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The effects of pharmacologic inhibition of NADPH oxidase or iNOS on pro-inflammatory cytokine, palmitic acid or H₂O₂ -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 have investigated the effects of prolonged exposure (24 hr) to pro-inflammatory cytokines, H_2O_2 or palmitic acid, on beta cell insulin secretion, ATP, the NADPH oxidase component $p47^{phox}$ and iNOS levels using primary mouse islets or clonal rat BRIN-BD11 beta cells. Addition of a pro-inflammatory cytokine mixture (IL-1 β , TNF- α and IFN- γ) or H_2O_2 (at sub-lethal concentrations) inhibited chronic (24hr) levels of insulin release by at least 50% (from islets and BRIN-BD11 cells), while addition of the saturated fatty acid palmitate inhibited acute (20min) stimulated levels of insulin release from mouse islets. H_2O_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 the antioxidants superoxide dismutase, catalase and N-acetylcysteine attenuated H_2O_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_2O_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_2O_2 can increase levels of the latter two proteins suggesting a key role for positive-feedback redox sensitive regulation of beta cell dysfunction.

Abbreviations:

Cat, catalase; GPx, glutathione dependent peroxidase; GSIS, glucose stimulated insulin secretion; iNOS, inducible nitric oxide synthase; NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; NEFA, non essential fatty acids; ROS, reactive oxygen species; SOD, superoxide dismutase

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 non-esterified fatty acids (NEFA). The metabolic interaction between glucose and palmitate (one of the most abundant NEFAs in plasma) may generate lipid intermediates (diacylglycerol, phosphatidylserine) that activate specific isoforms of protein kinase C (PKC), thereby enhancing insulin secretion [1, 2]. Indeed, it was recently 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 in non-phagocytic cells [4–6]. The NADPH oxidase consists of six hetero-subunits, which associate in a stimulus-dependent manner to form the active enzyme complex and produce superoxide. Two NADPH oxidase subunits, gp91^{phox} and p22^{phox}, 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 the other subunits. The additional subunits are required for regulation and are located in the cytosol during the resting state. They include the proteins p67^{phox}, p47^{phox} and p40^{phox} as well as the small GTPase Rac. p47^{phox} 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 beta cell exposure to pro-inflammatory cytokines or high concentrations of fatty acids at non-lethal concentrations will lead to an elevated generation of ROS 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. In this work we have 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 palmitic acid, by use of specific inhibitors of NADPH oxidase and iNOS respectively. We demonstrate that both beta 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 have utilised primary mouse islets and the clonal rat beta 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 palmitic acid and cytokine effects on insulin secretion and p47^{phox} expression, utilising this cell line [9, 13, 19].

Materials and methods

Animals and Materials

C57/BL6 mice were obtained from the Conway Institute of Biomolecular and Biomedical Research (UCD, Dublin, Ireland) and the Biotechnikum Greifswald (Greifswald, Germany). Rat insulinoma BRIN-BD11 beta cells were obtained from Prof. Peter Flatt and Dr. Neville McClenaghan of the University of Ulster, Coleraine, N. Ireland. Collagenase type XI was obtained from Sigma. Anti-GAPDH rabbit polyclonal antibody was purchased from Abcam (Cambridge, UK). Goat polyclonal antibody directed against beta-actin was obtained from SantaCruz Biotechnology (Heidelberg, Germany). Rabbit polyclonal antibody directed against p47^{phox} from rat or mouse was obtained from SantaCruz Biotechnology (Heidelberg, Germany). Rabbit monoclonal antibody directed against iNOS from rat or mouse was purchased from Sigma. Cytokines were obtained from Roche (Mannheim, Germany). Glucose,

palmitic acid, superoxide dismutase (PEG-SOD), catalase (PEG-CAT), N-acetyl-cysteine were all obtained from Sigma (Germany or Ireland). 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 media supplemented with 10% (v/v) foetal bovine serum and 0.1% antibiotics (100U/ml penicillin and 0.1 mg/ml streptomycin). Cells (1.5×10^5) were seeded in 24-well plates containing 1ml of media or 1.5×10^6 cells were seeded in 6-well plates containing 5ml of media and allowed to adhere overnight, before treatment in the presence or absence of various concentrations of cytokines, palmitic acid or H_2O_2 . The cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 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 Gey buffer buffer (which consisted of 112 mM NaCl, 27 mM $NaHCO_3$, 4.96 mM KCl, 0.294 mM KH_2PO_4 , 0.3 mM $MgSO_4$, 4 mM glucose, and 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4 gassed with CO_2/O_2 for 20 min and adjusted to pH 7.4) and chopped into small pieces. After spin (1000 rpm for 2 sec) the pellet with 2 mg collagenase XI (Sigma) per pancreas and a 1:1 ratio of Gey Gey buffer was shaken in water bath at 37 °C until there are no more lumps present in the solution followed by a subsequent spin step (1000 rpm for 5 sec). The islets were resuspended in fresh buffer, picked and cultured for 24 hours in RPMI-1640 medium supplemented with 10 % (v/v) heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and with or without cytokines (31.25 U/ml TNF- α , 0.3125 U/ml IL1- β , 15.625 U/ml INF- γ), or 88 µM H_2O_2 , or 25 mM glucose, or 0.1 mM palmitic acid in the presence or absence of antioxidants such as superoxide dismutase (20 U/ml), catalase (100 U/ml), N-acetyl-cysteine (0.2 mM), NADPH oxidase inhibitor Apocynin (0.2 mM), iNOS inhibitor 1400W (0.2 mM) at 37°C in a humidified atmosphere containing 95 % (v/v) air and 5 % (v/v) CO_2 . After 24h, an aliquot of culture media was removed for determination of insulin concentration (see below). In some experiments islets were subsequently pre-incubated for 10 minutes and then challenged with 16.7 mM glucose for 20min, after which insulin secretion was determined (see below).

Western blot

BRIN-BD 11 were seeded in 6 well-plates (1.5×10^6 cells/well) in RPMI medium and scraped with 150 µl RIPA buffer (50 mM Tris-HCl, pH 7.4; 1 % (v/v) NP-40; 0.25 % (w/v) Na-deoxycholate; 150 mM NaCl; 1 mM EDTA, protease inhibitors), shock frozen in liquid nitrogen, and centrifuged (5 min, 12,000 rpm, 4°C).

Isolated islets were lysed by the addition of 50 µl RIPA buffer (50 mM Tris-HCl, pH 7.4; 1 % (v/v) NP-40; 0.25 % (w/v) Na-deoxycholate; 150 mM NaCl; 1 mM EDTA; protease inhibitors) to 100 islets and freeze in liquid nitrogen, followed by a centrifugation step after thawing.

Proteins (10 or 20 µg) were separated by 12.5 % (w/v) SDS-PAGE and electroblotted onto nitrocellulose membranes using a semidry blotter. The blot was blocked with Roti®-Block (Roth, Karlsruhe, Germany) for 1 h at room temperature and subsequently incubated in a 1:1,000 dilution of a polyclonal rabbit anti-p47^{phox} or anti-GAPDH antiserum in TBST buffer with 3 % (w/v) BSA, 1: 10,000 dilution of monoclonal rabbit anti-iNOS antibody or 1: 1,000 dilution of polyclonal goat antibody directed against beta-actin. After washing the blot with TBS/Tween 0.05 % (v/v), a HRP-conjugated anti-rabbit IgG (1:10,000) or anti-goat IgG (1:10000) was used as secondary antibody. Detection of bound antibody was performed with LumiGLO reagent and peroxide (Cell Signaling Technology, Danvers, USA).

Insulin Secretion

BRIN-BD 11 cells were seeded in 24-well plates (1.5×10^5 cells/ well) in RPMI medium followed by treatment as mentioned above. After 24 h incubation media was removed and centrifuged at 400 g and analysed for basal cell culture insulin secretion using Mercodia

Ultrasensitive Rat Insulin ELISA kit (Uppsala, Sweden). Where indicated, mouse islet cells were washed with phosphate buffered saline and preincubated at 37°C for 10 min in Krebs-Ringer Bicarbonate (KRB) buffer comprising 115 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 10 mM NaHCO₃, 5 g/l bovine serum albumin (pH 7.4) supplemented with 1.1 mM D-glucose. After 10 min cells were stimulated with 16.7 mM glucose, prepared in Krebs Ringer buffer pH 7.4. After 20 min incubation media was removed and centrifuged at 400 g for 10 min, and analysed for insulin using Mercodia Ultrasensitive Rat Insulin ELISA kit. RIPA solution (lysis buffer) was used for protein extraction.

Protein determination

Cellular protein was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA kit), which utilizes a modification of the Biuret reaction. The method combines the reduction of Cu²⁺ to Cu⁺ by protein in an alkaline medium. The product is determined by absorbance at 562 nm.

ATP measurement

ATP was measured using an ATP determination kit from Proteinkinase (Kassel, Germany). BRIN-BD 11 cells were seeded into 24-well plates (4x10⁴) in RPMI medium in the presence of treatment mentioned above. After 24 h incubation, Media was removed from wells and the cells were washed with PBS in ice. ATP-releasing agent was added to each well and the resulting solution was transferred into Eppendorf tubes. The experimental sample/ standard was added to a well in the plate and the final reagent (prepared of reaction buffer containing DTT, D-Luciferin, and Luciferase stock solution following the manufacturer's protocol) was added. The microplate was covered in tinfoil and then incubated for 15 min before luminescence was measured.

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 analyzed using a 1 way ANOVA test. Differences are considered statistically significant at p< 0.05.

Results

Effects of pro-inflammatory cytokines or hydrogen peroxide in the presence or absence of various antioxidants or inhibitors, on release of insulin over 24h from mouse pancreatic islets or clonal BRIN-BD11 beta cells.

We report, as expected based on results from previous studies utilizing clonal BRIN BD11 beta cells [9], that culture of mouse islets for 24 hr in the presence of pro-inflammatory cytokines (31.25 U/ml TNF-α, 0.3125 U/ml IL1-β, or 15.625 U/ml INF-γ) decreased chronic (24h) insulin secretion by almost 80% (Figure 1A). The basal control cell culture level of insulin secretion was 100 ± 9 μg / mg protein over the 24h period. The addition of antioxidants (superoxide dismutase, catalase, N-acetylcysteine) 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 (24h) insulin secretion (Fig 1A).

When the clonal pancreatic beta cell line BRIN-BD11 was tested under similar conditions, the addition of antioxidants (superoxide dismutase, catalase, N-acetylcysteine) 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 protein over the 24h 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 (Fig 1B).

To determine the effect of oxidative stress on insulin secretion, we made an addition of 88 μM H_2O_2 to cell culture media for 24 h and observed reduced chronic insulin secretion from mouse islets by at least 60 % (Fig 2A). This concentration did not alter cell viability as assessed by MTS assay [9]. The addition of antioxidants (superoxide dismutase, catalase, N-acetylcysteine) significantly attenuated H_2O_2 induced inhibition of chronic (24hr) insulin secretion as did the addition of the iNOS inhibitor 1400W or the NADPH oxidase inhibitor Apocynin (Fig 2A). Addition of H_2O_2 to BRIN-BD11 beta cell culture media for 24 h reduced chronic insulin secretion by approximately 50 % (Fig 2B). The addition of antioxidants or apocynin and 1400W did not significantly reduce the effect of H_2O_2 .

Effect of 24h exposure to palmitic acid, 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 beta cell lines to saturated long chain-fatty acids is known to reduce acute glucose stimulated insulin secretion [10, 19]. To avoid potential problems with respect to interpretation of islet insulin release data obtained over a 24h period of incubation where both stimulatory and inhibitory effects of fatty acids will occur, we incubated mouse islets in the presence of 0.1 mM palmitic acid 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 palmitic acid for 24 hr resulted in a subsequent reduction of acute (20min) glucose stimulated insulin secretion, by at least 80 % (Fig 3). Following 24 hr incubation in the absence of palmitic acid, the basal control (20min) insulin secretion was 0.75 μg / mg protein which increased to 15 μg / mg protein on challenge with 16.7 mM glucose. A similar effect of palmitic acid has been previously reported with respect to BRIN-BD11 cells [19]. Antioxidants as well as the chemical inhibitors of iNOS and NADPH oxidase were able to completely prevent the inhibitory effects of palmitic acid in mouse islets (Fig. 3).

Effects of pro-inflammatory cytokines or hydrogen peroxide, 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 beta cells.

Culture of mouse islets or BRIN-BD11 cells for 24 hours in the presence of IL-1 β , 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_2O_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_2O_2 treated BRIN-BD11 cells was reduced by at least 60 % (Fig.4). The addition of antioxidants (superoxide dismutase, catalase, N-acetylcysteine) attenuated H_2O_2 induced reduction in ATP levels as did the addition of the NADPH oxidase inhibitor apocynin or the iNOS inhibitor 1400W (Fig 4).

p47^{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 beta cells under inflammatory conditions [5]. p47^{phox} protein expression was increased by more than 2-fold in response to IL-1 β , IFN- γ plus TNF- α in mouse islets (Fig 5). The NADPH oxidase inhibitor apocynin or the iNOS inhibitor 1400W or both significantly attenuated the pro-inflammatory cytokine dependent increase in p47^{phox} expression (Fig 5) suggesting that O_2^- and H_2O_2 may enhance the expression of components of the enzyme responsible for their production.

p47^{phox} protein expression was increased by approximately 2-fold or 3-fold in response to H₂O₂ in mouse islets or BRIN BD11 cells respectively following 24h of incubation (Fig 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-1 β , IFN- γ plus TNF- α or approximately 2-fold in response to 24h of incubation in the presence of H₂O₂ (Fig 7).

Discussion

Compared to many other cell types, the β -cell may be at high risk for 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 beta cell NADPH oxidase activity and NO generation from iNOS (related to the findings described in this paper) and (iii) failure of antioxidant defenses. With respect to T2DM, beta 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 beta cell dysfunction. Thus any explanation for molecular integration at the level of the beta 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 beta cell NADPH oxidase is activated by glucose, saturated fatty acids (such as palmitic acid), endocrine factors (angiotensin II) and pro-inflammatory cytokines [11].

Impairment of beta-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 so insulin secretion [11]. Thus the data presented in this paper have described the important role for ROS and NO generation in pancreatic beta 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 (Fig. 4), and insulin secretion (Fig. 1-3). While the impact of anti-oxidants or inhibitors may not have been identical in terms of magnitude of response in islets compared with BRIN BD11 beta cells, this may reflect different kinetics of response to cytokines as we have previously demonstrated that the level of the NADPH oxidase component p47^{phox} 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 anti-oxidants or inhibitors might be expected to be greater in islet cells, as reported in Fig 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, p47^{phox} (Fig. 6) and also iNOS (Fig. 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 beta cell metabolism by ROS was also demonstrated using alternative experimental approaches - using a patch-clamp technique a decrease in K_{ATP}-channel activity and a suppression of plasma membrane electrical excitability induced by hydrogen peroxide 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, pancreatic and duodenal homeoboxprotein-1 (PDX-1) and MafA, are targets of ROS regulation [28]. These beta cell-specific transcriptional factors play a crucial role in pancreas development, beta 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 beta 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, clearly visible in the nuclei of islet cells after the antioxidant treatment [29]. With respect to pancreatic beta cell lines, RINm5F cells overexpressing catalase, glutathione peroxidase, and Cu/Zn superoxide dismutase, were protected from a lethal pro-inflammatory cytokine mixture (IL-1 β , tumor necrosis factor- α and γ -interferon) and exhibited a significantly lower level of ROS-dependent protein damage [30]. Indeed earlier reports utilizing primary rat islets described the importance of redox enzymes to the regulation of beta cell metabolism include a study describing the inhibition of glutathione reductase (GR, a GSSG reducing enzyme) which impaired glucose metabolism and insulin secretion [31]. Additionally, GSIS was reported to be dependent on the level of intracellular reduced glutathione [32]. In this study the addition of superoxide dismutase, catalase and N-acetylcysteine was sufficient to prevent all damages caused by H₂O₂ or palmitic acid and they partially attenuated the inhibitory effects of the pro-inflammatory cytokines IL1- β , TNF- α and IFN- γ on insulin secretion in BRIN-BD11 cells. The effect of ROS (H₂O₂) on increased expression of p47^{phox} and iNOS (Figs 6 and 7) suggests that ROS may act at a transcriptional level. The promoter studies of the p47^{phox} gene are still at an early stage. There is some evidence for induction by cytokines [33]. iNOS induction and activation resulted in an increase in translocation of p47^{phox} and activation of NADPH oxidase in macrophages [34]. Detail on the transcriptional regulation of iNOS is at a more advanced stage. For direct activation there are NF κ B and STAT binding sites present in the iNOS promoter [35]. Indeed it has been reported that iNOS expression was dependent on NADPH oxidase-dependent redox signaling in microvascular endothelial cells [36]. While NF κ B activation was required for iNOS induction in the latter study, it was not dependent on ROS signaling. However, the induction of NADPH oxidase components appears to be amplified by iNOS generated product. It was shown, that in coronary artery smooth muscle cells the NADPH oxidase activity was enhanced by IL-1 β (67%) and a NO donor SNP (45%) [33]. This indicates that beside the direct cytokine effects, NO is involved in the activation of NADPH oxidase. The present study has provided evidence that NO is involved in the transcriptional activation of p47^{phox} by cytokines, utilizing the specific inhibitor of iNOS 1400W (Fig. 5). On the other hand, ROS production by NADPH oxidase was shown to be an important activator of NF κ B (which activates iNOS) after stress with IL-1 β in monocytic cells [37]. In our experiments with insulinoma cells the effect of H₂O₂ or cytokines included an increase the expression of p47^{phox} and iNOS (Figs 5, 6 and 7). We have attempted to synthesize an overview to explain our findings in the context of previously published results and our resulting hypothesis is outlined in Figure 8.

Considering the potent effects of apocynin and 1400W (in contrast to the combination of SOD, catalase and N-acetylcysteine antioxidants), on the protection against cytokine mediated inhibition of insulin secretion in isolated islets (Fig. 1A), the specific inhibition of iNOS and/or NADPH oxidase in pancreatic beta 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 beta 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 beta cell essential to maintain beta 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 beta cell dysfunction and death. The same may also apply to the role of NO. Glucose and insulin activate beta cell nNOS, which produces NO [40]. This second messenger was shown to be important for insulin secretion and synthesis [41]. However cytokine or saturated non-esterified fatty acid induced stress situations result in up-regulation of iNOS in pancreatic beta 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 but maintenance of nNOS activity could be helpful for beta cells to survive such attack 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 addition of GLP-1 which reduced iNOS activity and expression [44].

Finally we hypothesize that antioxidants are very effective protectors against over-nutritional or 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 beta cell dysfunction or destruction. In this situation a selective inhibition of stress enzymes, like NADPH oxidase and iNOS, may be more effective to delay diabetes manifestation *in vivo*.

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Figures legends

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

Pro-inflammatory cytokines (31.25 U/ml TNF- α , 0.3125 U/ml IL1- β , 15.625 U/ml INF- γ) and antioxidants: superoxide dismutase (20 U/ml), catalase (100 U/ml), N-acetylcysteine (0.2 mM), or iNOS inhibitor 1400W (0.2 mM) or/and NADPH oxidase inhibitor apocynin (0.2 mM) were added to the medium of isolated mouse islets (A) or BRIN-BD 11 cells (B). Chronic insulin secretion over a chronic 24 h period was determined by ELISA. Basal control islet culture insulin secretion was 100 ± 9 μ g / mg protein over the 24h period. Basal control BRIN BD11 cell culture insulin secretion was 264 ± 51.0 ng / mg protein over the 24h period. Statistical analyses were performed using 1 way ANOVA test. Differences are considered statistically significant at $p < 0.05$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Fig. 2: Effects of hydrogen peroxide in the presence or absence of various antioxidants or inhibitors on basal cell culture release of insulin over 24h.

Chronic insulin secretion from mouse islets (A) or BRIN-BD 11 cells (B) after 24 h treatment with 80 μ M H₂O₂ in the presence or absence of various antioxidants (superoxide dismutase (20 U/ml), catalase (100 U/ml), N-acetylcysteine (0.2 mM), iNOS inhibitor 1400W (0.2 mM), NADPH oxidase inhibitor apocynin (0.2 mM) was determined by ELISA. Basal control islet culture insulin secretion was 100 ± 9 μ g / mg protein over the 24h period. Basal control BRIN BD11 cell culture insulin secretion was 264 ± 51.0 ng / mg protein over the 24h period. Statistical analyses were performed using 1 way ANOVA test. Differences are considered statistically significant at $p < 0.05$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Fig. 3: Effects of palmitic acid in the presence or absence of various antioxidants or inhibitors on acute (20min) release of insulin.

Effect of antioxidant treatment for 24h (superoxide dismutase (20 U/ml), catalase (100 U/ml), N-acetylcysteine (0.2 mM), iNOS inhibitor 1400W (0.2 mM) or NADPH oxidase inhibitor apocynin (0.2 mM) on the subsequent 20 min acute glucose (16.7 mM) stimulated insulin secretion following 24 h treatment with palmitic acid (PA) in mouse islets. Following 24 hr incubation in the absence of palmitic acid, the basal control (20min) insulin secretion was 0.75 μ g / mg protein which increased to 15 μ g / mg protein on challenge with 16.7 mM glucose. Insulin secretion was determined by ELISA. Statistical analyses were performed using 1 way ANOVA test. Differences are considered statistically significant at $p < 0.05$. * $p < 0.05$

Fig. 4: Effects of hydrogen peroxide in the presence or absence of various antioxidants or inhibitors on ATP levels in BRIN-BD11 cells.

ATP levels after chronic (24 h) exposure to 80 μ M hydrogen peroxide and antioxidants (superoxide dismutase (20 U/ml), catalase (100 U/ml), N-acetylcysteine (0.2 mM), NADPH oxidase inhibitor apocynin (0.2 mM) or iNOS inhibitor 1400W (0.2mM) were detected using a bioluminescent Luciferase assay. The control ATP level following 24 h incubation was 46.2 ± 11.7 μ mol / mg of protein. The RLUs are expressed relative to non treated cells (control = 100 %). Statistical analyses were performed using 1 way ANOVA test. Differences are considered statistically significant at $p < 0.05$. *** $p < 0.001$

Fig. 5: Effects of pro-inflammatory cytokines, in the presence or absence of various antioxidants or inhibitors on p47^{phox} expression in mouse islets.

A mixture of cytokines (31,25U/ml TNF- α , 0,3125U/ml IL1- β , 15,625U/ml INF- γ) and different antioxidants: superoxide dismutase (20U/ml), catalase (100U/ml), N-acetylcysteine (0.2mM), iNOS inhibitor 1400W (0.2mM) or NADPH oxidase inhibitor apocynin (0.2mM) on production of NADPH oxidase components p47^{phox} were added to the medium of mouse islets for 24h. Expression of p47^{phox} was determined by western blots. Statistical analyses from the grey scale values of all experiments were performed using 1 way ANOVA test. Differences are considered statistically significant at $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$

Fig. 6: Effects of hydrogen peroxide on level of p47^{phox} expression.

Hydrogen peroxide (88 μ M) was added to the medium of mouse islets or BRIN-BD11 cells for 24h. Production of NADPH oxidase components p47^{phox} was determined by western blot. Statistical analyses from the grey scale values of all experiments were performed using 1 way ANOVA test. Differences are considered statistically significant at $p < 0.05$. * $p < 0.05$, ** $p < 0.01$

Fig. 7: Effects of pro-inflammatory cytokines or hydrogen peroxide on iNOS levels in BRIN-BD11 cells.

A mixture of cytokines (31.25 U/ml TNF- α , 0.3125 U/ml IL1- β , 15.625 U/ml INF- γ) or hydrogen peroxide (88 μ M) was added to the culture medium of BRIN BD11 cells for 24h. Induction of iNOS was determined by western blot. Statistical analyses from the grey scale values of all experiments was performed using 1 way ANOVA test. Differences are considered statistically significant at ** $p < 0.01$

Fig. 8: Overview of potential mechanisms of action and mechanistic cooperation of pro-inflammatory cytokines, hydrogen peroxide and palmitic acid in pancreatic beta cells.

Proinflammatory cytokines, via transcriptional regulation, increase expression of iNOS and the p47^{phox} subunit of NADPH oxidase which results in NO, O₂⁻ and H₂O₂ generation. O₂⁻ and H₂O₂ further increase expression and activity of iNOS and the p47^{phox} subunit of NADPH oxidase enzymes so stimulating NO and ROS production. PA may also increase expression of iNOS and the p47^{phox} 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 (superoxide dismutase, catalase, N-acetylcysteine) as well as the NADPH oxidase inhibitor apocynin (Apo) or the iNOS inhibitor (1400W) act at the sites identified in the Figure, so reducing oxidative stress.

Fig. 1A

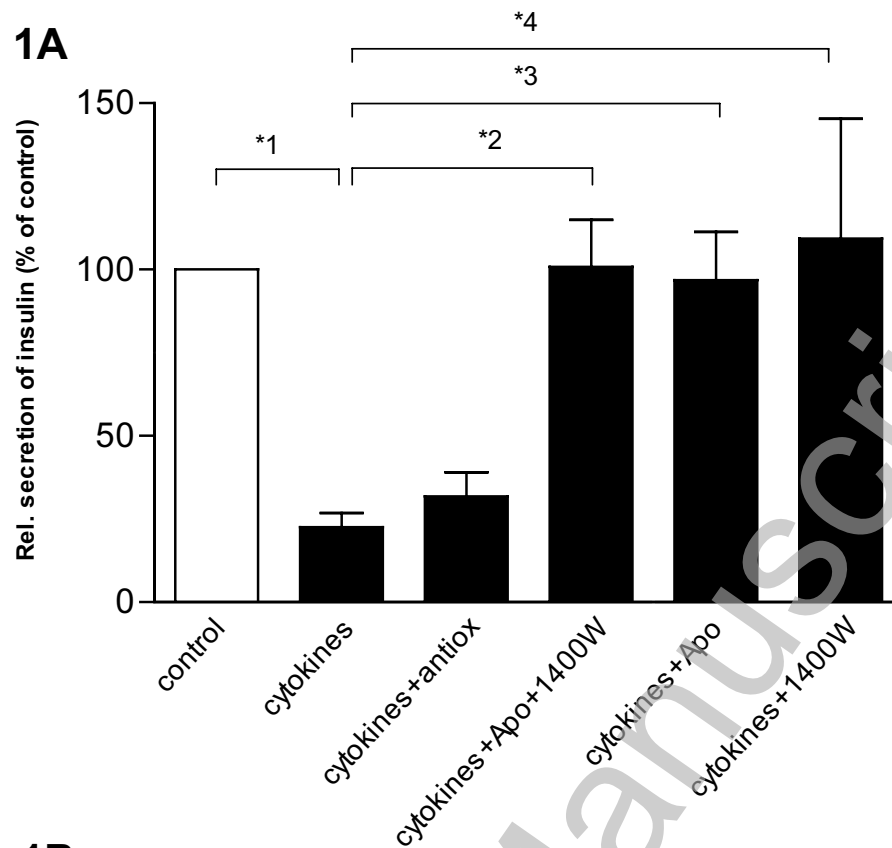


Fig. 1B

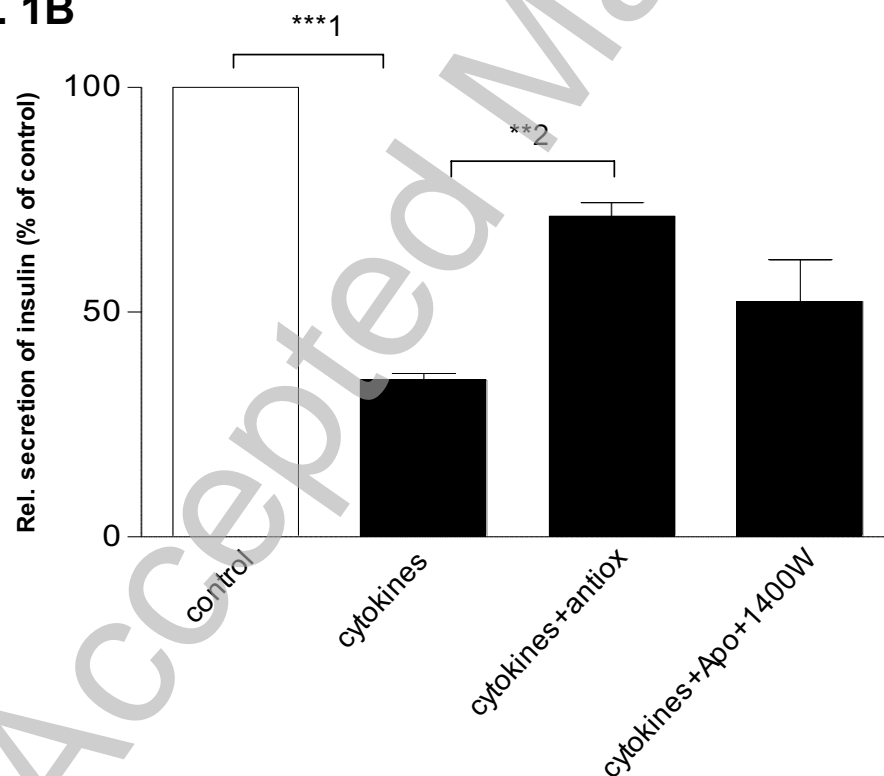


Fig. 2A

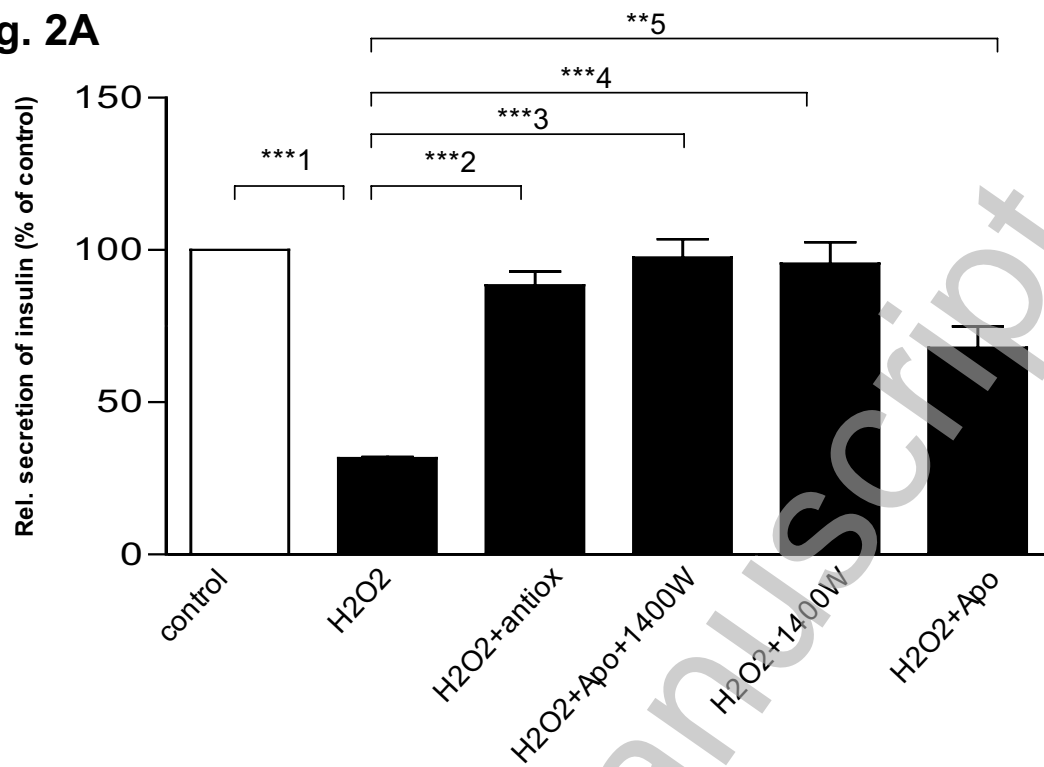


Fig. 2B

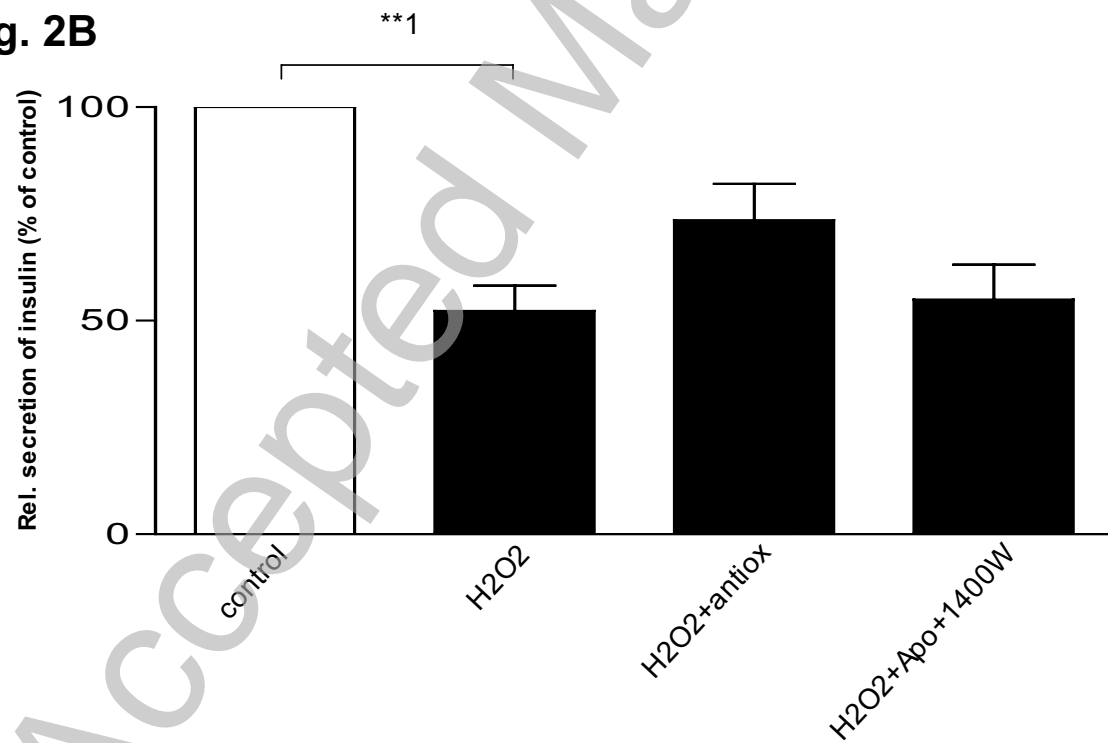


Fig. 3

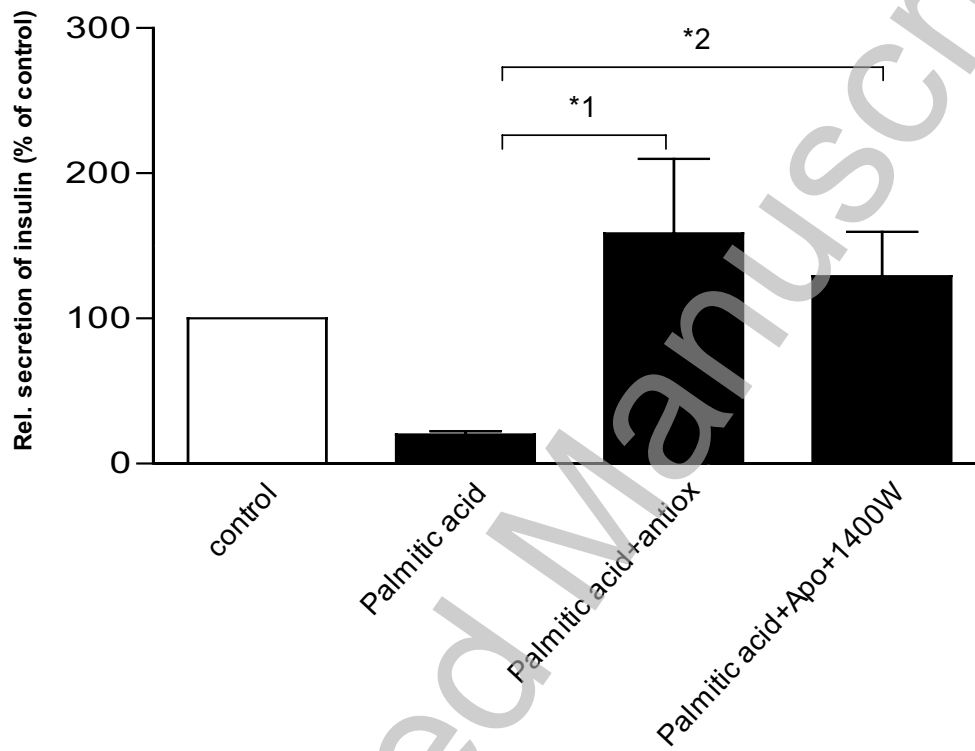


Fig. 4

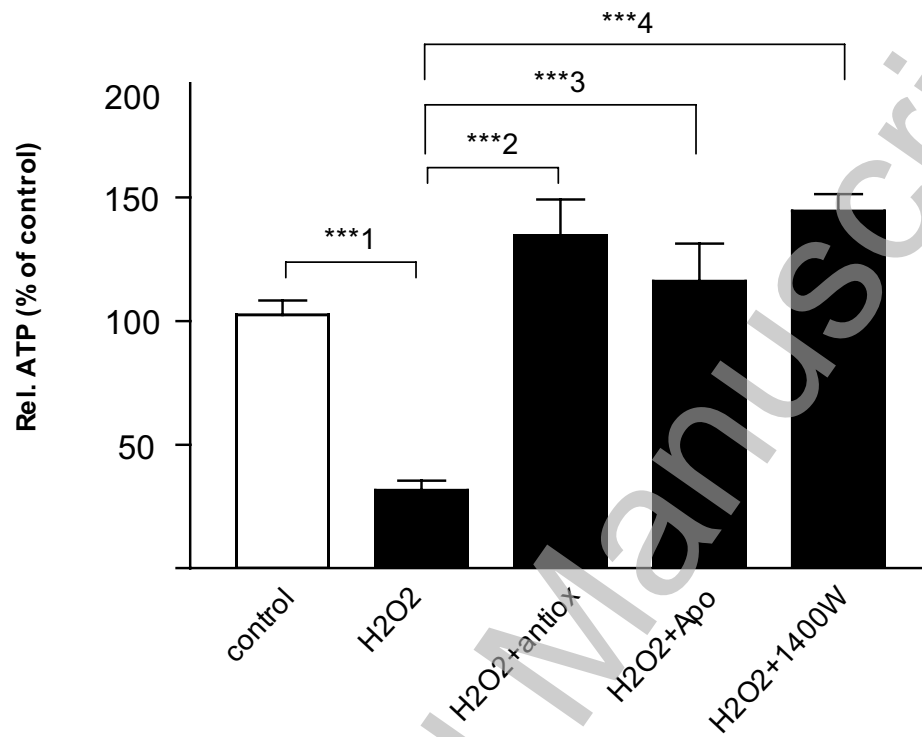


Fig. 5

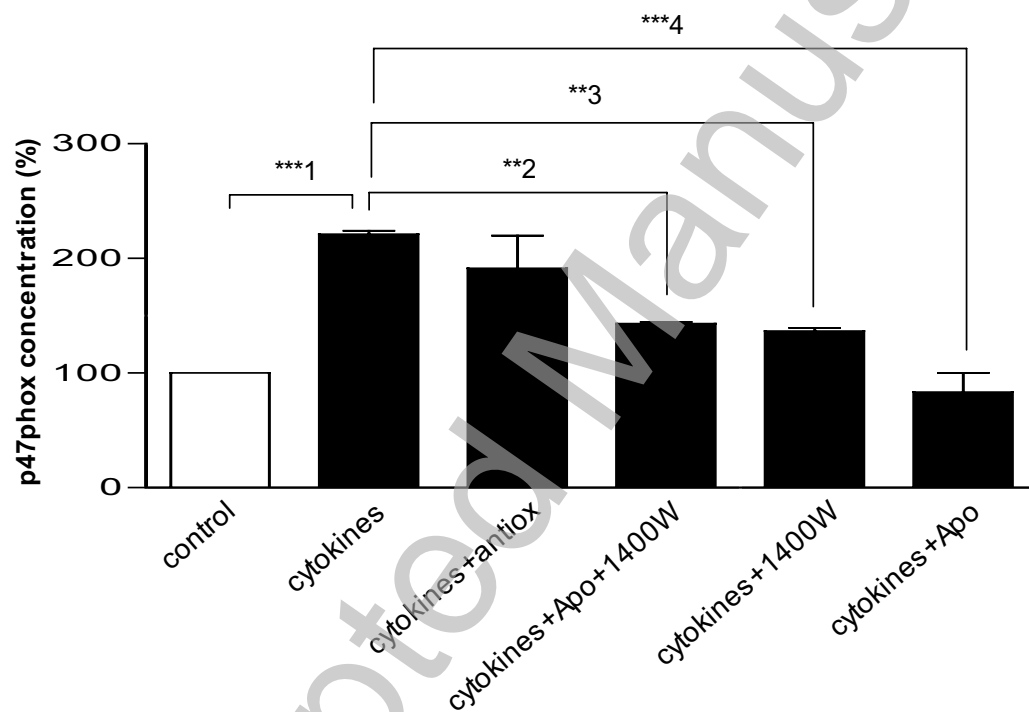
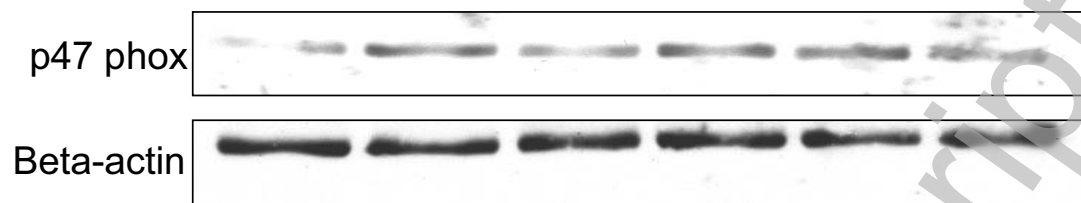


Fig. 6

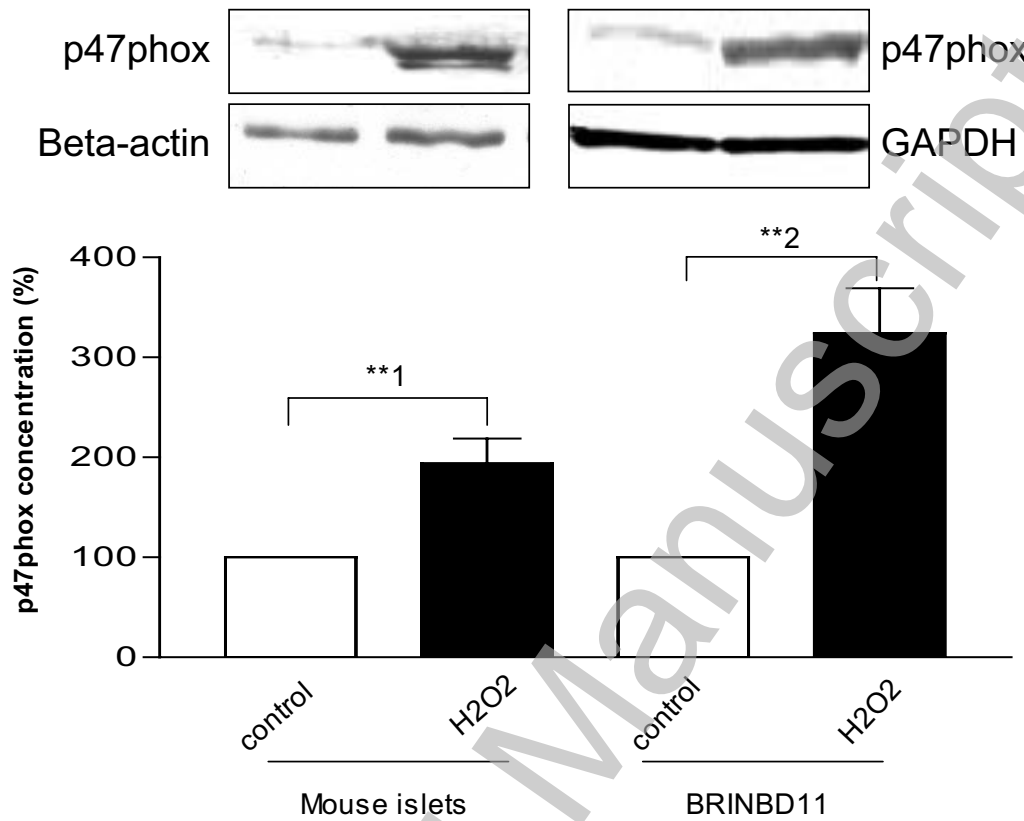


Fig. 7

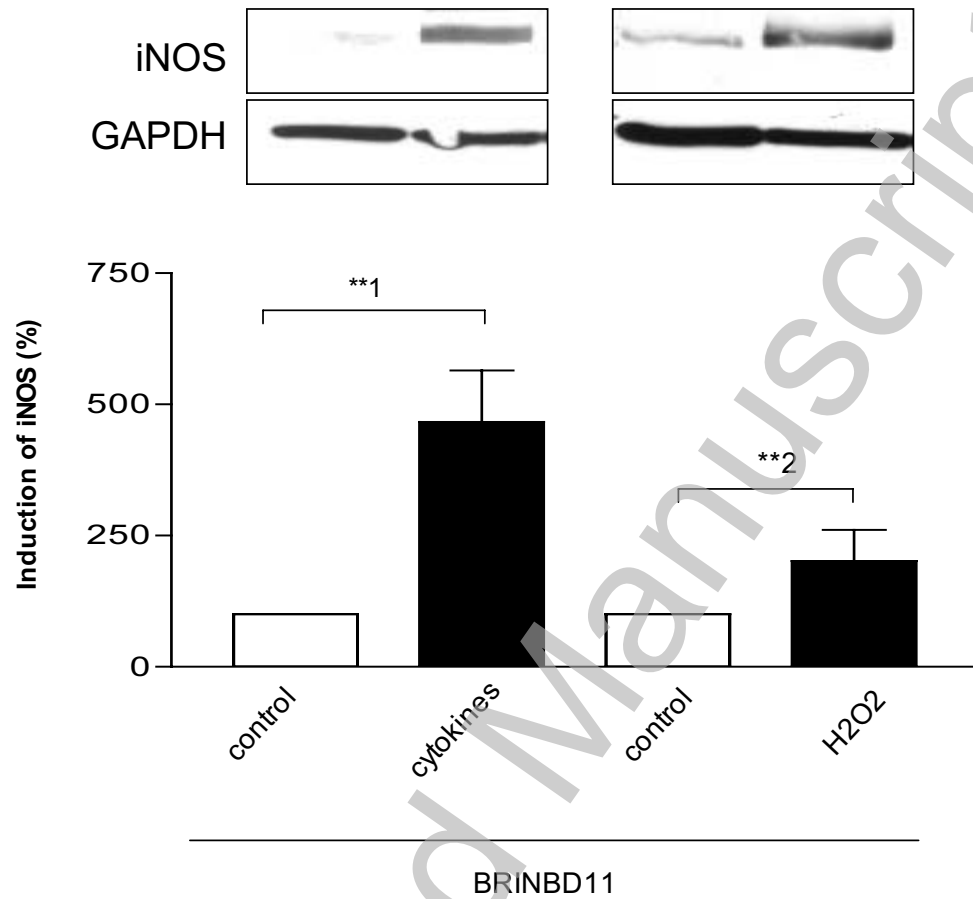


Fig. 8

