Distinct differences in the responses of the human pancreatic β-cell line EndoC-βH1 and human islets to proinflammatory cytokines

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Insulinoma, produced by pancreatic β-cells found in the islets of Langerhans, regulates whole body glucose metabolism. Diabetes develops when β-cells are not capable of providing the appropriate amount of insulin to control blood glucose levels. The two major forms of diabetes, Type 1 and Type 2, are characterized by an impairment in β-cell function. The impairment in function (reduced insulin secretion) results from a loss of β-cell mass and an inability of β-cells to produce sufficient amounts of insulin to induce uptake of blood glucose by peripheral tissues in insulin-resistant individuals. Insulinoma cells have proven to be exceptionally important in studies designed to understand the mechanisms regulating glucose-stimulated insulin secretion (GSIS) and those controlling β-cell fate. To date, nearly all insulinoma cells have been derived from rodents. From the first RIN lines derived from tumors formed in irradiated rats to the generation of INS1 that were derived from the same tumors but cultured with 2-mercaptoethanol to maintain glutathione levels (2, 8, 22), these insulinoma cells have proven to be an extremely important source of insulin-producing tissue for molecular studies of β-cell function and viability.

Cytokines, such as macrophage-derived IL-1 and TNF, and lymphocyte-derived IFN-γ, are inflammatory mediators implicated in the loss of functional β-cell mass during the development of Type 1 and Type 2 diabetes (7, 19, 20, 46). Insulinoma cells have proven extremely useful in determining the mechanisms by which cytokines impair β-cell function. RINm5F cells were originally used to demonstrate that IL-1 stimulates β-cell expression of inducible nitric oxide synthase (iNOS) and production of nitric oxide by electron paramagnetic resonance spectroscopy (15). The production of nitric oxide by these cells has also been used as a selective bioassay for human and mouse IL-1 (25). Clonal selection was used to generate INS cells lines sensitive and resistant to killing by proinflammatory cytokines, and these cells have been extremely valuable in determining the type of β-cell death induced by cytokines (9). Insulinoma cells have been the primary source of insulin-producing cells used to identify the kinases, transcription factors, and genes that contribute to the response of β-cells to cytokines. The ease of molecular analysis, from gene targeting to overexpression, and very close similarities in response to isolated islets and primary β-cell purified by fluorescence-activated cell sorting has led to the widespread use of insulinoma cells to study the actions of cytokines on β-cells.

It has been difficult to translate findings from rodent islets and insulinoma cell lines to human islets because of the limited availability of human tissue and the lack of suitable β-cell lines of human origin (36, 44). Recently, Ravassard et al. (49) developed a human insulinoma cell line from fetal pancreatic buds transduced with simian virus 40 larger tumor antigen. While EndoC-βH1 cells have a very slow doubling time, they are glucose-responsive human insulin-producing cells (49). In this report, we have compared the cytokine responses of EndoC-βH1 cells to the responses of human islets. Recent

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studies have shown that the combination of IL-1β, IFN-γ, and TNF-α reduces human islet cell viability in a time-dependent manner (1, 13, 38, 48). Cell death is associated with increased iNOS expression and nitric oxide production by β-cells and is prevented by inhibitors of nitric oxide synthase (NOS) (1, 13, 57). We show that IL-1β, IFN-γ, and TNF-α alone do not modify EndoC-βH1 cell viability; however, when combined, these cytokines reduce cell viability in a time-dependent manner with ~40% loss following a 48-h incubation. EndoC-βH1 cell death in response to cytokine treatment is associated with cell shrinkage and a fivefold increase in caspase cleavage, consistent with cell death by apoptosis. Unlike primary human islet cells, the loss in viability of EndoC-βH1 in response to cytokines is not sensitive to NOS inhibition. Furthermore, EndoC-βH1 cells do not express iNOS or produce nitric oxide following cytokine treatment. Stressed human islets, or human islets expressing heat shock protein 70 (HSP70), fail to respond to cytokines (3, 4, 29, 54, 55) or produce nitric oxide following cytokine treatment. Inhibition. Furthermore, EndoC-βH1 cells do not express iNOS or produce nitric oxide following cytokine treatment. Stressed human islets, or human islets expressing heat shock protein 70 (HSP70), fail to respond to cytokines (3, 4, 29, 54, 61) and, similar to stressed human islets, EndoC-βH1 cells constitutively express HSP70. These findings suggest that EndoC-βH1 cells do not faithfully recapitulate the response of human islets to cytokines and caution should be exercised in interpreting results from studies focused on cytokine-mediated damage when using this new human insulinoma line.

RESEARCH DESIGN AND METHODS

Materials and animals. Insulinoma EndoC-βH1 cells were obtained from Raphael Scharffman (Paris Descartes University, France) under Material Transfer Agreements made with both the Medical College of Wisconsin and the University of Florida (49). Male Sprague-Dawley rats (250–300 g) were purchased from Harlan (Indianapolis, IN). Human islets were obtained from the Integrated Islet Distribution Program (Irvine, CA). Dulbecco’s modified Eagle’s medium (1 g/l glucose), Connaught Medical Research Laboratories (CMRL)-1066 medium, penicillin, and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (fraction V) was obtained from Raphael Scharffman (Paris Descartes University, France) and the University of Florida (C.E.M., 201301189).

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Western blot analysis. Cells/islets were washed with PBS and lysed in Laemmli buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and blocked in 5% milk in Tris-buffered saline-Tween 20 for 30 min. Membranes were incubated overnight at 4°C with the following dilutions of primary antibodies in 1% BSA/Tris-buffered saline-Tween 20: iNOS (1:1,000), COX-2 (1:1,000), HSP70 (1:1,000), iκBα (1:1,000), p-STAT1 (1:1,000), phosphorylated p38 (1:1,000), phosphorylated JNK (1:2,000), cleaved caspase-3 (1:500), total JNK (1:100), total p38 (1:100), and GAPDH (1:10,000). Membranes were then incubated with secondary antibodies for 45 min at the following concentrations: donkey anti-mouse horseradish peroxidase (1:5,000) and donkey anti-rabbit-horseradish peroxidase (1:7,000), followed by detection using chemiluminescence, as previously described (31).

Nitrite determination. Nitric oxide formation was determined by measuring the accumulation of its stable metabolite nitrite in culture supernatants using the Griess assay (23).

Cell viability and death assays. Cell viability was measured using the neutral red uptake assay, as previously described (50, 57). Following treatment, EndoC-βH1 cells were incubated with neutral red (40 μg/ml) for 1 h at 37°C. The supernatant was then removed by aspiration, and the cells were fixed with 1% formaldehyde/1% CaCl2. Neutral red was extracted with 50% ethanol/1% acetic acid, and optical density was measured at 540 nm. Neutral red assays were performed in a 96-well plate with six technical replicates for each condition. Cell death was determined by assessing fluorescence of the SYTOX Green nucleic acid stain (Invitrogen). SYTOX Green was used at 5 μM, with duplicate wells for each condition receiving 5 μM SYTOX Green and 120 μM digitonin. Cells were incubated at 37°C for 30 min, and fluorescence was read at excitation/emission of 504 nm/523 nm. Percent cell death was calculated by normalizing to fluorescence in SYTOX Green + digitonin duplicate wells for each condition (set as 100% cell death).

EndoC-βH1 cell insulin secretion and bioenergetics. EndoC-βH1 cells were plated 70,000 cells per well in either flat-bottom 96-well plates (Costar) or 24-well XF24 Cell Culture Microplates (Seahorse Biosciences) in complete EndoC-βH1 media. For cytokine treatments, either IFN-γ alone or the combination of IL-1β (75 U/ml), IFN-γ (750 U/ml), and TNF-α (1,000 U/ml) were added, and the cultures incubated for 72 h. At this time, EndoC-βH1 cells were either subjected to GSIS analysis or extracellular flux bioenergetic analysis using a Seahorse XF24 bioanalyzer (Seahorse Bioscience). To measure static glucose-stimulated insulin secretion, EndoC-βH1 cells were washed with Krebs-Ringer bicarbonate-HEPES (KRBH) twice, and then incubated in 200 μl of KRBH with 2 mM glucose for 1 h. After this hour, 100 μl of media were removed, and 100 μl of KRBH containing 40 mM glucose were added (20 mM glucose final concentration), and the cells were incubated for an additional hour. Samples removed after culture of EndoC-βH1 cells in low or high glucose were tested for insulin levels using an insulin enzyme immunoassay (Alpco).

Cytokine-induced changes in mitochondrial respiration [oxygen consumption rate (OCR) or glycolysis [extracellular acidification rate (ECAR)]] were measured using an XF24 bioanalyzer. After 72 h of exposure to cytokines, the EndoC-βH1 cells were prepared for analysis by washing and then equilibrating in modified Seahorse XF assay medium (2 mM glucose; pH 7.4). The assay protocol took readings at 7-min intervals. Compounds were added at the indicated times. These compounds were glucose (port A: 20 mM final), oligomycin (port B: 5 μM final), carbonyl cyanide p-trifluoromethoxyphenylhydrazone.
Real-time PCR. Total RNA was isolated from EndoC-βH1 cells and islets using the RNeasy kit (Qiagen). First-strand cDNA synthesis was performed using oligo(dTs) and the SuperScript premplification system per manufacturer’s instructions (Life Technologies). Quantitative RT-PCR was performed using SsoFast EvaGreen supermix (Bio-Rad) and a Bio-Rad CFX96 Real-Time system per manufacturer’s instructions. PCR products were then separated on a 2% agarose gel and visualized with ethidium bromide staining. All primers were purchased from Integrated DNA Technologies (Coralville, IA) and are as follows: iNOS (forward) 5'-CGTTGAGACGGGAAGAAGT-3', (reverse) 5'-CTTAATGTGACCGAGGCA-3'; GAPDH (forward) 5'-CTATACAGGCGAGGCA-3', (reverse) 5'-GCCGCAAATACGACCAAATC-3'.

Statistics. Statistical analysis was performed using one-way analysis of variance with a Tukey-Kramer post hoc test. The minimum level of significance was set at $P < 0.05$.

RESULTS

Cytokines induce EndoC-βH1 cell death in a nitric oxide-independent manner. To determine whether EndoC-βH1 cells respond to cytokines in a manner similar to human islets, EndoC-βH1 cells were treated with a cytokine combination of IL-1, IFN-γ, and TNF-α that is known to induce human islet cell death following 24- or 48-h treatments (13). In a time-related manner, this cytokine combination decreases EndoC-βH1 cell viability by 25% following a 24-h incubation and ~45% following a 48-h treatment (Fig. 1A). Staurosporine (STS) is shown as a positive control for apoptosis. Morphological analysis of cytokine-treated EndoC-βH1 cells by phase-contrast microscopy revealed that death is associated with cell shrinking and rounding before eventual detachment (Fig. 1B).

These morphological changes are indicative of apoptotic cell death, and, consistent with this interpretation, cytokines stimulate approximately fivefold increase in cleavage of caspase-3 following a 24-h treatment (Fig. 1, C and D). Single or dual combinations of cytokines (IL-1β + IFN-γ, TNF-α + IFN-γ, or IL-1β + TNF-α) do not reduce EndoC-βH1 viability following 24- and 48-h incubations (Fig. 1E). Nitric oxide does not appear to contribute to cytokine-induced death of EndoC-βH1 cells, as the NOS inhibitor L-NMMA does not modify the response of these human insulinoma cells to the cytokine combination (Fig. 1A).

The effects of cytokines on iNOS and COX-2 expression in EndoC-βH1 cells. Since nitric oxide mediates the damaging actions of cytokines on human islet function and viability (13), and NOS inhibition does not modify cytokine-induced EndoC-βH1 cell death, we examined whether these cells express iNOS in response to cytokine treatment. EndoC-βH1 cells were treated for 24 and 48 h with the cytokine combination of IL-1, IFN-γ, and TNF-α, and then the cells were isolated and iNOS expression was examined by Western blot analysis. Consistent with the absence of an effect of the NOS inhibitor on cell viability, EndoC-βH1 cells do not express iNOS following 24- or 48-h cytokine treatment (Fig. 2A). In addition to iNOS, the effects of cytokines on the expression of the inducible isoform of COX [COX-2, (10)] were examined by Western blot analysis. Similar to iNOS, EndoC-βH1 cells do not express COX-2 in response to cytokine treatment; however, this cytokine combination stimulates COX-2 expression in human islets (Fig. 2, A–D). There is an accumulation of iNOS mRNA in

**Fig. 1.** Cytokines cause EndoC-βH1 cell death in a nitric oxide-independent manner. EndoC-βH1 cells were treated with cytokines (75 U/ml IL-1β, 750 U/ml IFN-γ, and 10 ng/ml TNF-α) with or without L-NMMA (2 mM) for the indicated times. A: cell viability was measured using the neutral red uptake assay. Staurosporine (STS; 1 μM for 24 h) was used as a positive control for cell death. B: EndoC-βH1 cell morphology was examined by phase contrast microscopy. Cleavage of caspase-3 was examined using Western blot analysis (C) and quantified by densitometry (D). Individual effects of 75 U/ml IL-1β, 750 U/ml IFN-γ, and 10 ng/ml TNF-α, or combinations of IL-1β + IFN-γ, IL-1β + TNF, or TNF + IFN-γ on EndoC-βH1 cell viability are shown (E). GAPDH was used as a control for equal protein loading. Results are representative or the average ± SE of three independent experiments with statistically significant differences vs. control. *$P < 0.05$. 

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EndoC-βH1 cells following cytokine treatment (Fig. 2E); however, the levels of mRNA accumulation are ~3.5-fold lower in EndoC-βH1 cells than the levels that accumulate in cytokine-treated human islets (Fig. 2E). Furthermore, translation of iNOS mRNA appears to be attenuated in EndoC-βH1 cells compared with human islets. Consistent with the reduction in iNOS mRNA accumulation, cytokines fail to stimulate nitrite production by EndoC-βH1 cells, either alone (data not shown) or in combination (Fig. 2F). While cytokine-stimulated EndoC-βH1 cells do not produce nitric oxide, these cells are still sensitive to this free radical, as a 4-h treatment with the nitric oxide donor dipropylentetriamine/nitric oxide (DPTA/NO) at the indicated concentrations for 4 h, and cell viability was measured by the neutral red uptake assay. Results are representative or the average ± SE of 2 (B) or 3 (A, E–G) independent experiments with statistically significant differences vs. control: *P < 0.05.

The effects of cytokines on insulin secretion and cellular bioenergetics in EndoC-βH1 cells. Cytokines inhibit insulin secretion from β-cells in a nitric oxide-dependent manner (11, 56, 62). As EndoC-βH1 cells do not produce nitric oxide following cytokine exposure, we examined whether cytokine treatment led to a decrease in GSIS in the EndoC-βH1 cells.

EndoC-βH1 cells were treated for 72 h with the cytokines IL-1, IFN-γ, and TNF-α, and insulin secretion was measured as described in RESEARCH DESIGN AND METHODS. In untreated cells, there was a statistically significant increase in GSIS, whereas, in cytokine-treated cells, GSIS was prevented (Fig. 3A). Importantly, the decrease in GSIS in the absence of nitric oxide is consistent with the ability of these cytokines to induce EndoC-βH1 cell death. In addition to insulin secretion, the effects of cytokines on glycolytic flux and mitochondrial respiration were examined using the Seahorse XF24 bioanalyzer. ECAR, an index of glycolytic flux (64), was increased more than three-fold in EndoC-βH1 cells exposed to high glucose compared with cells exposed to low glucose (Fig. 3B). Cytokine treatment had no effect on ECAR under both low- and high-glucose conditions (Fig. 3C). Taken together, these data suggest that, while GSIS is inhibi-
Edited, cytokines do not affect glycolytic and mitochondrial metabolism in EndoC-βH1 cells.

**EndoC-βH1 cells express HSP70 under basal conditions.** While our results (Fig. 2) suggest that there are differences in the cytokine-responsiveness of EndoC-βH1 cells compared with human islets, previous studies by our laboratory and others have shown that islets (rodent and human) undergoing various forms of stress do not respond normally to cytokines (3, 29, 54, 61). The defects in the response to cytokines include a failure of cytokines to signal and induce new gene expression; specifically of genes associated with inflammation such as iNOS (54, 57, 63). The inhibition of cytokine action on islets is associated with elevated levels of HSP70; however, HSP70 does not mediate the inhibition. We have shown that antisense knockdown of HSP70 does not prevent stress-associated impairment in the β-cell response to cytokines (60, 61). These findings suggest that elevated levels of HSP70 mark islets with diminished responsiveness to cytokines (29, 57). Indeed, the basal levels of HSP70 are elevated in EndoC-βH1 cells, consistent with the inability of these cells to express iNOS and COX-2 in response to cytokine treatment (Fig. 4A). In contrast, rat islets, INS 832/13 cells, and human islets are all cytokine responsive and do not express HSP70 under basal conditions (data not shown and Fig. 4A). The elevated levels of HSP70 under basal conditions are consistent with inhibition in cytokine-induced iNOS expression in EndoC-βH1 cells (Fig. 1). While cytokines stimulate expression of HSP70 in islets (21), a 24-h cytokine treatment leads to a reduction in HSP70 in EndoC-βH1 cells (Fig. 4B). Another difference between the EndoC-βH1 cells and other insulinoma cell lines and islets is the
composition of culture media. In particular, the EndoC-βH1 cells are cultured in the absence of serum, whereas most islet/insulinoma cell line media formulations contain 10% FBS. When FBS is added to EndoC-βH1 cell media to 10%, basal levels of HSP70 are markedly decreased, although an increase in cell death is also observed (Fig. 4B and unpublished observation).

Cytokines activate NF-κB, STAT1, and MAPK signaling pathways in EndoC-βH1 cells. Elevated levels of HSP70 expression are also associated with an inhibition or attenuation in the signaling of cytokines from receptor binding to activation of signaling cascades. In rodent and human islets expressing HSP70 (stressed islets), the activation of JAK-STAT signaling by IFN-γ and NF-κB and MAPK by IL-1β is attenuated (3, 4, 54, 60). Since EndoC-βH1 cells express HSP70 under basal conditions, the effects of IL-1β on NF-κB and MAPK activation, and IFN-γ on STAT1 activation, were examined. In contrast to our laboratory’s previous studies (54), HSP70 expression in EndoC-βH1 cells did not lead to an inhibition of transcription factor activation. IFN-γ stimulates p-STAT1, and IL-1β stimulates IκB degradation (an index of NF-κB activation) and JNK and p38 phosphorylation following 30-min incubation (Fig. 5). In contrast to stressed human and rodent islets expressing HSP70 (54, 60), cytokines are capable of activating signaling pathways required for the expression of iNOS, as well as MAPK signaling in EndoC-βH1 cells.

EndoC-βH1 cells are insensitive to the endoplasmic reticulum stress inducer thapsigargin. The elevated levels of HSP70 observed in the EndoC-βH1 cells suggest that these cells may have abnormal activation of endoplasmic reticulum (ER) stress responses. To determine the capacity to respond to the ER stress-inducing agent thapsigargin, EndoC-βH1 cells were treated with the ER stress-inducing agent thapsigargin, an inhibitor of sarco/endoplasmic reticulum calcium ATPase. In response to thapsigargin, phosphorylation of eukaryotic initiation factor-2α (p-eIF-2α; a marker of ER stress) does not increase, while thapsigargin stimulates p-eIF-2α in the INS 832/13 cell line (Fig. 6, A and B). Additionally, the nitric oxide donor fails to stimulate p-eIF-2α in the EndoC-βH1 cells, although it does in the INS 832/13 cells (Fig. 5, A and B). If cellular ER stress cannot be resolved, the activated ER stress pathways will ultimately mediate cell death (59). Consistent with the lack of eIF-2α phosphorylation, thapsigargin treatment for 24 h does not cause cell death in the EndoC-βH1 cells, whereas ~75% of INS 832/13 cells are killed in response to this treatment (Fig. 6C). These data suggest that, despite an elevated expression of basal HSP70, EndoC-βH1 cells may have a dysfunctional ER stress response and thus are insensitive to ER stress-inducing agents.

DISCUSSION

Over the past 20 years, insulinoma cell lines have proven to be extremely useful in studies designed to determine the mechanisms that control β-cell function and viability in response to stressors such as cytokines. Until recently, human insulinoma cells have not been available for this purpose, as
the cell lines generated before the EndoC-βH1 cells exhibited very poor or inconsistent GSIS (36, 44). In this study, we have examined whether the new human pancreatic β-cell line, EndoC-βH1, responds to cytokines in a manner consistent with human islets (13, 17, 47, 48). Cytokine treatment of human islets results in an inhibition GSIS and the loss of viability in a concentration-dependent manner (13, 48). Nitric oxide is a primary mediator of cytokine-induced β-cell damage (16, 24, 33, 46). Inhibition of nitric oxide generation by human and rodent islets of Langerhans attenuates the inhibitory effects of cytokines on protein synthesis, oxidative metabolism, insulin secretion, the induction of DNA damage, and islet cell death (24, 33, 40, 41, 46). In addition to preventing damage, the inhibition of nitric oxide generation can promote a full recovery of β-cell function following cytokine-mediated damage. The addition of NOS inhibitors to human or rodent islets cultured for 24 h with cytokines, followed by the continued culture for 8 h in the presence of both the cytokine and NOS inhibitor, results in a complete recovery of insulin secretion, oxidative metabolism, protein synthesis, and the repair of damaged DNA (12, 27, 51, 55). This functional recovery occurs in the presence of the proinflammatory cytokines. These findings indicate that nitric oxide mediates the initial cytokine-mediated damage, and that removal of nitric oxide (NOS inhibition) in cytokine-treated islets is essential for repair/recovery of β-cell function. These findings, which have been shown in rodent and human islets, indicate that nitric oxide is the primary mediator of cytokine-induced β-cell damage (12, 27, 46, 51, 55).

While nitric oxide has been shown to be a primary mediator of cytokine-induced damage, a number of studies have reported that cytokines damage β-cells by mechanisms independent of nitric oxide, or that human islets are less sensitive to nitric oxide than rodent islets (18, 20, 37, 47). Much of this confusion comes from previous studies that provide evidence for a small amount of cytokine-induced islet cell death following a 9-day incubation of islets isolated from iNOS-deficient mice (7%) that may result from caspase-dependent mechanisms (37). It should be noted that the 7% of islet death in iNOS-deficient islets is miniscule compared with the >80% death of islet isolated from wild-type mice under similar conditions (37). These findings serve to both confirm and highlight the importance of iNOS in cytokine-mediated β-cell death. The rationale that human islets are less sensitive to nitric oxide than rodent islets is based on studies showing that aminoguanidine, a selective iNOS inhibitor (14, 42), attenuates cytokine killing of human islets by only 50%, yet the concentration of aminoguanidine used in these studies decreased human islet viability by 50%, independent of cytokine treatment (20). Further complicating human islet studies are the findings that stressed islets, or more specifically human islets expressing HSP70, fail to express iNOS or produce nitric oxide in response to cytokines. As heat shock of islets blunts the effects of cytokines through the induction of HSP70 or HSP27 and inhibition of iNOS activity (3, 29, 54, 61), any studies using human islets to determine the mechanisms of cytokine toxicity should assess the levels of protective proteins, such as HSP27 or HSP70, as a routine procedure.

The availability of a human insulinoma cell line that responds to cytokines in a manner similar to human islets would provide an experimental system to address many of the issues highlighted above and could serve as a tissue source to perform more mechanistic studies regarding cytokine actions on these cells, as well as to examine a means to prevent the damaging effects of cytokines. Rodent insulinoma cell lines have provided investigators an opportunity to attenuate the expression of target molecules or express specific molecules in insulin-producing cells by transfection. Of the many insulinoma cell lines studied (mouse-MIN6, βTC; and rat-RINm5F, INS-1, INS 832/13), nonobese diabetic mouse-derived NIT-1 cells have been the only insulinoma cell line that does not express iNOS or produce nitric oxide in response to cytokine treatment (58). In this paper, we examined whether the human insulinoma EndoC-βH1 cells respond to cytokines in a manner consistent with human islets. Treatment of EndoC-βH1 cells with the combination of IL-1β, IFN-γ, and TNF-α results in ~30% and ~40% decreases in cell viability following 24 and 48 h exposure, respectively. Unlike human islets, EndoC-βH1 cell death under these conditions was not mediated by nitric oxide, as inhibition of iNOS with L-NMMA does not attenuate this death. EndoC-βH1 cell death induced by cytokines appears to be apoptotic, as evident by morphological changes consistent with this type of death (cell rounding and shrinking) and by a more than fivefold increase in the cleavage of caspase-3. Although cytokine exposure leads to
cell death, EndoC-βH1 cells do not express iNOS or COX-2 [a second gene known to be induced in response to inflammatory cytokine treatment (10)] or produce nitric oxide. This effect contrasts the actions of this cytokine combination on human islets, which induce iNOS and COX-2 expression, nitric oxide production, and nitric oxide-dependent inhibition of function and loss in viability (10, 13, 57).

The inability of cytokines to stimulate nitric oxide production from EndoC-βH1 cells is similar to the response NIT-1 cells, which do not produce nitrite or accumulate iNOS mRNA, yet are killed by combined cytokines (58). It has been suggested that cytokines kill NIT-1 cells through a TNF-α-dependent process that is potentiated by IFN-γ and occurs by nitric oxide-independent mechanisms (58). NIT-1 cells have proven highly useful in studies to understand β-cell death by Fas receptor activation or by cytotoxic CD8+ T cells (6). In contrast to NIT1 cells, cytokines stimulate low levels of iNOS mRNA accumulation in EndoC-βH1 cells; however, the levels of accumulation are much lower than the levels that accumulate in cytokine-treated human islets. In both NIT-1 (28) and EndoC-βH1 cells (this study), cytokines appear to stimulate cell death by apoptosis. Importantly, nitric oxide is a known inhibitor of apoptosis (32, 35, 43, 52). In cells producing high levels of nitric oxide, caspase activity is attenuated due to the S-nitrosation of an active site cysteine residue on this protease (32, 35, 43, 52).

We have shown that cytokines are capable of reducing caspase activity in rodent and human islets; however, this only occurs when the cells no longer produce high levels of nitric oxide following prolonged incubations of 36 h or greater (5, 26). Therefore, apoptosis of EndoC-βH1, like that in NIT-1 cells, is possible, as these cells do not produce the endogenous caspase inhibitor nitric oxide.

Exposure of human or murine islets to cytokines results in inhibition of oxidative metabolism and insulin secretory function (39, 53). The impairment of insulin secretion is a consequence of nitric oxide-dependent inhibition of mitochondrial aconitase through disruption of the iron-sulfur clusters required for its enzymatic activity, resulting in a decrease in oxidative metabolism and ultimately insufficient levels of cellular ATP for GSIS (11, 56, 62). Unlike primary β-cells and many established insulinoma cell lines, EndoC-βH1 do not increase mitochondrial respiration in response to glucose, although flux through glycolysis is increased (Fig. 3). In light of this observation, it is likely that insulin secretion is maintained by ATP derived from glycolysis. As insulin secretion in normal cytokine-treated β-cells is impaired via nitric oxide-dependent inhibition of oxidative phosphorylation, and the EndoC-βH1 J) do not make nitric oxide and 2) do not rely on oxidative phosphorylation, the observed attenuation in GSIS is likely a consequence of cell death in cytokine-treated EndoC-βH1 cells (Fig. 3).

Stress is a major factor that controls the ability of human islets to respond to cytokines. Islets experiencing cellular stresses, such as heat shock or ER stress, become insensitive to cytokine stimulation (3, 29, 54, 61). Elevated basal levels of HSP70 have been used as an index of islet stress, as human islets expressing HSP70 do not express iNOS in response to cytokine treatment (54, 57). Like human islets, rodent islets that have been heat stressed or treated with ER stressors do not express iNOS in response to cytokine treatment (54, 60, 61). HSP70 does not appear to mediate the inhibitory effects of stress, as antisense RNA depletion of this heat shock protein does not prevent the inhibitory actions of heat shock on β-cell responses to cytokines (60). Much like stressed human and rodent islets, EndoC-βH1 cells express elevated levels of HSP70; however, EndoC-βH1 cells express elevated HSP70 under basal conditions. Unlike in human and rodent islets, however, cytokines decrease the expression of HSP70 in EndoC-βH1 cells. The inhibition of iNOS expression and nitric oxide production is associated with a significant attenuation in the accumulation of iNOS mRNA following cytokine treatment compared with the response of isolated human islets; however, this attenuation in mRNA accumulation does not appear to be associated with defects in cell signaling. IL-1β stimulates IκB degradation, an indication of NF-κB activation, and p38 and JNK phosphorylation, and IFN-γ stimulates p-STAT1. Collectively, the failure of EndoC-βH1 cells to express iNOS and produce nitric oxide in response to cytokines, despite intact signaling pathways required for iNOS expression, likely is the result of defects in transcriptional activation, as iNOS mRNA accumulates to levels that are approximately fourfold lower in EndoC-βH1 cells compared with human islets and an inhibition in translation of iNOS mRNA. The translational inhibition of iNOS may be due to the changes in the hypusination of eukaryotic translation initiation factor 5A, as iNOS is a target that is regulated by this initiation factor (45). Similar to the abnormal response to proinflammatory cytokines, the EndoC-βH1 cells additionally appear to have a dysfunctional ER stress response. The lack of phosphorylation of eIF-2α in response to thapsigargin or nitric oxide suggests that the EndoC-βH1 cells may be irregular at sensing or transducing signals associated with ER stress. This is supported by the observed resistance of EndoC-βH1 cells to thapsigargin-induced cell death, a classical inducer of ER stress that is ultimately toxic to human and rodent β-cells (34, 65). Although many rodent-derived insulinoma cell lines have been successfully used to study β-cell biology, and many of these lines appear to mimic some of the responses of primary β-cells, the death of glucose-responsive human insulinoma lines has hampered a detailed characterization of the impact of cytokines on human β-cells. EndoC-βH1 cells represent one of the first consistently glucose-responsive insulinoma lines derived from human β-cells. Unfortunately, these cells do not appear to mimic the response of human islets to proinflammatory mediators (Table 1) and caution should be used when drawing conclusions from studies focused on the actions of cytokines when EndoC-βH1 cells are the primary source of iNOS.

Table 1. Summary of the responses of human islets and EndoC-βH1 cells to cytokines

<table>
<thead>
<tr>
<th>Human Islets</th>
<th>EndoC-βH1 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible to cytokine-induced cell death</td>
<td>+</td>
</tr>
<tr>
<td>Produce nitric oxide in response to cytokines</td>
<td>+</td>
</tr>
<tr>
<td>Nitric oxide mediates cytokine-induced cell death</td>
<td>+</td>
</tr>
<tr>
<td>Respond to glucose with increase in mitochondrial respiration</td>
<td>+</td>
</tr>
<tr>
<td>Signaling pathways (NF-κB, STAT, MAPK) activated in response to cytokine treatment</td>
<td>+</td>
</tr>
<tr>
<td>Basal HSP70 expression</td>
<td>-</td>
</tr>
<tr>
<td>Respond to ER stress inducers</td>
<td>+</td>
</tr>
</tbody>
</table>

Human islets and EndoC-βH1 cell similarities and differences are given. +, Yes; –, no; HSP70, heat shock protein 70; ER, endoplasmic reticulum.
human tissue. Additional studies are required to determine the potential use for EndoC-BH1 cells as human β-cell surrogate in areas pertinent to β-cell function and viability, for example, fatty acid and glucose toxicity, or cytotoxicity when co-incubated with human-derived cytotoxic CD8+ T cells.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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