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Expression of NADPH oxidase in human pancreatic islets

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ABSTRACT

Aims: NADPH oxidase (NOX) is a known source of superoxide anions in phagocytic and non-phagocytic cells. In this study, the presence of this enzyme in human pancreatic islets and the importance of NADPH oxidase in human β-cell function were investigated.

Main methods and key findings: In isolated human pancreatic islets, the expression of NADPH oxidase components was evidenced by real-time PCR ($p22^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$), Western blotting ($p47^{PHOX}$ and $p67^{PHOX}$) and immunohistochemistry ($p47^{PHOX}$, $p67^{PHOX}$ and $gp91^{PHOX}$). Immunohistochemistry experiments showed co-localization of $p47^{PHOX}$, $p67^{PHOX}$ and $gp91^{PHOX}$ (isoform 2 of NADPH oxidase—NOX2) with insulin secreting cells. Inhibition of NADPH oxidase activity impaired glucose metabolism and glucose-stimulated insulin secretion.

Significance: These findings demonstrate the presence of the main intrinsic components of NADPH oxidase comprising the NOX2 isoform in human pancreatic islets, whose activity also contributes to human β-cell function.

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Introduction

The classical NADPH oxidase (NOX) is a heteromultimeric enzyme which was first described in phagocytes (Bedard and Krause, 2007) composed of the membrane-bound cytochrome b_{558} ($gp91^{PHOX}$ also referred to as NOX2, and $p22^{PHOX}$), three cytosolic components ($p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$) and a low-molecular-weight Rho GTPase (Rac1 or Rac2) (Babior, 2004). The transmembrane NOX2 protein, which is the catalytic core of this enzyme, generates superoxide upon its activation by interaction with the cytosolic $p47^{PHOX}$, $p67^{PHOX}$, $p40^{PHOX}$ subunits, and one of the small Rho GTP-binding proteins (Sorce and Krause, 2009). Recruitment of the cytosolic components to the membrane occurs through protein kinase C (PKC)-mediated $p47^{PHOX}$ serine phosphorylation, in which the serine residues S303, S304, S358 and S370 play a key role for recruitment (Babior, 2004). The interaction of these cytosolic components with cytochrome b_{558} is mediated by its $p22^{PHOX}$ subunit. NOX2 is then activated by $p67^{PHOX}$, assembling the oxidase activity (Sorce and Krause, 2009).

The presence of the NADPH oxidase isoform whose activity is dependent on $p47^{PHOX}$ translocation, i.e. NOX2, and of other NOX isoforms (NOX1 and NOX4) has been reported in rat pancreatic islets (Oliveira et al., 2003; Uchizono et al., 2006). In β-cells NOX2 appeared to be involved in oxidative stress induced by high chronic glucose levels in glucotoxicity (Yuan et al., 2010). Interestingly, although NOX2 deficiency protected against streptozotocin-induced β-cell destruction (Xiang et al., 2010), the suppression of its activity also impaired the mechanisms of glucose-stimulated insulin secretion (GSIS) in rodent islets (Imoto et al., 2008; Morgan et al., 2009). These observations suggest the involvement of NADPH oxidase in the function and dysfunction of pancreatic islets cells, which may depend on the stimulus presented.

Despite the fact that reports on the involvement of NADPH oxidase in the function and dysfunction of rodent pancreatic islets are increasing, there is limited information regarding human islets. A possible implication of NADPH oxidase in human β-cell dysfunction was raised by the report of high $p22^{PHOX}$ mRNA levels in pancreatic islets from type 2 diabetic patients (Marchetti et al., 2004). Protein expression ($gp91^{PHOX}$ and $p47^{PHOX}$) has been recently demonstrated, whereas no changes were observed between normal and diabetic human islets (Syed et al., 2011). However, there is no investigation concerning the presence of the functional NADPH oxidase complex and the possible implications of this enzyme in human β-cell physiology.

In this study, we investigated the presence and participation of NADPH oxidase in human islet function. The components of the NOX2 isoform are expressed in human pancreatic islets and the inhibition of the enzyme activity suppressed glucose metabolism and GSIS. Thus, the presence and activity of this enzyme appears to be significant for human pancreatic β-cell functioning.

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Materials and methods

Isolation and culture of human pancreatic islets

Human pancreas from adult brain-dead donors (mean age 45 ± 3 years, n = 19) were harvested in accordance with Brazilian regulations and the local institutional ethics committee. Pancreatic islets were isolated after ductal distension of the pancreas and digestion of the tissue with Liberase H1 (Roche Diagnostics, Indianapolis, IN) or collagenase NB1 premium grade and neutral protease NB (Serva Electrophoresis GmbH, Heidelberg, Germany), according to the automated method of Ricordi et al. (1988) with modifications (Shapiro et al., 2000). Islet purification was achieved using a continuous Ficoll density gradient in a COBE 2991 cell processor (Gambro, Lakewood, CO). The islet preparations used in this study exhibited 70 ± 4% purity as determined by dithizone staining. Islet cell viability, which was usually greater than 80%, was evaluated using the live/dead ratio fluorescent method, based on the incorporation of either acridine orange (Sigma, St. Louis, MO) by live cells or propidium iodide (Sigma) by dead cells. After isolation and purification, human islets were cultured in CMRL-1066 medium (Mediatech, Inc., Manassas, VA) supplemented with 5.6 mM glucose, 0.5% human serum albumin (HSA), 10 mM nicotinamide, 6 μM vitamin E and antibiotics for at least 24 h at 37 °C and 5% CO_2.

Real-time polymerase chain reaction (real-time PCR)

Total RNA from 48 h-cultured human pancreatic islets and from fresh human peripheral blood mononuclear cells (PBMC) was prepared as previously described (Sogayar et al., 2004) with minor modifications. RNA integrity was electrophoretically verified by both ethydium bromide staining and 260/280 nm absorption ratio > 1.95. cDNA was generated from the RNA samples using SuperScript (Invitrogen, Carlsbad, CA). The primers used for gene amplification by real-time PCR were designed using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA) and fed into a 7300 real-time PCR system thermocycler (Applied Biosystems, Foster City, CA). PCR reactions were quantified using the SYBR® Green dye reagent (Applied Biosystems, Foster City, CA). For each primer, the real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ (Rasmussen et al., 2001). In order to guarantee an mRNA expression level that ensures specific amplification, the PCR reactions were only used for analysis when the CT was less or equal to 30. The following primers were used in this study: human $gp91^{PHOX}$ (F: 5′ GTCGCCTATGGGATAGCTGTTAA 3′; R: 5′GGTAGCGTTCCAGGTTGAAG AA3′), human $p22^{PHOX}$ (F: 5′TCCGGCCTG ATCCTCATC 3′; R: 5′AATGGA GTAGGCACCAAAGTACCA3′), human $p47^{PHOX}$ (F: 5′GCTGGTGGGTCATC AGGAAA3′; R: 5′GCCCTGACTTTTGCAGGTACA3′), human $p67^{PHOX}$ (F:5′ CCTG CAACTACCTTGAACCAGTT3′; R:5′GGACTGCGG AGAGCTTTCC3′), and hypoxanthine phosphoribosyl transferase (HPRT) (F: 5′GAACGTC TTGCTCGAGATGTGA3′; R: 5′TCCAGCAGGTCAGCAAAGAAT3′).

Management of the thermocycler and assessment of the results generated during amplification were performed using the SDS software version 1.3.1 (Applied Biosystems, Foster City, CA). All real-time PCR reactions were performed in triplicate for each independent experiment. The relative expression of target genes was determined in comparison to a reference gene (Livak and Schmittgen, 2001), which was chosen to be a stable and unregulated transcript, the HPRT housekeeping gene transcript.

Western blotting

Eighty micrograms of proteins from 48 h-cultured human islet extracts and forty micrograms of proteins from fresh human neutrophil extracts were solubilized in a sample buffer (60 mM Tris–HCl [pH: 6.8]), 2% sodium dodecyl sulfate (SDS, 10% glycerol, 0.01% bromophenol blue) and subjected to SDS-polyacrylamide gel (7.5%) electrophoresis (PAGE). Proteins were transferred to nitrocellulose membranes, which were blocked and then incubated with polyclonal anti-human antibodies: $p47^{PHOX}$ (07-001; Upstate Biotechnologies, Temecula, CA) and $p67^{PHOX}$ (sc-15342; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA). The proteins were visualized by an enhanced chemiluminescence assay performed according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry

Human pancreatic islets were fixed in 1% paraformaldehyde dissolved in 100 mM phosphate buffer (PB), pH 7.3, for 30 min and cryoprotected in 10% sucrose solution at 4 °C. The islets were frozen in tissue freezing medium (TBS, Durham, NC) and cut (12 μm) in a cryostat. The sections were kept at 37 °C for 30 min in gelatinized slides and stored at −20 °C until use. For the immunohistochemistry assays, the islet sections were blocked for 2 h in a solution containing 5% normal goat serum and 0.3% Triton X-100 in PB. In order to determine the expression of $p47^{PHOX}$, $p67^{PHOX}$ and $gp91^{PHOX}$, antibodies against the respective proteins were used (Upstate Biotechnology, Temecula, CA) in a 1:50 dilution with 0.3% Triton X-100 in PB. Guinea pig insulin antibody (1:200) was used for the double-labeling experiments. The islet sections were incubated with the primary antibodies overnight at room temperature. After several washes, sections were incubated for double- and single-labeling experiments with secondary antibodies conjugated to fluorescein isothiocyanate (FITC; Jackson Labs, West Grove, PA; 1:100), and to tetramethyl rhodamine isothiocyanate (TRITC; Jackson Labs; 1:100) in 0.3% Triton X-100 in PB. After washing, the tissue was mounted using Vecta Shield (Vector Labs, Burlingame, CA). Protein expression and co-localization were analyzed in 0.5 μm optical sections on a confocal microscope (LSM 510, Axiovert 100 M, Carl Zeiss, Jena, Germany). Controls for the experiments consisted of the omission of primary antibodies, with no staining being observed.

Measurement of superoxide production by human islets

Batches of 20 human islets were incubated for 30 min at 37 °C in 500 μL Krebs–Henseleit medium containing 16.7 mM glucose in the absence and in the presence of diphenyleneiodonium (DPI − 10 μM) or polyethylene glycol-conjugated superoxide dismutase (PEG-SOD 100 U/mL). For the experiments using PEG-SOD the islets were pre-incubated for 1 h at 5.6 mM glucose in the absence or presence of 100 U/mL PEG-SOD followed by incubation for 30 min in 16.7 mM glucose also in the absence and in the presence of PEG-SOD (100 U/mL). Hydroethidine (HEt) at a final concentration of 100 μM was added and the islets were incubated for an additional 20 min protected from light at room temperature. After this period, the islets were washed twice with Krebs–Henseleit buffer without glucose and analyzed by confocal microscopy using an excitation wavelength set at 488 nm and an emission wavelength between 560 and 615 nm (LSM 510, Axiovert 100 M, Carl Zeiss). HEt oxidation was quantified by the fluorescence emission intensity corrected for the islet area as previously published (Morgan et al., 2007). The images were acquired using a 40× objective and the same parameters were used for the analysis of all samples in each experiment.

Measurement of [U–^{14}C]-glucose oxidation

Groups of 100 human islets were incubated in 2.4 mL Krebs–Henseleit buffer containing albumin (0.2%), at 37 °C, for 1 h in glass vials containing a filter paper and 400 μL phenylethylamine, diluted to 1:1 v/v in methanol, in a separated compartment. The incubation buffer

contained 0.1 μCi/mL [U–^{14}C]-glucose for 2.8 mM glucose and 0.6 μCi/mL [U–^{14}C]-glucose for 16.7 mM glucose in the absence and in the presence of 10 μM DPI or of 300 μM apocynin. Incubation was stopped by adding 400 μL HCl (10 M) and the vials were shaken for an additional 90 min. The filter paper with phenylethylamine was transferred to a plastic tube containing 1.8 mL biodegradable scintillation liquid and the $^{14}CO_2$ adsorbed was measured in a scintillation counter (Beckman Instruments, Fulerton, CA, USA).

Measurement of glucose-stimulated insulin secretion in the presence of NADPH oxidase inhibitors

Cultured human islets were washed with PBS, resuspended in RPMI 1640 medium without glucose and pre-incubated for 30 min at 37 °C. Triplicate batches of 100 islet equivalents (IEQs) were incubated at 37 °C for 2 h in 1.5 mL RPMI 1640 medium supplemented with 0.5% human serum albumin in the presence of 2.8 mM or 16.7 mM and 20 mM glucose in the absence or presence of one of the following NADPH oxidase inhibitors: 10 μM DPI, and 60 or 300 μM apocynin. This conditioned medium was collected from each well and insulin was measured by electrochemiluminescence assay ELECSYS (Sapin et al., 2001) (Roche Diagnostics, Indianapolis, IN). The limit of detection of this assay is 0.2 μU/mL (1.39 pM). The results were normalized on the basis of the DNA content and presented as the relative value to that in 2.8 mM glucose from five independent experiments (triplicate measurements for each experiment).

Statistical analysis

For the measurements of superoxide content and static insulin secretion a glucose condition was set as the control for the adjustment

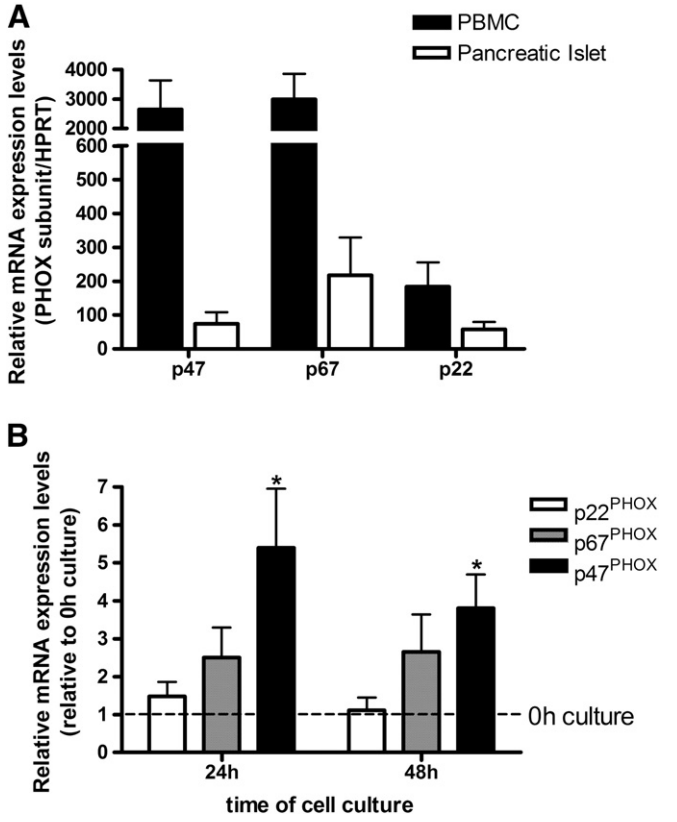

Fig. 1. Gene expression of NADPH oxidase components. A) The mRNA levels of NADPH oxidase subunits were analyzed by real-time PCR using 1 μg of RNA from human peripheral blood mononuclear cells (PBMC) and human pancreatic islets. B) Measurement of mRNA levels of $p22^{PHOX}$; $p67^{PHOX}$ and $p47^{PHOX}$ at different periods of time in culture (24 and 48 h), relative to the 0 h culture (n = 7). *P<0.05 vs 0 h.

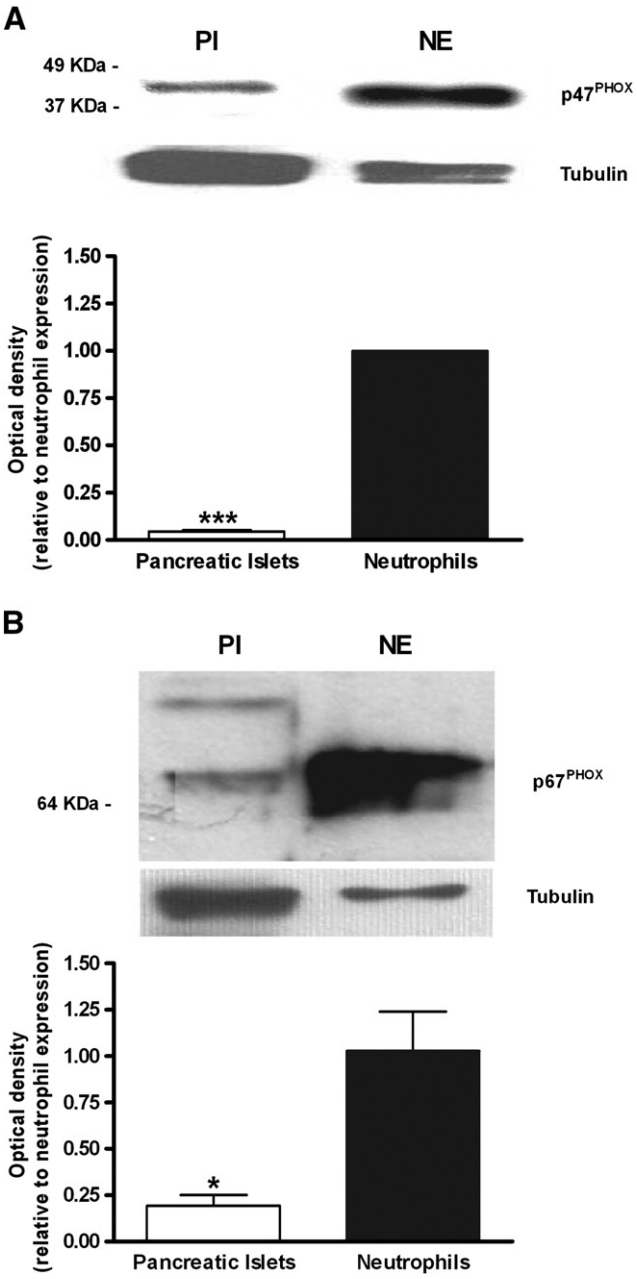

Fig. 2. Protein levels of the NADPH oxidase components. Primary cultures of human pancreatic islets (48 h) were lysed. The protein levels of $p47^{PHOX}$ (A) and $p67^{PHOX}$ (B) were measured by Western blotting. Eighty micrograms of proteins from human pancreatic islets (PI) and forty micrograms of proteins from human neutrophils (NE) were loaded into the gel. Tubulin was used as loading control. The immunoblots are representative of five independent experiments. The densitometry graphs shown are corrected for the different amounts of protein loaded between samples. *P<0.05 and ***P<0.001 vs NE.

of each independent experiment. The results are presented as means ± SEM. Statistical analysis was performed with Student's *t* test or one-way ANOVA followed by Tukey's multiple comparison post-test as appropriate. Differences were considered to be significant for P<0.05.

Results

The gene expression of the NADPH oxidase membrane-bound component $p22^{PHOX}$ and of the cytosolic components $p47^{PHOX}$ and $p67^{PHOX}$ in human pancreatic islets was examined by real time PCR, using human peripheral blood mononuclear cells (PBMC) as positive

control. The mRNA levels of p47^{PHOX}, p67^{PHOX} and p22^{PHOX} were very similar in pancreatic islets (Fig. 1A). The mRNA levels of p22^{PHOX} and p67^{PHOX} in pancreatic islets remained stable during the culture period (Fig. 1B). However, the mRNA levels of p47^{PHOX} were increased after 24 h in culture (Fig. 1B). Therefore, the experiments were performed in human islets cultured for at least 24 h, when the mRNA of p47^{PHOX} attained stable levels. The protein expression of the cytosolic components p47^{PHOX} and p67^{PHOX} of NADPH oxidase was evaluated by Western blotting (Fig. 2A and B). Although the protein amount used for human islet labeling was twice that for human neutrophils (positive control), the content of p47^{PHOX} and p67^{PHOX} protein was much lower in human islets than in human neutrophils. In three of five human pancreatic extracts, p67^{PHOX} was identified as a double band instead of the single one detected in the extract of human neutrophils (Fig. 2B), as previously observed in rat islets (Oliveira et al., 2003). Immunohistochemistry analysis confirmed the presence of p47^{PHOX}, p67^{PHOX} and gp91^{PHOX} proteins in human pancreatic islets, with no fluorescence being observed in the negative control (Fig. 3A). The green-labeling in the upper panel of Fig. 3B also refers to the p47^{PHOX}, p67^{PHOX} and gp91^{PHOX} protein expressions in human pancreatic islets, which are overlaid with insulin labeling shown in red (middle panel). The co-localization of NADPH oxidase components within insulin secreting cells from human pancreatic islets is demonstrated by the yellow color in the lower panel (Fig. 3B).

The NADPH oxidase activity was evaluated by superoxide production, that was decreased by DPI (10 μM), a pharmacological inhibitor of NADPH oxidase (Fig. 4). Hydroethydine (HEt) oxidation by superoxide was evaluated by the addition of membrane-permeable superoxide dismutase (PEG-SOD 100 U/mL), a treatment that decreased HEt oxidation by 50% (Fig. 4). DPI (10 μM) or apocynin (300 μM), an inhibitor of p47^{PHOX} translocation, suppressed glucose metabolism at 16.7 mM glucose by 70% and 62%, respectively (Fig. 5A). Additionally, these inhibitors, DPI (10 μM) and apocynin (60 and 300 μM), also impaired GSIS by 57%, 34% and 52%, respectively, at 16.7 mM glucose; and by 56%, 44% and 52%, respectively, at 20 mM glucose (Fig. 5B). However, no effect on insulin secretion was observed with

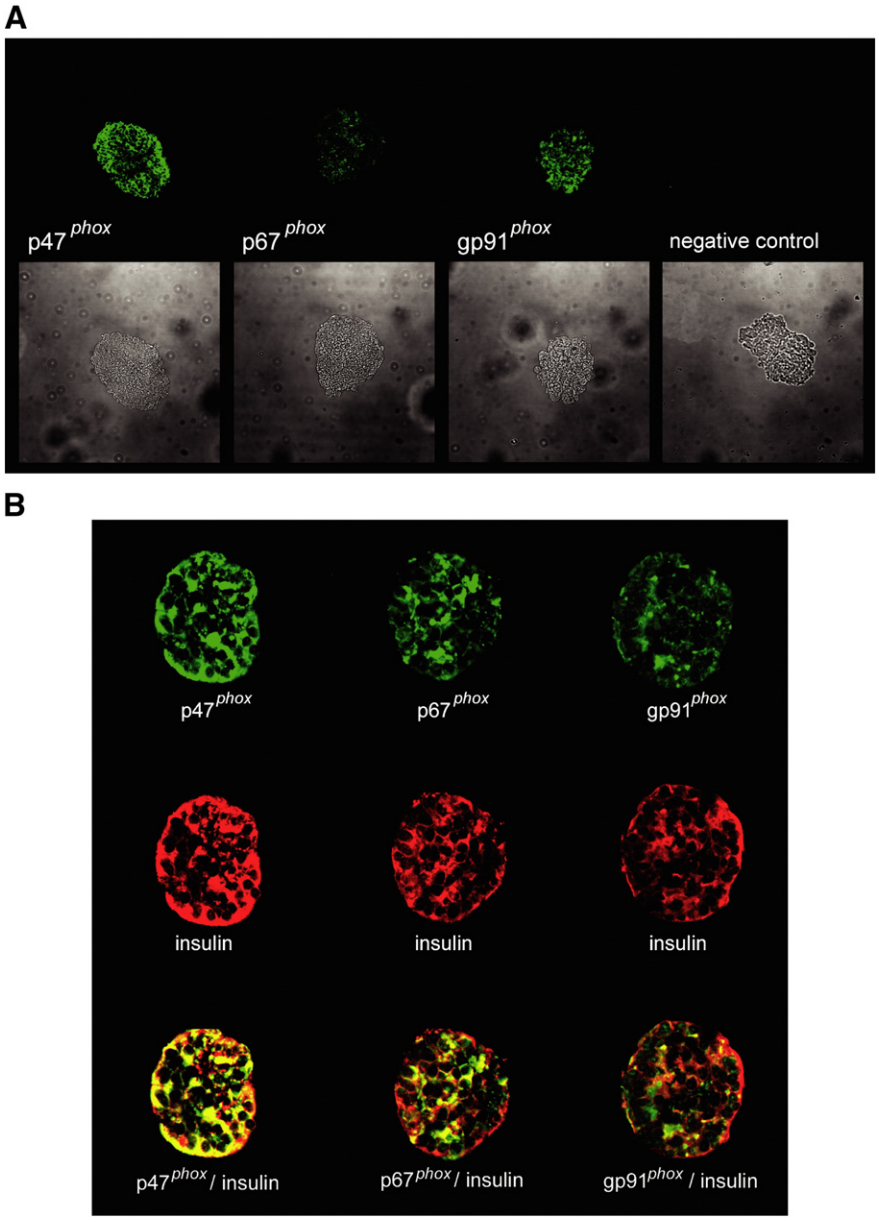

Fig. 3. Immunofluorescence of NADPH oxidase components. A) Fluorescence detection (green) of p47^{PHOX}, p67^{PHOX} and gp91^{PHOX} proteins in 12 μm-thick frozen islet sections (top panel) and corresponding phase contrast images of the islets (lower panel). B) Co-localization (yellow) of NADPH oxidase components p47^{PHOX}, p67^{PHOX} and gp91^{PHOX} (green) with insulin staining (red). The images are representative of six independent experiments.

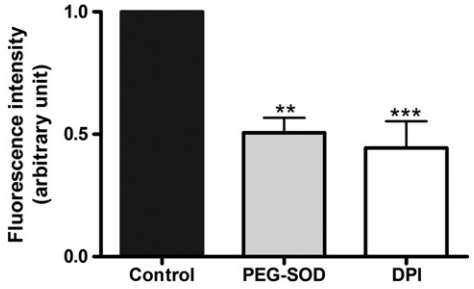

Fig. 4. Blockade of NADPH oxidase activity. The effect of PEG-SOD (100 U/mL) or DPI (10 μM) on superoxide levels in islets (n = 3–5). **P<0.01 and ***P<0.001 vs control.

DPI (10 μM) and apocynin (60 and 300 μM) at 2.8 mM glucose (data not shown).

Discussion

In the present study, the presence of the NADPH oxidase components in human islets was observed at mRNA (Fig. 1) and protein (Figs. 2 and 3) levels. Similar to that observed for rats (Oliveira et al., 2003), a lower protein expression of NADPH oxidase components in pancreatic islets compared to phagocytes would also be expected for humans. In effect, this low expression of NADPH oxidase components in human pancreatic islets was confirmed by the low protein levels of $p47^{PHOX}$ and $p67^{PHOX}$ (Fig. 2A and B). Immunohistochemistry analysis showed the presence of $p47^{PHOX}$, $p67^{PHOX}$ and $gp91^{PHOX}$ (Fig. 3A) in human islets, and the expression of these proteins in insulin-containing cells (Fig. 3B). These observations, together with those from a recent study showing the expression of $gp91^{PHOX}$, $p47^{PHOX}$ and the phosphorylated form of $p47^{PHOX}$ in normal and diabetic human islets (Syed et al., 2011), provide further evidence of the presence of a functional NOX2 isoform, whose activity is driven by $p47^{PHOX}$ phosphorylation that leads to the translocation of the $p47^{PHOX}$/$p67^{PHOX}$ complex to the plasma membrane (Bedard and Krause, 2007). In islets from rodents, $p47^{PHOX}$ phosphorylation is a PKC-dependent process (Oliveira et al., 2003). The anchoring of phosphorylated $p47^{PHOX}$ to the C-terminal cytoplasmic tail of $p22^{PHOX}$ would then allow the subsequent activation of NOX2 through a direct protein–protein interaction with $p67^{PHOX}$ (Bedard and Krause, 2007).

NADPH oxidase was previously reported to have a role in rat pancreatic β-cell function. Impairment of glucose metabolism, intracellular calcium oscillations and GSIS were observed as a consequence of the inhibition of this enzymatic activity (Imoto et al., 2008; Morgan et al., 2009). To evaluate the effects of the inhibition of the enzyme in human beta cells, a pharmacological inhibitor was used (Fig. 4). Considering the non-selectivity of DPI for NOX, another NOX inhibitor, apocynin, was also used. The blockade of enzyme activity by DPI or of the $p47^{PHOX}$ translocation to the plasma membrane by apocynin reduced glucose oxidation (Fig. 5A), concomitantly with the suppression of GSIS (Fig. 5B). Thus, by different approaches we observed similar final effects, suggesting that the activity of this enzyme is involved in GSIS. Nonetheless, the molecular mechanism by which NADPH oxidase activity contributes to islet function is not yet known. However, considering the importance of the redox control for GSIS (Rebelato et al., 2011; Martens et al., 2005) and that even the intracellular level of H_2O_2 acts as negative regulator of glucose metabolism (Rebelato et al., 2011), this effect of NADPH oxidase inhibition on β-cell function could occur via local action in the plasma membrane, as NOX activity has also been implicated in the control of intracellular pH and transmembrane ion fluxes (Bedard and Krause, 2007), or a redox microenvironment in the vicinity of the membrane.

Despite the rising interest in the implications of NADPH oxidase in non-phagocytic cells, the reports were mainly carried out in rodents. The present description of the main components of NADPH oxidase in human islets draws attention to further investigations on its implication also on the mechanisms underlying human β-cell dysfunction.

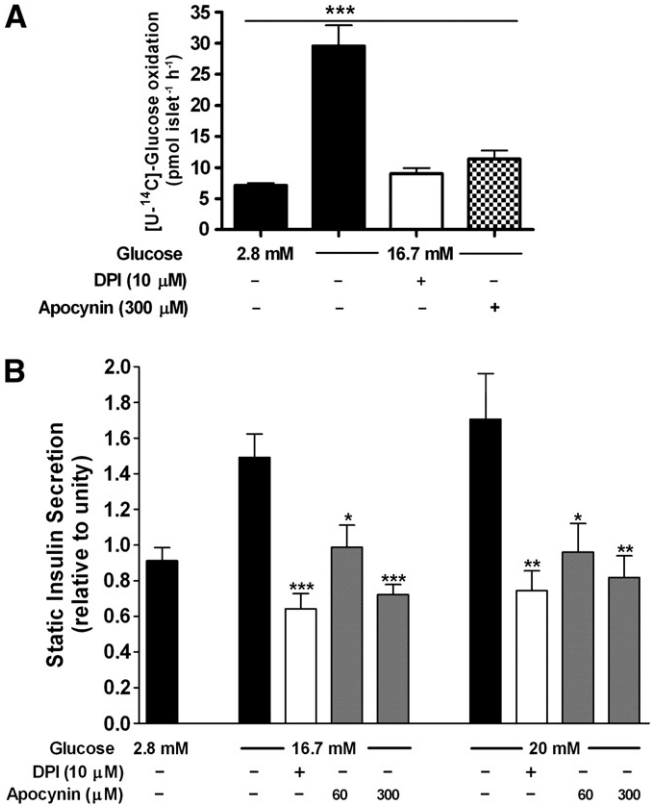

Fig. 5. Involvement of NADPH oxidase activity in glucose oxidation and insulin secretion. A) [U-^{14}C]-Glucose oxidation in islets incubated in the absence or presence of 10 μM DPI and 300 μM apocynin (n = 5). B) Effects of DPI and apocynin on insulin secretion (n = 5). *P<0.05, **P<0.01, and ***P<0.001 vs the respective condition without the inhibitors.

Conclusion

The present study demonstrated the presence of the main intrinsic components of NADPH oxidase comprising the NOX2 isoform in human pancreatic islets. It was also shown here that the activity of NADPH oxidase is significant for glucose metabolism and glucose-stimulated insulin secretion in human β-cells.

Conflict of interest statement

The authors have nothing to declare.

Acknowledgments

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