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LI, Ning, et al.

Abstract

In insulin-secreting cells, expression of NADPH oxidase (NOX), a potent source of ROS, has been reported, along with controversial findings regarding its function. Here, the role of NOXs was investigated: first by expression and cellular localization in mouse and human pancreatic islets, and then by functional studies in islets isolated from Nox isoform-specific knockout mice. Both human and mouse β-cells express NOX, in particular NOX2. With use of Nox isoform-specific knockout mice, functional analysis revealed Nox2 as the predominant isoform. In human islets, NOX2 colocalized with both insulin granules and endosome/lysosome membranes. Nox2-deficient islets stimulated with 22.8 mmol/L glucose exhibited potentiation of insulin release compared with controls, an effect confirmed with in vitro knockdown of Nox2. The enhanced secretory function in Nox2-deficient islets was associated with both lower superoxide levels and elevated cAMP concentrations. In control islets, GLP-1 and other cAMP inducers suppressed glucose-induced ROS production similarly to Nox2 deficiency. Inhibiting cAMP-dependent protein kinase reduced the [...]
NADPH Oxidase NOX2 Defines a New Antagonistic Role for Reactive Oxygen Species and cAMP/PKA in the Regulation of Insulin Secretion

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In insulin-secreting cells, expression of NADPH oxidase (NOX), a potent source of ROS, has been reported, along with controversial findings regarding its function. Here, the role of NOXs was investigated: first by expression and cellular localization in mouse and human pancreatic islets, and then by functional studies in islets isolated from Nox isoform–specific knockout mice. Both human and mouse β-cells express NOX, in particular NOX2. With use of NOX isoform–specific knockout mice, functional analysis revealed Nox2 as the predominant isoform. In human islets, NOX2 colocalized with both insulin granules and endosome/lysosome membranes. NOX2-deficient islets stimulated with 22.8 mmol/L glucose exhibited potentiation of insulin release compared with controls, an effect confirmed with in vitro knockdown of Nox2. The enhanced secretory function in Nox2-deficient islets was associated with both lower superoxide levels and elevated cAMP concentrations. In control islets, GLP-1 and other cAMP inducers suppressed glucose-induced ROS production similarly to Nox2 deficiency. Inhibiting cAMP-dependent protein kinase reduced the secretory response in Nox2-null islets, although not in control islets. This study ascribes a new role for NOX2 in pancreatic β-cells as negative modulator of the secretory response, reducing cAMP/PKA signaling secondary to ROS generation. Results also show reciprocal inhibition between the cAMP/PKA pathway and ROS. Diabetes 61:2842–2850, 2012

NOX enzymes generate superoxide by transferring one electron from NADPH to oxygen (1). The best known NOX isoform is the phagocyte NADPH oxidase (NOX), a multicomponent complex comprising a membrane catalytic heterodimer, the flavocytochrome b558, formed by p40phox (also referred to as NOX2) and p22phox (where phox is phagocyte oxidase). The cytosolic regulatory subunits are composed of p40phox, p47phox, p67phox, and GTPases Rac1 or Rac2 (1). Assembly of cytosolic elements to membrane catalytic core initiates the activation of NOX. To date, seven isoforms of NOX (NOX1–5 and dual oxidases DUOX1–2) have been identified with different activation mechanisms and heterogeneous tissue distribution (1). In addition to microbial attack by professional phagocytes, physiopathological roles of NOX have recently attracted attention in nonphagocytic cells, including pancreatic β-cells (2–7). Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, might participate in β-cell dysfunction (8). The redox imbalance favored by high metabolic rate and a relatively low detoxifying system has contributed to the general concept that β-cells are sensitive to ROS, although they can handle rather high concentrations of H2O2 (9).

NOX family represents one of the potential sources of ROS in insulin-secreting cells (4). Both rat islets and insulinoma express membrane-associated catalytic components Nox1, Nox2, Nox4, and p22phox, as well as cytosolic regulators p40phox, p47phox, and p67phox and their homologs Noxo1 and Noxa1 (3,5,6). Regarding their putative function in the β-cell, NOXs have been implicated in glucose-induced ROS production in MIN-6 cells (10). Knockdown of p47phox results in total inhibition of glucose-stimulated insulin secretion and lowers ROS (11). In animal models of type 2 diabetes, islets exhibit increased NOX components Nox2 and p22phox, correlating with increased oxidative stress (12). Activation of Nox and accompanying ROS generation were demonstrated in Zucker diabetic fatty (ZDF) rat and diabetic human islets (13). However, inhibition of Nox using diphenyleneiodonium (DPI) impairs glucose-stimulated insulin secretion (6) along with blunted glucose-induced superoxide production (5,10). These conflicting findings regarding NOX activity and β-cell function might be attributed to poor specificity of old-generation NOX inhibitors, such as apocynin and DPI (14). The former has been shown to function as a general ROS scavenger, and the latter is a nonspecific inhibitor of electron transporters (1,15).

In the current study, we first investigated relative expression levels of the different catalytic subunits of NOXs in both human and mouse pancreatic islets. Then, subcellular distribution of the identified predominant NOX isoform NOX2 was assessed in human islet cells. For avoidance of poor specificity of NOX inhibitors, islets isolated from Nox isoform–specific–deficient mice were used to investigate the contribution of NOXs in insulin secretory function.

RESEARCH DESIGN AND METHODS

Nox2-deficient mice were originally generated by Pollock et al. (16) and were purchased from The Jackson Laboratory (Bar Harbor, MA). We recently generated knockout mice for Nox1 (17) and Nox4 (18). Overall phenotype of Nox2 knockout (Nox2ko) mice is available at www.jax.org under the reference B6.129S-Clyb<sup>Mm</sup>tb/J. Regarding infection susceptibility, there is no evidence for increased infection rate in Nox1- and Nox4-deficient mice, while...
Nox2-null mice may exhibit immune defects. In order to avoid potential risks of infectious problems, animals were kept under specific pathogen-free conditions (Centre Médical Universitaire-Zootechnie, Geneva, Switzerland) and systematically examined for signs of skin infection or weight loss during the period preceding the experiments. Knockout immunoblotting on extracts of islets were age matched with C57BL/Jl (wild-type [WT]) control mice. The study was approved by the official ethics committee.

Pancreatic islet isolation and β-cell purification. Mouse pancreatic islets were isolated by collagenase digestion and cultured overnight free floating in RPMI-1640 medium before handpicking for experiments as previously described (19). Freshly isolated human islets from three different donors, who had provided written informed consent, were obtained from D. Bosco and T. Berney (Cell Isolation and Transplantation Center, Department of Surgery, Geneva University Hospital). The use of human islets for research was approved by the institutional ethics committee. Human islets were maintained in CMLR-1066 for 24 h before experiments (20). Mouse pancreatic β-cells were purified by fluorescence-activated cell sorting as previously described (21).

Expression analyses by reverse transcriptase-PCR. Total RNA from 600 mouse islets and 1,200 human islets was extracted using the RNeasy microkit with DNase treatment (Qiagen, Hilden, Germany). First-strand cDNA synthesis was performed with 1 μg RNA, 200 units of reverse transcriptase (SuperScript II Reverse Transcriptase; Invitrogen), and 250 ng random primers (Promega, Madison, WI). PCR was performed at 35 and 40 cycles for each component tested in whole islets and at 40 cycles for Nox2 in sorted mouse β-cells. PCR products were detected using a 1% (v/v) agarose gel stained with ethidium bromide. Details of PCR conditions are summarized in Supplementary Table 1 (mouse) and Supplementary Table 2 (human), with GAPDH serving as the housekeeping gene. Quantitative PCR was performed using a StepOnePlus Real-Time PCR system (Applied Biosystems; Life Technologies), and PCR products were quantified fluorometrically using the FastStart Universal SYBR Green Master (ROX; Roche Diagnostics). Negative PCR controls were conducted with RNA without reverse transcriptase (RT) reaction. Details of primers used in quantitative RT-PCR are summarized in Supplementary Table 3. The values obtained were normalized to values of the reference cDNA of cyclophilin.

NOX2 subcellular localization in human islets. Human islets were dispersed at 37°C with 0.05% trypsin-EDTA solution for 15 min with regular pipetting on cooling with ice-cold RPMI-1640 (Sigma-Aldrich) and left for overnight attachment. First, for endosome labeling, islet cells were incubated with 2.5 mg/mL 10 kDa fluorescent fluid-phase marker rhodamine-dextran (Molecular Probes, Eugene, OR) for 15 min at 37°C in culture medium containing 1% FCS and then fixed with 4% para-formaldehyde. For subsequent immunofluorescence, fixed cells were permeabilized with 0.1% Triton X-100 in PBS. Slides were blocked with PBS/0.1% BSA/0.1% Tween-20 for 30 min before incubation for 2 h with monoclonal mouse antibody against human NOX2 (1:250 dilution; kindly provided by D. Roos, University of Amsterdam, Academic Medical Centre), polyclonal rabbit antibody against human lysosome–associated membrane protein (LAMP)-1 (1:150 dilution; Thermo Scientific), and polyclonal porcine antiguinea pig insulin (1:400 dilution; DAKO, Carpinteria, CA) antibodies. Specificity of the LAMP-1 antibody was verified in two independent equal fractions. Each fraction was placed on poly-1-ornithine–precoated 48-well plates followed by transfection with 100 nmol/L scramble oligos or an equal mixture of four small interfering (si)RNA oligos targeting Nox2 (Supplementary Table 4) (ON-TARGETplus SMARTpool siRNA; Thermo Scientific) using 10 μg/mL LipoFectamine 2000 (Invitrogen) in 1% FCS RPMI-1640 medium. After 4 h, the transfection medium was replaced by complete culture medium. The dispersed islet cells were kept in standard culture conditions for 48 h before RNA extraction and insulin secretion assay.

Ca2+ measurement. Cellular Ca2+ changes were monitored as ratiometric measurements of Fura-2 fluorescence. Isolated mouse islets were cultured on poly-l-lysine (Sigma)-treated glass coverslips that were placed in a thermostatic chamber (Harvard Apparatus) before incubation with 2 μmol/L Fura-2/acetoxymethyl ester (AM) for 60 min. After washing, Fura-2 fluorescence of a single islet was monitored with alternate 1:600 scans at 340/380 nm excitation and 510 nm emission using an Axiovert S100 TV through a 40 × 1.3 NA oil immersion objective (Carl Zeiss).

Ilet cAMP content. Islets in groups of 40 were handpicked and preincubated for 1 h at 2.8 mmol/L glucose in KRHBH/BSA before stimulation with 22.8 mmol/L glucose for 1 h. Then, supernatants were removed and islets washed once with ice-cold KRHBH, and ice-cold HCl (100 μL at 0.1 mmol/L) was added to islet pellets followed by 10-min agitation at 4°C. After neutralization with 100 μL 0.1 mmol/L NaOH, islet cAMP content was determined using an ELISA kit (Amersham Biosciences) following the manufacturer’s instructions.

Statistical analysis. Data are presented as means ± SEM. Differences between the NOX knockout and the WT controls were analyzed by one-way ANOVA followed by Turkey or least significant difference posttest with IBM SPSS statistics software (version 19). P ≤ 0.05 was considered statistically significant.

RESULTS

Expression and cellular localization of NOX in human islet cells. Conventional RT-PCR showed that human islet cells express NOX isoforms NOX2, -4, and -5 at the mRNA level (Fig. 1A). Expression was confirmed by quantitative reverse transcriptase-PCR, giving a cycle threshold (Ct) of 28.9 for NOX2 and >30 for other isoforms, compatible with higher NOX2 expression. Immunofluorescence on dispersed insulin measurement and islet pellets were resuspended in acid-ethanol to determine insulin contents by radioimmunoassay (Linco Research, St. Charles, MO).

Superoxide generation. Superoxides were detected by both nitroblue tetrazolium assay using high-performance liquid chromatography (HPLC) and fluorescence (24). For nitroblue tetrazolium assay, yellow water-soluble nitroblue tetrazolium was reduced to dark-blue water-insoluble formazan by superoxide generated in 15 islets/mL KRHBH/BSA buffer with 0.2% nitroblue tetrazolium (23). The samples were incubated for 90 min at 37°C with either basal 2.8 mmol/L or stimulatory 22.8 mmol/L glucose in three independent absence of cAMP inducers as mentioned above. Islets were centrifuged (13,000 rpm, 5 min, 4°C), the supernatant was discarded, and then cells were broken in 2 mol/L KOH and formazan (nitroblue tetrazolium reduced to insoluble) dissolved in DMSO. Absorbance was determined at 620 nm in a microtiter plate reader (Wallac 1420; Perkin Elmer, Courtabeuf, France), and data were expressed as optical density per 15 islets.

Superoxide generation was also monitored using dihydroethidine, which becomes fluorescent upon reaction with superoxides (24). After preincubation for 1 h at 2.8 mmol/L glucose in KRHBH/BSA, groups of 50 islets were stimulated with 22.8 mmol/L glucose for 30 min before exposure to 50 μmol/L dihydroethidine for 30 min. Then, islets were pelleted down, resuspended in methanol, and homogenized. The homogenate was centrifuged at 13,000 rpm for 5 min, and the supernatant was collected for speed vacuum centrifugation (Micro 14 R; Eppendorf, Hamburg, Germany). Then, 100 μL of the supernatant was added to 100 μL of dihydroethidine (Sigma-Aldrich) and left for overnight attachment. The dispersed islet cells were kept in standard culture medium containing 1% FCS and then fixed with 4% paraformaldehyde. For subsequent immunofluorescence, fixed cells were permeabilized with 0.1% Triton X-100 in PBS. Slides were blocked with PBS/0.1% BSA/0.1% Tween-20 for 30 min before incubation for 2 h with monoclonal mouse antibody against human NOX2 (1:250 dilution; kindly provided by D. Roos, University of Amsterdam, Academic Medical Centre), polyclonal rabbit antibody against human lysosome–associated membrane protein (LAMP)-1 (1:150 dilution; Thermo Scientific), and polyclonal porcine antiguinea pig insulin (1:400 dilution; DAKO, Carpinteria, CA) antibodies. Specificity of the LAMP-1 antibody was verified in two independent equal fractions. Each fraction was placed on poly-l-ornithine–precoated 48-well plates followed by transfection with 100 nmol/L scramble oligos or an equal mixture of four small interfering (si)RNA oligos targeting Nox2 (Supplementary Table 4) (ON-TARGETplus SMARTpool siRNA; Thermo Scientific) using 10 μg/mL LipoFectamine 2000 (Invitrogen) in 1% FCS RPMI-1640 medium. After 4 h, the transfection medium was replaced by complete culture medium. The dispersed islet cells were kept in standard culture conditions for 48 h before RNA extraction and insulin secretion assay.

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human islet cells revealed the presence of NOX2 in insulin-positive cells (Fig. 1B–E). Intriguingly, NOX2 labeling displayed colocalization with both insulin granule and endosome-like structures. Regarding the latter, two approaches were used to study this specific subcellular localization. First, rhodamine-conjugated dextran (10 kDa), whose internalization and accumulation have been applied to identify the fluid phase of early endosomal structure (25), revealed vesicles of ~1–2 μm in human β-cells. These dextran-positive structures were recognized by antibody against human NOX2 (Fig. 1B). Consistently, pattern of NOX2-containing vesicles was similar to that of late endosome/lysosome marker LAMP-1 (Fig. 1D). Apart from endosomal/lysosomal localization, NOX2 staining was also found in the periphery of β-cells, overlapping with insulin staining (Fig. 1C and E).

Expression of NOX in mouse pancreatic islets. With the exception that NOX5 is exclusively expressed in tissues of human origin (1), expression profile of catalytic Nox enzymes in mouse islets was similar to human islets, with Nox2 being the only isoform efficiently amplified after 35 PCR cycles (Fig. 2A). Consistently, quantitative real-time RT-PCR analysis on WT islets revealed CT values of 29.8 ± 0.9 for Nox2, 30.5 ± 0.6 for Nox4, and 33.7 ± 0.2 for Nox1 compared with 23.3 ± 1.3 for the housekeeping gene cyclophilin (N = 5 independent experiments). As negative control, CT values with versus without reverse transcriptase showed the strongest difference for Nox2 (Nox2−RT 35.6 ± 0.2, Δ5.8; Nox4 33.0 ± 0.2, Δ2.5; and Nox1 34.6 ± 0.5, Δ0.9), suggesting relatively high expression of Nox2.

FIG. 1. Gene expression of NOX components and NOX2 localization in human islets. A: Expression of catalytic NOX subunits in human islets (upper panel) was detected at the mRNA level by RT-PCR (for each gene, 35/40 cycles in the left and right lanes, respectively). Positive controls (lower panel) were performed on cDNA from human colon carcinoma cell line Caco-2 for NOX1 (#1), human neutrophils for NOX2 (#2), human embryonic kidney (HEK) cells transduced with human NOX3 gene (HEK-NOX3, #3), human microglia cell line clone 3 (HMC3) for NOX4 (#4), and HEK cells transduced with human NOX5 gene (HEK-NOX5, #5). B–E: Cellular localization by immunofluorescence of NOX2 (green) in β-cells from dissociated human islets combined with detection in red of rhodamine-conjugated dextran (Rh-dextran)-labeled early endosome (B) and insulin (C), late endosome and lysosome marker LAMP-1 (D), and insulin (E). Nuclei were stained in blue with DAPI. Fluorescence staining was performed with three independent batches of human pancreatic islet cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (A high-quality digital representation of this figure is available in the online issue.)

FIG. 2. Gene expression of NOX components in mouse islets and purified β-cells. A, B, and C: Expression of catalytic Nox subunits in mouse islets (left panels of A–C) was detected at the mRNA level by RT-PCR (for each gene, 35 and 40 cycles for the left and right lanes, respectively). Positive controls (right panels of A–C) were performed with cDNA from mouse colon for Nox1, Nox1α (homolog of p67phox), and Nox1β (homolog of p47phox); from spleen for Nox2, p22phox, p40phox, and p67phox; and from kidney for Nox4. D: Nox2 expression (40 cycles of PCR) in purified β-cells from two C57BL/6J mice with cDNA from mouse spleen as positive control. Gapdh, glyceraldehyde-3-phosphate dehydrogenase.
and near absence of Nox1. In Nox2-deficient islets, high 32.4 C<sub>T</sub> value was required to get a positive signal, indicative of background unspecific amplification, while no differences in Nox1 and Nox4 expression were recorded, arguing against compensatory mechanisms. The mRNA of p22<sub>phox</sub> and cytosolic elements p40<sub>phox</sub>, p47<sub>phox</sub>, and p67<sub>phox</sub> and their homologs Noxo1 and Noxa1 were all expressed in mouse islets (Fig. 2B and C). Nox2 expression was specific for β-cells, as confirmed using cDNA obtained from β-cells of mouse islets sorted by fluorescence-activated cell sorter (Fig. 2D). Thus, both human and mouse β-cells express NOX, with NOX2 being expressed in both species.

Islet morphology and insulin content of Nox2-deficient mice. No significant differences in the size or organization of islets were observed between WT and Nox2-deficient mice, which exhibited the expected distribution of α and β-cells (Supplementary Fig. 1). Islet insulin contents were similar between WT (25.1 ± 9.8 ng/islet) and Nox2-deficient (22.5 ± 10.3 ng/islet) mice (N = 4), indicating that Nox2 may not be involved in the regulation of insulin synthesis.

Nox2 deficiency reduces ROS generation and potentiates the glucose response. For investigation of the role of Nox enzymes in islet function, glucose-stimulated superoxide generation and insulin secretion were simultaneously measured.
determined in the same islets obtained from WT and Nox1-, Nox2-, and Nox4-deficient mice. In WT islets, nitroblue tetrazolium assay showed that superoxide levels were enhanced 1.9-fold when glucose was raised from 2.8 to 22.8 mmol/L (P < 0.05). In Nox2-deficient islets, superoxide levels at 22.8 mmol/L glucose were reduced by 56% compared with WT (P < 0.05) (Fig. 3A), whereas no significant changes were recorded in islets of Nox1- and Nox4-deficient mice.

Regarding insulin release in the presence of nitroblue tetrazolium, stimulatory 22.8 mmol/L glucose induced a 2.8-fold secretory response compared with basal 2.8 mmol/L glucose (P < 0.01) in WT islets (Fig. 3B). Responses in Nox-deficient islets were 3.5-fold (P < 0.01) for Nox1ko, 2.5-fold (P < 0.01) for Nox4ko, and 4.4-fold for Nox2ko (P < 0.01) mice, with the latter exhibiting significant enhancement of the glucose response compared with WT (+55%, P < 0.05) (Fig. 3B). Superoxide levels and secretory responses pointed to Nox2 as the functionally predominant Nox isofrm in mouse islets. Previous studies using DPI as Nox inhibitor reported almost absence of glucose-induced insulin release (6,11). Combining specific Nox deficiency and 10 μmol/L DPI exposure (based on a dose response of DPI [Supplementary Fig. 2A]), we observed an overall suppression of islet secretory function in WT and all Nox-deficient isofroms (Supplementary Fig. 2B). Thus, data showing that Nox deficiency exhibited opposite effects compared with DPI treatment suggest Nox-independent inhibition of insulin secretion by DPI.

It was intriguing that the presence of nitroblue tetrazolium during secretion assay blunted the glucose response in WT islets (Fig. 3C), indicating inhibitory properties of nitroblue tetrazolium. We therefore reexamened glucose-induced insulin secretion without nitroblue tetrazolium on islets of mice deficient in Nox2 and Nox4, two isoforms being expressed in both human and mouse islets. Compared with WT islets, which exhibited a 9.4-fold secretory response, glucose-stimulated insulin secretion in Nox2- and Nox4-deficient islets was potentiated in the absence of nitroblue tetrazolium (123 and 38% [Fig. 3D and C, respectively]). The glucose response tested without nitroblue tetrazolium confirmed inhibitory properties of Nox2 and uncovered similar, but much more modest, effects of Nox4.

Superoxide levels at 22.8 mmol/L glucose were further evaluated by dihydroethidine-based HPLC technique. Compared with WT, Nox2-deficient islets exhibited 52% less superoxide-specific EOH signal (P < 0.05) (Fig. 3D), in agreement with the nitroblue tetrazolium assay. This effect was also consistent with dihydroethidine-based direct fluorometric assay, showing a 35% reduction of oxidized dihydroethidine in Nox2ko versus control islets (P < 0.01) (Supplementary Fig. 3).

In vivo, basal blood glucose levels of Nox2-deficient mice were comparable with those of control animals. However, in the first minutes of a glucose tolerance test, glycemia was lower in Nox2ko mice compared with that in controls (Fig. 3E). Plasma insulin levels remained similar to those in controls (data not shown), an observation that does not rule out Putative transient increased insulin signaling in Nox2-deficient mice, according to the fast insulin clearance from the circulation (26). At time 15 min and onward, glycemia and insulin levels were similar between groups (Supplementary Fig. 4).

**Knockdown of Nox2 in islet cells by siRNA.** The potentiated insulin release observed in Nox2 knockout islets might be a primary effect of Nox2 deficiency, or alternatively, secondary to developmental or metabolic changes induced by Nox2 deletion. To address this point, we carried out in vitro siRNA-mediated Nox2 silencing in primary mouse β-cells. Efficient knockdown (~93%) of Nox2 in dispersed mouse islet cells (Fig. 4A) resulted in a 2.8-fold secretory response (P < 0.05) compared with 1.7-fold (P < 0.05) insulin secretion in the scramble control condition (P < 0.05) (Fig. 4B). This is consistent with data obtained in islets from Nox2-deficient mice.

**Second messengers in Nox2-deficient islets.** We next addressed at which step Nox2 might interfere with insulin exocytosis by first investigating glucose-induced [Ca2+]i responses, which have been reported to be inhibited by DPI (11). Similar elevations of [Ca2+]i were recorded in WT and Nox2-deficient islets in response to 22.8 mmol/L glucose (Fig. 5A), suggesting alternative signals being implicated in the potentiated secretory response. In this context, cAMP triggered our attention because 1) it potentiates glucose-evoked insulin exocytosis and 2) it inhibits NOXs in neutrophils (27). As expected, in WT islets the cAMP inducers GLP-1 as well as forskolin plus IBMX markedly potentiated glucose-stimulated insulin secretion (Fig. 5B). In Nox2-deficient islets, the enhanced secretory response conferred by the lack of Nox2 was not further elevated by the cAMP inducers, reaching similar levels compared with WT islets treated with the same compounds without additive effects (Fig. 5B). These observations suggest a link between cAMP levels and the redox state of the cell. Indeed, when measured in control WT
islets, the cAMP inducers suppressed glucose-induced ROS production \( (P < 0.05) \) (Fig. 5C), which is an effect similar to Nox2 deficiency (Fig. 3A). In agreement with these data, cellular cAMP concentrations were found to be higher in Nox2-deficient islets compared with WT after glucose stimulation (34.3%, \( P < 0.05 \)) (Fig. 5D). Overall, data indicate a link between cAMP levels and ROS production, with the lack of the ROS-generator Nox2 preserving cAMP levels and in turn enhancing the secretory response. Elevation of cAMP leads to activation of PKA, which phosphorylates and upregulates components of exocytosis machinery. In this context, PKA inhibitor H89 (22) was added during glucose stimulation (Fig. 5E). H89 had no effect on glucose-induced insulin exocytosis in WT control islets, which is in agreement with a previous study (28). However, in Nox2-deficient islets insulin secretion was reduced by H89, indicating a link between PKA and Nox2.

**DISCUSSION**

The data demonstrate that both human and mouse β-cells express NOX catalytic subunits, consistent with previous...
observations in rat islets (6). Among the different NOXs, NOX2 is functionally the predominant isoform. Regarding subcellular localization, NOX enzymes have been found in membranes of various cell organelles, in relationship with tissue function and the associated NOX activity (29–32). In human β-cells, we observed immune-reactive NOX2 in both endosome/lysosome membranes and insulin granules. The association of NOX2 with insulin-secretory vesicles suggests a redox control of exocytosis as previously described (33). Regarding the presence of NOX2 on late endosome/lysosome membranes, it might reveal the coordinated exocytosis followed by endocytosis of insulin granules as described long ago (34). In human β-cells, residence of NOX2 in a population of vesicles containing endosome/lysosome markers suggests a recruitment of NOX2 from exocytosed secretory vesicles.

Activation of NOX complex requires the assembly of different cytosolic factors, including p47phox which serves as stabilizer of the complex (35). In rat islets, silencing p47phox by 50% results in total abrogation of glucose-stimulated insulin secretion (11), suggesting implication of other targets. Function of NOXs in pancreatic β-cells has been addressed in previous studies, mostly by use of chemical inhibitors (DPI or apocynin) in an attempt to inhibit NOX, leading to the conclusion that lowering NOX activity impairs insulin secretion (6,36). Here, mouse islets genetically deficient for Nox isoforms did not show any impairment in glucose-stimulated insulin secretion. On the contrary, Nox2-null islets exhibited enhanced secretory responses, while DPI inhibited insulin release in both WT and Nox-deficient islets. DPI, a flavoenzyme inhibitor, interferes not only with Nox activity but also with other flavin-dependent enzymes, such as those required for mitochondrial electron transport chain activity (37), which is mandatory for β-cell function (38). Accordingly, DPI lowers glucose oxidation and intracellular Ca2+ signals in rat islets (11), an effect correlating with impaired mitochondrial function (2). Overall, data show that use of DPI might be misleading regarding function of NOXs in β-cells.

To avoid potential off-target NOX inhibition, we used isoform-specific–deficient mice, revealing potentiated glucose-stimulated insulin secretion in islets lacking Nox2 and, to a lesser extent, in Nox4-deficient islets. Deletion of the functionally predominant isoform Nox2 did not induce compensatory upregulation of other Nox catalytic isoforms, which maintained low transcript levels. Although low levels of ROS, possibly generated by mitochondria, have been reported to positively correlate with glucose-stimulated insulin secretion (39), present data argue for an inhibitory effect of Nox-derived ROS on the secretory function. In line with our results, Nox2 deficiency attenuates β-cell destruction and preserves islet function in streptozotocin-induced diabetes, partially through the reduction of ROS generation (7). Additionally, Nox2-derived ROS contribute to dysfunction and apoptosis of NIT-1 β-cells induced by free fatty acids, effects counteracted by the suppression of Nox2 (40). In rats fed a high-fat diet, the adaptive elevated insulin release secondary to peripheral insulin resistance is associated with lower levels of Nox and ROS in their islets (41), raising the question of the phenotype of Nox2 knockout on an obese background. In a recent study, Nox4 deficiency was shown to render mice more susceptible to diet-induced obesity, along with higher blood insulin levels (42). This phenotype is compatible with the observed increased insulin release from Nox4 knockout islets, although the increase was much less pronounced than that from Nox2 knockout islets (Fig. 3). The potentiated secretory response in islets isolated from Nox2-deficient mice can be considered a direct consequence of Nox2 deficiency, since acute in vitro knockdown of Nox2 resulted in similar effects (Fig. 4).

Previously, the potential role of NOX in the regulation of glucose-induced insulin secretion has been associated with [Ca2+]i, possibly as a result of impaired mitochondrial metabolism with use of DPI, as discussed above. In contrast, Nox2-deficient islets exhibited normal [Ca2+]i changes indicating alternative signals being implicated. Among them, cAMP is well-known to strongly potentiate the secretory response. Different neurotransmitters and hormones, including glucagon and GLP-1, increase cAMP levels in the β-cell by activating adenylyl cyclase (43). Here, the lack of Nox2 pointed to a cAMP/PAK-dependent pathway because Nox2 deletion mimicked GLP-1 effects in terms of both cAMP rise and potentiation of insulin release. Previously, it was shown that PKA inhibition prevents potentiation of insulin secretion induced by a cAMP-raising agent (28). In the current study, inhibition of PKA completely blocked the fraction of enhanced secretory response associated with Nox2 deficiency. Consequently, and according to the absence of [Ca2+]i changes, implication of PKA-independent mechanisms, such as Epac (44), is unlikely but not impossible. We did not observe additive effects of Nox2 deficiency plus GLP-1 treatment. Therefore, the elevated cAMP levels measured in Nox2-deficient islets, combined with lower ROS, might explain effects on the secretory response, although implication of other signaling molecules cannot be ruled out. On the basis of present and previous data, activation of an adenylyl cyclase/cAMP/PAK pathway seems to be redox responsive. At present, there is no evidence for direct interaction of Nox2 with adenylyl cyclase. However, both Nox2 (45) and adenylyl cyclase (46) have been localized in lipid rafts, suggesting possible cross-talk between these two enzymes. In stimulated neutrophils, cAMP has been proposed as a physiological suppressor of superoxide production (27). Likewise, we observed much lower glucose-induced superoxide production when WT islets were incubated with adenylyl cyclase activators. Thus, such a pathway may exert ROS scavenging properties, possibly through protein kinase activation. Recent studies in vascular experimental systems have highlighted adenylyl cyclase/cAMP/PAK signaling in the regulation of ROS production, showing inverse correlation between cAMP and ROS levels mediated by adenylyl cyclase and PAK (47,48).

Overall, data indicate that in β-cells the adenylyl cyclase/cAMP/PAK pathway modulates redox homeostasis and vice versa, since this pathway is in turn redox sensitive. Indeed, oxidants can regulate adenylyl cyclase, thereby altering cAMP availability (49), and site-specific oxidation of PKA inhibits its catalytic activity (50). In β-cells, lowering of ROS production would preserve adenylyl cyclase activity and cAMP molecules, in turn activating PKA and increasing the secretory response (Fig. 6).

In conclusion, we propose a new role for NOXs in pancreatic β-cells as negative modulators of the secretory response through ROS generation, in turn reducing adenylyl cyclase/cAMP/PAK signaling. Thus, the reduced secretory response associated with ROS production would
be contributed by changes in cAMP levels, the most powerful amplifying signal of insulin exocytosis.

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N.L. conducted experiments, analyzed data, and wrote the manuscript. B.L., T.B., C.D.-D., Z.M., Y.D., and X.-J.M. generated data. K.-H.K. and P.M. supervised the project, analyzed data, and wrote the manuscript. K.-H.K. and P.M. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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