Palmitate and Oleate Induce the Immediate-Early Response Genes c-*fos* and *nur*-77 in the Pancreatic β-Cell Line INS-1

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To better understand the link between fatty acid signaling and the pleiotropic effects of fatty acids in the pancreatic β-cell, we investigated whether fatty acids regulate immediate-early response genes (IEGs) coding for transcription factors implicated in cell proliferation, differentiation, and apoptosis. Palmitate and oleate, but not long-chain polyunsaturated fatty acids, caused a pronounced accumulation of c-*fos* and *nur*-77 mRNAs in β-cells (INS cells) to an extent similar to that produced by the protein kinase C (PKC) activator phorbol myristate acetate (PMA). The effect was dose dependent and occurred at concentrations between 0.1 and 0.5 mmol/l in the presence of 0.5% albumin. The action of the fatty acid occurred at the transcriptional level, and the mRNA accumulation displayed a bell-shaped kinetics with a maximal effect at 1 h. 2-Bromopalmitate was ineffective, indicating that fatty acids must be metabolized to cause their effect. Neither fatty acid was able to induce c-*fos* and *nur*-77 in PKC-downregulated cells or cells incubated in the presence of the Ca^{2+} channel blocker nifedipine or the Ca^{2+} chelator EGTA, suggesting involvement of the PKC and Ca^{2+} signaling pathways. Palmitate and oleate also increased c-*fos* protein expression and DNA binding activity of the transcription factor AP-1. Oleate, but not palmitate, increased [^{3}H]thymidine incorporation in INS cells. Finally, both palmitate and oleate caused c-*fos* and *nur*-77 mRNA accumulation in isolated rat islets. It is suggested that IEG induction by the most abundant circulating fatty acids plays a role in the adaptive process of the β-cell to hyperlipidemia. These results have implications for our understanding of obesity-associated diabetes and the link between fatty acids and tumorigenesis. *Diabetes* 48:2007–2014, 1999

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BSA, bovine serum albumin; DTT, dithiothreitol; IEG, immediate-early response gene; KRBB, Krebs-Ringer bicarbonate buffer; LCFA, long-chain fatty acid; NEFA, nonesterified fatty acid; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PPAR-α, peroxisome proliferator activating receptor α.

Long-chain fatty acids (LCFAs) have a dual effect on insulin secretion. Acute administration of LCFAs to the β-cell potentiates glucose-induced insulin release, whereas long-term exposure causes elevated secretion at low glucose concentrations in association with reduced insulin biosynthesis (1–3). In addition, LCFAs appear to be implicated in the etiology of obesity-associated type 2 diabetes (4). First, by sensitizing the β-cell to the action of glucose, they may participate in the β-cell compensation of the prevalent insulin resistance, allowing enough insulin to be secreted to maintain euglycemia (4–6). As suggested from in vitro and animal studies, LCFAs may in addition be implicated in susceptible individuals in the decompensation leading to β-cell failure and overt diabetes through apoptotic cell death (7). Much remains to be learned about the mechanisms underlying these actions of LCFAs. Since these are long-term processes, LCFAs likely exert these effects through changes in the expression of a number of genes that remain to be identified. To date, LCFAs have been shown to increase the expression of enzymes of fat oxidation, in particular carnitine-palmitoyltransferase I and acyl-CoA oxidase (8,9), and to reduce acetyl-CoA carboxylase (10) in both normal rat islets and the β-cell line INS-1, but not in islets from *fa/fa* rats (9). In addition, LCFAs induced the LCFA-ligand transcription factor peroxisome proliferator activating receptor α (PPAR-α) in rat islets (9) and reduced IDX-1 expression (3). Whether LCFAs regulate the expression of other transcription factors and immediate-early response genes (IEGs) in the β-cell, which in turn might influence the expression of secondary genes, is not known.

Because cytosolic Ca^{2+} increases in response to β-cell stimulation by nutrients (11) and has recently been implicated in the control of β-cell apoptosis (12), we chose to study, as a paradigm of IEG regulated by LCFAs, two candidate genes that in other systems have been shown to be particularly induced by Ca^{2+} agonists, c-*fos* and *nur*-77. Members of the Fos and Jun families dimerize to form the AP-1 transcription factor, which regulates many secondary genes, including some coding for metabolic enzymes and genes involved in cell cycle progression and apoptosis (13–15). Nur-77 is an orphan nuclear receptor that is markedly induced in immature T-cell apoptosis (16). Contrary to other members of the nuclear receptor superfamily, which bind to DNA as dimers, Nur-77 binds as a monomer (17,18). In addition, our previous work shows that the c-*fos* and *nur*-77 genes are strongly induced in the β-cell by glucose in combination with physio-

logic cAMP-elevating agents, such as glucagon-like peptide (GLP)-1 and pituitary adenylate cyclase-activating polypeptide (PACAP) (19).

To link fatty acid signaling to the late phenotypic changes induced by these nutrients, we investigated whether the main dietary fatty acids modulate the expression level of c-*fos* and *nur*-77. To perform this study, we used the β-cell line INS-1, which displays a phenotype very close to primary β-cells, providing at the same time a homogeneous population of β-cells (20,21). In the present study, we show that palmitate and oleate cause a rapid and transient transcriptional induction of c-*fos* and *nur*-77 genes. Furthermore, this induction appears to be mediated by certain isoforms of protein kinase C (PKC) and requires elevated $[Ca^{2+}]_i$.

RESEARCH DESIGN AND METHODS

Cell culture conditions. INS-1 cells were grown in monolayer cultures in RPMI 1640 medium containing 11.2 mmol/l D-glucose supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 µmol/l β-mercaptoethanol, 10 mmol/l HEPES, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere (5% CO_2, 95% air) (21). Cells (10×10^6) were seeded 7 days before use in 75-cm^2 culture flasks. When cells reached 80% confluence, they were washed twice with Krebs-Ringer bicarbonate buffer (KRBB) containing 5 mmol/l glucose and 0.07% bovine serum albumin (BSA). Afterward, cells were incubated for 2 days in RPMI medium with 10% serum, but containing 5 mmol/l D-glucose. The medium was changed every day. The day of the experiment, medium was removed and cells were incubated for various times at 37°C in RPMI without serum and with 5 mmol/l glucose in the presence of defatted BSA (control condition) or BSA-bound fatty acids. After their isolation (22), rat islets (200 per condition) were preincubated at 37°C for a period of 60 min in KRBB without serum and with 5 mmol/l glucose and 0.5% BSA. Medium was changed, and islets were incubated for 1 h in the same medium with or without fatty acids bound to BSA (0.5%). The stock solutions of fatty acids bound to BSA were prepared as follows. The sodium salt of the corresponding fatty acid was dissolved at 37°C for 8 h under nitrogen atmosphere in KRBB containing 5% fatty acid–free BSA to obtain a 5 mmol/l fatty acid stock solution. The molar ratio of fatty acid to BSA was 6:1 for most fatty acids or slightly lower for some long-chain polyunsaturated fatty acids. At the end of the incubation, the pH of the fatty acid solution was adjusted to 7.4, and the stock solution was filtered through a 0.2-µm filter. Finally, the fatty acid solutions were stored at –20°C under nitrogen to prevent oxidation. The fatty acid stock solutions were diluted in culture medium to obtain various concentrations of fatty acids ranging from 0.05 to 0.5 mmol/l at a fixed concentration of 0.5% BSA. The nonesterified fatty acid (NEFA) concentrations in the medium were verified using the NEFA kit (Wako Chemicals, Richmond, VA).

mRNA measurements and in vitro transcription assay. Total RNA was extracted from INS-1 cells or rat islets using the guanidium isothiocyanate method (23). RNA samples (15 µg for INS cells and 10 µg for rat islets) were denatured by incubation with glyoxal, loaded in 1.2% agarose gels, separated by electrophoresis, and transferred by capillary action to a nylon membrane. The corresponding mRNAs were detected by hybridization with a [^{32}P]cDNA probe labeled by random priming. The fragments used as templates to make the probes were 2.1-kb *Eco*RI fragment of rat c-*fos* (positions 1–2116) subcloned in pSP65, 1.9-kb *Eco*RI fragment of mouse *jun*-B (positions 1–1917) subcloned in pGEM-2, 1.5-kb *Eco*RI fragment of mouse *nur*-77 (positions 1056–2456) subcloned in pGEM-2 (provided by Dr. L. Lau, University of Illinois), 3.2-kb *Eco*RI fragment of mouse *zif*-268/*erg*-1 (positions 1–3200) subcloned in pBSKS$^+$, and 1.78-kb *Eco*RI-*Bam*HI fragment (positions 1–1780) of mouse rRNA 18S cDNA subcloned in pUC830.

Run-on in vitro transcription assays were performed using isolated INS-1 nuclei as described (8). Nascent transcripts were elongated in the presence of [^{32}P]UTP and 2.1 mg/ml heparin. The resulting [^{32}P]RNAs were subjected to mild alkaline hydrolysis (30 min at 50°C in the presence of 50 mmol/l Na_2CO_3) and hybridized to 2 µg/dot of the same DNA constructions described for Northern blot analysis.

Electrophoretic mobility shift assays

Preparation of nuclear extracts. Cells (40×10^6) were harvested and washed in cold phosphate-buffered saline (PBS) and lysed with 1 ml buffer A (15 mmol/l KCl, 2 mmol/l $MgCl_2$, 10 mmol/l HEPES, 0.1% phenylmethylsulfonyl fluoride [PMSF], and 0.5% Nonidet P-40). After an incubation of 10 min on ice, nuclei were collected by centrifugation and washed with buffer A without Nonidet P-40. Nuclei were lysed in a buffer containing 2 mmol/l KCl, 25 mmol/l HEPES, 0.1% EDTA, and 1 mmol/l dithiothreitol (DTT). After an incubation of 15 min on ice, a dialysis buffer (25 mmol/l HEPES, 1 mmol/l DTT, 0.1% PMSF, 2 µg/ml aprotinin, 0.1 mmol/l EDTA, and 11% glycerol) was added to the nuclei preparation. Samples were centrifuged, and the supernatant, containing the nuclear proteins, was divided into aliquots and kept frozen at –80°C for further analysis.

DNA probes. Oligonucleotides for the DNA retardation assay were purchased from ACGT (Toronto, Ontario, Canada). A 28-mer oligonucleotide containing a consensus AP-1 recognition sequence was used (24). After annealing, the double-stranded oligonucleotide was labeled with γ[^{32}P]ATP using the DNA 5′ end-labelling kit from Boehringer Mannheim (Indianapolis, IN).

DNA retardation assay. Nuclear proteins (5 µg) were incubated with a radiolabeled probe (20,000 cpm per sample) for 20 min at room temperature in a buffer containing 25 mmol/l HEPES, 10% glycerol, 50 mmol/l NaCl, 0.05% Nonidet P-40, and 1 mmol/l DTT. For competition, an excess (10- to 50-fold) of cold oligonucleotide was added at the same time. Samples were loaded on a 4% nondenaturing polyacrylamide gel containing 0.01% Nonidet P-40 for analysis by autoradiography.

c-Fos protein analysis. After two washes of cells with ice-cold KRBB, 0.5 ml extraction buffer (50 mmol/l Tris, pH 7.5, 5 mmol/l EDTA, 5 mmol/l EGTA, 0.5 mmol/l PMSF, 10 µg/ml leupeptin, 10 µg ml aprotinin, 5 µg/ml antipain, 5 µg/ml pepstatin, and 10 mmol/l mercaptoethanol) was added to the cells. Cells were scraped from the dishes, transferred to an Eppendorf tube, and disrupted by sonication. Extracts (60 µg) of total cellular protein were resolved on 10% SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes (Bio-Rad, Richmond, CA). c-Fos protein was detected by immunoblotting with specific antibodies obtained from Calbiochem (Toronto, Ontario, Canada).

[^{3}H]thymidine incorporation assay. Cells were seeded 2 days before use in 96-well plates as described above (8×10^4 cells per well). Cells were washed with PBS and starved in a minimal RPMI medium (without serum and glucose but with

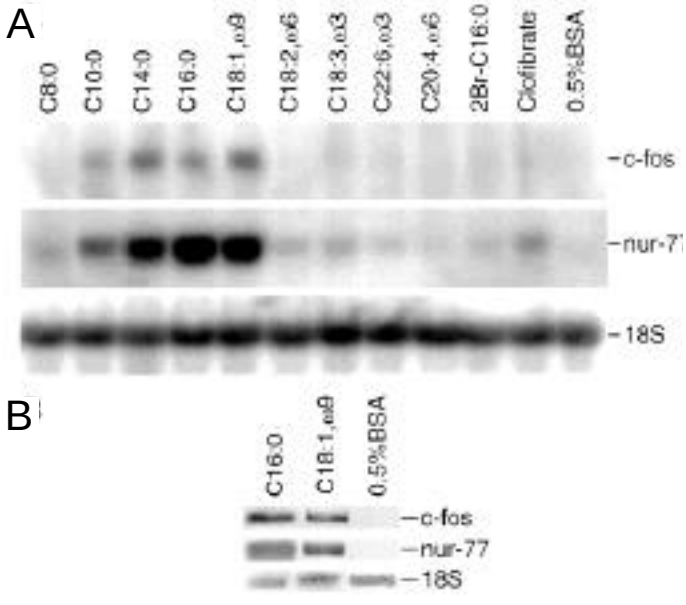

FIG. 1. Effect of different fatty acids and agonists on c-*fos* and *nur*-77 mRNA expression. *A*: Northern blot analysis of total RNA (15 µg) extracted from INS cells incubated for 1 h with one of the following substances: 2.5 mmol/l octanoic acid (C8:0), 2.5 mmol/l decanoic acid (C10:0), 0.4 mmol/l myristate (C14:0), 0.4 mmol/l palmitate (C16:0), 0.4 mmol/l oleate (C18:1, ω9), 0.4 mmol/l linoleic acid (C18:2, ω6), 0.4 mmol/l linolenic acid (C18:3, ω3), 0.3 mmol/l docosahexaenoic acid (C22:6, ω3), 0.25 mmol/l arachidonic acid (C20:4, ω6), 0.4 mmol/l 2-Br-palmitate (2Br-C16:0), or 0.5 mmol/l clofibrate. Serum albumin (0.5% BSA) was present under all conditions. Docosahexaenoic and arachidonic acids were used at their maximal concentrations that could be bound to BSA, thus explaining why cells were incubated in their presence at slightly lower concentrations than the other fatty acids. The 18S blot is shown as invariant control of the amount of RNA present in each lane. Similar results were observed in four separate experiments. *B*: Northern blot analysis of total RNA (10 µg) extracted from isolated rat islets incubated for 1 h in the absence or presence of oleate (0.45 mmol/l) and palmitate (0.45 mmol/l) bound to BSA (0.5%). The figure shows a representative blot from two independent experiments.

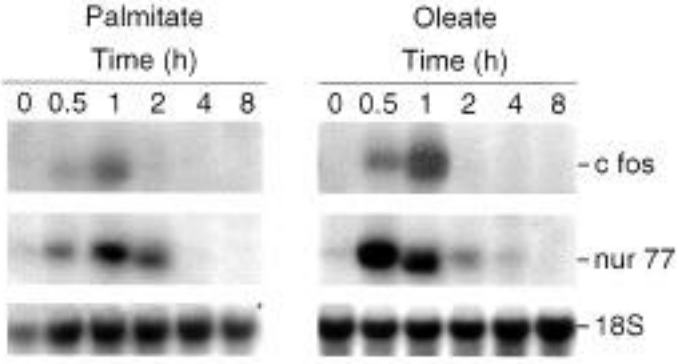

FIG. 2. Time dependence of c-*fos*, *nur*-77, and 18S induction. Northern blot analysis of total RNA (15 µg) extracted from INS cells incubated for different times in the presence of 0.5 mmol/l palmitate or 0.5 mmol/l oleate bound to 0.5% defatted BSA. The 18S blot is shown as invariant control. The figure shows a representative blot from three different experiments.

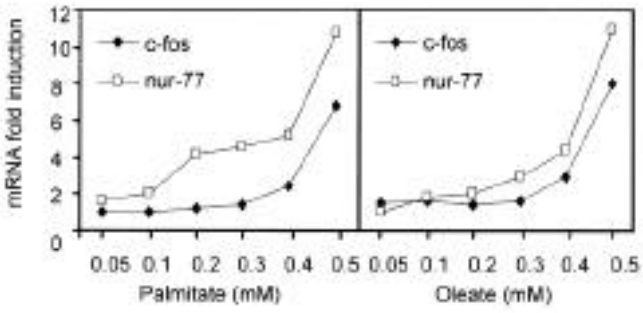

FIG. 3. Dose dependence of palmitate and oleate on the induction of c-*fos* and *nur*-77 mRNA. Each point represents the mean of two or three independent experiments quantified by densitometry. The PMA/18S densitometric value is arbitrarily referred to as 10.

0.1% BSA) for 24 h as described (25). Then they were incubated in this minimal RPMI medium with the various substances to be tested (glucose, palmitate, and oleate) for 24 h. Tritiated thymidine was added (1 µCi/well) 4 h before harvesting the cells with a PHD cell harvester from Cambridge Technology (Watertown, MA), and radioactivity was measured.

Reagents. RPMI 1640 medium and supplements including fetal calf serum were purchased from Gibco BRL. [^{32}P]dCTP, [^{32}P]UTP, and γ[^{32}P]ATP were purchased from Amersham (Buckinghamshire, U.K.). Methyl [^{3}H]thymidine was from ICN (Costa Mesa, CA). Phorbol 12-myristate 13-acetate (PMA), sodium palmitate, sodium oleate, and other fatty acids were purchased from Sigma. Nifedipine was from RBI (Natick, MA). All other biochemicals were of analytical grade.

RESULTS

The main dietary fatty acids palmitate and oleate induce the accumulation of the c-*fos* and *nur*-77 transcripts. The results in Fig. 1*A* indicate that the most common saturated and monounsaturated long-chain fatty acids—myristate (C14:0), palmitate (C16:0), and oleate (C18:1, ω9)—are very effective in inducing the accumulation of *nur*-77 and c-*fos* mRNAs. No change was observed in the rRNA 18S level, which was used as invariant control. On the other hand, the polyunsaturated fatty acids linoleate (C18:2, ω6), linolenate (C18:3, ω3), docosahexaenoate (DHA) (C22:6, ω3), arachidonate (C20:4, ω6); the nonmetabolizable fatty acid analog 2-Br-palmitate (2Br-C16:0); and short-chain fatty acids decanoate (C10:0) and octanoate (C8:0) barely changed the expression level of c-*fos* and *nur*-77. The PPAR-α agonist clofibrate had only a slight effect on *nur*-77 mRNA expression. BSA, which was used to solubilize the different fatty acids in the culture medium, had no effect in the induction of these transcripts. These results show that the induction of c-*fos* and *nur*-77 is mainly observed with the two most abundant dietary fatty acids, palmitate and oleate.

The accumulation of c-*fos* and *nur*-77 RNAs was not due to lipid peroxide generation, since no production of malonyldialdehyde (a marker of these toxic agents) (26) was detected in fatty acid–treated cells (data not shown).

Concerning other tested candidate IEGs, no change in transcript accumulation was observed for *jun*-B, whereas a modest (twofold) increase in *zif*-268/*egr*-1 was detected under the same experimental conditions (data not shown). We therefore further studied only the c-*fos* and *nur*-77 genes in the next series of experiments.

Figure 1*B* shows that oleate and palmitate also caused a prominent accumulation of c-*fos* and *nur*-77 mRNA in isolated rat islets. For tissue availability reasons, the mechanism of this inductive process was further studied only with INS-1 cells.

Kinetics and dose dependence of *nur*-77 and c-*fos* mRNA accumulation. Figure 2 shows that the c-*fos* and *nur*-77 transcripts were detected after a lag time of 30 min. Transcript accumulation reached a maximum at 1 h and returned to basal level after 2–4 h of stimulation. Therefore, the activation of c-*fos* and *nur*-77 by palmitate and oleate displayed a characteristic bell-shaped kinetics, which is often observed in IEG induction in other cell types (27,28).

The transcript accumulation of these IEGs was dose dependent in the pathophysiologic range of concentration. A strong accumulation occurred at high concentration of palmitate and oleate (0.4–0.5 mmol/l), whereas a modest accumulation was observed at low concentrations of fatty acids (~0.1–0.2 mmol/l) in the presence of 0.5% BSA (Fig. 3). By measuring the action of the fatty acids at different ratios of palmitate or oleate to albumin, it appears that the free fraction of the fatty acid, rather than the total concentration, is causally implicated in the transcript accumulation process. Thus, the action of 0.5 mmol/l palmitate at 0.5% BSA was identical to that of 1 mmol/l palmitate in the presence of 1% BSA (data not shown).

Palmitate and oleate cause transcriptional activation of the *nur*-77 and c-*fos* genes. To assess the possibility that the accumulation of the studied IEGs is due to a transcriptional activation, we performed run-on assays with isolated INS-1 cell nuclei. Figure 4 shows that elevated concentrations of fatty acids caused a pronounced transcriptional activation of the c-*fos* and *nur*-77 genes. Therefore, it appears that the main site of action of palmitate and oleate on the expression of these IEGs is at the transcriptional level.

Palmitate and oleate increased Fos protein expression and the DNA-binding activity of the AP-1 complex. Figure 5 shows that oleate and palmitate increased the expression level of the Fos protein by 30 and 80%, respectively. DNA retardation assays were carried out to determine whether the accumulations of the inducible c-*fos* transcript and protein were associated with a parallel increase in the DNA-binding activity of the AP-1 complex. The results in Fig. 6 show that an active AP-1 complex was formed when INS-1 cells were incubated in the presence of palmitate or oleate, confirming the results obtained by Northern and Western blots.

Role of PKC and Ca^{2+} signaling in the induction of *nur*-77 and c-*fos* genes. To better understand the mechanism of the induction of these early genes by fatty acids, we

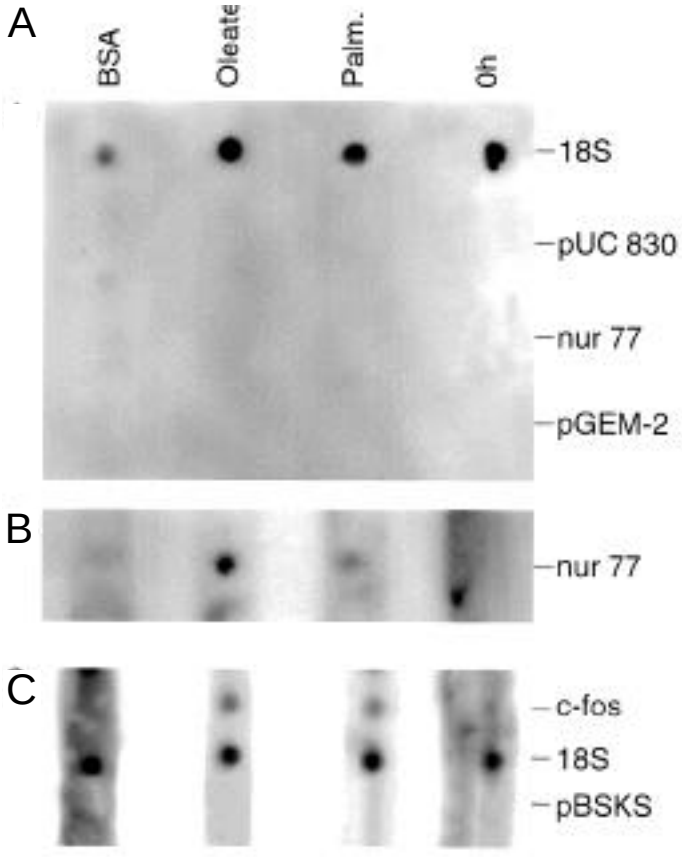

FIG. 4. Palmitate and oleate induce the transcription of the c-*fos* and *nur*-77 genes. INS cells were incubated for 1 h in the presence of 0.5% defatted BSA, 0.5 mmol/l oleate + 0.5% BSA, and 0.5 mmol/l palmitate + 0.5% BSA. At the end of the incubation, nuclei were isolated, and run-on transcription assays were performed. The [^{32}P]-labeled transcripts were hybridized to a *nur*-77 probe (1 day of exposure [*A*] and 3 days of exposure [*B*]), a c-*fos* probe (1 day of exposure [*C*]), and an 18S probe (*A* and *C*). The figure is representative of three separate experiments.

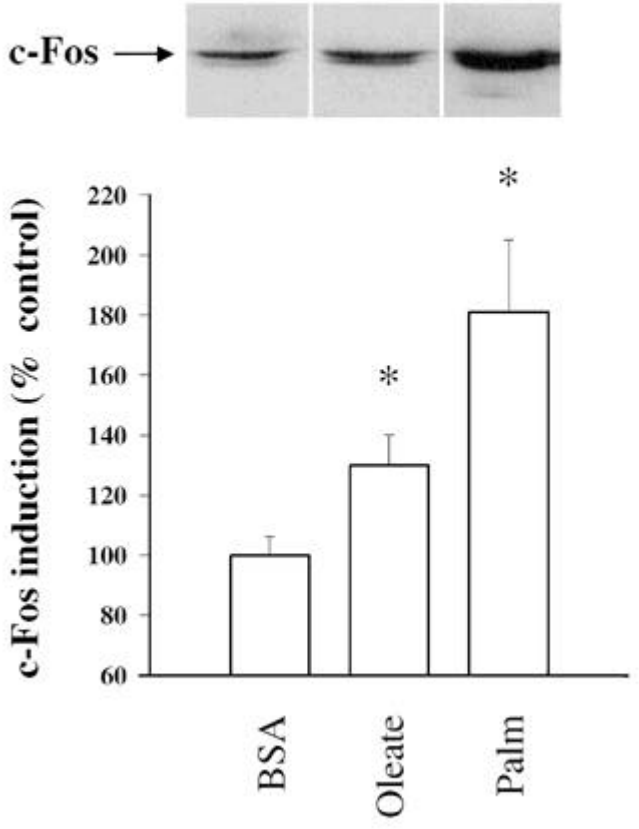

FIG. 5. Palmitate and oleate increase the expression level of c-Fos protein. Immunoblot analysis of proteins (60 µg) extracted from INS cells incubated for 90 min in the presence of 0.4 mmol/l palmitate or 0.4 mmol/l oleate bound to 0.5% defatted BSA. The figure shows a representative blot. Means ± SE of three independent experiments. *$P < 0.05$.

performed experiments to dissect the transduction machinery implicated in this process. Previous work has shown that fatty acids and their CoA derivatives activate certain PKC isoforms (29–31). To test this possibility, we carried out PKC-downregulation experiments by incubating INS-1 cells in the presence of stimulatory concentrations of the phorbol ester PMA for 8–12 h. This treatment induced a rapid degradation of certain PKC isoforms and a loss of sensitivity of β-cells to phorbol ester stimulation (32). Figure 7 shows that PKC-downregulated cells did not display an accumulation of *nur*-77 and c-*fos* transcripts on incubation with PMA (positive control), palmitate, or oleate. However, the action of the adenylate cyclase activator forskolin and elevated KCl remained intact. This result suggests that the PKC transduction pathway is implicated in the action of palmitate and oleate. Interestingly, the action of palmitate and oleate on *nur*-77 and c-*fos* was similar to that caused by the well-known tumor promoter PMA (Figs. 7 and 8), which activates specific PKC enzymes (33).

Fatty acids cause the entry of extracellular Ca^{2+} into the β-cell (11). To directly test the role of extracellular Ca^{2+} in the induction of *nur*-77 and c-*fos*, cells were incubated in the presence of the L-type Ca^{2+} channel inhibitor nifedipine (Fig. 8). Nifedipine almost abolished the induction of these IEGs in response to high KCl (positive control), PMA, and fatty acids. These results indicate the importance of Ca^{2+} signaling in the induction of *nur*-77 and c-*fos*. At the same time, it appears that Ca^{2+} is also necessary in the PKC activation pathway by fatty acids, since nifedipine and EGTA also suppressed the action of PMA. This finding suggests that classical Ca^{2+}-dependent kinase C isoforms are involved in this process of gene induction. Finally, similar Ca^{2+} dependence was observed also by using nimodipine, another specific voltage-gated channel inhibitor, and the Ca^{2+} chelator EGTA (data not shown), confirming the key role of this ion in c-*fos* and *nur*-77 induction process by LCFAs.

Oleate, but not palmitate, promotes [^{3}H]thymidine incorporation in β-cells. To examine whether the fatty acid–induced rise in the expression level of c-*fos* and *nur*-77 and AP-1 activity in INS-1 cells is associated with functional phenotypic changes, [^{3}H]thymidine incorporation experiments were performed as an index of DNA replication and cell proliferation (Fig. 9). As expected from previous results (20), glucose stimulated [^{3}H]thymidine incorporation in this cell type. Interestingly, oleate increased [^{3}H]thymidine incorporation to an extent similar to that of high glucose. In contrast, palmitate had no effect.

DISCUSSION

To understand how fatty acids cause their long-term adaptive effects on the β-cell in obesity and possible toxic action in the

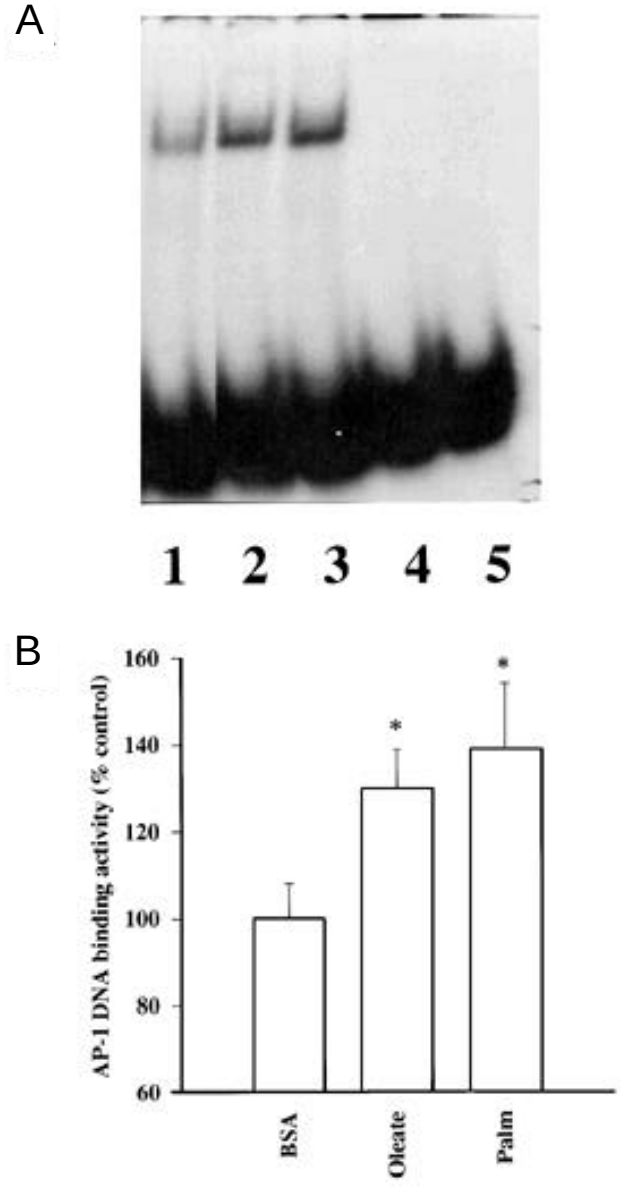

FIG. 6. Palmitate and oleate increase AP-1 DNA binding activity. *A*: Representative DNA retardation assay performed with nuclear extracts of INS-1 cells cultured for 2 h at 5 mmol/l glucose in the presence of oleate or palmitate bound to 0.5% BSA. *Lane 1*, 5 mmol/l glucose, 0.5% BSA; *lane 2*, 0.4 mmol/l oleate; *lane 3*, 0.4 mmol/l palmitate; *lane 4*, no cell extract added in the assay; *lane 5*, replicate of *lane 3* with a 100-fold excess of cold probe. *B*: Quantification of the DNA retardation assays. Data represent means ± SE of three different experiments. *$P < 0.05$.

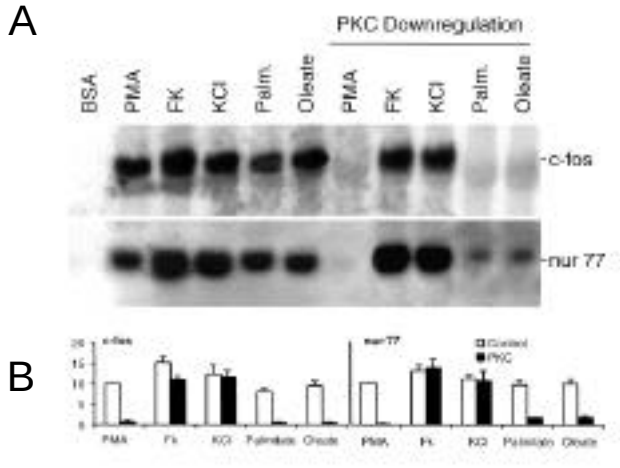

FIG. 7. Palmitate and oleate are unable to induce c-*fos* and *nur*-77 mRNA accumulation in PKC-downregulated cells. INS cells were incubated in the presence or absence of 0.1 µmol/l PMA for 10 h to downregulate PKC expression. Control and PMA-treated cells were incubated for 1 h in the presence of the following agonists: 0.1 µmol/l PMA, 2 µmol/l forskolin (FK), 30 mmol/l KCl, 0.5 mmol/l palmitate (Palm.), and 0.5 mmol/l oleate. BSA (0.5%) was present under all conditions. *A*: Representative Northern blot. *B*: Means ± SE of densitometric analysis of four independent experiments normalized to the invariant control 18S RNA. The PMA/18S densitometric value is arbitrarily referred to as 10.

development of diabetes, there is considerable interest in identifying fatty acid–regulated genes. Previous work has shown that fatty acids modulate the expression of a number of "late" genes of fatty acid metabolism in the β-cell, including acetyl-CoA carboxylase and fatty acid synthase (10). Enzymes of fat oxidation, in particular carnitine palmitoyltransferase I and fatty acyl-CoA oxidase, are induced (8,9), an event that may be linked to lipid detoxification. Whether fatty acids modulate IEGs encoding transcription factors was, before this study, unknown in the β-cell. The identification of IEGs regulated by fatty acids is relevant to our understanding of fatty acid stimulus-transcription coupling and to delineating the pathways of pleiotropic actions of these nutrients. Indeed, several IEGs regulate secondary response genes and have been implicated in the control of cell differentiation, growth, and apoptosis (13). The present study shows that, in β-cells (INS cells) and isolated rat islets, fatty acids regulate the expression of c-*fos*, which is known to act as a master switch in gene regulation. Additionally, they regulate expression of *nur*-77, a gene that in other systems has been used as a paradigm of Ca^{2+} regulation and that encodes a transcription factor that may be involved in the control of apoptosis (16,34).

The action of the fatty acids occurs at 0.1–0.5 mmol/l, which is in the physiologic range of free fatty acid concentrations. Interestingly, it is specific for some fatty acids, since none of the long-chain polyunsaturated fatty acids examined are effective. Among the many long-chain fatty acids tested, only palmitate, oleate, and myristate, which are abundant in the circulation, are effective. Fatty acid metabolism beyond their activation as CoA esters is required for the induction of the studied genes, since the nonmetabolizable Br-palmitate is ineffective. We favor the view that metabolism of fatty acids to complex lipids, rather than their mitochondrial oxidation, is implicated. Thus, octanoate, which is well oxidized in the β-cell, barely induces these IEGs.

The precise mechanism of these IEG induction processes remains to be defined. Nonetheless, the evidence indicates that the Ca^{2+} and PKC transduction systems are implicated. Thus, the phorbol ester PMA induces both IEGs to a similar extent compared with palmitate and oleate, and the actions of both fatty acids are suppressed in PKC-downregulated cells. In addition, L-type Ca^{2+} channel blockers and chelation of medium Ca^{2+} with EGTA suppressed the action of palmitate and oleate. The implication of the Ca^{2+} and C-kinase systems is in accord with two previous reports. Thus, palmitate was shown to promote the translocation of PKC to a particulate fraction in islet tissue, an event requiring metabolism of

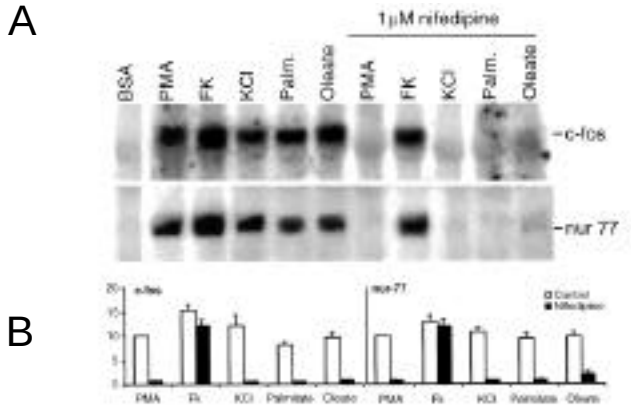

FIG. 8. Palmitate and oleate are unable to induce c-*fos* and *nur*-77 mRNA accumulation in nifedipine-treated INS-1 cells. INS cells were incubated in the presence or absence of 1 μmol/l nifedipine for 15 min before adding the following agonists: 0.1 μmol/l PMA, 2 μmol/l forskolin (FK), 30 mmol/l KCl, 0.5 mmol/l palmitate (Palm.), and 0.5 mmol/l oleate. BSA (0.5%) was present under all conditions. *A*: Representative Northern blot. *B*: Means ± SE of densitometric analysis of four independent experiments normalized to the invariant control 18S RNA. The PMA/18S densitometric value is arbitrarily referred to as 10.

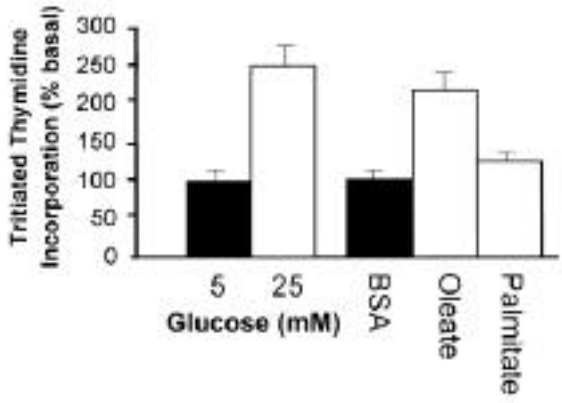

FIG. 9. Effects of glucose, palmitate, and oleate on DNA synthesis in INS-1 cells. DNA synthesis was determined by [^{3}H]thymidine incorporation. Cells were cultured in serum-free RPMI medium containing 5 and 25 mmol/l palmitate and 0.5 mmol/l oleate bound to 0.5% BSA for 24 h. To each well, 1 μCi [^{3}H]thymidine was added during the final 4 h of the 24-h incubation period. Data represent means ± SE of four independent experiments (each comprising three or four wells).

the fatty acid (31). In addition, it has been documented that palmitate promotes Ca^{2+} influx via L-type Ca^{2+} channels in rat islets (11). Hence, the available evidence suggests the following possibility as far as the mechanism of c-*fos* and *nur*-77 gene induction is concerned. Fatty acids would promote a rise in cytosolic Ca^{2+} after the opening of voltage-gated Ca^{2+} channels. In addition, they would be transformed to complex lipids, such as phosphatidylserine or others, activating classical PKC isoforms. The rise in Ca^{2+} in association with complex lipid formation would contribute to full PKC activation and a resulting induction of these genes via the interaction of AP-1 transcription factors with tetraphorbol acetate (TPA)/serum-responsive elements, which are present in the promoters of both genes (28).

A more challenging question concerns the physiologic significance of the induction of c-*fos* and *nur*-77 genes by dietary fatty acids. This question can be answered by considering the late genes that are regulated by the c-Fos and Nur-77 transcription factors. However, the exact target genes modulated by c-Fos and Nur-77 remain to be identified in β-cells. AP-1/c-Fos binding elements are very pleiotropic, activated by different pathways, and probably involved in different aspects of β-cell function, such as cell proliferation, apoptosis, and induction of metabolic genes (13). On the other hand, the target genes for Nur-77 are still poorly identified, although the experimental evidence suggests a role of this transcription factor in thymocyte apoptosis (16,34,35).

Three possibilities can be considered. First, cell growth control. The stimulation of [^{3}H]thymidine incorporation by oleate supports this idea. Several reports support such a role for c-*fos* induction in many cell types (28). On the other hand, members of the Nur-77 family are strongly implicated in proliferative processes. In this context, RNR-1 (a nuclear receptor displaying high homology to Nur-77) is strongly induced within 1 h of partial hepatectomy (36). The reason oleate increases [^{3}H]thymidine incorporation into DNA in β-cells, whereas palmitate is ineffective, is unclear. Palmitate can enter the ceramide pathway, which has been implicated in β-cell apoptosis, whereas oleate does not directly enter the pathway (37,38). Possibly, the balance between cell growth and apoptosis is differentially controlled by these fatty acids. Further studies are required to evaluate this possibility.

Second, apoptosis regulation. Previous reports suggest that fatty acids may induce β-cell apoptosis in Zucker diabetic fatty rats through the induction of NO synthase and NO production (7,39). Interestingly, the NOS genes contain an AP-1 response element (40). On the other hand, different studies have well established an increase of expression of Nur-77 in T-cell apoptosis (16,34,35). In addition, it has been reported that sustained expression of c-Fos mediates cellular apoptosis (15). Furthermore, induction of c-Jun–estrogen receptor chimeras in cultured fibroblasts is also involved in programmed cell death (14). These observations suggest that IEG induction leading to apoptosis must be considered as a key process in β-cell pathology and deserves more attention.

Third, control of insulin, metabolic, and detoxifying enzyme gene expression. In this context, an AP-1 binding site has been identified in the rat insulin (41) and GLUT2 promoters (42). Interestingly, palmitate downregulates the GLUT2 and insulin genes (3). On the other hand, two clear target genes have been identified for Nur-77 in adrenocortical and neuroblastoma cells. One of these is the gene encoding for steroid 21-hydroxylase, an enzyme related to the cytochrome P-450 superfamily, which is involved in several cell functions such as detoxification, steroid hormone biosynthesis, and stress adaptation (43). The second known target for Nur-77 is the gene encoding for fructose-bisphosphatase aldolase C, which catalyzes the cleavage of fructose 1,6-bisphosphate into dihydroxyacetone phosphate and D-glyceraldehyde phosphate (44).

If extended to other pathologies, such as tumor promotion, this report suggests a mechanism to link dietary fatty acids to oncogenesis. Tumor promoters are not necessarily mutagens. For instance, some phorbol esters that activate PKC (45) or the sesquiterpene lactone thapsigargin, which causes Ca^{2+} mobilization from internal stores (46), are potent tumor promoters and induce IEGs (27,47,48). Their mode of action in

carcinogenesis is uncertain, but it has been proposed that they might stimulate growth of initiated cells carrying mutations that would otherwise be quiescent (49). It is noteworthy that fatty acids, like phorbol esters, markedly activate PKC (29,30) and elevate Ca^{2+} in insulin-secreting cells (11). It is therefore attractive to hypothesize that elevated circulating fatty acids under some circumstances act like tumor promoters by stimulating the growth of mutated cells. The mechanism might implicate activation of various transduction systems, in particular the PKC and Ca^{2+}/calmodulin transduction systems, with resulting activation of some IEGs encoding proto-oncogenes or transcription factors implicated in cell growth regulation. The results of this study are compatible with this view.

In conclusion, palmitate and oleate, at concentrations in the physiologic range, cause a rapid and transient transcriptional induction of the c-*fos* and *nur*-77 genes in β-cells (INS-1 cells) and isolated rat islets. This induction appears to be mediated by classical isoforms of PKC, requiring elevated $[Ca^{2+}]_i$. This may prove to be of interest from the standpoint of nutrition, which is causally linked to the development of certain pathologies such as type 2 diabetes, obesity, and cancer.

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