

Evidence Against the Involvement of Oxidative Stress in Fatty Acid Inhibition of Insulin Secretion

Patrick C. Moore,¹ Marco A. Ugas,¹ Derek K. Hagman,¹ Susan D. Parazzoli,¹ and Vincent Poytout^{1,2}

Prolonged exposure to elevated levels of fatty acids adversely affects pancreatic β -cell function. Here we investigated 1) whether ceramide synthesis, which we reported to mediate fatty acid inhibition of insulin gene expression, also inhibits insulin secretion and 2) whether fatty acid inhibition of insulin secretion involves the generation of reactive oxygen species (ROS), nitric oxide (NO), or prostaglandin E₂ (PGE₂). A 72-h culture of islets in the presence of palmitate or oleate resulted in a marked decrease in glucose-induced insulin release assessed in 1-h static incubations. This effect was reproduced by exogenous diacylglycerol, but not by a cell-permeable analog of ceramide. Culture in the presence of fatty acids was not associated with an increase in intracellular peroxide or NO levels, neither was insulin secretion restored by antioxidants or an inhibitor of NO production. Exposure to fatty acids led to an increase in PGE₂ release, but an inhibitor of cyclooxygenase 2 was unable to prevent fatty acid inhibition of insulin secretion. These results indicate that fatty acid inhibition of insulin secretion 1) is not mediated by de novo ceramide synthesis, ROS, NO, or PGE₂, and 2) is likely to be caused by the generation of signals or metabolites downstream of diacylglycerol. *Diabetes* 53:2610–2616, 2004

Fatty acids acutely amplify glucose-induced insulin secretion from the pancreatic β -cell, but they become harmful when present at elevated levels for prolonged periods of time (reviewed in 1). Chronic, deleterious effects of fatty acids on β -cell function, collectively referred to as lipotoxicity, include inhibition of insulin secretion (2–5) and gene expression (6–10), as well as promotion of cell death by apoptosis (11–16). The cellular and molecular mechanisms of lipotoxicity are only partly understood. It has been proposed that the simultaneous elevation of fatty acids and glucose concentrations leads to inhibition of fatty acid oxidation and the generation of lipid-derived cytosolic signals that in turn adversely affect β -cell function (17). Indeed, lipotoxic

effects of fatty acids are only observed in the presence of high glucose (8,9,16,18). Such cytosolic signals might be derived either from the esterification pathway or, when the fatty acid is palmitate, from de novo synthesis of ceramide. We have shown that lipotoxicity in isolated islets is associated with increased esterification into cellular lipids (9) and that forcing triglyceride synthesis by overexpression of diacylglycerol acyltransferase 1 (DGAT-1) inhibits glucose-induced insulin secretion (19). Surprisingly, insulin gene expression was not impaired by DGAT-1 overexpression, suggesting that the mechanisms whereby chronic fatty acids affect insulin secretion and insulin gene expression might be distinct. Indeed, we recently showed that palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis (10). Whether ceramide generation also plays a role in fatty acid inhibition of insulin secretion is unknown. Alternatively, the nature of the downstream signals that are activated by fatty acid metabolites and that eventually inhibit insulin secretion is unknown. Several studies have suggested the potential involvement of oxidative stress, either in the form of reactive oxygen species (ROS) (20–24) or reactive nitrogen species (22,25), a hypothesis supported by the protection against lipotoxicity afforded by molecules with antioxidant properties (26–28). In addition, it is conceivable that excessive fatty acid levels, either via remodeling of the plasma membrane and arachidonic acid release (29) or via generation of ceramide (30), induce prostaglandin E₂ (PGE₂) production, which would in turn inhibit insulin secretion (31).

Therefore, the aims of this study were 1) to assess whether ceramide generation plays a role in fatty acid inhibition of insulin secretion; 2) to evaluate whether prolonged exposure of isolated rat islets to elevated levels of fatty acids is associated with production of ROS, nitric oxide (NO), or PGE₂; and, if so, 3) to determine whether fatty acid inhibition of insulin secretion can be prevented by antioxidants, inhibition of NO synthesis, or inhibition of PGE₂ formation.

RESEARCH DESIGN AND METHODS

Reagents. α -[³²P]UTP and γ -[³²P]ATP were from Amersham Biosciences (Piscataway, NJ). Propidium iodide and dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) were from Molecular Probes (Eugene, OR). Interleukin-1 β (IL-1 β) was from R&D Systems (Minneapolis, MN). C2-ceramide and dihydro-C2-ceramide were from Avanti Polar Lipids (Alabaster, AL). 1,2-dioctanoyl-sn-glycerol (DAG) was from Biomol (Plymouth Meeting, PA). Collagenase, palmitic acid (sodium salt), oleic acid (sodium salt), fatty acid-free BSA, Griess reagent (modified), and all other reagents (analytical grade) were from Sigma-Aldrich (St. Louis, MO). Silica gel 60 thin-layer chromatography plates were from Whatman (Clifton, NJ).

Animals. Six-week-old male Wistar rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Animals were housed on a 12-h light/dark

From the ¹Pacific Northwest Research Institute, Seattle, Washington; and the ²Department of Medicine, University of Washington, Seattle, Washington.

Address correspondence and reprint requests to Vincent Poytout, DVM, PhD, Pacific Northwest Research Institute, 720 Broadway, Seattle, WA 98122. E-mail: vpoitout@pnri.org.

Received for publication 22 March 2004 and accepted in revised form 16 July 2004.

carboxy-H₂DCFDA, dichlorodihydrofluorescein diacetate; DAG, dioctanoyl-sn-glycerol; DETAPAC, diethylenetriamine-penta-acetic acid; DGAT-1, diacylglycerol acyltransferase-1; IL, interleukin; L-NAME, N-nitro-L-arginine-methyl ester; NAC, N-acetyl-cysteine; PGE₂, prostaglandin E₂; ROS, reactive oxygen species.

© 2004 by the American Diabetes Association.

cycle with free access to water and standard laboratory food. The Pacific Northwest Research Institute Institutional Animal Care and Use Committee approved all procedures using animals.

Rat islet isolation and culture. Rat islets were isolated by collagenase digestion as described (9). After an overnight culture in RPMI 1640 containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 11.1 mmol/l glucose, batches of 100–200 islets were resuspended in fresh media and incubated in various experimental conditions as described in RESULTS. Preparation of culture media containing fatty acids was performed as previously described (9). The final molar ratio of fatty acids to BSA was 5:1. All control conditions contained the same amount of BSA and vehicle (EtOH and H₂O, 1:1 dilution) as those with fatty acids.

Insulin secretion and insulin content. For measurements of cumulative secreted insulin, small aliquots of culture media were sampled at the end of the culture period. For assessment of glucose-induced insulin secretion in static incubations, batches of 10 islets each were washed twice in Krebs-Ringer buffer containing 0.1% BSA and 2.8 mmol/l glucose for 20 min at 37°C and then incubated for 60 min in the presence of 2.8 or 16.7 mmol/l glucose. Each condition was run in duplicate. Intracellular insulin content was measured in duplicate batches of 20 islets after acid-alcohol extraction. Insulin was measured using a sensitive rat insulin radioimmunoassay kit (Linco Research, St. Charles, MO). Total proteins were measured in duplicate batches of 20 islets using a BCA assay (Pierce, Rockford, IL).

Ribonuclease protection assay. Ribonuclease protection assays were carried out using the Direct Protect lysis ribonuclease protection assay kit (Ambion, Austin, TX) as described (10). Briefly, batches of 100 cultured islets were washed twice in Hank's balanced salt solution, suspended in 150 μ l of lysis buffer (provided in the kit), and sonicated 4×1 s at low power. α -[³²P]UTP-labeled antisense probes were transcribed from a T7 promoter to a 360-base pair (bp) sequence for the rat II preproinsulin gene cloned into a pBLUEScript plasmid (Stratagene, La Jolla, CA). Control probes were made to a 245-bp sequence of mouse β -actin (Ambion). Probes were hybridized directly in the lysate overnight at 42°C. The lysate was then treated as directed by the manufacturer, and protected fragments were resolved by electrophoresis on 5% denaturing gels and analyzed by autoradiography and phosphorimaging. The probes were determined to be in excess for each experiment.

Determination of DAG content. Batches of 100 islets each were washed in ice-cold PBS, and lipids were extracted by chloroform and methanol (1:2 vol:vol). Extracted lipids were dried under N₂, and DAG content was measured by the DAG kinase assay and thin-layer chromatography analysis as described (32). Dried lipids were solubilized in 20 ml of detergent solution (7.5% n-octyl- β -glucopyranoside/5 mmol/l cardiolipin in 1 mmol/l diethylenetriamine-penta-acetic acid [DETAPAC]). After adding 50 μ l of assay buffer (120 mmol/l HEPES/100 mmol/l LiCl/25 mmol/l MgCl₂/2 mmol/l EGTA), 10 μ l of 20 mmol/l dithiothreitol, and 10 μ l of DAG kinase and enzyme diluent at 1:1 dilution (1 mmol/l DETAPAC/10 mmol/l imidazole), the reaction was started by adding 10 μ l of 10 mmol/l γ -[³²P]ATP (specific activity 80 mCi/mmol) prepared in 100 mmol/l imidazole/1 mmol/l DETAPAC. After mixing, the reaction was continued for 30 min at room temperature. Lipids were extracted again and dried under N₂. Samples were resuspended in 100 μ l chloroform, spotted onto thin-layer chromatography plates, and developed with chloroform, methanol and acetic acid (65:15:5 vol:vol:vol). The radioactive spot corresponding to [³²P]phosphatidic acid was identified using autoradiography. 1,2-DAG standards were run on each plate.

Measurement of ROS formation and propidium iodide staining. The fluorescent probe carboxy-H₂DCFDA was used to measure intracellular peroxide in dissociated islets by flow cytometry as previously described (33). At 3 h before the end of the culture period, batches of 100 islets each were incubated for 1 h in 100 μ mol/l of freshly prepared carboxy-H₂DCFDA in PBS at 37°C for 1 h and then further exposed to the culture conditions for another 2 h. Islets were then washed with PBS and incubated in 300 μ l 0.25% trypsin/1 mmol/l EDTA for 5 min at 37°C, followed by gentle mechanical dispersion through a 1-ml pipette tip. The reaction was stopped by the addition of 700 μ l cold PBS, and islet cells were then collected by centrifugation and resuspended in 500 μ l PBS. Propidium iodide was added to a final concentration of 6 μ g/ml, and the cells were incubated in the dark at 4°C for 30 min. Single-cell populations, defined based on size on a forward-versus-size scatter, were analyzed for carboxy-H₂DCFDA fluorescence and propidium iodide exclusion using an EpicsXL flow cytometer (Coulter, Miami, FL). Results are presented as the fold increase from control islets.

PGE₂ and nitrite assay. PGE₂ levels were measured in culture media using an enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI). In preliminary experiments, we determined that the fatty acid solutions did not interfere with the assay (data not shown). Nitrite levels were measured in 200- μ l aliquots of culture media using Griess reagent (34). Because of reported interference of fatty acids with this assay (14), wells containing culture media

but no islets were cultured side by side with the experimental conditions. Values obtained from these blanks were subtracted from the sample values. **Expression of data and statistics.** Data are expressed as the means \pm SE. Statistical analysis was performed by ANOVA followed by two-by-two comparisons using Dunnett's method against the control group (Figs. 1, 4, 6, and 7A) or Tukey's method, with equal weight between all conditions (Figs. 5 and 7B). $P < 0.05$ was considered significant.

RESULTS

Exogenous DAG inhibits insulin secretion, whereas exogenous ceramide impairs insulin mRNA levels but not insulin secretion. We have previously demonstrated that de novo ceramide generation from palmitate inhibits insulin gene expression (10). To determine whether the same mechanism underlies fatty acid inhibition of insulin secretion, we cultured isolated rat islets for 72 h in the presence of 0.5 mmol/l palmitate, 0.5 mmol/l oleate, 0.5 mmol/l DAG, or 50 μ mol/l C2-ceramide, a cell-permeable analog of ceramide (Fig. 1), and we investigated glucose-induced insulin secretion in 1-h static incubations at the end of the culture period. None of the culture conditions affected subsequent insulin release in response to 2.8 mmol/l glucose (ANOVA; NS; $n = 4$ in each group) (Fig. 1A). In contrast, culture in the presence of palmitate or oleate led to a significant decrease in 16.7 mmol/l glucose-induced insulin secretion: 120.8 ± 8.5 μ U/islet in the controls ($n = 4$) vs. 49.5 ± 9.2 μ U/islet with palmitate ($P = 0.002$, $n = 4$) and 45.8 ± 14.7 μ U/islet with oleate ($P = 0.001$, $n = 4$) (Fig. 1A). Culture in the presence of DAG had a similar inhibitory effect on glucose-stimulated insulin release (57.7 ± 10.1 μ U/islet, $P = 0.004$, $n = 4$) (Fig. 1A). The presence of C2-ceramide did not significantly affect insulin secretion (100.1 ± 13.6 μ U/islet, NS, $n = 4$) (Fig. 1A). Intracellular insulin content was significantly diminished after culture with palmitate (2.45 ± 0.19 vs. 3.94 ± 0.45 mU/islet, $P = 0.03$, $n = 5$) (Fig. 1B) and oleate (2.10 ± 0.34 vs. 3.94 ± 0.45 mU/islet, $P = 0.01$, $n = 4$) (Fig. 1B), but not DAG (3.64 ± 0.46 vs. 3.94 ± 0.45 mU/islet, NS, $n = 5$) (Fig. 1B). Cumulative insulin secreted in the medium during the 72-h culture was not affected by DAG, as compared with 16.7 mmol/l glucose alone (71.0 ± 9.2 vs. 71.2 ± 9.8 mU/islet, NS, $n = 4$) (Fig. 1C). Intracellular DAG content was increased after culture in the presence of palmitate and DAG (Fig. 2). In contrast to its lack of effect on insulin secretion, C2-ceramide significantly reduced the amount of preproinsulin mRNA in islets after a 72-h culture, whereas the non-cell-permeable analog dihydro-C2-ceramide had no effect (Fig. 3A). Consistent with this finding, only palmitate reduced preproinsulin mRNA levels, whereas oleate had no effect (Fig. 3B). Indeed, only palmitate, but not oleate, can serve as a substrate for ceramide synthesis (35). These results indicate that inhibition of insulin secretion is also observed with oleate and exogenous DAG but is not replicated by exogenous ceramide.

Fatty acids do not increase ROS production in isolated islets, and neither do antioxidants protect against inhibition of insulin secretion. To investigate the role of ROS in fatty acid inhibition of insulin secretion, we first determined whether prolonged culture of isolated islets in the presence of fatty acids was associated with increased production of ROS. Isolated rat islets were cultured for 72 h in the presence of 0.5 mmol/l palmitate, 0.5 mmol/l oleate, or 50 mmol/l ribose, which is known to

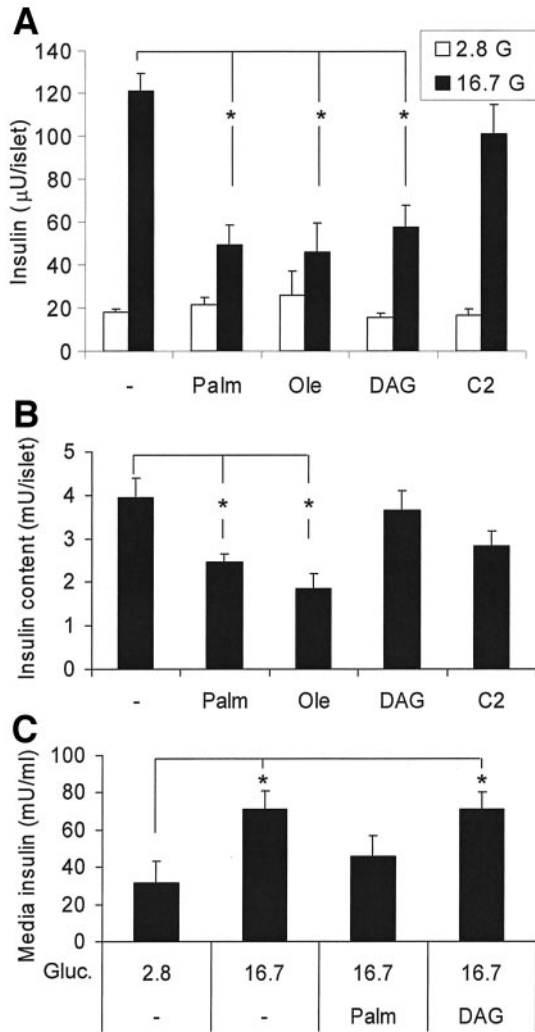


FIG. 1. Effects of fatty acids and ceramide on insulin secretion and insulin content in isolated rat islets. **A:** Insulin secretion in response to 2.8 or 16.7 mmol/l glucose (G) after a 72-h culture with 16.7 mmol/l glucose and 0.5 mmol/l palmitate (Palm), 0.5 mmol/l oleate (Ole), 0.5 mmol/l DAG, or 50 µmol/l C2-ceramide (C2). Results are the means ± SE of four replicate experiments. **P* < 0.01. **B:** Insulin content after a 72-h culture with 16.7 mmol/l glucose and 0.5 mmol/l palmitate (Palm), 0.5 mmol/l oleate (Ole), 0.5 mmol/l DAG, or 50 µmol/l C2-ceramide (C2). Results are the means ± SE of 4–5 replicate experiments. **P* < 0.05. **C:** Cumulative insulin levels in the culture medium at the end of a 72-h culture with 2.8 or 16.7 mmol/l glucose (Gluc.) and 0.5 mmol/l palmitate (Palm) or 0.5 mmol/l DAG. Results are the means ± SE of four replicate experiments. **P* < 0.05.

induce peroxide generation (33). Intracellular peroxide levels and propidium iodide staining were measured by flow cytometry after dissociation of the islets into single cells, as described in RESEARCH DESIGN AND METHODS (Fig. 4). The carboxy-H₂DCFDA probe used in these experiments measures the overall oxidative stress level, including hydrogen peroxide, peroxy radical, and peroxyxynitrite anion. The presence of ribose increased intracellular peroxides 2.1 ± 0.6-fold over the control values (*P* < 0.05, *n* = 6) (Fig. 4A). However, neither palmitate (1.1 ± 0.2-fold increase, NS, *n* = 6) nor oleate (1.1 ± 0.1-fold increase, NS, *n* = 6) affected peroxide levels (Fig. 4A). In addition, none of the culture conditions significantly affected cell viability, as assessed by the percentage of propidium iodide-positive cells (all NS, *n* = 6) (Fig. 4B). To further exclude the involvement of oxidative stress, we then

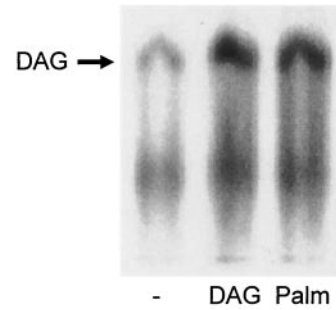


FIG. 2. Effects of palmitate (Palm) and DAG on intracellular DAG content in isolated rat islets. DAG content was measured as described in RESEARCH DESIGN AND METHODS after a 72-h culture with 16.7 mmol/l glucose and 0.5 mmol/l palmitate (Palm) or 0.5 mmol/l DAG. A representative autoradiogram is shown. Similar results were obtained in three replicate experiments.

evaluated whether the presence of antioxidants during the 72-h culture could prevent fatty acid inhibition of insulin secretion (Fig. 5). We tested the effects of 10 mmol/l *N*-acetyl-cysteine (NAC), which protects β-cells from oxidant stress induced by glucotoxicity (36), and that of 1 mmol/l pyridoxamine, an inhibitor of the formation of advanced lipid peroxidation end products (37) (Fig. 5). In static incubations performed at the end of the culture period, none of the culture conditions affected insulin release in response to 2.8 mmol/l glucose (all NS, *n* = 4–8). Both NAC and pyridoxamine tended to decrease glucose-induced insulin secretion, but this effect was not statistically significant (NS; *n* = 4 and 7, respectively). As expected, palmitate inhibited glucose-induced release (47.7 ± 7.3 vs. 107.2 ± 11.9 µU/islet, *P* < 0.001, *n* = 8). However, neither NAC (25.9 ± 4.1 vs. 91.2 ± 18.5 µU/islet, *P* < 0.05, *n* = 4) nor pyridoxamine (43.5 ± 13.4 vs. 92.8 ± 12.2 µU/islet, *P* < 0.05, *n* = 7) prevented the inhibitory effect of palmitate. Altogether, these results suggest that

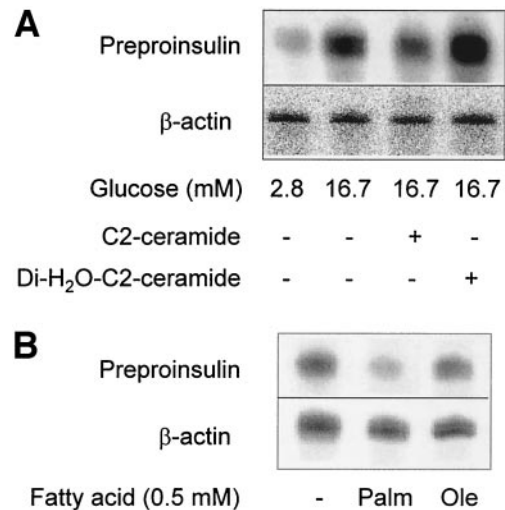


FIG. 3. Effects of fatty acids and ceramide analogs on preproinsulin mRNA in isolated rat islets. **A:** Representative ribonuclease protection assay of preproinsulin and β-actin mRNA after a 72-h culture in the presence of 2.8 or 16.7 mmol/l glucose with or without 50 µmol/l C2-ceramide or dihydro-C2-ceramide (a non-cell-permeable analog). Similar results were obtained in three replicate experiments. **B:** Representative ribonuclease protection assay analysis of preproinsulin and β-actin mRNA after a 72-h culture in the presence of 16.7 mmol/l glucose with or without 0.5 mmol/l palmitate (Palm) or oleate (Ole). Similar results were obtained in three replicate experiments.

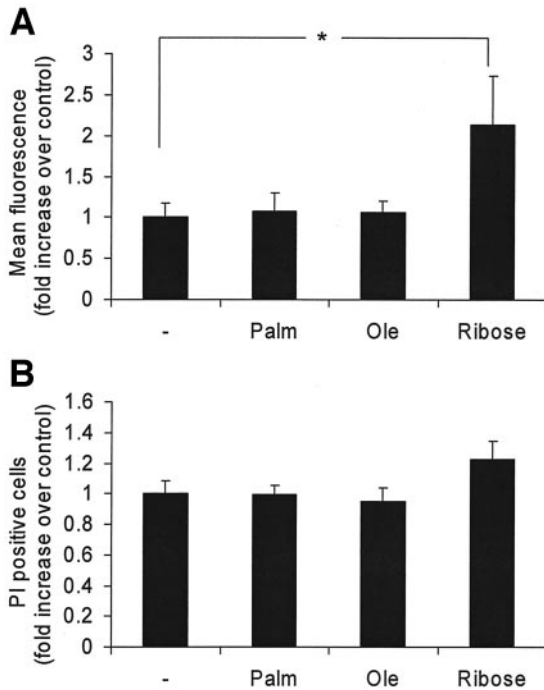


FIG. 4. Effects of fatty acids and ribose on ROS production and cell viability in isolated rat islets. Intracellular peroxide levels and propidium iodide staining were assessed by flow cytometry after dissociation of the islets into single cells at the end of the 72-h culture in 16.7 mmol/l glucose with 0.5 mmol/l palmitate (Palm), 0.5 mmol/l oleate (Ole), or 50 mmol/l ribose. **A:** Carboxy- H_2DCFDA fluorescence. **B:** Percentage of propidium iodide (PI)-positive cells. Results are expressed as the fold increase over the control condition in the absence of fatty acids and are the means \pm SE of six replicate experiments. * $P < 0.05$.

the mechanisms underlying fatty acid inhibition of insulin secretion do not involve the generation of ROS.

Fatty acids do not increase NO production in isolated islets, nor does *N*-nitro-*L*-arginine-methyl ester, an inhibitor of the inducible form of NO synthase, protect against inhibition of insulin secretion. Nitrite levels were measured in the media after 72 h of islet culture in the presence of palmitate, oleate, or 5 ng/ml interleukin (IL)-1 as a positive control (Fig. 6A). Wells

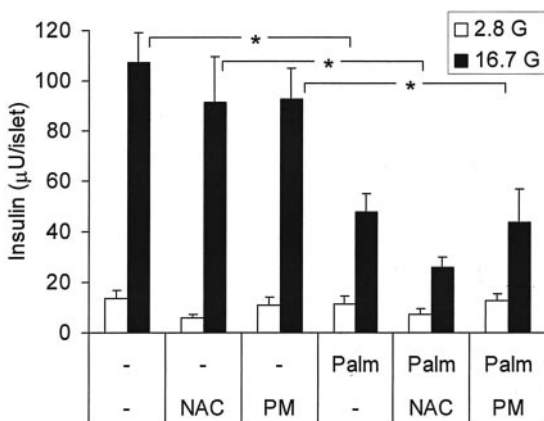


FIG. 5. Lack of protection from palmitate inhibition of insulin secretion by antioxidants. Insulin secretion in response to 2.8 or 16.7 mmol/l glucose (G) was assessed in 1-h static incubations after a 72-h culture in 16.7 mmol/l glucose with or without 0.5 mmol/l palmitate (Palm), 10 mmol/l NAC, or 1 mmol/l pyridoxamine (PM). Results are the means \pm SE of four to seven replicate experiments. * $P < 0.05$.

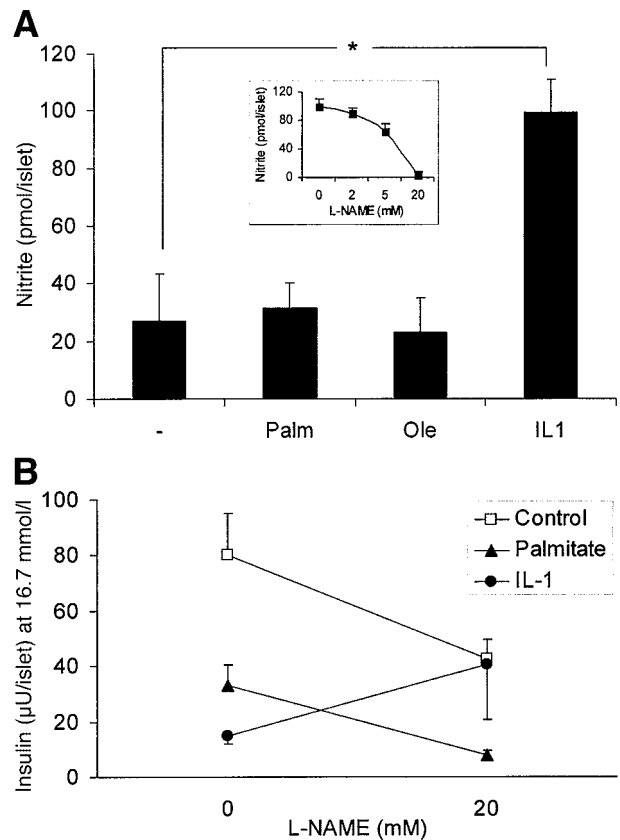


FIG. 6. Effects of fatty acids on NO release and lack of protection from palmitate inhibition of insulin secretion by L-NAME. **A:** Nitrite levels were measured in the media after 72-h of culture with 0.5 mmol/l palmitate (Palm), 0.5 mmol/l oleate (Ole), or 5 ng/ml IL-1. Results are the means \pm SE of 6–11 replicate experiments. * $P < 0.001$. **Insert:** nitrite levels were measured after culture with 5 ng/ml IL-1 and increasing concentrations of L-NAME. Results are the means \pm SE of 2–8 replicate experiments for each concentration (ANOVA, $P < 0.0001$). **B:** Insulin secretion was assessed in 1-h static incubations after a 72-h culture in 16.7 mmol/l glucose with or without 0.5 mmol/l palmitate or 5 ng/ml IL-1 in the absence or presence of 20 mmol/l L-NAME. Results are shown as insulin release at 16.7 mmol/l glucose and are the means \pm SE of three to seven replicate experiments.

containing the same culture medium and cultured side by side with the islets were used as blank samples subtracted from the islet values. As shown in Fig. 6A, nitrite levels were increased by IL-1 (99.4 ± 11.4 vs. 27.1 ± 16.1 pmol/islet, $P = 0.001$, $n = 8$) but not by palmitate (31.4 ± 8.9 pmol/islet, NS, $n = 11$) or oleate (23.0 ± 12.1 pmol/islet, NS, $n = 6$). Addition of *N*-nitro-*L*-arginine-methyl ester (L-NAME), an inhibitor of the inducible form of NO synthase, dose-dependently prevented IL-1-induced NO generation (ANOVA: $P < 0.0001$, $n = 2-8$) (insert, Fig. 6A). The maximally effective concentration of L-NAME (20 mmol/l) was then added to the culture medium for 72 h in the presence of 0.5 mmol/l palmitate or 5 ng/ml IL-1, and insulin secretion was measured in static incubations (Fig. 6B). Both palmitate (33.1 ± 7.4 vs. 80.9 ± 14.8 μ U/islet, $P < 0.01$, $n = 7$) and IL-1 (15.0 ± 3.1 vs. 80.9 ± 14.8 μ U/islet, $P < 0.001$, $n = 7$) inhibited glucose-induced insulin secretion. The presence of 20 mmol/l L-NAME reduced glucose-induced insulin release from both control islets and islets cultured with palmitate; however, this effect only reached statistical significance in the palmitate condition (7.9 ± 1.5 vs. 33.1 ± 7.3 , $P = 0.04$, $n = 3$). The

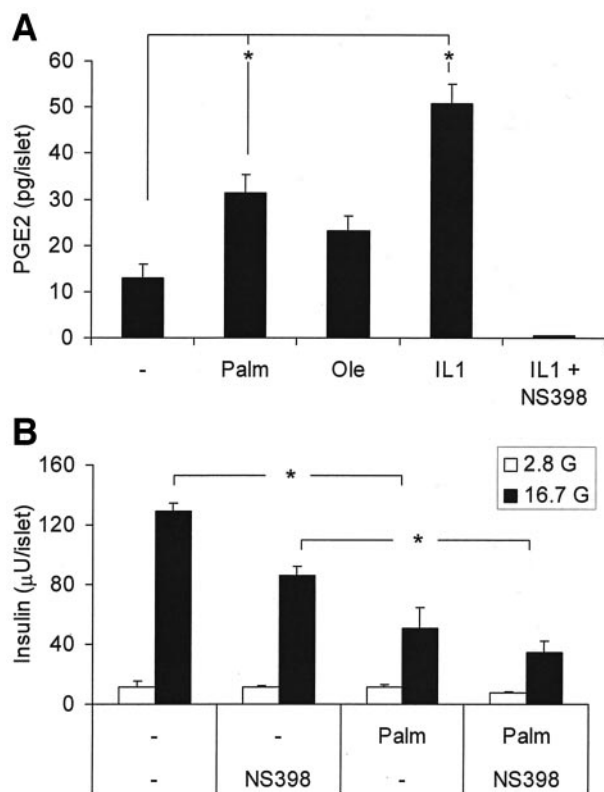


FIG. 7. Effects of fatty acids on PGE₂ release and lack of protection from palmitate inhibition of insulin secretion by NS398. **A:** PGE₂ levels were measured in the media after 72 h of culture with 0.5 mmol/l palmitate (Palm), 0.5 mmol/l oleate (Ole), 5 ng/ml IL-1, or 5 ng/ml IL-1 + 10 µmol/l NS398. Results are the means ± SE of five replicate experiments. **P* < 0.01. **B:** Insulin secretion in response to 2.8 and 16.7 mmol/l glucose (G) was assessed in 1-h static incubations after a 72-h culture in 16.7 mmol/l glucose with or without 0.5 mmol/l palmitate in the absence or presence of 10 µmol/l NS398. Results are the means ± SE of three replicate experiments.

reasons for this inhibitory effect of L-NAME are unclear, but in any case, L-NAME did not prevent palmitate inhibition of glucose-induced insulin release. In contrast, L-NAME restored glucose-induced insulin secretion in IL-1-cultured islets to the levels observed in control islets treated with L-NAME alone (40.4 ± 19.8 vs. 42.4 ± 7.0 µU/islet, NS, *n* = 3). Altogether, these results indicate that NO generation is not involved in fatty acid inhibition of insulin secretion.

PGE₂ release is increased in the presence of fatty acids, but inhibition of PGE₂ synthesis does not protect against fatty acid inhibition of insulin secretion.

Endogenous PGE₂ release by the islets is known to inhibit insulin secretion under conditions of exposure to IL-1 (31). To test whether prolonged exposure to fatty acids induces PGE₂ release, PGE₂ levels were measured in the media after 72 h of culture with 16.7 mmol/l glucose in the absence or presence of 0.5 mmol/l palmitate, 0.5 mmol/l oleate, or 5 ng/ml IL-1 (Fig. 7A). As expected, IL-1 induced a marked increase in PGE₂ release (50.9 ± 4.2 vs. 13.0 ± 2.9 pg/islet, *P* < 0.0001, *n* = 5). PGE₂ release was also significantly increased in the presence of palmitate (31.4 ± 3.9 pg/islet, *P* = 0.002, *n* = 5) and tended to be increased by oleate, although the difference did not reach statistical significance (23.2 ± 3.2 pg/islet, NS, *n* = 6). In the presence of 10 µmol/l NS398, a specific inhibitor of

cyclooxygenase 2, IL-1-induced PGE₂ release was completely blocked (0.5 ± 0.03 vs. 50.9 ± 4.2 pg/islet, *P* < 0.0001, *n* = 3) (Fig. 7A). The presence of NS398 during the 72-h culture significantly lowered subsequent glucose-induced insulin release in control islets, as assessed in static incubations (86.3 ± 6.7 vs. 129.2 ± 5.7 µU/islet, *P* < 0.01, *n* = 3) (Fig. 7B). However, NS398 did not prevent palmitate inhibition of insulin secretion (34.9 ± 7.6 vs. 51.1 ± 13.2 µU/islet, NS, *n* = 3) (Fig. 7B). We therefore conclude that the moderate increase in endogenous PGE₂ release observed upon culture with fatty acids does not mediate their inhibition of insulin secretion.

DISCUSSION

The aims of this study were to investigate whether ceramide synthesis represents a mechanism whereby fatty acids impair insulin secretion and to ascertain the potential involvement of ROS, NO, and PGE₂. Our results demonstrate that fatty acid inhibition of insulin secretion is not related to ceramide synthesis but is mimicked by provision of exogenous DAG. In addition, our findings suggest that neither the generation of oxidative stress nor the production of PGE₂ play an important role in this process.

We have previously demonstrated that de novo ceramide generation from palmitate mediates fatty acid inhibition of insulin gene expression (10). Ceramide has also been proposed as a mechanism for fatty acid-induced β-cell death (13,15,38,39). We observed that exogenous ceramide, at a concentration at which it inhibits insulin mRNA levels (10) (Fig. 3A), does not affect insulin secretion, indicating that ceramide generation is not a mechanism for fatty acid inhibition of insulin secretion. Consistent with this finding, only palmitate, the precursor of de novo ceramide synthesis, inhibits insulin mRNA levels (Fig. 3B), whereas both palmitate and oleate impair insulin secretion (Fig. 1A). In contrast, provision of exogenous DAG mimics palmitate and oleate inhibition of glucose-induced insulin release (Fig. 1A), but it does not affect insulin content (Fig. 1B) or cumulative insulin release during the culture period (Fig. 1C). Importantly, exogenous DAG results in an increase in intracellular DAG content similar to that observed after culture with palmitate (Fig. 2). We conclude from these results that signals or metabolites generated downstream of DAG, rather than the ceramide synthesis pathway, mediate fatty acid inhibition of insulin secretion. This is consistent with the results of an earlier study in which we overexpressed DGAT-1 via an adenovirus in primary islets (19). We found that DGAT-1 overexpression impaired glucose-induced insulin secretion after 72 h of culture in high glucose, but that insulin gene expression was unaffected (19). In addition, triglyceride content in islets is markedly increased after 72 h of exposure to high glucose and fatty acids (9). These results thus provide the first evidence that the mechanisms whereby chronic fatty acids affect insulin secretion and insulin gene expression are distinct.

The mechanisms whereby fatty acids inhibit insulin secretion are unknown. Several potential candidates were considered in the present study. Oxidative stress is known to play a role in the pathogenesis of diabetes complications (40). Mitochondrial ROS (41) inhibit insulin secre-

tion, and the role of ROS as mediators of glucose toxicity in the β -cell is well established (reviewed in 42). Here, we found that a 72-h exposure of islets to palmitate or oleate did not increase intracellular peroxide levels (Fig. 4A) and that neither NAC nor pyridoxamine was able to prevent fatty acid inhibition of insulin secretion (Fig. 5). These results are in conflict with prior investigations showing that fatty acids increase intracellular ROS production in isolated islets (20) and insulin-secreting cells (22–24). In the case of the studies by Maestre et al. (22), Koshkin et al. (23), and Wang et al. (24), it is conceivable that the discrepancies may result from differences between transformed insulin-secreting cell lines and primary islets. In islets, the slight increase in ROS production observed by Carlsson et al. (20) after 24 h of exposure to palmitate might no longer be detectable after 72 h. Although there might be differences in the sensitivities of the assays, we were able to readily detect an increase in ROS production in response to ribose (Fig. 4A). Interestingly, Wang et al. (24) reported that NAC protects insulin-secreting MIN-6 cells from the proapoptotic effect of oleic acid but not from its inhibition of insulin secretion. This raises the possibility that generation of oxidative stress might be a mechanism of fatty acid-induced β -cell death but does not play a role in the impairment of insulin secretion. This model is consistent with the fact that cell death was not observed under our culture conditions (10) (Fig. 4B).

An alternative possibility is that fatty acid-induced production of NO, which is known to inhibit insulin secretion (43), might be a mechanism for lipotoxicity in β -cells (22,25). However, other studies have been controversial (20), and these discrepancies have been attributed to a nonspecific interference of the fatty acids with nitrite measurements by the Griess reagent (14). To address this issue, we systematically subtracted the values of blank samples cultured side by side with the islet samples. We did not observe any increase in nitrite production after culture with fatty acids (Fig. 6A), and neither were we able to prevent fatty acid inhibition of insulin secretion with L-NAME (Fig. 6B) at a concentration at which it completely blocks IL-1-induced NO release (*insert*, Fig. 6A).

Arachidonic acid, the precursor of PGE₂, represents approximately one-third of the fatty acyl groups in islet phospholipids (44). Because PGE₂ inhibits insulin secretion (31), we investigated the possibility that prolonged exposure to fatty acids might be associated with remodeling of the plasma membrane, endogenous release of arachidonic acid, PGE₂ synthesis, and inhibition of insulin secretion. Interestingly, we found that prolonged exposure to palmitate increased PGE₂ release (Fig. 7A). However, a concentration of the cyclooxygenase-2 inhibitor NS398 that completely blocked IL-1-induced PGE₂ release (Fig. 7A) was unable to prevent palmitate inhibition of insulin secretion (Fig. 7B), although these results should be interpreted with caution because of the apparent inhibitory effect of NS398 on glucose-stimulated insulin release (Fig. 7B).

In conclusion, the results of this study uniquely demonstrate that distinct mechanisms underlie fatty acid inhibition of insulin secretion and gene expression. De novo synthesis from ceramide impairs insulin gene expression but does not affect insulin secretion. Generation of oxida-

tive stress has been linked to fatty acid-induced β -cell death but was not observed in our studies, under conditions in which cell viability was not affected. Rather, our observation that exogenous DAG recapitulates inhibition of insulin secretion by fatty acids suggests the involvement of metabolites generated from, or signaling pathways activated by, DAG, the nature of which remain to be identified. Although these findings cannot be generalized to other models of lipotoxicity and their relevance to β -cell failure in humans remains to be demonstrated, they identify important distinctions between the various mechanisms underlying the pleiotropic effects of fatty acids on the β -cell, which could have implications for devising selective therapeutic targets aimed at preventing the deterioration of β -cell function during the course of type 2 diabetes.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (R01 DK 58096) and the American Heart Association (Pacific Mountain Affiliate). V.P. is the recipient of the 2003 Thomas R. Lee Career Development Award from the American Diabetes Association.

REFERENCES

- Poitout V: Lipid partitioning in the pancreatic beta-cell: physiologic and pathophysiologic implications. *Curr Opin Endocrinol Diabetes* 9:152–159, 2002
- Sako Y, Grill VE: A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and β -cell oxidation through a process likely coupled to fatty acid oxidation. *Endocrinology* 127:1580–1589, 1990
- Elks ML: Chronic perfusion of rat islets with palmitate suppresses glucose-stimulated insulin release. *Endocrinology* 133:208–214, 1993
- Zhou Y-P, Grill V: Long term exposure to fatty acids and ketones inhibits B-cell functions in human pancreatic islets of Langerhans. *J Clin Endocrinol Metab* 80:1584–1590, 1995
- Zhou YP, Grill VE: Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose-fatty acid cycle. *J Clin Invest* 93:870–876, 1994
- Gremlich S, Bonny C, Waeber G, Thorens B: Fatty acids decrease IDX-1 expression in rat pancreatic islets and reduce GLUT2, glucokinase, insulin, and somatostatin levels. *J Biol Chem* 272:30261–30269, 1997
- Ritz-Laser B, Meda P, Constant I, Klages N, Charollais A, Morales A, Magnan C, Ktorza A, Philippe J: Glucose-induced preproinsulin gene expression is inhibited by the free-fatty acid palmitate. *Endocrinology* 140:4005–4014, 1999
- Jacqueminet S, Briaud I, Rouault C, Reach G, Poitout V: Inhibition of insulin gene expression by long-term exposure of pancreatic beta-cells to palmitate is dependent upon the presence of a stimulatory glucose concentration. *Metabolism* 49:532–536, 2000
- Briaud I, Harmon JS, Kelpe CL, Segu VB, Poitout V: Lipotoxicity of the pancreatic β -cell is associated with glucose-dependent esterification of fatty acids into neutral lipids. *Diabetes* 50:315–321, 2001
- Kelpe CL, Moore PC, Parazzoli SD, Wicksteed B, Rhodes CJ, Poitout V: Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis. *J Biol Chem* 278:30015–30021, 2003
- Pick A, Clark J, Kubstrup C, Levisetti M, Pugh W, Bonner-Weir S, Polonsky K: Role of apoptosis in failure of β -cell mass compensation for insulin resistance and β -cell defects in the male Zucker diabetes fatty rat. *Diabetes* 47:358–364, 1998
- Shimabukuro M, Higa M, Zhou YT, Wang MY, Newgard CB, Unger RH: Lipopoptosis in beta-cells of obese prediabetic fa/fa rats: role of serine palmitoyltransferase overexpression. *J Biol Chem* 273:32487–32490, 1998
- Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY: Distinct effects of saturated and monounsaturated fatty acids on β -cell turnover and function. *Diabetes* 50:69–76, 2001
- Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG: Inverse

- relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* 50:1771–1777, 2001
15. Maedler K, Oberholzer J, Bucher P, Spinas GA, Donath MY: Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic β -cell turnover and function. *Diabetes* 52:726–733, 2003
 16. El-Assaad W, Buteau J, Peyot ML, Nolan C, Roduit R, Hardy S, Joly E, Dbaibo G, Rosenberg L, Prentki M: Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology* 144:4154–4163, 2003
 17. Prentki M, Corkey BE: Are the β -cell signaling molecules malonyl-CoA and cytosolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? *Diabetes* 45:273–283, 1996
 18. Briaud I, Kelpe CL, Johnson LM, Tran PO, Poitout V: Differential effects of hyperlipidemia on insulin secretion in islets of Langerhans from hyperglycemic versus normoglycemic rats. *Diabetes* 51:662–668, 2002
 19. Kelpe CL, Johnson LM, Poitout V: Increasing triglyceride synthesis inhibits glucose-induced insulin secretion in isolated rat islets of Langerhans: a study using adenoviral expression of diacylglycerol acyltransferase. *Endocrinology* 143:3326–3332, 2002
 20. Carlsson C, Borg LA, Welsh N: Sodium palmitate induces partial mitochondrial uncoupling and reactive oxygen species in rat pancreatic islets in vitro. *Endocrinology* 140:3422–3428, 1999
 21. Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, Aoki T, Etoh T, Hashimoto T, Naruse M, Sano H, Utsumi H, Nawata H: High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 49:1939–1945, 2000
 22. Maestre I, Jordan J, Calvo S, Reig JA, Cena V, Soria B, Prentki M, Roche E: Mitochondrial dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in the beta-cell line INS-1. *Endocrinology* 144:335–345, 2003
 23. Koshkin V, Wang X, Scherer PE, Chan CB, Wheeler MB: Mitochondrial functional state in clonal pancreatic beta-cells exposed to free fatty acids. *J Biol Chem* 278:19709–19715, 2003
 24. Wang X, Li H, De Leo D, Guo W, Koshkin V, Fantus IG, Giacca A, Chan CB, Der S, Wheeler MB: Gene and protein kinase expression profiling of reactive oxygen species-associated lipotoxicity in the pancreatic β -cell line MIN6. *Diabetes* 53:129–140, 2004
 25. Shimabukuro M, Ohneda M, Lee Y, Unger RH: Role of nitric oxide in obesity-induced β -cell disease. *J Clin Invest* 100:290–295, 1997
 26. Patane G, Piro S, Rabuazzo AM, Anello M, Vigneri R, Purrello F: Metformin restores insulin secretion altered by chronic exposure to free fatty acids or high glucose: a direct metformin effect on pancreatic β -cells. *Diabetes* 49:735–740, 2000
 27. Yoshikawa H, Tajiri Y, Sako Y, Hashimoto T, Umeda F, Nawata H: Effects of biotin on glucotoxicity or lipotoxicity in rat pancreatic islets. *Metabolism* 51:163–168, 2002
 28. Piro S, Anello M, Di Pietro C, Lizzio MN, Patane G, Rabuazzo AM, Vigneri R, Purrello M, Purrello F: Chronic exposure to free fatty acids or high glucose induces apoptosis in rat pancreatic islets: possible role of oxidative stress. *Metabolism* 51:1340–1347, 2002
 29. Nowatzke W, Ramanadham S, Ma Z, Hsu FF, Bohrer A, Turk J: Mass spectrometric evidence that agents that cause loss of Ca^{2+} from intracellular compartments induce hydrolysis of arachidonic acid from pancreatic islet membrane phospholipids by a mechanism that does not require a rise in cytosolic Ca^{2+} concentration. *Endocrinology* 139:4073–4085, 1998
 30. Claycombe KJ, Wu D, Nikolova-Karakashian M, Palmer H, Beharka A, Paulson KE, Meydani SN: Ceramide mediates age-associated increase in macrophage cyclooxygenase-2 expression. *J Biol Chem* 277:30784–30791, 2002
 31. Tran POT, Gleason CE, Poitout V, Robertson RP: Prostaglandin E₂ mediates inhibition of insulin secretion by interleukin-1 β . *J Biol Chem* 274:31245–31248, 1999
 32. Preiss J, Loomis CR, Bishop WR, Stein R, Nidel JE, Bell RM: Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and ras- and sis-transformed normal rat kidney cells. *J Biol Chem* 261:8597–8600, 1986
 33. Tanaka Y, Tran PO, Harmon J, Robertson RP: A role for glutathione peroxidase in protecting pancreatic beta cells against oxidative stress in a model of glucose toxicity. *Proc Natl Acad Sci U S A* 99:12363–12368, 2002
 34. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR: Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 126:131–138, 1982
 35. Mathias S, Pena LA, Kolesnick RN: Signal transduction of stress via ceramide. *Biochem J* 335:465–480, 1998
 36. Tanaka Y, Gleason CE, Tran POT, Harmon JS, Robertson RP: Prevention of glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats by antioxidants. *Proc Natl Acad Sci U S A* 96:10857–10862, 1999
 37. Onorato JM, Jenkins AJ, Thorpe SR, Baynes JW: Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions: mechanism of action of pyridoxamine. *J Biol Chem* 275:21177–21184, 2000
 38. Shimabukuro M, Zhou YT, Levi M, Unger RH: Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proc Natl Acad Sci U S A* 95:2498–2502, 1998
 39. Lupi R, Dotta F, Marselli L, Del Guerra S, Masini M, Santangelo C, Patane G, Boggi U, Piro S, Anello M, Bergamini E, Mosca F, Di Mario U, Del Prato S, Marchetti P: Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that β -cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* 51:1437–1442, 2002
 40. Baynes JW, Thorpe SR: Role of oxidative stress in diabetic complications: a new perspective on an old paradigm (Review). *Diabetes* 48:1–9, 1999
 41. Sakai K, Matsumoto K, Nishikawa T, Suefuji M, Nakamaru K, Hirashima Y, Kawashima J, Shirota T, Ichinose K, Brownlee M, Araki E: Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic beta-cells. *Biochem Biophys Res Commun* 300:216–222, 2003
 42. Robertson RP, Harmon J, Tran PO, Tanaka Y, Takahashi H: Glucose toxicity in β -cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. *Diabetes* 52:581–587, 2003
 43. Corbett JA, Sweetland MA, Wang JL, Lancaster JR Jr, McDaniel ML: Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. *Proc Natl Acad Sci U S A* 90:1731–1735, 1993
 44. Ramanadham S, Bohrer A, Gross RW, Turk J: Mass spectrometric characterization of arachidonate-containing plasmalogens in human pancreatic islets and in rat islet beta-cells and subcellular membranes. *Biochemistry* 32:13499–13509, 1993