Hydrogen peroxide alters mitochondrial activation and insulin secretion in pancreatic beta cells

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Abstract
The effects of a transient exposure to hydrogen peroxide (10 min at 200 microM H(2)O(2)) on pancreatic beta cell signal transduction and insulin secretion have been evaluated. In rat islets, insulin secretion evoked by glucose (16.7 mM) or by the mitochondrial substrate methyl succinate (5 mM) was markedly blunted following exposure to H(2)O(2). In contrast, the secretory response induced by plasma membrane depolarization (20 mM KCl) was not significantly affected. Similar results were obtained in insulinoma INS-1 cells using glucose (12.8 mM) as secretagogue. After H(2)O(2) treatment, glucose no longer depolarized the membrane potential (DeltaPsi) of INS-1 cells or increased cytosolic Ca(2+). Both DeltaPsi and Ca(2+) responses were still observed with 30 mM KCl despite an elevated baseline of cytosolic Ca(2+) appearing approximately 10 min after exposure to H(2)O(2). The mitochondrial DeltaPsi of INS-1 cells was depolarized by H(2)O(2) abolishing the hyperpolarizing action of glucose. These DeltaPsi changes correlated with altered mitochondrial morphology; the latter was not preserved by the overexpression of the [...]
Hydrogen Peroxide Alters Mitochondrial Activation and Insulin Secretion in Pancreatic Beta Cells

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The effects of a transient exposure to hydrogen peroxide (10 min at 200 μM H₂O₂) on pancreatic beta cell signal transduction and insulin secretion have been evaluated. In rat islets, insulin secretion evoked by glucose (16.7 mM) or by the mitochondrial substrate methyl succinate (5 mM) was markedly blunted following exposure to H₂O₂. In contrast, the secretory response induced by plasma membrane depolarization (20 mM KCl) was not significantly affected. Similar results were obtained in insulinoma INS-1 cells using glucose (12.8 mM) as secretagogue. After H₂O₂ treatment, glucose no longer depolarized the membrane potential (ΔΨ) of INS-1 cells or increased cytosolic Ca²⁺. Both ΔΨ and Ca²⁺ responses were still observed with 30 mM KCl despite an elevated baseline of cytosolic Ca²⁺ appearing ~10 min after exposure to H₂O₂. The mitochondrial ΔΨ of INS-1 cells was depolarized by H₂O₂ abolishing the hyperpolarizing action of glucose. These ΔΨ changes correlated with altered mitochondrial morphology; the latter was not preserved by the overexpression of the antiapoptotic protein Bcl-2. Mitochondrial Ca²⁺ was increased following exposure to H₂O₂ up to the micromolar range. No further augmentation occurred after glucose addition, which normally raises this parameter. Nevertheless, KCl was still efficient in enhancing mitochondrial Ca²⁺. Cytosolic ATP was markedly reduced by H₂O₂ treatment, probably explaining the decreased endoplasmic reticulum Ca²⁺. Taken together, these data point to the mitochondria as primary targets for H₂O₂ damage, which will eventually interrupt the transduction of signals normally coupling glucose metabolism to insulin secretion.

The control of insulin secretion in the pancreatic beta cell depends on the precise tuning of glucose metabolism leading to signal transduction (1, 2). Indeed, impaired metabolism secretory coupling results in inappropriate insulin release potentially causing defective blood glucose homeostasis. Dysfunction of the beta cell signal transduction may be of various origin, among which oxidative stress has been proposed to play a critical role.

Type I diabetes, or insulin dependent diabetes mellitus, is an autoimmune disease characterized by altered function and beta cell death subsequent to exposure to inflammation products (3). During insulitis macrophages infiltrate the islets of Langerhans and generate reactive oxygen species such as hydrogen peroxide (H₂O₂), which exert deleterious actions on the beta cells and on mitochondrial oxidative metabolism. It is noteworthy that activated phagocytes can produce as much as 47 nmol of H₂O₂/10⁶ cells within 30 min corresponding to a concentration of 47 μM H₂O₂ in a diluted volume of 1 ml (4). Nitric oxide (NO), another free radical precursor produced by macrophages, suppresses mitochondrial activity leading to a defective insulin release in response to nutrient secretagogues (5). Moreover, it has been shown that NO damages islet cell DNA (6) and mitochondrial DNA in beta cells (7). In general, mitochondrial DNA is more sensitive to oxidative stress than nuclear DNA (8, 9). The mitochondria play a key role in the control of nutrient-induced insulin exocytosis by generating 1) ATP to raise cytosolic Ca²⁺ concentration ([Ca²⁺]c) through membrane depolarization (1, 2) and 2) additional mitochondrial factor(s) triggering insulin exocytosis (10, 11).

A defective secretory response to nutrients can be encountered in aged patients (12). In normal rats, insulin secretion is maintained throughout life (13), whereas in perinatal malnourished rats aging results in hypoinsulinemia and hyperglycemia (14). Aging has been shown to be associated with the alteration of beta cell function independent of that seen in noninsulin-dependent diabetes mellitus (15). A reduced translation of the mitochondrial genome (16) and of the cytochrome c oxidase activity (17) has been reported in elderly subjects, pointing to an age-related mitochondrial dysfunction in this highly oxidative organelle. Mitochondrial aconitase, a tricarboxylic acid cycle enzyme, is susceptible to oxidative modification during aging in vivo (18). Moreover, it has recently been demonstrated that the mitochondrial adenine-nucleotide translocase is modified oxidatively during aging together with loss of functional activity (19). In cells the mitochondrion is the main source of oxidants. Indeed, imperfect electron transport generates superoxide anions, which are spontaneously dismutated to H₂O₂ (9, 20). Thus, the mitochondria are pivotal in the control of insulin secretion, whereas at the same time generating reactive oxygen species in the cell mostly in the form of H₂O₂.

Of particular importance is the high sensitivity of pancreatic beta cells to oxidative stress. Moreover, the diabetic state is associated with increased oxidative stress and free radical damage (20). In fact, the expression of the H₂O₂-inactivating enzymes catalase and glutathione peroxidase in rat pancreatic islets is twenty times lower than in the liver (21). As a conse-
sequence the basal catalase activity in islets is very low (1.3 units/mg protein in the rat) and was reported to result in high susceptibility to cytotoxicity during a 16-h exposure to H$_2$O$_2$ (10–500 μM) (22). The rat beta cell line INS-1, which was used in the present study, exhibits similar low baseline catalase levels (1.0 units/mg protein) as primary rat islets (22). Taken together, these findings call for a better understanding of the cellular mechanisms linking oxidative stress to impaired insulin secretion.

In mouse pancreatic beta cells H$_2$O$_2$ hyperpolarizes the cell membrane coupled with an increase of cell membrane conductance (23). Moreover, it has recently been shown that H$_2$O$_2$ increases intracellular Ca$^{2+}$, decreases the ATP/ADP ratio, and inhibits glucose-stimulated insulin secretion from isolated mouse islets (24). The present work was designed to dissect in more detail oxidative stress-induced cell damage and to parallel mitochondrial parameters with the secretory response in insulin-secreting cells stimulated with glucose after exposure to H$_2$O$_2$. We used H$_2$O$_2$ as a well established model of a bioel† mitochondrial parameters with the secretory response in insulin-secreting cells stimulated with glucose after exposure to H$_2$O$_2$. We used H$_2$O$_2$ as a well established model of a biologically active oxygen-derived intermediate (20) at the concentration of 200 μM. Rat islets and the beta cell line INS-1 have been shown to be sensitive to this H$_2$O$_2$ concentration without exhibiting major toxicity (22). Our results show that the defective glucose-induced insulin secretion observed after H$_2$O$_2$ treatment correlates with altered mitochondrial activation seen as a loss of mitochondrial membrane potential (Δψ$_m$), decreased ATP generation (measured on-line in living cells), and impaired responses of mitochondrial Ca$^{2+}$ concentration ([Ca$^{2+}]_{m}$). In addition, the mitochondrial morphology, which depends mainly on Δψ$_m$, was examined. In PC12 cells, the antiapoptotic protein Bcl-2 (for a review see Ref. 25) was reported to prevent the H$_2$O$_2$-induced Δψ$_m$ loss and the subsequent apoptosis (26). Here we also examined whether overexpression of Bcl-2 could prevent the alteration of mitochondrial morphology during H$_2$O$_2$ treatment.

**EXPERIMENTAL PROCEDURES**

*Materials—* Coelenterazine, Mitotracker, rhodamine-123, and bisoxonol were obtained from Molecular Probes (Eugene, OR); luciferin was from Promega (Madison, WI); bovine serum albumin, doxycline, methyl succinate, firefly lantern extract, and FCCP were from Sigma; anti-insulin antibody was from Linco (St. Charles, MO); Bcl-2 mouse monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); G418 and hygromycin were from Calbiochem.

**Cell Culture—** INS-1 cells were cultured in RPMI 1640 medium as described previously (27–29). Stable clones of INS-1 cells expressing the Ca$^{2+}$-sensitive photoprotein aequorin in the cytosol (INS-1/C-29) (28) or to the endoplasmic reticulum (ER) (INS-1/ER#18) (30) were cultured in the presence of 250 μg/ml G418 for continuous selection of cells expressing the plasmid with the associated neomycin resistance. Clonal INS-1 lines expressing cytosolic luciferase under the control of doxycycline-dependent transcriptional transactivator (INS-r-d-RU7) were used for cytosolic ATP monitoring in living cells (29). Pancreatic islet cells were isolated by collagenase digestion from male Wistar rats weighing ~200 g (31) and were cultured free floating in RPMI 1640 medium (11.1 mM glucose) for 2–4 days.

**Insulin Secretion—** For perfusion protocols, islets or trypsinized cells were kept in spinner culture for 2 h in glucose-free RPMI 1640 supplemented with 25 mM HEPES and 1% new born calf serum prior to perfusion in modified Krebs-Ringer bicarbonate HEPES buffer (KRBH) composed of 135 mM NaCl, 3.6 mM KCl, 10 mM HEPES, pH 7.4, 5 mM NaHCO$_3$, 0.5 mM NaH$_2$PO$_4$, 0.5 mM MgCl$_2$, 1.5 mM CaCl$_2$, and 2.8 mM glucose. Cells were placed in a thermostatted chamber (35 rat islets or 10$^6$ clonal cells/chamber) and perfused at a flow rate of 1 ml/min. For static incubations, INS-1 cells (2 × 10$^6$ cells/well in polycarbonate-treated 24-well plates) were seeded and cultured for 3–5 days in complete RPMI 1640 medium. Prior to the experiments, cells were main-

![Fig. 1](image_url)
Effect of $H_2O_2$ on insulin secretion (% of cell content) in INS-1 cells stimulated with glucose following different preincubation conditions

<table>
<thead>
<tr>
<th>Preincubation conditions</th>
<th>$H_2O_2$ exposure</th>
<th>Insulin secretion during $H_2O_2$ exposure</th>
<th>Glucose incubation</th>
<th>Insulin secretion during glucose incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>10 min</td>
<td>30 min</td>
<td></td>
</tr>
<tr>
<td>a. Glucose-free</td>
<td>Nil</td>
<td>1.30 ± 0.12</td>
<td>2.8 mm (basal)</td>
<td>1.93 ± 0.20</td>
</tr>
<tr>
<td>b. Glucose-free</td>
<td>Nil</td>
<td>1.34 ± 0.15</td>
<td>12.8 mm (stimulated)</td>
<td>16.74 ± 1.41</td>
</tr>
<tr>
<td>c. Glucose 11.1 mM</td>
<td>Nil</td>
<td>1.57 ± 0.06</td>
<td>2.8 mm (basal)</td>
<td>5.14 ± 0.12</td>
</tr>
<tr>
<td>d. Glucose 11.1 mM</td>
<td>Nil</td>
<td>1.70 ± 0.15</td>
<td>12.8 mm (stimulated)</td>
<td>16.81 ± 0.75</td>
</tr>
<tr>
<td>e. Glucose 11.1 mM 200 $\mu M$ $H_2O_2$</td>
<td>200 $\mu M$ $H_2O_2$</td>
<td>3.83 ± 0.35</td>
<td>2.8 mm (basal)</td>
<td>3.43 ± 0.49</td>
</tr>
<tr>
<td>f. Glucose 11.1 mM 200 $\mu M$ $H_2O_2$</td>
<td>200 $\mu M$ $H_2O_2$</td>
<td>p &lt; 0.001 versus c.</td>
<td>12.8 mm (stimulated)</td>
<td>NS versus c.</td>
</tr>
</tbody>
</table>

RESULTS

Effect of $H_2O_2$ on Insulin Secretion in Rat Islets and INS-1 Cells—Rat pancreatic islets were maintained in culture (11.1 mM glucose) for 2–4 days prior to the experiments. Insulin secretion was stimulated with 16.7 mM glucose for 10 min after basal perfusion at 2.8 mM glucose (Fig. 1A). When 200 $\mu M$ $H_2O_2$ was added for 10 min a transient release of insulin was observed. The cells were then washed for 5 min in the presence of catalase to block any remaining extracellular $H_2O_2$. The subsequent stimulation with 16.7 mM glucose was largely blunted (Fig. 1B), because only 30% of the secretory response was preserved in terms of area under the curve (see Table I). Control representative traces of islets perfused without glucose stimulation are shown in Fig. 1, A and B (thin lines).

The tricarboxylic acid cycle intermediate succinate, rendered cell permeant by the ester binding of a methyl group (34), evoked an insulin secretory response (Fig. 1C), which was significantly inhibited by 74% after $H_2O_2$ treatment (Fig. 1D and Table I). A nonnutrient stimulation of insulin release was induced by KCl, which triggers insulin exocytosis by a simple rise of $[Ca^{2+}]_c$ consequent to membrane depolarization (Fig. 1E). In this case
a similar secretory response to KCl was observed after H$_2$O$_2$ exposure compared with the control stimulation (Fig. 1F). In static incubations at basal 2.8 mM glucose, the insulinoma cells INS-1 released insulin during a 10-min exposure to H$_2$O$_2$ in a dose-dependent manner (Fig. 2A). The threshold for the action of H$_2$O$_2$ on insulin secretion was 200 μM (152%, p < 0.02; **, p < 0.002; ***, p < 0.0005 versus A, control value without H$_2$O$_2$ (1.75 ± 0.11% content); B, basal conditions in control groups). Following the 10-min treatment with 200 μM H$_2$O$_2$, the basal secretion (at 2.8 mM glucose) measured during a 30-min incubation was elevated (Fig. 2B). The clonal cells were more sensitive to H$_2$O$_2$ than primary cells, because stimulation with 12.8 mM glucose after the oxidative stress period was unable to evoke any secretory response (Fig. 2B). Moreover, because the basal release was elevated, no further effect of 30 mM KCl was observed above the secretory level reached in control preparations. It should be noted that these experiments were performed following a 2-h preincubation period in glucose-free medium, which slightly decreases basal insulin release (Table II). This could possibly sensitize the cells to oxidative stress because of fuel depletion. Therefore, secretion experiments were repeated without a starvation period and showed a similar pattern as in Fig. 2, A and B, in terms of H$_2$O$_2$ sensitivity and blunted glucose (12.8 mM) response (Table II).

**Effect of H$_2$O$_2$ on ΔΨ$_m$ in INS-1 Cells**—Bisoxonol fluorescence was used to monitor the ΔΨ$_m$ in a suspension of INS-1 cells. After a baseline at 2.8 mM glucose, 10 mM sugar was added (12.8 mM final), which produced a depolarization of ΔΨ$_m$ and it was further depolarized by the subsequent addition of 30 mM KCl (Fig. 3A). When 200 μM H$_2$O$_2$ was first added to the cuvette, a transient hyperpolarization of ΔΨ$_m$ was followed by a slight, gradual depolarization (Fig. 3B). After a 10-min inter-
val, 100 units/ml catalase was added to neutralize the remaining extracellular H₂O₂. Subsequent exposure to 10 mM glucose caused no significant depolarization of the DC

In contrast, KCl (30 mM) evoked further depolarization (Fig. 3B). When H₂O₂ treatment was applied after glucose stimulation, the glucose-induced cell depolarization was reversed without inhibition of the effect of KCl added subsequently (Fig. 3C).

Effect of H₂O₂ on [Ca²⁺]c in INS-1 Cells—Attached INS-1 cells stably expressing cytosolic aequorin, a Ca²⁺-sensitive photoprotein, were used to measure [Ca²⁺]c in a perifusion setup. Glucose (12.8 mM) and KCl (30 mM) raised [Ca²⁺]c (Fig. 4A). The perifusion of 200 µM H₂O₂ for 10 min resulted in a retarded and sustained increase of [Ca²⁺]c to ~400 nM (Fig. 4B). When glucose (12.8 mM) was added after H₂O₂ treatment, no further augmentation of [Ca²⁺]c was observed (Fig. 4C). In contrast, KCl (30 mM) caused a normal rise in [Ca²⁺]c even after the oxidative stress (Fig. 4D).

Effect of H₂O₂ on ΔΨm in INS-1 Cells—The ΔΨm was measured in a suspension of INS-1 cells by monitoring rhodamine-123 fluorescence. The addition of 10 mM glucose (12.8 mM final) potently hyperpolarized the ΔΨm, and 1 µM protonophore FCCP depolarized it (Fig. 5A). H₂O₂ (200 µM) induced an initial moderate and rapid depolarization of ΔΨm followed by a slow, progressive further depolarization (Fig. 5B). After catalase

treatment, 10 mM glucose (12.8 mM final) failed to hyperpolarize ΔΨm and even accelerated the depolarization. The subsequent addition of 1 µM FCCP completed the ΔΨm depolarization first initiated by H₂O₂. As a control of enzymatic efficacy, catalase was added before H₂O₂ treatment. In these conditions, H₂O₂ failed to alter the ΔΨm, and glucose added thereafter hyperpolarized the ΔΨm in a normal manner (Fig. 5B, thin line). When H₂O₂ was applied after glucose stimulation, the hyperpolarization of ΔΨm resulting from glucose metabolism
was counteracted leading to depolarization of $\Delta \Psi_m$ (Fig. 5C). $H_2O_2$ almost completely depolarized the $\Delta \Psi_m$ because FCCP had only minor effects when added after the oxidative stress.

**Effect of $H_2O_2$ on $[Ca^{2+}]_m$ in INS-1 Cells—**$[Ca^{2+}]_m$ was monitored in perfused INS-1 cells stably expressing mitochondrially targeted aequorin. In control recordings glucose (12.8 mM) raised $[Ca^{2+}]_m$ up to 1.3 $\mu M$ (Fig. 6A). The addition of 200 $\mu M$ $H_2O_2$ for 10 min rapidly increased the $[Ca^{2+}]_m$ from a baseline of 200 nM to the micromolar range followed by a continuous augmentation up to 1.2 $\mu M$ (Fig. 6B). After the removal of $H_2O_2$ and its extracellular depletion by catalase (100 units/ml for 2 min), $[Ca^{2+}]_m$ stabilized at a plateau of approximately 1.1 $\mu M$.

This oxidative stress completely inhibited the glucose response (Fig. 6C). In contrast, $H_2O_2$ treatment did not affect the $[Ca^{2+}]_m$ rise evoked by FCCP-induced cell depolarization (Fig. 6D), which also augments $[Ca^{2+}]_m$ (Fig. 4D).

**Table III**

<table>
<thead>
<tr>
<th>Groups</th>
<th>ATP pmol/mg protein</th>
<th>Differences versus control</th>
<th>Differences versus $H_2O_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.9 ± 1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2.8 mM glucose)</td>
<td></td>
<td>+34%</td>
<td></td>
</tr>
<tr>
<td>Glucose (12.8 mM)</td>
<td>46.7 ± 1.5</td>
<td>-57%</td>
<td></td>
</tr>
<tr>
<td>$H_2O_2$-control</td>
<td>15.0 ± 1.1</td>
<td>+34%</td>
<td>-57%</td>
</tr>
<tr>
<td>(Oxidative stress)</td>
<td>p &lt; 0.0005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (12.8 mM)</td>
<td>20.9 ± 2.6</td>
<td>-40%</td>
<td>+39%</td>
</tr>
<tr>
<td>After oxidative stress</td>
<td>p &lt; 0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

Mitochondria play a key role in the control of nutrient-induced insulin secretion by coupling glycolysis to distal events leading to exocytosis (1, 2, 35). However, this highly aerobic organelle also produces $H_2O_2$ from dismutated superoxide anions (9) generating an intracellular oxidative stress that may damage neighboring molecules. Among these, mitochondrial enzymes such as aconitase (18) and adenine-nucleotide trans-
Oxidative Stress and Pancreatic Beta Cell Function

loca (19) are susceptible to oxidative modification. Mitochondrial DNA is also highly sensitive to oxidative stress (8, 9). In the present study, we used H2O2 as a recognized biologically important oxidant (20). The cells were exposed to 200 μM H2O2, a level seen during activation of phagocytes in vitro (4).

Exposure of rat islets to H2O2 resulted in a transient increase in insulin release at basal nonstimulatory glucose concentration and impaired the glucose-induced secretory response tested subsequent to the oxidative stress. A similar pattern was obtained using the mitochondrial substrate methyl succinate as a secretagogue, suggesting that pathways downstream of glycolysis are susceptible to oxidative alterations. On the contrary, the KCl-evoked cell depolarization was still able to promote insulin exocytosis following exposure to H2O2 in the islet perifusion experiments. This points to the mitochondrion rather than to the exocytotic process as the sensitive site. These results are in accordance with a recent report from our laboratory (36) using the diabetogenic compound alloxan known to generate H2O2 and free radicals (20). Exposure of INS-1 cells to alloxan for 5 min resulted in elevated basal insulin release; thereafter glucose failed to stimulate oxidative metabolism and insulin secretion, whereas the secretory response to KCl was largely preserved (36). Taken together, these data suggest that oxidative stress primarily alters nutrient-evoked insulin secretion and that the elevated basal insulin release is the consequence of an increase in [Ca2+]i. Indeed, treatment of INS-1 cells with either H2O2 (present study) or alloxan (36) results in the elevation of [Ca2+]i. The steady state [Ca2+]i attained under these conditions was similar to that measured with KCl on the plateau phase, which could explain why the effects of H2O2 and KCl on insulin secretion in static incubation were not additive.

In the mitochondria, exposure to H2O2 caused a rapid elevation of [Ca2+]i, even at early time points when [Ca2+]i was still at basal level, excluding a simple transfer between these two compartments. Rather, the first phase of [Ca2+]i rise might be explained by a direct inhibition by H2O2 of the Na+/Ca2+ antiporter that controls the efflux of Ca2+ from the mitochondrial matrix (37). Transfer of reducing equivalents to the electron transport chain increases ΔΨm, which enhances the driving force for mitochondrial Ca2+ uptake mediated by a low affinity uniporter (38). The failure of glucose to hyperpolarize ΔΨm following oxidative stress undoubtedly explains the lack of the normal [Ca2+]i increase during glucose stimulation (28). These mitochondrial alterations led to the diminished ATP synthesis and probably to an insufficient generation of additional mitochondrial factors required for nutrient-evoked insulin exocytosis (10). The depolarized ΔΨm explains the altered mitochondrial morphology, which was not prevented by overexpression of Bcl-2 (Fig. 8). Both alterations are also observed in mitochondrial DNA-deficient INS-1 cells associated with impaired nutrient-induced insulin secretion (39). It is noteworthy that a mutation in the human mitochondrial tRNALeod(UUR) gene, which is associated with mitochondrial diabetes, also yields a similar mitochondrial morphology (40).

[Ca2+]i, was elevated following H2O2 treatment, and glucose added thereafter failed to elicit a [Ca2+]i response. This was not the case for the KCl-evoked depolarization of ΔΨm, which resulted in normal [Ca2+]i rise, suggesting that the voltage sensitive i-type Ca2+ channels were still functional. Similar
effects of H$_2$O$_2$ have been reported recently in the CRI-G1 insulin secreting cell line (41) and in mouse islets (24). Krippert-Drews et al. (24) postulated that the first phase of intracellular Ca$^{2+}$ rise is because of Ca$^{2+}$ mobilization from the mitochondria, whereas the second phase would reflect Ca$^{2+}$ influx through pathways distinct from L-type Ca$^{2+}$ channels. Herson et al. (41) suggested that the first phase is caused by mobilization of intracellular Ca$^{2+}$ stores insensitive to thapsigargin and therefore proposed the mitochondria as the source of the initial [Ca$^{2+}$]$_i$ rise. Regarding the second phase, these authors (41) postulated that H$_2$O$_2$ opens a nonselective cation channel in the plasma membrane, which was described in a previous study as a novel Ca$^{2+}$ influx pathway activated by oxidative stress (42). We did not observe the second phase [Ca$^{2+}$]$_i$ rise probably because of the 50 times lower concentration of H$_2$O$_2$ used in the present study. The measurements of Ca$^{2+}$ changes both in the mitochondrial and ER compartments permitted us to conclude that the first phase of [Ca$^{2+}$]$_i$ elevation is probably because of diminished pumping of Ca$^{2+}$ into the ER as a consequence of a decrease in cytosolic ATP (30, 43). Indeed, ER Ca$^{2+}$ was lowered by H$_2$O$_2$ treatment secondary to the marked decrease in cytosolic ATP and before the elevation of [Ca$^{2+}$]$_i$. These results substantiate the critical role of cytosolic ATP in determining the filling state of the ER Ca$^{2+}$ stores.

The following sequence of events for the actions of H$_2$O$_2$ can be proposed. We suggest that hydrogen peroxide or derivative products such as free radicals directly inhibits the mitochondrial Na$^+$/Ca$^{2+}$ antiporter, as previously observed (37), blocking the efflux of Ca$^{2+}$ from the mitochondria. This inhibition would lead to the rapid rise in [Ca$^{2+}$]$_i$ independent of any changes in the cytosolic compartment, an effect recently reported in endothelial cells (37). Meanwhile, oxidative stress inhibits tricarboxylic acid cycle enzymes such as aconitase (18), attenuating the generation of reducing equivalents, in particular NADH. This would lead to inactivation of the electron transport chain with the concomitant loss of $\Delta$Ψ$_m$ and the decline in ATP generation. Moreover, oxidative modification of the adenine-nucleotide translocase would inhibit the translocation of ATP to the cytosol (19). The depolarization of $\Delta$Ψ$_m$ is unlikely to be because of the opening of the transition pore, at least on this short time scale, because overexpression of Bcl-2 did not prevent the mitochondrial effects evoked by H$_2$O$_2$ treatment.

The retarded elevation in [Ca$^{2+}$]$_i$, following exposure to H$_2$O$_2$ may well be explained by the observed decrease in cytosolic ATP. Indeed, lowering of cytosolic ATP by the mitochondrial uncoupler FCCP results in a marked decrease of ER Ca$^{2+}$ as monitored in INS-1 cells expressing aequorin in this cellular compartment (30). Reduction of cytosolic ATP results in impaired pumping of Ca$^{2+}$ into the ER, an effect mimicked by the sarco/endoplasmic reticulum Ca$^{2+}$-ATPase inhibitors. The latter effect has been demonstrated in insulin-secreting cell lines (30, 41) and has also been shown to lead to apoptosis (44, 45). Thus, the lowering of ER Ca$^{2+}$ by H$_2$O$_2$ treatment is another demonstration of the close functional coupling between mitochondria and ER Ca$^{2+}$ stores (45, 46). This effect on ER Ca$^{2+}$ certainly accounts for the rise in [Ca$^{2+}$]$_i$ observed shortly after H$_2$O$_2$ treatment. Ca$^{2+}$ influx from the extracellular space was also reported to occur following exposure to H$_2$O$_2$ in mouse islets (24) or to alloxan (generating free radicals) in INS-1 cells (36). It has been postulated that a nonselective cation channel mediates this retarded Ca$^{2+}$ influx, which is promoted by oxidative stress (41). Whatever the mechanism, it can be surmised that both the inhibition of Ca$^{2+}$ pumping by the sarco/endoplasmic reticulum Ca$^{2+}$-ATPase into the ER and the Ca$^{2+}$ influx from the extracellular space contribute to the elevation of [Ca$^{2+}$]$_i$.

Dysfunction of the beta cell by oxidative stress is implicated in type I diabetes and in aging. The present study provides evidence for the involvement of the mitochondria in impaired

**FIG. 8. Effect of H$_2$O$_2$ on mitochondrial morphology in INS-1 cells.** Cells were cultured on glass coverslips and (D–I) transfected with Bcl-2. Mitochondria were stained with Mitotracker prior to the appropriate treatment for 10 min followed by fixation and visualization under a laserscan confocal microscope. A, control cells; B, cells treated with 200 μM H$_2$O$_2$; C, cells treated with 1 μM of the uncoupler FCCP. In D–I, cells were transiently transfected with human Bcl-2. Cells expressing the transgene were identified by immunostaining using anti-human Bcl-2 antibody (E, G, and I). D, F, and H show the mitochondrial staining of untransfected cells and Bcl-2-transfected cells (arrow) for control, H$_2$O$_2$, and FCCP conditions, respectively.
signal transduction, coupling glucose metabolism to insulin secretion following an oxidative stress. Moreover, a model is suggested for the sequence of events leading to the elevation of [Ca^{2+}]_{i} further work will address the identification of factors normally required for nutrient-induced insulin secretion but are missing in the damaged beta cell.

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REFERENCES