Long-Term Elevation of Free Fatty Acids Leads to Delayed Processing of Proinsulin and Prohormone Convertases 2 and 3 in the Pancreatic β-Cell Line MIN6

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To explore the role of chronically elevated free fatty acids (FFAs) in the pathogenesis of the hyperproinsulinemia of type 2 diabetes, we have investigated the effect of FFAs on proinsulin processing and prohormone convertases PC2 and PC1/PC3 in MIN6 cells cultured in Dulbecco's modified Eagle's medium with or without 0.5 mmol/l FFA mixture (palmitic acid:oleic acid = 1:2). After 7 days of culture, the percent of proinsulin in FFA-exposed cells was increased (25.9 ± 0.3% intracellular and 75.4 ± 1.2% in medium vs. 13.5 ± 0.2 and 56.2 ± 4.1%, respectively, in control cells). The biosynthesis and secretion of proinsulin and insulin were analyzed by comparing the incorporation of [^{3}H]Leu and [^{35}S]Met. In pulse-chase studies, proinsulin-to-insulin conversion was inhibited, and proinsulin in the medium was increased by 50% after 3 h of chase, while insulin secretion was decreased by 50% after FFA exposure. Levels of cellular PC2 and PC3 analyzed by Western blotting were decreased by 23 and 15%, respectively. However, PC2, PC3, proinsulin, and 7B2 mRNA levels were not altered by FFA exposure. To test for an effect on the biosynthesis of PC2, PC3, proinsulin, and 7B2, a protein required for PC2 activation, MIN6 cells were labeled with [^{35}S]Met for 10–15 min, followed by a prolonged chase. Most proPC2 was converted after 6 h of chase in control cells, but conversion was incomplete even after 6 h of chase in FFA-exposed MIN6 cells. Media from chase incubations showed that FFA-exposed cells secreted more proPC2 than controls. Similar inhibitory effects were noted on the processing of proPC3, proinsulin, and 7B2. In conclusion, prolonged exposure of β-cells to FFAs may affect the biosynthesis and posttranslational processing of proinsulin, PC2, PC3, and 7B2, and thereby contribute to the hyperproinsulinemia of type 2 diabetes. The mechanism of inhibition of secretory granule processing by FFAs may be through changes in Ca^{2+} concentration, the pH in the secretory granules, and/or other factors that may influence the activation and function of the convertases. *Diabetes* 48:1395–1401, 1999

The concept that elevated levels of plasma free fatty acids (FFAs) produce peripheral and hepatic insulin resistance in obesity and type 2 diabetes was first proposed by Randle et al. (1) more than 30 years ago. They proposed the "glucose-fatty acid cycle" based on their demonstration that the increased oxidation of FFAs inhibits the uptake and oxidation of glucose in muscle (1). Subsequent studies have supported the hypothesis that FFA oxidation stimulates gluconeogenesis and inhibits glucose uptake and glycolysis in liver (2–4).

Previous studies have examined mainly the short-term effects of FFAs on insulin secretion. FFAs may serve as fuel for pancreatic β-cells (5), and acute exposure of islets to FFAs stimulates insulin secretion (5–7). This stimulatory effect of FFAs has been attributed to increased ATP derived from fatty acid oxidation or to activation of protein kinase C by long-chain acyl-CoA (8). Recently, chronic effects of FFAs on insulin secretion have been demonstrated; these include elevated basal insulin secretion at low glucose concentrations, accompanied by inhibition of glucose-stimulated insulin secretion (GSIS) (9–11). The effects of long-term exposure to FFAs on β-cell glucose metabolism described so far are an apparent increase in hexokinase activity (12) and indications of a decrease in the activity of pyruvate dehydrogenase (13,14). On the other hand, since glucose-related ATP generation does not parallel the change in insulin release in response to glucose, it has been suggested that impaired energy production from both glucose and FFA metabolism in pancreatic islets is not the major cause of inhibited GSIS (15). The inhibition of islet glucose oxidation after long-term exposure to FFAs (10,11,15) is consistent with the operation of the glucose fatty acid cycle.

Plasma FFAs are elevated in most obese and type 2 diabetic subjects (16–19), and it has been observed that ~85% of patients with type 2 diabetes in the U.S. are obese (20). Elevated levels of plasma FFAs may, thus, play a key role in the pathogenesis of type 2 diabetes. Also, the existence of disproportionate hyperproinsulinemia in type 2 diabetes is well established (21–27). For the most part, hyperproinsulinemia accompanies hyperglycemia (21,23,25), and the

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BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FFA, free fatty acid; GSIS, glucose-stimulated insulin secretion; KRB, Krebs-Ringer bicarbonate; mpII, mouse proinsulin II; PBS, phosphate-buffered saline.

proportion of circulating proinsulin rises from ~10% to 15–22% (21).

To determine whether prolonged elevations of FFAs may induce increased levels of proinsulin, we have investigated the long-term effect of FFAs on proinsulin processing and on the levels and activation state of the prohormone convertases PC2 and PC1/PC3, as well as the neuroendocrine protein 7B2, a helper protein for PC2 activation (28,29), in cultured MIN6 cells. This cell line was derived from SV-40–induced mouse insulinomas (30). The MIN6 cell line has very similar characteristics to isolated islets (31) and displays coordinate regulation of the biosynthesis of proinsulin and of the prohormone convertases PC2 and PC3 by glucose at the translational level (32,33). Therefore, the MIN6 cell line is an appropriate model for studying the mechanism of GSIS and PC2, PC3, and proinsulin processing.

RESEARCH DESIGN AND METHODS

Reagents. Palmitic acid, oleic acid (sodium salts), and bovine serum albumin (BSA; fraction V) were purchased from Sigma (St. Louis, MO). L-[4,5-^{3}H]Leucine (148 Ci/mmol), L-[^{35}S]methionine (1,000 Ci/mmol), and [^{14}C]-methylated protein molecular weight markers were from Amersham (Amersham Pharmacia Biotech, Newark, NJ). [^{32}P]dCTP (3 mCi/mmol) was from Du Pont-NEN (Boston, MA). Immobilized protein A agarose beads were from Pierce (Rockford, IL). Rabbit polyclonal PC2 antiserum raised against a COOH-terminal peptide, PC3 antibody directed against the NH_2-terminal region of mature PC3, and guinea pig anti-insulin antisera coupled to Affi-gel 10 agarose beads (Bio-Rad, Hercules, CA) were as previously described (33,34). 7B2 antiserum was kindly provided by Iris Lindberg (Louisiana State University Medical Center, New Orleans, LA).

Cell culture. MIN6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mg/ml glucose and 0.39 mmol/l mercaptoethanol and supplemented with 10% fetal bovine serum, 100 U/ml penicillin sulfate, 70 µmol/l streptomycin, and 50-µg/ml gentamycin at 37°C in a humidified atmosphere of 5% CO_2/95% air (30,31). MIN6 cell cultures were used between passages 19 and 28, wherein no change in the characteristics of proinsulin biosynthesis was observed.

Immunoreactive proinsulin and insulin. MIN6 cells were cultured in DMEM with or without FFAs for 7 days. Cells were extracted with acidic ethanol as described (35), and medium was extracted using Sep-Pak cartridges (Millipore, Milford, MA). Extracts of cells and medium dissolved in 3 mol/l acetic acid were then chromatographed over 1 × 50 cm columns of Bio-Gel (Bio-Rad, Hercules, CA) P-30 eluted with 3 mol/l acetic acid containing 50 µg/ml BSA. The fractions containing proinsulin and insulin were evaporated to dryness, redissolved in radioimmunoassay buffer (0.13 mol/l sodium borate, pH 8.0, 3 mmol/l NaN3, and 5 mg/ml BSA), and analyzed by insulin radioimmunoassay (35). Recovery of insulin-related material was >85%, and no significant degradation of added proinsulin or insulin was observed in incubated media.

Insulin biosynthesis. MIN6 cells were cultured in 60-mm plastic dishes in DMEM with or without 0.5 mmol/l fatty acid mixture (palmitic acid:oleic acid = 1:2) for 7 days. The calculated level of FFAs in this medium is 0.37 mmol/l, corrected for BSA binding according to the method of Spector et al. (36). Cells were rinsed with HEPES-balanced Krebs-Ringer bicarbonate (KRB) buffer containing 1 mg/ml BSA, preincubated in KRB buffer containing 0.5 mg/ml (2.8 mmol/l) glucose and 1 mg/ml BSA for 60 min, and then labeled for periods of 30 min by incubation at 37°C in KRB buffer containing 3.0 mg/ml (16.7 mmol/l) glucose, 1 mg/ml BSA, and 200 µCi of [^{3}H]Leu and [^{35}S]Met. Some dishes were then chase-incubated in KRB buffer containing 3.0 mg/ml glucose and unlabeled amino acids for various time periods. MIN6 cells exposed to FFAs during culture were included in each experiment. After incubation, the medium was removed and saved for analysis. The cells were rinsed with cold KRB buffer, removed from the dish with lysis buffer (25 mmol/l Tris-HCl, pH 7.4, 300 mmol/l NaCl, 1 mmol/l $CaCl_2$, 10 µg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride, 1,000 kallekrein U/ml aprotinin, 1.5 µmol/l pepstatin A, 15 µmol/l E64, and 1% Triton X-100), and then sonicated. Medium and the cell lysates were immunopurified with insulin antibody linked to Affi-gel 10 (33). The protein pellet after immunopurification was dissolved in a small volume of 3 mol/l acetic acid and applied to 1 × 50 cm columns of Bio-Gel P-30 eluted with 3 mol/l acetic acid containing 50 µg/ml BSA. Radioactivity in a small aliquot of each fraction was measured in a liquid scintillation counter. In these experiments, total proinsulin:insulin radioactivity tended to increase during the chase period for unknown reasons, but this did not influence the interpretation of the results.

Immunoprecipitation. Cells were incubated for 60 min in methionine-free DMEM with 1 mg/ml BSA, then labeled for 15 min in 0.5 mCi/ml [^{35}S]Met and 1 mg/ml BSA. Some of the plates were further incubated in DMEM with unlabeled methionine for various time periods. The media were collected, centrifuged to remove cellular debris, and stored at –20°C. The cells were washed twice with cold phosphate-buffered saline (PBS), lysed as above, diluted into 50 mm sodium phosphate with 1% Triton X-100 buffer, sonicated, and stored at –20°C. Immunoprecipitations were performed on 500 µl of media and aliquots of cell lysates adjusted to equal protein content. The solutions were precleared with 2 µl rabbit serum and 20 µl of protein A beads. After 2-h incubation, the suspension was centrifuged, and the supernate was subjected to serial immunoprecipitations with antisera to PC2, PC3, insulin, and 7B2, as described (33). Immunoprecipitates were washed, solubilized in Laemmli buffer containing 5% 2-mercaptoethanol, and boiled at 95°C for 5 min. Samples were electrophoresed on 7.5% (for PC2 and PC3), 15% (for 7B2) SDS-polyacrylamide gels, or a 16% Tricine-buffered polyacrylamide gel for proinsulin and insulin. Gels were fixed in 10% acetic acid and 25% isopropyl alcohol for 30 min and soaked in a fluorographic solution for 20 min. Dried gels were exposed to X-ray film with an intensifying screen for 1–3 days.

Western blotting. Cells were homogenized in 0.275 mol/l sucrose, and aliquots, adjusted for protein content measured by a dye-binding technique (37), were electrophoresed on 7.5% SDS-PAGE. Proteins were transferred to Immobilon-P membrane (Millipore). Membranes were incubated in blocking buffer (PBS, 0.2% [vol/vol] Tween 20 and 5% [wt/vol] nonfat dry milk) for 4 h, incubated overnight in blocking buffer with PC2 and PC3 antibody (1:10,000 dilution), and then washed twice with PBS with 2% Tween 20. Membranes were incubated in blocking buffer with protein A–horseradish peroxidase (1:10,000 dilution) for 1 h, washed with PBS with 2% Tween 20 for 3 h, and then detected with enhanced chemiluminescence reagents (Amersham).

Northern blot analysis. MIN6 cells cultured in DMEM with or without 0.5 mmol/l FFAs for 7 days were washed twice with PBS to remove any detached cells. RNA was extracted using the acid guanidinium thiocyanate/phenol/chloroform extraction method (38). Total RNA (20 µg per sample) was run on 1% (wt/vol) agarose, 6% (vol/vol) formaldehyde/MOPS (3-[N-morpholino]

TABLE 1
Effect of long-term elevation of fatty acids on insulin content and proinsulin secretion

	Control	0.5 mmol/l fatty acids	*P*
Insulin content	254 ± 3	189 ± 12	<0.05
Secreted proinsulin	19 ± 2	51 ± 4	<0.01
Secreted insulin	15 ± 1	17 ± 1	NS

Data are means ± SE of four determinations and are expressed in microunits per 10^6 cells. Radioimmunoassayable proinsulin and insulin in MIN6 cells and medium were measured. MIN6 cells were cultured in DMEM containing 3.0 mg/ml glucose with or without 0.5 mmol/l fatty acid mixture (palmitate:oleate = 1:2) for 7 days. Medium and acid-ethanol extracts of cells were fractionated by Biogel P30 gel chromatography and analyzed by insulin radioimmunoassay.

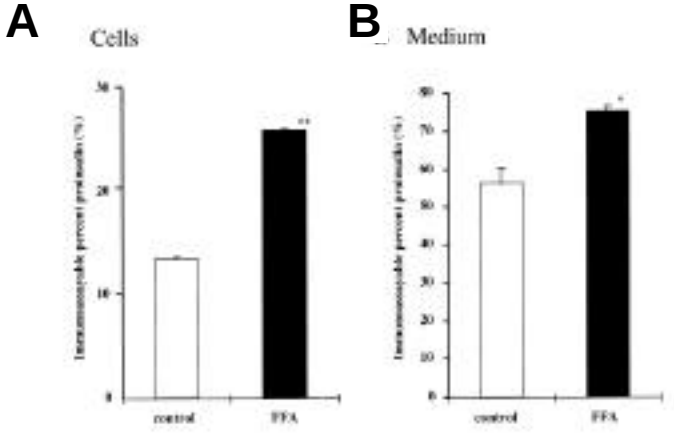

FIG. 1. Effect of long-term elevation of FFAs on percent immunoreactive proinsulin in MIN6 cells (*A*) and culture medium (*B*). MIN6 cells were cultured in DMEM with or without 0.5 mmol/l FFAs for 7 days. Medium or acid-ethanol extracts of cells were fractionated into proinsulin and insulin by Bio-Gel P-30 gel chromatography and quantitated by insulin radioimmunoassay. Data are expressed as means ± SE (n = 4). *P < 0.05, ** P < 0.001 vs. controls.

TABLE 2
Effect of prolonged fatty acid exposure on the biosynthesis, processing, and secretion of (pro)insulin

	Cells		Medium	
	Proinsulin	Insulin	Proinsulin	Insulin
[^{3}H]Leu label				
Control				
1 h	172 ± 29	280 ± 58	490 ± 74	250 ± 38
2 h	134 ± 29	360 ± 66	650 ± 180	258 ± 13
3 h	87 ± 15	464 ± 30	643 ± 220	688 ± 118
FFAs				
1 h	363 ± 53	205 ± 35	414 ± 39	109 ± 40*
2 h	340 ± 81	295 ± 53	661 ± 45	136 ± 35*
3 h	221 ± 44	422 ± 82	854 ± 173	448 ± 90*
[^{35}S]Met label				
Control				
1 h	392 ± 47	382 ± 70	1,012 ± 320	262 ± 56
2 h	246 ± 19	386 ± 34	1,095 ± 250	340 ± 73
3 h	329 ± 52	623 ± 55	1,065 ± 221	970 ± 182
FFAs				
1 h	933 ± 223	328 ± 73	828 ± 118	172 ± 45
2 h	752 ± 116	517 ± 53	1,217 ± 312	241 ± 33
3 h	555 ± 89	508 ± 72	1,530 ± 240	457 ± 90†

Data are means ± SE and are expressed in disintegrations per minute per 10^6 cells. n = 8 for 1 h and 3 h; n = 5 for 2 h. Replicate pulse-chase experiments were carried out at 3.0 mg/ml glucose, as described in METHODS. *P < 0.05, †P < 0.001 vs. control.

propanesulfonic acid; Sigma Aldrich, St. Louis, MO) gels and blotted onto Hybond-N membrane (Amersham) and probed with [^{32}P]-labeled preproinsulin, PC2, PC3, 7B2, and β-actin cDNA. Northern blots were quantitated with a densitometer and normalized for loading differences by reference to the relative strength of the β-actin mRNA signal on the same blot.

Statistics. Data are expressed as means ± SE. Statistical differences were analyzed using the appropriate Student's t test, where P < 0.05 was considered significant.

RESULTS

Effect of long-term elevation of FFAs on percent immunoreactive proinsulin content and secretion. The content and secretion of insulin-related peptides were studied in cultured MIN6 cells exposed to fatty acids and parallel controls at stimulatory concentrations of glucose (16.7 mmol/l) (Table 1). The FFA-exposed MIN6 cells showed an increased proportion of proinsulin (25.9 ± 0.3% intracellular and 75.4 ± 1.2% medium) compared with controls (13.5 ± 0.2 and 56.2 ± 4.1%, respectively), confirming that proinsulin is accumulated in significantly increased amounts within FFA-exposed cells (Fig. 1). Previous studies have shown that FFA treatment leads to similar decreases in cellular insulin content in FFA-exposed β-cell lines or islets (11,39).

Insulin biosynthetic studies in FFA-exposed and control MIN6 cells. The biosynthesis of insulin was studied in cultured MIN6 cells by comparing the incorporation of [^{3}H]Leu and [^{35}S]Met in pulse-chase experiments (Table 2). There are eleven leucine residues in each mouse proinsulin, five within the C-peptide and six in the insulin moiety, and the data shown in Table 2 have been corrected accordingly. On the other hand, only one methionine is present in mouse (pro)insulin II. The incorporation of [^{35}S]Met thus monitors the biosynthesis and conversion of mouse proinsulin II (mpII), while incorporation of [^{3}H]Leu monitors both proinsulin I and II. The data in Fig. 2 show the decline in percent of labeled proinsulin in the FFA-exposed and control MIN6 cells during chase incubations carried out at 16.7 mmol/l glucose. The FFA-exposed MIN6 cells showed a slower rate of conversion of proinsulin to insulin, as reflected in both the [^{3}H]Leu and [^{35}S]Met data. The half-life for conversion to insulin is 2.3 h for [^{3}H]Leu- and >3 h for [^{35}S]Met-labeled proinsulin in the FFA-exposed cells, in contrast to corresponding values of 0.9 and 1.3 h, respectively, in control cells. These data show that mpII is processed more slowly in both groups, most likely due to the presence in mpII of Met rather than Lys in the P4 position four residues upstream of the B-chain–C-peptide cleavage site, as also observed in normal rodent islets (40,41).

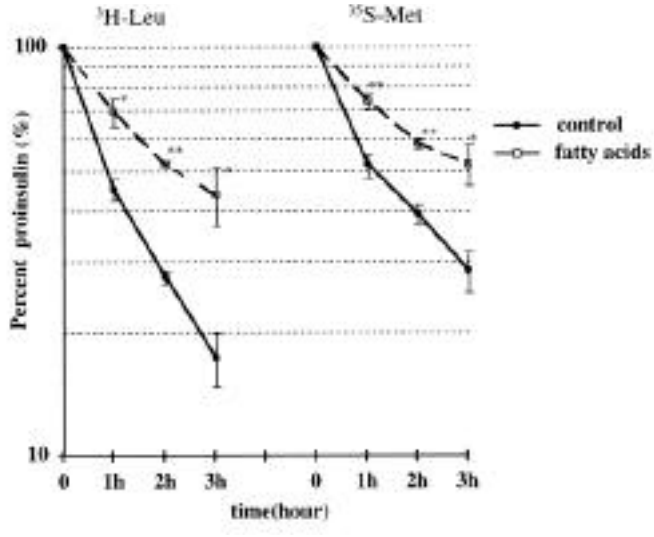

FIG. 2. Semilog plots of the decline in percent proinsulin during pulse-chase labeling studies. MIN6 cells were cultured in DMEM with or without 0.5 mmol/l FFAs for 7 days. Cells were pulse-labeled for 30 min with [^{35}S]Met and [^{3}H]Leu and chased for 1, 2, or 3 h, all at 3.0 mg/ml glucose. See METHODS for further details. Data are expressed as means ± SE (n = 5). *Left graph*: [^{3}H]Leu-labeled material (proinsulins I and II); *right graph*: [^{35}S]-labeled material (proinsulin II). *P < 0.005, ** P < 0.001 vs. controls.

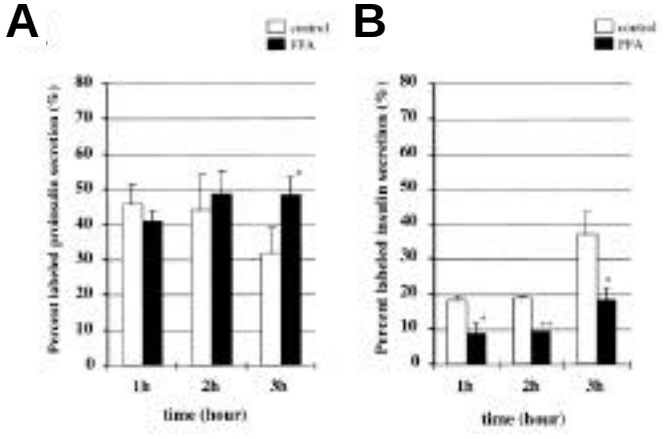

FIG. 3. Effects of FFAs on [^{3}H]Leu-labeled proinsulin (*A*) and insulin (*B*) secretion during pulse-chase studies. MIN6 cells were cultured in DMEM with or without 0.5 mmol/l FFAs for 7 days, then subjected to pulse-chase labeling as in Fig. 2. Percent proinsulin or insulin secretion represents the proportion of total insulin and proinsulin counts (in cells plus medium and corrected for leucine content) appearing in the medium as either proinsulin (*A*) or insulin (*B*). Data are expressed as means ± SE (n = 5). *P < 0.05, ** P < 0.001 vs. controls.

Effect of long-term exposure to fatty acids on proinsulin and insulin secretion in pulse-chase studies. Previous studies have demonstrated that long-term exposure of islets to elevated FFAs leads to inhibition of GSIS (9–11, 42). In the present studies, carried out at 16.7 mmol/l glucose, the percentage of proinsulin secreted in FFA-exposed cells is increased at 3 h of chase, while secreted insulin is proportionately decreased during the chase (Fig. 3). In contrast, when we examined constitutive proinsulin secretion at 2.7 mmol/l glucose during a similar pulse-chase protocol, substantial amounts of proinsulin were released during the first hour of chase (~60% of total), but there were no significant differences between the FFA-exposed and control cells at 1 or 3 h (data not shown). Thus, taken altogether, our findings indicate that the material stored in secretory granules is less mature, resulting in elevated proinsulin secretion from the FFA-treated cells.

Effect of long-term elevation of fatty acids on proinsulin, PC2, PC3, and 7B2 mRNA levels. Controls and MIN6 cells exposed to FFAs for 7 days at 16.7 mmol/l glucose were analyzed for preproinsulin, PC2, PC3, and 7B2 mRNA levels. The data in Fig. 4 show that there are no significant differences between the two groups. These data indicate that under the conditions of FFA exposure used in these experiments, the transcription of these genes is not affected.

PC2 and PC3 protein levels in cultured MIN6 cells. Figure 5 shows representative Western blots of PC2 (Fig. 5*A*) and PC3 (Fig. 5*B*) protein in FFA-exposed and control MIN6 cells. These show a small but significant difference in the level of PC2 and PC3 protein; PC2 and PC3 levels are 23 ± 7 and 15 ± 8% lower (P < 0.05), respectively, in FFA-exposed cells, perhaps reflecting decreased biosynthesis and/or reduced numbers of storage granules (consistent with reduced total stored immunoreactive insulin; Table 1) in FFA-treated cells.

Demonstration of delayed processing of PC2, PC3, proinsulin, and 7B2 after 7 days of culture with 0.5 mmol/l FFA mixture. The processing of PC2, PC3, proinsulin, and 7B2 were studied in MIN6 cells by comparing the incorporation of [^{35}S]Met into these proteins by immunoprecipitation with selected antisera. The precipitated proteins were then fractionated on polyacrylamide gels and fluorographed, and the bands were quantitated with a densitometer. Most of the proPC2 is converted to mature PC2 after 6 h of chase in the control cells, but conversion is incomplete after a 6-h chase in FFA-exposed cells. Media from chase incubations showed that FFA-exposed MIN6 cells secrete more proPC2 than controls (Fig. 6A). The time required for 50% conversion of proPC2 to mature PC2 is 3 h in control cells versus >6 h in FFA-exposed cells (Fig. 7*A*). The processing of PC3 is even more dramatically delayed in FFA-exposed cells than in the controls. As shown in Fig. 7*B*, the halftime for 50% conversion of full-length PC3 to 66kDa PC3 is 2.5 h in control cells and >6 h in FFA-exposed cells. Most of the full-length PC3 is converted after 4 h of chase in the control cells, but processing is far from complete even after 6 h of chase in FFA-exposed cells. Media from chase incubations show that FFA-exposed cells secrete a relatively much greater proportion of full-length PC3 than do controls (Fig. 6*B*). Similar effects are noted on the processing (Fig.6*C*) and rate of conversion (Fig. 7*C*) of proinsulin. Similarly, the conversion of pro7B2 to mature 7B2 in control cells is complete after 30 min of chase; however, in FFA-exposed cells, it requires 45–60 min of chase (Fig. 6*D*). These results indicate that long-term exposure to FFAs significantly inhibits the posttranslational processing of PC2, PC3, proinsulin, and 7B2.

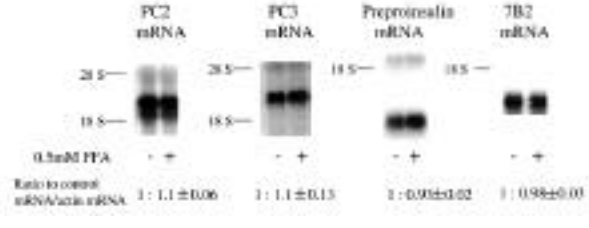

FIG. 4. Northern blot analysis of preproinsulin, PC2, PC3, and 7B2 mRNA content in MIN6 cells. MIN6 cells were cultured in DMEM with or without 0.5 mmol/l FFAs for 7 days. RNA was extracted using the acid guanidinium thiocyanate extraction method. Autoradiographs were analyzed quantitatively by densitometry and normalized for loading differences to corresponding actin mRNA. The PC2/β-actin, PC3/β-actin, preproinsulin/β-actin, and 7B2/β-actin ratios are expressed as means ± SE (n = 3).

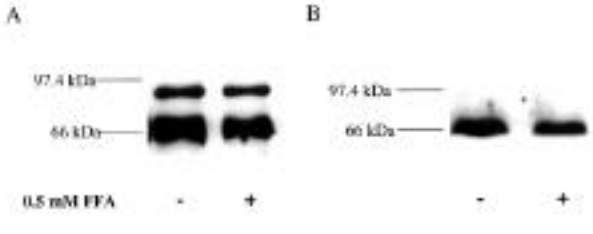

FIG. 5. Western blot analysis of PC2, PC3 in cultured MIN6 cells. MIN6 cells were cultured in DMEM with or without 0.5 mmol/l FFAs for 7 days. Cells were homogenized in 0.275 mol/l sucrose and protein measured in aliquots by the Bradford method. Aliquots of similar protein content were analyzed by polyacrylamide gel electrophoresis followed by enhanced chemiluminescence detection reagents and densitometric scanning. *A*: PC2, *B*: PC3 protein bands. A representative blot from three replicate experiments is shown.

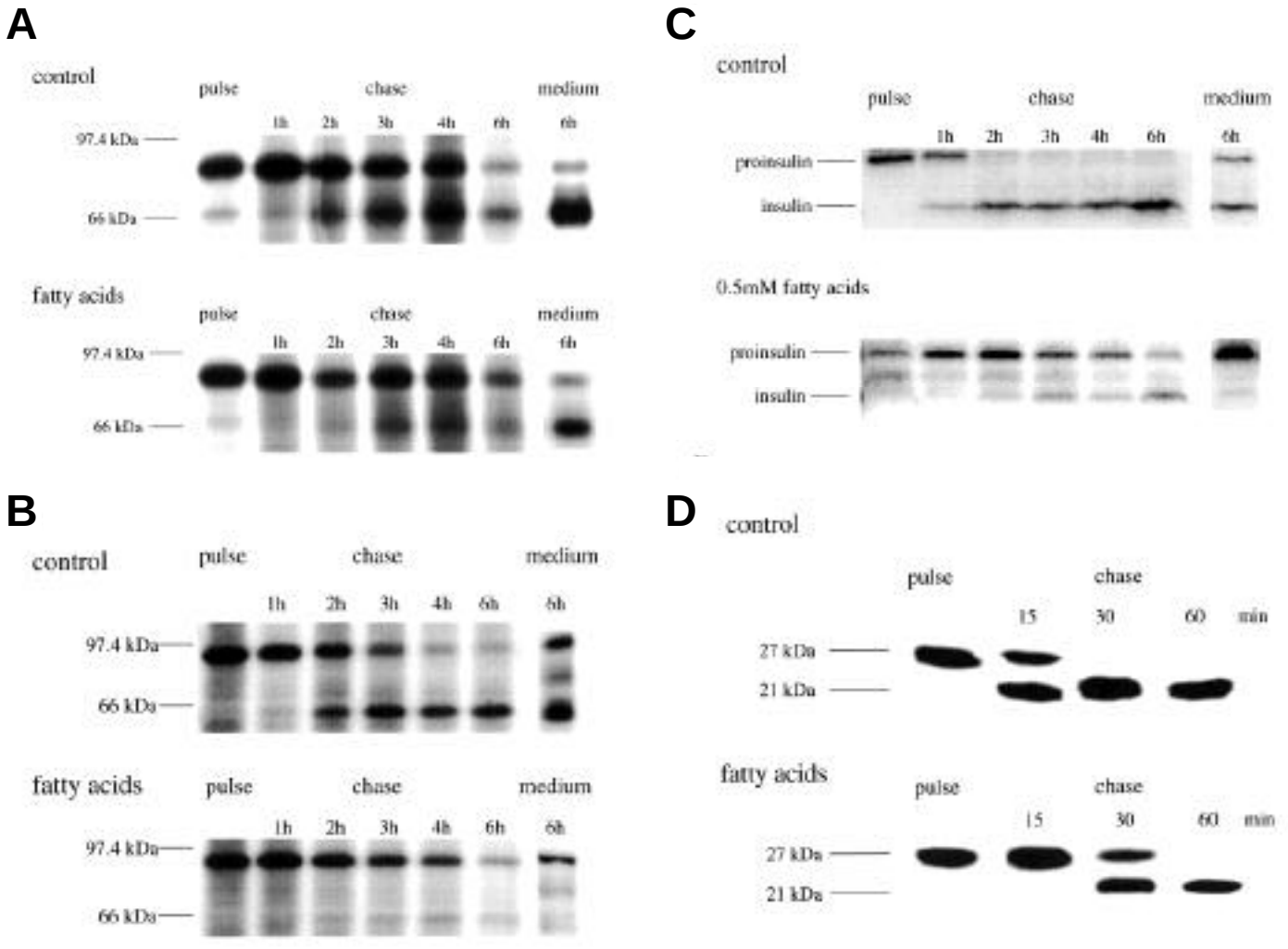

FIG. 6. Effect of 7 days' exposure to FFAs on PC2, PC3, insulin, and 7B2 biosynthesis. Control cells and MIN6 cells exposed to FFAs for 7 days were preincubated in methionine-free DMEM containing 4.5 mg/ml glucose and 1 mg/ml BSA for 60 min, then labeled for 15 min in 0.5 mCi/ml [^{35}S]Met, 4.5 mg/ml glucose, and 1 mg/ml BSA. Some of the plates were further incubated in DMEM with unlabeled methionine containing 3.0 mg/ml glucose and 1 mg/ml BSA for different time periods. The media and cell lysates were subjected to serial immunoprecipitations with selected antisera against PC2, PC3, insulin, and 7B2. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis followed by fluorography and densitometric scanning. *A*: PC2; *B*: PC3; *C*: insulin; *D*: 7B2 biosynthetic studies.

DISCUSSION

Plasma FFAs are elevated in most obese and type 2 diabetic subjects (16–19). Also, the existence of disproportionate hyperproinsulinemia in type 2 diabetes is well established (21–27). To explore whether prolonged elevations of FFAs may induce increased levels of proinsulin, we have analyzed the biosynthesis and processing of proinsulin, PC2, and PC3 in cultured MIN6 cells. We chose the MIN6 cell line as a model to investigate this issue because it has more characteristics that are similar to isolated islets than other available cell lines (31) and because it displays coordinate regulation of the biosynthesis of PC2, PC3, and proinsulin by glucose at the translational level (32).

The present study shows that long-term elevation of FFAs led to decreased insulin content and secretion (9–11,15), as well as markedly increased proinsulin secretion. Such an effect on proinsulin secretion has not previously been documented. Clinically, elevated levels of FFAs and disproportionate hyperproinsulinemia often accompany type 2 diabetes. Our results are thus consistent with clinical findings. However, FFAs did not affect the secretion of proinsulin via the constitutive (unregulated) pathway. Thus, our findings indicate that one effect of FFAs may be to increase immature granules intracellularly, resulting in the release of larger amounts of incompletely processed materials.

Several studies have been reported on the metabolic effects of chronically elevated FFAs on GSIS. These have indicated that FFAs induce an increase in the activity of hexokinase (12), inhibition of pyruvate dehydrogenase activity (13,14), and depletion of protein kinase C (15). However, it is unlikely that these metabolic effects can explain the increased proinsulin accumulation and secretion without conversion.

The conversion of proinsulin to insulin in secretory granules requires the two Ca^{2+} -dependent prohormone convertases, PC2 and PC3. Normally, PC2 and PC3 mRNA levels in the β-cells coordinately increase with proinsulin mRNA (43). Also, both short- and long-term exposure to elevated glucose greatly augments proinsulin biosynthesis at the level of translation (44,45). Similar translational effects have been noted for PC2 and PC3 biosynthesis in isolated islets (33). Our

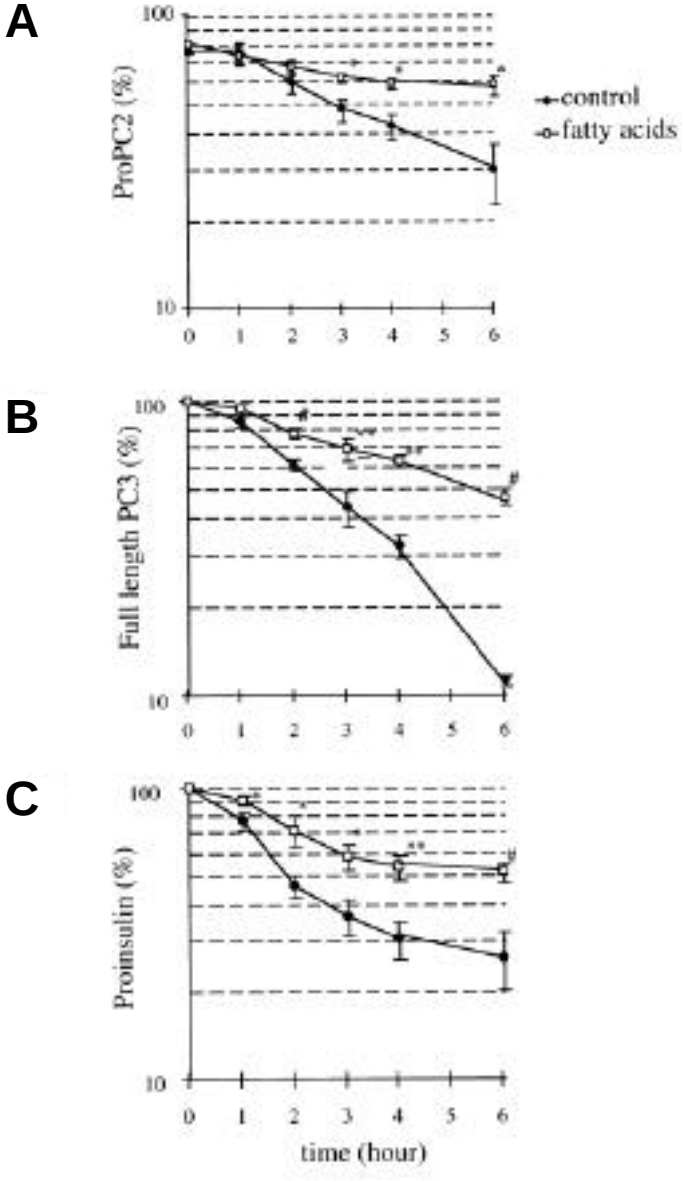

FIG. 7. Semilog plots of rates of conversion of precursors of PC2, PC3, and proinsulin. Control cells and MIN6 cells exposed to FFAs for 7 days were preincubated in methionine-free DMEM, then subjected to immunoprecipitation as in Fig. 6. *A*: The conversion of proPC2 to mature PC2; *B*: the conversion of full-length PC3 to 66 kDa PC3; *C*: the conversion of proinsulin to insulin. Data are expressed as means ± SE (n = 4). *P < 0.05, **P < 0.01, #P < 0.005 vs. control.

results indicate that FFAs do not affect preproinsulin, PC2, PC3, or 7B2 mRNA content. A previous study (39) showed that islets cultured with 0.125 mmol/l oleate for 24 h contained increased preproinsulin mRNA levels; however, in that study, the islets were cultured under normoglycemic conditions and for a much shorter time period than in our study.

The amounts of PC2 and PC3 protein assessed by Western blotting were decreased by 23 and 15%, respectively, in FFA-exposed cells. Whether these modest decreases resulted from reduced rates of biosynthesis of the convertases or reflect changes in stored enzyme in secretory granules is unclear. However, our data on the processing of proinsulin and of the prohormone convertases in cultured MIN6 cells indicate that long-term exposure to FFAs leads to delayed processing of proinsulin, as well as of the precursors of PC2, PC3, and 7B2. These findings provide a plausible causative link to the hyperproinsulinemia of diabetes. Previous studies have demonstrated that the processing of proinsulin, PC2, and PC3 are dependent on the secretory granule Ca^{2+} level (46) and require conditions of moderately acidic pH (47). Recent work in isolated inside-out patches of *ob*/*ob* islet cells has shown that long-chain acyl-CoA affects ATP-sensitive K^+ channel activity. The ability of oleoyl-CoA to open the channel was seen in the presence of ATP and ADP (48). Long-term exposure to FFAs may thus interfere in the depolarization of the plasma membrane and the opening of voltage-dependent calcium channels, resulting in reduction of cytoplasmic and granular free calcium and leading to inhibition of the processing of proinsulin and other granule constituents, including the precursors of the convertases, PC2 and PC3, as well as the PC2 helper protein 7B2. Another interesting possibility may be that FFAs may increase the pH in secretory granules through uncoupling of the pH gradient, as they uncouple proton transport in mitochondria (N. Welsh, personal communication). Alternatively, the reduced insulin content in treated cells may reflect more rapid granule turnover with an attendant shift toward immaturity in all the granule components.

We observed that, during long-term exposure of MIN6 cells, the cell number was decreased by 25% compared with controls, and we saw some ballooning of the cells and accumulation of fat droplets in the cytoplasm by phase microscopy. We suggest that intracellular fat accumulation may also influence membrane composition with functional consequences in various subcellular compartments. Previous studies have shown that elevation of triglyceride content in islets coincides precisely with the onset of hyperglycemia in prediabetic ZDF rat islets (49). Triglyceride stores may be elevated in ZDF rat islets in part because of a genetic defect in the leptin receptor, which underlies the diabetic phenotype of the ZDF rat. Leptin has been shown to regulate intracellular fat metabolism (50). Exposure to FFAs may induce islet changes similar to those of the leptin receptor defect. Further effects of FFAs may include the induced expression of an isoform of nitric oxide synthase and the accumulation of nitric oxide in the β-cells, leading to increased rates of apoptosis (51). Such a mechanism could explain the increased numbers of detached cells and decrease in total cell number in FFA-exposed MIN6 cells.

In conclusion, we suggest that long-term exposure to FFAs may inhibit secretory granule maturation through a variety of mechanisms and thereby contribute to the hyperproinsulinemia of diabetes.

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