurred in the linear range, and equal starting amounts of each sample were used.

rAd Vectors and Gene Transduction of Rodent Islets. The rAd vector expressing A20 (rAd.A20) was a gift from Dr. V. Dixit (Department of Molecular Oncology, Genentech, Inc., South San Francisco, CA); the control vector expressing β-galactosidase (rAd.β-gal) was a gift from Dr. R. Gerard (Department of Biochemistry, University of Texas, Southwestern Medical Center, Dallas, TX). Islets were infected with rAd vectors immediately after isolation as described previously for other cell types (28). After infection, islets were cultured for an additional 24 h before being used for further experiments. For all experiments (unless otherwise stated), 200 islets were cultured in 500 μl of media in 24-well tissue culture plates.

Analysis of A20 Protein Expression and Islet Viability after rAd Infection. Expression of A20 protein after rAd.A20 gene transduction in islets was determined by Western blotting using standard techniques. A20 protein expression was detected with a polyclonal A20 antisera (A20-NT) raised against an NH2 terminus peptide sequence of human A20 (IR ERT PED F KP T). N. A20 viability after viral infection was assessed by staining with propidium iodide (10 μg/ml) and calcein-AM (2 μM; Molecular Probes), then determined by two-color fluorescence microscopy.

Flow Cytometric Analysis of A20 Expression. Islet cultures were stimulated with recombinant murine IL-1β (10 U/ml) and recombinant rat IFN-γ (300 U/ml) (R&D Systems) for 40 h. Islets were then harvested, dispersed, fixed in 70% ethanol, and suspended into DNA staining buffer (PBS, pH 7.4, containing 0.1% Triton X-100, 0.1 mM EDTA, 50 μg/ml propidium iodide, 50 μg/ml RNase A). Islet DNA content was analyzed on a FACScan™ using CELLQuest™ acquisition software (Becton Dickinson Immunocytometry Systems). Islets with a normal DNA content (2N = 2N) were scored as viable, whereas islets with a hypodiploid DNA content (<2N, termed A20) were scored as apoptotic. To exclude debris and apoptotic cell-free fragments, all experiments with an FL-2 area profile below that of chicken erythrocyte nuclei were excluded from analysis.

Determination of iNOS Protein Expression. To determine the effects of A20 expression on iNOS protein induction, islets were stimulated with IL-1β (10 U/ml) for 24 h. iNOS protein expression was determined by Western blotting using the polyclonal anti-iNOS Ab (Santa Cruz). To determine the role of NO in cytokine-induced apoptosis, islets were treated with IL-1β (10 U/ml) and IFN-γ (300 U/ml) in the presence or absence of the NOS inhibitor L-NAME (10 mM) used at the optimal concentration of 1 mM. The extent of iNOS expression and NO generation was determined as described above.

Transient Transfection of the Murine β-TC3 Cells. β-TC3 cells (29) were plated at a density of 1.5 × 106 cells/well into 6-well tissue culture plates and transfected 24 h later using the Lipofectamine-Plus reagent (Gibco BRL) with 1 μg total DNA. Specifically, β-TC3 cells were transfected with 0.6 μg of the iNOS reporter (pGLH/H2; containing 1,755 bp of the murine iNOS promoter linked to a luciferase gene [30]), a gift of Dr. W. J. M. Murphy (Wilkinson Laboratory of the Kansas Cancer Institute, University of Kansas Medical Center, Kansas City, KS); 0.3 μg of an expression plasmid containing the human A20 gene (pCDNA3/HA-A20) or the control empty plasmid pCDNA3; and 0.1 μg of a β-gal reporter (driven by the CMV promoter), used to correct for transfection efficiency. 24 h after transfection, cells were analyzed for NO levels (measured as nitrite) by adding 50 μl of Griess reagent (equal volume of 1% sulfanilamide in 0.1 M HCl and 0.1% N-1-naphthyl-ethylenediamine dihydrochloride) to 50 μl of culture media. NO concentration was determined by spectrophotometry (560 nm) from a standard curve (0–200 μM) derived from NaNO2. NO data are expressed as mean ± SD [nitrite] in μM per 200 islets.

Analysis of the Role of NOS in Cytokine-induced Apoptosis. To examine whether NO could directly induce apoptosis, islets were treated for 24 h with the NO donors 5-nitrosothioglutathione (GSNO) or N-(2-aminoethyl)-N-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (NOSO) at a range of concentrations (0.001–10 mM). To determine the role of NOS in cytokine-induced apoptosis, islets were treated with IL-1β (10 U/ml) and IFN-γ (300 U/ml) in the presence or absence of the NOS inhibitor L-Nω-(1-iminoethyl) ornithine, dihydrochloride (L-NωNO) used at the optimal concentration of 1 mM. The extent of iNOS expression and NO generation was determined as described above.

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were stimulated with IL-1β (100 U/ml) for 36 h. These conditions were shown to be optimal in preliminary experiments (data not shown). Luciferase and β-gal activity were assessed as described (27). Data are expressed as relative luciferase activity according to the formula: luciferase light units/β-gal light units × 100.

Electrophoretic Mobility Shift Assay. To determine the effect of A20 overexpression on the transcription factor NF-κB, islets (1,000 islets/1 ml media in 24-well tissue culture plates) were stimulated with IL-1β (100 U) for 1 h. Islet nuclei were recovered by an isoosmotic/NP-40 lysis procedure, and nuclear proteins were extracted as described (31). DNA binding reactions were performed by incubating 5 μg of nuclear proteins with 1 μg of poly(dI-dC) and 10^5 cpm of radiolabeled NF-κB consensus oligonucleotide, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (Promega Corp.). For competition assays, 1.75 pmol of either unlabeled NF-κB or an unrelated oligonucleotide was added to the reaction mixture. Supershift analysis was conducted by adding 0.1 μg of Ab specific for p50/NF-κB1, p65/R elA, R-el-B, c-R el, or Ets-1 (Santa Cruz) to the reaction 1 h before the addition of radiolabeled oligonucleotide. The DNA binding reactions were resolved on a 6% polyacrylamide gel and analyzed by autoradiography.

Determination of β-gal Degradation. The effect of A20 expression on β-gal protein degradation was determined by Western blot analysis, after treatment with IL-1β (100 U/ml) for 0, 15, and 60 min. β-gal protein expression was detected using the polyclonal anti-β-gal Ab, C-20 (Santa Cruz).

Statistical Analysis. All statistical analysis was conducted using the alternate W elch’s method.

Results

A20 Is Induced in Islets of Langerhans in Response to Inflammatory Stimuli. We first examined if A20 was expressed constitutively in islets and whether A20 expression could be induced by cytokine stimulation. No weak constitutive A20 mRNA was detected in rat and human islets as analyzed by reverse transcription (RT)-PCR (Fig. 1, a and b). A20 mRNA was rapidly induced (within 1–2 h) in both rat and human islets after IL-1β stimulation (Fig. 1, a and b). Rat β A20 mRNA is induced in human and rat islets after stimulation with IL-1β. After isolation, 500–1,000 islets were cultured overnight and then stimulated with IL-1β (100 U/ml). A20 mRNA expression was determined by RT-PCR in (a) human islets, 1 h after stimulation; (b) rat islets, 2 h after stimulation; and (c) rat insulinoma cells (R.in5F), 1 and 2 h after stimulation. A20 mRNA was rapidly induced after IL-1β activation in both species. media, no IL-1β stimulation; TC, template control without cDNA.

Figure 1. A20 mRNA is induced in human and rat islets after stimulation with IL-1β. After isolation, 500–1,000 islets were cultured overnight and then stimulated with IL-1β (100 U/ml). A20 mRNA expression was determined by RT-PCR in (a) human islets, 1 h after stimulation; (b) rat islets, 2 h after stimulation; and (c) rat insulinoma cells (R.in5F), 1 and 2 h after stimulation. A20 mRNA was rapidly induced after IL-1β activation in both species.

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indicates that adenoviral infection of islets per se does not alter their function.

A20 overexpression Protects Islets from Cytokine-induced Apoptosis. Previous work has demonstrated that A20 is an early response gene that protects cells against cytokine-mediated cytotoxicity (25, 26). The proinflammatory cytokine IL-1β is cytotoxic to β-cells and represents a significant mediator of β-cell apoptosis in IDDM, especially in combination with IFN-γ (10). Therefore, we examined whether A20 would protect islets against IL-1β- and IFN-γ-mediated toxicity. IL-1β and IFN-γ were used at the optimal dose of 10 and 300 U/ml, respectively, induced a significant percentage of apoptosis in rat islets after 40 h in culture (Fig. 3). This percentage (mean ± SD, n = 4 independent experiments) reached 57.58 ± 16.51 and 55.08 ± 18.35% in both noninfected and control rAd.β-gal-infected islets, respectively (P < 0.01, n = 4), as evaluated by FACS® analysis of DNA content (Fig. 3). In contrast, rAd.A20-infected islets were protected from IL-1β- and IFN-γ-mediated apoptosis; the percentage of apoptosis in these islets was not significantly different (P = 0.714, n = 4) from that observed in non–cytokine-activated control islets (Fig. 3). These data demonstrate that A20 protects islets from cytokine-mediated apoptosis.

A20-mediated Protection from Apoptosis Correlates with Suppression of NO Production. There is substantial evidence that A20 protects islets from cytokine-mediated apoptosis. Noninfected and rAd.β-gal-infected islets produced equally high levels of NO after stimulation with IL-1β and IFN-γ (Fig. 4). In contrast, NO production in rAd.A20-infected islets was totally suppressed (P < 0.0001, n = 4) compared with noninfected and rAd.β-gal-infected islets and was not significantly different (P = 0.099, n = 4) from background levels observed in non-cytokine-activated groups (Fig. 4). Thus, the percentage of islets undergoing apoptosis for each treatment correlated with their production of NO.

IL-1β- and IFN-γ-induced Apoptosis Is Mediated by NO. Our data demonstrate that A20 can protect islets from cytokine-induced apoptosis. Furthermore, they show that the antiapoptotic effect of A20 correlates with suppression of cytokine-induced NO production, suggesting that A20 is protecting islets through effects on NO generation. This hypothesis is in accordance with data from the literature showing that NO is a key mediator of cytokine-induced islet cytotoxicity (9, 14, 33). To determine whether the antiapoptotic effect of A20 was a direct result of its ability to suppress NO production, we examined the role of NO in cytokine-induced apoptosis of islets. We first examined if NO could directly induce apoptosis in rat islets. Rat islets were cocultured with one of two NO donors, NO NO or GSNO, at various concentrations ranging from 0.01 μM to 10 mM. 16 h later, islets were examined for induction of apoptosis (Fig. 5 a). Both NO NO and GSNO, in a dose-dependent manner, induced significant levels of apoptosis in rat islets. However, NO NO was 10-fold more potent than GSNO due to its higher release of NO in the medium (Fig. 5 a, and data not shown). Given that NO is able to directly induce apoptosis in rat islets, we next examined whether NO was the agent responsible for islet apoptosis after cytokine stimulation. The NOS inhibitor L-NIO (500 μM) was added to cytokine-stimulated islets. Islets stimu-

![Figure 3](image-url)  
**Figure 3.** A20 protects rat islets against cytokine-induced apoptosis. Noninfected (NI), rAd.β-gal-, and rAd.A20-infected islets were cultured in the presence or absence of IL-1β (10 U/ml) and IFN-γ (300 U/ml) for 40 h, and the percentage of apoptotic cells was determined by flow cytometry. The percentage of apoptosis in each treatment (given in upper right corner) was calculated by analysis of the percentage of events in the subdiploid region (termed Apoptosis®, where DNA content < 2 N) from the FL-2 area histogram (total of 10,000 events collected). The data presented are representative of four independent experiments conducted. Results demonstrate that expression of A20 in islets protects them from cytokine-mediated apoptosis.

![Figure 4](image-url)  
**Figure 4.** A20 inhibits production of NO by cytokine-activated rat islets. Noninfected (NI), rAd.β-gal-, and rAd.A20-infected islets were cultured in the presence or absence of IL-1β (10 U/ml) and IFN-γ (300 U/ml) for 40 h, and NO levels were determined in the culture medium. There was no significant difference in IL-1β-stimulated NO production by rAd.β-gal-infected islets compared with noninfected islets (P = 0.315). However, NO production was totally abrogated in A20-expressing islets compared with noninfected or rAd.β-gal-infected islets (P < 0.0001). Nitrite levels (μM/200 islets) are the mean ± SD of triplicate determinations, pooled from four independent experiments.
iNOS protein expression, steady state mRNA levels, and regulation of gene transcription. For these and subsequent experiments, islets were stimulated with IL-1β alone, as IFN-γ by itself had little or no effect on NO induction (data not shown).

We examined whether A20 overexpression would modulate the induction of iNOS protein after cytokine stimulation. Noninfected and rAd.β-gal–infected islets expressed high levels of iNOS protein 24 h after activation with IL-1β (Fig. 6 a). These data are in accordance with previous studies demonstrating that in islets, cytokine treatment results in de novo production of iNOS mRNA and protein (34). In contrast, IL-1β–mediated upregulation of iNOS protein was totally suppressed in A20-expressing islets (Fig. 6 a). Accordingly, NO generation after IL-1β stimulation was highly suppressed (≈90%) in A20-expressing islets compared with the significant NO levels detected in noninfected and rAd.β-gal infected islets (data not shown).

To determine the underlying mechanism by which A20 was suppressing iNOS protein upregulation, we examined, by RT-PCR analysis, iNOS steady state mRNA levels after IL-1β activation. No iNOS mRNA was detected in nonstimulated islets, whereas iNOS transcript was induced 5 h after IL-1β stimulation in both noninfected and rAd.β-gal–infected islets (Fig. 6 b). In contrast, no iNOS mRNA was detected in rAd.A20-infected islets (Fig. 6 b).

It has been established that induction of iNOS mRNA expression by IL-1β is regulated at the transcription level (30, 34, 35). Therefore, we questioned whether the inhibitory effect of A20 on inos gene upregulation occurred at the level of gene transcription. To address this possibility, β-TC3 cells were cotransfected with a murine iNOS reporter (30) and a human A20 expression plasmid or the control plasmid, pcDNA3. β-TC3 cells were stimulated with IL-1β (100 U/ml) for 36 h after transfection, and luciferase activity was calculated as described in Materials and Methods. As shown in Fig. 6 c, IL-1β stimulation resulted in a significant two- to threefold induction of the iNOS reporter in A20–infected β-TC3 cells (mean fold induction ± SD, 2.24 ± 0.75; P < 0.0001, n = 5). In contrast, IL-1β induction of the iNOS reporter in A20-expressing β-TC3 cells was totally suppressed (P < 0.0001, n = 5) to the extent that there was no difference relative to background levels in pcDNA3–infected β-TC3 cells (P = 0.75, n = 5). Interestingly, A20 overexpression also significantly reduced the basal (nonstimulated) iNOS reporter activity by ~50% (P < 0.005, n = 5) compared with β-TC3 cells transfected with pcDNA3.

A20 Inhibits NF-κB Activation at a Level Upstream of IκBα Degradation. Our data indicate that A20 can suppress the IL-1β–dependent activation of the iNos gene. Previous reports have implicated the transcription factor NF-κB as an essential component of this activation (34, 36). Therefore, we examined whether A20 was suppressing iNos transcription via modulation of NF-κB activation. To check whether A20 expression was altering NF-κB translocation to the nucleus, we performed electrophoretic mobility shift assays (EMSA) using nuclear extracts isolated from noninfected, rAd.β-gal–, and rAd.A20–infected islets after IL-1β stimulation.

Figure 5. NO mediates islet apoptosis induced by IL-1β and IFN-γ. (a) NO donors induce apoptosis in rat islets. Islets were left untreated or were stimulated with GSN O (1.0 mM) or NONOate (0.1 mM) for 16 h, and the percentage of apoptotic cells was determined by flow cytometry. The percentage of apoptotic events was calculated as described and is given in the upper right corner. Data are from a representative experiment of three independent experiments conducted. (b) The L-arginine analogue L-NIO inhibits both apoptosis and NO generation in rat islets. Islets were cultured in the presence or absence of IL-1β (10 U/ml) and IFN-γ (300 U/ml) for 40 h with or without L-NIO (2.2 μM), and the percentage of apoptosis for each condition was measured by flow cytometry. Data from three independent experiments were pooled and are given as the percentage of apoptosis (mean ± SD). NO production (mean ± SD, [nitrite] μM) was measured in the culture medium from each condition and is given in the chart. Suppression of NO production correlated with protection from apoptosis.
Discussion

IDDM is an autoimmune disease characterized by the specific destruction of β cells in islets of Langerhans (3).
LPS, CD40 ligation, the LMP1 protein of EBV, and the Tax protein of HIV (42–45). The rapid induction of A20 mRNA by these diverse stimuli requires the activation of the transcription factor NF-κB. Two κB binding elements map within the A20 promoter and are essential for its expression (46). Here we show that expression of A20 is rapidly induced in β cells in response to IL-1β. This is the first report showing the induced expression of the antiapoptotic gene A20 in β cells. Further, our data show that IL-1β induces the activation of NF-κB in islets, which concurs with its ability to upregulate the expression of A20. The rapid kinetics of A20 expression in islets suggests that, as in endothelial cells, it may be a component of their physiological protective response to injury (47).

Having established that A20 is a rapid response gene in β cells, we examined whether A20 maintained its antiapoptotic function in islets. Expression of A20 in islets by means of an rAd protects them from apoptosis induced by IL-1β and IFN-γ. The protective effect of A20 against IL-1β- and IFN-γ-induced apoptosis is critical given the central role of IL-1β in β cell dysfunction and destruction during IDDM (9, 48). IL-1β inhibits glucose-dependent insulin secretion, impairs glucokinase synthesis, and induces cell death by apoptosis (49, 50). Inhibition of IL-1β using neutralizing mAbs prevents diabetes progression in NOD mice (51). The pathway by which IL-1β mediates β cell death and toxicity has recently been clarified. IL-1β is produced by activated resident macrophages within the islets (48, 21, 52, 53). Once produced, IL-1β acts directly and selectively upon β cells to induce iNOS, leading to the production of high and sustained levels of NO and to a lesser extent superoxide (12, 54). NO directly induces apoptosis of β cells and is the mediator of the multiple toxic effects of IL-1β on β cells (55–57). We confirmed the apoptotic potential of NO in our system with the NOS inhibitors GSN O and NO3Oate, which rapidly induced apoptosis of rat islets. Furthermore, the addition of the NOS inhibitor L-NIO to cytokine-activated islets prevented both NO production and apoptosis. These data demonstrate that endogenously generated NO is the mediator of cytokine-induced islet apoptosis in our system.

The central role of NO in cytokine-mediated β cell toxicity prompted us to examine whether the protective effect of A20 in islets was associated with modulation of NO levels. We found that expression of A20 in islets abrogated NO production in response to cytokines. Taken together with our data showing that pharmacologic suppression of NO production also protects from cytokine-induced apoptosis, these data establish the suppression of NO production as one mechanism by which A20 protects islets (58). The suppression of NO production by A20 could also impact on T cell–dependent β cell destruction. Indeed, NO facilitates T cell–dependent killing via upregulation of Fas on human islets (15, 59). Ongoing work in our laboratory is aiming at determining whether expression of A20 in islets will also protect β cells against T cell–mediated cytotoxicity via the perforin/granzyme or the Fas/FasL pathway.

The mechanism by which A20 suppresses cytokine-induced NO production is shown to be via inhibition of IL-1β–induced iNOS mRNA and protein expression. Expression of iNOS protein in islets is regulated by de novo transcription of the nos gene (30, 34, 35). We reasoned that the absence of iNOS protein and mRNA after cytokine
NF-κB activation and inos mRNA induction can be suppressed in islets by antioxidants such as pyrrolidine dithiocarbamate (PDTC) (34). Moreover, NF-κB is a redox-sensitive transcription factor, as indicated by the fact that NF-κB activation can be induced by H₂O₂ or, conversely, NF-κB nuclear translocation is blocked by antioxidants such as PDTC (71, 72). The potential for A20 to interfere at the oxidative step in NF-κB activation is currently being tested. Interestingly, several studies have addressed the protective potential of antioxidants in islets by overexpressing free radical scavenging enzymes (41, 73–75). The overexpression of MnSOD in an engineered β cell resulted in selective protection from IL-1β-induced cytotoxicity as well as a reduction in cytokine-induced NO generation (75). In addition, transgenic expression of the antioxidant thioredoxin in β cells of NOD mice reduced the incidence of spontaneous diabetes and protected from streptozotocin-induced diabetes (76).

Interestingly, thioredoxin has been shown to inhibit NF-κB by interfering with a redox-sensitive step required for its activation (77, 78). Thus, in the model of Hotta et al. (76), the protective effect of thioredoxin may involve inhibition of NF-κB activation, given the role of NF-κB activation in NO generation and islet destruction (36, 54, 79). Together, these results illustrate a novel concept whereby protection of the target (in this case, β cells) would offer a potent therapeutic strategy to inhibit disease occurrence even in the presence of the effector mechanisms (cellular and soluble mediators). This approach might constitute an alternative to systemic modulation of the immune system as currently practiced using diverse immunosuppressants, such as costimulation blockade (80–83). Along with this approach, other antiapoptotic genes such as bcl-2 have been proposed as gene therapy tools to protect islets from cytokine-mediated apoptosis. Expression of Bcl-2 in a murine β cell line did provide modest protection from cytokine-mediated apoptosis (84, 85). Interestingly, bcl genes have, like A20, antiinflammatory properties through blockade of transcription factors, such as NF-κB in endothelial cells (86–88). We are currently testing whether they maintain this dual function in islets and could synergize with A20 to protect β cells. However, in contrast to A20, Bcl-2 is expressed constitutively in islets and is not induced upon cytokine activation (data not shown). We propose that constitutively expressed antiapoptotic proteins such as Bcl-2 may function to protect cells from baseline cellular stress, whereas induced cytoprotective proteins such as A20 protect cells from greater stress caused by inflammatory reactions (47). We suggest that A20 could be a more relevant gene therapy candidate for protection of β cells against the additional stress encountered in the setting of transplantation and autoimmunity. Future experiments will determine the efficacy of A20 in both islet transplant and autoimmune diabetes models.
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