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and 1% BSA (fraction V, Sigma) containing the different glucose concentrations and/or pharmacological agents 1 mmol/l tetroxol, 100 μmol/l rotenone, 20 μmol/l antimycin, 1 mmol/l glutathione ethylester, and 1 μmol/l CCCP. Rotenone and antimycin partially block the electron transfer in the mitochondrial chain (depending on concentration), favoring the reaction with molecular O₂, and thus an intracellular superoxide formation, which is itself being rapidly converted into H₂O₂ (via the mitochondrial manganese superoxide dismutase [MnSOD]). The islets were further incubated for 30 min at 37°C. At the end of this period, the medium was stored at −20°C until assayed for insulin, except for reseretion tests. The islets were sonicated, and ATP and NADH contents were respectively determined using a biosimunens kit (Sigma-Aldrich) and a spectrophotometer to measure NADH through the absorption peak at 340 nm. In experiments to determine intracellular ROS, islets were loaded with a fluorescent probe that corresponds to intracellular H₂O₂ increase, because the probe used (CM-H₂DCFDA) is a classical H₂O₂ marker. The incubation was conducted as previously described (6). At the end of the experiments, islets were rinsed and disrupted using sterile water. After a 5-min centrifugation (3,000 × g), islet supernatants containing the oxidized product fluorescent due to ROS were analyzed as previously described (6).

Evaluation of insulin release during perifusion experiments. Kinetics of insulin release in vitro were studied using the perifusion procedure already described (8). Four columns were run at the same time, each containing 85–120 freshly isolated islets of Langerhans. The perifusion medium containing the basal buffer [2.8 mmol/l glucose in KRBH containing 140 mmol/l NaCl, 3.6 mmol/l KCl, 0.5 mmol/l NaH₄PO₄, 0.5 mmol/l MgSO₄, 2 mmol/l NaHCO₃, 1.5 mmol/l CaCl₂, 115 mmol/l NaCl, 5 mmol/l KCl, 1.28 mmol/l CaCl₂, 1.2 mmol/l MgCl₂, and 5.5 mmol/l glucose and 5 mg/ml BSA]. After loading, eight islets at a time were allowed to attach onto a polylysine-treated covered glass transferred to a perifusion chamber placed on the stage of an inverted fluorescent microscope (Nikon Diaphot, Champigny sur Marne, France). Canulas feeding into the chamber were connected to a peristaltic pump and allowed a continuous superfusion of the islets at a flow rate of 1 ml/min with a 25 mmol/l HEPES-buffered medium maintained at 37°C containing 125 mmol/l NaCl, 5.9 mmol/l KCl, 1.28 mmol/l CaCl₂, 1.2 mmol/l MgCl₂, 5.5 mmol/l glucose, and 1 mg/ml BSA. Intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ) was determined as previously described (9,10). Briefly, a selected area of the islets was excited at 340 and 380 nm alternatively (every 2 s), and the fluorescence intensity emitted at 510 nm was measured by using a Photonas II microfluorometer (Photon Technology International, Biotek Kontron, St. Quentin Yvelines, France). The measurements of successive 340/380 fluorescence ratios (R) reflect the cytosolic free calcium concentration that is abbreviated as follows: R = F(Fluo 380)/F(Fluo 340), where F(Fluo 380) is the fluorescence of an intracellular Ca²⁺ activity in presence of 1 μmol/l thapsigargin or 1 mmol/l EGTA. Effects of ROS on depolarization were done in presence of the antioxidant trolox (1 mmol/l) by stimulating islets with 30 mmol/l KCl. Before iset measurement, background fluorescence was recorded for both wave-lengths in areas devoid of islets, and the data were subtracted from the corresponding measurements of fura-2-loaded islets.

Insulin reselection test. After antimycin or rotenone exposition (static condition) for 30 min, islets were replaced for 30 min in 5.5 mmol/l glucose, insulin was measured, and then islets were rinsed and re-exposed to 16.7 mmol/l glucose for the same lapse of time; de novo insulin secretion was evaluated on supernatants.

Insulin radioimmunoassay. Insulin was measured by means of a radioimmunoassay kit (Cis-Bio International, Gif-sur-Yvette, France) using ¹²⁵I-labeled porcine insulin tracer and tube coated with anti-porcine guinea pig antiserum. Rat insulin standard was obtained from Linco Research (St. Charles, MO). The lower limit of the assays was 75 pmol/l with a variation coefficient of 6% within the assays and of 8% between the assays.

Lipid peroxidation determinations. Hydroperoxides in biological samples were estimated using the Lipid Hydroperoxide Assay kit (Cayman; Alexis Biochemicals). This method is based on lipid extraction into chloroform, eliminating interference caused by hydrogen peroxide or endogenous ferric ions.

Statistical analysis. Values are expressed as means ± SE. The statistical significance of differences between two groups was determined by Student’s t test, and multiple comparisons were made by ANOVA followed by Student-Newman-Keuls test. For the regressions, a Pearson correlation matrix using SYSTAT software was used, followed by a matrix of Bonferroni probabilities.

RESULTS

Glucose induces ROS production in isolated rat islets. A glucose challenge (16.7 mmol/l) during 30-min incubation triggered a threefold increase of ROS fluorescence as shown in Fig. 1A. This ROS production was accompanied by a classical GSIS (Fig. 1B). We then investigated whether a causal link exists between these two events by quenching ROS rise using an antioxidant. As expected, ROS production was completely abolished by trolox, a vitamin E analog (Fig. 1A). Strikingly, this was associated with a complete blunting of GSIS, suggesting the involvement of ROS in GSIS (Fig. 1B). To confirm these data, we performed perifusion experiments, which allow a dynamic view of insulin secretion. As was the case previously, the presence of the antioxidant induced a pronounced and significant reduction of GSIS (Fig. 1C). We then investigated whether a dose response might be established between ROS production and insulin secretion. This was achieved in static incubation by gradual antioxidant levels to modulate ROS production under GSIS (Fig. 1D and E). Under these conditions, the regression linking ROS production to insulin secretion (r = 0.899) was highly significant (P < 0.001; Fig. 1F). Moreover, kinetics of both ROS production and insulin secretion were measured under GSIS and revealed the ROS elevation shown at 5 min coincided with the start of insulin secretion under static conditions. A plateau was maintained for both ROS production and insulin secretion during the 30 min that lasted the experiment (Fig. 2A and B). Altogether, these results indicated that ROS was produced under acute glucose stimulation and was required for insulin secretion. Moreover insulin was dose dependently released, reinforcing the fact that ROS could be an important part of the in GSIS signaling.

mROS mimic GSIS. To test whether mROS have the ability to mimic GSIS, specific mitochondrial blockers that increase mROS production were used. Rotenone and antimycin, complex I and III inhibitors, respectively, were added to the 30-min static incubation with no glucose challenge in basal glucose condition (5.5 mmol/l). The concentrations (100 μmol/l rotenone and 20 μmol/l antimycin) were chosen to mimic a similar ROS production to that obtained with a 16.7 mmol/l glucose stimulus (Fig. 3A). Trolox (1 mmol/l) or 1 mmol/l glutathione reduced ethyl ester cotreatment blunted the ROS fluorescence (Fig. 3A) and the insulin release (Fig. 3B). To assess this response in a dynamic model, perifusion experiments were also conducted. The presence of the mitochondrial blocker rotenone perfectly mimicked GSIS (Fig. 3C), which was significantly reduced in the presence of the antioxidant trolox (Fig. 3C). To test our hypothesis in static incubation, we coadministered an antioxidant in a dose response–dependent manner. It consequently diminished ROS production (Fig. 3D and E). ROS production was clearly correlated to insulin secretion (r = 0.883), and the regression was highly significant (P < 0.001; Fig. 3F). To confirm the mitochondrial origin of glucose-induced ROS, cells were cotreated with 16.7 mmol/l glucose and the mild uncoupler CCCP (1 μmol/l), which makes it possible to block the ATP rise without decreasing its level (11). This uncoupler accelerates electron transit by dissipating the H⁺ gradient, decreasing the probability of the electrons reacting with oxygen and thus leading to the
Glucose challenge (16.7 mmol/l) in a 30-min static incubation triggered a threefold increase in ROS fluorescence. A: Quenching ROS by trolox completely blunted GSIS. A and B: Three independent experiments, n = 6 per group; **P < 0.001, glucose 5.5 vs. 16.7 mmol/l; ###P < 0.001, glucose 16.7 vs. 16.7 mmol/l + trolox. C: Dynamic experiments using perifusion demonstrating the antioxidant-induced reduction of GSIS. Three independent experiments, P < 0.001 between vehicle and treated groups and between 16.7 mmol/l glucose vs. 16.7 mmol/l glucose + trolox. D and E: Modulation of glucose-induced ROS production and GSIS by gradual antioxidant doses. Three independent experiments, n = 6 per group; **P < 0.01 or ***P < 0.001, glucose 16.7 mmol/l trolox vs. 16.7 mmol/l glucose + 1 µmol/l trolox; #P < 0.05 or ##P < 0.001, 1 µmol/l trolox vs. 1 µmol/l trolox + 1 mM trolox. F: In these conditions, the regression linking ROS production to insulin secretion (r = 0.899) was highly significant P < 0.001. A.U., arbitrary unit.
FIG. 3. mROS mimic GSIS in β-cells islets. A: Effect of treatments on intracellular H$_2$O$_2$ production measured with H$_2$DCFDA probe. Treatments were as follows: 100 µmol/l rotenone or 20 µmol/l antimycin, inhibitors of complexes I and III, respectively, added to the 30-min static incubation in 5.5 mmol/l glucose; 1 mmol/l trolox or 1 mmol/l glutathione reduced ethyl ester (GE) cotreatment with 16.7 mmol/l glucose or rotenone (rot) or antimycin (ant); 16.7 mmol/l glucose coadministrated with the uncoupler CCCP. C: Insulin release measurement in the same conditions. A and C: Three independent experiments, n = 6 per group. ***P < 0.001, glucose 5.5 vs. 16.7 mmol/l glucose or rotenone (rot) or antimycin (ant); ‡‡‡P < 0.001, 16.7 mmol/l glucose vs. 16.7 mmol/l glucose + trolox. B and E: Dynamic insulin secretion using perifusion model under rotenone alone or in presence of the antioxidant trolox. Three independent experiments, with one perifused column per case, P < 0.001 between vehicle and treated groups and between rotenone vs. rotenone + trolox.

B: 1600 900 800 700 600 500 400 300 200 100 0 ROS fluorescence (A.U.)

C: 6 5 4 3 2 1 0 Insulin secretion (ng/islet/30 min)

D: 4 3 2 1 0 Insulin secretion (ng/30 min/islet)

E: 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 Insulin secretion (pg/islets/min)

F: rotenone stimulation + trolox 1mM/ 1µM/ 1nM

r=0.883
that mROS are robust stimulators of insulin secretion independently of these other products linked to glucose metabolism.

**Effect of quenching ROS production on intracellular calcium profiles after glucose, mitochondrial blocker, or KCl treatment.** Switching the extracellular glucose concentration from 5 to 16.7 mM led to an increase in \([\text{Ca}^{2+}]_i\). The subsequent addition of 1 mM trolox led to a marked fall in \([\text{Ca}^{2+}]_i\) to basal level in the following 5 min, suggesting that ROS quenching led to impaired \([\text{Ca}^{2+}]_i\) mobilization (Fig. 5A). Switching the extracellular glucose concentration from 5 mM to 16.7 mM plus 100 µmol/l rotenone first led to a transient fall in \([\text{Ca}^{2+}]_i\) in the first 8 min, followed by a large sustained increase (Fig. 5B). The addition of the antioxidant trolox (1 mM/l) to rotenone completely abolished the \([\text{Ca}^{2+}]_i\) mobilization (Fig. 5B), showing that mROS production might importantly serve as signals mediating the necessary Ca\(^{2+}\) recruitment for insulin secretion. Finally, Fig. 5C and D show one representative example of three experiments for the \([\text{Ca}^{2+}]_i\) mobilization under either 1 mM/l EGTA (chelating extracellular Ca\(^{2+}\) ions) or 1 µmol/l thapsigargin (irreversible inhibition of Ca\(^{2+}\) pumps from intracellular stores). Increasing the glucose concentration or adding rotenone first triggered a typical biphasic response; second, the presence of EGTA (calcium-free medium) resulted in a profound decrease of fluorescence fura-2 ratio 340:380, i.e., intracellular calcium mobilization, for both glucose and rotenone, suggesting the calcium might be mainly of extracellular origin (Fig. 5C). Figure 5D shows the complementary experiment using thapsigargin, which did not suppress the massive increase of \([\text{Ca}^{2+}]_i\), whatever

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*FIG. 4. NADH and ATP increases are not necessary for mROS-induced insulin secretion. Ratio of either ROS production as measured with H\(_2\)-DCFDA (A), NADH (B), insulin secretion (C), or ATP (D) compared with basal condition (5.5 mM/l glucose) in three independent experiments, *n* = 4 per group. A and C: Static islet incubation for 30 min in 16.7 mM/l glucose cotreated with trolox abolished ROS production and blunted insulin secretion; ***<i>P</i> < 0.001, glucose 16.7 vs. 5.5 mM/l; ###<i>P</i> < 0.001, 16.7 mM/l glucose + trolox vs. 16.7 mM/l glucose alone. B and D: Both NADH and ATP were increased, although insulin secretion was abolished when ROS were blunted; *<i>P</i> < 0.05 or **<i>P</i> < 0.001, 16.7 mM/l glucose + trolox vs. 16.7 mM/l glucose alone. Conversely, 100 µmol/l rotenone (rot) stimulated ROS production and insulin secretion (A and C), independently of NADH or ATP (B and D), which were unchanged compared with controls. ***<i>P</i> < 0.001, 5.5 mM/l glucose + rotenone vs. 5.5 mM/l glucose; §§§<i>P</i> < 0.001 or §<i>P</i> < 0.05, 5.5 mM/l glucose + rotenone vs. 5.5 mM/l glucose + rotenone + trolox.*

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\(D\): Modulation of rotenone-induced ROS production and GSIS by gradual antioxidant doses. ROS-dependent response might be established with insulin secretion in static incubation. Three independent experiments, *n* = 6 per group; ***<i>P</i> < 0.001, rotenone + 1 mM/l trolox vs. rotenone + 1 µmol/l trolox; ###<i>P</i> < 0.001, rotenone + 1 µmol/l trolox vs. rotenone + 1 mM/l trolox. \(F\): In these conditions, the regression linking ROS production to insulin secretion (\(r = 0.883\)) was highly significant \(P < 0.001\). ⊗, vehicle; ○, glucose 16.7 mM; ▲, rotenone; □, rotenone + trolox.
the stimulus, glucose or rotenone. However, the typical biphasic rise disappeared in both cases, emphasizing that the first peak might require calcium of intracellular origin. In the next experiment, we tested whether the ROS scavenger trolox (1 mmol/l) would abrogate the calcium effect of KCl. It had no inhibitory effect on KCl-induced \([\text{Ca}^{2+}]_i\) mobilization (Fig. 5E).

**No toxic effects are present in the model used.** To rule out a putative damaging effect on membrane integrity due to peroxidation and a nonspecific oxidative stress of ROS, lipid peroxidation was evaluated. Hydroperoxide measurements were conducted on glucose and pharmacologically treated islets. No difference in peroxidation in the pharmacologically treated groups compared with glucose was observed (Fig. 6A). To definitively exclude a toxic effect, \(\beta\)-cells previously treated with the mitochondrial blockers for 30 min were replaced in a fresh medium, and insulin was reevaluated before and after a glucose challenge. Under these conditions, only a slight insulin release was detected in the fresh medium under basal conditions, and the ability of glucose to stimulate insulin secretion was totally maintained after the pharmacological treatments (Fig. 6B). Altogether, these results exclude the possibility of damaging effects of rotenone or antimycin on islets.

**DISCUSSION**
It is now accepted that small fluctuations in the steady-state concentration of ROS may play a role in intracellular signaling (3,12). Their pivotal role in nutrient sensing is
beginning to be considered in the literature since we highlighted their requirement in hypothalamic glucose and lipid sensing (5,6). Here, we expand the question to the gold standard, and the main glucose-sensitive cell type, the pancreatic β-cells. The present results undoubtedly demonstrate that glucose-induced mitochondrial ROS production is an obligatory stimulus for insulin secretion.

In cells, mitochondria are the main source of oxidants. Transient-accelerated electron transport on glucose stimulation generates an H$_2$O$_2$ burst in many cell types, including the β-cells (13). Metabolism of substrates leads to reduced formation of equivalents (NADH and FADH$_2$) that predispose to increased mROS through a direct effect on the electron-transfer chain (14). Preceding studies point to the crucial role of H$_2$O$_2$, which either acts as a transduction signal, normally coupling glucose metabolism to insulin secretion, or interrupts it depending on its concentration (7,15–17).

In this study, the pharmacological approach was highly justified because transient, moderate, and reversible mROS production was required (11). Currently, no genetic construction, even through a conditional control, permits this fine regulation. In this study, we not only found that insulin is released in a mROS dose-dependent fashion but also that mROS are absolutely required for the secretion, using different and complementary pharmacological manipulations. Our results are coherent and convergent, they moreover are consistent with those of Collins and colleagues (7), who demonstrated that glucose increases intracellular accumulation of H$_2$O$_2$, stimulating insulin secretion in both isolated mouse islets and INS-1(832/13) cells. These results enhance the emerging view that glucose induces a transient and moderate H$_2$O$_2$ production in β-cells. This light production is made possible because a fine adapted system makes it possible to regulate the process (antioxidant defenses) (7). The discrepancies observed in the levels of ROS (either increase or decrease) in β-cells exposed to glucose reported in numerous studies (15,18,19) might be explained by the different exposure times and glucose-culture conditions; it now appears clearly that cyclic ROS production occurs in a time-dependent manner (7). One of the regulating systems operates through the nucleotide nicotinamide transhydrogenase, rapidly and actively regenerating redox balance through the supply of NADPH, which simultaneously dissipates the proton gradient (20). The light and necessary production of ROS for insulin secretion during glucose exposure might rapidly become inadequate when time exposure is no longer controlled, due to the unbalance of the redox-status regeneration, a process that is exacerbated in culture conditions.

The present data clearly point out the importance of ROS from mitochondrial origin. First, mitochondrial ROS mimic the glucose effect. Using mitochondrial blockers that increase mROS at a level similar to that of glucose, insulin secretion was identical to that produced by glucose. Second, no extramitochondrial ROS was detected in glucose and CCCP-cotreated β-cells. This compound increases respiration and diminishes mROS generation. If glucose has the ability to induce ROS production independently of the mitochondria, it might be detected in a condition that only abolishes mitochondrial ROS production (CCCP). This means that ROS produced in the time course of glucose metabolism might be solely due to mitochondria in our detection conditions. It strongly suggests that mROS are robust stimulators of insulin secretion independently of other products linked to glucose metabolism, such as NADH or ATP. Thus, even in the presence of an NADH or ATP rise, GSIS was systematically

![Graph](image-url)
blunted when ROS production was abolished. Unfortunately, NADPH, which is a potential mediator of GSIS, was not detectable in our experimental conditions. We cannot exclude its level changed by the manipulations used here, maybe independently of ROS.

Mitochondrial blockers triggering insulin release in our study are in contradiction with the results of Collins and colleagues (7), who conclude that the ROS might not be of mitochondrial origin. The difference is the concentrations we used, and as a result, the sole difference is based on ATP levels that were unchanged in our model but down-regulated in their study. The downregulation of ATP content suggests a strong inhibition of oxidative phosphorylation and might explain the discrepancies between the two studies. The additive experiments on [Ca$^{2+}$], suggest mROS production being of importance, because the addition of the antioxidant trolox to either glucose or rotenone completely abolished the Ca$^{2+}$ mobilization. The results of Fig. 5C and D suggest mROS are important for the biphasic effect on [Ca$^{2+}$] because they totally mimicked the Ca$^{2+}$ mobilization profile seen with glucose. The exploration of the calcium origin suggests the first peak of Ca$^{2+}$ might be of intracellular origin even through the second massive increase appears to be of extracellular one. These results are in concordance with part of the study of Krippeit-Drews et al. (17) who had extensively studied the origin of Ca$^{2+}$ under a concentrated H$_2$O$_2$ stimulus. Whether the Ca$^{2+}$ influx might be due to ATP-sensitive K$^+$ channel (K$_{ATP}$ channel)-induced depolarization was undertaken in this study. In this case, H$_2$O$_2$ caused the opening of the channel (the sulfonylurea tolbutamide was able to reverse the effect), and the sustained extracellular Ca$^{2+}$ influx was shown to be due to a direct activation of L-type Ca$^{2+}$ channels in mouse $\beta$-cells (21–23) and recently in dopamine neurons (24). These results are not in favor of the participation of the K$_{ATP}$ channels in the second sustained increase of [Ca$^{2+}$]. Moreover, even if ATP levels were critical in these studies, here, the pharmacological doses of rotenone or antimycin A used were without effect on ATP levels when compared with glucose controls (5.5 mmol/l).

The $\beta$-cell depolarization is a crucial event to activate the insulin exocytotic machinery in the early phase of GSIS, and that is the reason why the ROS dependence of depolarization-stimulated Ca$^{2+}$ mobilization was investigated. Our results indicate that mROS-stimulated insulin secretion is an extracellular Ca$^{2+}$-dependent process, suggesting that mROS may be involved in Ca$^{2+}$ influx. In contrast, the evidence that the exogenous antioxidant trolox does not inhibit KCl-induced [Ca$^{2+}$] increase suggests that the voltage pathway works independently of the mROS pathway. These last results are in perfect concordance with those of Collins and colleagues, who did not detect an inhibition of the antioxidants on KCl-induced insulin release. Our results, combined with those of Collins and colleagues, reinforce the idea that mROS belong to the essential coupling factors for stimulating insulin secretion.

Moreover, in our experiments, putative toxic effects can be ruled out; both results of lipid peroxidation and functional tests (GSIS) were in keeping with intact islet preservation. These data support that mROS signaling is of prime importance, although the underlying mechanism(s) leading to insulin secretion is(are) far from being identified.

Strikingly, one is led to think that the classical ATP elevation-induced K$_{ATP}$ closure is not as determinant as has been suggested. Different putative targets might be involved, linking mROS to insulin production. First, the mitochondria itself: mitochondrial Ca$^{2+}$ rise is in part H$_2$O$_2$ sensitive and absolutely required for insulin secretion (16,17). Second, the channels, which are involved in membrane depolarization (25). For example, transient receptor potential channels may also be potential targets because many of them are H$_2$O$_2$ sensitive (26–31).

Altogether, these data point to the complex involvement of mitochondrial metabolism in GSIS. Moreover, the consensual model of ATP as a coupling factor of nutrient-induced insulin secretion is no longer as evident as previously accepted. Finally, our findings support the idea that mROS are obligatory signaling pathways of GSIS. The mROS appear as the necessary balanced signal between NADH supply on one hand and the ATP production on the other hand. The understanding of this signaling might be of prime importance in nutritional care of energy balance and health.

ACKNOWLEDGMENTS

This research was supported by the Centre National de la Recherche Scientifique, by the Agence Nationale pour la Recherche (grant 05-PNRA 004-01), and by the Programme National de Recherche sur le Diabète (grant 0602).

No potential conflicts of interest relevant to this article were reported.

We thank Michael Healey for careful manuscript reading.

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