Overexpression of glutathione peroxidase 4 prevents β-cell dysfunction induced by prolonged elevation of lipids in vivo

Khajag Koulaian,1* Alexander Ivovic,1* Kaitai Ye,1 Tejas Desai,1 Anu Shah,1,2,3 I. George Fantus,1,2,3,7,8,9 Qitao Ran,4,5,6 and Adria Giacca1,7,8,9

1Department of Physiology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada; 2Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada; 3Toronto General Research Institute, University Health Network, Toronto, Ontario, Canada; 4Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, Texas; 5Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center at San Antonio, San Antonio, Texas; 6Department of Veterans Affairs, South Texas Veterans Health Care System, San Antonio, Texas; 7Department of Medicine, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada; 8Banting and Best Diabetes Centre, University of Toronto, Toronto, Ontario, Canada; and 9Institute of Medical Sciences, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada

Submitted 25 September 2012; accepted in final form 15 May 2013

Chronic exposure of pancreatic β-cells to free fatty acids (FFA) impairs β-cell function (21). Although not all studies are concordant (45), a growing body of evidence implicates oxidative stress as a mechanism of FFA-induced β-cell dysfunction (9, 38, 46, 60, 67). Pancreatic β-cells have low antioxidant defenses (34) and are thus susceptible to reactive oxygen species (ROS)-induced decrease in function and viability (37, 47). We have demonstrated previously that FFA-induced impairment of β-cell function assessed in vitro, ex vivo and in vivo. These results implicate lipid peroxidation in FFA-induced β-cell dysfunction.

Oxidative stress; lipid peroxide; glutathione peroxidase 4; lipotoxicity; β-cell dysfunction; in vivo

Overexpression of glutathione peroxidase 4 prevents β-cell dysfunction induced by prolonged elevation of lipids in vivo

Overexpression of glutathione peroxidase 4 prevents β-cell dysfunction induced by prolonged elevation of lipids in vivo. Am J Physiol Endocrinol Metab 305: E254–E262, 2013. First published May 21, 2013; doi:10.1152/ajpendo.00481.2012.—We have shown that oxidative stress is a mechanism of free fatty acid (FFA)-induced β-cell dysfunction. Unsataturated fatty acids in membranes, including plasma and mitochondrial membranes, are substrates for lipid peroxidation, and lipid peroxidation products are known to cause impaired insulin secretion. Therefore, we hypothesized that mice overexpressing glutathione peroxidase-4 (GPx4), an enzyme that specifically reduces lipid peroxides, are protected from fat-induced β-cell dysfunction. GPx4-overexpressing mice and their wild-type littermate controls were infused intravenously with saline or oleate for 48 h, after which reactive oxygen species (ROS) were imaged, using dihydrodichlorofluorescein diacetate (H2DCF-DA), prevents the impairing effects of FFA on β-cell function (51). Zhang et al. (72) confirmed our findings in rats (51) in a different model of prolonged FFA elevation. Our group has also shown that the antioxidant taurine alleviates FFA-induced impairment in β-cell function in humans (68).

The type and cellular localization of ROS in FFA-induced β-cell dysfunction are still unclear. We have demonstrated that cytosolic superoxide plays a causal role in fat-induced β-cell dysfunction (32); however, since the antioxidants N-acetylcysteine and taurine, which do not decrease superoxide, were also effective in restoring β-cell function (51), other ROS are implicated in addition to superoxide. These other ROS appear to be detected by H2DCF-DA, since the level of H2DCF-DA measured ROS correlated with the impairment in β-cell function (51). Unsaturated fatty acids in membranes, including plasma and mitochondrial membranes, are substrates for lipid peroxidation (27). Lipid peroxides are detected by H2DCF-DA (33), and lipid peroxidation products are known to cause impaired insulin secretion (44). Therefore, we hypothesized that lipid peroxidation is involved in FFA-induced β-cell dysfunction.

Glutathione peroxidases (GPx) are a group of selenium-containing proteins that catalyze the reduction of peroxides generated by ROS at the expense of glutathione (8). GPx1 is the most abundant glutathione peroxidase, and GPx4 is a membrane-associated glutathione peroxidase that, in addition to peroxides, reduces hydroperoxide groups on phospholipids, lipoproteins, and cholesterol esters. Unlike the other glutathione peroxidases, which are tetrameric enzymes, GPx4 is a monomeric enzyme and is rich in hydrophobic amino acid residues that make GPx4 interact with complex lipids in membranes and reduce membrane lipid hydroperoxides (64). GPx4 is considered to be the primary enzymatic defense system against oxidative damage to cellular membranes (8).

The other pathway for removing membrane lipid peroxides from membranes is through the coupled actions of phospholipase A2 and GPx1 (66).
in vivo. Against FFA-induced zyme that selectively decreases lipid peroxides, protects mice vitro. The results show that overexpression of GPx4, an en-

of GPx4-overexpressing mice and littermate controls to FFA in during hyperglycemic clamps. Additionally, we exposed islets without GPx4 overexpression both in isolated islets and in vivo. The data represent means ± SE; n = 3/group. WT, wild type; GINF, glucose infusion rate.

To investigate the involvement of lipid peroxides in β-cell lipotoxicity, we intravenously infused FFA in GPx4-overexpressing mice, which were reported to have two- to threefold overexpression of GPx4 protein in different tissues and to be protected from oxidant-induced liver injury (33). The effect of FFA infusion on β-cell function was evaluated in mice with or without GPx4 overexpression both in isolated islets and in vivo during hyperglycemic clamps. Additionally, we exposed islets of GPx4-overexpressing mice and littermate controls to FFA in vitro. The results show that overexpression of GPx4, an enzyzme that selectively decreases lipid peroxides, protects mice against FFA-induced β-cell dysfunction in vitro, ex vivo, and in vivo.

MATERIALS AND METHODS

Animals

All procedures were conducted in accordance with the Canadian Council of Animal Care Standards and were approved by the Animal Care Committee of the University of Toronto. Male 11- to 13-wk-old GPx4-overexpressing mice (transgenic (Tg)) or their wild-type (WT) littermate controls were used for the experiments. The Tg mice that overexpress GPx4 in all tissues were generated using a genomic clone containing the human gpx4 gene. These mice, on a C57BL6 background (genotype of hemizygous and homozygous mice cannot be differentiated), do not show any alterations in the activities of GPx1 and catalase, and hence, their primary phenotype is their ability to remove membrane lipid peroxides (53). The animals were housed in the University of Toronto’s Department of Comparative Medicine. They were exposed to a 12:12-h light-dark cycle and were fed rodent chow (Teklad Global 2018; Harlan-Teklad, Madison, WI).

Surgery

An indwelling catheter was inserted into the right internal jugular vein of anesthetized mice for infusion. Polyethylene catheters (PE-10; Cay Adams, Boston, MA), each extended with a segment of silastic tubing (length of 1.7 cm; Dow Corning, Midland, MI), were used. The catheter was extended to the level of the right atrium, tunneled subcutaneously, and exteriorized. The catheter was filled with heparin (1,000 U/ml) in normal saline to maintain patency. The tail vein was used for sampling.

Intravenous Infusions

After a minimum 3- to 4-day period of postsurgery recovery, mice were connected to the infusion apparatus. The infusion lines ran inside a tether that was fitted to the subcutaneous implant. Each mouse was placed in a cage, and the infusion lines were protected by a tether and run through a swivel, which was suspended on the top of the cage to give complete freedom of movement to the mouse. Forty-eight-hour infusions were started through the jugular vein. The mice were infused for 48 h with either l) saline (WT-SAL, Tg-SAL) or 2) oleate (WT-OLE, Tg-OLE) prepared in FFA-free BSA (41, 51) to elevate plasma FFA by 1.5- to twofold (0.4 µmol/min). We have not used BSA as a control, as we have shown previously that rats infused with either BSA or saline for 48 h had similar insulin and C-peptide levels (41). Our data show that this observation is also true in mice (Table 1).

Pancreatic Islet Isolation

Pancreatic islets were isolated in 5-h-fasted mice following a 48-h infusion, as described previously (16).

Determination of the Level of Overexpression of GPx4 Protein

Islets of WT and Tg mice were collected and frozen. Afterward, the protein levels of the GPx4 gene (mouse GPx4 in WT, mouse, and human GPx4 in Tg) were quantified by Western blot analysis (53, 54).

ROS Measurements

Islet ROS was measured with H2DCF-DA (30), which detects most ROS, including lipid peroxides and peroxynitrite anion (33). Briefly, following 1 h of preincubation at 37°C in Krebs-Ringer buffer containing 10 mM HEPES buffer (KRBH) containing 2.8 mM glucose, islets were incubated with 10 µM H2DCF-DA (Sigma) in KRBH containing 2.8 mM glucose for 15 min at 37°C. After washing with KRBH, islet fluorescence was measured at 480 nm excitation and 510 nm emission using an Olympus fluorescent BX51W1 microscope. Approximately 10 islets/mouse were measured (n). Data are expressed as percentage of SAL ± SE. Data were analyzed using ImagePro.

Table 1. GINF, insulin, and C-peptide levels and sensitivity and disposition indexes during the hyperglycemic clamp in WT mice after 48-h saline or 2% BSA (vehicle for oleate) infusion

<table>
<thead>
<tr>
<th></th>
<th>Saline Group (n = 3)</th>
<th>BSA Group (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GINF, µmol·kg⁻¹·min⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clamp</td>
<td>338.27 ± 24.59</td>
<td>385.82 ± 11.55</td>
</tr>
<tr>
<td>Insulin, pM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>85.50 ± 10.50</td>
<td>80.27 ± 3.77</td>
</tr>
<tr>
<td>Clamp</td>
<td>106.91 ± 20.25</td>
<td>126.27 ± 26.30</td>
</tr>
<tr>
<td>C-peptide, nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.41 ± 0.1</td>
<td>0.285 ± 0.04</td>
</tr>
<tr>
<td>Clamp</td>
<td>0.81 ± 0.12</td>
<td>0.63 ± 0.1</td>
</tr>
<tr>
<td>Sensitivity index</td>
<td>3.26 ± 0.55</td>
<td>3.44 ± 0.58</td>
</tr>
<tr>
<td>Disposition index</td>
<td>2.02 ± 0.38</td>
<td>2.06 ± 0.16</td>
</tr>
</tbody>
</table>

Fig. 1. Glutathione peroxidase 4 (GPx4) in islets of wild-type (WT) and transgenic (TG) mice. A: total GPx4 protein levels in islets isolated from GPx4-overexpressing Tg mice (n = 8) and their littermate WT control mice (n = 9), as determined by Western blot. Data are means ± SE. ***p < 0.001. B: representative immunoblot.
Table 2. FFA, glucose, and insulin levels during the 48-h infusions

<table>
<thead>
<tr>
<th>Mouse and Treatment (Time, h)</th>
<th>FFA, µM</th>
<th>Plasma glucose, mM</th>
<th>Insulin, pM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Significance vs. SAL</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>WT-SAL 0</td>
<td>415 ± 59</td>
<td></td>
<td>8.51 ± 0.38</td>
</tr>
<tr>
<td>WT-SAL 48</td>
<td>372 ± 50</td>
<td></td>
<td>8.67 ± 0.57</td>
</tr>
<tr>
<td>WT-OLE 0</td>
<td>668 ± 64</td>
<td>P &lt; 0.001 vs. WT-SAL</td>
<td>8.29 ± 0.21</td>
</tr>
<tr>
<td>WT-OLE 48</td>
<td>1,215 ± 108</td>
<td></td>
<td>8.50 ± 0.41</td>
</tr>
<tr>
<td>Tg-OLE 0</td>
<td>719 ± 43</td>
<td>P &lt; 0.01 vs. Tg-SAL</td>
<td>8.88 ± 0.32</td>
</tr>
<tr>
<td>Tg-OLE 48</td>
<td>1,286 ± 183</td>
<td></td>
<td>8.65 ± 0.33</td>
</tr>
<tr>
<td>Tg-SAL 0</td>
<td>400 ± 48</td>
<td></td>
<td>8.59 ± 0.20</td>
</tr>
<tr>
<td>Tg-SAL 48</td>
<td>430 ± 148</td>
<td></td>
<td>8.05 ± 0.17</td>
</tr>
</tbody>
</table>

Data represent means ± SE. FFA, free fatty acids; WT, wild type; Tg, transgenic; SAL, saline; OLE, oleate.

Thiobarbituric Acid-Reacting Substances Assay

Lipid peroxidation in islets was measured indirectly by the measurement of the secondary product malondialdehyde (MDA), using the thiobarbituric acid method. MDA forms a colored complex in the presence of thiobarbituric acid, which is detectable by measurement of absorbance at 532 nm spectrophotometrically (39). Islets of WT and Tg mice treated with saline/oleate were washed with PBS, and MDA concentrations were measured using the thiobarbituric acid reacting substances assay kit (Cayman Chemical, Ann Arbor, MI). The levels of MDA were expressed as micromoles per milligram of islet protein.

Lipid Hydroperoxide Assay

Lipid hydroperoxides were measured directly, utilizing the redox reactions with ferrous ions (43). Islets of WT and Tg cultured in control/oleate medium were washed with PBS, and lipid hydroperoxide concentrations were measured using the lipid peroxidation assay kit (Cayman Chemical).

Ex Vivo Evaluation of β-Cell Function

Isolated islets of saline/oleate-infused mice were preincubated for 1 h at 37°C in KRBH and 2.8 mM glucose. Thereafter, 10 islets of approximately the same size were incubated in duplicate at 6.5 or 22 mM glucose for 2 h at 37°C. Insulin concentration in the medium was analyzed using a radioimmunnoassay kit specific for rat/mouse insulin (Linco, St. Charles, MO). The islets used for secretion were then subjected to acid ethanol extraction (62) for insulin content measurement.

In Vivo Evaluation of β-Cell Function (Hyperglycemic Clamp)

The hyperglycemic clamp was performed in 5-h-fasted conscious mice. After 46 h of saline/oleate infusion, basal insulin and C-peptide were measured (t = 0), and then an infusion of 37.5% glucose was started. Blood glucose was maintained at 22 mM by adjusting the rate of glucose infusion according to frequent (every 10 min) glycemic determination from tail blood. Samples for insulin and C-peptide were taken during the last 20 min of the 120-min hyperglycemic clamp. Blood glucose was measured using a Hemocue Analyzer II (HemoCue, Lake Forest, CA).

Calculations

Insulin sensitivity index. Insulin sensitivity during the hyperglycemic clamp is calculated as insulin sensitivity index (M/I). M/I is calculated according to the following formula: \( M/I = G_{INF}/\text{insulin} \), where \( G_{INF} \) is the rate of glucose infusion and insulin is the plasma insulin concentration at individual time points during the last 20 min of the hyperglycemic clamp.

Disposition index. Insulin secretion in vivo has to be evaluated in the context of insulin sensitivity since the normal β-cell compensates for insulin resistance. In normal subjects, the relationship between insulin sensitivity and insulin secretion is hyperbolic (5, 31); i.e., the product of insulin sensitivity and insulin secretion is constant defined as disposition index (DI) and considered as a measurement of β-cell function. DI was calculated according to the following formula: \( DI = M/I \times C\text{-peptide} \), where M/I is calculated as described above and during the last 20 min of the hyperglycemic clamp.

Table 3. Pancreatic β-cell mass and individual β-cell area following 48-h infusion

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pancreatic Weight, g</th>
<th>%β-Cell Area</th>
<th>β-Cell Mass, mg/pancreas</th>
<th>Individual β-Cell Area, μm²/β-cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-SAL (n = 5)</td>
<td>0.191 ± 0.05</td>
<td>0.742 ± 0.240</td>
<td>1.051 ± 0.140</td>
<td>130.360 ± 3.320</td>
</tr>
<tr>
<td>WT-OLE (n = 5)</td>
<td>0.182 ± 0.02</td>
<td>0.742 ± 0.132</td>
<td>1.042 ± 0.326</td>
<td>131.598 ± 7.115</td>
</tr>
<tr>
<td>Tg-OLE (n = 5)</td>
<td>0.151 ± 0.02</td>
<td>0.686 ± 0.177</td>
<td>1.086 ± 0.330</td>
<td>120.708 ± 5.844</td>
</tr>
<tr>
<td>Tg-SAL (n = 4)</td>
<td>0.175 ± 0.02</td>
<td>0.573 ± 0.039</td>
<td>0.976 ± 0.047</td>
<td>143.696 ± 2.034</td>
</tr>
</tbody>
</table>

Data represent means ± SE.
Plasma Assays

Plasma FFA were measured with an enzymatic colorimetric kit (Wako Industries, Neuss, Germany). Radioimmunoassay specific for mouse insulin and C-peptide (Linco) was used to determine plasma concentrations (interassay coefficient of variation <10%).

Statistics

Data are means ± SE. One-way nonparametric ANOVA for repeated measurements, followed by Duncan’s test, was used to compare treatments. Calculations were performed using SAS (Cary, NC).

RESULTS

Human GPx4 is Overexpressed in Islets of the Tg Mice

Western blots performed in islet lysates of WT and Tg mice show that the protein level of GPx4 in islets of Tg mice is 50% higher than in the WT animals ($P < 0.001$; Fig. 1, A and B).

FFA Levels are Increased After 48-h Oleate Infusion

After the 48-h infusions, the oleate-treated mice had higher plasma FFA than mice treated with saline, as expected (Table 2). The infusion of oleate did not affect plasma glucose or insulin (Table 2).

Islet Morphology Is Not Changed In Any Group

Islet morphological studies were performed in the isolated pancreas after 48-h infusion and hyperglycemic clamps. β-Cell mass and individual area were similar in WT-SAL and TG-SAL mice. Oleate infusion did not have any effect on these parameters (Table 3).

Islets of Tg Mice Infused With Oleate are Protected From ROS and MDA Elevation and β-Cell Dysfunction Ex Vivo

Forty-eight hours of oleate infusion increased intracellular ROS levels in islets of WT mice ($P < 0.001$). Overexpression of GPx4 abolished the oleate-induced increase in ROS ($P < 0.05$, Tg-OLE vs. WT-OLE, not significant vs. WT-SAL; Fig. 2A). Likewise, transgenic mice infused with oleate were protected against the increased levels of the lipid peroxidation product MDA (Fig. 2B).

Forty-eight-hour oleate infusion impaired GSIS significantly at 22 mM glucose in WT mice ($P < 0.05$). In contrast, Tg mice infused with oleate had similar GSIS compared with Tg-SAL mice (Fig. 2C). Likewise, the islet insulin content was significantly decreased in WT-OLE mice compared with WT-SAL ($P < 0.001$). Tg mice were protected from the decrease in islet insulin content induced by oleate ($P < 0.05$; Fig. 2D).

Tg Mice are Protected From Oleate-Induced β-Cell Dysfunction In Vivo

After 48 h of infusion, plasma FFA levels were twofold higher in oleate-infused WT and Tg mice than in saline-treated WT and Tg mice (data not shown). During the hyperglycemic clamp, plasma glucose was elevated to 20–22 mM (Fig. 3A) in all groups. In WT-OLE mice, a lower GINF was needed to clamp glucose at 20–22 mM ($P < 0.05$ vs. all), indicating that the circulating insulin was inadequate to compensate for insulin resistance. In contrast, GINF in Tg-OLE was similar to Tg-SAL (Fig. 3B).

No significant difference was observed in basal insulin or C-peptide levels among the different groups. In the oleate-treated WT mice, clamp insulin ($P < 0.001$; Fig. 4A) and insulin secretion ($P < 0.05$ vs. all) were increased compared with WT-SAL mice (Fig. 4B). In contrast, insulin secretion and clamp insulin content in Tg-OLE were not significantly different from those in Tg-SAL mice (Fig. 4C and D).

Fig. 2. Effect of 48-h oleate infusion in freshly isolated islets of WT and GPx4 Tg mice. Islets of GPx4-overexpressing TG mice and their WT littermate control mice were treated intravenously for 48 h with an infusion of saline (SAL) or oleate (OLE; 0.4 μmol/min) to elevate plasma free fatty acid levels 1.5- to 2-fold. A: dihydrodichlorofluorescein diacetate (H$_2$DCF-DA)-detected reactive oxygen species (ROS). Data are expressed as %WT-SAL ± SE; $n = 4$ in all groups. Representative fluorescent images of the islets are shown. B: malondialdehyde (MDA) levels detected by thiobarbituric acid-reacting substances assay. Data are expressed as %WT-SAL ± SE; WT-SAL, $n = 8$; WT-OLE, $n = 5$; Tg-OLE, $n = 6$; Tg-SAL, $n = 5$. C and D: insulin secretory response to glucose (C) and islet insulin content (D) in freshly isolated islets of mice treated as described above. Data are means ± SE; WT-SAL, $n = 8$; WT-OLE, $n = 6$; Tg-OLE, $n = 6$; Tg-SAL, $n = 5$. *$P < 0.05$ vs. WT-SAL; †$P < 0.001$, WT-OLE vs. WT-SAL; **$P < 0.05$, WT-OLE vs. Tg-OLE; ‡$P < 0.05$ vs. all; ¶$P < 0.05$, WT-OLE vs. Tg-SAL and Tg-OLE.
GPx4 OVEREXPRESSION AND β-CELL FUNCTION

C-peptide \((P < 0.05; \text{Fig. } 4B)\) levels were significantly higher compared with the saline-treated groups.

WT-OLE mice had a significantly lower sensitivity index compared with the saline-infused mice \((P < 0.001 \text{ vs. WT-SAL and Tg-SAL})\), indicating that oleate induced insulin resistance. However, in Tg-OLE mice the sensitivity index was higher than that in WT-OLE \((P < 0.01, \text{Tg-OLE vs. WT-OLE})\) but lower than that in mice infused with saline \((P < 0.05, \text{Tg-OLE vs. WT-SAL and Tg-SAL})\), indicating that overexpression of GPx4 partially protected the mice from oleate-induced insulin resistance \((\text{Fig. } 4C)\). The DI, an index of β-cell function, was significantly reduced only in the WT-OLE group \((P < 0.05 \text{ WT-OLE vs. all; Fig. } 4D)\) but not in Tg-OLE, indicating that GPx4-overexpressing mice are protected against oleate-induced β-cell dysfunction in vivo.

Islets of Tg Mice Cultured With Oleate are Protected From ROS and Lipid Hydroperoxide Elevation and β-Cell Dysfunction In Vitro

Forty-eight hours of culture in oleate increased the levels of ROS and lipid hydroperoxides in WT islets compared with culture in control medium \((P < 0.05)\). Islets of mice overexpressing GPx4 were protected from the effects of oleate to increase ROS \((\text{Fig. } 5A)\) and lipid hydroperoxides \((\text{Fig. } 5B)\). Oleate decreased GSIS \((P < 0.01 \text{ WT-OLE vs. all; Fig. } 5C)\) and tended to decrease insulin content \((\text{Fig. } 5D)\) in islets of WT mice. These effects of oleate were absent in the islets of GPx4-overexpressing mice.

DISCUSSION

Here, we show that whole body overexpression of the enzyme GPx4 in mice prevents the increase in ROS caused by 48-h FFA infusion and prevents β-cell dysfunction both ex vivo and in vivo.

The attack of free radicals on biological membranes results in the generation of the primary products lipid hydroperoxides, which are short-lived and thus decompose to form toxic and more stable secondary end products, and the lipid aldehydes such as MDA and 4-hydroxynonenal \((17)\). Since lipid aldehydes are rather long-lived, they can reach and attack targets such as phospholipids, DNA, and proteins distant from their original site of formation \((3, 17, 40, 52)\).

GPx activity is very low in islets \((22, 56, 63)\), in agreement with the low antioxidant defenses in β-cells \((34)\). Harmon et al. \((25)\), Tanaka et al. \((61)\), Lepore et al. \((35)\), and Mysore et al. \((48)\) have shown that GPx1 overexpression in β-cells or islets improves their function. However, another report has shown that whole body overexpression of GPx1 in mice results in insulin resistance \((42)\). The current study is the first report of the effects of GPx4 on β-cell function.

We show that mice with systemic overexpression of GPx4 in whole body have decreased levels of ROS and MDA induced by 48-h oleate infusion and are protected against the decrease in insulin secretion caused by oleate ex vivo in isolated islets and in vivo during hyperglycemic clamps.

During the hyperglycemic clamp, unlike the rat model in our previous studies \((41, 51)\), absolute insulin and C-peptide levels were elevated in mice that received 48-h oleate infusion. However, insulin resistance was also induced in these animals, as shown by the lower M/I. This is in contrast with our studies in rats, where oleate did not affect insulin sensitivity \((41, 51)\). Besides species-specific effect difference, the reason behind this finding may be the strain of mice, as C57BL/6 mice are very susceptible to fatty-induced insulin resistance \((59)\). Insulin secretion in vivo has to be evaluated in the context of insulin sensitivity, since the normal β-cell compensates for insulin resistance by increasing insulin secretion independent of plasma glucose. In subjects with normal glucose tolerance, insulin secretion and sensitivity are linked through a hyperbolic relationship \((5, 31)\); i.e., the product of insulin sensitivity and insulin secretion is a constant. This constant is defined as the DI and is a well-established measure of β-cell function \((18, 31)\). DI was decreased in oleate-infused WT mice, demonstrating that β-cell dysfunction was induced by 48-h oleate infusion. Transgenic mice overexpressing GPx4 and infused with oleate were completely protected from β-cell dysfunction despite their partial prevention from insulin resistance. During hyperglycemic clamp, the sensitivity index reflects the sensitivity in the peripheral tissues (muscle and fat). The partial protection from insulin resistance can be explained by the overexpression of GPx4 in these tissues.

With regard to the results of the hyperglycemic clamp, the limitation of the calculated indices should be acknowledged. The M/I is obtained by normalizing the GINF for plasma glucose level. There are limitations to using this method, as it has been reported that the relationship between circulating insulin levels and insulin action is not linear at high insulin concentrations \((50)\). However, it is impossible to perform additional
Fig. 4. Effect of 48-h oleate infusion on plasma insulin, C-peptide, sensitivity index, and disposition index during hyperglycemic clamps in WT and GPx4 TG mice. Insulin (A) and C-peptide levels (B) before and during the last 20 min of the hyperglycemic clamp and sensitivity (M/I/GINF/insulin; C) and disposition indexes (DI = M/I × C-peptide; D) during the last 20 min of hyperglycemic clamp in mice treated as described in the legend to Fig. 2. Data are means ± SE. WT-SAL, n = 6; WT-OLE, n = 7; TG-OLE, n = 6; TG-SAL, n = 6. ***P < 0.001 vs. saline-treated groups; *P < 0.05 vs. saline-treated groups; ††P < 0.01, WT-OLE vs. TG-OLE; #P < 0.05 vs. all.

Fig. 5. Effect of 48-h OLE exposure in cultured islets of WT and GPx4-overexpressing TG mice. Islets of GPx4-overexpressing TG mice and their WT littermate control mice were cultured for 48 h in free fatty acid-free BSA (CON) or OLE (0.4 mM). A and B: H$_2$DCF-DA-detected ROS (A) and hydroperoxide levels (B). Data are expressed as %WT-CON ± SE and means ± SE. WT-CON, n = 3; WT-OLE, n = 4; TG-OLE, n = 4; TG-CON, n = 3. The representative fluorescent images of the islets are shown. C and D: insulin secretory response to glucose (C) and islet insulin content in cultured islets of mice (D), as described above. Data are means ± SE. WT-CON, n = 4; WT-OLE, n = 5; TG-OLE, n = 5; TG-CON, n = 4. *P < 0.05, WT-OLE vs. WT-CON; ###P < 0.01 vs. all.
hyperinsulinemic euglycemic clamp studies (the gold standard method for assessing insulin sensitivity) in the same small animal. Also, C-peptide levels were taken as an index of absolute insulin secretion, as calculations of insulin secretion by C-peptide deconvolution, which is commonly performed in humans (10, 11, 65), cannot be performed in the mouse. This is because the parameters of C-peptide kinetics cannot be derived in the mouse, as mouse C-peptide (species specific) is not available in amounts sufficient for in vivo injections. However, studies in humans have shown that the kinetics of C-peptide are not influenced by glucose or fat (4, 24).

Throughout the 48-h infusion period, blood glucose and plasma insulin levels were unchanged among the different treatments despite the insulin resistance and the decrease in GSIS in the wild-type mice infused with oleate. This indicates that the effect of FFA on GSIS is independent of hyperglycemia and also of β-cell overstimulation (28, 57). It should be noted that β-cell dysfunction upon glucose stimulation can be present before hyperglycemia is apparent, as shown by many studies in offspring of type 2 diabetes mellitus (20, 58). Our studies in lipid-infused humans support our findings (68–71), although in some of these studies, a small elevation in fasting plasma glucose was observed (10).

A limitation of our study is the lack of β-cell-specific overexpression of GPx4. Harmon et al. (25) established the first in vivo model of β-cell-specific overexpression of GPx1 and showed that the overexpression of the transgene protected the mice from streptozotocin-induced diabetes and ameliorated hyperglycemia in db/db mice. They attributed these beneficial effects to the preservation of musculoaponeurotic fibrosarcoma oncogene homolog A in the transgenic db/db mice. These studies support the concept that progressive dysfunction of β-cells induced by chronic elevation of glucose and chronic oxidative stress can be compensated by enhancing GPx1. GPx4 is more specific to lipid peroxides than GPx1 (64). In fact, the breakdown of lipid peroxides by GPx1 also requires the action of phospholipase A2, whose affinity to lipid peroxides is 104-fold less than GPx4 (2). Since our model is a whole body transgenic mouse, in order for us to prove that the beneficial effects of GPx4 can be explained by its protective effects on the β-cell, we cultured islets isolated from wild-type and the transgenic mice in control/oleate medium. Our in vitro results are consistent with the in vivo results.

However, how can lipid peroxides, which occur in all membranes, cause a decrease in insulin secretion? Lipid peroxides generate soluble products that can cause β-cell dysfunction (55). We have shown that islets of rats infused with oleate for 48 h have increased levels of cytosolic superoxide and the lipid peroxidation product MDA. Both cytosolic superoxide and MDA were decreased when apocynin, an NADPH oxidase inhibitor, was coinjected with oleate (32). This observation suggests that oleate induces oxidative stress in islets through NADPH oxidase-generated cytosolic superoxide, which is converted to lipid peroxides and finally to reactive aldehydes (23). These lipid peroxidation products impair glucose-stimulated insulin secretion probably through affecting glycolysis and glucose oxidation (44). There is also evidence that lipid peroxidation products activate the IKKβ/NF-κB pathway (6, 19). Our unpublished data are consistent with the involvement of the IKKβ/NF-κB pathway in FFA-induced β-cell dysfunction.

We have not found an increase in mitochondrial superoxide in this model (32). However, since lipid peroxides are formed by the attack of superoxide-generated hydroxyl radical on membranes (23, 27), the mitochondrial membranes can also be a target of this attack by cytosolic superoxide (1). Lipid peroxidation products are known to decrease mitochondrial membrane fluidity (13), causing mitochondrial dysfunction. Hence, overexpression of GPx4 can also protect mitochondria from dysfunction caused by lipid peroxidation products.

Contrary to our finding of the involvement of oxidative stress in impairing β-cell function, Moore et al. (45) demonstrated evidence against the involvement of oxidative stress in fatty acid inhibition of insulin secretion. However, the time of culture, the presence of antioxidant in the medium, the probe used for detecting ROS, and the use of dispersed islets vs. whole islets account for the differences in the results (51). Similarly, a body of evidence that includes a study in Zucker diabetic fatty rats treated with either a lipid-lowering or glucose-lowering agent (26) raises the concern that fat alone does not have deleterious effects on β-cell function unless concurrent high glucose levels are also present (7, 26, 29). However, there are ample data in the literature that show the existence of an FFA-induced decrease in β-cell function without glucose-induced β-cell dysfunction, at least in genetically predisposed individuals (14, 36).

In conclusion, we show for the first time that overexpression of GPx4 prevents the increase in lipid peroxides and protects against the FFA-induced decrease in β-cell secretory function in an in vivo model. Therefore, GPx4 activation by mimetics (12, 15, 49) may be of therapeutic interest in the prevention of β-cell failure in obesity-associated type 2 diabetes, where FFA levels are elevated.

ACKNOWLEDGMENTS

We thank Loretta Lam, Department of Physiology, University of Toronto, for excellent technical assistance.

GRANTS

This work was supported by Canadian Institutes of Health Research Grants MOP-69018 (A. Giacca). K. Koulaian was supported by Scholarships from the Banting and Best Diabetes Centre (University of Toronto), an Ontario Graduate Scholarship, and an Ontario Graduate Scholarship for Science and Technology.

DISCLOSURES

The authors declare that there is no dualty of interest associated with this article.

AUTHOR CONTRIBUTIONS

K.K., A.I., K.Y., T.D., and A.S. performed the experiments; K.K. and A.I. analyzed the data; K.K., A.I., I.G.F., Q.R., and A.G. interpreted the results of the experiments; K.K. prepared the figures; K.K. drafted the manuscript; K.K., A.I., K.Y., T.D., A.S., I.G.F., Q.R., and A.G. revised the manuscript; K.K., A.I., K.Y., T.D., A.S., I.G.F., Q.R., and A.G. approved the final version of the manuscript; A.G. contributed to the conception and design of the research.

REFERENCES


Sako Y, Grill VE.

Robertson RP, Harmon JS.


