Do β-Cells Generate Peroxynitrite in Response to Cytokine Treatment?*

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Background: The species that mediates cytokine-induced β-cell death, be it nitric oxide or peroxynitrite, is unknown.

Results: Cytokines stimulate the formation of nitric oxide but fail to stimulate peroxynitrite production by β-cells.

Conclusion: Terminally differentiated β-cells, unlike macrophages, do not produce peroxynitrite.

Significance: Peroxynitrite does not contribute to cytokine-induced β-cell damage.

The purpose of this study was to determine the reactive species that is responsible for cytokine-mediated β-cell death. Inhibitors of inducible nitric oxide synthase prevent this death, and addition of exogenous nitric oxide using donors induces β-cell death. The reaction of nitric oxide with superoxide results in the generation of peroxynitrite, and this powerful oxidant has been suggested to be the mediator of β-cell death in response to cytokine treatment. Recently, coumarin-7-boronate has been developed as a probe for the selective detection of peroxynitrite. Using this reagent, we show that addition of the NADPH oxidase activator phorbol 12-myristate 13-acetate to nitric oxide-producing macrophages results in peroxynitrite generation. Using a similar approach, we demonstrate that cytokines fail to stimulate peroxynitrite generation by rat islets and insulinoma cells, either with or without phorbol 12-myristate 13-acetate treatment. When forced to produce superoxide using redox cyclers, this generation is associated with protection from nitric oxide toxicity. These findings indicate that: (i) nitric oxide is the likely mediator of the toxic effects of cytokines, (ii) β-cells do not produce peroxynitrite in response to cytokines, and (iii) when forced to produce superoxide, the scavenging of nitric oxide by superoxide is associated with protection of β-cells from nitric oxide-mediated toxicity.

Cytokines (IL-1β and IFNγ), released during islet inflammation, are believed to participate in the loss of insulin-producing β-cells during the development of diabetes (1, 2). This hypothesis is based on the ability of cytokines to impair β-cell function and to induce β-cell death (3–5). The inhibitory actions of cytokines are mediated through elevated expression of inducible nitric oxide synthase (iNOS) and production of micromolar levels of nitric oxide by β-cells (6–8). Excessive production of reactive oxygen species (ROS) has also been observed in type 1 diabetes and may contribute to the destruction of pancreatic islets (9–11). Although the production of nitric oxide by IL-1-treated β-cells correlates with an inhibition of glucose-stimulated insulin secretion, and inhibitors of NOS prevent this damage (12–14), the importance of this free radical in mediating the inhibitory actions of cytokines on β-cell function and the chemical nature of damaging species are still debated (6, 15–17). Much of the discussion concerning the toxic molecule that mediates cytokine-induced damage is whether nitric oxide is toxic (6, 15) or whether toxicity is mediated by the production of peroxynitrite (the product of the reaction between nitric oxide and superoxide) (16, 17).

Major sources of superoxide in β-cells include NADPH oxidase (NOX) (18) and electron leaks from the mitochondrial electron transport chain (19). The expression of phagocyte-like NOX (NOX2) has been reported in β-cells, and evidence suggests it may also contribute to β-cell dysfunction (18, 20, 21). This multiprotein complex, consisting of membrane-associated and cytosolic subunits, is activated in response to phosphorylation of p47phox in the cytosol by PKC (22, 23).

The diffusion-controlled reaction between superoxide and nitric oxide generates peroxynitrite, a highly reactive nitrogen species (RNS). Peroxynitrite interacts with lipids, DNA, and proteins and is a potent inducer of cell death (24, 25). In addition, β-cells have been considered to be particularly vulnerable to oxidant-induced damage caused by relatively low levels of antioxidant enzymes in comparison to the levels expressed in the liver or kidney (26).

Although peroxynitrite was discovered over two decades ago (27), only a limited number of methods with sufficient sensitivity and selectivity have been developed to detect its formation. The primary method used to detect this reactive and short-lived species in biological systems relies on formation of nitrated tyrosine residues on proteins (28), including islet proteins (29, 30). However, protein nitration is an indirect marker and is produced by nitrogen dioxide (NO2), a decomposition product of peroxynitrite (28) that can also be formed in the

bol 12-myristate 13-acetate; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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‡The abbreviations used are: iNOS, inducible nitric oxide synthase; 2-OH-E, 2-hydroxyethidium; DPTA/NO, dipropylenetriamine NONOate; HE, hydroethidine; L-NMMA, L-N6-monomethyl arginine; MARCKS, myristoylated alanine-rich protein kinase C substrate; NOX, NAPDH oxidase; PMA, phorbol 12-myristate 13-acetate; RNS, reactive nitrogen species; ROS, reactive oxygen species.
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absence of peroxynitrite (31). Therefore, tyrosine nitration of proteins is possible without intermediacy of peroxynitrite. Recently, boronate-based fluorescent probes have been developed for the detection of peroxynitrite in cells (32). Coumarin-7-borionate reacts rapidly and directly with peroxynitrite, yielding the fluorescent product that can be detected in real time (32, 33).

The goal of the current study was to determine whether β-cells produce peroxynitrite in response to cytokine treatment. To address this question, peroxynitrite formation was evaluated using the selective boronate probe. Activated macrophages, which have been shown to produce peroxynitrite, were used as a positive control (33). In this report, we provide experimental evidence that pancreatic β-cells do not generate peroxynitrite in response to cytokines. This is in contrast to activated macrophages (pretreated with LPS and IFNγ), which produce peroxynitrite when NOX is activated with PKC agonists. In addition, when nitric oxide-treated β-cells are forced to produce superoxide, it affords protection against nitric oxide-mediated toxicity. These findings indicate that cytokine-mediated β-cell damage is dependent on NOS activity and that the reactive species responsible for the toxicity is likely to be nitric oxide or products of nitric oxide oxidation, such as nitrogen dioxide.

EXPERIMENTAL PROCEDURES

Materials and Animals—IL-1 and IFNγ were purchased from ProProTech (Rocky Hill, NJ). Dipropylendiamine NONOate (DPTA/NO) and SIN-1 were supplied by Cayman Chemical (Ann Arbor, MI). Hydroethidine (HE) was obtained from Molecular Probes (Grand Island, NY). RPMI 1640, DMEM, and CMRL-1066 tissue culture media were from Invitrogen. FBS and fetal calf serum were purchased from Biological Industries (Kibbutz, Israel). Male Sprague-Dawley rats (250–300 g) were from Harlan (Indianapolis, IN). RPMI 1640, DMEM, and CMRL-1066 tissue culture media were from Invitrogen. FBS and fetal calf serum were purchased from HyClone (Logan, UT). All other chemicals were of analytical grade and purchased from Sigma-Aldrich.

Cell Culture—INS 832/13 cells were grown in RPMI supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 μg/ml β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C under an atmosphere of 95% air and 5% CO2. To induce iNOS expression and production of nitric oxide in cells, INS832/13 cells were exposed to IL-1 (10 units/ml) and IFNγ (150 units/ml) for 18 h, and RAW 264.7 cells were treated with lipopolysaccharide (1 μg/ml) and IFNγ (150 units/ml) for 18 h. For real time monitoring of peroxynitrite and superoxide, the cells were washed to remove the tissue culture media and then incubated with digitonin (0.007% w/v in 20 mM HEPES containing 250 mM sucrose, 10 mM KH2PO4, and 1 mM EGTA, pH 7.4) for 15 min on ice. Following centrifugation (2,000 × g for 15 min), pellets were lysed (30 mM Tris, 30 mM NaCl, 0.2% Triton X-100, pH 7.0), and acetonitrile activity was measured in a reaction mixture containing 20 mM citrate, 0.2 mM NADPH, 6 mM MnCl2, 50 mM Tris-HCl, pH 7.4, 0.6 unit of isocitrate dehydrogenase, and 25 μl of cell extract in a total volume of 100 μl. The increase in absorbance at 340 nm was followed for 30 min at room temperature. Aconitase activi-
ity was quantified as 1 pmol of NADPH formed per min per microgram of protein.

Western Blot Analysis—Equal amounts of cell lysate proteins were resolved using reducing SDS/PAGE and transferred on to nitrocellulose membranes (GE Healthcare). Phosphorylation of myristoylated alanine-rich protein kinase C substrate (MARCKS) was detected using a phosphospecific antibody (Cell Signaling, Danvers, MA; 1:1,000 dilution) and visualized with enhanced chemiluminescence.

Statistical Analysis—Statistical comparisons were made between groups using one-way analysis of variance with a Tukey post hoc test. The minimum level of significance was set at \( p < 0.05 \).

RESULTS

Effects of Peroxynitrite Scavenger and NOS Inhibitor on Cytokine-induced Toxicity—To elucidate the reactive species that mediate cytokine-induced β-cell damage, the effects of iNOS inhibition and scavengers of the nitric oxide metabolite peroxynitrite on cell viability were examined. Treatment of INS832/13 cells for 18 h with IL-1 and IFNγ results in 50% reduction in viability (Fig. 1A). The damaging actions of cytokines on INS832/13 cells are not modified in the presence of the peroxynitrite scavenger phenylalanine boronate (40); however, inhibition of iNOS activity with \( L-N^G \)-monomethyl arginine (L-NMMA) completely prevents the loss of INS832/13 cell viability in response to IL-1 + IFNγ (Fig. 1, A and B). These findings indicate that a product of iNOS (nitric oxide or other nitrogen oxides) mediates the loss of β-cell viability in response to cytokine treatment and that a scavenger of peroxynitrite does not prevent this damage. Because peroxynitrite production requires the reaction of nitric oxide with superoxide, INS832/13 cells were treated with the NADPH oxidase (NOX) activator PMA to facilitate conditions favorable for peroxynitrite generation. In this experiment, cytokine-induced iNOS expression would provide nitric oxide, and PMA, through NOX activation, would induce superoxide production. The addition of PMA does not modify the damaging actions of cytokines on INS832/13 cells. Also, PMA alone does not alter INS832/13 cell viability (Fig. 1B). Because PMA stimulates superoxide formation by NOX through a PKC-dependent pathway (23), the effects of PMA on PKC activation were evaluated. As shown in Fig. 1C, PMA stimulates the time-related phosphorylation of MARCKS (41). These findings show that PMA is capable of stimulating PKC activation in β-cells, even though it does not modify the loss of β-cell viability in response to cytokine treatment. These findings suggest that PMA fails to stimulate superoxide production by β-cell, a finding that is confirmed in studies shown later in this manuscript.

The concentration-dependent effects of chemical donors that liberate nitric oxide (DPTA/NO), hydrogen peroxide, superoxide (menadione), and peroxynitrite (SIN-1) on INS832/13 cell viability were measured to directly examine the toxicity of each of these reactive species (Fig. 2). The nitric oxide donor DPTA/NO induces a concentration-dependent decrease in cell viability that is maximal at 400 μM (Fig. 2A). There is a steep increase in the toxicity of hydrogen peroxide that occurs at concentrations between 100 and 200 μM, with minimal increases in toxicity above 200 μM (Fig. 2B). Menadione, which generates superoxide by one-electron redox cycling (42, 43), does not affect cell viability at concentrations up to 25 μM; however, at concentrations of 50 μM and greater, this superoxide generator is highly toxic to INS832/13 cells (Fig. 2C). In contrast, SIN-1, which produces nitric oxide and superoxide during decomposition and is generally considered a peroxynitrite donor, does not reduce the viability of INS832/13 cells at concentrations of 500 μM (Fig. 2D). The generation of peroxynitrite has been confirmed using coumarin-7-boronate (Fig. 2E). This novel probe allows for the quantification of per-
Peroxynitrite by the increase in fluorescence that occurs when coumarin-7-boronate is oxidized by peroxynitrite (32). Peroxynitrite at concentrations or fluxes as low as 4 nM/min (produced from co-generated nitric oxide and superoxide) can be detected using this probe (33). The increase in fluorescence was inhibited by addition of superoxide dismutase, confirming that superoxide generation was necessary to observe an increase in signal (Fig. 2F). Taken together, these findings suggest that β-cells are sensitive to agents that generate nitric oxide, superoxide, and hydrogen peroxide but do not appear to be sensitive to agents that can generate peroxynitrite.

Formation of Peroxynitrite in β-Cells and in Macrophages—Consistent with previous studies (33), the addition of PMA to RAW 264.7 cells pretreated for 18 h with LPS/IFNγ results in a time-dependent increase in fluorescence from coumarin-7-boronate oxidation, indicating production of peroxynitrite (Fig. 3A). Only modest increase in fluorescence intensity is observed when RAW 264.7 macrophages are treated with LPS/IFNγ or with PMA alone. Inhibition of iNOS activity using L-N^G^-nitroarginine methyl ester attenuates peroxynitrite formation by RAW 264.7 macrophages (Fig. 3B). Similar to the effects of iNOS inhibition, the decomposition of superoxide to

FIGURE 2. The effects of ROS and RNS on β-cell viability. A–D, INS832/13 cells were exposed to indicated concentrations of the nitric oxide donor DPTA/NO, H2O2, superoxide generator menadione, and peroxynitrite donor SIN-1 for 4 h. Cell viability was determined using neutral red assay. E, INS832/13 cells were treated with SIN-1, and peroxynitrite generation was determined by the oxidation of coumarin-7 boronate. RFU, relative fluorescence units. F, rates of peroxynitrite formation were determined from the data presented in E and from treatments in the presence of superoxide dismutase (SOD, 0.1 mg/ml). The results are the averages of three independent experiments ± S.D. *, p < 0.05 compared with untreated control.
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FIGURE 3. Quantification of peroxynitrite generation in RAW 264.7 macrophages and INS832/13 cells. A, RAW 264.7 cells were treated for 18 h with LPS (1 μg/ml), then PMA (1 μM) was added, and peroxynitrite generation was determined by the oxidation of coumarin-7-borionate. RFU, relative fluorescence units. B, rates of peroxynitrite formation were determined from the data presented in A and from treatments in the presence of L-Nω-nitroarginine methyl ester (L-NAME, 4 μM) and ROS-detoxifying enzymes (SOD, superoxide dismutase, 0.1 mg/ml; and CAT, catalase, 100 units/ml). C, nitrite accumulation was assayed after 18 h of incubation and normalized to protein content. D–F, insulinoma INS832/13 cells were treated with IL-1 + IFNγ + PMA, and peroxynitrite generation was evaluated as outlined for A–C. The results are the averages of three independent experiments ± S.D. *, p < 0.001 compared with untreated control; #, p < 0.001 compared with the same treatment without L-NMMA or superoxide dismutase.

hydrogen peroxide by the addition of superoxide dismutase results in 60% decrease in the rate of peroxynitrite formation by RAW 264.7 cells activated with LPS, IFNγ, and PMA. As anticipated, catalase, which decomposes hydrogen peroxide, does not modify fluorescence signal, confirming assignment of the oxidizing species to peroxynitrite and not hydrogen peroxide (Fig. 3B).

Unlike macrophages, INS832/13 cells do not produce peroxynitrite after treatment for 18 h with IL-1 and IFNγ to induce iNOS expression, followed by the addition of PMA and continued culture, because there is no change in fluorescence from coumarin-7-borionate oxidation (Fig. 3, D and E). In response to IL-1 + IFNγ, INS832/13 cells produce as much nitric oxide as LPS + IFNγ-treated macrophages (Fig. 3, C and F), indicating that the lack of peroxynitrite production by INS832/13 cells is not due to deficiencies in nitric oxide production.

Peroxynitrite Formation in β-Cells Exposed to a Superoxide Generating Redox Cycler—Because cytokine- and PMA-stimulated INS832/13 cells do not appear to generate peroxynitrite, additional experiments were performed to determine whether the lack of peroxynitrite generation is the result of an absence of superoxide formation in response to PMA or enhanced superoxide scavenging, such that it is removed prior to its reaction with nitric oxide. To test these hypotheses, superoxide was generated endogenously in INS832/13 cells using the redox cycler, menadione, in the presence or absence of IL-1 + IFNγ treatment. In response to the cytokine treatment, INS832/13 cells generate 87.0 ± 4.4 pmol of nitrite/μg of protein, indicating that the cells are producing high levels of nitric oxide. When cytokine-induced INS832/13 cells are exposed to menadione, at a concentration that does not decrease INS832/13 cell viability, there is an increase in the rate of peroxynitrite generation (Fig. 4A). The formation of peroxynitrite is inhibited by L-NMMA and by superoxide dismutase, confirming that both nitric oxide and superoxide are necessary for peroxynitrite production (Fig. 4B). These findings indicate that insulinoma cells have the capacity to produce peroxynitrite when superoxide is chemically generated, but peroxynitrite does not appear to be produced in response to cytokines alone or in the presence of activators of NOX (such as PMA).

Consistent with insulinoma cells, rat islets treated for 18 h with IL-1 (which stimulates the production of 21.7 pmol of nitrite/μg of protein) or treated with IL-1 and then exposed to PMA do not generate detectable levels of peroxynitrite (Fig. 4C). However, peroxynitrite generation occurs when the IL-1 treated islets are exposed to menadione. Similar to the effects of endogenously produced nitric oxide following cytokine stimulation; rat islets generate peroxynitrite when exposed to exogenously supplied nitric oxide following treatment with DPTA/NO and to superoxide using menadione (Fig. 4D). The rates of peroxynitrite formation in this experiment are shown in Fig. 4E. These data demonstrate that both insulinoma cells and isolated rodent islets have the capacity to produce peroxynitrite when forced to generate superoxide and when they produce nitric oxide (e.g., as a result of cytokine stimulation). However, the classical activation of NOX using the PKC activator PMA does not appear to stimulate superoxide production to levels sufficient to generate peroxynitrite in cytokine-treated islets and insulinoma cells.
Real Time Monitoring of Superoxide in β-Cells—To determine whether INS832/13 cells and rat islets produce superoxide in response to PMA treatment, its formation was evaluated in real time by measuring the oxidation of hydroethidine to a fluorescent product. PMA does not increase superoxide formation above the levels observed in untreated INS832/13 cells, and superoxide generation is not altered by the addition of superoxide dismutase or catalase (Fig. 5, A and B). At the same time, PMA effectively induces the phosphorylation of the PKC substrate MARCKS (Fig. 1C). In contrast to β-cells, macrophages produce superoxide after PMA stimulation (Fig. 5C), and this is attenuated by superoxide dismutase, but not catalase (Fig. 5D). The lack of superoxide production by INS832/13 cells in response to PMA is consistent with the lack of peroxynitrite production in cytokine- and PMA-treated insulinoma cells and isolated islets (Fig. 2).

Although INS832/13 cells fail to produce superoxide in response to PMA; menadione, in a concentration-related manner, stimulates the formation of detectable levels of superoxide by β-cells (Fig. 6A). At levels of 20–100 μM, menadione increases the rate of hydroethidine oxidation in insulinoma cells by 2–5-fold (Fig. 6B). The generation of superoxide by menadione is cell-dependent because this compound does not stimulate superoxide formation in the absence of cells. Much like INS832/13 cells, rat islets do not appear to generate superoxide following PMA stimulation (Fig. 6C); however, they produce superoxide in response to menadione (20 and 100 μM). For islet experiments, superoxide formation was quantified by HPLC detection of the 2-OH-EI. A concentration-dependent increase in the formation of the superoxide-specific product 2-OH-EI was detected in islets as well as the extracellular medium of samples treated with menadione in the absence of PMA (Fig. 6, C and D). These findings indicate that superoxide can be generated by β-cells after treatment with a redox cycler but not in response to NOX activation using PMA.

The Effects of Superoxide and Nitric Oxide on β-Cells—Because the concomitant production of superoxide and nitric oxide can lead to formation of peroxynitrite (27), and peroxynitrite has been implicated as the reactive species that mediates cytokine-induced β-cell damage (2, 16, 24), the effects of combined exposure to nitric oxide and superoxide on β-cell viability were evaluated. To characterize the functional effects of these
radical species alone and in combination, we exposed INS832/13 cells to nitric oxide using DPTA/NO and to superoxide by treating with menadione. Under these conditions, mitochondrial aconitase activity, ATP levels and insulinoma cell viability were determined (Fig. 7). Treatment with the nitric oxide donor DPTA/NO inhibits aconitase activity by ~80%, and this is associated with a 10-fold decrease in ATP levels and a 50% loss of INS832/13 cell viability. Alone, menadione does not modify aconitase activity, ATP levels, or the viability of INS832/13 cells; however, when INS832/13 cells are treated with both DPTA/NO and menadione, the inhibitory actions of nitric oxide on aconitase activity, reductions in ATP levels, and the loss of viability are prevented (Fig. 7). The protective actions of menadione in nitric oxide-treated INS832/13 cells are associated with levels of ATP that are above the levels measured in untreated control cells. Surprisingly, the protective effects of menadione on DPTA/NO-induced toxicity (Fig. 7) are associated with the detection of peroxynitrite in β-cells (Fig. 4).

DISCUSSION

Previous studies have identified a role for nitric oxide or oxidative products of nitric oxide metabolism as the primary mediators of β-cell damage during cytokine exposure (7, 8, 44).

Using islets isolated from rat, mouse, and human primary β-cells purified by FACS, we and others have shown that cytokines such as IL-1 (in rat islets) or IL-1 + IFNγ (in mouse and human islets) inhibit glucose-induced insulin secretion, the oxidation of glucose to CO2, and protein synthesis; induce DNA damage; and kill β-cells (1, 2, 36, 45). To date, only inhibitors of NOS completely prevent these damaging actions of cytokines (12–14). Furthermore, transgenic mice expressing iNOS under control of the insulin promoter develop diabetes that can be prevented by administration of the selective iNOS inhibitor aminoguanidine (46). Although these studies have identified nitric oxide as the mediator of cytokine-induced β-cell damage, the identity of the terminal effector of this cellular injury (e.g., nitric oxide or a product of nitric oxide oxidation such as peroxynitrite) is unknown. Complicating the determination of the toxic molecule is the relatively short biological half-life of nitric oxide and products of its metabolism, as well as the absence of assays with sensitivity and selectivity to detect nitric oxide metabolites that are formed under physiological conditions. For example, the nitration of tyrosine residues on proteins has been used to mark peroxynitrite generation (28), and the identification of nitrotyrosine staining in cytokine-treated islets has led to the conclusion that peroxynitrite is the
end effector of cytokine-mediated damage (17, 29). However, tyrosine nitration is possible in the absence of peroxynitrite (31, 47), indicating that nitrotyrosine may not be an ideal marker for peroxynitrite.

The objective of this study was to use state of the art methodologies to determine the forms of RNS that are responsible for cytokine-mediated β-cell damage, with a specific focus on the generation of peroxynitrite because this form of RNS has been implicated as the damaging agent (17, 48). Also, the actions of cytokines were compared with the effects of endogenous and exogenous sources of ROS and RNS to confirm that the reactive species that mediates cytokine-induced β-cell injury also causes damage in the absence of cytokines. Recently, sensitive and selective methodologies for the detection of peroxynitrite have been developed (40) and applied for the detection of this oxidant from cellular sources (33). We have reproduced these previous findings showing that murine macrophages generate peroxynitrite after iNOS induction (following endotoxin treatment) and NOX activation by the co-stimulation with PMA (33). In addition, we show that the formation of peroxynitrite is prevented by the addition of iNOS inhibitors or by scavengers of superoxide, providing support for the reaction of superoxide and nitric oxide in the formation of this reactive species. Pancreatic β-cells do not respond to LPS; however, in response to IL-1 (or IL-1 + IFNγ), these cells produce nitric oxide to levels comparable to the levels produced by activated macrophages. Unlike macrophages, IL-1-stimulated rat islets or INS832/13 insulinoma cells do not generate superoxide or peroxynitrite in response to PMA. Even in the absence of IL-1 treatment, PMA fails to stimulate superoxide production by β-cells; however, PMA is effective at stimulating the activation of PKC, a NOX activator, as evidenced by enhanced MARCKS phosphorylation. These findings suggest that under the conditions examined in our study, pancreatic β-cells do not appear to have the capacity to produce peroxynitrite in response to cytokine treatment, nor do they respond to PKC activators to stimulate NOX activation. It is possible that there are treatment conditions that are capable of stimulating the production of peroxynitrite or the activation of NOX by β-cells; however, we have yet to identify conditions in which cytokines are capable of stimulating peroxynitrite production by β-cells.

We found that β-cells are capable of producing peroxynitrite when stimulated with cytokines and co-treated with a redox cycling agent that generates superoxide. We were not surprised by the lack of NOX activation following PMA treatment of islets and INS832/13 cells exposed to IL-1, because we have shown that signal transduction pathways activated by extracellular agonists are impaired in β-cells that produce or are exposed to nitric oxide (49). The mechanistic by which nitric oxide attenuates cell signaling is associated with induction of
endoplasmic reticulum stress and the subsequent activation of the unfolded protein response (49). It is rather surprising that PMA alone does not stimulate superoxide formation in the absence of nitric oxide (or cytokine treatment), because expression of NOX2 has been demonstrated in INS832/13 cells (20), and in rodent and human islets NOX2 localizes in insulin-con-
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islets are cultured for additional 8 h with both the cytokines and NOS inhibitor, all of the damaging effects of cytokines are completely repaired (12, 14). These findings and results obtained in the current studies indicate that NOS activity and subsequent production of nitric oxide mediate the toxicity of cytokines. Finally, the effects of nitric oxide and hydrogen peroxide on cell signaling, gene expression, and cell survival have been directly compared in islets and insulinoma cells (62). Pathways activated by nitric oxide differ dramatically from the pathways activated by hydrogen peroxide (62). Nitric oxide activates MAP kinases, transcription factor FoxO1, and the unfolded protein response. Hydrogen peroxide stabilizes p53 and activates poly-(ADP-ribose) polymerase but does not stimulate any of the pathways activated by nitric oxide. The only shared response is the activation of AMPK, and this occurs by different mechanisms. Nitric oxide activates AMPK by an IRE-1α-dependent pathway (63), whereas poly(ADP-ribose) polymerase is required for AMPK activation in response to hydrogen peroxide (62). Because cytokine treatment does not activate poly-(ADP-ribose) polymerase but is associated with the activation of each of the pathways listed for nitric oxide, the mediator of the actions of cytokines on β-cell function and viability is most likely nitric oxide.

To gain further insights as to the identity of the effector that mediates cytokine-induced β-cell damage, the effects of individual reactive species were compared with the effects of cytokine treatment on INS832/13 cell viability. As previously described, the toxicity (~40% increase in death) of an 18-h incubation with IL-1 and IFNγ is prevented by the NOS inhibitor L-NMMA (64, 65). Individually, exposure of pancreatic β-cells to nitric oxide, superoxide, and hydrogen peroxide results in a concentration-dependent decrease in cell viability, much like the effects of cytokine treatment. Interestingly, the liberation of peroxynitrite using the donor SIN-1 is not cytotoxic. These findings are consistent with previous studies showing that treatment with SIN-1 or authentic peroxynitrite leads to DNA damage and inhibition of mitochondrial function; however, these effects require much higher concentrations of 1 mM and above, whereas at concentrations of 400–500 μM, there is no toxicity (57, 66). These are surprising results, because peroxynitrite is a powerful oxidant that is considered to be far more noxious than nitric oxide or superoxide alone (25). To further add to this surprising result, we show that the inhibitory actions of nitric oxide on mitochondrial aconitase activity and decreased ATP levels are prevented by superoxide (induced using the redoxycler menadione). Importantly, superoxide alone, at the concentrations tested, does not modify aconitase activity or ATP levels in INS832/13 cells. These data suggest that superoxide acts as a scavenger of nitric oxide and, by doing so, protects β-cells from nitric oxide-mediated toxicity. Others have shown that concurrent generation of nitric oxide and superoxide can decrease the steady-state levels of nitric oxide, and this appears to modify cell signaling pathways in tumor cells (67). Also, ROS generated by the redox cycling agent 2,3-dimethoxy-1,4-naphthoquinone have been shown to scavenge nitric oxide and attenuate HIF-1α stabilization in epithelial cells (68). The nitric oxide/superoxide reaction can generate peroxynitrite; however, the efficiency of this reaction is greatest when both nitric oxide and superoxide are produced at equal fluxes (69). Although we are able to detect peroxynitrite generation by β-cells treated with cytokines and with superoxide, the levels of production are lower than the levels produced by macrophages under similar conditions. Thus, the interaction between nitric oxide and superoxide may limit, in this case, the toxic effects of nitric oxide. The relative sensitivity of a given cell type or tissue toward nitric oxide-induced damage is likely to be a factor in determining whether superoxide co-generation is going to exert protective or damaging effects. For example, we have observed that neither nitric oxide donor nor superoxide is toxic to endothelial cells, but when these two compounds are combined, there is synergistic toxic effect that is manifested by an impairment in mitochondrial function, depletion of ATP levels, and cell death (38). These striking differences in responses to oxidative and nitrative stress between various cell types emphasize how important it is to test and measure the identity of toxic species rather than simply assume its presence based on observed effects.

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