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-1β (IL-1β) 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
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 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 μg/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 conditions contained the same amount of BSA and vehicle (E1OH 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. A 1-h static incubation was started after culture with carboxy-H2DCFDA, whereas oleate had no effect (Fig. 3C). Consistent with this finding, only palmitate reduced preproinsulin mRNA levels (0.45 mU/islet, NS, P = 0.005 vs. 3.94 mU/islet, NS, n = 4) (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 (71.2 ± 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.

**PGE2 and nitrate 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). PGE2 levels were measured in 200-μl 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.

**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 μmol/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 μU/islet in the controls (n = 4) vs. 49.5 ± 9.2 μU/islet with palmitate (P = 0.002, n = 4) and 45.8 ± 14.7 μU/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 μU/islet, P = 0.004, n = 4) (Fig. 1A). The presence of C2-ceramide did not significantly affect insulin secretion (100.1 ± 13.6 μU/islet, NS, n = 4) (Fig. 1A). Intracellular insulin content was significantly diminished after culture with palmitate (2.45 ± 0.19 vs. 3.94 ± 0.46 μg/islet, P = 0.03, n = 5) (Fig. 1B) and oleate (2.10 ± 0.34 vs. 3.94 ± 0.45 μg/islet, P = 0.01, n = 4) (Fig. 1B), but not DAG (3.64 ± 0.46 vs. 3.94 ± 0.45 μg/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 μU/islet, NS, n = 4) (Fig. 1C). Intracellular DAG content was increased after culture in the presence of palmitate and DAG (Fig. 2). 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.
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-H$_2$DCFDA probe used in these experiments measures the overall oxidative stress level, including hydrogen peroxide, peroxyl radical, and peroxynitrite anion. The presence of ribose increased intracellular peroxides $2.1 \times 1006 \pm 0.6$–fold over the control values ($P < 0.05$, $n = 6$) (Fig. 4A). However, neither palmitate ($1.1 \pm 0.2$–fold increase, NS, $n = 6$) nor oleate ($1.1 \pm 0.1$–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 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 ($47.7 \pm 7.3$ vs. $107.2 \pm 11.9$ μU/islet, $P < 0.001$, $n = 8$). However, neither NAC ($25.9 \pm 4.1$ vs. $91.2 \pm 18.5$ μU/islet, $P < 0.05$, $n = 4$) nor pyridoxamine ($43.5 \pm 13.4$ vs. $92.8 \pm 12.2$ μU/islet, $P < 0.05$, $n = 7$) prevented the inhibitory effect of palmitate. Altogether, these results suggest that...
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 culture with 0.5 mmol/l palmitate (Palm), 0.5 mmol/l oleate (Ole), or 5 ng/ml IL-1. Results are expressed as the fold increase over the control condition in the absence of fatty acids and are the means ± SE of six replicate experiments. *P < 0.05.

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 μU/islet, P < 0.01, n = 7) and IL-1 (15.0 ± 3.1 vs. 80.9 ± 14.8 μU/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).
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.

**PGE<sub>2</sub> release is increased in the presence of fatty acids, but inhibition of PGE<sub>2</sub> synthesis does not protect against fatty acid inhibition of insulin secretion.** Endogenous PGE<sub>2</sub> 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 PGE<sub>2</sub> release, PGE<sub>2</sub> levels were measured in the media after 72 h of culture with 0.5 mmol/l palmitate (Palm), 0.5 mmol/l olate (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.

**FIG. 7. Effects of fatty acids on PGE<sub>2</sub> release and lack of protection from palmitate inhibition of insulin secretion by NS398.** A: PGE<sub>2</sub> levels were measured in the media after 72 h of culture with 0.5 mmol/l palmitate (Palm), 0.5 mmol/l olate (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.

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 PGE<sub>2</sub>. 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 PGE<sub>2</sub> 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 olate impair insulin secretion (Fig. 1A). In contrast, provision of exogenous DAG mimics palmitate and olate 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 oxidative 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.

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