Differential responses of pancreatic β-cells to ROS and RNS

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Meares GP, Fontanilla D, Broniowska KA, Andreone T, Lancaster JR Jr, Corbett JA. Differential responses of pancreatic β-cells to ROS and RNS. Am J Physiol Endocrinol Metab 67: E614–E622, 2013. First published January 15, 2013; doi:10.1152/ajpendo.00424.2012.—Reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as nitric oxide and hydrogen peroxide (H2O2) induce DNA damage, control the viability of pancreatic β-cells, and participate in the regulation of a number of fundamental cellular processes under physiological conditions, including insulin secretion and insulin action. In this study, the pathways activated and the mechanisms by which ROS and RNS control the viability of pancreatic β-cells were examined. Although both nitric oxide and hydrogen peroxide (H2O2) induce DNA damage, reduce cell viability, and activate AMPK, the mechanisms of AMPK activation and cell death induction differ between each reactive species. Nitric oxide activates the unfolded protein and heat shock responses and MAPK kinase signaling, whereas H2O2 stimulates p53 stabilization and poly(ADP-ribose) polymerase (PARP) activation but fails to induce the unfolded protein or heat shock responses or MAPK activation. The control of cell fate decisions is selective for the form of stress. H2O2-mediated reduction in β-cell viability is controlled by PARP, whereas cell death in response to nitric oxide is PARP independent but associated with the nuclear localization of GAPDH. These findings show that both ROS and RNS activate AMPK, induce DNA damage, and reduce cell viability; however, the pathways controlling the responses of β-cells are selective for the type of reactive species.

reactive oxygen species; reactive nitrogen species; diabetes; endoplasmic reticulum stress; DNA damage; nitric oxide; AMP-activated protein kinase; poly(ADP-ribose) polymerase

REACTIVE OXYGEN SPECIES (ROS) such as hydrogen peroxide (H2O2) and reactive nitrogen species (RNS) such as nitric oxide participate in the regulation of a number of fundamental cellular processes under physiological conditions, including insulin secretion and insulin action (22, 62). ROS function as signaling intermediates in response to cytokines and growth factors and, when produced during the oxidative burst, are essential for proper immune function (6). In contrast to the physiological roles of these oxidants, enhanced production of ROS and RNS can contribute to disease development.

Previous studies have identified roles for ROS and RNS as mediators of β-cell damage during the onset of insulin-dependent diabetes mellitus (28). Resident intraislet macrophages and invading autoreactive immune cells release proinflammatory cytokines in and around pancreatic islets of Langerhans (35, 42). These proinflammatory cytokines, such as interleukin-1β (IL-1β), stimulate β-cell expression of inducible nitric oxide synthase (iNOS), leading to the generation of nitric oxide (14, 18). Produced in micromolar concentrations by β-cells, nitric oxide inhibits mitochondrial oxidation of glucose to CO2 and glucose-stimulated insulin secretion (12, 14, 16, 17, 31). Following prolonged exposures, cytokines cause β-cell death (12, 14, 16, 17, 31). When supplied exogenously using donor compounds, nitric oxide induces biological responses that are similar to those observed following the induction of iNOS in cytokine-treated β-cells (28, 43, 44, 59). β-Cells are reported to be highly sensitive to free radical-induced damage and cell death because of low levels of free radical defense enzymes such as catalase, superoxide dismutases, and glutathione peroxidase (38). As such, endogenously produced or exogenously supplied nitric oxide results in the inactivation of metabolic enzymes, the inhibition of insulin secretion, and β-cell death (12, 14, 16, 17, 31).

Mammalian cells have the capacity to respond quickly and specifically to damage from environmental and endogenous stress. Two classic examples of stress-specific signaling are the unfolded protein response (UPR) and the DNA damage response (DDR). When misfolded proteins accumulate in the endoplasmic reticulum (ER), the UPR is activated and sends signals from the ER via eukaryotic translation initiation factor 2α kinase 3 (PERK), inositol-requiring enzyme-1 (IRE1), and activating transcription factor 6 that are designed to attenuate protein synthesis and enhance the expression of repair enzymes as a means of adaptation. If the cell is unable to repair the damage, this same pathway may initiate apoptosis (54). The DDR is activated following extensive DNA damage and, like the UPR, functions to limit additional damage by signaling for cell cycle arrest. In addition, the activated DDR signals for repair of DNA damage through the expression and activation of specialized repair enzymes such as poly(ADP-ribose) (PAR) polymerase (PARP) and ataxia telangiectasia mutated (ATM). When necessary, the DDR signals for elimination of the irreparably damaged cell. In many cases, the DNA damage-induced cell death is controlled by the tumor suppressor and transcription factor p53 (10).

Although free radicals are damaging to β-cells, we have shown that β-cells maintain the ability to survive from and repair nitric oxide- and cytokine-induced damage (13, 15, 58, 59). Although these survival mechanisms are just beginning to be elucidated, they include the activation of JNK and the forkhead transcription factor FoxO1 and the enhanced expression of protective enzymes such as GADD45α, PGC-1α, and heat shock proteins (HSP) (30, 44). Additionally, we have shown that AMP-activated protein kinase (AMPK) plays a
central role in enhancing metabolic recovery from cytokine-mediated damage and in attenuating β-cell death following nitric oxide-induced damage (30). AMPK is a conserved heterotrimeric serine/threonine kinase that modifies anabolic and catabolic processes through the phosphorylation of multiple substrates in an effort to restore and maintain cellular energy balance (27). The signaling pathways leading to AMPK activation in response to ROS/RNS are not fully understood. We have shown that the upstream kinase LKB1 is dispensable during the activation of AMPK by nitric oxide. The activation of AMPK by nitric oxide is associated with induction of the unfolded protein response and requires the RNase activity of IRE1 (45). In response to ROS, activation of the NAD⁺-dependent enzyme PARP causes the depletion of cellular energy and the activation of AMPK (29, 53). DNA damage induced by ROS treatment results in the overactivation of PARP, leading to an energy-consuming cycle of ADP-ribosylation of proteins (including PARP itself), followed by the ATP-dependent hydrolysis of this modification (53, 55). The net result is ATP and NAD⁺ depletion and cell death by programmed necrosis (25). Additionally, PARP activation in response to H₂O₂ can lead to LKB1-mediated AMPK activation and promotion of autophagy (29, 53).

Although ROS and RNS modify the function and survival of β-cells (21), the intracellular signaling pathways involved in controlling the cellular responses to each oxidant have yet to be fully elucidated. In this study, the mechanistic differences between the stress-induced signaling pathways activated by RNS and ROS in pancreatic β-cells have been examined. We show that ROS and RNS activate distinct cellular stress responses. H₂O₂ induces a p53- and PARP-dependent DDR. In response to nitric oxide, p53 and PARP are not activated in β-cells. In contrast, nitric oxide activates the unfolded protein and heat shock responses in β-cells. Although both oxidants activate AMPK, it is by different mechanisms. These findings show that selective pathways are activated in β-cells in response to ROS and RNS and that the cellular response, be it protective or damaging, is dependent on the source and length of exposure to ROS/RNS.

MATERIALS AND METHODS

Materials and animals. Male Sprague-Dawley rats (250–300 g) were purchased from Harlan (Indianapolis, IN). The Institutional Animal Care and Use Committees at The Medical College of Wisconsin and University of Alabama at Birmingham approved all animal care and experimental procedures. INS832/13 cells were provided by Chris Newgard (Duke University, Durham, NC). RPMI 1640, CMRL-1066 tissue culture medium, t-glutamine, streptomycin, and penicillin were purchased from Ambion (Austin, TX). Horseradish peroxidase-conjugated donkey anti-rabbit and donkey anti-mouse antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA), PGC-1α, CCAAT/enhancer-binding protein homologous protein (CHOP), GADD45α, HSP70, p53-upregulated modulator of apoptosis (PUMA), and GAPDH primers were obtained from IDT DNA Technologies (Coralville, IA).

Islet isolation and cell culture. Islets were isolated from male Sprague-Dawley rats (250–300 g) by collagenase digestion, as described previously (34). Islets were cultured overnight in complete CMRL-1066 (containing 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin) at 37°C under an atmosphere of 95% air and 5% CO₂ before experimentation. INS832/13 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 55 µM β-mercaptoethanol, 10 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37°C under an atmosphere of 95% air and 5% CO₂.

Immunoblotting. Cells were washed twice with PBS and lysed with IP lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml leupeptin, 25 µg/ml aprotinin, and 1X phosphatase inhibitor cocktail; Pierce, Rockford, IL), as described previously (47). Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). Equal amounts of protein from each sample were solubilized in Laemmli sample buffer (2% SDS) and heated for 5 min at 95°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and the membranes were blocked in 1% BSA and 5% dry milk, followed by an overnight incubation at 4°C with primary antibodies. Horseradish peroxidase-conjugated donkey anti-rabbit or donkey anti-mouse (1:4,000 dilution) secondary antibodies were incubated for 1 h at room temperature, followed by detection with enhanced chemiluminescence.

Subcellular fractionation. Nuclear and cytosolic fractions were prepared as described previously (3, 46), with minor modifications. Cells were washed twice with PBS and then harvested in 200 µl of lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 0.05% Nonidet P-40, 1 mM EGTA, 1 mM sodium orthovanadate, 100 µM phenylmethylsulfonyl fluoride, 0.1 µM okadaic acid, 50 mM sodium fluoride, and 10 µg/ml each of leupeptin, aprotinin, and pepstatin). Following lysis, the cells were centrifuged at 2,700 g for 10 min at 4°C. The supernatant was collected and centrifuged at 20,800 g for 15 min at 4°C to obtain the cytosolic fraction. The pellet containing nuclei was washed twice in 200 µl of wash buffer (5 mM HEPES, pH 7.4, 3 mM MgCl₂, 1 mM EGTA, 250 mM sucrose, and 0.1% BSA with protease and phosphatase inhibitors). The pellet was collected, and the nuclei were centrifuged through a 1 M sucrose cushion (with protease and phosphatase inhibitors) at 2,700 g for 10 min at 4°C, washed in lysis buffer containing 0.05% Nonidet P-40, and suspended in nuclear extraction buffer (20 mM HEPES, pH 7.9, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 100 µM phenylmethylsulfonyl fluoride, 0.1 µM okadaic acid, 50 mM sodium fluoride, and 10 µg/ml each of leupeptin, aprotinin, and pepstatin). Following a 30-min incubation on ice, nuclei were extracted by centrifugation at 20,800 g for 15 min at 4°C, with the supernatant retained as the nuclear extract.

TUNEL assay and immunocytochemistry. DNA strand breaks were identified using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Following treatment, cells were collected in PBS and centrifuged onto slides. The cells were fixed in 4% paraformaldehyde for 30 min, washed in PBS, and permeabilized with 0.1% Triton X-100 in 0.1% citrate for 3 min. The samples were labeled according to the manufacturer’s instructions (Roche, Manheim, Germany). Cellular localization of GAPDH was performed by immunocytochemistry, as described previously (30, 31), using a 1:200 dilution of anti-GAPDH. Images were obtained using a Nikon 90i confocal microscope and are at ×20.

Real-time PCR. RNA was isolated using the RNeasy kit (Qiagen), and cDNA synthesis was performed using oligo(dT) and the reverse transcription protocol. The PCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and analyzed using an Applied Biosystems 7500 Fast Real-Time PCR System. The mRNA levels of the target genes were normalized to the expression of the housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, using the 2^-ΔΔCt method. The primers used for real-time PCR are listed in Table 1.
transcriptase Superscript Preamplification System (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was performed using the Light Cycler 480 (Roche Applied Science) with SYBR Green incorporation for product detection. Values were normalized to β-actin and fold change calculated by the ΔΔCt method. Primer sequences were as follows: GADD45 forward (TGGCTGCGGATGAAGATGAC), GADD45 reverse (GTGGGGAGTGAACGTGGTGAAC); HSP70 forward (CATGAGACTGGCCCTTCC), HSP70 reverse (CGAGGATCAGGTCGC); PUMA forward (GCACCTGATGGAGATACGTTG), PUMA reverse (ATGAAGGTGAGGCACATTG); β-actin forward (ATGAGGATGAGGATGCCATTG), PUMA reverse (ATGAAGGTGAGGCACATTG); CHOP forward (AAATAACAGGGGACCTGA), CHOP reverse (GGGGATGCAGGGTCAAGAGT).

RESULTS

ROS and RNS induce DNA damage and the activation of stress responses in INS832/13 cells. Treatment of INS832/13 cells with the nitric oxide donor DEANO (1 mM) or H2O2 (100 μM) for 30 min results in extensive DNA strand breaks in >95% of the cells, as assessed using TUNEL staining (Fig. 1A). Similar findings were obtained using the Comet assay of DNA damage (data not shown). In response to the nitric oxide donor or H2O2, the cells did not display apoptotic morphology, as the entire nucleus contains damaged DNA (light blue nuclear staining on the merged images). We have shown previously that forced induction of apoptosis in insulinoma cells using camptothecin results in chromatin condensation with punctate TUNEL-positive staining (8).

To determine the cellular responses to RNS and ROS, INS832/13 cells were treated with DEANO (1 mM) or H2O2 (100 μM) for 30–90 min; the cells were harvested, and cell signaling was examined by immunoblotting. Both DEANO and H2O2 stimulate AMPK phosphorylation and phosphorylation of the AMPK substrate ACC (Fig. 1B). Whereas both ROS and RNS activate AMPK, the induction of ER stress, as evidenced by PERK and eIF-2α phosphorylation, is stimulated in response only to the nitric oxide donor. H2O2 fails to stimulate PERK or eIF-2α phosphorylation. Much like ER stress (61), DEANO stimulates JNK phosphorylation, whereas H2O2 does not activate this MAPK (Fig. 1B). We have shown previously that, in response to nitric oxide (produced endogenously by INS832/13 cells or supplied exogenously), there is a reduction in Akt-dependent phosphorylation of FoxO1, resulting in the nuclear localization of this transcription factor (32). Consistent with these previous findings, treatment with DEANO results in a decrease, whereas H2O2 does not modify Akt-dependent...
FoxO1 phosphorylation. In response to DNA damage, p53 is activated via protein stabilization and posttranslational modifications such as phosphorylation (39). DEANO treatment does not modify the steady-state levels of p53, whereas H2O2 treatment stimulates the time-related accumulation of p53 (Fig. 1B). PARP is also activated in response to DNA damage and translocates to sites of damaged DNA, where it undergoes auto ribosylation (PARP formation) during DNA repair (19). In response to H2O2, there is a rapid and transient increase in PAR immunoreactivity that is apparent after 30 min and lasts at later time points (60 and 90 min). This transient response likely reflects the rapid consumption of the substrate NAD+ and ATP during the cycles of PARP-mediated ribosylation and hydrolysis of this modification (19). In contrast to the actions of H2O2, nitric oxide fails to stimulate the accumulation of PAR in INS832/13 cells. These findings are consistent with previous studies by our laboratory showing that PARP deficiency does not protect β-cells from cytokine-induced damage (2). These studies show that both ROS and RNS cause DNA damage and the activation of AMPK; however, additional pathways are activated in a stress-selective fashion. Nitric oxide stimulates a stress response that is characterized by the induction of the unfolded protein response (PERK/eIF-2α) and activation of the transcription factor FoxO1, whereas H2O2 induces a DNA damage repair type response characterized by p53 stabilization and PAR accumulation.

PARP participates in the activation of AMPK in response to H2O2. Because both ROS and RNS cause DNA damage (Fig. 1A), the potential exists for a common signaling pathway to converge on the AMPK signaling node. PARP appears to be a potential player in this pathway, as extensive DNA damage can lead to the overactivation of PARP, resulting in depletion of NAD+ and ATP to levels sufficient for the activation of AMPK (53, 65). To test this hypothesis, INS832/13 cells were treated with DEANO or H2O2 in the presence or absence of the PARP-selective inhibitor PJ34. The inhibition of PARP does not modify DEANO-induced AMPK phosphorylation (Fig. 2A) but attenuates H2O2-induced AMPK phosphorylation (Fig. 2B). These results were confirmed using a second nitric oxide donor, PAPANO, which stimulates the phosphorylation of AMPK and the AMPK substrate ACC in a PARP-independent fashion, whereas PJ34 attenuates H2O2-stimulated AMPK and ACC phosphorylation (Fig. 2C).

In addition to the activation of AMPK, nitric oxide also attenuates the basal levels of Akt phosphorylation (32, 64), and this effect is not modified by the presence of PJ34 (Fig. 2C), whereas H2O2 treatment does not modify basal Akt phosphorylation in the presence or absence of PJ34. These findings are consistent with previous reports showing that H2O2 treatment stimulates the overactivation of PARP due to extensive DNA damage (41) and the accumulation of PAR in INS832/13 cells treated with 100 μM H2O2 (Fig. 2C). A second nitric oxide donor PAPANO, like DEANO, also does not stimulate PAR accumulation in INS832/13 cells. Furthermore, DEANO treatment does not alter, whereas H2O2 decreases, the levels of NAD+ in INS832/13 cells (data not shown). Similarly to INS832/13 cells, treatment with DEANO results in PARP-independent activation of AMPK, whereas H2O2 activates AMPK in a PARP-dependent manner in rat islets (Fig. 2D). These data indicate that both RNS and ROS activate AMPK; however, the regulatory mechanisms responsible for this activation are stimulus selective.

Differential gene expression in response to nitric oxide and H2O2 treatment. Much like the differences in the mechanisms of activation of AMPK, gene expression is also differentially regulated in response to RNS and ROS. Exposure to increasing concentrations of DEANO results in the accumulation of mRNA for the ER stress-responsive transcription factor CHOP (GADD153; Fig. 3), and this effect is consistent with PERK and eIF-2α phosphorylation (Fig. 1B). DEANO treatment also activates a heat shock response, as evidenced by the accumulation of HSP70 mRNA. In addition to the induction of stress response genes, nitric oxide stimulates the expression of protective genes such as GADD45α [DNA repair (49)] and PGC-1α [mitochondrial metabolism (40)]. H2O2 treatment does not modify the steady-state levels of any of these stress-responsive or protective genes (<2-fold increase in mRNA accumulation for all targets under all conditions); however, H2O2 and DEANO stimulate the accumulation of the bcl2...
We hypothesized that PUMA expression is regulated through distinct mechanisms. PUMA is induced typically in a p53-dependent manner (48), and, consistent with this hypothesis, H2O2 stimulates p53 stabilization in INS832/13 cells (Fig. 1B). In response to nitric oxide, PUMA expression is regulated in a p53-independent, FoxO1-dependent manner (30). Consistent with this mechanism of action, nitric oxide treatment results in the attenuation of Akt-dependent phosphorylation of FoxO1 (Fig. 1B) but does not enhance p53 phosphorylation or accumulation in INS832/13 cells or rat islets (Fig. 1 and Ref. 30). These data demonstrate that ROS and RNS elicit divergent transcriptional programs.

**Role of PARP in β-cell death triggered by exposure to ROS and RNS.** The loss of cell viability is a common feature associated with the exposure of cells to RNS and ROS (57), and the mechanisms responsible for cell death differ depending on the reactive species. In response to ROS, there is an overactivation of PARP, as evidenced by PAR formation (Figs. 1 and 2) and PARP-dependent AMPK activation (Fig. 2). Overactivation of PARP contributes to cell death in response to H2O2, as the PARP inhibitor PJ34 attenuates the loss of INS832/13 cell viability following a 4-h treatment with 100 and 300 µM H2O2 (Fig. 4A). In contrast, increasing concentrations of nitric oxide, supplied exogenously using DEANO, reduce cell viability (~20% at 0.3 and 1 mM), and this action is PARP independent because PJ34 does not modify the loss in cell viability (Fig. 4B). These findings indicate that ROS treatment and the resulting DNA damage activate a number of pathways, leading to PARP-dependent AMPK activation and PARP-dependent cell death.

The role of PARP overactivation in β-cell death appears to be selective for ROS. In response to RNS, PARP is not overactivated (PAR does not accumulate; Figs. 1 and 2), and PARP inhibition does not alter the loss of cell viability in response to RNS (Fig. 4). While exploring pathways by which RNS mediates β-cell damage, we observed that nitric oxide stimulates the nuclear accumulation of GAPDH (Fig. 5). Consistent with previous reports that nitric oxide stimulates cell death by a pathway that is associated with S-nitrosation and nuclear localization of GAPDH (5, 26, 60), DEANO induces the nuclear accumulation of GAPDH under conditions in which PARP is not overactivated (as evidenced by the absence of nuclear PAR formation; Fig. 5A). In contrast, H2O2 does not stimulate the nuclear accumulation of GAPDH; however, it is effective at stimulating the accumulation of PAR in the nucleus of INS832/13 cells (Fig. 5A). IL-1β is an effective stimulator of iNOS expression and the production of micromolar levels of nitric oxide by β-cells following 18- to 24-h incubations (15, 16, 18). Similar to the effects of nitric oxide donors, IL-1β stimulates the nuclear localization of GAPDH following a 20-h incubation, and nuclear GAPDH persists to 48 h (Fig. 5B). The stimulation of GAPDH nuclear localization appears to be reversible, as a 1-h incubation with DEANO stimulates nuclear
localization of GAPDH, and washing to remove the nitric oxide donor, followed by continued culture for 5 h, results in a loss of GAPDH in nuclear extracts (Fig. 5C). Immunohistochemical analysis was used to confirm these biochemical findings, as PAPANO stimulates the nuclear localization of a fraction of the cellular GAPDH in ~50% of INS832/13 cells following 30-min exposure. In response to a similar exposure to H$_2$O$_2$, GAPDH does not translocate to the nucleus (Fig. 5, D and E). These data indicate that nitric oxide and IL-1$\beta$ stimulate nuclear GAPDH accumulation and may point toward a role for nuclear GAPDH as a mediator of $\beta$-cell destruction in response to proinflammatory cytokines.

**DISCUSSION**

In this study, we have examined the differential signaling stimulated by RNS and ROS. Consistent with differential signaling, RNS and ROS have divergent effects on $\beta$-cell function. Whereas RNS inhibits oxidative metabolism and subsequent glucose-stimulated insulin secretion (12, 16), ROS, as a second messenger in response to glucose, stimulates insulin secretion. However, at pathological concentrations, ROS also leads to $\beta$-cell dysfunction and cell death (52). A large body of evidence has shown that ROS and RNS modify cellular function and viability by multiple pathways, including PARP activation and energy depletion, caspase induction through mitochondrial directed pathways, prolonged ER stress, and the associated induction of apoptosis (11, 23, 41, 63). A target of both ROS and RNS is the rapid induction of DNA damage that is followed by cell death. Although the net effect of each reactive species can be the loss of viability, the process by which individual cells sense and respond to ROS and RNS is largely unknown for an individual cell type. In this report, the effects of RNS and ROS on the viability of pancreatic $\beta$-cells were explored. Cytokines, released from invading inflammatory cells during islet inflammation, are believed to contribute to the loss of pancreatic $\beta$-cell function and viability during the development of autoimmune diabetes (36). Previous studies have indicated that production of RNS and ROS is a major mediator in the process by which cytokines cause $\beta$-cell damage (37, 43), and yet the pathways that are activated and the mechanisms responsible for the loss of function and viability have not been fully elucidated. Complicating these issues is the induction of protective mechanisms, such as the heat shock and unfolded protein responses that assist in alleviating cell stress and repairing cellular damage. By directly comparing the effects of RNS and ROS on the activation of signaling cascades that control $\beta$-cell viability, we now show differential activation of signaling cascades that are consistent with protection from cellular damage (RNS) or the induction of programmed necrosis and/or the more recently described PARP-mediated parthanatos (20) as a mechanism of cell death (ROS).

In response to nitric oxide, there is a rapid and pronounced activation of signaling cascades/pathways that afford protection of cells from stress or participate in $\beta$-cell recovery from cytokine-mediated damage. Nitric oxide stimulates induction of ER stress and the activation of the UPR, including enhanced phosphorylation of PERK and its substrate eIF2$\alpha$ and the accumulation of CHOP mRNA. The induction of ER stress in response to RNS has been described in a number of cell types (9, 24, 33, 63), including $\beta$-cells (50), and occurs in response to exogenously supplied and endogenously produced nitric oxide (7, 45). Although prolonged activation of the UPR can be associated with the induction of apoptosis (56), previous studies using insulinoma cells and isolated islets have dissociated UPR activation from the loss of $\beta$-cell viability in response to the production of nitric oxide following cytokine treatment (1, 7). Even though signaling pathways that are associated with protection from oxidant damage are activated by nitric oxide, prolonged exposure to this RNS does result in cell death in a PARP-independent manner. In contrast to RNS, H$_2$O$_2$ does not activate the UPR or stimulate the heat shock response.

Although there appear to be a number of differences in the response of $\beta$-cells to RNS compared with ROS, the AMPK cascade is one pathway that is modified in a similar fashion. In a time-dependent manner, both nitric oxide and H$_2$O$_2$ activate AMPK; however, the mechanisms of activation differ. In response to H$_2$O$_2$, AMPK activation correlates with the formation of PAR and is sensitive to inhibitors of PARP. In this mechanism of AMPK activation, double-stranded DNA breaks stimulate the overactivation of PARP, the rapid poly(ADP)-ribosylation of target proteins that include PARP itself (note...
When the NAD or H2O2 (100 µM) are treated with PAPANO (1 mM) or DEANO (0.5 mM) for 1 h. The levels of nuclear GAPDH were determined by Western blot. ATP-dependent resynthesis of NAD and the rapid ATP-dependent hydrolysis of the PAR modification result in a cycle of PARP-mediated NAD and ATP consumption, leading to the depletion of cellular ATP (25, 41). Nitric oxide also stimulates AMPK activation, but it does this in a PARP-independent fashion. Recently, we identified a requirement for the RNase activity of IRE1 in the activation of AMPK by nitric oxide (45). Furthermore, inhibitors of PARP attenuate H2O2-induced cell death, whereas they have no effect on the loss of INS832/13 cell viability in response to nitric oxide. In contrast, nitric oxide-induced INS832/13 cell death is associated with the translocation of GAPDH to the nucleus. Much like β-cells, nitric oxide stimulates the nuclear localization of GAPDH in neurons (26), and nuclear GAPDH appears to activate the histone acetyltransferase p300/CPB to stimulate cell death (60). This pathway may also participate in the control of β-cell fate in response to nitric oxide, as we have identified an acetylation-dependent role for FoxO1-dependent transcription in determining the response of β-cells to cytokine treatment (32). When the NAD-dependent deacetylase Sirt1 is more active, nuclear FoxO1 is deacetylated and directs a transcriptional program that results in functional recovery and repair of damaged DNA (4, 32). This pathway includes the expression of genes such as GADD45α and PGC-1α that participate in the repair of DNA and mitochondrial damage. When less active, FoxO1 is acetylated, likely by p300, and directs a transcriptional program that results in apoptosis via the expression of PUMA (32, 51). Although both nitric oxide and H2O2 stimulate PUMA expression (Fig. 4), in response to ROS the accumulation of PUMA mRNA is likely mediated by p53, as H2O2 activates p53, as evidenced by its stabilization. Nitric oxide also stimulates PUMA mRNA accumulation; however, it fails to stabilize p53 or stimulate p53 phosphorylation (30). In contrast, nitric oxide-induced PUMA mRNA accumulation is dependent on FoxO1 (32).

In comparing our findings with previously reported actions of ROS and RNS, it appears that the responses to different forms of cellular stress are distinct and selective for individual cell types. In β-cells, the actions of RNS appear to be protective early; however, if cell damage becomes too extensive and the cells can no longer repair damaged DNA or recover oxidative metabolic capabilities, an apoptotic cascade appears to be triggered, as evidenced by the expression of PUMA (31,
In contrast, oxidative stress appears to stimulate programmed necrosis/parthanatos that is associated with PARP overactivation, reduced ATP levels (as evidenced by PARP-dependent AMPK activation), stabilization of p53, and PARP-dependent loss of viability. Much like the response of β-cells to exogenously supplied nitric oxide, cytokine treatment stimulates the nitric oxide-dependent activation of AMPK, accumulation of PGC-1α and GADD45α mRNA, and induction of the UPR (1, 7, 30, 44, 45). These findings suggest that, in response to cytokines, the induction of RNS directs the response of β-cells, controlling both the protective (GADD45α and PGC-1α expression) and detrimental actions of cytokines (inhibition of mitochondrial oxidation, PUMA expression) on β-cell function.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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