Glucose-Stimulated Insulin Secretion Fundamentally Requires $H_2O_2$ Signaling by NADPH Oxidase 4

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NADPH facilitates glucose-stimulated insulin secretion (GSIS) in pancreatic islets (PIs) of β-cells through an as yet unknown mechanism. We found NADPH oxidase isoform 4 (NOX4) to be the main producer of cytosolic $H_2O_2$, which is essential for GSIS; an increase in ATP alone was insufficient for GSIS. The fast GSIS phase was absent from PIs from NOX4-null, β-cell–specific knockout mice (NOX4ΔKO) (though not from NOX2 knockout mice) and from NOX4-silenced or catalase-overexpressing INS-1E cells. Lentiviral NOX4 overexpression or $H_2O_2$ rescued GSIS in PIs from NOX4ΔKO mice. NOX4 silencing suppressed Ca$^{2+}$ oscillations, and the patch-clamped $K_{ATP}$ channel opened more frequently when glucose was high. Mitochondrial $H_2O_2$, decreasing upon GSIS, provided alternative redox signaling when 2-oxo-isocaproate or fatty acid oxidation formed superoxides through electron-transfer flavoprotein–Q-oxidoreductase. Unlike GSIS, such insulin secretion was blocked with mitochondrial antioxidant SkQ1. Both NOX4 knockout and NOX4ΔKO mice exhibited impaired glucose tolerance and peripheral insulin resistance. Thus, the redox signaling previously suggested to cause β-cells to self-check hypothetically induces insulin resistance when it is absent. In conclusion, increases in ATP and $H_2O_2$ constitute an essential signal that switches on insulin exocytosis for glucose and branched-chain oxoacids as secretagogues (it does so partially for fatty acids). Redox signaling could be impaired by cytosolic antioxidants; hence, those targeting mitochondria should be preferred for clinical applications to treat (pre)diabetes at any stage.

Insulin, which is released from pancreatic β-cells, controls the blood glucose level in healthy individuals, and insulin release is impaired in those with diabetes (1–4). An understanding of the pathophysiology of the insulin release mechanism is indispensable for clinical innovation. The consensus mechanism of glucose sensing in pancreatic β-cells involves the elevation of ATP synthesis by mitochondria upon increased glucose metabolism enabled by the human GLUT1– or rodent GLUT2–mediated glucose uptake and fast glycolysis, which generates pyruvate, leading to ATP production by oxidative phosphorylation (OXPHOS) (1).

The increased ATP-to-ADP ratio in the subplasmalemmal cytosol in β-cells should cause the $K_{ATP}$ channel to close (4–8), depolarizing the plasma membrane and activating voltage-gated L-type Ca$^{2+}$ ($C_{a,L}$) channels. The resulting Ca$^{2+}$ entry stimulates Ca$^{2+}$-dependent exocytosis of the insulin-containing secretory granules (8). Glucose-stimulated insulin secretion (GSIS) is facilitated by an increase in cytosolic NADPH (4.9–12), as various redox shuttles affecting metabolism generate cytosolic NADPH upon glucose intake, at the expense of NADH in the mitochondrial matrix (4,9–12). Also, the first enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PDH), might contribute to the NADPH surplus upon GSIS (13–15). The resulting NADPH may be supplied to NADPH oxidase (NOX) isoforms 1 (NOX1) and 2 (NOX2) (16,17), but only if they are properly assembled. Nevertheless, the only constitutively expressed and assembled isoform is NOX4, and it is the only one that produces $H_2O_2$ directly (18,19). Inhibition of an unidentified NOX isoform was reported to attenuate GSIS when an antisense $p47^{PHOX}$ oligonucleotide (20), the nonspecific NOX (plus complex I) inhibitor diphenyleneiodonium (DPI) (21,22), or an inhibitor of two isoforms (23) was used. Hence, if any NOX isoform participates in GSIS, it must be definitively recognized.

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Only reactive oxygen species (ROS) of mitochondrial origin, such as ROS resulting from the addition of monooctyl-glycerol (23), have been suggested to modulate insulin secretion (24). An effect of antioxidants has been reported, with glutathione being decreased by diethylmaleate in INS-1(823/13) cells and acting as an unspecific link between GSIS and external H₂O₂ (25). However, it has never been considered that elevated ATP is insufficient for GSIS, nor that any redox signaling may be essential for GSIS. Thus, the elevation of the ATP-to-ADP ratio was thought to close K_ATP channels without any additional requirement for parallel redox signaling. A rather weak antioxidant defense and low redox buffer capacity, but high thioredoxin and peroxiredoxin content (26), provide an ideal, delicate ROS homeostasis in β-cells, although this homeostasis might be disturbed by a relatively weak insult and may spread within the cytosol (2,27).

This work revisited a mechanism of GSIS, and this article describes a novel mechanism of NOX4-mediated redox stimulation of insulin secretion, which, together with increased ATP, is essential for GSIS. Moreover, the absence of this redox signaling affects insulin sensitivity in peripheral tissues.

**RESEARCH DESIGN AND METHODS**

**Tests on Mice**

Experiments were approved by the committee of the Institute of Molecular Genetics (Prague, Czech Republic) and complied with the 2010/63/EU directive (U.S. National Institutes of Health publication no. 85-23, revised in 1996) and the guidelines laid out in Animal Research: Reporting of In Vivo Experiments.

NOX4-null (knockout) (NOX4KO) mice were generated through targeted deletion of the translation initiation site and exons 1 and 2 of the gene (Research Resource Identifiers [RRID]: Mouse Genome Informatics [MGI]:4838554) (28,29). Control (NOX4Flox/Flox) mice were obtained by intercrossing NOX4-null progeny with C57BL/6 mice for 10 generations. NOX2-null (knockout) (NOX2KO) mice were from the Jackson Laboratory (Bar Harbor, ME) (B6.129S-Cybbtm1Din/J, RRID:IMSR_JAX:002365). The β-cell-specific NOX4KO (NOX4βKO) mouse was created by crossing NOX4Flox/Flox mice with B6.Cg-Tg(Ins2-cre)25Mgn/J mice (RRID:IMSR_JAX:003573) (30,31). Tail genotyping selected congenic NOX4Flox+/+ Cre+/+ (B6.Cg-Nox4<tm1Ams>Tg[Ins2-cre]25Mgn) mice (NOX4βKO).

NOX4 deletion, specifically in mouse β-cells, was verified (Supplementary Fig. 3A and B). At least 64% of β-cells exhibited the β-knockout alleles (~80% of the β-cell fraction of pancreatic islet [PI] cells). Peripheral insulin resistance was evaluated as described by Alán et al. (32).

An intraperitoneal (i.p.) glucose tolerance test and a parallel insulin assay were performed after the i.p. injection of glucose (1 mg/g body weight; ~111 μmol glucose per mouse) into mice deprived of food overnight. Blood was taken from a blood vessel in the ocular plexus. Tests were also performed with 2-oxoisocaproate (OIC) (1 mg/g body weight; ~153 μmol OIC per mouse). Blood glucose was determined with a glucometer (Roche, Basel, Switzerland); insulin was determined with an ELISA kit (Merodia, Uppsala, Sweden). Samples were taken at two or three time points for both insulin and glucose estimates from each mouse. Mean data from 10–27 mice (sex is specified in the various figure legends) enabled the construction of time dependencies.

**Pancreatic Islet Isolation and Perfusion**

Two male and two female mice with each genotype were anesthetized. The pancreases were digested with collagenase, and the PIs were isolated subsequently on a Ficoll gradient (33). The yield was 100–200 islets per mouse.

Dynamic insulin release from PIs was determined by using perfusion. Approximately 100 islets were placed in a column with a flow adaptor (1 × 7 cm Econo-Column; Bio-Rad, Hercules, CA) attached, and the islets were immobilized with Bio-Gel P4 (Bio-Rad). PIs were washed for 60 min in a continuous flow of glucose-free Krebs-Ringer HEPES (KRH) buffer (135 mmol/L NaCl, 3.6 mmol/L KCl, 10 mmol/L HEPES, 0.5 mmol/L MgCl₂, 1.5 mmol/L CaCl₂, 0.5 NaH₂PO₄, 0.1% BSA [pH 7.4]). Insulin secretion was stimulated with KRH buffer containing stimulatory compounds. The perfusate was collected at rates of 0.5 ± 0.1 mL/min. Insulin was detected with an Insulin Mouse ELISA High Sensitive kit (BioVendor, Brno, Czech Republic). Islets were lysed, and their DNA content was quantified by using a PicoGreen Assay (Thermo Fisher Scientific, Eugene, OR).

**NOX4 Overexpression by Using Lentiviral Particles**

PIs were seeded on laminin-covered 96-well plates (Bio-Lamina, Stockholm, Sweden). NOX4 was overexpressed in PIs isolated from control (NOX4Flox/Flox) and NOX4βKO mice by using 72-h lentiviral transduction with LentioRF particles (OriGene, Rockville, MD) that contain pLenti-C-mGFP as a control, or pLenti-mouse NOX4-C-mGFP for NOX4 overexpression. Insulin release was assayed after 6 min.

**Cell Cultivation and Transfection**

Rat insulinoma INS-1E cells (C0018009; AddexBio, San Diego, CA) (31) were routinely cultivated with 11 mmol/L glucose in RPMI 1640 medium supplemented with 10 mmol/L HEPES, 1 mmol/L pyruvate, 5% (v/v) FCS, 50 μmol/L mercaptoethanol, 50 IU/mL penicillin, and 50 μg/mL streptomycin. The 1- and 15-h incubations were also performed with 3 mmol/L glucose (34).

Cells were transfected twice in 24 h with Oligofectamine (Thermo Fisher Scientific) with two predesigned Ambion siRNAs against rat NOX4 (exons 14 and 10; Thermo Fisher Scientific) in a serum- and antibiotic-free medium. Their 5’ to 3’ sequences were CCGUUUGCAUCGAUUAATT and CAUUAUCGAGUAAUAUCAUTT. Also, different pairs of predesigned Ambion siRNAs against rat SUR1 (Aabc8), G6PDH, and branched-chain α-ketoacid dehydrogenase
EGTA, 4 mmol/L MgCl₂, 2.4 mmol/L CaCl₂ [pH 7.15]) were glucose (130 mmol/L KCl, 20 mmol/L HEPES, 10 mmol/L HEPES, 1.2 mmol/L CaCl₂ [pH 7.2]). Cells were incubated with an isotonic solution (150 mmol/L KCl, 10 mmol/L glutamine) by using a Rat Insulin ELISA Kit (U-E type; Shibayagi Co., Shibukawa, Japan) after a 5-min preincubation phase before glucose (25 mmol/L) was added.

Cell Auxiliary Assays

H₂O₂ monitoring with TCS-SP2 or TCS-SP8 confocal microscopes (Leica) used an overexpressed H₂O₂-selective fluorescent protein, either Hyper-C (35) or heat shock protein–fluorescence resonance energy transfer (HSP-FRET) (36). A sample chamber (37°C) was supplied with 5% CO₂. INS-1E cells were transfected with an X-tremeGene DNA reagent (Roche), and the coexpression of NOX4 siRNAs was facilitated by using Lipofectamine 2000 in a serum-free and antibiotic-free medium for 24 h. An ATP Bioluminescence Assay kit HS II (Roche) was used to quantify ATP.

Monitoring of Cell K⁺ and Ca²⁺ Influx

A FluxOR Potassium Channel Assay (Thermo Fisher Scientific) was used to assay Ti⁺ influx rates. INS-1E cells were preloaded with a “stimulus buffer” containing Ti⁺. The emission of FluxOR was monitored at 525 nm on an RFS301 spectrofluorometer (Shimadzu). Fluorescent Ca²⁺ in the cell cytosol was monitored with Fura-2.

Cell Patch Clamp Recording

Patch pipettes made of borosilicate glass (Harvard Apparatus, Kent, U.K.), with a resistance of 15–20 mol/LΩ, were filled with an isotonic solution (150 mmol/L KCl, 10 mmol/L HEPES, 1.2 mmol/L CaCl₂ [pH 7.2]). Cells were incubated in a solution without glucose (134 mmol/L NaCl, 6 mmol/L KCl, 10 mmol/L HEPES, 1.2 mmol/L MgCl₂, 1.2 mmol/L CaCl₂ [pH 7.2]) for 2 h. Single-channel currents with no glucose (130 mmol/L KCl, 20 mmol/L HEPES, 10 mmol/L EGTA, 4 mmol/L MgCl₂, 2.4 mmol/L CaCl₂ [pH 7.15]) were recorded in the cell-attached mode with an EPC-7 amplifier (HEKA Electronics, Lambrecht, Germany); these currents were low-pass filtered at a corner frequency of 0.5 kHz and sampled at a frequency of 2.5 kHz by using Clampex software version 9.2. Analyses were done in Clampfit software version 9.2 (Axon Instruments, Foster City, CA).

Statistical Analysis

Biological (N) and experimental (n) replicates are listed. ANOVA (t test) used the Tukey and Holm-Sidak tests (for P values, see the Supplementary Material) on the prevalidated data (normality test). Calculations were performed in SigmaStat software version 3.1 (Systat Software, San Jose, CA).

Data and Resource Availability

Data are available from the corresponding author upon request.

RESULTS

GSIS Suppression in NOX4-Silenced INS-1E Cells

To evaluate the effects of NOX4-produced H₂O₂, two NOX4 siRNAs were validated (Supplementary Fig. 1A); these siRNAs were always tested in combination in INS-1E cells. When stimulated with 25 mmol/L glucose, a linear, steady release of insulin (2.9 ± 0.8 ng · min⁻¹ · 10⁻⁶ cells [N = 10]) was observed in nontransgenic (ntg) cells (scrambled siRNA), whereas rates were 4% ± 3% without glucose (positive and negative glucose differences represent the rate of GSIS). In NOX4-silenced cells, GSIS rates were 16% ± 12% (N = 10) (Fig. 1A and B); they were 30% ± 20% after preincubation with 3 mmol/L glucose for 15 h (N = 5) (Fig. 1B). NOX activity (evaluated with the nitroblue tetrazolium assay; Fig. 1C) and the amount of NOX4 in protein (Fig. 1D) were predominantly reduced in silenced cells. GSIS rates (N = 3) were inhibited to 10% ± 8% with stigmatellin (which blocks mitochondrial respiration and hence ATP synthesis), to 8% ± 5% with DPI (which nonspecifically inhibits NOX), and to 38% ± 20% with 6-aminonicotin-amide (which blocks the pentose phosphate pathway).

Transfections with SUR1 siRNAs, disassembling K₄ATP (37), decreased GSIS rates to 20% ± 1% (N = 3) (Fig. 1A and Supplementary Fig. 1B). Silencing G6PDH (Supplementary Fig. 2C), thereby diminishing NADPH, reduced GSIS by ~50% (N = 3) (Fig. 1E). Catalase overexpression (Fig. 1F and Supplementary Fig. 1F) and the NOX4-selective inhibitor GKT-137831 (Fig. 1G) almost completely blocked GSIS (N = 3). Besides the ntg cells’ and NOX4-silenced cells’ equal responses to KCl, the ability of NOX4-silenced cells to release insulin was demonstrated by using glibenclamide to close K₄ATP channels, which restored insulin secretion (N = 3) (Fig. 1G).

Next we demonstrated that NOX4 predominantly participates in accelerated cytosolic ROS/H₂O₂ release upon GSIS. Rates and levels of ROS release in the cytosol were significantly less elevated in NOX4-silenced INS-1E cells (Fig. 2A–H). Independent of NOX4 absence, mitochondrial ROS declined (Fig. 2F), whereas cytosolic ATP was elevated (Fig. 1H).

NOX4 Is Essential for the Initial GSIS Phase In Vivo

To confirm that NOX4 is essential for GSIS in vivo, NOX4KO mice (Fig. 3A) and NOX4KO mice (Fig. 3B) were studied. (For genotype verification and RIP-Cre effects (30,31), see Supplementary Figs. 3 and 4.) Mice (12 weeks old) were starved overnight, blood was collected from the ocular plexus, and then an i.p. glucose tolerance test was performed using that blood, while also assaying for secreted insulin. Samples from two or three time points for each mouse (Supplementary Fig. 5) allowed for constructions of
average time dependencies. In both NOX4KO mice (N = 34 time courses) and NOX4βKO mice (N = 37 time courses), the initial fast phase of insulin release into the blood circulation was abolished; this was not the case in age-matched controls: the "backcrossed" mice (N = 19 time courses) (Fig. 3A) and NOX4Flox/Flox mice (N = 30 time courses) (Fig. 3B), respectively. Concomitant glycaemia was reduced more slowly in knockout mice, manifesting impaired glucose tolerance (IGT) (Fig. 3C, D, K, and L and Supplementary Figs. 6 and 7). Substantial insulin was released upon stimulation with glibenclamide without glucose in both knockout mouse strains (four time courses each, accompanied by glycaemia, which was probably elevated because of stress; Fig. 3E–H) and upon glucose stimulation in NOX2KO mice (N = 3 time courses; N = 5 time courses for backcrossed controls) (Fig. 3I and J).

Perfusion of PIs (the typically predominant β-cell population; Supplementary Fig. 8) isolated from knockout mice reflected profoundly diminished GSIS in its first fast phase (N = 5) (Fig. 4A–D), sensitive to GKT-137831 (N = 3) (Fig. 4D). However, significant responses to glibenclamide (N = 3) were found when it was added alone (Fig. 4E and F) and with glucose (Fig. 4C and D). Glucose-induced release of ROS in cytosol was attenuated in NOX4-deficient PIs (N = 3) (Fig. 4I–K). Insulin release was not suppressed when induced by fatty acids with 5 mmol/L glucose (N = 2) (Fig. 4G and H and Supplementary Fig. 9), because the majority of fatty acid-stimulated insulin secretion (FASIS) is dependent on GPR40 and independent from KₐTP (2,4,27) (Fig. 6C, d a r k r e d ) .

To investigate the possibility of restoring GSIS, mouse NOX4 was overexpressed in PIs by using lentiviral transduction (N = 3) (Fig. 4I). This overexpression rescued GSIS at the 6th minute in NOX4KO PIs, whereas in the control cells (NOX4Flox/Flox PIs), it increased GSIS by 1.4-fold (Fig. 4L). GSIS could also be rescued in PIs from knockout mice by perifusing with H₂O₂ for 10 min (Fig. 4M and N). In conclusion, our data show NOX4-mediated H₂O₂ release to be an essential comediator of GSIS, in parallel with ATP (Fig. 4O), and thus both must be elevated for GSIS to occur (Fig. 8A).

(A, purple), G6PDH-silenced INS-1E cells (E, blue), and cells with overexpressed catalase (F, dark red). B: **P < 0.05 (ANOVA, Tukey test) for 2nd vs. 3rd bar; ***P < 0.001 (ANOVA, Tukey test) for all combinations except for 2nd vs. 3rd bar. (For the Holm-Sidak test, see the Supplementary Appendix). C: NOX4 enzyme activity assayed by a nitroblue tetrazolium reduction (19) in ntg cells (black bars) and NOX4-silenced INS-1E cells (red dashed bars) normalized to the mean NOX4 activity in control ntg cells and in "scrambled" cells, respectively, cultivated in 11 mmol/L Glc (N = 3). D: Western blot illustrating the reduction of NOX4 protein. NOX4-silenced cells ("si NOX4") were transfected with two NOX4 siRNAs (see RESEARCH DESIGN AND METHODS) by using Oligofectamine. The bar graph indicates the estimated percentage of NOX4 silencing from the normalized integral density, quantified by using ImageJ software. H: Total glucose-induced ATP increase (N = 3) (color coding as above).

Figure 1 — GSIS suppression in INS-1E cells upon NOX4 silencing. A, E, F, and G: Typical time courses of insulin release in INS-1E cells after glucose (Glc) was added to a final concentration of 25 mmol/L; without glucose (quantified in E); without glucose but with 1 μmol/L glibenclamide (G, green); with 30 mmol/L KCl (G, gray); or after preincubation with the NOX4-selective inhibitor GKT-137831 (15 μmol/L) before the addition of glucose (G, magenta). B: Time courses were quantified for standard cultivation (11 mmol/L Glc; N = 10 time courses) and for preincubation with 3 mmol/L Glc for 15 h (N = 5 time courses). Also tested were SUR1-silenced INS-1E cells
NOX4 Ablation Induces Insulin Resistance

In addition to IGT, both NOX4KO strains developed insulin resistance, which was evaluated as insulin-dependent 14C-glucose uptake into glycogen in the diaphragm (Fig. 3K and Supplementary Fig. 10A and B) and into lipids in epididymal adipose tissue (N = 7) (Fig. 3L and Supplementary Fig. 10C).

The initial stress signal originating from β-cells lacking an ability to check redox identity (38) can be predicted regarding the source of such insulin resistance caused by the ablation of a single gene (Fig. 8B and C).

K<sub>ATP</sub> Channel Status Upon NOX4 Silencing

Patch clamp recordings in the cell-attached mode elucidated single-channel currents through the K<sub>ATP</sub> channel (Fig. 5A–D). INS-1E cells were depleted of glucose in modified glucose-free Ringer solution for 2 h to increase the probability that the K<sub>ATP</sub> channel would be open in membrane patches attached to ntg cells (Fig. 5A). Whereas in ntg cells glucose addition caused significant K<sub>ATP</sub> channel closure (that is, reducing the probability that the channel is entirely open [NP<sub>a</sub>]), the K<sub>ATP</sub> channel was only partially closed in NOX4-silenced cells (Fig. 5C and D). The fully open state (L3) of 73 pS contrasted with the partially open K<sub>ATP</sub> channel states (L2 and L1). In ntg cells, there was a profound shift from the larger (L3, L2) to smaller (L1) states, whereas in the NOX4-silenced cells, the shift to smaller states was much less pronounced (Fig. 5E–G).
Figure 3 — GSIS suppression, IGT, and peripheral insulin resistance (IR) in NOX4KO mice (orange) and NOX4\(\text{b}\)KO mice (red). Insulin released to blood circulation (A, B, E, F, I) and glycemia (C, D, G, H, J) were both evaluated in blood from the ocular plexus after i.p. injection of a glucose bolus (1 mg/g body weight) in mice that had been deprived of food overnight (wild-type backcrossed mice [9 males, 10 females] [black] or NOX4KO mice [20 males, 14 females] (A and C) and NOX4\(\text{Flox/Flox}\) (16 males, 14 females) (black) or NOX4\(\text{b}\)KO mice (19 males, 18 females) (B and D). E–H: Glibenclamide (5 ng/g body weight) without glucose was also injected i.p. (12 males and 12 females for each strain). I and J: Likewise, the corresponding tests were performed in NOX2KO mice (two males, one female; green) and mice backcrossed from them (three males, two females; black). Areas under the curve (AUCs) constructed from means (or the mean ± SD for controls) of insulin release are 38–53% for NOX4KO vs. wild-type backcrossed mice (138–162% for glycemia) and 12–25% for NOX4\(\text{b}\)KO mice vs. NOX4\(\text{Flox/Flox}\) mice (141–184% for glycemia). After glibenclamide was administered, AUCs were 126–97% (up to the mean ± SD for knockout mice) for NOX4KO vs. wild-type backcrossed mice (79–135% for glycemia) and 43–81% (up to the mean ± SD for knockout mice) for NOX4\(\text{b}\)KO mice vs. NOX4\(\text{Flox/Flox}\) mice (634–670% for glycemia). GSIS in NOX2KO mice (green) exhibited an AUC that was 89–93% of that for the corresponding backcrossed mice (108–105% for glycemia). Samples from two or three time points from each mouse were used to estimate insulin and glycemia. Time dependencies were constructed from numerous groups of mice (comprising different littermates, both males and females; 19–37 mice were in each group; 3 for NOX2KO, 5 for its backcrossed mice), covering all the required time points. The different littermates are indicated with different symbols. This setup represents the only way to perform fast sampling of insulin release by using the most sensitive insulin ELISA kit (Mercodia).\(^*\) 

For notable \(P\) values, see the Supplementary Appendix.) K and L: Peripheral IR indicated by inhibited \(^{14}\)C-glucose uptake into the glycogen of the diaphragm (K) and lipids of epidydimal adipose tissue (L). \(^{**}P < 0.001,\ ANOVA (Tukey test) (N = 7; always male mice). (For original data, see Supplementary Fig. 10.) Note that the Holm-Sidak test yielded \(P\) values of \(2.3 \times 10^{-7}\) and \(2.5 \times 10^{-10}\) in \(K\) and values of \(3.3 \times 10^{-5}\) and \(0.0036\) in \(L\).
Figure 4—GSIS suppression in PIs isolated from NOX4KO and NOX4βKO mice, NOX4-dependent H₂O₂ cytosolic release, and rescue of GSIS with Nox4 overexpression or H₂O₂. Colors of bars and symbols are as presented in Fig. 3. A–H: Perfusion of PIs. Mean time courses (N = 3 isolations each from two males and two females, or the typical course from a single isolation) of insulin release in PIs (preincubated in 2.5 mmol/L glucose) with initial additions of 25 mmol/L (A–D) or 5 mmol/L (G and H) glucose; in some cases, 25 μmol/L palmitic acid and 25 μmol/L oleic acid were coinjected within a medium containing 2 mmol/L glutamine (G and H). When indicated, the NOX4-selective inhibitor GKT-137831 (5 μmol/L; magenta) was added to control PIs, or 100 μmol/L glibenclamide was added. The marks at 60 min indicate the levels when 30 mmol/L KCl was added at the end of the perfusate. Until 18 min, areas under the curve (AUCs) were 38% (85% with fatty acids) in PIs from NOX4KO mice and 12% (70% with fatty acids) in PIs from NOX4βKO mice. AUCs obtained from the data were related between PIs from NOX4KO mice and those from NOX4βKO mice, as follows: 38% until 18 min (A), 12% until 18 min (B), 31% until 14 min (74% for glibenclamide phase) (C), 18% until 14 min (73% for glibenclamide phase) (D), 113% (E), 55% (F), 85% (G), and 96% (H); *P < 0.1, **P < 0.05, ***P < 0.001, ANOVA (Tukey test) (N = 3; n = 5–7). With the Holm-Sidak test, notable P values varied. In A, P < 0.001 at 4 min, P = 0.006 at 6 min, P = 0.011 at 8 min, and P = 0.047 at 10 min; in B, P < 0.001 at 4, 6, and 8 min; P = 0.003 at 10 min; and P = 0.009 at 12 or 14 min. In D, P = 0.001 at 4 or 6 min and P = 0.004 at 8 min (otherwise see the Supplementary Appendix). J–K: Cytosolic ROS release monitored in PIs with DCF, as described in Fig. 2. Both exemplar traces (I and J) and quantification of rates (K) are shown. ***P < 0.001, ANOVA (Tukey test) (n = 10). L: Rescue experiment in PIs. Lentiviral NOX4 overexpression (N = 3; blue) in PIs of NOX4βKO and NOX4βFlx/Flox mice (NOX4β-/- [dashed bar]) vs. in “empty” green fluorescent protein reporter vector expression (GFP). Obtained statistics: magenta (yellow) points. ***P = 0.007, ANOVA (Tukey test). For the Holm-Sidak test, notable values were P = 0.0016, GFP vs. NOX4βKO NOX, and P = 0.00032, NOX4βKO vs. NOX4βFlx/Flox rescue. The identity of values was indicated by P = 0.321 for NOX4β-/- and NOX4βKO rescue, and P = 0.222 for GFP and NOX4βKO rescue. (Otherwise, see the Supplementary Appendix.) M and N: Rescue with H₂O₂ in PIs from NOX4KO mice. Rescue was evaluated as in A and B, but the perfusate contained 100 μmol/L H₂O₂ for the first 10 min after glucose was added. Typical
Calcium Channel Status Upon NOX4 Silencing

Nimodipine, inhibiting the Ca\textsubscript{L} channel, decreased the rate of GSIS to 12% ± 11% (Fig. 6C). In ntg-INS-1E cells, oscillations of nimodipine-sensitive Ca\textsuperscript{2+} influx reflected the opening of the Ca\textsubscript{L} channel after glucose was set to 25 mmol/L \((N = 5)\) (Fig. 6H–M). In NOX4-silenced INS-1E cells, the rates of nimodipine-sensitive Ca\textsuperscript{2+} influx fell to 19% ± 3%, and the cells exhibited fewer Ca\textsuperscript{2+} oscillations (Fig. 6K and L). The first oscillation peaked (after a delay) after glucose was added (Fig. 6M). Typically, cromakalim prevented Ca\textsuperscript{2+} influx in controls and blocked the remaining Ca\textsuperscript{2+} oscillations in NOX4-silenced INS-1E cells (Fig. 6H–J). Hence, the K\textsubscript{ATP} channel was indeed affected by the NOX4 deficiency, whereas the Ca\textsubscript{L} channel was affected only indirectly. Rates of oligomycin-sensitive Ca\textsuperscript{2+} influx remained at 22% without the addition of glucose.

2-Oxoacid Metabolism Supplies ATP and Mitochondrial H\textsubscript{2}O\textsubscript{2} to Stimulate Insulin Secretion

When stimulated by OIC, a well-described distinct secretagogue, the secretion of insulin was highly inhibited by the mitochondrial matrix–targeted antioxidant SkQ1 in ntg cells, but it was preserved in NOX4-silenced INS-1E cells (\(N = 3\)) (Fig. 6G and Fig. 7A–C and L) and in NOX4KO mice (\(N = 35\) time courses); it was, however, partially abolished in NOX4\textsuperscript{4KO} mice (\(N = 35\) time courses) (Fig. 7E and F). The same pattern was found in PIs (typical

The holding potential was 0 mV at similar K\textsuperscript{+} concentrations on both sides of the membrane. Hence, the K\textsubscript{ATP} current was driven by a resting potential of −60 mV, resulting in inward currents (downward deflections). It was possible to inhibit all three K\textsubscript{ATP} open states (L1–L3) with glibenclamide. The single-channel conductance of the fully open state (L3) did not change by adding glucose or silencing NOX4 (Fig. 5G). The fully open state (L3) in the ntg cells started to close immediately after the addition of glucose. This closure was completed during the 3rd minute of continuous recording. The NOX4-silenced cells exhibited an increased probability of each K\textsubscript{ATP} state being open \((P_o)\), even without glucose. With glucose, the fully open state (L3) remained open similar to L2 and L1 in the 3rd minute.

Cromakalim or diazoxide, which open K\textsubscript{ATP} channels, were ineffective in redox (H\textsubscript{2}O\textsubscript{2})-stimulated insulin secretion (RSIS) \((N = 11)\) (Fig. 6A and B); these vasodilators decreased the insulin release (GSIS) rate to 6% ± 5% and 13% ± 5%, respectively (Fig. 6C). Diazoxide neither activated nor inhibited residual GSIS at silenced NOX4.

Probing K\textsubscript{ATP} activity, defined as the rate of glibenclamide-sensitive Ti\textsuperscript{+} influx (Ti\textsuperscript{+} being a surrogate for K\textsuperscript{+}) into ntg-INS-1E cells, we found that the Ti\textsuperscript{+} influx was inhibited to ∼20%, indicating a characteristic closure of the K\textsubscript{ATP} channel with 25 mmol/L glucose \((N = 4)\) (Fig. 6D and G). Predominant K\textsubscript{ATP} participation was verified by the rate acceleration that manifested upon application of the K\textsubscript{ATP} channel openers cromakalim and diazoxide, and inhibition of that acceleration by glibenclamide (to 10% ± 13%). However, NOX4-silenced or catalase-overexpressing INS-1E cells did not respond to glucose and still exhibited 70–95% of the maximum rates of glibenclamide-sensitive Ti\textsuperscript{+} influx.

A requirement for both NOX4-produced H\textsubscript{2}O\textsubscript{2} and a higher ATP-to-ADP ratio (OXPHOS) was also suggested by the effects of oligomycin, which left ∼50% of the K\textsubscript{ATP} channel open in control cells but up to 95% of it open in NOX4-silenced cells (Fig. 6D), with a concomitant blockage of GSIS (Fig. 6C). The SUR1-silenced INS-1E cells exhibited largely suppressed Ti\textsuperscript{+} influx rates \((N = 3)\) (Fig. 6E), a result of the incorrect assembly of the remaining Kir6.2, which lacks the SUR1 partner (34). This result is consistent with inhibited GSIS (Fig. 1A and Fig. 6B).

Next, fluorescence monitoring with a plasma membrane potential indicator demonstrated a profound reduction of depolarization in the plasma membrane of ntg cells after the addition of glucose but not in NOX4-silenced or catalase-overexpressing INS-1E cells \((N = 2)\) (Fig. 6F).

Runs are shown \((N = 3)\). Gray traces represent only glucose, without H\textsubscript{2}O\textsubscript{2}. M and N: AUCs of H\textsubscript{2}O\textsubscript{2} rescue in PIs from NOX4\textsuperscript{4KO} (NOX4\textsuperscript{4KO}) mice vs. H\textsubscript{2}O\textsubscript{2} plus glucose or only glucose in wild-type backcrossed (NOX4\textsuperscript{lox/lox}Flx/Flox) mice were 107% or 157% until 10 min (M) and 66% or 90% until 10 min (N). O: Glucose-induced increase in total ATP in PIs. Values were normalized to the initial levels at 3 mmol/L glucose.
results from N = 3 isolations are shown in Fig. 7I and J). OIC administration to mice increased glucose above fasting levels (Fig. 7G and H).

Note that GSIS itself was not sensitive to SkQ1 (Fig. 7K). With OIC, only a negligible fraction of H$_2$O$_2$ comes from NOX4; this H$_2$O$_2$ is supplied with NADPH by the basal activities of redox shuttles (otherwise activated by glucose). In contrast, a source of mitochondrial superoxide (transformed to H$_2$O$_2$) is essential for OIC-stimulated insulin secretion, as inferred from its nearly complete inhibition with SkQ1, which was paralleled by a decreasing release of superoxide in the matrix (N = 3) (Fig. 7C and D).

Mitochondrial H$_2$O$_2$ originates from a superoxide converted from manganese-dependent superoxide dismutase; this superoxide arises at the E$_{5}$ site of electron-transferring flavoprotein:Q-oxidoreductase (ETF:QOR) (40) upon the oxidation of isovaleryl-CoA. Isovaleryl-CoA is produced from OIC by the BCKDH complex. Indeed, BCKDH E1α silencing led to ~70% suppression of OIC-stimulated insulin release (N = 4) (Fig. 7B and C and Supplementary Fig. 1E), and it prevented the accompanying redox signal (Fig. 7D). Aminoxyacetate, an aminotransferase inhibitor upstream of BCKDH, did not affect the OIC-induced release of superoxide in the matrix (Fig. 7D), but it did slightly inhibit OIC-stimulated insulin secretion (Fig. 7B and C). Thus, OIC metabolism in INS-1E cells provides both ATP (from OXPHOS) and H$_2$O$_2$ (Fig. 7M) to stimulate insulin release.

In conclusion, the essential requirement of redox signaling for insulin secretion in vivo can be provided either by NOX4 (GSIS) or by mitochondrial sources (Fig. 8A and C).

**DISCUSSION**

**H$_2$O$_2$ Signaling Is Essential for GSIS**

We found that the elevation of ATP (ATP-to-ADP ratio) alone is insufficient for the fast GSIS phase in pancreatic channel, the program used $N_{\text{P}} = T_{L} (T_{L} + T_{D})$, where $N$ is the minimum number of channels, and $T_{L} = \sum T_{L}$, where $L$ is the number of openings. For comparison, $N_{\text{P}}$ values were normalized to the $N_{\text{P}}$ value for ntg cells, and the different $P_{\text{O}}$ values were normalized to $T_{L}$ under those same conditions. The resting potential of the ntg cells was -70 mV before and -40 mV after glucose was added. This demonstrated the depolarization of the plasma membrane by glucose in ntg cells. All the open states were, again, completely closed by glibenclamide. Once the glucose was applied, the excision of the patch to inside-out mode allowed K$_{\text{ATP}}$ activity to recover. The recovered K$_{\text{ATP}}$ activity was inhibited after the addition of glibenclamide. $G$: L1–L3 contribution to total $P_{\text{O}}$. Glucose reduced the percentage contribution of L3 and L2 states to the benefit of L1, and it did so strongly in ntg cells. In F, ANOVA (N = 5; n = 5–7) yielded notable $P$ values according to the Holm–Sidak test: $P = 0.08$ (0.31 for Tukey test) for ntg vs. siRNA NOX4, indicating equal initial $N_{\text{P}}$ values before glucose was added; and $P = 0.00028$ (**$P = 0.002$, Tukey test), indicating significantly high $N_{\text{P}}$ values after glucose was added in siRNA NOX4 samples. For ntg cells before and after glucose, $P = 6.4 \times 10^{-6}$ indicates significance.

![Figure 5](https://example.com/f5.png)


Figure 6 — GSIS or RSIS vs. the status of the KATP channel in INS-1E cells. A-C: Normalized rates of GSIS or RSIS (H2O2) for the indicated agents (C). The RSIS time courses after 98 μmol/L H2O2 (with or without 25 mmol/L glucose [Glc] added at the beginning) (A); related quantifications of insulin released after 60 min (B). In B, **P < 0.05, ANOVA (Tukey test; n = 7). In C, ***P = 0.001 (N = 3; n = 5–7) relative to GSIS in respective ntg controls. (For the Holm-Sidak test see the Supplementary Appendix.) D, E, and G: KATP status as reflected by Ti⁺ influx into ntg control (black bars), NOX4-silenced (NOX4 siRNA; red bars), and catalase-overexpressing INS-1E cells, with 25 mmol/L Glc added or after direct addition of H2O2 (98 and 196 μmol/L, as indicated), without or with 50 μmol/L diazoxide (white bars), 1.3 μg/mL oligomycin (H2O2Olig.), or only oligomycin (Olig.); or with 15 mmol/L OIC or 20 μmol/L palmitic acid (G). ***P < 0.001, ANOVA (Tukey test) (N = 3–5; n = 10; n = 5 with reagents; related to GSIS in D). (For the Holm-Sidak test see the Supplementary Appendix.) F: Plasma membrane depolarization, as monitored with PPMP 5 min after 25 mmol/L Glc was added; values were normalized to those in ntg control cells. H2O2 after glc, 25 mmol/L Glc is already present; H2O2 before glc, addition of H2O2 alone; values were normalized to those in the Supplementary Appendix.) H–M: GSIS or RSIS vs. the status of the KATP channel as the Target of H2O2 Signaling Upon GSIS. The KATP channel has been suggested to be regulated by redox in vascular smooth muscle cells (43), but this has not yet been suggested in pancreatic β-cells. Plausible proposals related to a Ca²⁺-induced (44,45) or H2O2-induced exocytosis of insulin granules suggested the activation of depolarizing TRPM2 channels (46,47). Our results excluded β-cells. Parallel redox signaling must also occur. Coupling of glucose stimulus and secretion requires H2O2-mediated redox signaling that originates from NOX4. The essential part played by H2O2 in facilitating KATP-dependent insulin exocytosis (Fig. 8A) calls for an extensive revision of the GSIS mechanism (1–4,1,27,41). Our findings stimulate a re-thinking of the origins of impaired insulin secretion in pancreatic β-cells and of strategies for treating type 2 diabetes. Essential dual signaling by the increased ATP-to-ADP ratio around the plasma membrane and elevated H2O2 is required to close the KATP channel and induce glucose- and OIC-stimulated insulin secretion. Such dual signaling allows fine-tuned regulation of insulin release. We demonstrated that the main target of redox signaling is KATP, but currently we cannot exclude parallel participation of redox-sensitive kinase signaling. Redox signaling might also target KATP via peroxiredoxins (26).

Consequently, not only a cell’s metabolic status (reflected by the increased ATP-to-ADP ratio) but also its redox status, determined by existing rates of H2O2 release into the cytosol, is linked to insulin exocytosis. Constitutively expressed and assembled NOX4 provides such an H2O2 branch of the mechanism of GSIS stimulation. G6PDH allows the glycolytic flux to increasingly branch out into the pentose phosphate shuttle (42).

KATP Channel as the Target of H2O2 Signaling Upon GSIS

The KATP channel has been suggested to be regulated by redox in vascular smooth muscle cells (43), but this has not yet been suggested in pancreatic β-cells. Plausible proposals related to a Ca²⁺-induced (44,45) or H2O2-induced exocytosis of insulin granules suggested the activation of depolarizing TRPM2 channels (46,47). Our results excluded
Figure 7 — NOX4-independent, oxoacid-stimulated insulin secretion requires a mitochondrial redox burst plus ATP. Throughout these graphs, NOX4-silenced INS-1E cells or NOX4KO mice are indicated with the color red; NOX4KO mice, orange; backcrossed mice as the control for NOX4KO mice, black; NOX4Flox/Flox mice as the control for NOX4KO mice, gray; BCKDH-silenced INS-1E cells, cyan; 500 nmol/L SkQ1, yellow. A and B: Insulin secretion responding to 15 mmol/L OIC in INS-1E cells, with or without 1 nmol/L SkQ1 or 4 mmol/L aminooxyacetate (AOA).

C: Quantification of the rate of insulin secretion. ***P < 0.001 vs. ntg GSIS values, ANOVA (Tukey test) (N = 4, n = 5–7). For the Holm-Sidak test, see the Supplementary Appendix.)

E–J: Insulin in the blood (E and F) and glycemia (G and H) after i.p. injection of 1 mg OIC/g body weight (154 μmol per mouse), or in PIs perfused with 15 mmol/L OIC (2.5 mmol/L glucose); for simplicity, a typical example is shown from N = 3 time courses/isolations for each situation (I and J). Areas under the curves (AUC) constructed from means of insulin release were 106% until 15 min for NOX4KO vs. wild-type backcrossed mice in E (177% for glycemia in G), and 58–77% (between means, up to between the mean ± SD) for NOX4KoKO mice vs. NOX4Flox/Flox mice in F (232% for glycemia in H). For PIs, AUCs were related to proper control: 112% until 10 min and 60% until 60 min (I) or 47% until 60 min (J). Here, 15 male and 20 female NOX4KO mice and 15 male and 20 female backcrossed mice were used, as were 57 male and 32 female NOX4KO mice and 25 male and 36 female NOX4Flox/Flox mice (though data from only 35 are displayed). **P < 0.05, ***P < 0.001, Student t test (n = 8–15 vs. n = 8–15 data pairs for NOX4KO mice and n = 13–21 vs. n = 13–21 data pairs for NOX4KO mice at each time point). The different littermate groups are indicated by the different symbols. For notable P values, see the Supplementary Appendix.

D: Superoxide release into the matrix with OIC (see L), normalized to rates of superoxide release in cells preincubated with 3 mmol/L glucose with nothing else added (Jm). Note that the addition of 25 mmol/L glucose causes the Jm rate to decrease (Fig. 2f), whereas the ETF:QOR contribution of OIC metabolism, which is dependent on BCKDH, causes the superoxide release to be sensitive to the mitochondrial matrix antioxidant SkQ1. ***P < 0.001 vs. ntg GSIS values yielded, ANOVA (Tukey test) (N > 3; n = 5–7). For the Holm-Sidak test, see the Supplementary Appendix.)

K: Typical insulin release with 1 nmol/L SkQ1 (yellow) after the addition of 25 mmol/L glucose. L: The chemical structure of OIC. M: OIC-stimulated insulin secretion, which is predominantly independent from NOX4 and uses ROS generated by mitochondrial ETF:QOR. BCAT, branched-chain α-ketoacid amino transferase; IVD, isovaleryl-CoA dehydrogenase; MCC, methylcrotonyl-CoA carboxylase; MGCoAH, methyl-glutocyan-CoA hydratase; HMGCoAL, 3-hydroxy-3-methylglutaryl-CoA lyase.
the Ca\textsuperscript{2+}\textsuperscript{-independent, H\textsubscript{2}O\textsubscript{2}}\textsuperscript{-induced exocytosis of insulin granules. Hence, if a TRPM2-dependent mechanism exists, it contributes only marginally to GSIS. We demonstrated that increases in only ATP or H\textsubscript{2}O\textsubscript{2} cannot close the K\textsubscript{ATP} channel and initiate the membrane potential events that lead to Ca\textsubscript{L} activation and subsequent insulin exocytosis.

**Other Secretagogues as Sources for Redox Signaling**

Another mode of redox signaling, coming from mitochondria, was found for the ketoacid OIC, a leucine metabolite. Also, intramitochondrial redox signaling was reported previously for FASIS (4,39). In both cases, H\textsubscript{2}O\textsubscript{2} is produced by manganese-dependent superoxide dismutase from the superoxide that is probably formed by ETF:QOR (40) upon OIC oxidation or \(\beta\)-oxidation (Fig. 8A). Branched-chain \(\alpha\)-ketoacid dehydrogenase (BCKDH) can also be a source of superoxide (40) (Fig. 7M). Mitochondria also contribute to a portion of the increase of cytosolic NADPH through thoroughly described redox shuttles (4,9). Their operation is reflected by the insulin release that remains when a partial blockage of GSIS occurs upon G6PDH silencing.

However, the GPR40-stimulated pathway or the glycerol/fatty acid cycle (whereby monoacylglycerol activates the exocytosis-promoting protein Munc13-1), which might be independent of K\textsubscript{ATP}, could predominate in FASIS (3,4,48,49). Upon FASIS, the H\textsubscript{2}O\textsubscript{2} supplied by ETF:QOR/superoxide dismutase simultaneously activates the redox-activated phospholipase iPLA\textsubscript{2}\textgreek{y}, cleaving both saturated and unsaturated fatty acids from mitochondrial phospholipids (27,39). GPR40 stimulation and the resulting K\textsubscript{ATP}\textsuperscript{-independent insulin secretion are subsequently amplified by these mitochondrial free fatty acids migrating to the plasma membrane (27,39). A minor portion of FASIS is stimulated via the K\textsubscript{ATP} channel as a result of increased OXPHOS by fatty acid \(\beta\)-oxidation, which also provides H\textsubscript{2}O\textsubscript{2} (Fig. 8A). When fatty acids and glucose are metabolized in \(\beta\)-cells to various extents, the impact of glucose is diminished as GPR40 stimulation increases.

**Consequences for Diabetes Etiology and Treatment**

The IGT/insulin resistance phenotype of NOX4KO and NOX4\textsuperscript{\beta}KO mice might have translational potential,
as these two strains may represent models of early (pre)diabetes. Even though the human growth hormone minigene (transferred from RIP-Cre mice) (30,31) amplifies the insufficiency of GSIS in NOX4KO mice (more profoundly on a systemic level) (Supplementary Fig. 4), the extensive inhibition is caused by a lack of NOX4 activity.

Surprisingly, ablation of just a single gene generates the onset of insulin resistance. We can speculate that pancreatic β-cells must emit an as yet unknown stress signal, either directly or via the immune system (Fig. 8B and C), thereby inducing peripheral insulin resistance (Fig. 3K and L). We hypothesize that such a putative stress signal is induced by the insufficient identity checking or autocrine self-maintenance of β-cells in NOX4KO or NOX4KO mice. β-Cell identity checking can be mediated by the same redox signaling that acts upon KATP-dependent insulin exocytosis (38). However, Swisa et al. (38) did not know the source of such redox signaling. Here, we suggest that its source is H2O2 produced by NOX4 upon GSIS. This may hypothetically contribute to the "correct" β-cell identity-checking signal, which also primarily maintains sufficient insulin gene expression (38). For NOX4KO mice, diet over time stimulates insulin release via FASIS and OIC or other secretagogues; hence, these secretagogues might be sufficient. However, because the GSIS/NOX4-mediated redox signaling is impaired, such mice lack the "correct" NOX4-induced β-cell identity-checking signal. We speculate that the lack of this signal evokes an as yet unknown stress signal for the periphery (Fig. 8B and C).

Because antioxidant defense is diminished in pancreatic β-cells, the NOX4-produced H2O2 during GSIS in vivo, which can be repeated, could be gradually transformed into oxidative stress, reflecting high β-cell vulnerability. This could potentially contribute to diabetes (50). In light of our findings, cytosol-targeted antioxidant therapy, which should inevitably suppress GSIS, seems to be irrelevant in the early stages of diabetes. Tuning down the essential release of H2O2 during GSIS would amplify symptoms of prediabetes instead of preventing them. In contrast, we predict that mitochondria-targeted antioxidants would not harm physiological redox signaling (except that of o xoacids) and might avoid the premature oxidative stress in the matrix at the prediabetes stage. Also, the described repeating H2O2 burst upon GSIS might add to the oxidative stress resulting from the attack of macrophages recruited to the pancreas.

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