Free Fatty Acid–Induced Reduction in Glucose-Stimulated Insulin Secretion

Evidence for a Role of Oxidative Stress In Vitro and In Vivo

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OBJECTIVE—An important mechanism in the pathogenesis of type 2 diabetes in obese individuals is elevation of plasma free fatty acids (FFAs), which induce insulin resistance and chronically decrease β-cell function and mass. Our objective was to investigate the role of oxidative stress in FFA-induced decrease in β-cell function.

RESEARCH DESIGN AND METHODS—We used an in vivo model of 48-h intravenous oleate infusion in Wistar rats followed by hyperglycemic clamps or islet secretion studies ex vivo and in vitro models of 48-h exposure to oleate in islets and MIN6 cells.

RESULTS—Forty-eight–hour infusion of oleate decreased the insulin and C-peptide responses to a hyperglycemic clamp ($P < 0.01$), an effect prevented by coinfusion of the antioxidants *N*-acetylcysteine (NAC) and taurine. Similar to the findings in vivo, 48-h infusion of oleate decreased glucose-stimulated insulin secretion ex vivo ($P < 0.01$) and induced oxidative stress ($P < 0.001$) in isolated islets, effects prevented by coinfusion of the antioxidants NAC, taurine, or tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl). Forty-eight–hour infusion of olive oil induced oxidative stress ($P < 0.001$) and decreased the insulin response of isolated islets similar to oleate ($P < 0.01$). Islets exposed to oleate or palmitate and MIN6 cells exposed to oleate showed a decreased insulin response to high glucose and increased levels of oxidative stress (both $P < 0.001$), effects prevented by taurine. Real-time RT-PCR showed increased mRNA levels of antioxidant genes in MIN6 cells after oleate exposure, an effect partially prevented by taurine.

CONCLUSIONS—Our data are the first demonstration that oxidative stress plays a role in the decrease in β-cell secretory function induced by prolonged exposure to FFAs in vitro and in vivo. ***Diabetes*** **56:2927–2937, 2007**

Type 2 diabetes is characterized by both insulin resistance and defective insulin secretion (1). Obesity is the major predisposing factor for type 2 diabetes and is associated with excessive release of fatty acids from the expanded adipose tissue mass, leading to elevated plasma free fatty acids (FFAs), which are known to induce insulin resistance (2,3). Acute FFA exposure stimulates insulin secretion (4), but studies in vitro and in situ have shown that prolonged FFA exposure decreases glucose-stimulated insulin secretion (GSIS) (5). The effect of prolonged FFA elevation on β-cell function in vivo has been more controversial, as absolute GSIS was found to be increased (6–8), unchanged (9,10), or decreased (11–13) by FFA. However, in most of these studies β-cell function was inadequate to compensate for FFA-induced insulin resistance (9–13), at least in predisposed individuals (14). Although the mechanisms behind FFA-induced decrease in β-cell function are unclear, one possibility points toward oxidative stress. Pancreatic β-cells have low antioxidant defenses (15) and are thus susceptible to reactive oxygen species (ROS)–induced decrease in function and viability (16,17). Oxidative stress has been implicated in the decrease in GSIS induced by prolonged exposure to glucose (18,19), which is in many respects similar to that induced by prolonged exposure to FFA (4,20). However, whether oxidative stress plays a role in FFA-induced decrease in β-cell function is still unclear. The majority of studies have shown that FFAs generate ROS in islets (21) and β-cell lines (22,23), although in a recent paper, ROS were not increased by FFA in cultured islets. In the same paper, antioxidants did not prevent the FFA-induced defect in GSIS (24). By contrast, in our previous study, although the antioxidant *N*-acetylcysteine (NAC) did not restore secretion in MIN6 cells, it did prevent the decrease in insulin content induced by oleate (23). There are no other studies linking oxidative stress to FFA-induced decrease in β-cell function, although insulin sensitizers with antioxidant activity restored GSIS in vitro (25,26).

In this study, we investigated the effects of prolonged exposure to oleate, alone and in combination with antioxidants, on GSIS during hyperglycemic clamps in vivo, ex vivo in islets of oleate-treated rats, and in vitro in islets and MIN6 β-cells. Both hyperglycemic clamps and ex vivo studies in islets were performed in rats infused intrave-

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Received for publication 16 January 2007 and accepted in revised form 20 August 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 19 September 2007. DOI: 10.2337/db07-0075.

Additional information for this article can be found in an online appendix at http://dx.doi.org/10.2337/db07-0075.

CPT-1, carnitine palmitoyl transferase-1; DMEM, Dulbecco's modified Eagle's medium; FFA, free fatty acid; GINF, glucose infusion rate; GPx-1, gluthathione peroxidase-1; GSIS, glucose-stimulated insulin secretion; H_2DCF-DA, dihydro-dichlorofluorescein-diacetate; HO-1, heme oxygenase-1; KRB, Krebs-Ringer buffer; KRBH, KRB containing 10 mmol/l^{-1} HEPES; ROS, reactive oxygen species; SOD, superoxide dismutase; tempol, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl; UCP, uncoupling protein.

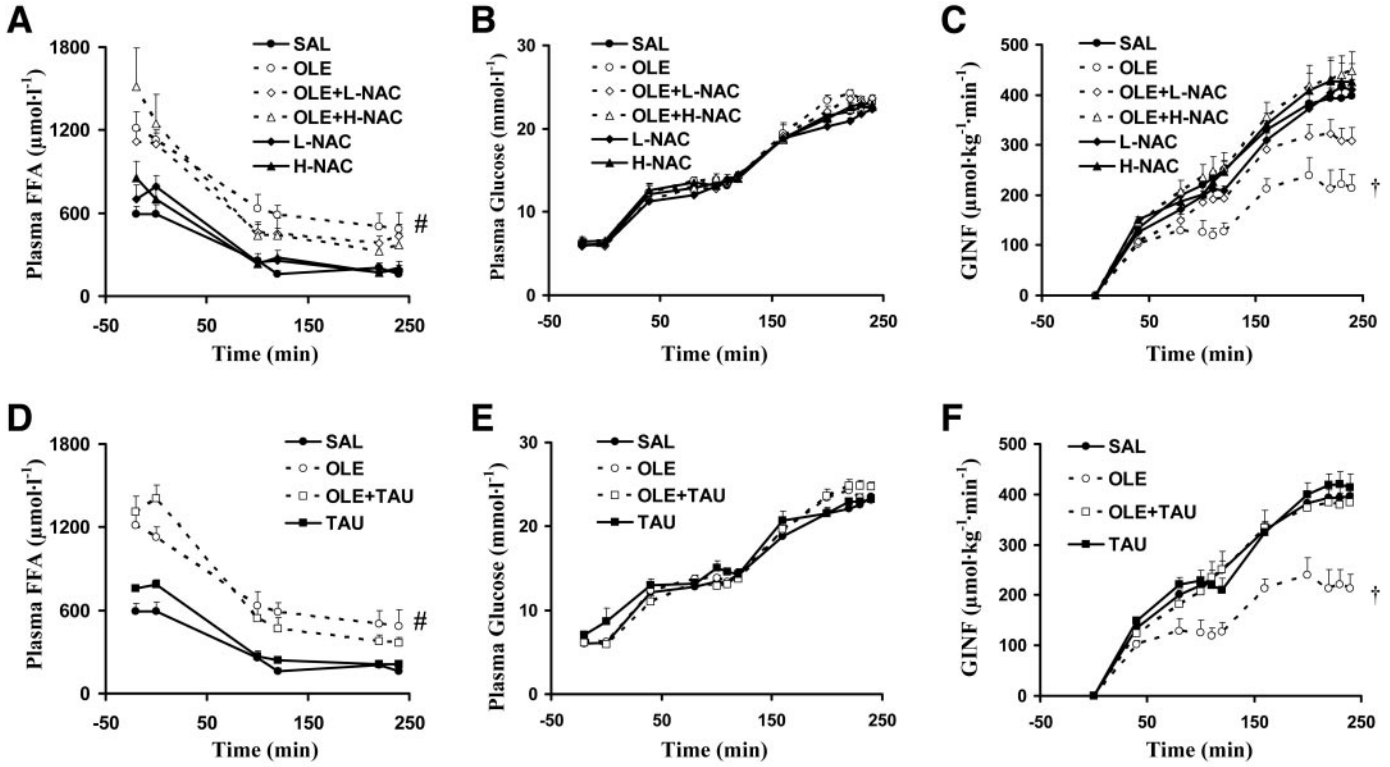

FIG. 1. Effects of low- and high-dose NAC (*A–C*) and taurine (*D–F*) on plasma FFA, glucose, and GINF during two-step hyperglycemic clamps with/without 48-h oleate infusion. Rats were treated with *1*) saline alone (SAL, $n = 11$ [●]), *2*) oleate alone (OLE, 1.3 μmol/min, $n = 10$ [○]), *3*) oleate plus low dose of NAC (OLE+L-NAC; oleate, 1.3 μmol/min; L-NAC, 2.14 μmol · kg^{-1} · min^{-1}; $n = 8$ [◇]), *4*) oleate plus high dose of NAC (OLE+H-NAC; oleate, 1.3 μmol/min; H-NAC, 2.76 μmol · kg^{-1} · min^{-1}; $n = 7$ [△]), *5*) oleate plus taurine (OLE+TAU; oleate, 1.3 μmol/min; taurine, 2.76 μmol · kg^{-1} · min^{-1}; $n = 14$; equimolar dose with H-NAC; □), *6*) low dose of NAC alone (L-NAC; $n = 8$; ♦), *7*) high dose of NAC alone (H-NAC; $n = 9$ [▲]), or *8*) taurine alone (TAU; $n = 13$ [■]). Data are means ± SE. Oleate increased plasma FFA, as expected. Plasma glucose levels were superimposable among groups. Oleate impaired the GINF during the two-step hyperglycemic clamp; the low dose of NAC partially prevented the effect of oleate, whereas the high dose of NAC or taurine completely prevented the impairment induced by oleate. #Oleate-infused groups vs. non–oleate infused groups, $P < 0.01$ throughout the hyperglycemic clamp. †Oleate-infused vs. all, except oleate plus low-dose NAC, $P < 0.001$ throughout the hyperglycemic clamp.

nously for 48 h with oleate/saline with or without antioxidants. We used three antioxidants: NAC, taurine, and 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (tempol). Oleate is the most prevalent circulating fatty acid, followed by palmitate. For in vitro studies in islets, both oleate and palmitate were used. Unfortunately, palmitate has low solubility and thus is not suitable for intravenous infusion. Oleate was used instead of the standard infusion of Intralipid and heparin because we have previously shown that a prolonged infusion of oleate impairs GSIS to a greater extent than Intralipid plus heparin (12). For in vitro studies in MIN6 cells, oleate was used, and we measured ROS and expression of genes induced by oxidative stress.

RESEARCH DESIGN AND METHODS

Studies in rats

Animals and surgery. Female Wistar rats (250–300 g; Charles River, Montreal, Canada) were cannulated as previously described (12). We used a jugular catheter for infusion and a carotid catheter for blood sampling. Infusions were started after 3–4 days of recovery after surgery. All procedures were approved by the Animal Care Committee of the University of Toronto.

Intravenous infusions. Rats were infused for 48 h with *1*) saline; *2*) oleate; *3*) oleate and lower dose of NAC; *4*) oleate and higher dose of NAC; *5*) oleate and taurine; *6*) lower dose of NAC; *7*) higher dose of NAC; or *8*) taurine, followed by a two-step hyperglycemic clamp. For ex vivo GSIS determinations in islets, we added three more treatments: *9*) olive oil/heparin alone; *10*) oleate and tempol; or *11*) tempol alone. We used the same infusion protocol as Mason et al. (12). Oleate (Sigma, St. Louis, MO) was prepared in fatty acid–free BSA (Sigma) according to the Bezman-Tarcher method (27) as modified by Miles et al. (28) and given at 1.3 μmol/min. The 20% olive oil emulsion was prepared by mixing chemical-grade (Sigma) olive oil (unrefined olive oil contains antioxidants) with phosphatidylcholine, glycerol, and penicillin-streptomycin, as described by Dobbins et al. (29). Heparin was added to a final concentration of 50 units/ml, and the emulsion was infused at 5 μl/min. Saline was given as control (equivolume) because we have shown no difference between saline and BSA infusion using the same experimental protocol (12). NAC was given at 2.14 μmol · kg^{-1} · min^{-1}, the dose that reversed insulin resistance induced by glucose in our previous study (30), and at 2.76 μmol · kg^{-1} · min^{-1}; taurine was given at this equimolar dose. Tempol was given at 2.41 μmol · kg^{-1} · min^{-1}, the dose that showed protection against ROS toxicity in experimental pancreatitis (31). All antioxidants (Sigma) were dissolved in saline at pH 7.4. After 48 h of infusion and ~12 h of fasting, we performed either in vivo studies, using two-step hyperglycemic clamps, or ex vivo studies in isolated islets.

Hyperglycemic clamps. GSIS in vivo was determined by measuring insulin and C-peptide during two-step hyperglycemic clamps. An infusion of 37.5% glucose was started at time = 0 min. We did not use a glucose prime because we wished to avoid possible arrhythmias caused by an oleate bolus from the dead space of the infusion line. Plasma glucose was maintained at 13 mmol/l by adjusting the rate of the glucose infusion according to frequent (every 5–10 min) glucose determinations. At 120 min, the glucose infusion was raised to achieve and maintain plasma glucose levels of 22 mmol/l until the end of experiments (time = 240 min).

Plasma assays. Plasma glucose was measured on a Beckman Analyzer II (Beckman, Fullerton, CA), and plasma FFAs were measured with an enzymatic colorimetric kit (Wako Industries, Neuss, Germany). Radioimmunoassay specific for rat insulin and C-peptide (Linco, St. Charles, MO) was used to determine plasma concentrations (interassay coefficient of variation <10%).

Islet isolation and secretion studies. Islets of in vivo–infused rats were isolated as performed by Joseph et al. (32) and preincubated for 1 h at 37°C in Krebs-Ringer buffer (KRB) containing 10 mmol/l HEPES (KRBH) and 2.8 mmol/l glucose. Thereafter, five islets of approximately the same size were

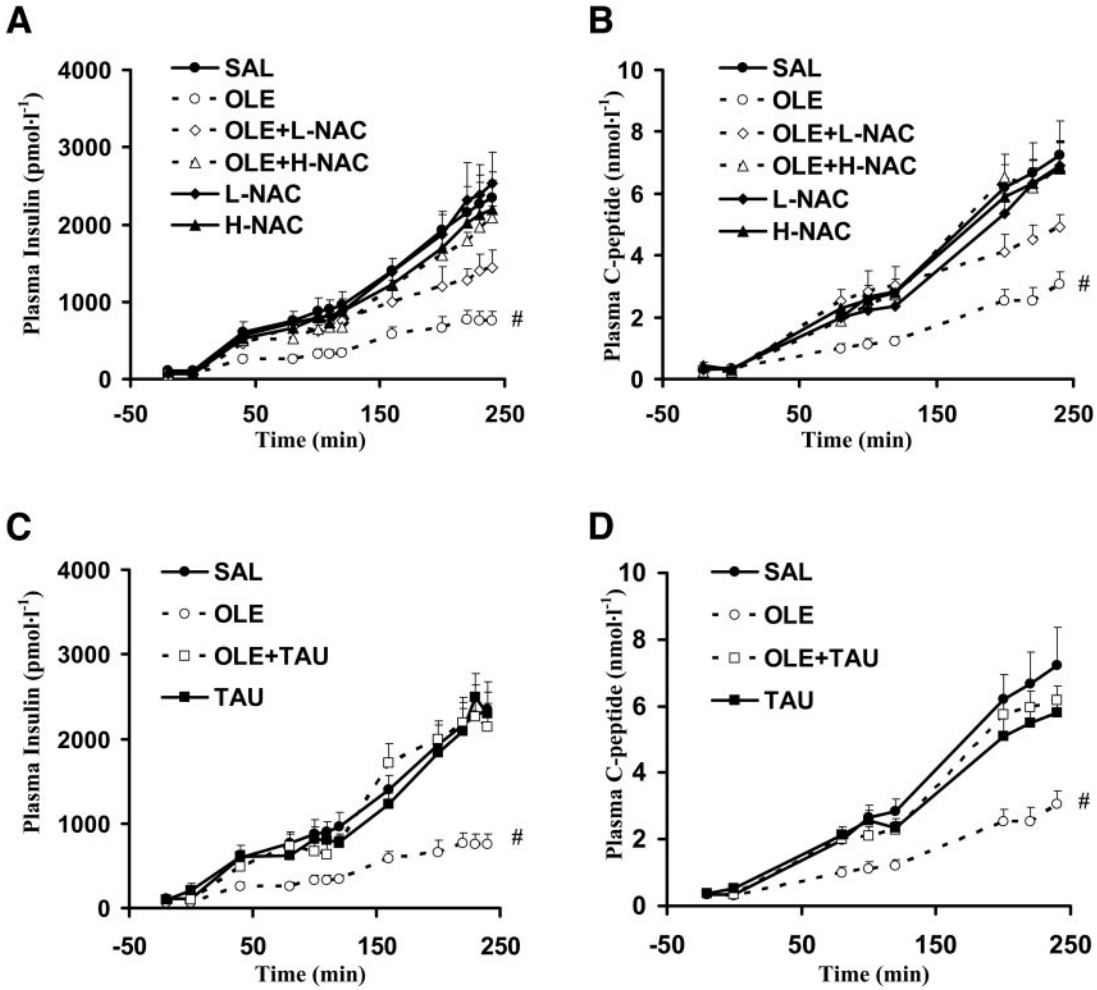

FIG. 2. Effects of low- and high-dose NAC (*A* and *B*) and taurine (*D* and *E*) on insulin and C-peptide during two-step hyperglycemic clamps with/without 48-h oleate infusion. Groups are described in the legend of Fig. 1. Oleate decreased both the insulin and C-peptide levels during the two-step hyperglycemic clamp; the low dose of NAC partially prevented the effect of oleate, whereas the high dose of NAC or taurine completely prevented the decrease induced by oleate. #Oleate-infused vs. all, except oleate plus low-dose NAC, $P < 0.01$ during the first and $P < 0.001$ during the second step of the hyperglycemic clamp.

incubated in triplicate at 2.8, 6.5, 13, and 22 mmol/l glucose for 2 h at 37°C. Insulin was measured in the supernatant with Linco's kit. For in vitro studies, before preincubation, islets of untreated rats were cultured for 48 h in RPMI-1640 without antioxidants, containing 0.4 mmol/l oleate or 0.4 mmol/l palmitate in 0.5% BSA, or 25 μmol/l H_2O_2, with or without 1 mmol/l taurine.

Studies in MIN6 Cells

Cell culture. Oleate, BSA, taurine, and KRBH were purchased from Sigma. Oleate bound to fatty acid–free BSA was prepared as described by Wang et al. (23). MIN6 β-cells (passage number 35–45) (33), a gift from Dr. S. Seino (Chiba University), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/l glucose and supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells (3.5×10^5) were then incubated overnight in DMEM containing 10 mmol/l glucose, followed by additional exposure to *1*) 0.5% (wt/vol) BSA, *2*) 0.4 mmol/l oleate in BSA, *3*) 0.4 mmol/l oleate in BSA plus 1 mmol/l taurine, or *4*) 1 mmol/l taurine in BSA for 48 h.

Insulin secretion. MIN6 cells were preincubated for two sequential periods of 30 min in KRBH with no glucose and incubated in KRBH containing 0 or 16.7 mmol/l glucose for 1 h. Insulin was determined in the supernatant with a Linco kit.

ROS measurements. Islets or MIN6 cells were incubated with 10 μmol/l dihydro-dichlorofluorescein-diacetate (H_2DCF-DA) (D6883; Sigma) in KRBH containing 2.8 mmol/l (islets) or 0 mmol/l (MIN6 cells) glucose for 30 min (32). Then, the medium was replaced with fresh KRBH containing no glucose, and fluorescence was measured at 480 nm excitation and 510 nm emission with an Olympus microscope. Data were analyzed using ImageMaster3.

RT-PCR. Primers were designed using Primer Express (Applied Biosystems, Foster City, CA). Total RNA was extracted using the RNeasy kit (Qiagen, Burlington, Canada). Total RNA was reverse transcribed using oligo-dT primers and Superscipt II (Invitrogen, Burlington, Canada). The real-time RT-PCR was monitored and analyzed by the Sequence Detection System (Applied Biosystems). All genes were normalized to β-actin. Primer sequences are available on request.

Statistics. Data are means ± SE. One-way nonparametric ANOVA for repeated measurements followed by Tukey's *t* test was used to compare treatments. Calculations were performed using SAS (Cary, NC).

RESULTS

In vivo clamp studies. During the 48-h infusions, as expected, the oleate-treated rats had higher plasma FFA than the rats treated with saline or antioxidant alone (supplemental Table 1, available in an online appendix at http://dx.doi.org/10.2337/db07-0075). The infusion of oleate or antioxidants did not affect plasma glucose or insulin (data not shown).

After the 48-h infusions, we evaluated insulin secretion in vivo during hyperglycemic clamps. As shown in Fig. 1*A* and *D*, basal plasma FFAs before the clamps were higher in oleate-treated than control rats or rats treated with antioxidant alone. FFA levels declined during the clamp

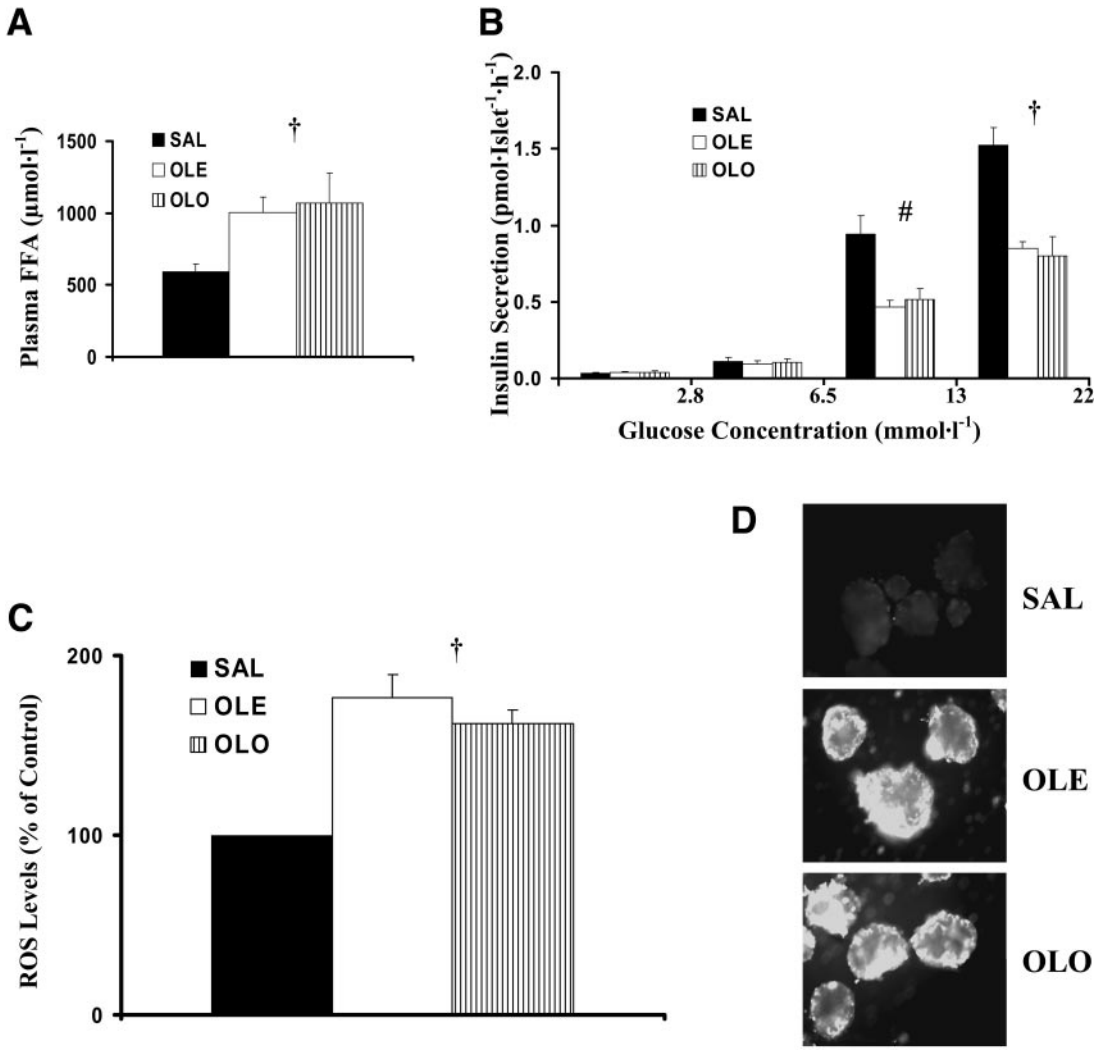

FIG. 3. Effects of 48-h oleate or olive oil infusion on plasma FFA (*A*) and insulin secretion (*B*) in islets. Islets were isolated from rats treated with saline (SAL; $n = 16$); oleate (OLE; 1.3 μmol/min; $n = 13$); and olive oil (OLO; 5 μl/min 20% olive oil and 50 units/ml heparin; $n = 10$). *A*: Both oleate and olive oil increased the plasma FFA levels to the same extent after 48 h compared with saline controls. *B*: Both oleate and olive oil infusions decreased the insulin response from isolated islets at 13 and 22 mmol/l glucose compared with control. *C*: Islets ROS (SAL, $n = 7$; OLE, $n = 6$; OLO, $n = 4$). †$P < 0.001$ for oleate and olive oil vs. saline. #$P < 0.01$ for oleate and olive oil vs. saline. *D*: Representative fluorescent images for ROS (magnification ×200). Light images are available on request from the authors.

because of hyperglycemia and hyperinsulinemia. However, FFA remained higher in oleate-treated rats.

Basal plasma glucose was similar in all groups. During the first step of the clamp, glucose levels rose to 13 mmol /l (upper physiological in rats) and during the second step, to 22 mmol/l (pathological but maximally stimulatory), with no differences among groups (Fig. 1*B* and *E*). The glucose infusion rate (GINF) necessary to maintain the target glucose level was lower in the oleate- than saline-treated group, consistent with decreased insulin secretion, decreased insulin sensitivity, or both (Fig. 1*C* and *F*). With the low NAC dose in combination with oleate, GINF was only partially restored (NS; oleate plus low-dose NAC vs. oleate or saline) (Fig. 1*C*). When the dose of NAC was increased by 25%, GINF was completely restored (Fig. 1*C*). Taurine, when coinfused with oleate at a dose equimolar to the higher NAC dose, also restored GINF to control levels (Fig. 1*F*). Antioxidants alone had no effect on GINF.

Basal insulin and C-peptide levels were similar in all groups. As expected, plasma insulin rose in response to increasing glucose levels (Fig. 2*A* and *C*). Plasma C-peptide also rose, indicating that the rise in insulin was due to increased secretion (Fig. 2*B* and *D*). Plasma insulin and C-peptide were lower in oleate- than saline-treated rats. In the group infused with oleate and low-dose NAC, plasma insulin and C-peptide were restored to control levels only at 13 mmol/l glucose (NS, oleate plus low-dose NAC vs. saline; $P < 0.05$, oleate plus low-dose NAC vs. oleate), whereas at 22 mmol/l glucose, the effect of oleate was only partially prevented (NS, oleate plus low-dose NAC vs. saline or oleate). When the higher dose of NAC or an equimolar dose of taurine was added to oleate, plasma insulin and C-peptide were completely restored (Fig. 2*A–B* and *C–D*; respectively). Antioxidants alone had no effect on insulin or C-peptide levels.

As evidenced by Figs. 1*C–F* and 2*A–C*, GINF and insulin levels were proportionally decreased in oleate versus saline groups; therefore, the sensitivity index M/I = GINF/insulin (34) was not significantly different during either the first step (0.404 ± 0.047 μmol · kg^{-1} · min^{-1}/pmol · l^{-1} for saline and 0.323 ± 0.023 μmol · kg^{-1} · min^{-1}/pmol · l^{-1} for oleate, NS) or the second step (0.260 ± 0.033 μmol · kg^{-1} · min^{-1}/pmol · l^{-1} for saline and 0.269 ± 0.030 μmol · kg^{-1}

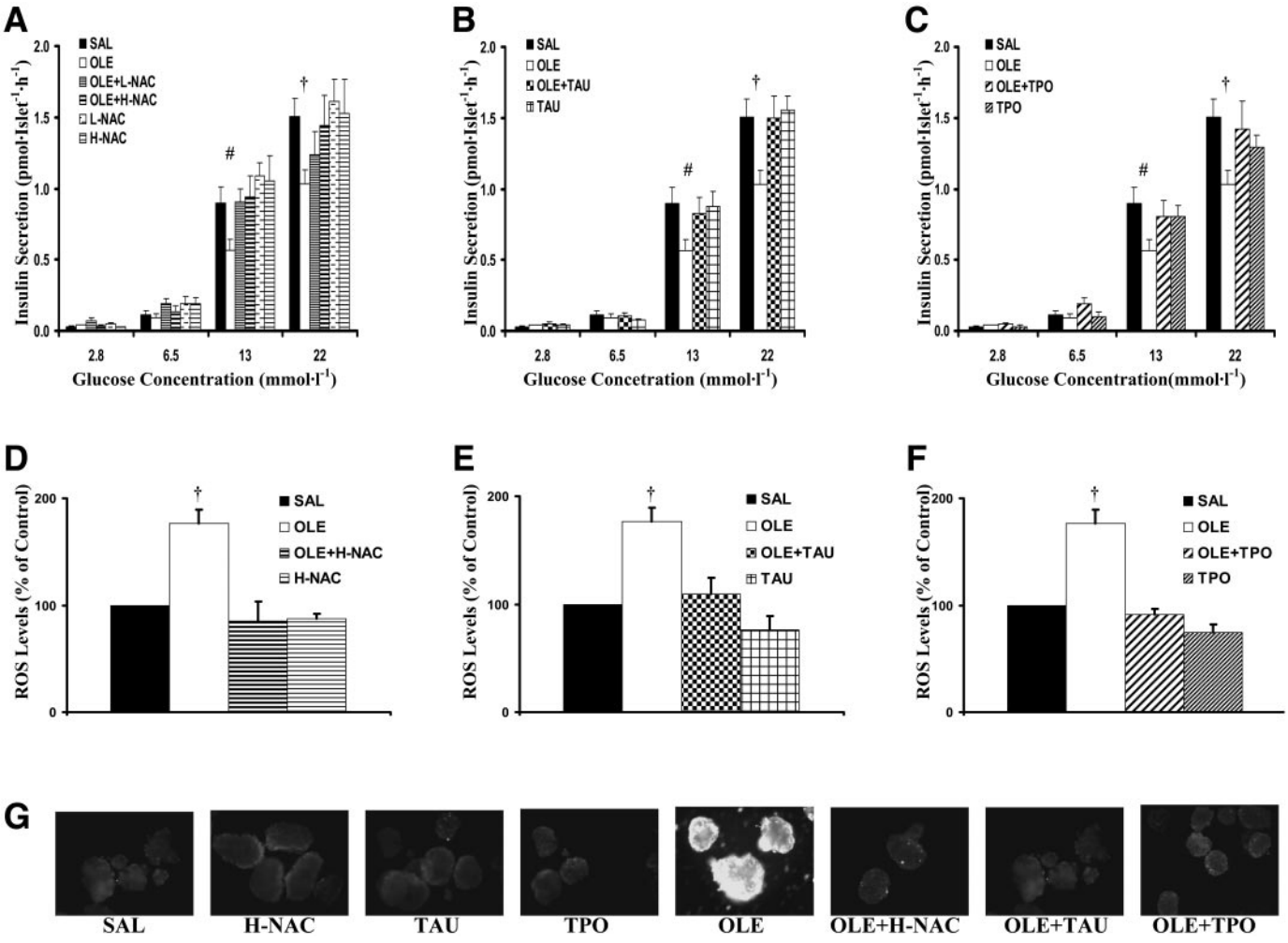

FIG. 4. Effects of oleate and low- and high-dose NAC (*A*), taurine (*B*), and tempol (*C*) on insulin secretion (*A–C*) and ROS (*D–F*) in islets. *A*: Rats were treated for 48 h with saline (SAL, $n = 16$), oleate (OLE, $n = 13$), oleate plus low-dose NAC (OLE+L-NAC, $n = 5$), oleate plus high-dose NAC (OLE+H-NAC, $n = 12$), low-dose NAC (L-NAC, $n = 6$), and high-dose NAC (H-NAC, $n = 6$). Oleate decreased the insulin secretion at both 13 and 22 mmol/l glucose; the low dose of NAC completely prevented the effect of oleate at 13 mmol/l glucose but had only a partial effect at 22 mmol/l glucose; the high dose of NAC completely prevented the GSIS decrease induced by oleate at both 13 and 22 mmol/l glucose. *B*: SAL as in *A*; OLE as in *A*; OLE+TAU ($n = 17$); TAU ($n = 12$). Taurine completely prevented the GSIS decrease induced by oleate at both 13 and 22 mmol/l glucose. *C*: SAL as in *A*; OLE as in *A*; oleate plus tempol (OLE+TPO, 2.41 μmol · kg^{-1} · min^{-1}, $n = 12$); tempol (TPO, $n = 12$). Tempol completely prevented the GSIS decrease induced by oleate at both 13 and 22 mmol/l glucose. *D–F*: ROS levels in cells exposed to treatments in *A–C*. (SAL, $n = 7$; OLE, $n = 6$; OLE+H-NAC, $n = 6$; OLE+TAU, $n = 4$; OLE+TPO, $n = 7$; H-NAC, $n = 5$; TAU, $n = 5$; TPO, $n = 6$). Data are means ± SE, calculated as fold change from control. Infusion of oleate increased ROS levels compared with saline control, whereas coinfusion of each antioxidant completely prevented the increase in ROS induced by oleate. Antioxidants alone had no significant effect. #$P < 0.01$ for oleate vs. saline. †$P < 0.001$ for oleate vs. saline. *G*: Representative fluorescent images for ROS (magnification ×200). Light images are available on request from the authors.

· min^{-1}/pmol · l^{-1} for oleate, NS) of the clamp. Both antioxidants had no effect on M/I.

Ex vivo studies in islets. Rats were infused for 48 h with oleate/saline at the same rate as that for our in vivo studies. At the end of the 48-h infusions, islets were isolated and incubated at the following glucose concentrations: 2.8 (nonstimulatory); 6.5 (basal glucose levels in rats); and 13 and 22 mmol/l (as in hyperglycemic clamps). The insulin secretory response of islets isolated from oleate-infused rats was markedly decreased compared with control at both 13 and 22 mmol/l glucose (Fig. 3*B*). To exclude any possible nonspecific effect on insulin secretion due to the surfactant activity of direct infusion of FFA, we infused for 48 h an emulsion of 20% olive oil, a triglyceride mixture containing 71% oleate. Heparin was added to the olive oil emulsion to activate lipoprotein lipase, which releases FFA from the triglycerides of olive oil. At the end of olive oil infusion, the increase in FFA levels was similar to that observed after the oleate infusion (Fig. 3*A*). Olive oil infusion decreased GSIS (Fig. 3*B*) and increased ROS (Fig. 3*C* and *D*) of isolated islets, similar to the oleate infusion. We also performed in vivo infusion of NAC and taurine alone and in combination with oleate, followed by evaluation of insulin secretion in isolated islets. Similar to our results in vivo, the lower NAC dose prevented the oleate-induced GSIS decrease at 13 mmol/l glucose (NS, oleate plus low-dose NAC vs. saline; $P < 0.05$, oleate plus low-dose NAC vs. oleate), whereas its protective effect at 22 mmol/l glucose was only partial (NS, oleate plus low-dose NAC vs. oleate or saline) (Fig. 4*A*). The higher NAC dose (Fig. 4*A*) and taurine (Fig. 4*B*) prevented the oleate-induced GSIS decrease at both 13 and 22 mmol/l glucose. Because NAC can be converted to taurine, which might affect insulin secretion independent of its antioxidant effect (35–37), we used another antioxidant, tempol, a superoxide dismutase (SOD) mimetic unrelated to NAC or taurine. Similar to the other antioxidants, when given alone, tempol did not affect plasma FFA during the 48-h

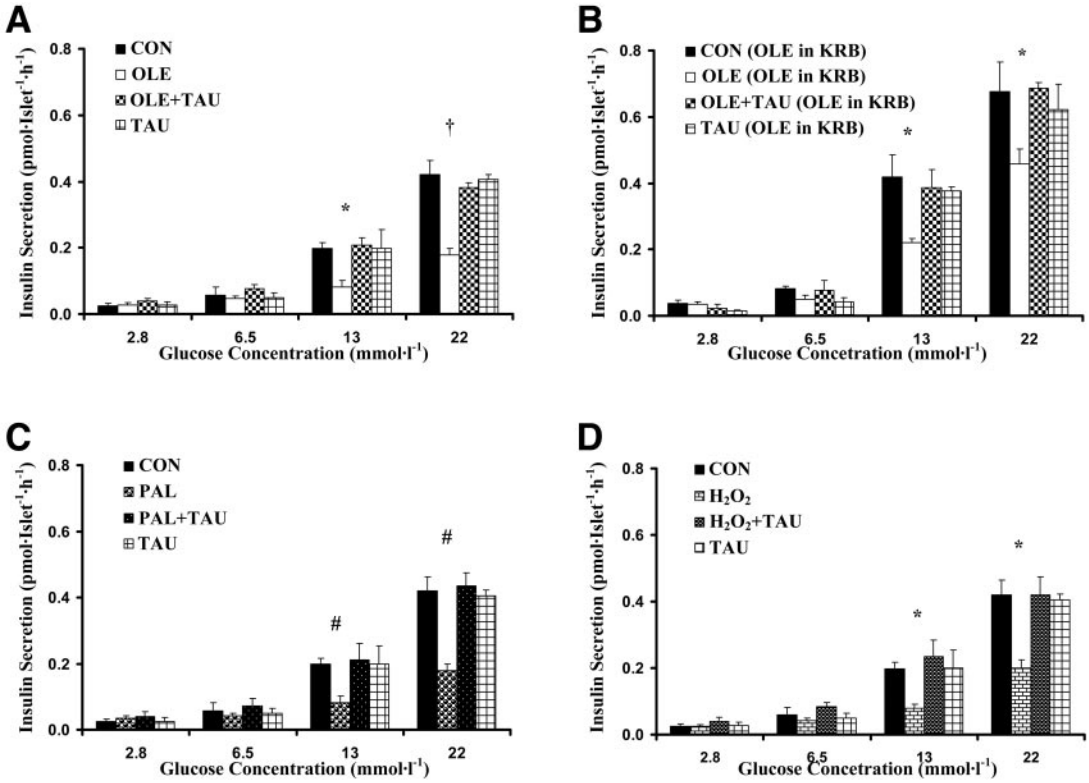

FIG. 5. Effects of oleate, palmitate, and hydrogen peroxide on insulin secretion in cultured islets. Islets were exposed for 48 h to the following conditions. *A*: 0.5% BSA (CON, $n = 7$); oleate (OLE, 0.4 mmol/l in 0.5% BSA, $n = 14$); oleate plus taurine (OLE+TAU; oleate, 0.4 mmol/l in 0.5% BSA; taurine, 1 mmol/l, $n = 10$); and taurine (TAU, 1 mmol/l, $n = 7$). *B*: Oleate (0.4 mmol/l in 0.5% BSA) was added to KRB during the 2-h static incubation; CON (OLE in KRB, $n = 5$); OLE (OLE in KRB, $n = 5$); OLE+TAU (OLE in KRB, $n = 4$); and TAU (OLE in KRB, $n = 4$). *C*: BSA as in *A*; palmitate (PAL, 0.4 mmol /l in 0.5% BSA, $n = 12$); palmitate plus taurine (PAL+TAU; palmitate, 0.4 mmol/l in 0.5% BSA; taurine, 1 mmol/l, $n = 10$); taurine as in *A*. *D*: BSA as in *A*; hydrogen peroxide (H_2O_2, 25 μmol/l in 0.5% BSA, $n = 5$); hydrogen peroxide plus taurine (H_2O_2+TAU; H_2O_2, 25 μmol/l in 0.5% BSA; taurine, 1 mmol/l, $n = 6$); taurine as in *A*. Oleate, palmitate, or hydrogen peroxide decreased the insulin secretion at both 13 and 22 mmol/l glucose, an effect completely prevented by taurine. Our results demonstrate that although the levels of insulin were increased in all the groups exposed to oleate in KRB, the previous exposure to oleate for 48 h still decreased GSIS—an effect prevented by taurine. *$P < 0.05$, #$P < 0.01$, and †$P < 0.001$, all vs. control.

infusion (oleate and tempol, 1,058 ± 108 μmol/l at 48 h, NS, vs. oleate, 1,093 ± 86 μmol/l; tempol, 713 ± 49 μmol/l at 48 h, NS, vs. saline, 663 ± 59 μmol/l). When tempol was added to the oleate infusion, it prevented the oleate-induced GSIS decrease in isolated islets, similar to NAC and taurine (Fig. 4*C*). No significant effect of any antioxidant alone was observed at any glucose concentration. Addition of high-dose NAC (Fig. 4*D*), taurine (Fig. 4*E*), or tempol (Fig. 4*F*) completely prevented the oleate-induced increase in ROS, whereas no significant change in ROS was observed with any antioxidant alone.

In vitro studies in islets. We performed in vitro studies in islets to investigate whether 48-h exposure to oleate had the same effect as in our in vivo or ex vivo studies; moreover, we also used palmitate, because it is commonly used in vitro, and hydrogen peroxide as a positive control for ROS induction (38). Concentrations of 0.4 mmol/l oleate or palmitate in 0.5% BSA decreased GSIS (Fig. 5*A* and *C*) to approximately the same degree, as did 25 μmol /l hydrogen peroxide (Fig. 5*D*), whereas coincubation with 1 mmol/l taurine restored GSIS. Taurine alone had no effect on insulin secretion. Oleate, palmitate, or hydrogen peroxide also increased ROS, which was prevented by the addition of taurine (Fig. 6*A*–*C*). Taurine alone did not affect ROS. Presence of 0.4 mmol/l oleate during the 2 h of GSIS increased the level of insulin in all groups, however, the inhibitory effect of 48-h oleate exposure was still present and prevented by addition of taurine (Fig. 5*B*).

In vitro studies in MIN6 cells. We also performed studies in MIN6 cells to investigate whether oleate upregulates genes of antioxidant enzymes and whether this effect is reversed by antioxidants. Oleate (0.4 mmol/l) in 0.5% BSA for 48 h increased insulin secretion at 0 glucose but decreased GSIS (Fig. 7*A*). Glucose concentrations of 2.8 mmol/l were not studied here because they were already stimulatory in MIN6 cells (23,39); however, our previous data suggest no effect of oleate at 2.8 mmol/l glucose (23). Coincubation with taurine restored GSIS and tended to normalize insulin secretion at 0 glucose. Taurine alone had no effect on insulin secretion. Oleate also increased ROS, which was prevented by the addition of taurine (Fig. 7*B*). Taurine alone did not affect ROS. To confirm the increase in ROS independent of the DCF method, we performed real-time RT-PCR for the following genes induced by oxidative stress: inducible heme oxygenase-1 (HO-1) (cytoplasmic or nuclear), gluthathione peroxidase-1 (GPx-1 mitochondrial and cytoplasmic), catalase (cytoplasmic), Cu/Zn-SOD (SOD3; extracellular), and Mn-SOD (SOD2; mitochondrial). We also evaluated the effect of oleate on the metabolic genes carnitine palmitoyl transferase-1 (CPT-1) and uncoupling protein 2 (UCP-2) as a control. Oleate increased the mRNA of CPT-1, UCP-2, Cu/Zn-SOD, GPx-1, and catalase (Fig. 8) but not SOD2 (data not shown). Addition of taurine did not affect the upregulation of CPT-1 mRNA as expected or of UCP-2 or GPx-1 mRNA but prevented the upregulation of catalase ($P < 0.05$,

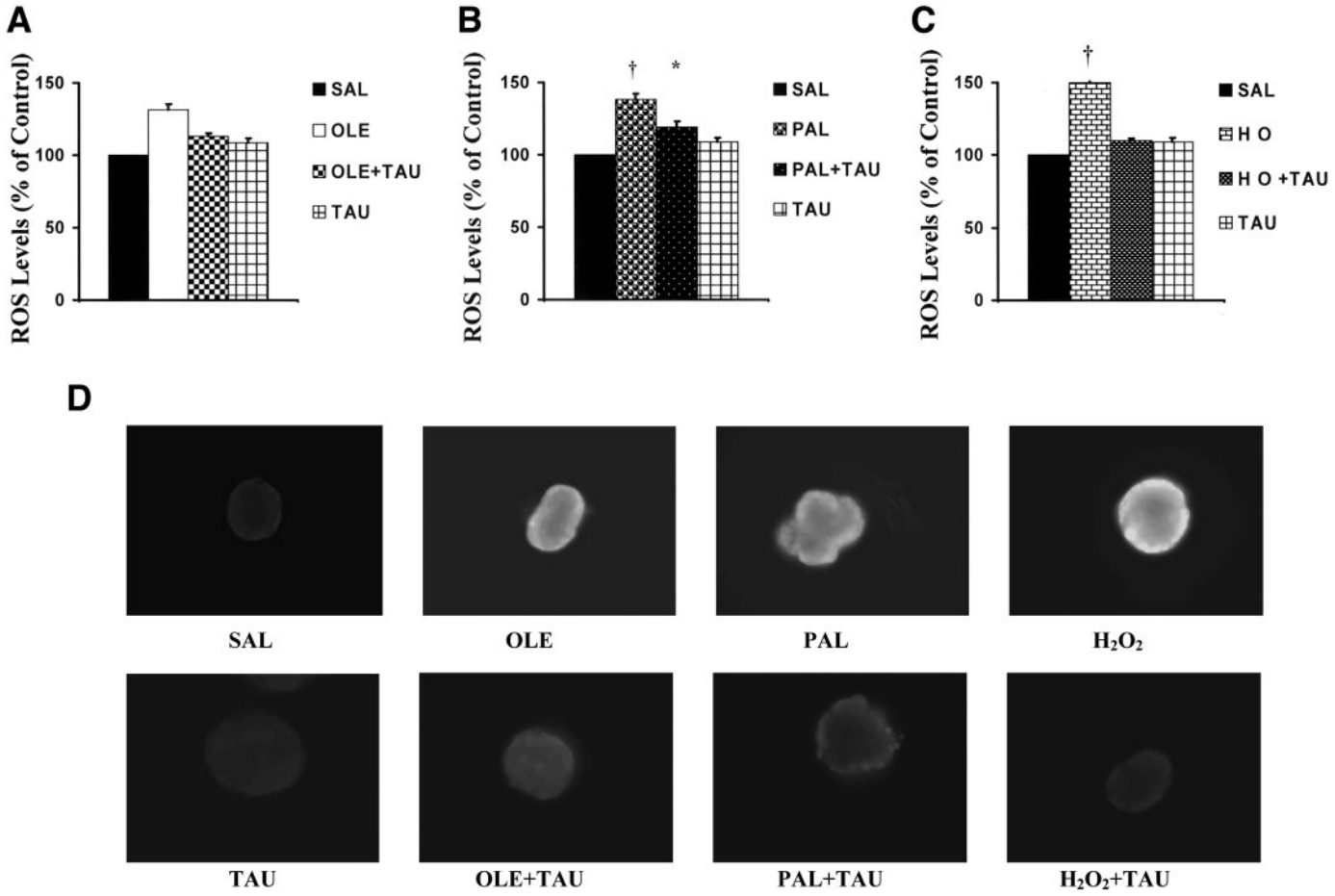

FIG. 6. Effects of oleate (*A*), palmitate (*B*), and hydrogen peroxide (*C*) on islet ROS. The groups are described in the legend of Fig. 5 (CON, n = 4; OLE, n = 6; PAL, n = 5; H_2O_2, n = 6; OLE+TAU, n = 5; PAL+TAU, n = 4; H_2O_2+TAU, n = 6; TAU, n = 5). Data are means ± SE, calculated as fold change from control. *D*: Representative fluorescent images for ROS (magnification ×200). Light images are available on request. Oleate, palmitate, and hydrogen peroxide increased ROS levels compared with BSA control, whereas taurine reversed this effect. *P < 0.05, #P < 0.01, and †P < 0.001, all vs. control.

oleate plus taurine vs. oleate), whereas the effect on Cu/Zn-SOD was only partial. HO-1 mRNA showed a tendency to increase in response to oleate, which appeared to be reversed by taurine.

DISCUSSION

We have examined the effects of prolonged FFA exposure with or without antioxidants on β-cell function in vivo, ex vivo, and in vitro. In our in vivo clamp studies, both insulin and C-peptide responses to glucose were lower in rats treated with oleate. These results indicate reduced insulin secretion, an effect consistent with that observed ex vivo and in vitro in isolated islets and in vitro in MIN6 cells. Insulin secretion during hyperglycemic clamps should be interpreted in the context of insulin sensitivity because in vivo, normal β-cells compensate for insulin resistance. Although FFAs induce insulin resistance, this effect was not observed in the present study. This was not surprising because we noticed only a trend for insulin sensitivity to decrease (12) in our previous studies performed using the same oleate infusion protocol in the same animal model. The lack of detectable insulin resistance may be related to a number of factors, including only moderate elevation of FFAs, their monounsaturation, the female sex of the rats, and the use of the hyperglycemic clamp rather than the gold-standard hyperinsulinemic-euglycemic clamp. Because there was no change in insulin sensitivity and therefore the β-cell did not have to compensate for insulin resistance, our results are similar in vivo and ex vivo.

The mechanism whereby prolonged exposure to fatty acids decreases β-cell function is not completely understood and is probably due to oxidative stress–dependent and –independent pathways (23). Studies have shown that fatty acids promote the generation of ROS in islets (21) and β-cell lines (22,23). Although ROS in minimal amounts can increase basal insulin secretion (40), both lipid peroxides (41) and hydrogen peroxide (16) decreased GSIS. Cytokine exposure (42) and chronic hyperglycemia (18,19) are two other conditions in which reduced β-cell function was found to be associated with oxidative stress, and antioxidants improved GSIS (18,19,43). Decreased GSIS in the presence of oxidative stress may be an adaptive response of the β-cell to limit further ROS generation and reduce endoplasmic reticulum stress, thereby allowing the β-cell to resist apoptosis. Thus, in terms of β-cell survival, decreased function can be interpreted as a beneficial adapting phenomenon (lipoadaptation as in ref. 4). In terms of whole-body physiology, however, decreased β-cell function can also be considered as maladaptive, and therefore lipotoxic, because it results in decreased glucose tolerance (as evidenced by the reduced GINF during our clamps), which can lead to hyperglycemia (9).

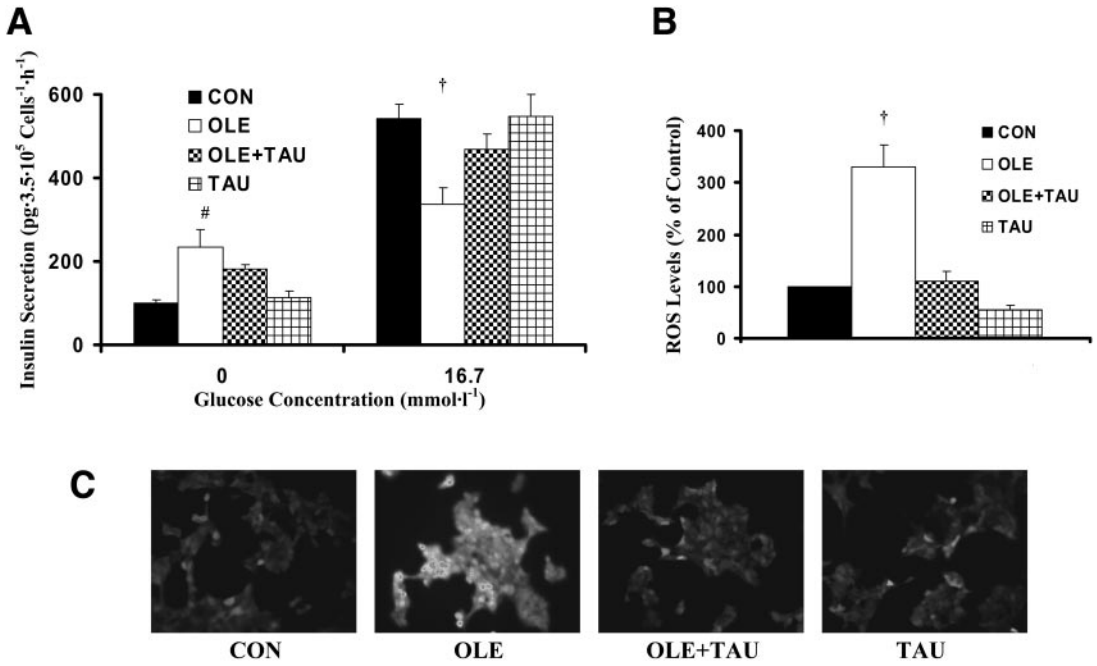

FIG. 7. Effects of oleate and taurine on insulin secretion (*A*) and ROS (*B*) in MIN6 cells. *A*: Cells were exposed for 48 h to 0.5% BSA (CON, n = 18); oleate (OLE, 0.4 mmol/l in 0.5% BSA, n = 18); oleate plus taurine (OLE+TAU; oleate, 0.4 mmol/l in 0.5% BSA; taurine, 1 mmol/l, n = 16); taurine (TAU, 1 mmol/l, n = 18). Glucose concentration of 0 was used to evaluate the non-GSIS because 2.8 mmol/l which is nonstimulatory for islets, is already stimulatory for MIN6 cells. Oleate decreased the insulin secretion at 16.7 mmol/l glucose; taurine completely prevented the decrease induced by oleate at 16.7 mmol/l glucose. Oleate increased insulin secretion at 0 glucose, an effect partially prevented by taurine. *B*: ROS levels in cells exposed to treatments in *A* (CON, n = 9; OLE, n = 9; OLE+TAU, n = 8; TAU, n = 8). Data are means ± SE, calculated as fold change from control. Oleate increased ROS levels compared with BSA control, whereas taurine completely prevented the increase in ROS induced by oleate. #P < 0.01 vs. control. †P < 0.001 vs. control. *C*: Representative fluorescent images for ROS of MIN6 cells (magnification ×200). Light images are available on request from the authors.

We here show that the FFA-induced decrease in GSIS was prevented by antioxidants. Both NAC and taurine were effective in vivo, and according to our results with NAC, the effect was dose dependent. To assess β-cell function independent of systemic factors influencing the β-cell response to glucose in vivo (e.g., not only the prevailing insulin sensitivity but also islet autonomic innervation), we examined GSIS ex vivo in isolated islets. We also measured islet ROS as direct proof of the presence of oxidative stress. Furthermore, to eliminate any indirect effect of in vivo treatments, we exposed islets and MIN6 cells directly to fat in vitro. Forty-eight–hour infusion of oleate or olive oil, which contains mostly oleate, increased ROS and impaired GSIS ex vivo in isolated islets, consistent with our in vivo findings. Treatment with NAC, taurine, or tempol protected the islets from the FFA-mediated impairment in GSIS. Direct exposure of islets for 48 h to oleate or palmitate decreased the insulin response at high glucose, and taurine restored GSIS, similar to our ex vivo model. The same results were obtained with oleate in MIN6 cells, where the oleate-induced increase in expression of antioxidant genes catalase and Cu/Zn-SOD was partially prevented by taurine. The reason for the only partial prevention by taurine is unclear but may be related to cell line variability.

Taurine and NAC are sulfur-containing amino acids that scavenge aldehydes and can directly or indirectly increase the content of glutathione. Besides its antioxidant properties, taurine has other effects that could influence insulin secretion. At high concentrations (>10-fold those used here), taurine can alter calcium flux (37) and interact with GABA receptors (35); however, whether this occurs in the β-cell is not known. At high concentrations, taurine can also close ATP-sensitive K^+ channels in β-cells (36). In support of the action of taurine as an antioxidant, the other antioxidant used, NAC, proved to be equally effective as taurine in preventing the FFA-induced decrease in GSIS; however, because NAC can be converted to taurine, we cannot totally exclude that the NAC effect may also be independent of its antioxidant properties. To address this possibility, we performed ex vivo studies with the antioxidant tempol, an SOD mimetic unrelated to NAC or taurine, which also prevented the oleate-induced decrease in GSIS and the increase in ROS.

Taken together, our findings demonstrate that oxidative stress mediates FFA-induced decrease in GSIS. Consistent with these findings, our previous study in MIN6 cells showed that NAC prevented the decrease in insulin content induced by oleate (23). In the above study, NAC had no effect on GSIS; however, a model of extreme FFA-mediated β-cell dysfunction was used because the cells were exposed for 72 h to oleate, which abolished GSIS. In another study in rat islets (24), NAC also failed to prevent the oleate-induced decrease in GSIS. However, the in vitro model used was also extreme (72-h oleate exposure), and much higher concentrations of NAC were used, raising the possibility of NAC toxicity. Furthermore, the islets were cultured in RPMI-1640, which contains antioxidants. The effect of NAC on insulin content was not examined in that study (24). The latter study of Moore et al. (24) also did not show an increase in ROS. However, the probe used for determination of ROS, carboxy-H_2DCF-DA, is a slightly different probe than ours (H_2DCF-DA), which raises the possibility of different detection levels. In this study, ROS were measured in dispersed islets. We analyzed ROS in situ, and islets or MIN6 cells did not go through any

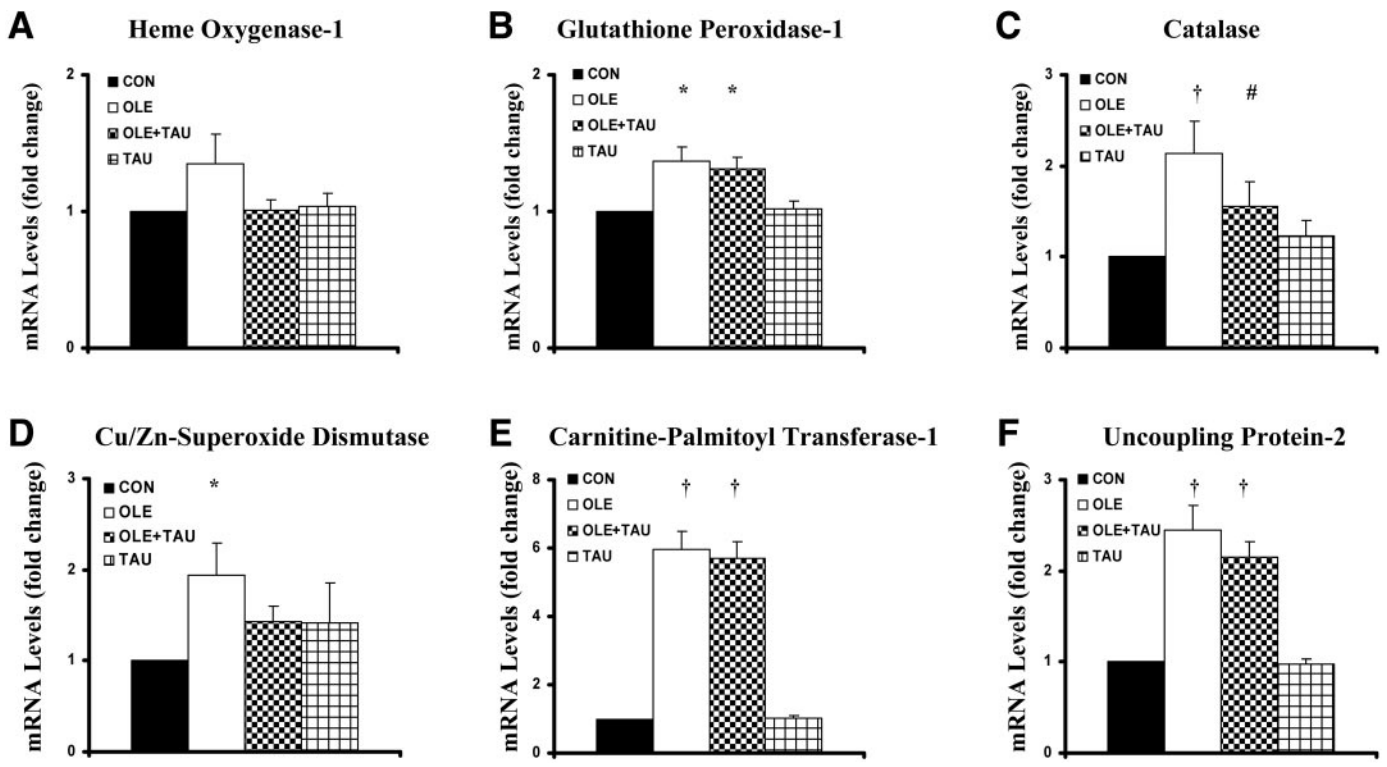

FIG. 8. Real-time RT-PCR from mRNA of MIN6 cells. The groups are described in the legend of Fig. 7 (CON, $n = 18$; OLE, $n = 18$; OLE+TAU, $n = 16$; and TAU, $n = 18$). The level of expression was measured for the following genes: HO-1 (*A*), GPx-1 (*B*), catalase (*C*), SOD (Cu/Zn-SOD, SOD3, and extracellular SOD) (*D*), CPT-1 (*E*), and UCP-2 (*F*). Data are means ± SE, calculated as fold change from control. Real-time RT-PCR of MIN6 cells treated with oleate showed induction of metabolic genes CPT-1 (*E*) and UCP-2 (*F*) and of oxidative stress response genes GPx-1 (*B*) and catalase (*C*) but not Mn-SOD (mitochondrial; not shown). Addition of taurine did not affect the upregulation of CPT-1 as expected (*E*), UCP-2 (*F*), or GPx-1 (*B*) but partially prevented the upregulation of catalase (*C*) and Cu/Zn-SOD (*D*). HO-1 (*A*) and Cu/Zn-SOD (*D*) showed a tendency to increase in response to oleate, which appeared to be reversed by taurine. *$P < 0.05$, #$P < 0.01$, and †$P < 0.001$, all vs. control.

latency or mechanical stress, which may allow clearance of the dye. Independent of the DCF method, we also showed induction of antioxidant genes, the functional consequence of which is uncertain, but which is evidence for the presence of oxidative stress in living cells.

The mechanisms whereby fat-induced oxidative stress affects β-cell function have yet to be clarified. Oxidative stress can inhibit glucose oxidation (41) and/or decrease ATP generation by inducing UCP-2 in β-cells (32). In our MIN6 cells, UCP2 mRNA was induced by oleate, but taurine did not prevent this effect, suggesting that ROS were not implicated. However, ROS could have increased the activity of UCP-2 (44). Oxidative stress can also decrease insulin gene expression (19) by inducing nuclear retention of FoxO1, with decreased pancreatic duodenal homeobox-1 but increased NeuroD expression (45). MaFA expression was shown to be increased by one group (45) but decreased by glucotoxicity, an oxidative stress–mediated event, by another group (46). In our study (23) in MIN6 cells, oleate did not affect insulin gene expression but reduced the insulin content, which was partially restored by NAC. This raises the possibility that antioxidants prevent the impairment in insulin biosynthesis induced by fat that was shown by previous studies (47–49). Another explanation for the decreased GSIS observed with oleate and its prevention by antioxidants may be a change in β-cell mass. However, our unpublished results show that 48-h exposure to fat is too short a period to induce a decrease in β-cell mass, which is in accordance with other authors' findings (7,50).

In summary, our study demonstrates that prolonged exposure to oleate, which induces oxidative stress in islets, decreases GSIS both in vitro and in vivo. These findings are the first direct demonstration that oxidative stress is involved in the FFA-induced decrease in β-cell secretory function and that antioxidants may be useful in its prevention.

ACKNOWLEDGMENTS

This work was supported by the Canadian Institutes of Health Research Grants MOP-69018 to A.G. and NET-54012 to I.G.F. and MOP-12898 to M.B.W. A.I.O. was supported by scholarships from the Banting and Best Diabetes Centre-Novo Nordisk and the Institute of Medical Science, University of Toronto.

We thank Loretta Lam for technical support and the Advanced Optical Microscopy Facility of the University Health Network (Toronto) for ROS data analysis.

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