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Free Fatty Acid-Induced Inhibition of Glucose and Insulin-Like Growth Factor I-Induced Deoxyribonucleic Acid Synthesis in the Pancreatic β-Cell Line INS-1*

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ABSTRACT

Pancreatic β-cell mitogenesis is increased by insulin-like growth factor I (IGF-I) in a glucose-dependent manner. In this study it was found that alternative β-cell nutrient fuels to glucose, pyruvate, and glutamine/leucine independently induced and provided a platform for IGF-I to induce INS-1 cell DNA synthesis in the absence of serum. In contrast, long chain FFA ($\geq C_{12}$) inhibited 15 mM glucose-induced [^{3}H]thymidine incorporation (±10 nM IGF-I) by 95% or more within 24 h above 0.2 mM FFA complexed to 1% BSA ($K_{0.5}$ for palmitate/1% BSA = 65–85 μM for 24 h; $t_{0.5}$ for 0.2 mM palmitate/1% BSA = ~6 h). FFA-mediated inhibition of glucose/IGF-I-induced β-cell DNA synthesis was reversible, and FFA oxidation did not appear to be required, nor did FFA interfere with glucose metabolism in INS-1 cells. An examination of mitogenic signal transduction pathways in INS-1 cells revealed that glucose/IGF-I induction of early signaling elements in SH2-containing protein (Shc)- and insulin receptor substrate-1/2-mediated pathways leading to downstream mitogen-activated protein kinase and phosphoinositol 3′-kinase activation, were unaffected by FFA. However, glucose-/IGF-I-induced activation of protein kinase B (PKB) was significantly inhibited, and protein kinase Cζ was chronically activated by FFA. It is possible that FFA-mediated inhibition of β-cell mitogenesis contributes to the reduction of β-cell mass and the subsequent failure to compensate for peripheral insulin resistance *in vivo* that is key to the pathogenesis of obesity-linked diabetes. (*Endocrinology* **142:** 229–240, 2001)

PANCREATIC β-CELL proliferation can be increased by several growth factors and certain nutrients (1, 2). However, it has not been until relatively recently that intracellular mitogenic signal transduction pathways, downstream of a growth factor receptor, have been investigated in pancreatic β-cells (3, 4). Insulin-like growth factor I (IGF-I), via activation of the intrinsic tyrosine kinase activity of the IGF-I receptor, tyrosine phosphorylates SH2-containing protein (SH2), insulin receptor substrate-1 (IRS-1), and IRS-2, resulting in downstream activation of mitogen-activated protein kinase [MAPK; extracellular regulated kinase-1 (Erk-1) and -2 isoforms], phosphatidylinositol 3′-kinase (PI3′K), and $p70^{S6K}$ (5). GH predominately signals via Janus kinase-2 (JAK2)/signal transducer and activator of transcription-5 (STAT5) activation, and in pancreatic β-cells there is no cross-talk to IRS-mediated signal transduction pathways (4). Unusually, and probably somewhat unique to the pancreatic β-cell, both GH and IGF-I are dependent on glucose being present in the physiologically relevant range (6–18 mM) to exert a mitogenic effect (3, 4). Intriguingly, glucose (>6 mM) is capable of independently activating IRS-1/2-mediated signal transduction (3, 4), but not JAK2/STAT5 signaling, despite GH-induced β-cell DNA synthesis being glucose dependent (4). However, the means by which glucose provides a permissive platform for IGF-I- and GH-induced β-cell mitogenesis is essentially unknown, other than that glucose metabolism is necessary (3, 4). In part this study examines whether other nutrient fuels, alternative to glucose, can provide a similar permissive basis for IGF-I- and GH-induced β-cell mitogenesis.

In states of peripheral insulin resistance, such as obesity, an increased demand for insulin can be compensated for by an increase in β-cell mass and relatively increased insulin production (6, 7). However, when pancreatic β-cell mass does not increase, there is a failure to compensate for the insulin resistance, and obesity-linked type II diabetes ensues (8). Thus, control of β-cell growth is a key factor contributing to the pathogenesis of type II diabetes. IRS-mediated signal transduction is important for glucose- and glucose-dependent growth factor-induced β-cell DNA synthesis, especially that via IRS-2 and PI3′K (3, 9). Moreover, it has been demonstrated that IRS-mediated mitogenic signal transduction pathways in the β-cell could play an important role in the pathogenesis of type II diabetes (10, 11). In the IRS-1 knockout mouse there is peripheral insulin resistance that is compensated for by an increase in β-cell mass, and there is no diabetes (10). However, in the IRS-2 knockout mouse the β-cell mass is markedly reduced, and in the face of additional peripheral insulin resistance, type II diabetes ensues (10, 11). Although there are likely genetic susceptibilities involved in the pathogenesis of type II diabetes (12), it appears that alterations in the IRS-2 primary gene structure do not contribute (13). However, this does not rule out that adverse

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biochemical affects in IRS-2 function could lead to a diabetic state. As such, an unresolved question is what are the pathophysiologically relevant factors that prevent an increase and/or decrease β-cell mass in type II diabetes so that there is no longer compensation for peripheral insulin resistance. It is thought that both prolonged hyperglycemia and hyperlipidemia are such contributing factors, and they can be detrimental to β-cell function and mass (6–8, 14).

In general, glucose has a tendency to increase β-cell mitogenesis (1, 2). In this study an examination of alternative nutrient fuels that might increase β-cell growth similar to the effect of glucose found that long chain FFA inhibited glucose-induced and glucose-dependent IGF-I-induced β-cell DNA synthesis. This in part was attributed to interference by FFAs in IRS-mediated signal transduction pathways downstream of PI3′-K. It is postulated that exposure to FFA leads to a reduction in β-cell mitogenesis as well as peripheral insulin resistance (7, 15, 16) that would contribute to decreased β-cell mass *in vivo* and presentation of type II diabetes.

Materials and Methods

Materials

[*methyl*-^{3}H]Thymidine (20 Ci/mmol) was obatined from NEN Life Science Products (Boston, MA), and [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Anti-active phospho-MAPK (Erk1/2), phospho-p38, phospho-c-Jun N-terminal kinase-1/2 (JNK1/2), as well as anti-total MAPK (Erk1/2), p38, and JNK1/2 antisera were purchased from Promega Corp. (Madison, WI). The IRS-1- and IRS-2 antisera were generated as previously described (17). The p70^{S6K} antisera were generated and used in immunoblot analysis as previously described (18). Anti-PI3′K p85, protein kinase B (PKB), mammalian Son of Sevenless (mSOS), growth factor bound protein 2 (Grb2), Shc, and antiphosphotyrosine antisera were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-protein kinase Cζ (anti-PKCζ) antisera was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The PKB activity assay kit was purchased from New England Biolabs, Inc. (Beverly, MA), and [Ser^{159}]PKCε-(153–164)-NH_2 PKC peptide substrate was obtained from Upstate Biotechnology, Inc. L-α-Phosphatidylserine and L-α-phosphatidylinositol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), 2-bromopalmitate was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI), methyl-palmitate was purchased from Sigma (St. Louis, MO), and all other FFA were purchased from Alltech (Deerfield, IL). FFA-free BSA was obtained from Roche Molecular Biochemicals (Indianapolis, IN), and C2 ceramide, *N*-acetyl-D-erythro-sphingosine, dihydro-*N*-acetyl-D-erythro-sphingosine, as well as IGF-I were purchased from Calbiochem-Novabiochem (La Jolla, CA). Transblot nitrocellulose membrane (0.2-μm pore size) were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). The immunoblot chemiluminescence detection kit was obtained from NEN Life Science Products (Boston, MA). Unless otherwise stated all other biochemicals were purchased from either Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were of the highest purity available.

Cell culture

The glucose-sensitive pancreatic β-cell line, INS-1 (19), was used in the experiments. INS-1 cells were maintained in RPMI 1640 medium containing 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS, and 11.2 mM glucose and incubated at 37 C in 5% CO_2 as previously described (19). Cells were subcultured at 80% confluence.

FFA/BSA complex solution preparation

A 100-mM FFA stock solution was prepared in 0.1 M NaOH by heating at 70 C in a shaking water bath. In an adjacent water bath at 55 C, a 10% (wt/vol) FFA-free BSA solution was prepared in H_2O. Altering the proportion of these two solutions when mixed together varied the concentration of FFA complexed to BSA. For example, a 5-mM FFA/10% BSA stock solution was prepared by adding 50 μl of the 100 mM FFA solution dropwise to 950 μl 10% BSA solution at 55 C in a shaking water bath, then vortex mixed for 10 sec followed by a further 10-min incubation at 55 C. The FFA/BSA complexed solution is cooled to room temperature and sterile filtered (0.45-μm pore size membrane filter). It can be stored at −20 C, where it is stable for 3–4 weeks. Stored 5-mM FFA/10% BSA stock solutions are heated for 15 min at 55 C, then cooled to room temperature before use.

[^{3}H]Thymidine incorporation

Incorporation of [^{3}H]thymidine was used as an indicator of DNA synthesis and INS-1 cell mitogenesis (20). A 96-well plate [^{3}H]thymidine assay was used as previously described (3, 4). Essentially, INS-1 cells were cultured on 96-well plates and incubated at 37 C in INS-1 medium to a density of approximately 2–5 × 10^4 cells/well. The medium was removed, and the cells were made quiescent by serum and glucose deprivation for 24 h in RPMI 1640 containing 0.1% BSA instead of serum and 0.5 mM glucose. The INS-1 cells were then incubated for a further 24 h in RPMI 1640 and 0.1% BSA at different glucose concentrations (0–24 mM glucose) with or without IGF-I (10 nM) and FFA (0.02–0.5 mM) complexed to 1% BSA. The last 4 h of this latter incubation period was carried out in the additional presence of 5 μCi [^{3}H]thymidine/ml to monitor the degree of DNA synthesis and assess the β-cell mitogenesis rate. After this final incubation period, the cells were centrifuged (3000 × *g*, 10 min, 4 C: Sorvall RT7 centrifuge (DuPont Medical Products, Newtown, CT) and RT750 rotor and 96-well plate holders), washed, and lysed using a semiautomatic cell harvester (Packard Instrumentation, Meriden, CT), and the cell lysates were transferred to Whatman glass-fiber micropore filters (Clifton, NJ). The [^{3}H]thymidine specifically incorporated into the INS-1 cell DNA trapped on glass-fiber filters was counted by liquid scintillation counting.

Protein immunoblot and coimmunoprecipitation analysis

INS-1 cells were subcultured on 10-cm plates to about 70% confluence as previously described (19). The cells were then incubated for a 24-h period of quiescence by serum and glucose deprivation in RPMI 1640 medium containing 0.1% BSA instead of serum and 0.5 mM glucose with or without 0.2–0.4 mM FFA complexed to 1% BSA. INS-1 cells were then incubated in fresh RPMI 1640 medium containing 0, 3, or 15 mM glucose with or without 10 nM IGF-I and 0.2–0.4 mM FFA/1% BSA for between 5 and 30 min as indicated. The cells were lysed in 0.5 ml ice-cold lysis as previously described (3, 4). Immunoprecipitation and immunoblot analysis of mitogenic signal transduction protein expression and protein tyrosine phosphorylation were performed as previously described (3, 4).

PI3′K assay

PI3′K was assayed as previously described (21, 22). Essentially, INS-1 cells were incubated at 0, 3, or 15 mM glucose with or without 10 nM IGF-I and 0.4 mM FFA/1% BSA for 15 min, then PI3′K was immunoprecipitated from 750 μg total protein of cell lysate with an anti-PI3′K p85 antibody. The immunoprecipitates were washed twice in 300 μl PBS (pH 7.4), 1% Nonidet P-40, and inhibitor cocktail (containing 1 mM Na_3VO_4, 20 mM NaF, 10 mM sodium pyrophosphate, 1 mM EDTA, 10 μM leupeptin, 10 μg/ml aprotinin, and 100 μM phenylmethylsulfonylfluoride); twice with 300 μl 0.1 M Tris-HCl (pH 7.5), 0.5 M LiCl, and inhibitor cocktail; and finally twice with 300 μl 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and inhibitor cocktail. Immunoprecipitates were then suspended in 70 μl 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 100 μM Na_3VO_4, and 100 μM 15 mM $MgCl_2$ containing 20 μg L-α- phosphatidylinositol. The assay was initiated by addition of 5 μl 10 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, and 1 mM ATP containing 20 μCi [γ-^{32}P]ATP and incubated for 10 min at 30 C in a shaking water bath. The reaction was stopped by addition 20 μl 8 M HCl, and the phospholipids were chloroform/methanol (1:1) extracted. [^{32}P]Phospholipids were then separated by TLC and detected by autoradiography as previously described (21, 22). Quantification of the phosphatidylinositol-3′-[^{32}P]phosphate produced was determined by densitometric scanning of the autoradiographs.

PKB assay

PKB activity was determined using a kit provided by New England Biolabs, Inc. Essentially, INS-1 cells were incubated as described for the

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PI3′-K assay described above. PKB was immunoprecipitated from 1 mg total protein equivalent of cell lysate with anti-PKB antibody linked to agarose beads overnight at 4 C with rotary mixing. Agarose beads were then pelleted by centrifugation (10,000 × *g*, 30 sec, 4 C), and the supernatant was removed. Immunoprecipitates were then washed twice in 500 μl ice-cold lysis buffer, then twice with 500 μl ice-cold PKB assay buffer consisting of 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 100 μM mM Na_3VO_4, and 10 mM $MgCl_2$ containing 20 μg L-α-phosphatidylinositol. PKB immunoprecipitates were suspended in 40 μl assay buffer additionally containing 200 μM ATP and 1 μg glycogen synthase-kinase (GSK)-3α/β fusion protein (containing the $Ser^{21/9}$ phosphorylation site), then incubated for 20 min at 30° C in a shaking water bath. The reaction was stopped by the addition of 20 μl 3 × SDS-electrophoresis sample buffer and then heated at 95 C for 5 min. The sample was run on SDS-PAGE, and the extent of GSK3 phosphorylation was analyzed with a specific phospho-GSK-3 antibody.

PKCζ assay

PKCζ activity was determined as previously described (23). Essentially, INS-1 cells were incubated as described for the PI3′K assay described above. PKCζ was then immunoprecipitated from 750 μg total protein equivalent of lysate (in a 500-μl final volume) with an anti-PKCζ antibody, followed by protein A-Sepharose as described previously (3, 4). Immunoprecipitates were then washed twice in 500 μl ice-cold lysis buffer, then twice with 500 μl ice-cold assay buffer consisting of 20 mM HEPES (pH 7.5), 1 mM Na_3VO_4, 20 mM NaF, 10 mM sodium pyrophosphate, 1 mM EDTA, 10 μM leupeptin, 10 μg/ml aprotinin, and 100 μM phenylmethylsulfonylfluoride. PKCζ immunoprecipitates were then suspended in 50 μl of the assay buffer, additionally containing 40 μM [Ser^{159}]PKC-ε-(153–164)-NH_2 peptide substrate, 50 μM ATP, and 40 μg/ml phosphatidylserine. The reaction was started by the addition of 5 μCi [γ-^{32}P]ATP and was incubated for 10 min at 30 C in a shaking water bath. A 25-μl aliquot of reaction mixture was then spotted onto a phosphocellulose p81 filter (Whatman) presoaked in 10% (vol/vol) acetic acid to trap the [Ser^{159}]PKC-ε-(153–164)-NH_2 peptide substrate. The filters were immediately extensively washed in 10% acetic acid by flow-through on a sample manifold apparatus (Millipore Corp., Bedford, MA), followed by repeated washes for an additional 10 min. The filters were then air-dried. Quantification of [^{32}P]Ser^{159}PKC-ε-(153–164)-NH_2 peptide was performed by liquid scintillation counting.

Other procedures

Protein assay was by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL). Data are presented as the mean ± SE, with the number of individual observations indicated (n). Statistically significant differences between groups were analyzed using Student's *t* test, where $P < 0.05$ was considered statistically significant.

Results

Alternative nutrient fuels stimulate β-cell DNA synthesis

The effects of pyruvate, leucine, and α-ketoisocaproate, all established nutrient fuels of pancreatic β-cells (24), on INS-1 cell DNA synthesis (with or without 10 nM IGF-I) was determined by [^{3}H]thymidine incorporation. INS-1 cells were chosen as a model to examine β-cell mitogenesis, because they respond to glucose in the physiologically relevant range (6–18 mM glucose) (3, 4, 19). A 15-mM glucose concentration significantly increased [^{3}H]thymidine incorporation in INS-1 cells between 20- to 25-fold above baseline ($P \leq 0.001$; Figs. 1-3). In the absence of glucose, IGF-I (10 nM) had no effect on INS-1 cell DNA synthesis and had a modest 3- to 4-fold increase at 3 mM glucose ($P \leq 0.05$), but had a marked significant 50- to 60-fold increase at a stimulatory 15 mM glucose (Figs. 1–3; $P \leq 0.001$), as previously observed (3, 4). The [^{3}H]thymidine incorporation assay was used as a marker of INS-1 cell mitogenesis. Cell counting revealed that a 20-fold increase in [^{3}H]thymidine incorporation in a 24-h period, as instigated by 15 mM glucose, was equivalent to a

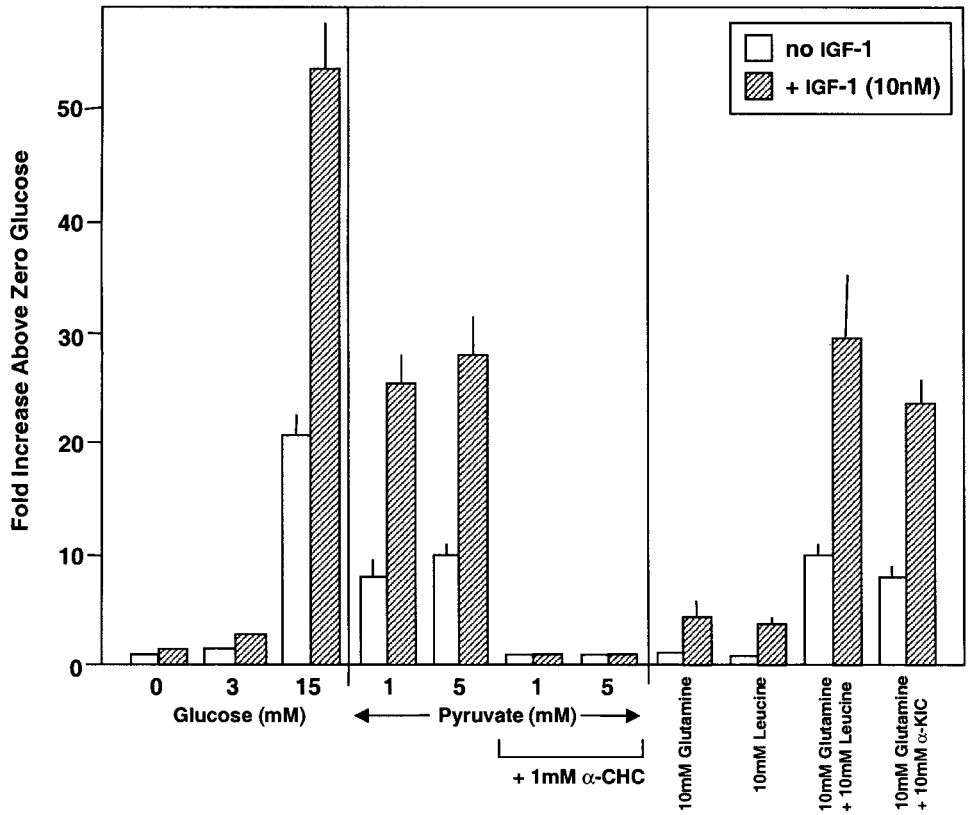

FIG. 1. Alternative metabolic fuels can stimulate DNA synthesis in INS-1 cells. DNA synthesis was determined at 0, 3, or 15 mM glucose with or without 10 nM IGF-I (as controls) or in the presence of alternative fuels [including 1 and 5 mM pyruvate with or without 1 mM α-cyano-4-hydroxycyanocinnamate (α-CHC), 10 mM glutamine, 10 mM leucine, 10 mM glutamine and 10 mM leucine, or 10 mM α-ketoisocaproate (α-KIC)] using a [^{3}H]thymidine incorporation assay as described in *Materials and Methods*. All experiments were performed in triplicate on at least six independent occasions. The data are expressed as the fold increase above the control observation in the absence of glucose and IGF-I and are depicted as the mean ± SE (n = 6).

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2-fold increase in cell number. A 50-fold in crease in [^{3}H]thymidine incorporation within 24 h, as stimulated by 15 mM glucose and 10 nM IGF-I increased cell number 3- to 4-fold.

Pyruvate (1 or 5 mM) independently increased INS-1 cell [^{3}H]thymidine incorporation between 8- to 10-fold ($P \leq 0.01$), and between 25- to 28-fold in the added presence of 10 nM IGF-I ($P \leq 0.001$; Fig. 1). The addition of 1 mM α-cyano-4-hydroxycyanocinnamate, which prevents pyruvate transport into β-cells and mitochondria (25, 26), prevented the pyruvate-induced increase in INS-1 cell DNA synthesis with or without IGF-I (Fig. 1). Glutamine (10 mM) and leucine (10 mM) had no independent effect on cell [^{3}H]thymidine incorporation into INS-1 cells, but in the added presence of 10 nM IGF-I there was a 3- to 4-fold increase in INS-1 cell DNA synthesis ($P \leq 0.05$; Fig. 1). However, when 10 mM leucine or 10 mM ketoisocaproate (a deaminated product of leucine) was combined with 10 mM glutamine (to activate glutamate dehydrogenase and increase intramitochondrial α-ketoglutarate) (26), a significant increase in INS-1 cell [^{3}H]thymidine incorporation was observed (8- to 10-fold; $P \leq 0.05$) that further increased 25- to 30-fold with 10 nM IGF-I added ($P \leq 0.01$; Fig. 1).

Long chain FFA inhibit glucose- and IGF-I-induced β-cell DNA synthesis

It was examined whether FFAs would affect β-cell mitogenesis and/or provide a platform for growth factors to evoke a mitogenic response as previously observed (3, 4). It was found that in the absence of glucose or at glucose concentrations of 5 mM or more, long chain FFA ($>C_{12}$) tended to slightly increase INS-1 cell [^{3}H]thymidine incorporation approximately 2-fold, but this was not statistically significant (data not shown). In contrast, it was found that FFAs inhibited glucose/IGF-I-induced INS-1 cell DNA synthesis (Fig. 2). FFAs of chain length C_8 to C_{18} [0.4 mM complexed to 1% (wt/vol) BSA] were incubated with INS-1 cells for 24 h at 15 mM glucose with or without 10 nM IGF-I. Linoleate ($C_{18:2}$), oleate ($C_{18:1}$), stearate ($C_{18:0}$), palmitate ($C_{16:0}$), myristate ($C_{14:0}$), laurate ($C_{12:0}$) significantly inhibited glucose-induced and glucose-dependent IGF-I-induced β-cell DNA synthesis by 95% or more ($P \leq 0.001$) compared with cells incubated in the absence of FFA (Fig. 2A). Caproate (C_{10}) also inhibited 15 mM glucose/IGF-I induced INS-1 cell [^{3}H]thymidine incorporation, but to a lesser extent (~80%; $P \leq 0.005$; Fig. 2). Capryloate (C_8) partially inhibited 15 mM glucose-induced INS-1 cell [^{3}H]thymidine incorporation (38.5 ± 3.3%; n = 5; $P \leq 0.05$), but had a negligible affect on glucose-dependent IGF-I induced β-cell DNA synthesis (20.3 ± 3.4%; n = 5; P = NS; Fig. 2).

In further experiments palmitate was used as a model FFA. Palmitate significantly inhibited β-cell DNA synthesis at 15 mM glucose with or without 10 nM IGF-I by 95% or more at a concentration of 0.2 mM or more complexed 1% BSA ($P \leq 0.001$; Fig. 3A). Titration inhibition curves indicated a K_{d50} of 86.0 ± 9.4 μM palmitate (complexed 1% BSA; n = 5) for inhibition of 15 mM glucose-induced INS-1 cell DNA synthesis and a K_{d50} of 64.3 ± 7.7 μM palmitate (complexed to 1% BSA; n = 5) for inhibition of 15 mM glucose-dependent

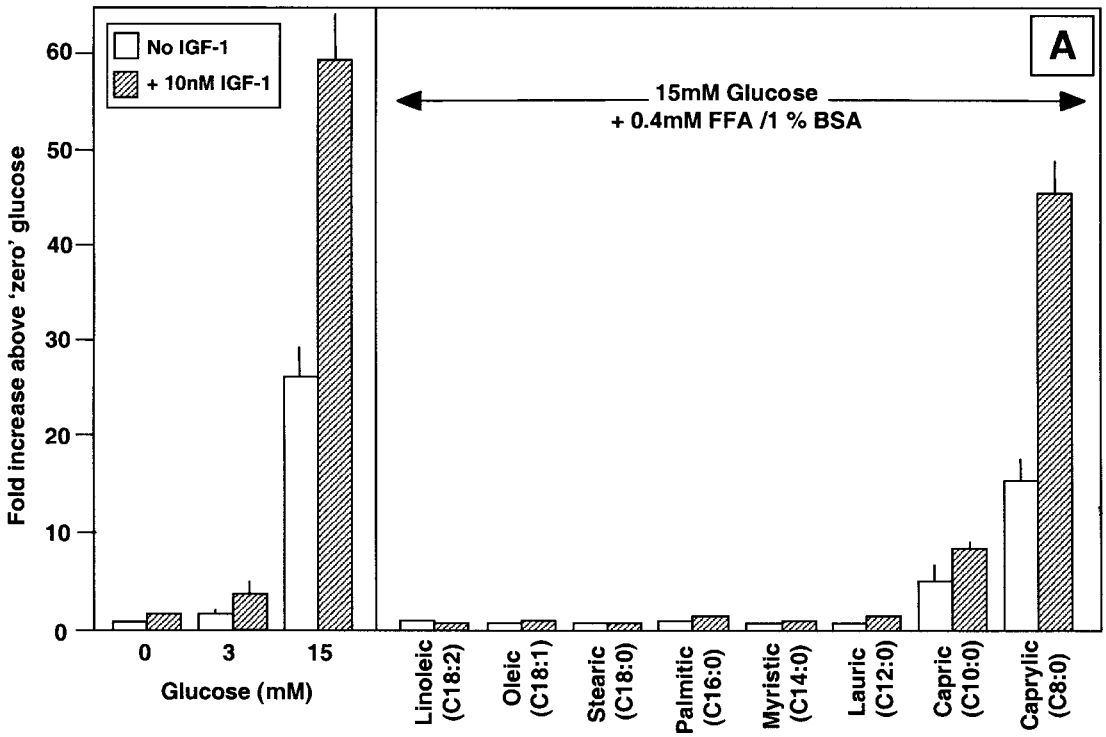

FIG. 2. Long chain FFA inhibit glucose- and IGF-I induced DNA synthesis in INS-1 cells. DNA synthesis was determined at 0, 3, or 15 mM glucose with or without 10 nM IGF-I (as controls) or at 15 mM glucose with or without 10 nM IGF-I in the additional presence of various acyl chain length FFAs (0.4 mM FFA complexed to 1% BSA) using a [^{3}H]thymidine incorporation assay as described in *Materials and Methods*. All experiments were performed in triplicate on at least six independent occasions. The data are expressed as the fold increase above the control observation in the absence of glucose, IGF-I, and 0.2 mM palmitate/1% BSA and are depicted as the mean ± SE (n = 6).

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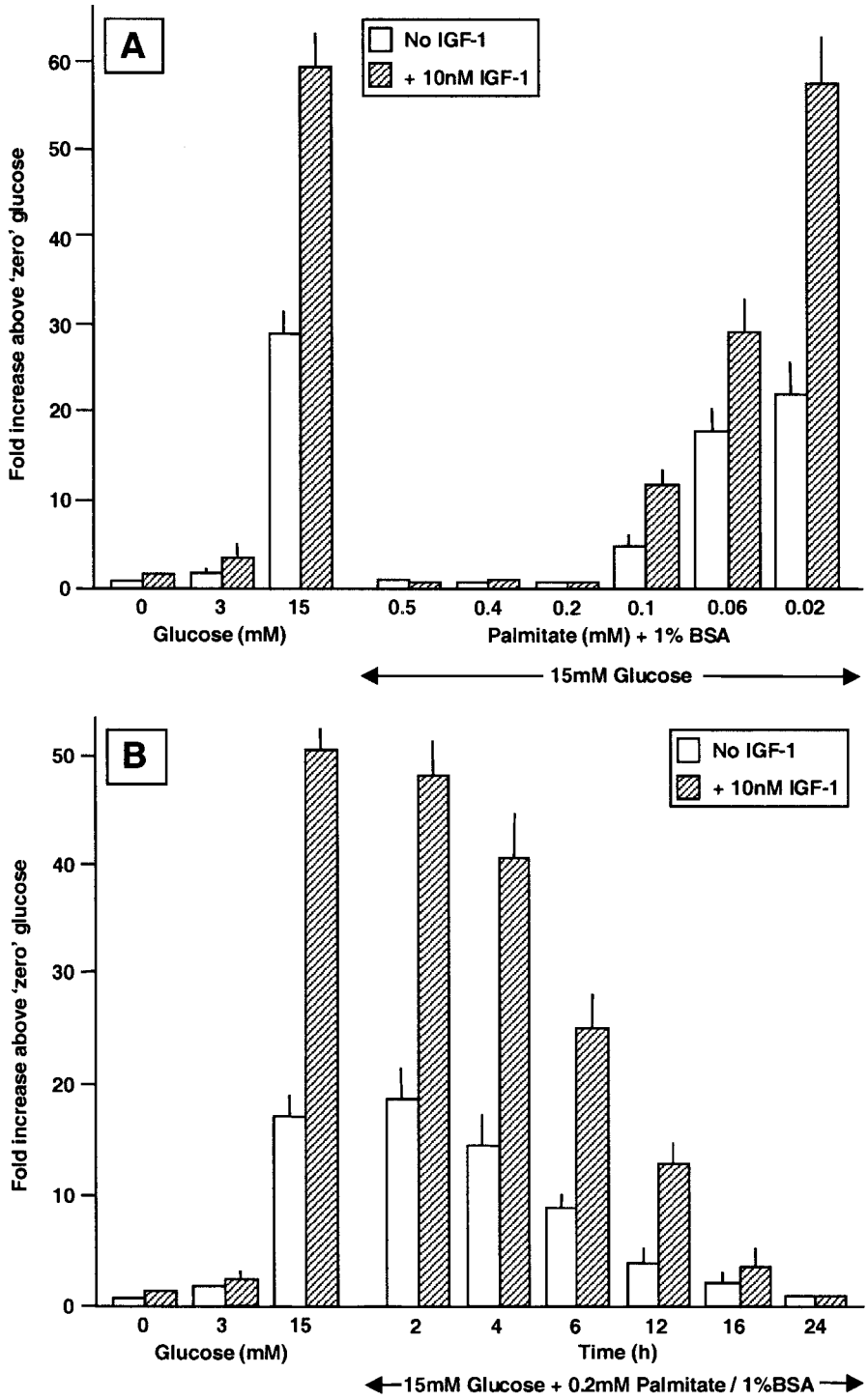

FIG. 3. Dose response and time course of palmitate-mediated inhibition of glucose- and IGF-I-induced DNA synthesis in INS-1 cells. DNA synthesis was determined at 15 mM glucose with or without 10 nM IGF-I over a 24-h period in the presence of various concentrations of palmitate (20–500 μM) complexed to a constant 1% BSA (A) or at 0.2 mM palmitate complexed to 1% BSA for various periods between 2–24 h (B), using a [^{3}H]thymidine incorporation assay essentially as described in *Materials and Methods*. To include shorter periods during the time course assay, the amount of [^{3}H]thymidine added was doubled in a shorter radiolabeling period of 2 h. All experiments were performed in triplicate on at least six independent occasions. The data are expressed as the fold increase above the control observation in the absence of glucose, IGF-I, and 0.2 mM palmitate/1% BSA and are depicted as the mean ± SE ($n \geq 6$).

IGF-I-induced INS-1 cell [^{3}H]thymidine incorporation (Fig. 3A). The time course for FFA-mediated inhibition of glucose-/IGF-I-induced INS-1 cell DNA synthesis was assessed using a slight modification of the 96-well plate [^{3}H]thymidine incorporation assay so as to assess early time points. Quiescent INS-1 cells were incubated in 15 mM glucose with or without 10 nM IGF-I in the added presence or absence of 0.2 mM palmitate complexed to 1% BSA for between 2–24 h, the last 2 h of which was in the presence of 10 μCi [^{3}H]thymidine/ml. For 0.2 mM palmitate inhibition of 15 mM glucose-induced β-cell DNA synthesis, $t_{0.5} = 6.3 \pm 0.4$ h, and for inhibition of 15 mM glucose-dependent IGF-I-induced β-cell DNA synthesis, $t_{0.5} = 5.8 \pm 0.9$ h. (Fig. 3B).

Palmitate inhibition of INS-1 cell DNA synthesis was reversible. If palmitate was removed from the medium after 24-h exposure to 0.2 mM palmitate/1% BSA and INS-1 cells, then further incubated in the absence of FFA, inhibition glucose/IGF-I-induced β-cell DNA synthesis recovered to 80% of that in control cells by 24 h and was fully recovered by 36 h (data not shown). Bromopalmitate (0.4 mM com-

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plexed to 1% BSA), an analog of palmitate that is not oxidized (26), significantly inhibited INS-1 cell [^{3}H]thymidine incorporation at 15 mM glucose with or without 10 nM IGF-I by 95% or more within 24 h ($P \leq 0.001$; data not shown), similar to that of palmitate (Figs. 2 and 3). This indicated that β-oxidation of FFA was not involved in FFA-induced inhibition of β-cell DNA synthesis. In contrast, methyl-palmitate (0.4 mM complexed to a constant 1% BSA), a modified form of palmitate that cannot form a coenzyme A (CoA) ester, only inhibited INS-1 cell [^{3}H]thymidine incorporation at 15 mM glucose with or without 10 nM IGF-I by 30% within 24 h (P = NS; data not shown). This latter observation suggested that formation of a fatty acyl-CoA moiety was necessary for FFA-mediated inhibition of glucose/IGF-I induced β-cell DNA synthesis. As ceramide can be derived from palmitoyl-CoA (27, 28), it was examined whether ceramide would affect glucose-induced and/or glucose-dependent IGF-I-induced INS-1 cell DNA synthesis in a similar fashion to that by FFA. However, addition of neither 10 μM C2-ceramide (*N*-acetyl-D-erythro-sphingosine) nor 10 μM C2-dihydroceramide (dihydro-*N*-acetyl-D-erythro-sphingosine; a biologically inactive analog of C2-ceramide) had any effect on glucose-induced or glucose-dependent IGF-I-induced INS-1 cell [^{3}H]thymidine incorporation.

FFA do not inhibit glucose or IGF-I activation of IRS-1/2 or Shc in INS-1 cells

Activation of IRS-mediated mitogenic signaling transduction pathways by glucose with or without IGF-I in INS-1 cells that were previously exposed to 0.4 mM palmitate/1% BSA (or 1% BSA alone as a control) for 24 h was investigated using coimmunoprecipitation and immunoblot analysis. Immunoprecipitation of the 85-kDa regulatory subunit of PI3′-K followed by immunoblot analysis for IRS-1 (Fig. 4A) or IRS-2 (Fig. 4B) revealed a significant increased association of PI3′-K with IRS-1 and IRS-2 instigated by stimulatory 15 mM glucose and IGF-I, similar to that previously observed (3, 4). However, palmitate had no adverse affect on gluocse/IGF-I-induced PI3′-K/IRS-1 or PI3′-K/IRS-2 interaction (Fig. 4, A

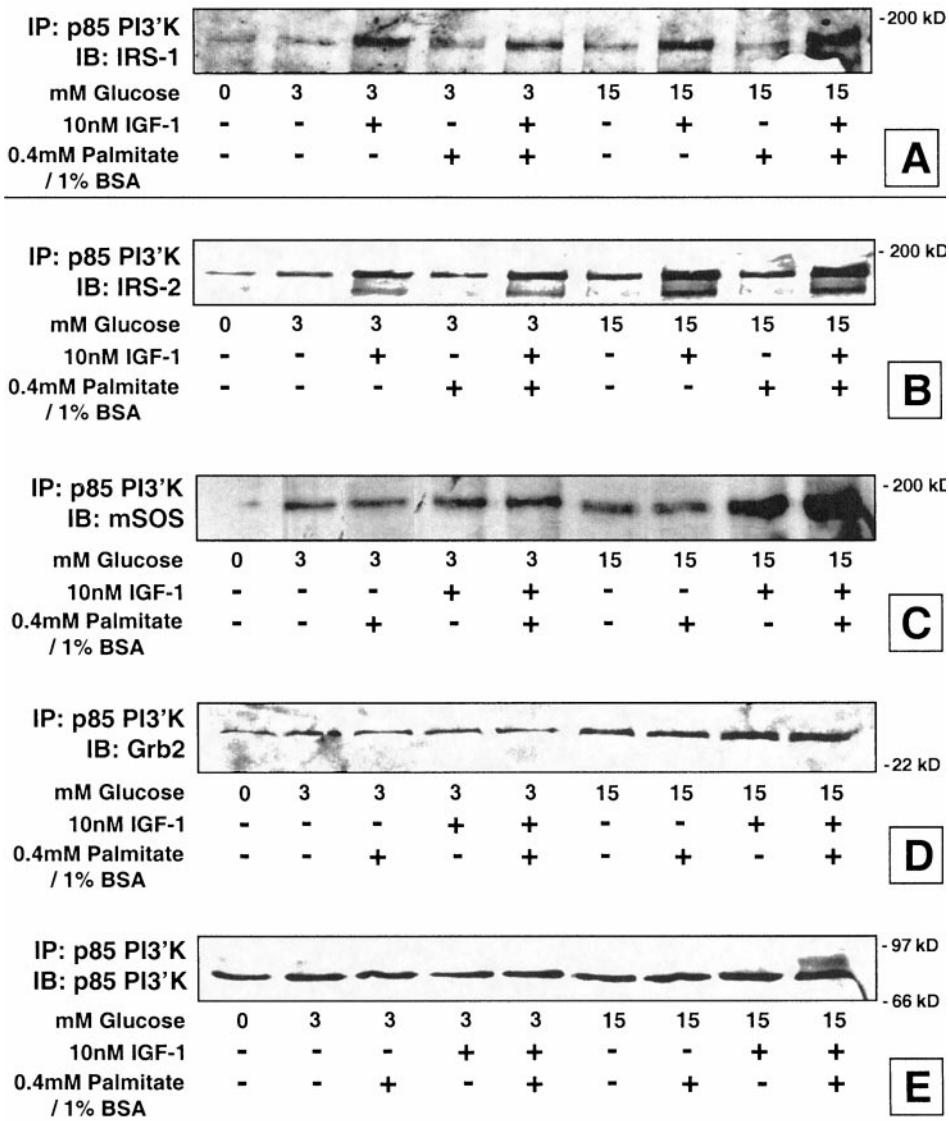

FIG. 4. Long chain FFA does not affect glucose- and IGF-I induced association of IRS-1/2 PI3′-K, Grb2, and mSOS. Recruitment of PI3′-K and Grb2/mSOS to an IRS-1/2 signaling complex was assessed by coimmunoprecipitation analysis as outlined in *Materials and Methods*. Essentially, INS-1 cells were then stimulated with 3 or 15 mM glucose with or without 10 nM IGF-I in the presence or absence 0.2 mM palmitate/1% BSA for 10 min, lysed, then subjected to immunoprecipitation (IP) with antiserum against the p85 regulatory subunit of PI3′K. Immunoprecipitates were then immunoblot (IB) analyzed with IRS-1 (A), IRS-2 (B), mSOS (C), mGrb2 (D), and p85 PI3′K (E) antibodies. An example blot for such coimmunoprecipitation analysis is shown.

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and B). Likewise, immunoblotting of the p85 PI3′-K immunoprecipitates with antiserum recognizing mSOS (Fig. 4C) or Grb2 (Fig. 4D) indicated an increased association of mSOS and Grb2 to a PI3′K/IRS signaling complex by 15 mM glucose, which was further increased in the presence of 10 nM IGF-I as previously described (3, 4). However, as for IRS-1/2 association with PI3′-K, palmitate had no adverse affect on glucose-induced or glucose-dependent IGF-I-induced association of Grb2/mSOS to the IRS signaling complex in β-cells (Fig. 4, C and D). The specific nature of this glucose/IGF-I-induced Grb2/mSOS, IRS-1, and IRS-2 interaction with PI3′-K was indicated, in that p85 PI3′-K immunoblot analysis of PI3′-K immunoprecipitates revealed that an equivalent amount of PI3′-K was present in each sample (Fig. 4E). In additional experiments, it was found that 15 mM glucose and IGF-I were able to significantly promote Shc association with Grb2/mSOS similar to that previously described (3, 4). However, addition of 0.4 mM palmitate/1% BSA for 24 h had no affect on the glucose/IGF-I-induced association of Grb2/mSOS to Shc (data not shown).

FFA do not inhibit glucose or IGF-I activation of MAPKs in INS-1 cells

The activation of mSOS, by its Grb2-mediated association with tyrosine phosphorylated IRS and/or Shc, results in downstream activation of Ras, which, in turn, stimulates Raf-1 serine kinase activity (5). Raf-1 then activates MEK, which, in turn, activates MAPK (Erk-1 and -2 isoforms) by serine phosphorylation (5). Activated MAPK can be detected with specific antiserum that only recognizes the phosphorylation-activated forms of Erk-1 and -2 (3, 4). Stimulatory 15 mM glucose independently increased Erk-1 and -2 phosphorylation activation above zero glucose, which was increased further in the presence of 10 nM IGF-I (Fig. 5A; $P \leq 0.01$). IGF-I also increased Erk-1 and -2 activation at a lower 3 mM glucose (Fig. 5A; $P \leq 0.05$). The glucose/IGF-I-induced phosphorylation activation of Erk-1 and -2 was unaffected by the presence of 0.4 mM palmitate/1% BSA (Fig. 5A). The total amount of Erk-1 and 2 in INS-1 cells, as ascertained by immunoblot analysis with antisera recognizing both active and inactive forms of Erk-1 and -2, was not significantly altered by glucose, IGF-I, or FFA treatment (Fig. 5A).

It was also examined whether FFA exerted an inhibitory effect on β-cell DNA synthesis via activation of stress kinase signaling pathways, which include other members of the MAPK family, such as p38 and JNK-1/-2 (29). As for Erk1 and -2, activation of p38 and JNK-1 and -2 can be detected with specific antiserum that specifically recognizes the phosphorylation-activated forms of these protein kinases (30). Phosphorylation activation of p38 was greatest in the absence

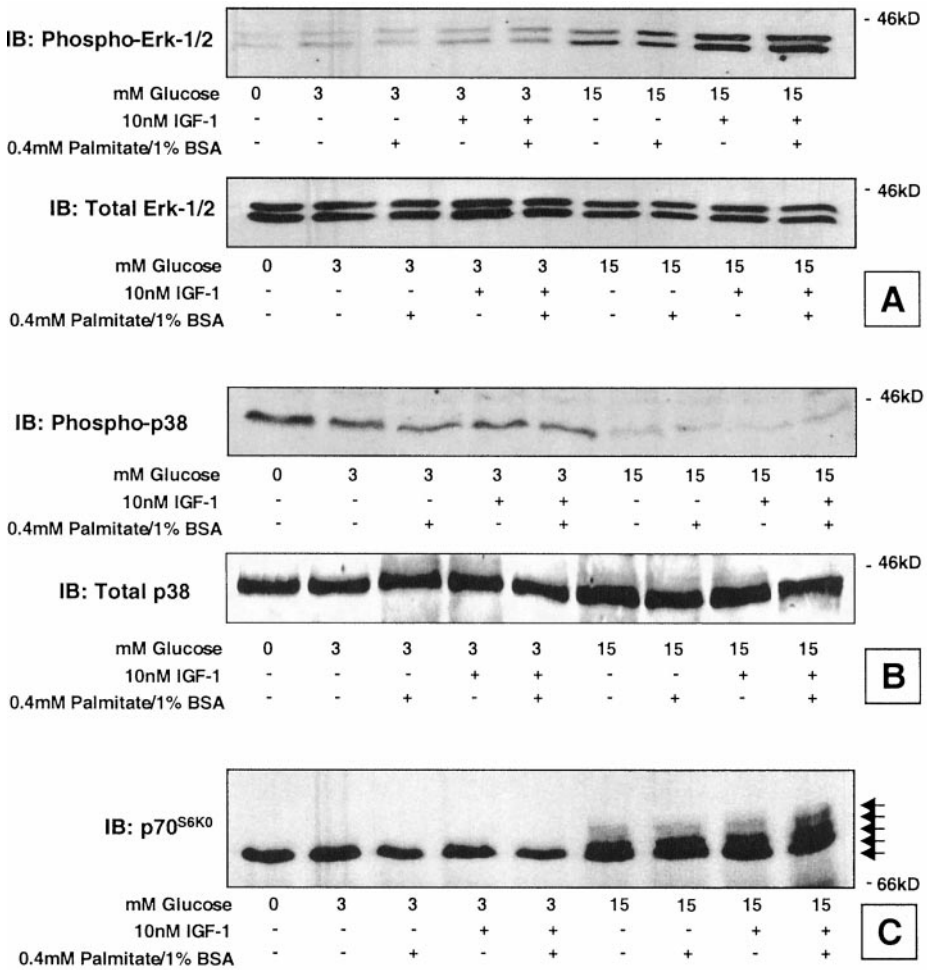

FIG. 5. Long chain FFA does not affect glucose- and IGF-I stimulated phosphorylation activation of MAPK, Erk-1/2, and p38 or $p70^{S6K}$ in INS-1 cells. INS-1 cells, incubated in the presence or absence of 0.2 mM palmitate complexed to 1% BSA for 24 h, were stimulated with 3 or 15 mM glucose with or without 10 nM IGF-I for 10 min (for Erk-1/2 and p38 analysis) or 30 min (for $p70^{S6K}$ analysis), then analyzed for phosphorylation activation of Erk-1/2, p38, and $p70^{S6K}$ by specific immunoblot analysis as described in *Materials and Methods*. Representative immunoblot (IB) analysis of activated phospho-Erk-1/2 and total Erk-1/2 (A) and activated phospho-p38 and total p38 (B) are shown. A representative immunoblot for $p70^{S6K}$ is shown (C), where phosphorylated forms of $p70^{S6K}$ become retarded on SDS-PAGE analysis, and these multiphosphorylated $p70^{S6K}$ forms are indicated by the *arrows*.

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of glucose (Fig. 5B). At 3 mM glucose, a p38 phosphorylation state was reduced by 40–50% compared with zero glucose, and by ore than 90% at 15 mM glucose (Fig. 5B). As such, the effect of glucose on p38 phosphorylation activation inversely correlated with glucose-induced activation of Erk-1 and -2 (Fig. 5A). No significant effect of IGF-I or 0.4 mM palmitate/1% BSA was observed on phosphorylation activation of p38 at any glucose concentration (Fig. 5B). Total p38 in INS-1 cells, as ascertained by immunoblot analysis with antisera recognizing both active and inactive forms of p38, was not significantly altered by glucose, IGF-I, or FFA treatment (Fig. 5B). Phosphorylation activation of JNK-1 and -2 was also assessed and was found to follow the same pattern as that for p38. JNK-1 and -2 phosphorylation inversely correlated to increasing glucose concentration. As for p38, IGF-I and FFA had no effect on the phosphorylation state of JNK-1 and -2 (data not shown).

FFA do not inhibit glucose or IGF-I activation of PI3′-K in INS-1 cells

PI3′-K activity was assessed in PI3′-K p85 immunoprecipitates as previously described (21, 22), derived from INS-1 cells previously exposed for 24 h with either 0.4 mM palmitate/1% BSA or 1% BSA, then incubated for 15 min at 3 or 15 mM glucose (with or without 10 nM IGF-I). An equivalent amount of p85 regulatory subunit of PI3′-K was immunoprecipitated from the variously treated INS-1 cell lysates (Fig. 6, *upper panel*). At 3 mM glucose, PI3′-K activity was increased 3.0 ± 0.5-fold (n = 3; $P \leq 0.05$) above that at zero glucose, and in the added presence of IGF-I was further increased 4.6 ± 0.7-fold (n = 3; $P \leq 0.05$; Fig. 6). At a stimulatory 15 mM glucose, PI3′-K activity was increased 4.7 ± 0.6-fold (n = 3; $P \leq 0.05$) above that at zero glucose, and in the added presence of IGF-I was further increased 7.2 ± 0.9-fold (n = 3; $P \leq 0.02$; Fig. 6). The presence of 0.4 mM palmitate/1% BSA had no effect on glucose- or IGF-I-induced activation of PI3′-K activity.

FFA inhibited glucose and IGF-I activation of PKB in INS-1 cells

PKB activity was assessed in PKB immunoprecipitates *in vitro* using an assay kit from New England Biolabs, Inc., based on a recombinant GSK3 protein substrate and subsequent detection of PKB-mediated GSK3 phosphorylation by specific phospho-GSK3 immunoblot analysis (Fig. 7, *middle panel*). Total PKB was immunoprecipitated from INS-1 cells previously exposed for 24 h to either 0.4 mM palmitate/1% BSA or 1% BSA, then incubated for 15 min at 3 or 15 mM glucose (with or without 10 nM IGF-I). An equivalent amount of PKB was immunoprecipitated from the variously treated INS-1 cell lysates (Fig. 7, *upper panel*). Extended electrophoresis and subsequent immunoblot analysis revealed that PKBα, PKBβ, and, to a lesser extent, PKBγ isoforms were present in β-cells (data not shown). At 3 mM glucose, PKB activity increased 3.6 ± 0.4-fold (n = 6; $P \leq 0.01$) above that at zero glucose, and in the added presence of IGF-I was further increased 5.6 ± 0.5-fold (n = 6; $P \leq 0.005$; Fig. 7). At a stimulatory 15 mM glucose, PKB activity increased 5.8 ± 0.5-fold (n = 6; $P \leq 0.01$) above that at zero glucose, and in the added presence of IGF-I was further increased 7 ± 0.8-fold (n = 6; $P \leq 0.01$; Fig. 7). However, in the presence of 0.4 mM palmitate/1% BSA, activation of PKB at 3 mM glucose was significantly reduced by 25% ($P \leq 0.05$) in the absence of IGF-I and by 40% ($P \leq 0.01$) in the presence of IGF-I (Fig. 7). The addition of 0.4 mM palmitate/1% BSA reduced 15 mM glucose-induced PKB activity by 28% ($P \leq 0.05$), but had a more marked effect on IGF-I-induced PKB activation at 15 mM glucose, where PKB activity was inhibited by 52% ($P \leq 0.01$; Fig. 7).

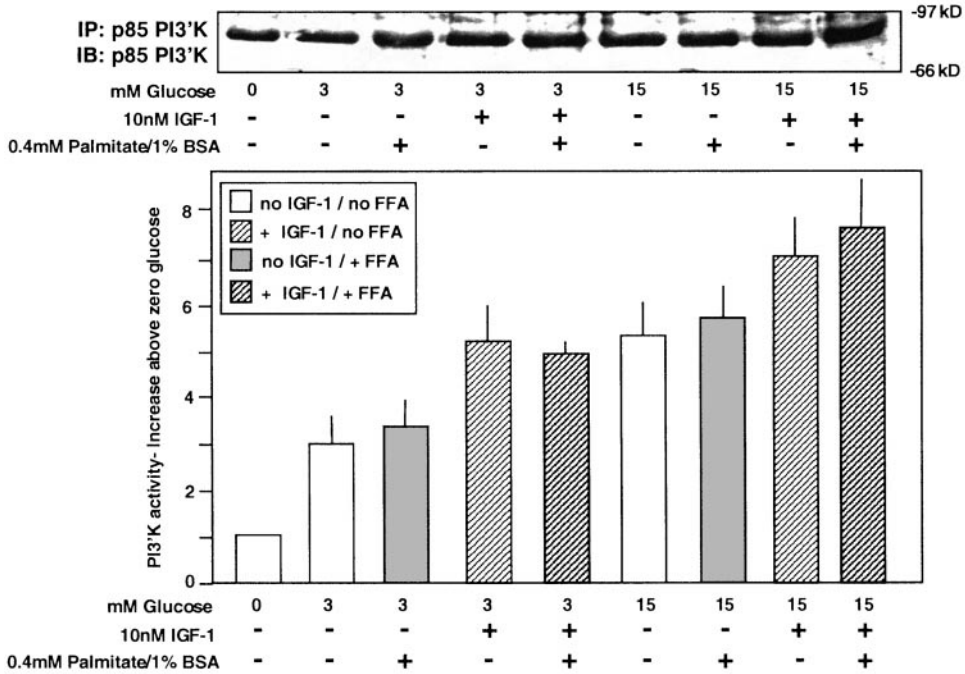

FIG. 6. Long chain FFA does not affect glucose- and IGF-I-stimulated PI3′K activity in INS-1 cells. INS-1 cells, incubated in the presence or absence of 0.2 mM palmitate complexed to 1% BSA, were stimulated with 3 or 15 mM glucose with or without 10 nM IGF-I for 10 min. INS-1 cell lysates were then subjected to immunoprecipitation (IP) with antiserum against the p85 regulatory subunit of PI3′K. PI3′K activity was measured in p85 immunoprecipitates as outlined in *Materials and Methods*. Equivalent p85 was immunoprecipitated between samples as assessed by immunoblot analysis of p85 immunoprecipitates, of which a representative immunoblot is shown. The data are expressed as the fold increase above the control observation in the absence of glucose, IGF-I, and 0.2 mM palmitate/1% BSA and are depicted as the mean ± SE (n ≥ 3).

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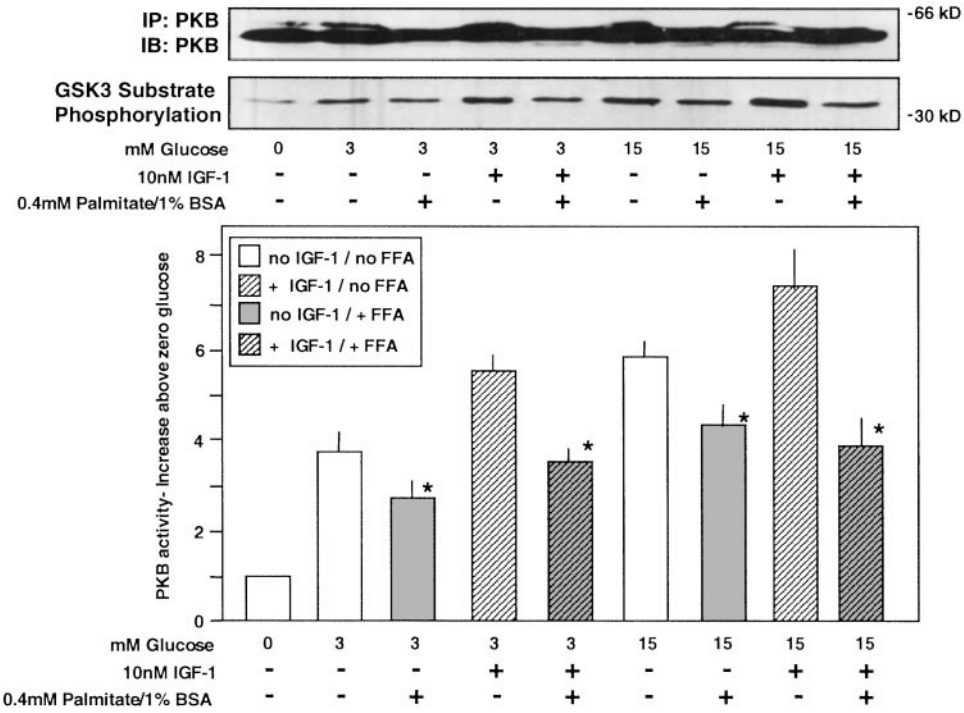

FIG. 7. Long chain FFA partly inhibit glucose/IGF-I-stimulated PKB activity in INS-1 cells. INS-1 cells, incubated in the presence or absence of 0.2 mM palmitate complexed to 1% BSA, were stimulated with 3 or 15 mM glucose with or without 10 nM IGF-I for 10 min. INS-1 cell lysates were then subjected to immunoprecipitation (IP) with antiserum against total PKB. PKB activity was then measured in PKB immunoprecipitates, as outlined in Materials and Methods, using recombinant GSK3 as a substrate and phosphorylation analysis with specific phospho-GSK immunoblot analysis (of which a representative phospho-GSK immunoblot is shown). Equivalent PKB was immunoprecipitated between samples, as assessed by immunoblot analysis of PKB immunoprecipitates, of which a representative immunoblot is shown. The data are expressed as the fold increase above the control observation in the absence of glucose, IGF-I, and 0.2 mM palmitate/1% BSA and are depicted as the mean ± SE (n ≥ 6).

FFA do not inhibit glucose or IGF-I activation of p70^{S6K} in INS-1 cells

p70^{S6K} is activated downstream of PI3′K/PKB activation (31). Phosphorylation activation of p70^{S6K} occurs on multiple sites, so p70^{S6K} phosphorylation can be detected on immunoblot analysis by apparent electrophoresis mobility retardation (32). INS-1 cells previously exposed for 24 h to either 0.4 mM palmitate/1% BSA or 1% BSA were then incubated for 30 min at 3 mM or stimulatory 15 mM glucose (with or without 10 nM IGF-I). Immunoblot analysis with p70^{S6K}-specific antiserum indicated that phosphorylation activation of p70^{S6K} in β-cells was not detected in the absence or presence of 3 mM glucose with or without IGF-I (Fig. 5C), similar to that previously observed (3, 4). However, phosphorylation activation of p70^{S6K} in response to 15 mM glucose was observed, which was further increased in the presence of IGF-I (Fig. 5C). The presence of 0.4 mM palmitate/1% BSA had no effect on glucose-induced or glucose-dependent IGF-I-induced phosphorylation activation of p70^{S6K}.

FFA-induced activation of PKCζ INS-1 cells

PKCζ activity was assessed in PKCζ immunoprecipitates *in vitro* as previously described (23). Total PKCζ was immunoprecipitated from INS-1 cells previously exposed for 24 h to either 0.4 mM palmitate/1% BSA or 1% BSA, then incubated for 15 min at 3 or 15 mM glucose (with or without 10 nM IGF-I). An equivalent amount of PKCζ was immunoprecipitated from variously treated INS-1 cell lysates (Fig. 8, *upper panel*). In the absence of glucose, but in the presence of 0.4 mM palmitate/1% BSA, PKCζ activity was elevated 5.1 ± 0.6-fold (n = 7; $P \leq 0.005$) above that in the presence of 1% BSA only (Fig. 8). This indicated increased activity of PKCζ during the prior 24-h incubation in the presence of FFA. For INS-1 cells incubated in the absence of FFA, 3 mM glucose increased PKCζ activity 3.2 ± 0.4-fold (n = 6; $P \leq 0.05$) above that at zero glucose, and in the added presence of IGF-I it was further increased to 4.6 ± 0.6-fold (n = 6; $P \leq 0.05$; Fig. 8). However, this modest incremental increase in PKCζ activity induced by IGF-I at 3 mM glucose was not statistically significant. In INS-1 cells exposed to 0.4 mM palmitate/1% BSA and incubated at 3 mM glucose for 15 min, PKCζ activity was significantly enhanced 7.5 ± 0.9-fold (n = 5; $P \leq 0.02$) above that at zero glucose, 2.3-fold higher than INS-1 cells incubated in the absence of FFA. Likewise, at 3 mM glucose and 10 nM IGF-I, 0.4 mM palmitate/1% BSA treatment of INS-1 cells, PKCζ activity was significantly enhanced 11.3 ± 1.7-fold (n = 5; $P \leq 0.05$) above that at zero glucose, 2.5-fold higher than equivalent INS-1 cells incubated in the absence of FFA. At a stimulatory 15 mM glucose, PKCζ activity was increased 9.6 ± 1.5-fold (n = 6; $P \leq 0.05$) above that at zero glucose, and in the added presence of IGF-I was further increased to 10.4 ± 1.1-fold (n = 6; $P \leq 0.02$; Fig. 8). As at 3 mM glucose, the slight incremental increase in PKCζ activity induced by IGF-I at 15 mM glucose was not statistically significant. Nonetheless, in the presence of 0.4 mM palmitate/1% BSA, activation of PKCζ by 15 mM glucose was significantly enhanced 13.1 ± 1.2-fold (n = 5; $P = 0.02$) above that at zero glucose, which was 36% higher than the level in INS-1 cells incubated in the absence of FFA. Likewise, PKCζ activation by 15 mM glucose and 10 nM IGF-I in the presence of 0.4 mM palmitate/1% BSA-treated INS-1 cells was significantly enhanced 15.3 ± 1.8-fold (n = 5; $P \leq 0.05$) above that at zero glucose, which was 47% higher than the level in equivalent INS-1 cells incubated in the absence of FFA (Fig. 8).

Discussion

Glucose is a major factor that regulates pancreatic β-cell mitogenesis (1, 2). It has recently been shown that the effect of IGF-I and GH to increase β-cell mitogenesis was depen-

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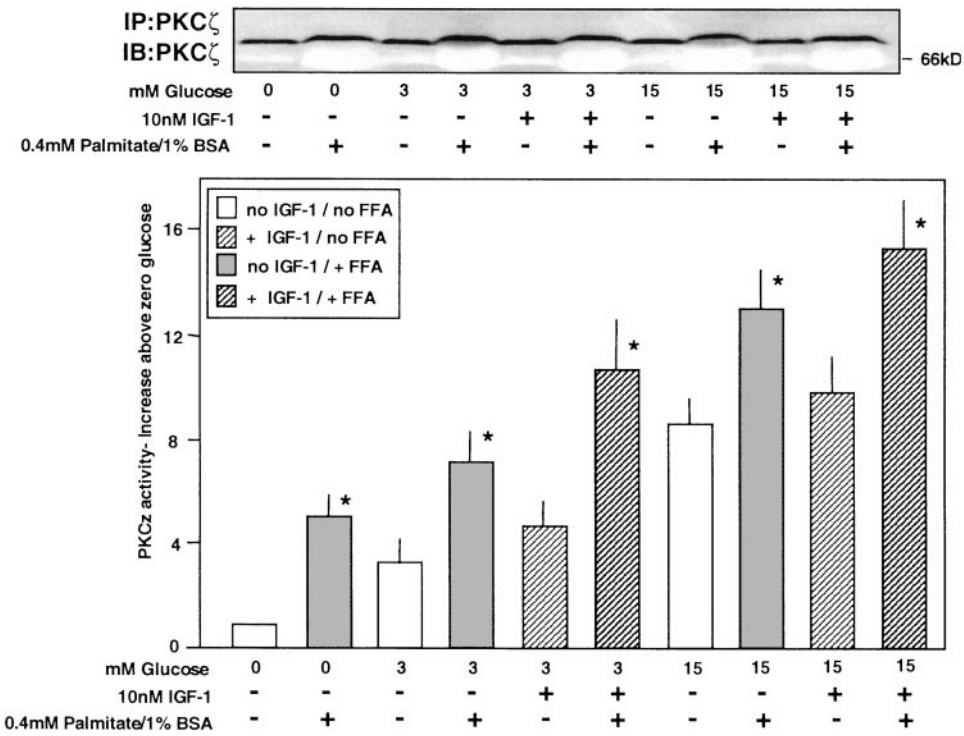

FIG. 8. Long chain FFA chronically increase PKCζ activity in INS-1 cells. INS-1 cells, incubated in the presence or absence of 0.2 mM palmitate complexed to 1% BSA, were incubated in the absence of glucose or with 3 or 15 mM glucose with or without 10 nM IGF-I for 15 min. INS-1 cell lysates were then subjected to immunoprecipitation (IP) with antiserum against PKCζ. PKCζ activity was measured in the immunoprecipitates as outlined in *Materials and Methods*. Equivalent PKCζ was immunoprecipitated between samples, as assessed by immunoblot analysis of PKCζ immunoprecipitates, of which a representative immunoblot is shown. The data are expressed as the fold increase above the control observation in the absence of glucose, IGF-I, and 0.2 mM palmitate/1% BSA and are depicted as the mean ± SE ($n \geq 6$).

dent on glucose metabolism in the physiologically relevant concentration range (6–18 mM) (3, 4). Therefore, it is conceivable that the pancreatic β-cell population is flexible and can adapt to the nutritional status of the organism (34), similar to the transient increase in β-cell mass that occurs during pregnancy (35). In this study we have examined whether certain intermediates of glucose metabolism and alternative metabolic fuels are capable of stimulating β-cell DNA synthesis independently as well as providing the necessary metabolic environment so that IGF-I can promote β-cell mitogenesis. Pyruvate, a product of glycolysis in β-cells, independently increased β-cell DNA synthesis at similar concentrations previously shown to specifically increase proinsulin biosynthesis and insulin secretion (26) and also enabled IGF-I to cause a marked increase in β-cell mitogenesis. Likewise, the combination of glutamine and leucine added to the medium to generate intracellular α-ketoglutarate (36) independently increased β-cell DNA synthesis and provided the environment for IGF-I-induced β-cell mitogenesis, similar to the effect of the glutamine/leucine combination on (pro)insulin synthesis and secretion (26). As both pyruvate and glutamate/leucine are used as mitochondrial fuels in β-cells, these data suggest that β-cell mitochondrial metabolism, downstream of glycolysis, is important for generating secondary coupling signals for glucose-induced β-cell DNA synthesis and for providing the permissive environment for IGF-I-induced β-cell mitogenesis.

However, not all metabolic fuels act as β-cell mitogens or provide a platform of growth factors to induce β-cell mitogenesis. In the absence of glucose (or at ≤5 mM glucose) FFAs were observed to have only a modest effect on INS-1 cell DNA synthesis compared with that of 15 mM glucose. Similar observations of the effect of FFA on β-cell mitogenesis have been previously made in islets (37), and INS-1 cells (38). In this study it was found that long chain (>C_{10}) FFA prevented 15 mM glucose-induced and glucose-dependent IGF-I-induced β-cell DNA synthesis within a 24-h period ($t_{0.5}$ = ~6 h). FFA-mediated inhibition of β-cell DNA synthesis occurred at a relatively low concentration of FFA ($K_{i0.5}$ = ~60–90 μM FFA complexed to 1% BSA) and was reversible upon removal of the FFA. Thus, it was unlikely that FFA-mediated inhibition of β-cell mitogenesis was due to a nonspecific detergent-like effect. FFA-induced inhibition of β-cell DNA synthesis might have been acting via FFA-derived intracellular ceramide formation, but this was unlikely, because ceramide had no effect on glucose/IGF-I-induced β-cell DNA synthesis. This would be consistent with the observation that both palmitate and oleate inhibited glucose/IGF-I induced β-cell mitogenesis, as ceramide is derived from palmitoyl-CoA, but not directly from oleoyl-CoA (39). Notwithstanding, long chain fatty acyl-CoA moieties were probably involved, as methyl-palmitate (which cannot form a CoA ester) was not as effective as other FFAs in mediating the inhibition of glucose/IGF-I-induced β-cell DNA synthesis. However, FFA-mediated inhibition of β-cell DNA synthesis was not due to changes in FFA oxidation, as a nonmetabolizable FFA analog (bromopalmitate) was also effective at inhibiting glucose/IGF-I-induced β-cell DNA synthesis. Moreover, it was found that in INS-1 cells that were incubated for 24 h in the presence of FFA (0.4 mM palmitate or oleate complexed to 1% BSA), the rate of glucose oxidation (at either 3 or 15 mM glucose) was unaffected by FFA (data not shown), as previously shown in pancreatic islets (40). Therefore, FFA were unlikely to inhibit glucose metabolism that, in turn, would affect the glucose-dependent aspect of growth factor-induced β-cell mitogenesis (3, 4).

IRS-mediated signal transduction in INS-1 cells (particularly via IRS-2) (9–11) has previously been shown to be

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necessary for glucose-induced and glucose-dependent IGF-I-induced β-cell DNA synthesis (3, 4). It was investigated whether FFA would interfere with glucose/IGF-I-induced signal transduction via IRS in a manner that correlated with FFA-mediated inhibition of glucose/IGF-I-induced β-cell DNA synthesis. Using palmitate as a model, it was found that FFA did not affect glucose/IGF-I-induced recruitment of PI3′-K or Grb2/mSOS to IRS-1/2, recruitment of Grb2/mSOS to Shc, activation of PI3′K activity, or phosphorylation activation of Erk-1/2 or $p70^{S6K}$. The stress kinases p38 and JNK-1/2 were phosphorylation activated by decreasing glucose concentrations, but were unaffected by FFA. Thus, it was unlikely that p38 or JNK-1/2 was involved in FFA-mediated inhibition of glucose/IGF-I-induced β-cell mitogenesis. In contrast, FFA significantly inhibited glucose/IGF-I-induced activation of PKB activity. However, it should be noted that FFA-mediated inhibition of PKB activity in β-cells was partial, and downstream glucose/IGF-I-induced phosphorylation activation of $p70^{S6K}$ was not affected by FFA. Nonetheless, although residual PKB activity in the presence of FFA was sufficient to promote full activation of $p70^{S6K}$, it is possible that phosphorylation of alternative protein substrates by PKB is inhibited in the presence of FFA, which, in turn, could lead to decreased β-cell mitogenesis (41–43).

It was also found that FFA activated PKCζ in INS-1 cells during a 24-h exposure, independently of glucose or IGF-I. In INS-1 cells previously exposed to FFA for 24 h, subsequent glucose/IGF-I-stimulated PKCζ activity was additive to the FFA-induced PKCζ activity. This raises the possibility that glucose/IGF-I and FFA activate β-cell PKCζ via distinct mechanisms. Indeed, PKCζ can be directly activated by both FFA, and phosphatidylinositol-3,4,5-trisphosphate can be generated by glucose/IGF-I induced activation of PI3′-K (44). Also, PKCζ activity can be further increased via phosphorylation by phosphoinositide-dependent protein kinase-1 (PDK-1), an enzyme that also lies downstream of PI3′-K (45, 46). Thus, PKCζ activity can be chronically activated by FFA-treated β-cells and further enhanced by increased glucose/IGF-I levels acutely. However, signaling proteins such as PKCζ are only transiently activated under normal circumstances (44). As such, chronic FFA-induced activation of PKCζ may lead to an abnormal prolonged phosphorylation state of certain protein substrates that could, in turn, lead to β-cell dysfunction, including an antagonism of glucose-induced mitogenesis. Interestingly, it has been shown that chronic activation of PKCζ can inhibit PKB activity (47, 48), and one might speculate that chronic FFA-induced activation of PKCζ contributed to FFA-mediated inhibition of glucose/IGF-I-induced activation of PKB in β-cells. However, although it is intriguing to contemplate that FFA-mediated inhibition of glucose/IGF-I-induced activation of PKB and/or chronic activation of PKCζ can contribute to FFA-induced inhibition of β-cell DNA synthesis, this needs to be demonstrated more directly by further experimentation. For instance, if this scenario is correct, one might predict that expression of a dominant negative PKB and/or constitutively active PKCζ would mimic FFA-mediated inhibition of glucose/IGF-I-induced β-cell DNA synthesis. Likewise, expression of a constitutively active PKB and/or dominant negative PKCζ would be predicted to alleviate FFA-mediated inhibition of glucose/IGF-I-induced β-cell DNA synthesis. Notwithstanding, FFA-mediated inhibition and/or chronic activation of other signal elements downstream of IRS, or in alternative signal transduction pathways cannot, for the moment, be ruled out as a mechanism for preventing glucose/IGF-I-induced β-cell mitogenesis.

It is currently thought that peripheral insulin resistance in obesity is compensated by an increase in pancreatic β-cell mass and consequential increased insulin production (8). However, a failure of β-cell mass to compensate for insulin resistance results in type II diabetes (6, 7, 11). It has previously been shown that hyperglycemia and hyperlipidemia contribute to β-cell dysfunction (6–8, 14). Chronic exposure of high concentrations of glucose can induce β-cell death (49) despite the common observation of glucose-induced β-cell growth (1, 2). It has also been proposed that FFA and triglyceride accumulation in β-cells derived from hyperlipidemia in obesity-linked type II diabetes contributes to β-cell dysfunction and reduction of β-cell mass (6, 8). Indeed, it has been previously shown that FFA and subsequent intracellular ceramide formation instigated β-cell apoptosis (8). The FFA-induced inhibition of glucose/IGF-I-induced β-cell mitogenesis outlined in this study (via a mechanism independent of ceramide) would additionally contribute to a FFA-induced decrease in β-cell mass and a failure to compensate for peripheral insulin resistance, which are keys to the pathogenesis of type II diabetes (6, 8). It is possible that FFA-induced inhibition of glucose/IGF-I-induced β-cell mitogenesis could be mediated by FFA-induced inhibition of PKB activation and/or chronic activation of PKCζ, although further experiments will be required to substantiate this observation. However, it should be noted that FFA also inhibit PKB (50, 51) and chronically activate certain PKC isoforms (52, 53) in skeletal muscle cells that, in turn, contribute to peripheral insulin resistance. Thus, similar adverse affects of FFA on insulin signal transduction in peripheral tissues and mitogenic signaling pancreatic β-cells both contribute to the pathogenesis of obesity-linked type II diabetes.

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