

# PPAR $\alpha$ suppresses insulin secretion and induces UCP2 in insulinoma cells

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**Abstract** Fatty acids may promote type 2 diabetes by altering insulin secretion from pancreatic  $\beta$  cells, a process known as lipotoxicity. The underlying mechanisms are poorly understood. To test the hypothesis that peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) has a direct effect on islet function, we treated INS-1 cells, an insulinoma cell line, with a PPAR $\alpha$  adenovirus (AdPPAR $\alpha$ ) as well as the PPAR $\alpha$  agonist clofibric acid. AdPPAR $\alpha$ -infected INS-1 cells showed PPAR $\alpha$  agonist- and fatty acid-dependent transactivation of a PPAR $\alpha$  reporter gene. Treatment with either AdPPAR $\alpha$  or clofibric acid increased both catalase activity (a marker of peroxisomal proliferation) and palmitate oxidation. AdPPAR $\alpha$  induced carnitine-palmitoyl transferase-I (CPT-I) mRNA, but had no effect on insulin gene expression. AdPPAR $\alpha$  treatment increased cellular triglyceride content but clofibric acid did not. Both AdPPAR $\alpha$  and clofibric acid decreased basal and glucose-stimulated insulin secretion. Despite increasing fatty acid oxidation, AdPPAR $\alpha$  did not increase cellular ATP content suggesting the stimulation of uncoupled respiration. Consistent with these observations, UCP2 expression doubled in PPAR $\alpha$ -treated cells. Clofibric acid-induced suppression of glucose-stimulated insulin secretion was prevented by the CPT-I inhibitor etomoxir. These data suggest that PPAR $\alpha$ -stimulated fatty acid oxidation can impair  $\beta$  cell function.—Tordjman, K., K. N. Standley, C. Bernal-Mizrachi, T. C. Leone, T. Coleman, D. P. Kelly, and C. F. Semenkovich. PPAR $\alpha$  suppresses insulin secretion and induces UCP2 in insulinoma cells. *J. Lipid Res.* 2002. 43: 936–943.

**Supplementary key words** lipotoxicity • fatty acid oxidation • type 2 diabetes •  $\beta$  cell failure • peroxisome proliferator-activated receptor  $\alpha$

Type 2 diabetes is increasing in prevalence in part because of an epidemic of obesity in developed countries. Although the mechanisms remain obscure, obesity impairs the ability of insulin to promote the uptake of glucose in peripheral tissues and suppresses the production of glucose by the liver (1). Pancreatic  $\beta$  cells compensate for insulin resistance by secreting more insulin, producing hyperinsulinemia sufficient to maintain normal levels of blood glucose. Humans with obesity and insulin resistance can maintain  $\beta$  cell hypersecretion chronically and not

develop diabetes. However,  $\beta$  cell compensation fails in many insulin resistant subjects, resulting in lower insulin levels, less transport of glucose into peripheral tissues, less suppression of hepatic glucose production, and an increase in glucose concentration leading to the diagnosis of diabetes. Type 2 diabetes generally does not occur without this failure of  $\beta$  cell compensation (2), a process that is poorly understood.

Diabetes is a disorder of lipid as well as glucose metabolism, and abnormal lipid metabolism may contribute to relative  $\beta$  cell failure (3). Plasma free fatty acids are elevated in insulin resistance long before  $\beta$  cell failure causes hyperglycemia (4). This occurs because of two early manifestations of insulin resistance: decreased suppression of hormone sensitive lipase in adipocytes (promoting lipolysis from lipid stores) and decreased re-esterification of fatty acids (5). Fatty acids fuel the dyslipidemia of insulin resistance by promoting VLDL production. VLDL is the major substrate for lipoprotein lipase, which is expressed by pancreatic  $\beta$  cells (6) and inversely related to insulin secretion (7).  $\beta$  cells thus have two abundant sources of lipid that might promote insulin secretory failure: plasma free fatty acids and fatty acids provided by lipoprotein lipase-mediated hydrolysis of VLDL. Chronic exposure of islets (8), intact rats (9), and obese non-diabetic humans (10) to elevated concentrations of free fatty acids impairs  $\beta$  cell function, but the mechanisms are unclear.

Peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) is an attractive potential mediator of the effects of fatty acids on  $\beta$  cell function. It is a ligand-activated nuclear

Abbreviations: Ad, adenovirus; ETYA, 5,8,11,14-eicosatetrayonic acid; PPAR $\alpha$ , peroxisome proliferator-activated receptor  $\alpha$ ; PPRE, peroxisome proliferator response element; UCP2, uncoupling protein-2; VLDL, very low density lipoprotein.

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transcription factor expressed at high levels in tissues adapted to metabolize fatty acids such as liver, heart, and kidney (11). Fatty acids are natural ligands for PPAR $\alpha$ , and its activation promotes fatty acid oxidation (12). PPAR $\alpha$  is known to be expressed at low levels in islets (13) but the direct effects of PPAR $\alpha$  on  $\beta$  cell function are unknown. To test the hypothesis that PPAR $\alpha$  affects insulin secretion, we compared the effects of adenoviral-mediated expression of PPAR $\alpha$  with clofibric acid (a PPAR $\alpha$  ligand) in INS-1 cells, a rat insulinoma cell line.

## MATERIALS AND METHODS

### Recombinant PPAR $\alpha$ adenovirus

The 2.1 kb mouse PPAR $\alpha$  cDNA was subcloned into pAC-CMV. Ten micrograms of this construct was combined with *Xba*I-digested AdRR5 in 560  $\mu$ l of HBS (10 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>). Fifty microliters of 2.5 M CaCl<sub>2</sub> was added, then the fine suspension was mixed with 293 cells grown in DMEM + 10% FBS. The suspension was removed the following day, and the medium was changed to DMEM + 2% FBS. One week later, cells were frozen and thawed three times, then spun at 200 g. Serial dilutions of the supernatant were combined with 2 $\times$  MEM containing 1.2% agar, then layered onto 293 cells at 85% confluency. Plaques appeared 2–3 weeks later. Positive clones (as determined by PCR) were expanded and purified by a standard protocol (14). For this virus, two different controls, Ad $\beta$ gal (expressing the bacterial  $\beta$ -galactosidase gene) and AdNull (the same replication-defective virus without an insert) were used. In some experiments, we also used a bicistronic green fluorescent protein (GFP)-PPAR $\alpha$  adenovirus (with a GFP-only virus as a control) generated as described (15). Transfection efficiency was 70–90% for both the PPAR $\alpha$  and control virus.

### Cell culture

INS-1 cells, a rat insulinoma cell line (16), were plated at 2–5  $\times$  10<sup>4</sup> cells/cm<sup>2</sup> in RPMI 1640 (containing 11 mM glucose) supplemented with 10% FBS, 1 mM pyruvate, 10 mM HEPES, 2 mM glutamine, 50  $\mu$ M  $\beta$ -mercaptoethanol, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin in a humidified atmosphere containing 5% CO<sub>2</sub>. Media were changed every 2 to 3 days. Cells at 80–90% confluence were infected with AdPPAR $\alpha$  or control viruses. Infections were performed at a titer of 10<sup>9</sup> pfu/ml, and virus-containing media remained in contact with cells for the duration of the experiment.

Clofibric acid (Sigma, St. Louis, MO) was dissolved in ethanol and used in most experiments at a final concentration of 500  $\mu$ M. Control cells received an equivalent volume of the vehicle. The + enantiomer of etomoxir, an inhibitor of carnitine palmitoyltransferase-I (CPT-I), was used at a concentration of 100  $\mu$ M.

### Northern blotting, RT-PCR, and sequencing

RNA was extracted from INS-1 cells using reagents from Promega (Madison, WI). Ten to twenty micrograms of RNA was subjected to Northern blotting and probed using cDNAs for mouse PPAR $\alpha$ , rat liver CPT-I (CPT-I $\beta$ ), and mouse pre-proinsulin. Probes were labeled with <sup>32</sup>P-dCTP, hybridization was carried out for 1 h at 68°C in ExpressHyb solution (Clontech, Palo Alto, CA), and washes were performed according to the manufacturer's recommendations. Blots were then analyzed using either a Biorad GS 525 Phosphorimager (Biorad Laboratories, Hercules, CA) or conventional autoradiography. For RT-PCR, first strand cDNA synthesis was performed using total RNA primed

with oligo(dT) and Superscript II reverse transcriptase (Life Technologies Inc., Rockville, MD). PCR (30 cycles of 94° for 1 min, 55° for 1 min, 72° for 1 min 30 sec) was conducted using primers derived from exons 6 and 8 of the mouse PPAR $\alpha$  gene (GenBank # X75292 and # X75294): upstream 5'-CGAAGCCT-ACCTGAAGAACT-3', downstream 5'-ACGTGCACAATCCCCT-CTG-3'. The predicted 580 bp product of several reactions was extracted from pooled gel slices (using reagents from Millipore, Bedford, MA) and used as template in dye terminator cycle sequencing reactions.

### Acyl-CoA oxidase reporter gene assay

INS-1 cells cultured in 12-well cluster dishes were infected with 10<sup>9</sup> pfu per well of AdPPAR $\alpha$  or the control virus AdNull. Three hours later, cells were transfected with p(ACO)<sub>3</sub>TKLuc, which contains three copies of the peroxisomal proliferator response element (PPRE) found in the ACO promoter (ACTG-GAAACAGGA) upstream of the luciferase reporter gene. Cells were co-transfected with a  $\beta$ -galactosidase plasmid to control for transfection efficiency. After overnight incubation, cells were washed and fed fresh medium in the presence or absence of 10  $\mu$ M 5,8,11,14-eicosatetraenoic acid (ETYA) (Biomol, Plymouth Meeting, PA) or 250  $\mu$ M oleic acid complexed with BSA (Sigma, St. Louis, MO). The cells were harvested 24 h later and luciferase and  $\beta$ -galactosidase activities were assayed (12).

### Catalase activity

As an index of peroxisome activation, the activity of the peroxisomal enzyme catalase was determined. Colorimetric catalase assays, based on the capacity of the enzyme to metabolize hydrogen peroxide, were performed exactly as described by Baudhuin and colleagues (17). Activity is expressed in arbitrary units calculated as the percent decrease in absorbance at 450 nm compared with assay blanks (with no cellular extract) and normalized for protein content.

### Fatty acid oxidation

$\beta$  oxidation was quantified by adding [<sup>14</sup>C]palmitate to cells and measuring the generation of labeled CO<sub>2</sub>. INS-1 cells were cultured in 25 cm<sup>2</sup> flasks. On the day of the assay, the medium was changed to glucose- and serum-free RPMI 1640. One hour later, the medium was changed to glucose- and serum-free RPMI 1640 containing 200 nCi/ml [<sup>14</sup>C]palmitate (American Radiolabeled Chemicals, St. Louis, MO) corresponding to a palmitate concentration of  $\sim$ 4 pM. The flasks were capped with a rubber septum fitted with a center well containing filter paper soaked with 250  $\mu$ l of 2 N NaOH. Reactions were terminated by injecting 2 ml of 6 N HCl through the septum. Flasks were kept horizontal for 15 min then placed upright for 12 h to trap liberated CO<sub>2</sub>. Filters were then transferred to scintillation vials pre-filled with 1 ml of water and 62  $\mu$ l of 2 N NaOH Aquasol II was added, the mixtures were shaken, chemiluminescence was allowed to subside, and the vials were counted in a scintillation counter.

### Determination of triglyceride content

Cells were washed extensively, pelleted by low speed centrifugation, transferred to glass tubes, dried and weighed, then lipids were extracted with 2:1 (v/v) chloroform-methanol. The organic phase was dried under nitrogen gas, lipids were resuspended in reaction mixtures for the determination of triglyceride content (Sigma), and triglycerides were assayed using a colorimetric assay as described previously (7).

### Insulin assay

INS-1 cells were cultured in 12-well cluster dishes and stimulated with glucose as previously described (7). On the day of the

assay, cells were rinsed with 1 ml KRBH buffer (134 mM NaCl, 4.7 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 10 mM HEPES pH 7.4, 2 mM NaHCO<sub>3</sub>, and 0.5% BSA), then incubated in 0.5 ml of glucose-free KRBH buffer for 1 h. The buffer was aspirated and the cells were incubated in either 0.5 ml glucose-free KRBH or KRBH containing 15 mM glucose for an additional hour. The buffer was then collected and assayed for insulin by radioimmunoassay using a rat insulin antibody (Linco Research, St. Charles, MO). Insulin results were normalized to cellular DNA content determined using a microfluorometer after adding 0.2 N NaOH to the cells and subjecting them to a freeze-thaw protocol.

#### ATP assay

ATP levels were determined within the linear response range of a luciferin-based assay (ApoGlow, BioWhittaker Inc., Rockland, ME). Cells were plated at a density of 10,000 cells/well of a 96-well plate and infected with AdPPAR $\alpha$  or control virus. Nucleotide releasing reagent (100  $\mu$ l) was added to each well containing 100  $\mu$ l of media, incubated for 5 min, then the resultant lysate was placed in a luminometer cuvette. Nucleotide monitoring reagent (20  $\mu$ l) was added and samples were read over 1 sec.

#### UCP2 expression

Quantitative real-time PCR was performed essentially as described (18). Data were normalized to the level of GAPDH message in the same sample. Primers and probe for UCP2 were identical to those used by Young et al. (19): (upstream) 5'-TCA-TCAAAGATACTCTCCTGAAAGC-3', (downstream) 5'-TGACG-GTGGTGCAGAAGC-3', (Probe) 5'-FAM-TGACAGACGACCTC-CCTTGCCACT-TAMRA-3'. For protein detection, extracts were subjected to SDS-PAGE and Western blotting by standard techniques then probed with a polyclonal goat antibody to UCP2 (SC6525, Santa Cruz Biotechnology Inc., Santa Cruz, CA).

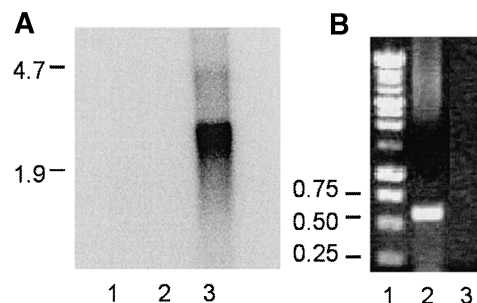
## RESULTS

### PPAR $\alpha$ expression in INS-1 cells

We generated a recombinant, replication-defective adenovirus for mouse PPAR $\alpha$  (AdPPAR $\alpha$ ) and infected INS-1 cells, a model for glucose stimulated insulin secretion. Forty eight hours after infection, PPAR $\alpha$  mRNA was detected by Northern blotting using RNA from cells infected with AdPPAR $\alpha$  (Fig. 1A, lane 3) but not in RNA from cells infected with the control virus Ad $\beta$ gal (Fig. 1A, lane 2) or mock-infected cells (lane 1). The failure to detect PPAR $\alpha$  message by Northern blotting in native INS-1 cells is expected. Levels of PPAR $\alpha$  mRNA are low in INS-1 cells and islets prompting the use of RT-PCR to document expression (13, 20). To confirm that PPAR $\alpha$  is expressed in native INS-1 cells, total RNA from these cells was subjected to RT-PCR. A fragment of the predicted size (580 bp) was detected (Fig. 1B, lane 2) that did not appear when the RT step was omitted from the PCR reaction (lane 3). Sequencing of this fragment confirmed its identity as rat PPAR $\alpha$ .

### AdPPAR $\alpha$ is transcriptionally active in INS-1 cells

To demonstrate that AdPPAR $\alpha$  expresses a functional nuclear receptor, we performed transactivation experiments in INS-1 cells using an acyl-CoA-oxidase (ACO)-luciferase



**Fig. 1.** Detection of peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) expression by Northern blotting in adenovirus (Ad)PPAR $\alpha$ -transfected INS-1 cells (A) and by RT-PCR in cells not exposed to adenoviruses (B). A: Total RNA was isolated from INS-1 cells 48 h after treatment with no virus (lane 1), 10<sup>9</sup> pfu/ml Ad $\beta$ gal, or 10<sup>9</sup> pfu/ml AdPPAR $\alpha$ . Ten micrograms of RNA was loaded in each lane and blots were hybridized with a <sup>32</sup>P-labeled mPPAR $\alpha$  cDNA. Size markers in kb are shown to the left of the panel. B: Total RNA was isolated from INS-1 cells not exposed to adenoviruses and subjected to RT-PCR using primers designed to span introns based on mouse genomic sequence. Lane 1 contains markers with sizes indicated in kb to the left of the panel. Lanes 2 and 3 contain the products of PCR reactions using INS-1 RNA in the presence (lane 2) and absence (lane 3) of RT. Sequencing of the 580 bp fragment from lane 2 confirmed its identity as rat PPAR $\alpha$ .

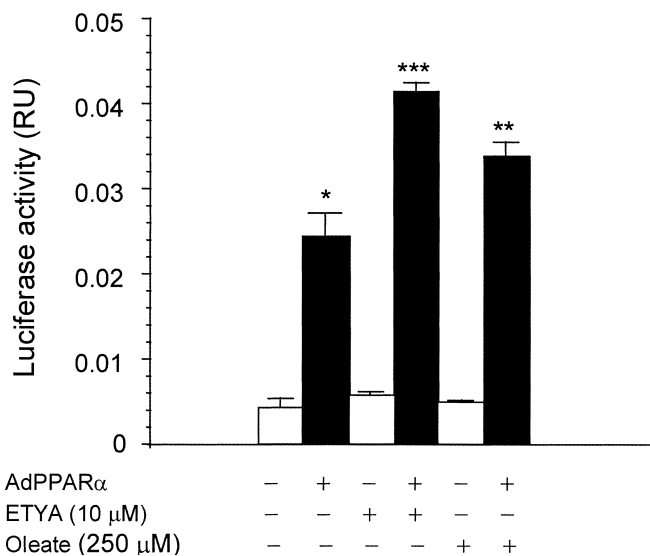
heterologous promoter-reporter construct. Cells infected with either AdPPAR $\alpha$  or AdNull were transfected with p(ACO)<sub>3</sub>TKLuc, a luciferase reporter plasmid containing three copies of the PPRE from the ACO promoter. Oleate (a likely natural activator of PPAR $\alpha$ ) and ETYA (an eicosanoid PPAR $\alpha$  ligand) were used to enhance responses. Infection with AdPPAR $\alpha$  increased luciferase activity ~6-fold in INS-1 cells, a response that was amplified by either ETYA or oleate (Fig. 2).

AdPPAR $\alpha$  was also capable of transactivating an endogenous target gene. The expression of CPT-I, rate-limiting for fatty acid oxidation, was determined by Northern blotting after AdPPAR $\alpha$  infection of INS-1 cells. CPT-I mRNA was difficult to detect despite prolonged exposure times in Ad $\beta$ gal-infected cells (Fig. 3, lane 1), but clearly present in AdPPAR $\alpha$ -treated cells (lane 2). In the same cells, PPAR $\alpha$  expression had no effect on insulin message levels (Fig. 3, bottom panel).

### Induction of catalase activity and fatty acid oxidation

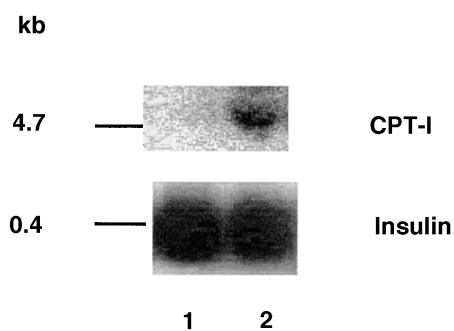
Catalase, a heme protein that degrades hydrogen peroxide, is found at high concentrations in peroxisomes. PPAR $\alpha$  is involved in peroxisomal proliferation in rodents (21). After 48 h, catalase activity was greater ( $P < 0.05$ ) in cells treated with AdPPAR $\alpha$  (Fig. 4A, solid bars) than cells treated with Ad $\beta$ gal (Fig. 4A, open bars). In the absence of adenoviruses, catalase activity was greater ( $P < 0.01$ ) after 48 h of treatment with clofibrate (Fig. 4B, solid bars) as compared with vehicle treatment (Fig. 4B, open bars). Catalase induction after both AdPPAR $\alpha$  and clofibrate treatment was time-dependent with increases notable after 12 h (not shown).

After 48 h, palmitate oxidation was increased ( $P < 0.01$ ) in AdPPAR $\alpha$ -treated cells (Fig. 5A, solid bars) as

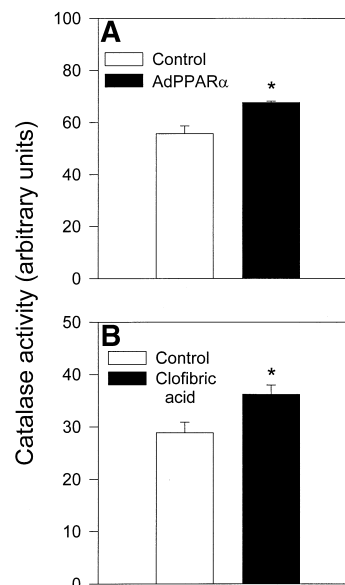


**Fig. 2.** AdPPAR $\alpha$  transactivates an acyl-CoA oxidase (ACO) reporter gene in INS-1 cells. Cells treated with either AdPPAR $\alpha$  (solid bars) or AdNull (open bars) were subsequently transfected with p(ACO)<sub>3</sub>TKLuc containing three copies of the peroxisome proliferator response element (PPRE) found in the ACO promoter upstream of the luciferase gene. All cells were also co-transfected with a  $\beta$ -galactosidase plasmid to control for transfection efficiency. After overnight incubation, the PPAR $\alpha$  ligands 5,8,11,14-eicosatetraenoic acid (ETYA) or oleate were added as indicated. Twenty-four hours later, cells were harvested and assayed for luciferase activity. Results are expressed in relative units as mean  $\pm$  SEM. \* $P$  < 0.05, \*\*\* $P$  < 0.001, \*\* $P$  < 0.005; each versus the open bar to the immediate left.

compared with AdNull-treated cells (Fig. 5A, open bars). In INS-1 cells not treated with adenoviruses, clofibrilic acid treatment for 48 h increased palmitate oxidation ( $P$  < 0.05) as compared with vehicle-treated cells. These assays were performed using labeled palmitate in serum-free



**Fig. 3.** Detection of CPT-I and insulin message levels by Northern blotting in INS-1 cells. Total RNA was extracted from INS-1 cells 48 h after transfection with either the control virus Ad $\beta$ -gal (Lane 1) or AdPPAR $\alpha$  (Lane 2). Ten micrograms of RNA were loaded in each lane, and blots were hybridized with probes for CPT-I $\beta$  (A) or preproinsulin (B). Molecular sizes are indicated to the left of the panels in kb. Identical results were seen in three independent experiments. The CPT-1 blots were exposed for 3–5 days while the insulin blots were exposed for less than 2 h. Detection of 18S and 28S rRNA confirmed the integrity of the samples and equal loading in each lane.



**Fig. 4.** Induction of catalase activity in INS-1 cells. Cells were treated for 48 h then catalase activity was measured by following the metabolism of hydrogen peroxide. In preliminary experiments, induction of catalase activity by either AdPPAR $\alpha$  or clofibrilic acid was shown to be time dependent. A: Cells were treated with Ad $\beta$ -gal (open bar) or AdPPAR $\alpha$  (solid bar). Results indicate mean  $\pm$  SEM. \* $P$  < 0.01 versus open bar. Identical results were seen in three independent experiments. B: Cells were treated with either carrier (open bar) or 500  $\mu$ M clofibrilic acid. Results indicate mean  $\pm$  SEM. \* $P$  < 0.05 versus open bar. Identical results were seen in four independent experiments.

and glucose-free media. The same proportional effects (at lower levels of oxidation) were seen when fatty acid oxidation studies were done in the presence of glucose.

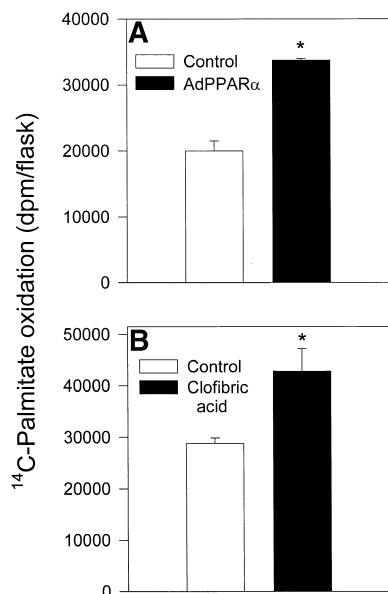
#### PPAR $\alpha$ suppresses insulin secretion independent of triglyceride content

Treatment with AdPPAR $\alpha$  decreased basal insulin secretion by  $\sim$ 50% and glucose-stimulated insulin secretion (GSIS) by  $\sim$ 57% (each  $P$  < 0.05, Fig. 6A, solid bars). Similar results were seen in seven independent experiments. In three independent experiments, parallel cells treated with AdPPAR $\alpha$  showed no effect on insulin message levels (Fig. 3). In the absence of adenoviruses, treatment with clofibrilic acid decreased basal insulin secretion by  $\sim$ 31% and GSIS by  $\sim$ 40% (each  $P$  < 0.05, Fig. 6B, solid bars). Similar results were seen in six independent experiments.

Triglyceride mass was determined in cells treated with AdPPAR $\alpha$  or clofibrilic acid (in the absence of adenoviruses). Triglyceride content was  $\sim$ 157% higher in AdPPAR $\alpha$ -treated as compared with AdNull-treated cells (Fig. 6C,  $P$  < 0.05), but there was no difference between cells treated with clofibrilic acid (Fig. 6D, solid bar) and vehicle (Fig. 6D, open bar).

#### Effects on ATP content and UCP2 expression

The generation of ATP from glucose metabolism can simulate insulin secretion in normal islets. Fatty acid oxidation can also stimulate ATP production. PPAR $\alpha$ -induced

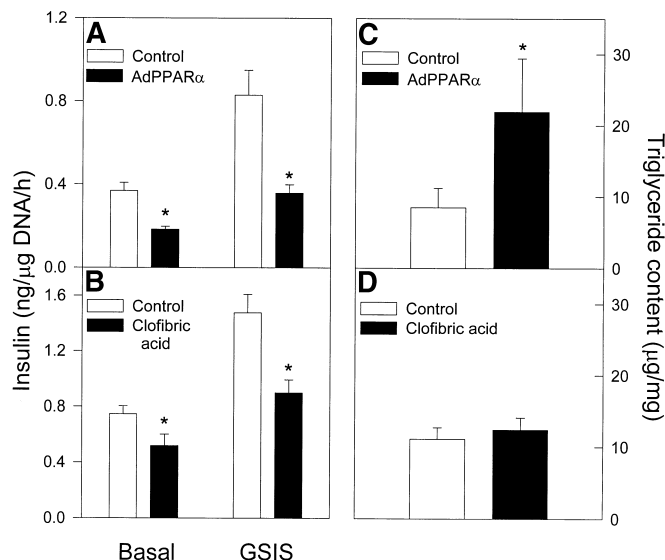


**Fig. 5.** Induction of [ $^{14}$ C]palmitate oxidation in INS-1 cells. Cells were treated for 48 h then fatty acid oxidation was measured by determining the ability of cells to produce radiolabeled  $\text{CO}_2$ . In preliminary experiments, induction of fatty acid oxidation by either AdPPAR $\alpha$  or clofibrilic acid was shown to be time dependent. A: Cells were treated with AdNull (open bar) or AdPPAR $\alpha$  (solid bar). Results indicate mean  $\pm$  SEM. \*  $P < 0.01$  versus open bar. Identical results were seen in three independent experiments. B: Cells were treated with either carrier (open bar) or 500  $\mu$ M clofibrilic acid. Results indicate mean  $\pm$  SEM. \*  $P < 0.05$  versus open bar. Identical results were seen in three independent experiments.

fatty acid oxidation had no significant effect on ATP levels in INS-1 cells (Table 1), suggesting that PPAR $\alpha$  activates respiration that is uncoupled from oxidative phosphorylation. Consistent with this notion, PPAR $\alpha$  increased levels of UCP2 mRNA (Fig. 7A) and protein (Fig. 7B) in INS-1 cells.

#### Etomoxir prevents clofibrilic acid-induced suppression of glucose-stimulated insulin secretion

Like AdPPAR $\alpha$ , clofibrilic acid induced catalase activity, increased fatty acid oxidation, and decreased insulin secretion, but unlike PPAR $\alpha$ , clofibrilic acid did not affect cellular triglyceride content. To determine if increased fatty acid oxidation is involved in the suppression of insulin secretion, INS-1 cells were treated with the specific CPT-1 inhibitor etomoxir. This treatment decreased [ $^{14}$ C]palmitate oxidation by 64% in cells not treated with clofibrilic acid ( $30,833 \pm 5,481$  dpm/flask without etomoxir;  $11,234 \pm 1,284$  dpm/flask + 100  $\mu$ M etomoxir;  $P < 0.05$ ) and by 70% in cells treated with clofibrilic acid ( $45,814 \pm 5,774$  dpm/flask without etomoxir;  $13,804 \pm 302 + 100 \mu$ M etomoxir;  $P < 0.01$ ). Consistent with a role for fatty acid oxidation in the suppression of insulin secretion, the decrease in glucose-stimulated insulin secretion caused by clofibrilic acid (Fig. 8B) was not seen when both clofibrilic acid and etomoxir were added to cells (Fig. 8B Clo + Eto).



**Fig. 6.** Effect of PPAR $\alpha$  activation on insulin secretion (A and B) and triglyceride content (C and D) in INS-1 cells. To test the effect of the PPAR $\alpha$  protein, cells were treated for 48 h with AdPPAR $\alpha$  (solid bars, A and C) or the control adenovirus (open bars, A and C). To test the effect of the PPAR $\alpha$  ligand, cells were treated for 48 h with 500  $\mu$ M clofibrilic acid solid bars, B and D) or its carrier (open bars, B and D). For the insulin secretion studies of A and B, basal secretion was assayed in glucose-free KRBH. Glucose stimulated insulin secretion (GSIS) was assayed in KRBH containing 15 mM glucose. Results indicate mean  $\pm$  SEM. \*  $P < 0.05$  compared with open bar to the left of the solid bar. The same results were seen in seven independent experiments for A and six for B. For purposes of clarity, not all significant comparisons are indicated. For example, insulin concentrations after glucose stimulation were significantly higher than in the basal state for each intervention. For the lipid content results of C and D, triglyceride content was assayed after drying cells and extracting lipids with chloroform-methanol. Results are presented as mean  $\pm$  SEM. In C, \*  $P < 0.05$  compared with control. The same results were seen in seven independent experiments.

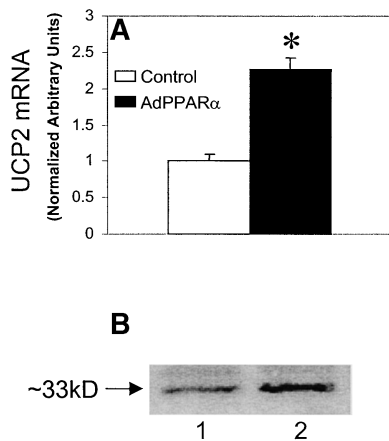
## DISCUSSION

Altered fatty acid metabolism in pancreatic  $\beta$  cells may contribute to the gradual failure of  $\beta$  cell compensation that frequently causes diabetes in humans with obesity and insulin resistance (22). Here we show that increasing fatty acid oxidation, either with adenoviral-mediated overexpression of PPAR $\alpha$  or with activation of endogenous PPAR $\alpha$  using clofibrilic acid, decreases insulin secretion.

TABLE 1. ATP content of INS-1 cells following treatment with AdPPAR $\alpha$

	Control	AdPPAR $\alpha$
Experiment 1 (n = 6)	139,116 $\pm$ 5,309	132,775 $\pm$ 6,471
Experiment 2 (n = 8)	220,016 $\pm$ 7,342	213,397 $\pm$ 5,397
Experiment 3 (n = 6)	121,235 $\pm$ 7,663	117,700 $\pm$ 11,440

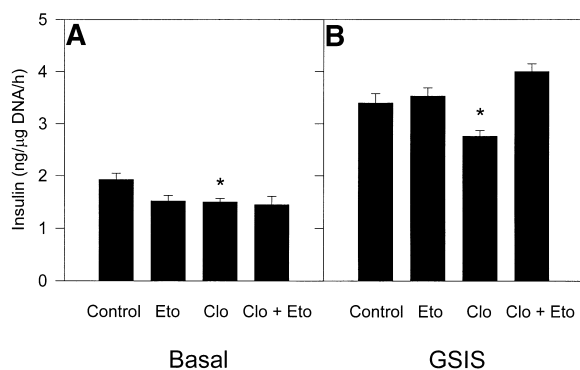
ATP content was determined using a luciferin-based assay 48 h after infection with a control adenovirus or an adenovirus expressing PPAR $\alpha$  (AdPPAR $\alpha$ ). Data are expressed as mean  $\pm$  SEM in relative light units.



**Fig. 7.** PPAR $\alpha$  induces UCP2 expression in INS-1 cells. Cells were studied 48 h following infection with control adenovirus or an adenovirus expressing PPAR $\alpha$  (AdPPAR $\alpha$ ). A: mRNA for UCP2 was determined by real-time PCR. Bars represent mean  $\pm$  SEM of UCP2 mRNA for four samples per condition. Results were normalized to levels in control samples and corrected to GAPDH mRNA levels. \*  $P < 0.01$  versus Control. The same results were seen in two independent experiments. B: Protein extracts were prepared from cells treated with control adenovirus (lane 1) or AdPPAR $\alpha$  (lane 2) for 48 h. Extracts were subjected to Western blotting using equal amounts of total protein in each lane and detected with a UCP2 antibody (Santa Cruz Biotechnology).

PPAR $\alpha$  induces the expression of UCP2 in insulinoma cells and does not increase intracellular ATP. The suppression of glucose-stimulated insulin secretion can be reversed by inhibition of fatty acid oxidation. Taken together, these findings suggest that excessive fatty acid oxidation may contribute to defects in  $\beta$  cell function.

Uncoupling proteins disrupt the electrochemical gradient across the inner mitochondrial membrane, producing



**Fig. 8.** Effect of etomoxir on clofibrilic acid-induced suppression of glucose-stimulated insulin secretion in INS-1 cells. Cells were treated with carrier (Control), 100  $\mu$ M etomoxir (Eto), 500  $\mu$ M clofibrilic acid (Clo), or both etomoxir and clofibrilic acid (Clo + Eto). After 48 h, cells were assayed for insulin secretion in the basal state (A) and after glucose stimulation (panel B, GSIS). Results are presented as mean  $\pm$  SEM. B: \*  $P < 0.05$  versus Control, Eto, and Clo + Eto by ANOVA. A: \*  $P < 0.05$  versus Control. For purposes of clarity, not all significant comparisons are indicated. For example, all glucose-stimulated insulin concentrations were significantly higher than in the basal state. The same results were seen in two independent experiments.

heat instead of ATP from respiration (23). PPAR $\alpha$  also induces UCP1 expression in brown fat (24) and UCP3 expression in the heart (19). The induction of UCP2 and suppression of insulin secretion in the current work are consistent with data showing that overexpression of UCP2 suppresses insulin secretion in normal islets (25) and that UCP2 deficiency improves insulin secretion in ob/ob mice (26). UCP2 enhances proton leak in isolated mitochondria from INS-1 cells (27), suggesting that PPAR $\alpha$ -mediated induction of UCP2 uncouples respiration and prevents a rise in ATP despite accelerated fatty acid oxidation.

If accelerated fatty acid oxidation decreases insulin secretion, decreasing fatty acid oxidation would be predicted to enhance insulin secretion. A recent human study has validated this prediction. Clayton and colleagues (28) described a subject with hypoglycemia and striking hyperinsulinemia in the setting of deficient short chain *L*-3-hydroxyacyl-CoA dehydrogenase activity, a key enzyme in the mitochondrial  $\beta$ -oxidation spiral. These results complement studies in several systems (29–31) showing that different inhibitors of CPT-I promote glucose-stimulated insulin secretion.

In Zucker diabetic fatty rats (fa/fa, animals with a mutation in the leptin receptor),  $\beta$  cell failure is associated with islet lipid accumulation (32). Message levels for PPAR $\alpha$ , CPT-I, and ACO are decreased and fatty acid oxidation is suppressed in these islets. Some of these defects are reversed by overexpressing the leptin receptor in fa/fa islets (13). These indirect findings suggest that increasing PPAR $\alpha$  activity would be associated with improved  $\beta$  cell function in Zucker diabetic islets. However, these islets may not be reflective of other models of insulin secretory failure since overexpression of UCP2 (which suppresses insulin secretion in normal islets, 25) improves insulin secretion in Zucker diabetic islets (33).

Glucose decreases PPAR $\alpha$  expression in  $\beta$  cells (20). Glucose and fatty acids compete as respiratory substrates in many cells, and PPAR $\alpha$  regulates fatty acid oxidation, so glucose-induced PPAR $\alpha$  suppression suggests the existence of a glucose-fatty acid cycle in the  $\beta$  cell. This issue is unresolved. Different groups report conflicting results regarding the effects of chronic fatty acid exposure on islet glucose metabolism (8, 34). Fatty acids do not have a sufficient effect on glucose metabolism in INS-1 cells to explain their effect on insulin secretion (35). Several lines of evidence implicate fatty acid metabolism in this process.

Fatty acids may be required for insulin secretion in the setting of starvation (36). Acute exposure to fatty acids increases insulin secretion (37, 38). Chronic exposure to fatty acids suppresses glucose-stimulated insulin secretion in insulinoma cells (39), as well as rat (8, 40), mouse (41), and human (42) islets. Fatty acids are PPAR $\alpha$  ligands, and induction of CPT-I by fatty acids is mediated by PPAR $\alpha$  (12). Our work extends these findings by providing direct evidence that PPAR $\alpha$  can affect insulin secretion.

While PPAR $\alpha$  promotes fatty acid oxidation, it also stimulates lipid uptake (43–45). Increased islet neutral lipid content (46) does not explain our findings. Both PPAR $\alpha$  and clofibrilic acid suppress insulin secretion, but clofibrilic

acid does so without affecting islet triglyceride content (Fig. 6). The reversal of insulin suppression by a fatty acid oxidation inhibitor (Fig. 8B) implicates excessive fatty acid oxidation in  $\beta$  cell dysfunction.

Excess fatty acid oxidation could decrease cytosolic levels of long chain acyl-CoA (47), a signaling molecule that might promote insulin secretion through phospholipid metabolism or activation of protein kinase C (48). Tests of the long chain acyl-CoA hypothesis have provided conflicting results (49, 50). Excess fatty acid oxidation could also desensitize the  $\beta$  cell to calcium fluxes. Glucose-stimulated insulin secretion is prompted by an increase in mitochondrial metabolism, leading to an increase in cytosolic calcium, the main trigger for insulin secretion. Repeated stimulation of mitochondria can alter calcium flux and suppress insulin secretion despite normal generation of ATP (51).

Yoshikasa and colleagues recently reported a striking decrease in glucose-stimulated insulin secretion and insulin content in rat pancreatic islets after treatment for 48 h with the PPAR $\alpha$  agonist bezafibrate (52). These results suggest that the effects of PPAR $\alpha$  on insulin secretion are not limited to insulinoma cells.

Given the complexities of  $\beta$  cell function, it is difficult to extrapolate observations made in INS-1 cells to defects in insulin secretion in human type 2 diabetes. However, chronic exposure of islets to fatty acids, PPAR $\alpha$  ligands, causes  $\beta$  cell dysfunction. Our results suggest a role for PPAR $\alpha$ -induced fatty acid oxidation in the suppression of insulin secretion. Future studies of lipid signaling molecules and mitochondrial calcium fluxes in PPAR $\alpha$ -treated cells could help clarify the mechanisms leading to  $\beta$  cell failure in the setting of insulin resistance. ■

This work was supported by NIH grants HL58427, DK53198, the Clinical Nutrition Research Unit (DK56341), the Diabetes Research and Training Center (DK20579), and by an American Diabetes Association/Johnson & Johnson Mentor-Based Postdoctoral Fellowship.

Manuscript received 2 October 2001 and in revised form 14 March 2002.

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