Effects of pharmacological inhibition of NADPH oxidase or iNOS on pro-inflammatory cytokine, palmitic acid or H$_2$O$_2$-induced mouse islet or clonal pancreatic $\beta$-cell dysfunction

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Synopsis

Various pancreatic $\beta$-cell stressors including cytokines and saturated fatty acids are known to induce oxidative stress, which results in metabolic disturbances and a reduction in insulin secretion. However, the key mechanisms underlying dysfunction are unknown. We investigated the effects of prolonged exposure (24 h) to pro-inflammatory cytokines, H$_2$O$_2$ or PA (palmitic acid) on $\beta$-cell insulin secretion, ATP, the NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase) component p47$^{phox}$ and iNOS (inducible nitric oxide synthase) levels using primary mouse islets or clonal rat BRIN-BD11 $\beta$-cells. Addition of a pro-inflammatory cytokine mixture [IL-1$\beta$ (interleukin-1$\beta$), TNF-$\alpha$ (tumour necrosis factor-$\alpha$) and IFN-$\gamma$ (interferon-$\gamma$)] or H$_2$O$_2$ (at sub-lethal concentrations) inhibited chronic (24 h) levels of insulin release by at least 50% (from islets and BRIN-BD11 cells), while addition of the saturated fatty acid palmitate inhibited acute (20 min) stimulated levels of insulin release from mouse islets. H$_2$O$_2$ decreased ATP levels in the cell line, but elevated p47$^{phox}$ and iNOS levels as did cytokine addition. Similar effects were observed in mouse islets with respect to elevation of p47$^{phox}$ and iNOS levels. Addition of antioxidants SOD (superoxide dismutase), Cat (catalase) and NAC ($N$-acetylcysteine) attenuated H$_2$O$_2$ or the saturated fatty acid palmitate-dependent effects, but not cytokine-induced dysfunction. However, specific chemical inhibitors of NADPH oxidase and/or iNOS appear to significantly attenuate the effects of cytokines, H$_2$O$_2$ or fatty acids in islets. While pro-inflammatory cytokines are known to increase p47$^{phox}$ and iNOS levels in $\beta$-cells, we now report that H$_2$O$_2$ can increase levels of the latter two proteins, suggesting a key role for positive-feedback redox sensitive regulation of $\beta$-cell dysfunction.

Key words: antioxidant, inducible nitric oxide synthase (iNOS), nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), oxidative stress, pancreatic $\beta$-cell, pro-inflammatory cytokine

INTRODUCTION

An increase in blood glucose concentration is quantitatively the most important regulatory stimulus for pancreatic $\beta$-cell insulin secretion. In addition to glucose, other nutrients are also important regulators of insulin secretion, e.g. NEFAs (non-esterified fatty acids). The metabolic interaction between glucose and palmitate (one of the most abundant NEFAs in plasma) may generate lipid intermediates (diacylglycerol and phosphatidylserine) that activate specific isoforms of PKC (protein kinase C), thereby enhancing insulin secretion [1,2]. Indeed, it has been demonstrated [3] that long-chain fatty acyl-CoA, mainly palmitoyl-CoA, potentiated glucose-stimulated insulin secretion from clonal $\beta$-cells through PKC activation.

Recent studies have reported the functional expression and activity of phagocyte-like NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase) in non-phagocytic cells [4–6]. The NADPH oxidase consists of six hetero-subunits that activate specific isoforms of PKC (protein kinase C), thereby enhancing insulin secretion [1,2]. Indeed, it has been demonstrated [3] that long-chain fatty acyl-CoA, mainly palmitoyl-CoA, potentiated glucose-stimulated insulin secretion from clonal $\beta$-cells through PKC activation.

Abbreviations used: Cat, catalase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSIS, glucose stimulated insulin secretion; IFN-$\gamma$, interferon-$\gamma$; IL-$1\beta$, interleukin-1$\beta$; iNOS, inducible nitric oxide synthase; KRB buffer, Krebs-Ringer bicarbonate buffer; NAC, N-acetylcysteine; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NEFA, non-esterified fatty acid; NF-$\kappa$B, nuclear factor-$\kappa$B; NP40, Nonidet P40; PA, palmitic acid; PDX-1, pancreatic and duodenal homeobox-1; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-$\alpha$, tumour necrosis factor-$\alpha$.  
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enzyme complex and produce superoxide. Two NADPH oxidase subunits, gp91phox and p22phox, are integral membrane proteins. They form a heterodimeric flavocytochrome b558 that forms the catalytic core of the enzyme, but exist in the inactive state in the absence of other subunits. The additional subunits are required for regulation and are located in the cytosol during the resting state. They include p67phox, p47phox and p40phox proteins as well as the small GTPase Rac. p47phox is phosphorylated at multiple sites by a number of protein kinases, including members of the PKC family, and as such plays the most important role in the regulation of NADPH oxidase activity [7,8].

Chronic exposure to pro-inflammatory cytokines [9] or high glucose or fatty acid levels, both in vivo and in vitro, results in suppression of insulin secretion and increased susceptibility to apoptotic stimuli [10,11]. It has been reported that pancreatic β-cell exposure to pro-inflammatory cytokines or high concentrations of fatty acids at non-lethal concentrations will lead to an elevated generation of ROS (reactive oxygen species) and NO over an extended period [10,11]. The source of ROS may be mitochondrial or cell membrane associated NADPH oxidase, while NO is produced from cytosolic iNOS (inducible nitric oxide synthase). In this work, we investigated the relative contribution of ROS generated from NADPH oxidase, and NO generated from iNOS, to the reduction in insulin secretion induced by pro-inflammatory cytokines or H2O2 or PA (palmitic acid), by use of specific inhibitors of NADPH oxidase and iNOS, respectively. We demonstrate that both β-cell NADPH oxidase and iNOS activity are critical for the suppression of insulin secretion induced by cytokines, H2O2 or excess fatty acids and thus form an integral part of the cellular response to external stressors.

We utilized primary mouse islets and the clonal rat β-cell line BRIN-BD11 as cell models in this work. BRIN-BD11 β-cells represent a useful model for such studies with stability in culture and well-characterized metabolic, signalling, insulin secretory and cell viability responses to glucose, amino acids and numerous other modulators of β-cell function [12–18]. Some preliminary work had been completed with respect to PA and cytokine effects on insulin secretion and p47phox expression, utilizing this cell line [9,13,19].

MATERIALS AND METHODS

Animals and materials
C57/BL6 mice were obtained from the Conway Institute of Molecular and Biomedical Research (UCD, Dublin, Ireland) and the Biotechnikum Greifswald (Greifswald, Germany). Rat insulinoma BRIN-BD11 β-cells were obtained from Professor Peter Flatt and Dr Neville McClenaghan of the University of Ulster, Coleraine, Northern Ireland. Collagenase type XI was obtained from Sigma. Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) rabbit polyclonal antibody was purchased from Abcam (Cambridge, U.K.). Goat polyclonal antibody directed against β-actin was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit polyclonal antibody directed against p47phox from rat or mouse was obtained from Santa Cruz Biotechnology. Rabbit monoclonal antibody directed against iNOS from rat or mouse was purchased from Sigma. Cytokines were obtained from Roche (Mannheim, Germany). Glucose, PA, SOD (superoxide dismutase) (PEG-SOD), Cat (catalase) (PEG-CAT) and NAC (N-acetylcysteine) were all obtained from Sigma. The NADPH oxidase inhibitor apocynin and the iNOS inhibitor 1400W (N-(3-(aminomethyl)benzyl)acetamide) were purchased from Calbiochem, Merck (Dublin, Ireland).

Cell culture
BRIN-BD11 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum and 0.1% antibiotics (100 units/ml penicillin and 0.1 mg/ml streptomycin). Cells (1.5 × 10^5) were seeded on to 24-well plates containing 1 ml of the medium or 1.5 × 10^6 cells were seeded on to 6-well plates containing 5 ml of the medium and allowed to adhere overnight, before treatment in the presence or absence of various concentrations of cytokines, PA or H2O2. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 and 95% air using a Forma Scientific incubator.

Isolation and incubation of pancreatic islets
The isolated pancreata from C57/BL6 mice were inflated with Gey and Gey buffer (which consisted of 112 mM NaCl, 27 mM NaHCO3, 4.96 mM KCl, 0.294 mM KH2PO4, 0.3 mM MgSO4, 4 mM glucose, and 1 mM CaCl2, 1 mM MgCl2 and 1 mM Na2HPO4 gassed with CO2/O2 for 20 min and adjusted to pH 7.4) and chopped into small pieces. After centrifugation (100 g for 5 min), the pellet was resuspended and digested with 2 mg of collagenase XI per pancreas in a 1:1 mixture with Gey and Gey buffer and shaken in a water bath at 37 °C until there were no more lumps present in the solution, followed by a subsequent centrifugation step (500 g for 5 min). The islets were resuspended in fresh buffer, picked and cultured for 24 h in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and with/without cytokines [31.25 units/ml TNF-α (tumour necrosis factor-α), 0.3125 units/ml IL-1β (interleukin-1β), 15.625 units/ml IFN-γ (interferon-γ)], or 88 µM H2O2, or 25 mM glucose, or 0.1 mM PA in the presence or absence of antioxidants such as SOD (20 units/ml), Cat (100 units/ml), NAC (0.2 mM), NADPH oxidase inhibitor apocynin (0.2 mM) and iNOS inhibitor 1400W (0.2 mM) at 37 °C in a humidified atmosphere containing 95% (v/v) air and 5% (v/v) CO2. After 24 h, an aliquot of culture medium was removed for determining insulin concentration (see below). In some experiments, islets were subsequently pre-incubated for 10 min and then challenged with 16.7 mM glucose for 20 min, after which insulin secretion was determined (see below).

Western blot analysis
BRIN-BD11 cells were seeded on to 6-well plates (1.5 × 10^6 cells per well) in RPMI 1640 medium and scraped with 150 µl of
RIPA buffer [50 mM Tris/HCl, pH 7.4, 1 % (v/v) NP40 (Nonidet P40), 0.25 % (v/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and protease inhibitors], shock frozen in liquid nitrogen and centrifuged (5 min, 13,400 g and 4 °C).

Isolated islets were lysed by the addition of 50 μl of RIPA buffer (50 mM Tris/HCl, pH 7.4, 1 % (v/v) NP40, 0.25 % (v/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and protease inhibitors) to 100 islets and frozen in liquid nitrogen, followed by a centrifugation step after thawing.

Proteins (10 or 20 μg) were separated by SDS/12.5 % (w/v) PAGE and electroblotted on to nitrocellulose membranes using a semidry blotter. The blot was blocked with Roti®-Block (Roth, Karlsruhe, Germany) for 1 h at room temperature (20 °C) and subsequently incubated in a 1:1000 dilution of a polyclonal rabbit anti-p47phox or anti-GAPDH antiserum in TBST buffer (0.02 M sodium phosphate buffer (pH 7.4), 1 % (v/v) NP40, 0.25 % (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and protease inhibitors) to 100 islets and frozen in liquid nitrogen, followed by a centrifugation step after thawing.

Statistical analyses
Statistical analyses were performed using GraphPad Prism version 3 software. Means ± S.E.M. were calculated from at least three different experiments. Data were analysed using a one-way ANOVA test. Differences are considered statistically significant at P < 0.05.

RESULTS
Effects of pro-inflammatory cytokines or H2O2 in the presence or absence of various antioxidants or inhibitors, on release of insulin over 24 h from mouse pancreatic islets or clonal BRIN-BD11 β-cells
We report, as expected based on results from previous studies utilizing clonal BRIN-BD11 β-cells [9], that culture of mouse islets for 24 h in the presence of pro-inflammatory cytokines (31.25 units/ml TNF-α, 0.3125 unit/ml IL1-β, or 15.625 units/ml IFN-γ) decreased chronic (24 h) insulin secretion by almost 80 % (Figure 1A). The basal control cell culture level of insulin secretion was 100 ± 9 μg/mg of protein over the 24 h period. The addition of antioxidants (SOD, Cat and NAC) did not attenuate cytokine-induced inhibition of insulin secretion. However, the addition of the NADPH oxidase inhibitor apocynin, or the iNOS inhibitor 1400W, or both, blocked the negative effect of cytokines on inhibition of chronic (24 h) insulin secretion (Figure 1A).

When the clonal pancreatic β-cell line BRIN-BD11 was tested under similar conditions, the addition of antioxidants (SOD, Cat and NAC) significantly attenuated cytokine-induced inhibition of insulin secretion. The basal control BRIN-BD11 cell culture level of insulin secretion was 264 ± 51.0 ng/mg of protein over the 24 h period. The addition of the NADPH oxidase inhibitor apocynin and the iNOS inhibitor 1400W reduced the negative effect of cytokines on inhibition of chronic (24 h) insulin secretion, but not significantly (Figure 1B).

To determine the effect of oxidative stress on insulin secretion, we added 88 μM H2O2 to cell culture media for 24 h and observed reduced chronic insulin secretion from mouse islets by at least 60 % (Figure 2A). This concentration did not alter cell viability as assessed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay [9]. The addition of antioxidants (SOD, Cat and NAC) significantly attenuated H2O2-induced inhibition of chronic (24 h)
insulin secretion as did the addition of the iNOS inhibitor 1400W or the NADPH oxidase inhibitor apocynin (Figure 2A).

Addition of H$_2$O$_2$ to BRIN-BD11 β-cell culture media for 24 h reduced chronic insulin secretion by approx. 50% (Figure 2B). The addition of antioxidants or apocynin and 1400W did not significantly reduce the effect of H$_2$O$_2$.

Effect of 24 h exposure to PA, in the presence or absence of various antioxidants or inhibitors, on subsequent release of insulin over 20 min from mouse pancreatic islets

The effects of high concentrations of saturated fatty acids on isolated islets and clonal pancreatic insulin-secreting cells are dependent on the period of exposure during cell culture. Acute exposure (1–3 h) of pancreatic islets to fatty acids enhances insulin secretion [20] and plays a critical role in modulating the stimulatory effect of glucose on insulin release [2,21]. However, chronic exposure of islets or β-cell lines to saturated long-chain fatty acids is known to reduce acute GSIS (glucose stimulated insulin secretion) [10,19]. To avoid potential problems with respect to interpretation of islet insulin release data obtained over a 24 h period of incubation where both stimulatory and inhibitory effects of fatty acids will occur, we incubated mouse islets in the

Figure 1 Effects of pro-inflammatory cytokines in the presence or absence of various antioxidants or inhibitors on basal cell culture release of insulin over 24 h

Pro-inflammatory cytokines (31.25 units/ml TNF-α, 0.3125 units/ml IL-1β, 15.625 units/ml IFN-γ) and antioxidants: SOD (20 units/ml), Cat (100 units/ml), NAC (0.2 mM), or iNOS inhibitor 1400W (0.2 mM) or/and NADPH oxidase inhibitor apocynin (Apo; 0.2 mM) were added to the medium of isolated mouse islets (A) or BRIN-BD11 cells (B). Chronic insulin secretion over a chronic 24 h period was determined by ELISA. Basal control islet culture insulin secretion was 100 ± 9 μg/mg of protein over the 24 h period. Basal control BRIN-BD11 cell culture insulin secretion was 264 ± 51.0 ng/mg of protein over the 24 h period. Statistical analyses were performed using the one-way ANOVA test. Differences are considered statistically significant at $P < 0.05$. **$P < 0.01$ and ***$P < 0.001$. For convenience, the data may be considered in numerical sequence.

Figure 2 Effects of H$_2$O$_2$ in the presence or absence of various antioxidants or inhibitors on basal cell culture release of insulin over 24 h

Chronic insulin secretion from mouse islets (A) or BRIN-BD11 cells (B) after 24 h treatment with 80 μM H$_2$O$_2$ in the presence or absence of various antioxidants: SOD (20 units/ml), Cat (100 units/ml), NAC (0.2 mM), iNOS inhibitor 1400W (0.2 mM) and NADPH oxidase inhibitor apocynin (Apo; 0.2 mM) was determined by ELISA. Basal control islet culture insulin secretion was 100 ± 9 μg/mg of protein over the 24 h period. Basal control BRIN-BD11 cell culture insulin secretion was 264 ± 51.0 ng/mg of protein over the 24 h period. Statistical analyses were performed using the one-way ANOVA test. Differences are considered statistically significant at $P < 0.05$. **$P < 0.01$ and ***$P < 0.001$. For convenience, the data may be considered in numerical sequence.
presence of 0.1 mM PA for 24 h and then insulin secretion was subsequently determined using an acute (20 min) stimulation test in the presence of a robust stimulus of 16.7 mM glucose. Incubation of islets with PA for 24 h resulted in a subsequent reduction of acute (20 min) GSIS, by at least 80\% (Figure 3). Following 24 h incubation in the absence of PA, the basal control (20 min) insulin secretion was 0.75 μg/mg of protein, which increased to 15 μg/mg of protein on challenge with 16.7 mM glucose. Insulin secretion was determined by ELISA. Statistical analyses were performed using the one-way ANOVA test. Differences are considered statistically significant at \(P < 0.05\). For convenience, the data may be considered in numerical sequence.

**Effects of pro-inflammatory cytokines or H\(_2\)O\(_2\), in the presence or absence of various antioxidants or inhibitors, on ATP levels, p47\(^{phox}\) or iNOS levels in mouse islets or clonal BRIN-BD11 β-cells**

Culture of mouse islets or BRIN-BD11 cells for 24 h in the presence of IL-1β, IFN-γ plus TNF-α has previously been reported to reduce ATP levels [9,22]. We have now extended this work to determine the effect of 88 μM H\(_2\)O\(_2\) (a non-lethal concentration) on ATP levels and possible protective effect of antioxidants. The control ATP level following 24 h incubation was 46.2 ± 11.7 μmol/mg of protein. The control ATP level in H\(_2\)O\(_2\) treated BRIN-BD11 cells was reduced by at least 60\% (Figure 4). The addition of antioxidants (SOD, Cat and NAC) attenuated H\(_2\)O\(_2\)-induced reduction in ATP levels as did the addition of the NADPH oxidase inhibitor apocynin or the iNOS inhibitor 1400W (Figure 4).

\[\text{p47}^{\text{phox}}\] is a key regulatory subunit of NADPH oxidase that translocates from a cytosolic location to the plasma membrane to associate with the two NADPH oxidase subunits, gp91\(^{phox}\) and p22\(^{phox}\), to regulate superoxide production in pancreatic β-cells under inflammatory conditions [5]. \text{p47}^{\text{phox}} protein expression was increased by more than 2-fold in response to IL-1β, IFN-γ plus TNF-α in mouse islets (Figure 5). The NADPH oxidase inhibitor apocynin or the iNOS inhibitor 1400W or both significantly attenuated the pro-inflammatory cytokine-dependent increase in \text{p47}^{\text{phox}} expression (Figure 5), suggesting that O\(_2^-\) and H\(_2\)O\(_2\) may enhance the expression of components of the enzyme responsible for their production. \text{p47}^{\text{phox}} protein expression was increased by approx. 2- or 3-fold in response to H\(_2\)O\(_2\) in mouse islets or BRIN-BD11 cells, respectively, following 24 h of incubation (Figure 6).

We extended the BRIN-BD11 cell study by determining the effect of stressors on iNOS protein expression, which was increased by more than 4-fold in response to IL-1β, IFN-γ plus TNF-α or approx. 2-fold in response to 24 h of incubation in the presence of H\(_2\)O\(_2\) (Figure 7).

**DISCUSSION**

Compared with many other cell types, the β-cell may be at high risk of oxidative damage with an increased sensitivity for apoptosis. This high risk may be due to (i) excessive levels of mitochondrial ROS generation, (ii) additional ROS generation...
through elevated β-cell NADPH oxidase activity and NO generation from iNOS (related to the findings described in this paper), and (iii) failure of antioxidant defences. With respect to Type 2 diabetes mellitus, β-cell dysfunction and associated depressed insulin secretion must be evident before hyperglycaemia develops [11]. It is clear that excessive levels of glucose, lipid, endocrine and various inflammatory factors interact at the level of the pancreatic islet to promote β-cell dysfunction. Thus any explanation for molecular integration at the level of the β-cell must include common mechanisms of dysfunction. One possible candidate mechanism is the activation of NADPH oxidase, iNOS activation and consequent ROS and NO production. The β-cell NADPH oxidase is activated by glucose, saturated fatty acids (such as PA), endocrine factors (angiotensin II) and pro-inflammatory cytokines [11].

Impairment of β-cell function in vivo may involve excessive generation of ROS through increased NADPH oxidase activity as described above and in [11]. Along with elevated levels of NO, this would subsequently affect mitochondrial function, reducing ATP production and hence insulin secretion [11]. Thus the data presented in this paper have described the important role of ROS and NO generation in pancreatic β-cells for subsequent dysfunction. What is particularly relevant is the specific inhibition of NADPH oxidase and iNOS, which results in partial or complete restoration of ATP levels (Figure 4), and insulin secretion (Figures 1–3). While the impact of antioxidants or inhibitors may not have been identical in terms of magnitude of response in islets compared with BRIN-BD11 β-cells, this may reflect different kinetics of response to cytokines, as we have previously demonstrated that the level of the NADPH oxidase component p47phox was increased much more rapidly in rat islets compared to BRIN-BD11 cells [13]. This may well apply to the response to H2O2 as well. If this is the case, then the protection afforded by antioxidants or inhibitors might be expected to be greater in islet cells, as reported in Figures 1 and 2. The inhibitors we have used in this study, apocynin and 1400W, have previously been shown to act specifically on their respective enzyme targets in biological systems [23,24]. Also, we provide evidence for the first time that H2O2 can actually increase levels of expression of the key regulator of NADPH oxidase, p47phox (Figure 6) and also iNOS (Figure 7). Thus it is possible that a positive-feedback system is in place, such that on moderate ROS generation, components of NADPH oxidase and iNOS are increased in expression (mediated by redox sensitive transcription factors) so resulting in enhanced ROS and NO generation, leading to a fall in ATP and other stimulus–secretion coupling factors and ultimately insulin secretion [9,13]. The impairment of β-cell metabolism by ROS was also demonstrated using alternative experimental approaches – using a patch-clamp technique, a decrease in KATP–channel activity and a suppression of plasma membrane electrical excitability induced by H2O2 was observed [25]. Similar experiments were performed in insulin-secreting cell lines, demonstrating inhibition of both metabolism and insulin secretion [26,27]. Moreover, two potent activators of insulin gene transcription, PDX-1 (pancreatic and duodenal homeobox-1) and MafA (musculoaponeurotic fibrosarcoma oncogene homologue A), are targets of ROS regulation [28].
These β-cell-specific transcriptional factors play a crucial role in pancreatic development, β-cell differentiation and function.

Antioxidant treatment exerts beneficial effects in diabetic models. Diabetic C57BL/KsJ-db/db mice were reported to have a significantly greater β-cell mass after antioxidant treatment, possibly due to suppression of apoptosis. They had significantly higher levels of insulin content and insulin mRNA, which was correlated with higher expression of PDX-1 and its translocation; it was clearly visible in the nuclei of islet cells after the antioxidant treatment [29]. With respect to pancreatic β-cell lines, RINm5F cells overexpressing Cat, glutathione peroxidase and Cu/Zn SOD were protected from a lethal pro-inflammatory cytokine mixture (IL-1β, TNF-α and IFN-γ) and exhibited a significantly lower β-cell differentiation and function. Pro-inflammatory cytokines, via transcriptional regulation, increase expression of iNOS and the p47phox subunit of NADPH oxidase, which results in NO, O₂⁻ and H₂O₂ generation. O₂⁻ and H₂O₂ further increase expression and activity of iNOS and the p47phox subunit of NADPH oxidase enzymes, thus stimulating NO and ROS production. PA may also increase expression of iNOS and the p47phox subunit of NADPH oxidase but probably the fatty acid is not as potent in action as pro-inflammatory cytokines. The oxygen- and nitrogen-free radicals inhibit ATP production and insulin secretion. Antioxidants (SOD, Cat and NAC) as well as the NADPH oxidase inhibitor apocynin (Apo) or the iNOS inhibitor (1400W) act at the sites identified in the Figure, thus reducing oxidative stress.

Figure 7 Effects of pro-inflammatory cytokines or H₂O₂ on iNOS levels in BRIN-BD11 cells
A mixture of cytokines (31.25 units/ml TNF-α, 0.3125 unit/ml IL1-β and 15.625 units/ml IFN-γ) or H₂O₂ (88 μM) was added to the culture medium of BRIN-BD11 cells for 24 h. Induction of iNOS was determined by Western blotting (upper panel). Statistical analyses from the greyscale values of all experiments were performed using a one-way ANOVA test. Differences are considered statistically significant at **P < 0.01. For convenience, the data may be considered in numerical sequence.

Figure 8 Overview of potential mechanisms of action and mechanistic cooperation of pro-inflammatory cytokines, H₂O₂ and PA in pancreatic β-cells
Pro-inflammatory cytokines, via transcriptional regulation, increase expression of iNOS and the p47phox subunit of NADPH oxidase, which results in NO, O₂⁻ and H₂O₂ generation. O₂⁻ and H₂O₂ further increase expression and activity of iNOS and the p47phox subunit of NADPH oxidase enzymes, thus stimulating NO and ROS production. PA may also increase expression of iNOS and the p47phox subunit of NADPH oxidase but probably the fatty acid is not as potent in action as pro-inflammatory cytokines. The oxygen- and nitrogen-free radicals inhibit ATP production and insulin secretion. Antioxidants (SOD, Cat and NAC) as well as the NADPH oxidase inhibitor apocynin (Apo) or the iNOS inhibitor (1400W) act at the sites identified in the Figure, thus reducing oxidative stress.

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secretion in isolated islets (Figure 1A), the specific inhibition of iNOS and/or NADPH oxidase in pancreatic β-cells could be a potent mechanism to delay diabetes manifestation in vivo. Indeed the action of NADPH oxidase and iNOS in cooperation with respect to inducing oxidative stress and downstream effects on metabolism and insulin secretion has not, as yet, been fully appreciated.

It was recently reported that H₂O₂ acts as a stimulating signal for GSIS, and its elimination by antioxidants abolished insulin secretion stimulated by glucose and high K⁺ levels [38]. However, high rates of glucose metabolism prevented the accumulation of superoxide-derived ROS in primary β-cells [25]. This suppressive effect was more pronounced in cells with a high metabolic response to glucose [25]. However, inhibition of NADPH oxidase suppresses glucose metabolism, calcium oscillation and GSIS in isolated islets [39]. This could indicate that there is a threshold range of ROS in the β-cell that is essential to maintain β-cell function, and a drastic variation of the redox state due to excessive levels of formation or reduction in disposal results in impairment in insulin secretion. It is also probable that the time of exposure to high glucose concentrations and rate of metabolism are important for provoking changes in ROS levels, prompting a re-evaluation of the concept that high glucose and/or fatty acid levels invariably cause ROS-induced β-cell dysfunction and death. The same may also apply to the role of NO. Glucose and insulin activate β-cell nNOS (neuronal NOS), which produces NO [40]. This second messenger was shown to be important for insulin secretion and synthesis [41]. However, cytokine or saturated NEFA-induced stress situations result in up-regulation of iNOS in pancreatic β-cells, causing generation of cytotoxic levels of NO. These dose-dependent effects of NO range from reduced insulin secretion [42] to apoptotic cell death [43]. Thus the specific inhibition of iNOS, despite maintenance of nNOS activity, could be helpful for β-cells to survive such attacks and to prevent dysfunction. Dysfunction of islets induced by exposure to high glucose concentrations was reported to be mediated by iNOS, an effect reduced by the addition of GLP-1 (glucagon-like peptide-1), which reduced iNOS activity and expression [44].

Finally, we hypothesize that antioxidants are very effective protectors against over-nutrition associated with mild oxidative stress (glucotoxicity, lipotoxicity resulting in elevated levels of ROS). However, after induction of immunological processes and the release of pro-inflammatory cytokines, such as in the progression of Type 1 diabetes, antioxidants may no longer be sufficient to prevent β-cell dysfunction or destruction. In this situation, a selective inhibition of stress enzymes, such as NADPH oxidase and iNOS, may be more effective to delay diabetes manifestation in vivo.

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