

# Gene and Protein Kinase Expression Profiling of Reactive Oxygen Species–Associated Lipotoxicity in the Pancreatic $\beta$ -Cell Line MIN6

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Oligonucleotide microarrays were used to define oleic acid (OA)-regulated gene expression and proteomic technology to screen protein kinases in MIN6 insulinoma cells. The effects of oxidative stress caused by OA and potential protective effects of *N*-acetyl-L-cysteine (NAC), a scavenger of reactive oxygen species (ROS), on global gene expression and  $\beta$ -cell function were investigated. Long-term exposure of MIN6 cells to OA led to a threefold increase in basal insulin secretion, a 50% decrease in insulin content, an inhibition of glucose-stimulated insulin secretion (GSIS), and a twofold increase in the level of ROS. The addition of NAC normalized both the OA-induced insulin content and ROS elevation, but it failed to restore GSIS. Microarray studies and subsequent quantitative PCR analysis showed that OA consistently regulated the expression of 45 genes involved in metabolism, cell growth, signal transduction, transcription, and protein processing. The addition of NAC largely normalized the expression of the OA-regulated genes involved in cell growth and differentiation but not other functions. A protein kinase screen showed that OA regulated the expression and/or phosphorylation levels of kinases involved in stress-response mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and cell cycle control pathways. Importantly, these findings indicate that chronic OA exposure can impair  $\beta$ -cell function through ROS-dependent and -independent mechanisms. *Diabetes* 53: 129–140, 2004

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CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; EST, expressed sequence tag; FFA, free fatty acid; GSK, glycogen synthase kinase; GSIS, glucose-stimulated insulin secretion; KRB, Krebs-Ringer HEPES buffer; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NAC, *N*-acetyl-L-cysteine; OA, oleic acid; PDK, 3-phosphoinositide-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; Rb, retinoblastoma; ROS, reactive oxygen species; SCD, stearoyl-CoA desaturase; Sdf, stromal cell-derived factor; TQ, trace quantity; UCP, uncoupling protein; VILIP, visinin-like protein.

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**A**cute exposure to elevated free fatty acids (FFAs) in the plasma leads to enhanced insulin secretion from the pancreatic  $\beta$ -cells, which then promotes the uptake of nutrients into the peripheral tissues (1). However, chronic exposure to elevated levels of plasma FFAs, such as that observed in obesity, has been implicated in the development of type 2 diabetes by the effects on insulin resistance in peripheral tissues and  $\beta$ -cell dysfunction (2,3). Long-term exposure to FFAs results in elevated basal insulin secretion with a concomitant suppression of glucose-stimulated insulin secretion (GSIS), elevated production of reactive oxygen species (ROS) (4), inhibition of insulin biosynthesis, and induction of  $\beta$ -cell death both in vivo and in vitro (5–9). Together with insulin resistance in peripheral tissues, it is these lipotoxic effects resulting in  $\beta$ -cell failure that are thought to precipitate the development of type 2 diabetes.

While the mechanism of lipotoxicity in  $\beta$ -cells is not well understood, previous studies have revealed that long-term exposure to FFAs results in changes in  $\beta$ -cell metabolism, abnormal signal transduction, and altered expression of proteins at the level of both transcription and translation (5,7,8). Studies of the effects of chronic FFA exposure on gene expression in islets and clonal  $\beta$ -cell lines have examined both specific candidate genes as well as global gene expression (6,9,10). These analyses have produced a complex picture of transcriptional regulation by FFAs. Long-term exposure to FFAs decreases acetyl-CoA carboxylase gene expression (11) and increases carnitine palmitoyltransferase 1 (CPT-1) gene expression leading to elevated FFA oxidation in INS-1 insulinoma cells (12). Microarray analysis of the effects of elevated concentrations of palmitate for up to 44 days has confirmed significant upregulation of genes related to fatty acid oxidation and downregulation of genes involved in glycolysis (10) consistent with the hypothesis of a glucose–fatty acid cycle (Randle cycle) (13,14). In islets cultured with palmitate for 48 h, pyruvate carboxylase (PC), peroxisome proliferator-activated receptor (PPAR)- $\alpha$ , GLUT2, glucokinase, preproinsulin, and pancreatic/duodenal homeobox-1 mRNA levels were significantly decreased (9,15), implying a significant effect of FFAs on glucose metabolism and insulin biosynthesis. Furthermore, uncoupling protein (UCP)-2 mRNA and protein levels are significantly upregulated in INS-1 cells following 48 h incubation with oleic

acid (OA), an effect that could be mediated through a PPAR- $\gamma$  pathway (16,17). We have shown that the level of UCP2 mRNA correlates negatively with both ATP synthesis and GSIS in islets (18–20) and correlate positively with the production of ROS (21). These observations implicate UCP2 upregulation as a potential mechanism linking chronic exposure to FFA with a diminished capacity for GSIS and a protective effect in the limitation of ROS production.

Elevated FFAs leading to increased  $\beta$ -oxidation and elevated ROS have been shown to contribute to the deterioration in islet function and  $\beta$ -cell viability (22). This might be due to the relatively low level of expression of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase in islets (23). Several studies have demonstrated that antioxidants such as *N*-acetyl-L-cysteine (NAC), aminoguanidine, and a combination of vitamins E and C can, at least in part, block the ROS-induced effects of chronic hyperglycemia (glucotoxicity) in  $\beta$ -cells (24,25), but the effectiveness of antioxidants in reversing FFA-mediated damage is less clear and hence the focus of the present study.

## RESEARCH DESIGN AND METHODS

**Reagents.** NAC, OA, cycloheximide (CHX),  $\beta$ -actin antibody, and BSA (fatty acid free) were purchased from Sigma-Aldrich (Oakville ON, Canada). OA bound to BSA was prepared by stirring 4 mmol/l OA with 5% (wt/vol) BSA in Krebs-Ringer HEPES buffer (KRB) (125 mmol/l NaCl, 5.9 mmol/l KCl, 1.28 mmol/l CaCl<sub>2</sub>, 5.0 mmol/l NaCO<sub>3</sub>, and 25 mmol/l HEPES) for 30 min at 50°C. All solutions were adjusted to pH 7.0, filtered through a 0.22- $\mu$ m filter, and stored at -20°C until required. TRIzol and SuperScript II RNase H-Reverse Transcriptase kits were from Invitrogen (Burlington ON, Canada). HotStarTaq PCR kits and RNeasy kits were purchased from Qiagen (Mississauga ON, Canada). SYBR Green was a product of Molecular Probes (Eugene, OR). Enzo BioArray High Yield RNA Transcript Labeling kits and U74AV2 murine genome microarrays were purchased from Affymetrix (Santa Clara, CA). Antibodies against phospho-p70 S6 kinase (Thr389), phospho-Akt (Thr308), and phospho-retinoblastoma (Rb) (Ser780) were purchased from Cell Signaling Technology (Beverly, MA). p38 mitogen-activated protein kinase (MAPK) and PKG were from Stressgen Biotechnologies (Victoria BC, Canada); p70 S6 kinase, raf-1, G protein-coupled receptor kinase 2, protein kinase C (PKC)- $\mu$ , cyclin-dependent kinase 7, and focal adhesion kinase antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Jun NH<sub>2</sub>-terminal kinase (JNK)-3/MAPK 10 antibody was from Upstate Biotechnology (Charlottesville, VA). MAPK kinase (MEK)-2 antibody was purchased from BD Biosciences (Mississauga ON, Canada); stromal cell-derived factor (Sdf)-1 antibody was from R&D Systems (Minneapolis, MN). VILIP-1 antibody was kindly provided by Dr. K.-H. Braunewell (Humboldt University, Berlin, Germany).

**Cell culture and treatment.** MIN6 cells, a gift from Dr. S. Seino, Chiba University (passage number 35–45), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/l glucose supplemented with 10% fetal bovine serum, 48.6  $\mu$ mol/l  $\beta$ -mercaptoethanol, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin, at 37°C in a humidified atmosphere (5% CO<sub>2</sub>, 95% air). Cells were grown in monolayer to 80% confluency in 100-mm<sup>2</sup> dishes or 24-well plates. Cells were then incubated overnight in DMEM containing 10 mmol/l glucose, followed by treatment for 72 h with either 1) 0.5% (wt/vol) BSA, 2) 0.4 mmol/l OA in BSA, 3) 0.4 mmol/l OA in BSA plus 1 mmol/l NAC, or 4) 1 mmol/l NAC in BSA.

**Insulin secretion and insulin content measurement.** MIN6 cells cultured in 24-well plates were preincubated for two sequential periods of 30 min in glucose-free KRB containing 0.1% (wt/vol) BSA in a 37°C humidified air incubator. The cells were then incubated in the same buffer with glucose concentrations ranging from 0 to 30 mmol/l for 30 min. At the end of the experiment, the supernatant was collected and centrifuged (300g/10 min) to remove cellular debris. The concentration of insulin in the supernatant was determined by radioimmunoassay as previously described (26). Insulin secretion measurements were normalized to total DNA content in the sample. The total cellular insulin content was extracted using 75% ethanol containing 1.5% (vol/vol) HCl. Insulin content was reported as the amount of insulin per microgram DNA.

**ROS measurement.** MIN6 cells were harvested following treatment by 0.05% (wt/vol) trypsin containing 0.5 mmol/l EDTA. The harvested cells were washed in Ca<sup>2+</sup>-free KRB buffer and permeabilized using 80  $\mu$ g/ml saponin as described elsewhere (27). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) production in  $\beta$ -cell mitochondria was measured in permeabilized cells fluorometrically by monitoring the catalase-sensitive conversion of dichlorofluorescein to dichlorofluorescein (excitation 490 nm, emission 530 nm) in the presence of horseradish peroxidase (28,29). The reaction was started by addition of 5 mmol/l glutamate and 5 mmol/l malate as a mitochondrial respiratory substrate and 1  $\mu$ mol/l antimycin A, an inhibitor of electron transfer. Dichlorofluorescein was obtained from the stable compound dichlorofluorescein diacetate by alkaline hydrolysis (28).

**RNA extraction and gene expression profiling.** Total RNA was isolated from MIN6 cells using TRIzol reagent and further purified with RNeasy kits according to the manufacturer's instructions. Double-stranded cDNA was prepared using SuperScript II RT and T7-(dT)<sub>24</sub> primers to initiate reverse transcription of mRNA in the sample. The biotin-labeled cRNA was then synthesized using the BioArray HighYield RNA transcript labeling kit, purified by RNeasy columns, fragmented to a mean size of ~50–100 nucleotides, and then hybridized to murine genome U74A microarrays version 2 according to the Affymetrix instructions. Microarrays were washed, stained with streptavidin-phycoerythrin, and scanned using Affymetrix GeneChip Fluidics Station 400 and Scanner. Expression of genes that were deemed significantly altered by OA treatment according to Microarray Suite version 5.0 statistical analysis software (Affymetrix, Santa Clara, CA) in at least three of four independent experiments were chosen and further classified according to biological function.

**Real-time PCR.** Total RNA was converted to cDNA using SuperScript II RT. Amplification of each target cDNA was performed with HotStarTaq PCR reagent kits in the ABI PRISM 7900HT sequence detection system according to the protocols provided by the manufacturer (PE Applied Biosystems, Foster City, CA). PCR products were quantified fluorometrically using SYBR Green.  $\beta$ -Actin expression in each sample was used as a control. Two different primer sets were designed and synthesized for each investigated gene or expressed sequence tag (EST) using Primer Express version 2.0 (Applied Biosystems). A standard curve of each primer set was generated using mouse genomic DNA. One primer set was chosen for each gene to perform all the subsequent PCR to ensure better PCR efficiency and standard curve lineage. Primer sequences will be provided on request.

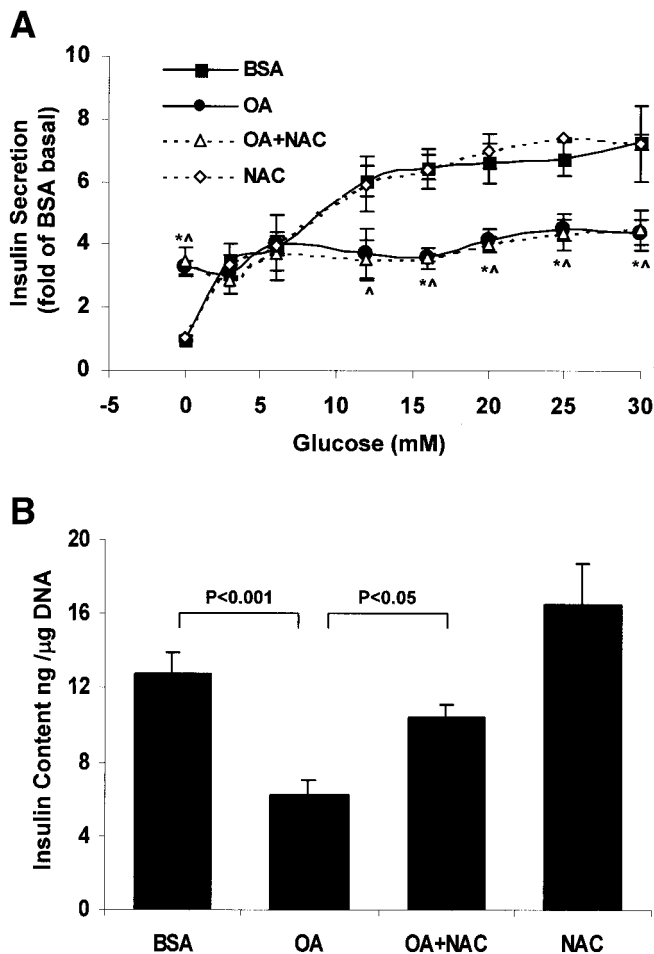
**Protein kinase and phosphorylation profiling.** Whole cell proteins were extracted from MIN6 cells using lysis buffer (20 mmol/l MOPS, pH 7.0, 2 mmol/l EGTA, 5 mmol/l EDTA, 30 mmol/l sodium fluoride, 40 mmol/l  $\beta$ -glycerophosphate, 10 mmol/l sodium pyrophosphate, 2 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulfonyl fluoride, 3 mmol/l benzamide, 5  $\mu$ mol/l pepstatin A, 10  $\mu$ mol/l leupeptin, 0.5% nonidet P-40, and 0.5% Triton X-100). The expression of 75 protein kinases was assessed by Kinetworks KPKS 1.0 Western blot analysis (Kinexus Bioinformatics, Vancouver, Canada). Phosphoprotein profiling was performed by Kinetworks KPPS 1.1 (36 different phosphorylation sites). Expression levels of kinase and phosphokinases in control cells were categorized according to the trace quantity (TQ) value with a TQ of 2,000 considered the threshold for detection (according to the service provider). Kinases with a TQ of 2,000–5,000 were considered to be expressed at low levels, whereas a TQ >5,000 was considered an indicator of high expression. Kinases with a TQ <2,000 were not included in the analyses. The criterion for a significant change in kinase or phosphorylation levels for treated samples was a consistent change in the relative quantity value of >1.25-fold over the control (BSA only) level for at least two of three independent experiments. The kinases consistently regulated by OA were then further validated with conventional Western blotting (30). The effects of NAC on OA-induced regulation of kinases were also examined by Western blotting. Band density was measured by densitometry, analyzed using image analysis software (Scion Image version 4.02; Scion, Frederick, MD), and normalized to  $\beta$ -actin content in parallel samples.

**Examination of posttranscriptional regulation of insulin levels.** CHX, an inhibitor of protein translation, was used to investigate protein translation and stability (31). Briefly, MIN6 cells were treated with OA and/or NAC as indicated above with or without 5  $\mu$ mol/l CHX for 36 h. Insulin content was measured as described above.

**Statistical analysis.** Data are expressed as means  $\pm$  SE. Statistical analysis was performed using ANOVA and Student's *t* test. Differences were deemed to be significant when *P* < 0.05.

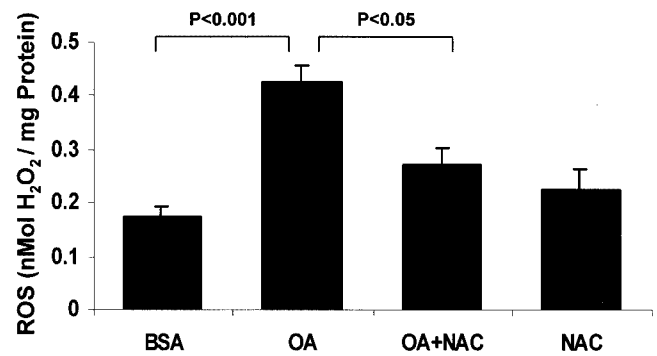
## RESULTS

**The effects of OA and/or NAC on insulin secretion and content in MIN6 cells.** OA increased basal insulin



**FIG. 1.** Effect of 72 h of OA and/or NAC treatment of MIN6 cells on basal and GSIS and insulin content. **A:** Insulin was measured in the media following incubation of MIN6 cells in 0 mmol/l glucose or concentrations increasing incrementally from 3 to 30 mmol/l. Insulin secretion is reported as the fold change over basal insulin secretion (BSA control). Results are the mean of three separate experiments performed in duplicate ( $\pm$ SE). \* $P < 0.05$  for OA vs. BSA and  $^{\wedge}P < 0.05$  for OA + NAC vs. BSA. **B:** Total cellular insulin was acid/ethanol extracted from MIN6 cells and quantified by insulin radioimmunoassay. Results were normalized to insulin per microgram DNA. Results are means of five separate experiments performed in duplicate ( $\pm$ SE).

secretion from  $115.7 \pm 16.8$  to  $369.4 \pm 26.7$  pg insulin/ $\mu$ g DNA, but decreased insulin secretion induced by glucose (Fig. 1A). Coincubation with NAC did not improve the insulin secretion in response to glucose or normalize increased basal insulin secretion in OA-exposed MIN6 cells. NAC in the absence of OA had no effect on insulin secretion. OA decreased insulin content in MIN6 cells by  $\sim 50\%$  compared with BSA control (from  $12.7 \pm 1.2$  to  $6.2 \pm 0.8$  ng insulin/ $\mu$ g DNA,  $P < 0.001$  for BSA vs. OA) (Fig. 1B). However, coincubation with NAC restored insulin content from  $6.22 \pm 0.81$  ng/ $\mu$ g DNA in OA-treated MIN6 cells to  $10.43 \pm 0.72$  ng/ $\mu$ g DNA in OA plus NAC-treated cells ( $P = 0.14$  for BSA vs. NAC + OA and  $P < 0.05$  for OA vs. NAC + OA;  $n = 5$ ). Insulin content in MIN6 cells treated with NAC alone was not significantly different from that of control cells ( $16.4 \pm 2.2$  ng insulin/ $\mu$ g DNA compared with  $12.7 \pm 1.16$  ng insulin/ $\mu$ g DNA,  $P = 0.19$ ;  $n = 5$ ). **ROS production induced by OA and/or NAC in MIN6 cells.** Preliminary experiments suggested that the mitochondrial respiratory chain is the principal source of ROS



**FIG. 2.** The effects of OA on ROS production. The cells were treated with 0.4 mmol/l OA bound to 0.5% BSA in the presence or absence of 1 mmol/l NAC for 72 h ( $n = 4$ ).

in MIN6 cells (data not shown). Total hydrogen peroxide production in mitochondrial respiratory complexes I and III was measured in MIN6 cells. OA increased ROS production by 2.6-fold ( $P < 0.001$  for OA vs. BSA), which importantly was prevented by the addition of NAC ( $P = 0.07$  for OA + NAC vs. BSA) (Fig. 2). NAC alone did not significantly change the ROS production compared with BSA control.

**The effects of OA and/or NAC on global gene expression in MIN6 cells.** As shown in Fig. 3A, of the 12,500 genes or ESTs analyzed by gene microarray, OA decreased expression of 845, 653, 529, or 639 genes or ESTs in four independent microarray analyses. Changes in gene expression were identified as being increased or decreased by comparing treatment to BSA control using Microarray Suite 5.0. Coincubation with NAC markedly reduced the number of OA-downregulated genes or ESTs from  $667 \pm 66$  to  $192 \pm 29$  ( $P < 0.05$  for OA vs. OA + NAC). The number of upregulated genes and ESTs was not significantly reduced ( $265 \pm 88$  to  $212 \pm 44$ ,  $P = 0.77$  for OA vs. OA + NAC). NAC independently upregulated  $\sim 240$  and downregulated 160 genes and ESTs.

Analyses of microarray results revealed that the expression of 62 genes was consistently regulated by OA from three to four independent experiments (Table 1). To verify OA-regulated expression of genes detected with microarray, these 62 genes were further characterized by real-time PCR. A total of 45 of 62 (73%) of the genes deemed significantly changed by the microarray analysis were further confirmed by real-time PCR (Tables 1 and 2). PCR confirmed all 16 genes changed by  $>1.8$ -fold, and the majority of the genes (28 of 38) shown to be changed between 1.4- to 1.7-fold, in microarray analysis. In contrast, only one of the eight genes that changed between 1.2- to 1.3-fold in the microarray was also found to be upregulated through RT-PCR analysis (Table 2B).

OA-regulated genes were clustered according to known function (Table 1 and Fig. 3B). The effects of NAC on the normalization of OA-regulated genes are shown in Table 1 and summarized in Table 2. Genes involved in metabolism comprised the largest functional cluster with 32% (14 of 45) of genes classified in this group. OA significantly upregulated genes involved in  $\beta$ -oxidation of lipids including carnitine palmitoyltransferase 1 ( $4.0 \pm 0.85$ -fold), 3-ketoacyl-CoA thiolase homolog ( $3.4 \pm 0.59$ -fold), long-chain acyl-CoA dehydrogenase ( $1.9 \pm 0.07$ -fold), and sol-

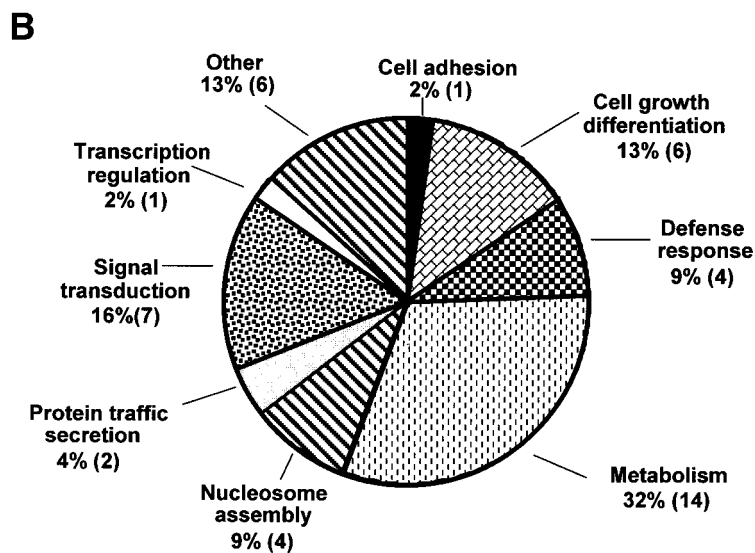
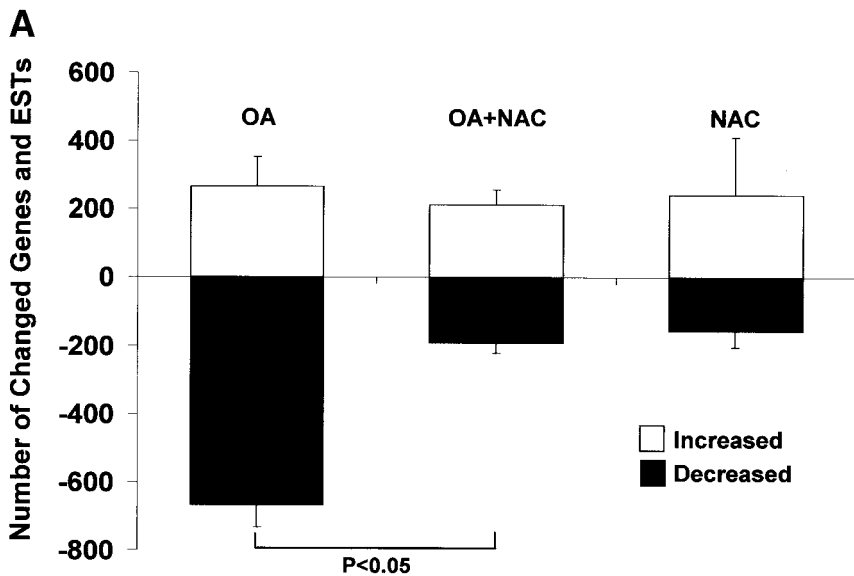


FIG. 3. A: Effects of OA on global gene expression in MIN6 cells. Number of changed genes or ESTs was evaluated (increased or decreased) by comparing test conditions to BSA control by Microarray Suite 5.0 ( $n = 4$ ). B: The distribution of OA-regulated genes into functional clusters. A total of 45 of OA-regulated genes confirmed by both microarray and real-time PCR were classified by function. Number in parentheses indicates number of genes in category. MIN6 cells were exposed to 0.4 mmol/l OA bound to 0.5% BSA, in the presence or absence of 1 mmol/l NAC or NAC alone for 72 h before any experiment.

ute carrier family 25 member 20/mCAC ( $2.7 \pm 0.38$ -fold), as well as genes involved in carbohydrate metabolism, such as fructose biphosphatase-1 ( $2.0 \pm 0.11$ -fold) and mannosidase-1 ( $2.4 \pm 0.32$ -fold). In contrast, OA downregulated stearoyl-CoA desaturase (SCD)-1 ( $-5.1 \pm 0.03$ -fold) and SCD2 ( $-2.7 \pm 0.04$ -fold), which encode proteins involved in the synthesis of unsaturated fatty acids, as well as the gene encoding an electron transporter NAD(P)H dehydrogenase ( $-2.2 \pm 0.04$ -fold). Importantly, all genes in the metabolism cluster differentially regulated by OA were not normalized by NAC treatment (Tables 1 and 2). Genes clustered into cell growth/differentiation and signal transduction groups represent 13% (6 of 45) and 16% (7 of 45) of the clustered genes, respectively. OA downregulated all the genes involved in cell growth and differentiation, all of which were normalized by coincubation with NAC. OA increased the expression of signal transduction genes encoding JNK3/MAPK10 ( $1.8 \pm 0.10$ -fold) and a calcium sensor protein, visinin-like protein (VILIP)-1 ( $2.2 \pm 0.07$ -fold), while decreasing the expression of genes encoding protein phosphatase 1 regulatory subunit 1A ( $-1.8 \pm$

0.04-fold). The addition of NAC failed to normalize most of the genes (86%, 6 of 7) mediating signal transduction. OA also regulated several genes involved in transcription, protein trafficking and secretion, nucleosome assembly, and cellular defense response, comprising 2–9% (1–4 of 45) of the clustered genes (Fig. 3B). In particular, the Sdf-1 gene ( $2.8 \pm 0.51$ -fold) was significantly upregulated in response to OA, effects not reversed by NAC. Conversely, as shown on Table 2, 30 of the OA-regulated genes or ESTs were normalized by the addition of NAC in microarray analysis. Among them, real-time PCR confirmed 13 NAC-normalized genes. PCR confirmed that most of NAC-normalized genes were involved in the cell growth/differentiation functional group, while most of genes involved in metabolism group could not be reversed by NAC (Tables 1 and 2).

**Kinase expression profiling in MIN6 cells.** Kinexus kinase analysis detected 46 of 75 screened kinases and 19 of 36 screened phosphorylated protein kinases at or above a TQ of 2,000, which is considered to be the threshold for detection (Table 3A and B). The expression levels of

TABLE 1  
Regulation of genes by OA in the presence or absence of NAC analyzed by microarray or real-time PCR

Gene	Treatment						Accession number
	OA		OA + NAC		NAC		
	Microarray	Real-time PCR	Microarray	Real-time PCR	Microarray	Real-time PCR	
<b>Cell adhesion</b>							
Secreted phosphoprotein 1 (Sppl)	-2.0 ± 0.35	-1.4 ± 0.01	-1.8 ± 0.66	-1.5 ± 0.07	I/NC	NC	X13986
<b>Cell growth differentiation</b>							
Ataxia telangiectasia mutated homolog (Atm)	-1.5 ± 0.09	-1.4 ± 0.07	NC	NC	D/NC	NC	U43678
Budding uninhibited by benzimidazoles 1 homolog (Bub1)	-1.4 ± 0.03	-1.4 ± 0.06	NC	NC	NC	NC	AF002823
Cell division cycle 6 homolog (Cdc6)	-1.4 ± 0.03	-1.3 ± 0.08	NC	NC	NC	NC	AJ223087
Cullin 1 (Cull1)	-1.2 ± 0.00	NC	NC	NC	NC	NC	AI849838
Lipin 1 (Lpin1)	-1.7 ± 0.17	-1.7 ± 0.06	NC	NC	NC	NC	AI846934
N-myc downstream regulated 1 (Ndr1)	-1.6 ± 0.08	-1.5 ± 0.02	NC	NC	NC	NC	U60593
N-myc downstream regulated-like (Ndr1)	-1.4 ± 0.14	-1.4 ± 0.05	NC	NC	NC	NC	U52073
<b>Defense response</b>							
B-cell linker protein (Blnc)	-1.4 ± 0.06	-1.4 ± 0.04	NC	NC	NC	NC	AF068182
CD24a antigen (Cd24a)	1.4 ± 0.03	1.6 ± 0.03	NC	NC	NC	NC	M58661
Stromal cell-derived factor (Sdf1)	2.2 ± 0.05	2.8 ± 0.51	2.7 ± 0.75	3.1 ± 0.49	NC	NC	L12029
D-dopachrome tautomerase (Ddt)	-1.4 ± 0.11	-1.4 ± 0.06	NC	-1.4 ± 0.05	NC	NC	AF068199
Lymphocyte antigen 6 complex, locus E (Ly6e)	1.5 ± 0.13	NC	NC	NC	NC	NC	U47737
<b>Metabolism</b>							
Acyl-Coenzyme A dehydrogenase, long-chain (Acadl)	2.1 ± 0.29	1.9 ± 0.07	2.3 ± 0.09	2.1 ± 0.11	NC	NC	U21489
Aldehyde dehydrogenase 9, subfamily A1 (Aldh9a1)	1.7 ± 0.13	1.6 ± 0.07	1.7 ± 0.17	1.6 ± 0.09	NC	NC	AW120804
Carnitine palmitoyltransferase 1, liver (Cpt1)	5.0 ± 0.70	4.0 ± 0.85	4.3 ± 0.95	3.9 ± 0.67	NC	NC	AF017175
Cysteine dioxygenase 1, cytosolic (Cdol)	-1.3 ± 0.02	-1.3 ± 0.02	-1.6 ± 0.23	-1.4 ± 0.10	NC	NC	A1854020
Homologous to 3-ketoacyl-CoA thiolase mitochondrial	3.6 ± 0.32	3.4 ± 0.59	3.8 ± 0.91	3.4 ± 0.45	NC	NC	A1849271
Enoyl coenzyme A hydratase 1, peroxisomal (Ech1)	1.8 ± 0.1	2.3 ± 0.24	2.1 ± 0.17	2.2 ± 0.28	NC	NC	AF030343
Fructose biphosphatase 1 (Fbp1)	2.0 ± 0.24	2.0 ± 0.11	1.6 ± 0.23	1.8 ± 0.17	NC	NC	A1790931
Mannosidase 1, alpha (Man1a)	2.2 ± 0.24	2.4 ± 0.32	2.0 ± 0.31	2.6 ± 0.36	NC	NC	U04299
NAD(P)H dehydrogenase, quinone 1 (Nqo1)	-2.3 ± 0.52	-2.2 ± 0.04	-2.5 ± 0.52	-2.0 ± 0.07	NC	NC	U12961
3-oxoacid CoA transferase (Oxct)	-1.4 ± 0.12	-1.4 ± 0.06	-1.4 ± 0.08	-1.2 ± 0.07	NC	NC	A1843232
Paraoxonase 3 (Pon3)	-1.4 ± 0.08	NC	NC	NC	NC	NC	L76193
Peroxisomal delta3, delta2-enoyl-Coenzyme A isomerase (Peci) pyruvate dehydrogenase (lipoamide) beta (Pdhb)	1.6 ± 0.09	1.5 ± 0.05	1.4 ± 0.08	1.5 ± 0.12	NC	NC	A1840013
Serine racemase (Srr)	-1.3 ± 0.03	NC	NC	NC	NC	NC	AW125336
Solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20 (Slc25a20)	-1.3 ± 0.08	NC	NC	NC	NC	NC	A1840579
Soluble carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20 (Slc25a20)	2.2 ± 0.1	2.7 ± 0.38	2.5 ± 0.26	2.7 ± 0.29	NC	NC	AB017112
Stearoyl-Coenzyme A desaturase 1 (Scd1)	-4.5 ± 0.90	-5.1 ± 0.03	-4.3 ± 1.04	-5.1 ± 0.04	NC	NC	M21285
Stearoyl-Coenzyme A desaturase 2 (Scd2)	-1.8 ± 0.24	-2.7 ± 0.04	-1.9 ± 0.20	-2.6 ± 0.06	NC	NC	M26270
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide (Ywhah)	-1.3 ± 0.03	NC	NC	NC	NC	NC	D87661
<b>Nucleosome assembly</b>							
Histone 1, H2bc (Hist1h2bc)	-1.4 ± 0.17	-1.3 ± 0.06	-1.3 ± 0.00	-1.3 ± 0.07	NC	NC	X05862
Nucleosome assembly protein 1-like 1 (Nap1l1)	-1.4 ± 0.17	-1.3 ± 0.07	NC	NC	NC	NC	X61449
RAD23b homolog	1.7 ± 0.07	1.7 ± 0.08	1.8 ± 0.09	1.9 ± 0.18	NC	NC	A1047107
Thyroid autoantigen 70 kDa (G22p1)	-1.5 ± 0.12	-1.3 ± 0.04	-1.4 ± 0.00	-1.2 ± 0.01	NC	NC	M38700
<b>Protein biosynthesis</b>							
G1 to S phase transition 1 (Gspt1)	-1.3 ± 0.03	NC	-1.3 ± 0.04	NC	NC	NC	AB003502

Continued on following page

TABLE 1  
Continued

Gene	Treatment						Accession number
	OA		OA + NAC		NAC		
	Microarray	Real-time PCR	Microarray	Real-time PCR	Microarray	Real-time PCR	
<b>Protein degradation/modification</b>							
Protective protein for beta-galactosidase (Ppqb)	-1.7 ± 0.21	NC	NC	NC	I/NC	NC	J05261
<b>Protein folding</b>							
Protein disulfide isomerase-related protein (P5-pending)	-1.7 ± 0.31	NC	NC	NC	I	NC	AW045202
Thioredoxin domain containing 4 (Txndc4)	-1.5 ± 0.17	NC	NC	NC	NC	NC	AW125408
<b>Protein traffic and secretion</b>							
Down syndrome critical region gene 3 (Dscr3)	-1.5 ± 0.07	-1.2 ± 0.04	NC	NC	NC	NC	AB001990
RAB7, member RAS oncogene family (Rab7)	-4.7 ± 3.30	-1.1 ± 0.01	NC	NC	I/NC	NC	Y13361
Vesicle-associated membrane protein 3 (Vamp3)	-1.4 ± 0.08	NC	NC	NC	D/NC	NC	A1847972
<b>Signal Transduction</b>							
Down syndrome critical region gene 1-like 2 (Dscr112)	1.4 ± 0.03	1.2 ± 0.01	1.4 ± 0.00	1.2 ± 0.03	NC	NC	A1847661
GTP cyclohydrolase 1 (Gch1)	-1.7 ± 0.10	-1.3 ± 0.06	-1.4 ± 0.10	NC	NC	NC	L09737
MAPK 10/JNK3 (Mapk10)	1.6 ± 0.09	1.8 ± 0.10	1.7 ± 0.04	1.8 ± 0.17	NC	NC	L35236
Phosphatidylinositol glycan, class A (piga)	-1.5 ± 0.15	-1.2 ± 0.03	-1.3 ± 0.04	-1.2 ± 0.03	NC	NC	D31863
Phospholipid scramblase 1 (Plscr1)	-1.4 ± 0.07	-1.2 ± 0.02	NC	NC	NC	NC	D78354
Protein phosphatase 1, regulatory (inhibitor) subunit 1A (Ppp1rla)	-1.6 ± 0.07	-1.8 ± 0.04	-1.6 ± 0.23	-1.9 ± 0.03	NC	NC	AW122076
Visinin-like protein 1 (VILIP-1)	3.1 ± 0.40	2.2 ± 0.07	3.7 ± 0.76	2.6 ± 0.21	NC	NC	D21165
<b>Transcription regulation</b>							
mini chromosome maintenance-deficient 4 homolog (Mcmd4)	-1.4 ± 0.06	NC	NC	NC	NC	NC	D26089
Nuclear receptor coactivator 4 (Ncoa4)	-1.6 ± 0.21	NC	NC	NC	NC	NC	AI834866
Transforming growth factor-β inducible early growth response (Tieg)	1.8 ± 0.26	1.4 ± 0.07	1.5 ± 0.25	1.4 ± 0.09	NC	NC	AF064088
<b>Other</b>							
Mus musculus, clone IMAGE: 1379624, mRNA, partial cds	-1.5 ± 0.09	NC	NC	NC	NC	NC	AI646098
RIKEN cDNA 1500032A09 gene	-1.4 ± 0.13	-1.8 ± 0.04	NC	NC	NC	NC	AI847069
RIKEN cDNA 2310016A09 gene	1.4 ± 0.03	1.7 ± 0.11	1.3 ± 0.00	1.6 ± 0.07	NC	NC	AW049373
RIKEN cDNA 2310035M22 gene	-1.5 ± 0.17	NC	NC	NC	D/NC	NC	AI851230
RIKEN cDNA 2410003A14 gene	-1.3 ± 0.05	NC	NC	NC	NC	NC	AA939576
RIKEN cDNA 2900035H07 gene	1.8 ± 0.16	1.8 ± 0.05	1.9 ± 0.21	1.8 ± 0.14	NC	NC	AW060889
RIKEN cDNA 4921515A04 gene	-1.5 ± 0.20	-1.3 ± 0.01	-1.5 ± 0.15	-1.2 ± 0.04	NC	NC	AI642098
RIKEN cDNA 4930415K17 gene	-1.3 ± 0.08	NC	NC	NC	NC	NC	AW124069
RIKEN cDNA 4933419D20 gene	-1.7 ± 0.17	-1.7 ± 0.02	-1.7 ± 0.06	-1.7 ± 0.03	NC	NC	AW124340
RIKEN cDNA B230114J08 gene	1.7 ± 0.12	1.7 ± 0.06	1.7 ± 0.17	1.9 ± 0.07	NC	NC	AW124185

MIN6 cells were treated with 0.4 mmol/l OA bound to 0.5% BSA in the presence or absence of 1 mmol/l NAC or 1 mmol/l NAC alone for 72 h ( $n = 3-4$ ). Microarray and real-time PCR protocols are outlined in the RESEARCH DESIGN AND METHODS section. Results are expressed as fold of change ± SE compared with cells treated with BSA only. Statistical analysis of raw microarray data was performed using Micorarray Suite version 5.0 ( $n = 3-4$ ), and real-time PCR data analysis was performed using Student's *t* test, and differences were deemed to be significant when  $P < 0.05$  ( $n = 3$ ). NC, no change compared with BSA control.

several kinase proteins correlated positively with a screen of βTC-3 and INS-1 cell lines (32) (Table 3A). Kinases comprising the three MAPK modules, Raf1/B→MAPK (MEK) 1/2→extracellular regulated kinase (Erk) 1/2, Cancer Osaka thyroid oncogene (COT)→MEK 4/7→JNK, and p21 activated kinase (PAK) 1→MEK6→p38 MAPK were detected in MIN6 cells. Kinases involved in phosphatidylinositol 3-kinase signaling pathways, 3-phosphoinositide-dependent protein kinase (PDK) 1→protein kinase B (PKB)→glycogen synthase kinase (GSK) 3 or →S6K p70

were also present. Multiple isoforms of PKC (-α, -β, -ε, and -μ), including atypical isoform PKC-ζ and cell cycle-regulating cyclin-dependent kinases (Cdk2, 4, 5, 6, and 7) were also detected by the kinase protein screening. We detected v-mos Moloney murine sarcoma viral oncogene homolog (MOS1) kinase, which had not been previously described in pancreatic β-cells. Phosphorylation of specific epitopes on Raf1→MEK1/2→Erk 1/2, PKB→GSK3, Src→STAT3, and PKC-α, -β, -ε signaling molecules were detectable by the kinase phosphorylation screen (Table 3B).

TABLE 2  
Effects of NAC on the number of genes or ESTs consistently regulated by OA in at least three of four independent microarrays clustered according to function or fold of change.

Gene function	Gene number (NAC normalized/total of OA regulated)	
	Microarray	PCR confirmed
Cell adhesion	0/1	0/1
Cell growth/differentiation	7/7	6/6
Defense response	4/5	2/4
Metabolism	4/18	0/14
Nucleosome assembly	1/4	1/4
Protein biosynthesis	0/1	0/0
Protein degradation/modification	1/1	0/0
Protein folding	2/2	0/0
Protein traffic and secretion	3/3	2/2
Signal transduction	1/7	1/7
Transcription regulation	2/3	0/1
Other	5/10	1/6
Sum	30/62	13/45
Fold of change		
1.8–5.0	0/16	0/16
1.4–1.7	24/38	13/28
1.2–1.3	6/8	0/1
Sum	30/62	13/45

**The effects of OA and/or NAC on protein kinase expression and phosphorylation in MIN6 cells.** The effects of OA on the expression and the phosphorylation states of the protein kinases listed in Table 4 were assessed in MIN6 cells. Kinase screen data showed that OA consistently regulated the expression of 10 protein kinases and the phosphorylation states of 3 protein kinases >1.25-fold (Table 4). Conventional Western blotting confirmed the OA-induced regulation of the protein level of six protein kinases (CDK7, GRK2, p38 MAPK, PKC- $\mu$ , JNK3/MAPK10, and p70S6K) and two of OA-induced changes in the phosphorylation states of kinases identified in the phospho-screen (p70S6K, Rb) (Table 4, Fig. 4). Western blotting also demonstrated that the addition of NAC normalized OA-induced regulation of most of the kinases but not OA-regulated JNK3/MAPK10 and p70S6K protein levels or phosphorylated Rb levels. Interestingly, both microarray and PCR data also show increased gene expression of JNK3/MAPK10 in OA-treated samples, effects not normalized by the addition of NAC. NAC alone did not have a significant effect on these OA-regulated kinases.

**Effects of OA and NAC on insulin protein synthesis and stabilization.** Our studies revealed that insulin content was decreased by OA and normalized by NAC treatment (Fig. 1B) without changes in insulin mRNA levels measured by microarray and real-time PCR (data not shown). This suggests that insulin content of MIN6 cells is regulated posttranscriptionally by OA and NAC. To investigate the effects of OA and NAC on insulin protein synthesis at the posttranscriptional level, insulin content was measured after 36 h incubation of MIN6 cells with various combinations of CHX (an inhibitor of protein translation), OA, and NAC. A 36-h incubation was used because it showed the same pattern of alteration in insulin

content as a 72-h incubation (Figs. 1B and 5). In control BSA-treated cells, a significant 60% decrease in insulin content was seen in the presence of CHX. In OA-treated cells, the results of three independent experiments demonstrated a trend toward decreased insulin content in the presence of CHX, but the difference was not significant (for BSA + CHX vs. OA + CHX,  $3.9 \pm 0.6$  vs.  $2.3 \pm 0.2$  ng insulin/ $\mu$ g DNA, respectively,  $P = 0.11$ ) (Fig. 5).

## DISCUSSION

In this study, we investigated the long-term effects of OA on MIN6  $\beta$ -cell function. In agreement with previous studies in islets and other  $\beta$ -cell lines (33,34), our results demonstrate that long-term exposure of MIN6 cells to OA leads to a threefold increase in basal insulin secretion with an inhibition of GSIS, and a greater than twofold increase in ROS. They are thought to contribute to the deterioration of islet function in the  $\beta$ -cell (24,35,36), and antioxidants such as NAC and a combination of vitamins E and C can restore to some degree the ROS-mediated effects of glucotoxicity in  $\beta$ -cells (24,25). However, in our studies, although ROS was elevated by OA and normalized by the addition of NAC, it failed to restore GSIS following OA treatment. This suggests that the increase in ROS production associated with chronic OA treatment of  $\beta$ -cells may not be directly implicated in the lipotoxic effects of OA on GSIS.

The exact pathophysiology of lipotoxicity is as yet unclear, however a number of hypotheses have been proposed: 1) increased FFA oxidation with a concomitant decrease in glucose metabolism resulting from substrate competition (14), 2) increased production of intermediate metabolites and triglycerides from FFA esterification (33,37), and 3) increased expression of mitochondrial uncouplers in  $\beta$ -cells (16,17,33). After prolonged exposure of MIN6 cells to OA, we have observed a significant upregulation of genes involved in  $\beta$ -oxidation of lipids including carnitine palmitoyltransferase 1, 3-ketoacyl-CoA thiolase homolog, long-chain acyl-CoA dehydrogenase, and solute carrier family 25 member 20/mCAC. In contrast, SCD1 and SCD2, which encode key rate-limiting enzymes involved in the synthesis of monounsaturated fatty acids (38,39), were significantly downregulated in response to OA treatment. These findings imply that after long-term exposure to OA, OA-catabolic pathways are upregulated while the endogenous production of OA is inhibited in an effort to normalize the OA level. Notably, the expression of genes involved in glycolysis and glucose oxidation was generally unchanged, in contrast with previous microarray studies (6,10). This may reflect differences in experimental protocol, such as the cell lines used, variations in glucose concentrations, or different incubation times with different FFAs. However, our study does support previous data that glucose oxidation is only minimally impaired by chronic exposure to elevated FFAs (40,41), suggesting that changes in glucose metabolism at the level of gene expression may not be an important effector of lipotoxicity in the  $\beta$ -cells.

Prolonged exposure to FFAs inhibits insulin biosynthesis and posttranslational processing of proinsulin and results in decreased insulin content and insulin maturation in vivo and in vitro (5,9,15,42,43). Our study showed that

TABLE 3  
Expression levels of protein kinase (A) and phosphoprotein kinases (B) in MIN6 cells

A			Protein kinases					
Not detected			Low expression			High expression		
Name	TQ		Name	TQ		Name	TQ	
BTK			BMX	2,275		CaMKK	5,636	
CaMK1	644		CaMK4	2,679		CDK5	7,950	
CDK1			CDK2	4,764		CDK7	9,309	
CDK9			CDK4	3,010		CK2	23,903	
CK1δ	897		CDK6	2,413		COT	13,690	
CK1ε			CSK	3,814		ERK1	18,336	
DAPK	1,352		GCK	3,459		ERK2	10,100	
DNAPK			GSK3α	3,820		ERK3	9,424	
FYN			GSK3β	3,965		ERK6	8,383	
IKKα			JAK2	4,938		FAK	7,238	
JAK1			MEK1	2,974		GRK2	11,203	
KSR1			MEK6	3,825		HPK1	11,508	
LCK			MEK7	2,005		JNK3/MAPK 10	5,836	
LYN	1,497		NEK2	2,079		MEK2	8,669	
MNK 2			PAK1	2,443		MEK4	33,802	
Mst 1			PKCα	3,677		MOS1	21,835	
PKA β			RSK2	4,687		P38 MAPK	14,210	
PKR			YES	3,495		PIM1	11,168	
PKCγ			ZAP70	3,352		PKA	6,461	
PKCλ						PKBα/Akt1	7,711	
PKCδ						PKG1	13,192	
PKCθ						PKCβ	5,914	
PYK2	1,427					PKCε	26,789	
ROK	977					PKCμ	5,897	
SRC						PKCζ	7,599	
SYK	1,277					RAF1	7,574	
						RAFB	12,742	
						RSK1	14,830	
						S6K p70	23,033	
						ZIP	9,131	

B			Phosphoprotein kinases					
Not detected			Low expression			High expression		
Name	Epitope	TQ	Name	Epitope	TQ	Name	Epitope	TQ
GSK 3β	S9		ERK 1	T202/Y204	4,094	Adducin	S662	17,204
CDK 1	Y15	1,717	ERK 2	T185/Y187	3,377	GSK 3α	S21	6,286
CREB	S133	1,022	MEK 3	S189/T193	2,298	GSK 3α	Y279	13,202
JAK 2	Y107/Y108		SRC	Y418	3,856	GSK 3β	Y216	17,931
JUN	S73	1,750	PKB α	T308	2,365	FAK	Y861	9,874
MEK6	S207/T211		S6K p70	T389	2,815	MEK 1/2	S221/S225	7,081
MSK 1/2	S376		STAT 3	S727	3,229	NRI	S896	6,689
P38 MAPK	T180/Y182		STAT 5	Y694	2,988	Raf 1	S259	17,712
PKC δ	T505	1,160				PKB α	S473	9,791
PKR	T451	1,479				PKC α	S657	30,207
Rb 1	S807/S811					PKC α/β	T638/641	16,137
RSK 1	T360/S364					PKC ε	S719	8,456
Src	Y529	1,164				Rb 1	S780	5,303
Smad 1	S463/465							
STAT 1	S701							

Kinases screened by Kinexus for 75 protein kinases (A) and 36 phosphorylation sites (B) were performed on MIN6 cells treated with BSA as outlined in the RESEARCH DESIGN AND METHODS section. Quantitative assessment was made based on TQ of band densities. BTK, Bruton agammaglobulinemia tyrosine kinase; Bmx, bone marrow X kinase; CaMKK, calmodulin-dependent kinase kinase; CaMK, calmodulin-dependent kinase; CDK, cyclin dependant kinase; CK, casine kinase; COT, cancer Osaka thyroid oncogene; CSK, c-SRC tyrosine kinase; DAPK, death-associated protein kinase; DNAPK, DNA-activated protein kinase; ERK, extracellular regulated kinase; FAK, focal adhesion kinase; FYN, fyn oncogene related to SRC; GCK, germinal centre kinase; GRK, G protein-coupled receptor kinase; GSK, glycogen synthase kinase; HPK, hematopoietic progenitor kinase; IKKα, inhibitor nuclear factor κB kinase α; JAK2, janus kinase; KSR, kinase suppressor of Ras; LCK, lymphocyte-specific protein tyrosine kinase; LYN, oncogene Lyn; MSK, mitogen and stress-activated protein kinase; MNK 2, MAP kinase interacting kinase 2; MOS1, v-mos Moloney murine sarcoma viral oncogene homolog; NEK, NIMA (never in mitosis)-related kinase; NR, N-methyl-D-aspartate glutamate receptor subunit; Mst1, mammalian sterile 20-like1; p38MAPK, p38 Hog MAP kinase; PAK1, p21 activated kinase 1; PIM1, provirus integration site for moloney murine leukemia virus; PDK, 3-phosphoinositide-dependent protein kinase; PKA, protein kinase A; PKB, protein kinase B/Akt; PKG, protein kinase G (cGMP-dependent protein kinase); PKR, dsRNA dependent protein kinase; PYK2, protein tyrosine kinase; Raf, oncogene Raf; RafB, v-raf murine sarcoma viral oncogene homolog; ROKa, RhoA kinase; RSK, ribosomal S6 kinase; S6K p70, S6 kinase p70; STAT, signal transducer and activator of transcription; SMAD, SMA- and MAD-related protein; SRC, oncogene SRC; SYK, spleen tyrosine kinase; YES, yamaguchi sarcoma viral oncogene homolog; TCR, zeta-chain; ZAP, associated protein kinase; ZIP, ZIP kinase.



TABLE 4  
Regulation of kinases by OA in the presence or absence of NAC analyzed by kinase screen or Western blotting

A	Fold change over BSA control			
	Kinase screen	Western blotting		
		OA	OA	OA + NAC
Kinase				
CDK7	1.5 ± 0.04	1.4 ± 0.09	NC	NC
FAK	2.0 ± 0.53	NC	NC	NC
GRK2	1.4 ± 0.04	1.3 ± 0.11	NC	NC
JNK3/MAPK10	1.3 ± 0.06	1.3 ± 0.05	1.3 ± 0.08	NC
MEK2	1.4 ± 0.18	NC	NC	NC
P38 Hog MAPK	1.6 ± 0.30	1.5 ± 0.06	NC	NC
PKG1	1.3 ± 0.02	NC	NC	NC
PKC $\mu$	1.4 ± 0.04	1.5 ± 0.05	NC	NC
RAF1	2.1 ± 0.96	NC	NC	NC
S6Kp70	0.7 ± 0.03	0.7 ± 0.02	0.7 ± 0.06	NC

B	Fold change over BSA control				
	Phosphokinase	Epitope	Kinase screen	Western blotting	
			OA	OA	OA + NAC
PKB $\alpha$	T308	1.9 ± 0.65	NC	NC	NC
RB1	S780	1.4 ± 0.22	1.4 ± 0.10	1.6 ± 0.15	NC
S6Kp70	T389	0.6 ± 0.22	0.7 ± 0.04	NC	NC

MIN6 cells were treated with 0.4 mmol/l OA bound to 0.5% BSA in the presence or absence of 1 mmol/l NAC or 1 mmol/l NAC alone for 72 h. Results of kinase screen are reported as the fold change of the RQ value for OA-BSA-treated cells compared with BSA only-treated cells from two or three independent experiments ( $\pm$ SE). Statistical analysis of Western blotting data was performed using Student's *t* test, differences were deemed to be significant when  $P < 0.05$  ( $n = 3-5$ ). NC, no change compared with BSA control.

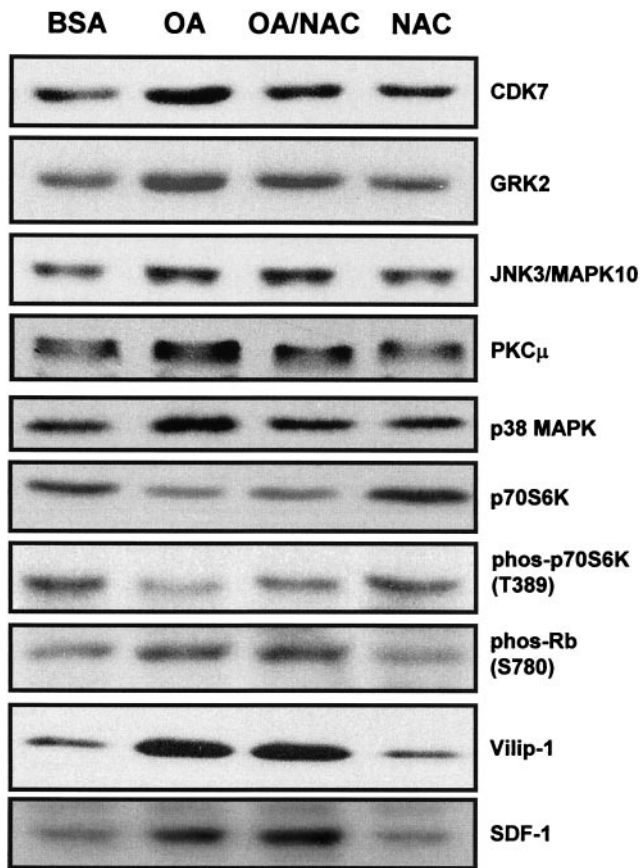
insulin content in MIN6 cells exposed to OA for 72 h was decreased 50% compared with that of control cells, and that this could be restored by cotreatment with NAC. The downregulation of insulin content by OA did not occur at the level of transcription, since both insulin I and insulin II mRNA levels were unchanged, as shown by both microarray analysis and real-time PCR ( $n = 3$  independent studies, data not shown). This raises the possibility that modulation of insulin content by OA and NAC may occur at the posttranscriptional level. This hypothesis is supported by experiments using CHX. These studies revealed that inhibitors of protein translation modified in part the actions of OA and NAC. However, OA and NAC may also have translation-independent effects on insulin content by affecting posttranslation pathways and/or degradation of insulin.

An alternate pathway that could mediate the changes in insulin content is the mTOR/p70S6K pathway. p70S6K is a kinase thought to control protein translation (44), and glucose-induced proinsulin biosynthesis has been shown to be mediated through ATP modulation of mTOR/p70S6K pathway in MIN6 cells and islets (45). Our studies demonstrated that protein and phosphorylation levels of p70S6K were significantly diminished by OA treatment in MIN6 cells (Fig. 4, Table 4), an effect normalized by concomitant administration of NAC. Thus, changes in p70S6K phosphorylation may also account for the reduced insulin content in OA-treated MIN6 cells, and its improvement by NAC. In addition to effects on proinsulin synthesis, it has been suggested that activation of the mTOR/p70S6K pathway, independent of PDK1/PKB activation, is a key requirement for glucose-dependent  $\beta$ -cell proliferation (46), consistent with the prominence of p70S6K in the control of protein translation. In our studies, genes and proteins involved in cell growth and survival showed a complex

pattern of regulation in response to chronic OA treatment. In general, genes involved in cell cycle regulation, differentiation, and cell survival were downregulated in response to OA treatment, an effect reversed by NAC. Given the importance of p70S6K in cell growth, and that OA suppresses p70S6K phosphorylation, lipid-induced suppression of p70S6K phosphorylation may be a key element in the failure to compensate for the  $\beta$ -cell loss observed in lipotoxic environments. Conversely, a number of kinases involved in the stimulation of cell cycle regulation and cell growth are stimulated by OA-treatment, including CDK7 and Rb, suggesting compensatory stimulation of  $\beta$ -cell growth in the face of elevated ROS and FFAs.

The MAPK pathways are crucial to cell differentiation, proliferation, and survival. We have confirmed the presence of three intact MAPK modules that have previously been detected in  $\beta$ TC-3 and INS-1 cell lines (32). A number of genes and kinases in stress-induced signaling pathways are upregulated in response to chronically elevated FFAs and elevated ROS, including MAPK pathways. This is illustrated by a recent study by Kaneto et al. (47) that shows activation of JNK, p38 MAPK, and PKC in response to oxidative stress in rat islets, which precedes the downregulation of insulin gene expression and secretion. In the present study, we see significant OA-induced upregulation of the JNK3 gene and JNK3, PKC $\mu$ , and p38 MAPK levels.

Interestingly, among the candidates significantly induced by OA was the VILIP-1 gene encoding a Ca<sup>2+</sup> sensor protein, which was upregulated three- to fourfold, a finding confirmed by Western blot. VILIP-1 belongs to a family of Ca<sup>2+</sup> sensor proteins, including neuronal Ca<sup>2+</sup> sensor-1 (NCS-1), which has been shown to enhance exocytosis in neurons and neuroendocrine cells (48). VILIP-1 has been implicated in the modulation of cAMP and cGMP signaling pathways in neural cells (49,50). However, despite being

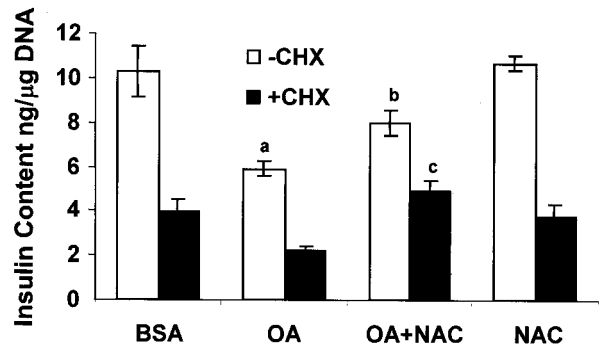


**FIG. 4.** Protein levels of OA-regulated protein kinases and proteins. Confirmatory Western blots were performed on protein kinases found to be upregulated in the protein kinase screen, as well as two genes found to be significantly upregulated in microarray analysis, VILIP-1 and Sdf-1. Representative Western blots of three to six independent experiments are shown. The fold change of these and other proteins identified in protein and gene profiling were analyzed by density scan and normalized with β-actin protein as shown in Table 4.

first identified in the pancreatic β-cell, its function in this cell type remains unknown.

OA also upregulated gene expression of Sdf-1 by two- to fourfold, as well as its protein levels (Table 1, Fig. 4). Upregulation of Sdf-1 gene expression in response to elevated FFAs has previously been noted by Busch et al. (6). Sdf-1 is a highly potent chemoattractant for monocytes and naive T-cells (51) and has been implicated in the recruitment of autoreactive B-cells in the development of type 1 diabetes (52). This raises the possibility that an increase in Sdf-1 could exacerbate the loss of β-cells seen with the progression of type 2 diabetes.

Reports have implicated elevated UCP2 levels as a mechanism for the blunted GSIS evident following chronic exposure to elevated FFA (17). Recent studies using Northern blot hybridization found increased levels of UCP2 gene expression after exposure to fatty acids in INS-1 cells (16,33,53). Expression of the UCP2 gene was not detectable by microarray analysis in this study, and there is no report of UCP2 regulation in response to elevated FFA levels in other studies using the microarray technique (6). However, real-time PCR detected UCP2 transcripts, and the level of UCP2 mRNA expression increased ~4.0-fold after OA treatment (data not shown).



**FIG. 5.** Posttranscriptional effects of OA and NAC on insulin content. MIN6 cells were treated with BSA only, OA-BSA, OA-BSA + NAC, and NAC + BSA in the presence or absence of 5 μmol/l CHX, an inhibitor of protein translation. Data shown represents mean ± SE (n = 3). <sup>a</sup>P < 0.05 for OA vs. BSA, <sup>b</sup>P < 0.05 for OA + NAC vs. OA, and <sup>c</sup>P < 0.05 for OA + NAC + CHX vs. OA + CHX.

These results demonstrate one limitation of microarray analysis, the detection of genes that are expressed at relatively low levels.

In summary, our findings suggest that chronic exposure to OA impairs β-cell function through ROS-dependent and -independent pathways. Downregulation of genes involved in cell growth/differentiation and protein synthesis may, in part, lead to decreased insulin content through a ROS-dependent pathway. OA-induced oxidative stress modulates kinase signaling pathways, including inactivation of p70S6K and upregulation of p38 MAPK, JNK3, and PKCμ may contribute to the deterioration of β-cells. Taken together, these findings have identified several candidate molecules for further investigation into the mechanism of lipotoxicity in pancreatic β-cells.

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