Differential Impact of Acute and Chronic Lipotoxicity on Gene Expression in INS-1 Cells

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Fatty acids induce abnormal insulin secretion, so-called lipotoxicity, which may develop over a period that can span from a few hours to several years. The relationship between insulin secretion patterns and the pace of lipotoxicity development is, however, sparse. In this study acute lipotoxicity was defined as the functional changes in clonal pancreatic β cells, INS-1 cells, cultured with 400 and 1,000 μmol/L palmitate for 2 days. Chronic lipotoxicity was demonstrated by exposure of INS-1 cells to 50 and 200 μmol/L palmitate for up to 10 weeks. During acute lipotoxicity, basal insulin secretion (BIS), as well as glucose- and fatty acid-stimulated insulin secretion, were reduced after 1,000 μmol/L palmitate exposure. Concomitantly, total cell protein and ^{3}H-thymidine incorporation were significantly reduced. In chronic lipotoxicity, BIS increased, whereas a decrease in insulin responsiveness to glucose and fatty acid (defined as fold increase in insulin compared with BIS) was observed after 5 weeks in cells cultured with 200 μmol/L palmitate. Carnitine palmitoyltransferase I gene expression was induced by palmitate upon acute, as well as chronic, exposure. Genes involved in the insulin signal pathway may play an important role in the pathogenesis of lipotoxicity in β cells. Thus, insulin receptor substrate-1 and 2 gene expressions were downregulated during acute lipotoxicity, while insulin receptor gene expression was suppressed in chronic lipotoxicity. In conclusion, insulin secretion and gene expression in INS-1 cells depends on palminate exposure time and concentration.

DYSLIPIDEMIA WITH AN increase in plasma nonesterified fatty acids is a common metabolic abnormality associated with type 2 diabetes,[1,2] in which high-lipid levels within the islets have been suggested to contribute to the abnormal β-cell function.[3]

A fatty acid–induced increase in basal insulin secretion and suppression of glucose-stimulated insulin secretion, so-called lipotoxicity, has been found both in vivo and in vitro.[4-6] The functional defects of β cells may be present in the prediabetic state and play a role in the pathogenesis of type 2 diabetes. The lipotoxicity apparently develops at a varying pace depending, among others, on which model is applied: (1) in perifusion studies, fatty acids inhibited insulin secretion after 2 hours in rat islets[7]; (2) intravenous infusion of fatty acids induced suppression of insulin secretion after 48 hours in both animals and humans[8,9]; (3) in Zucker diabetic fatty rats, a few weeks seemed to be required for lipotoxicity to develop[6]; and (4) at least in some humans, it may take a few years for lipotoxicity to develop.[10] The time needed for lipotoxicity to develop thus depends, eg, on the species, as well as the structure and concentration of the fatty acids.[6-8,10-12] The absolute level of insulin did not decline in the early stage of diabetes in male Zucker fatty rats.[6] However, it is obvious that the responsiveness to glucose-stimulation was reduced or lost. It seems that this relative loss may represent functional changes in the early stage of lipotoxicity.[12,13] The diminished absolute glucose-stimulated insulin secretion may therefore reflect the late events of lipotoxicity as documented in many studies.[7,13-15] This invites the question whether lipotoxicity developing after a few hours (acute lipotoxicity) is causing the same changes in β-cell gene expression as lipotoxicity developing after weeks or several years (chronic lipotoxicity).

We have sought an answer to this question by examining the impact of acute lipotoxicity induced by high concentrations of palmitate for 2 days and chronic lipotoxicity induced by low concentrations of palmitate for weeks using clonal pancreatic β cells, INS-1 cells. Apart from insulin secretion, we also investigated the expression of insulin 1, insulin receptor (IR), insulin receptor substrate-1 (IRS-1), and insulin receptor substrate-2 (IRS-2), type 1 hexokinase (HK1), carnitine palmitoyl transferase-1 (CPT-1), and glucose transporter 2 (GLUT2) genes. In the view of the different impact short- and long-term exposure to fatty acids has on insulin secretion, we hypothesize that changes in the gene expression of these key regulatory substances in INS-1 cells depend on the exposure time.

MATERIALS AND METHODS

This study comprises 2 sets of experiments. In the first, INS-1 cells were cultured with 0, 400, and 1,000 μmol/L palmitate for 2 days to investigate changes related to what we have defined as acute lipotoxicity. In the more long-term studies, INS-1 cells were cultured for up to 10 weeks in 0, 50, and 200 μmol/L palmitate. Studies on the insulin secretion were performed at weeks 1, 2, 4, 5, 6, and 10 (chronic lipotoxicity). INS-1 cells were simultaneously harvested for RNA preparation. Gene expression changes were determined by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). In addition, ^{3}H-thymidine incorporation was used to monitor cell growth.

Cell Culture

Acute Lipotoxicity Study

Prior to this study, the INS-1 cells were maintained under the culture condition described below with the exception that 10 mmol/L glucose was present and the cells were passed weekly.[16] The INS-1 cells were grown in a humidified atmosphere (5% CO_2, 95% air at 37°C) in monolayer in a modified RPMI 1640 medium. The medium was

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supplemented with 10% (vol/vol) fetal calf serum (FCS), 6.6 mmol/L glucose, 10 mmol/L HEPES, 100 IU/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamate, 50 μmol/L β-mercaptoethanol (all from GIBCO BRL, Paisley, UK), and 0.5% bovine serum albumin (BSA, fatty acid–free, Boehringer Mannheim, Mannheim, Germany). Equal numbers of cells were plated in each well in 6-well or 24-well plates (NUNC Brand Products, NUNC A/S, Roskilde, Denmark). A stock solution of palmitate (Sigma Chemical, St Louis, MO) was prepared by mixing and heating to 90°C equal molar amounts of NaOH and palmitic acid supplemented with distilled H_2O to a concentration of 400 mmol/L. This solution was further diluted with 5% BSA (fatty acid–free), which gave a concentration of 50 mmol/L. The solution was filter sterilized and stored at 4°C. A suitable amount of heated (about 40°C) 50 mmol/L solution was slowly added to a 37°C culture medium to reach final concentrations in the medium of 0 (P0), 400 (P400), and 1,000 μmol/L (P1,000). The cell number was counted after 48 hours of culture.

Chronic Lipotoxicity Study

This study was based on the results of the acute lipotoxicity study and relatively low concentrations, ie, 50 and 200 μmol/L of palmitate was added. INS-1 cells in flasks or 24-well plates were exposed to 50 μmol/L (P50) and 200 μmol/L (P200) palmitate. Basal conditions were as described in the acute lipotoxicity study, ie, culture growth was pursued in a medium supplemented with 6.6 mmol/L glucose. INS-1 cells cultured in 6.6 mmol/L glucose without palmitate served as controls. The cells were passed into new flasks weekly and continuously cultured under the same conditions and subcultured for 48 hours in 24-well plates prior to the secretion studies.

Insulin release and insulin content. In the long-term study, we measured the insulin release at different times after a 48-hour subculture under constant conditions. For insulin content determination, the cells were treated with trypsin (37°C, 2 minutes, GIBCO), harvested, centrifuged, and counted. A total of 2×10^5 cells were transferred to glycine-BSA buffer (glycine 100 mmol/L, 0.25% BSA, pH 8.8), and insulin was released by sonication (Branson Sonifier 250, Danbury, CT) on ice for 15 seconds 2 times. After centrifugation for 30 minutes at 16,000 rpm, the supernatant was collected and frozen at -20°C for later assay. The insulin content was corrected for protein concentration.

Insulin secretion study. Cells were cultured for 48 hours in the presence of 6.6 mmol/L glucose with or without palmitate and then preincubated at 37°C for 30 minutes in a modified Krebs-Ringer buffer (125 mmol/L NaCl, 5.9 mmol/L KCl, 1.28 mmol/L $CaCl_2$, 5.0 mmol/L $NaHCO_3$, 25 mmol/L HEPES, pH 7.4) (all from Sigma Chemical) containing 3.3 mmol/L glucose and 0.1% BSA. Cells were changed to a fresh buffer containing 3.3 and 16.7 mmol/L glucose, and the glucose-stimulated insulin secretion was tested after incubation for 60 minutes. For fatty acid stimulation, 200 or 400 μmol/L palmitate was added in the presence of 16.7 mmol/L glucose. At the end of the incubation, 300 μL medium was collected, centrifuged, and 200 μL of the supernatant was stored at –20°C for insulin assay.

Protein assay. Samples were collected for insulin assay, and the remaining medium was discarded. The cells were lysed in 0.1 mol/L NaOH, and the total protein was measured by a Bio-Rad detergent compatible protein kit (Bio-Rad Laboratories, Hercules, CA).

Insulin assay. Insulin was measured by radioimmunoassay (RIA) with a guinea pig, antiporcine insulin antibody, and mono-^{125}I- (Tyr-A14)-labeled human insulin as tracer and rat insulin as standard. Free and bound radioactivity were separated using ethanol. Both inter and intra-assay variation coefficients were less than 5%.

RNA extraction. Total RNA was isolated from the INS-1 cells using Trizol (GIBCO).

RT-PCR. Reverse transcription was performed with 25 ng total RNA with reverse transcriptase, random hexamer primers. The cDNA was amplified with Ampli Taq Gold DNA polymerase (Gene Amp kit, Perkin Elmer-Cetus, Norwalk, CT). With housekeeper gene β actin as internal reference, CPT-1, HK1, GLUT2, insulin 1, IRS-1, IRS-2, and IR gene expressions were investigated using semiquantitative multiplex PCR (1 target gene together with β actin). The primer sequences are listed in Table 1. Initial experiments were performed for each set of primers to determine the optimal number of cycles for exponential (linear range) amplification of cDNA (data not shown). PCR was performed at predenaturing first at 95°C for 5 minutes and then at 95°C for 30 seconds, at 56°C for 30 seconds, and at 72°C for 60 seconds in 1 cycle. The pair of primers that needed more cycles to obtain exponential amplification was put into the PCR reaction mixture first. Then the pair of primers that needed less cycles to obtain exponential amplification were dropped into the same reaction tube and coamplified (primer dropping method).[17] By this procedure, both the target gene and the housekeeper gene reached the exponential amplification phase at the same time. A total of 26 to 40 cycles for different abundant mRNA and 30 to 32 cycles for β actin were used in the multiplex PCR. Before termination of PCR, a 6-minute extension at 74°C was added. Products were separated by 3% agarose electrophoresis, stained by ethidium bromide, and scanned by Bio-Rad Gel 1000 system for quantification. The relative gene expression was determined as the ratio of target gene to β-actin gene band density of RT-PCR products. The coefficient of variation was approximately 10%. All levels of gene expression were expressed as ratios to β actin and were normalized with the corresponding controls.

^{3}H-Thymidine Incorporation in INS-1 Cells

^{3}H-thymidine incorporation was used to monitor INS-1 cell proliferation and DNA synthesis. A 96-well plate assay was used. Briefly, cells (2×10^4 each well) were plated on 96-well plates and cultured in 6.6 mmol/L glucose, RPMI 1640, 10% FCS for 24 hours. The medium was removed, and the cells were made quiescent by serum and glucose deprivation in RMPI 1640 containing 0.1% BSA instead of serum and

Table 1. Primer Sequences Used in RT-PCR

Name	Sense Primer	Antisense Primer	Base Pairs
CPT-1	5′-CAGCTCGCACATTACAAGGA-3′	5′-GGAGACACCATAGCCGTCAT-3′	398
HK1	5′-GTAAAGATCCGCAGTGGGAA-3′	5′-CTCTCTCCTCTTCACCGCAT-3′	290
Insulin I	5′-TGACCAGCTACAATCATAGACCA-3′	5′-CTCCAGTGCCAAGGTCTGA-3′	398
IR	5′-GACCTTCCTGGAAATCGTCA-3′	5′-GAAAAGAATCCTTCCCCGAG-3′	341
GK	5′-AGACCTGGGAGGAACCAACT-3′	5′-ACGATGTTGTTCCCTTCTGC-3′	296
GLUT2	5′-CAGCAATGATGAGAGCATGTG-3′	5′-GACACCCCACTCATAGTCACA-3′	271
IRS-1	5′-CACCCAGTTTTTCGACACCT-3′	5′-GCTTGTTCTTGGAGTCAGCC-3′	284
IRS-2	5′-GAGCCTTCAGTAGCCACAGG-3′	5′-CAGGCGTGGTTAGGGAGTAA-3′	212
β-actin	5′-CACGGCATTGTAACCAACTG-3′	5′-AGGAAGGAAGGCTGGAAGAG-3′	582

0.5 mmol/L glucose for 24 hours. The INS-1 cells were then incubated for another 24 hours in RMPI 1640 and 0.5% BSA and different concentrations of glucose and palmitate and 1 μCi [methy-^{3}H] thymidine (48 Ci/mol, Pharmacia, Buckinghampshire, UK). The cells were incubated and the plates were washed twice in cold phosphate-buffered saline (PBS). After applying 200 μL liquid scitillator, the incorporated [methy-^{3}H] thymidine was counted by a 1450 MicroBeta TRILUX (Wallac, Turku, Finland).

Statistics

Data are presented as mean ± SEM. Significance of difference was determined by 1-way analysis of variance (ANOVA) ($P < .05$).

RESULTS

Protein Concentration

In the acute lipotoxicity study, a similar number of cells (2×10^5 cells) was seeded in each well in a 24-well plate. After culture for 48 hours of 0, 400, and 1,000 μmol/L palmitate, the cellular protein concentrations were 130 ± 3 (P0), 115 ± 3 (P400), and 100 ± 1 μg/mL (P1,000) (n = 96 for each group, $P < .05$ for P400 and P1,000 *v* P0).

Insulin Secretion

The Effect of Short-Term Exposure (2 Days)

Basal insulin secretion. The presence of 3.3 mmol/L glucose in cells precultured with palmitate caused a decrease in basal insulin secretion (BIS) after adjusting for protein concentration (Fig 1A).

Glucose-stimulated insulin secretion. Glucose-stimulated insulin secretion (GSIS) was reduced in cells exposed to 1,000 μmol/L palmitate (Fig 1A). Maximum response was observed in the presence of 6.6 mmol/L glucose in all groups. Compared with BIS (in 3.3 mmol/L glucose) and quantified in relative terms as a response above baseline, GSIS increased in cells precultured in 400 μmol/L palmitate at 6.6 mmol/L and 16.7 mmol/L glucose, but only at 16.7 mmol/L in cells precultured with 1,000 μmol/L palmitate (Fig 1B).

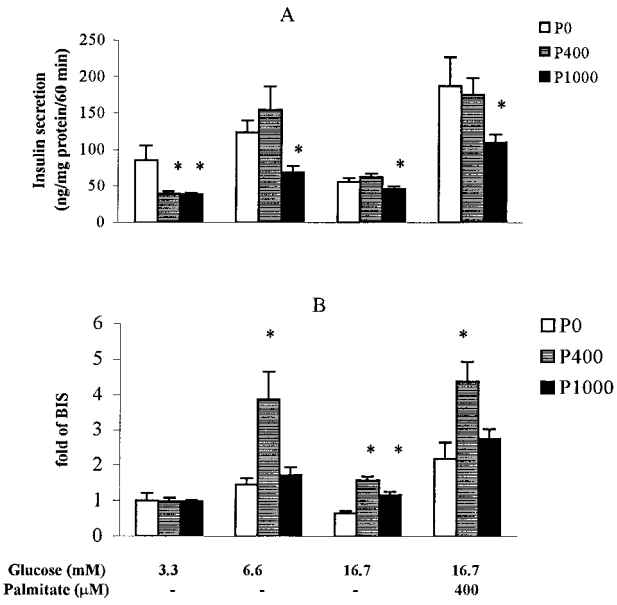

Fig 1. Insulin secretion from INS-1 cells (n = 24 for each column) exposed for 60 minutes to glucose (3.3 to 6.7 mmol/L) and glucose (16.7 mmol/L) plus 400 μmol/L palmitate. Cells were precultured for 48 hours in 0, 400, or 1,000 μmol/L palmitate and 6.6 mmol/L glucose. (A) Insulin concentration adjusted for protein concentration; (B) insulin secretion relative to secretion at 3.3 mmol/L glucose. * *P* < .05 *v* controls (without palmitate).

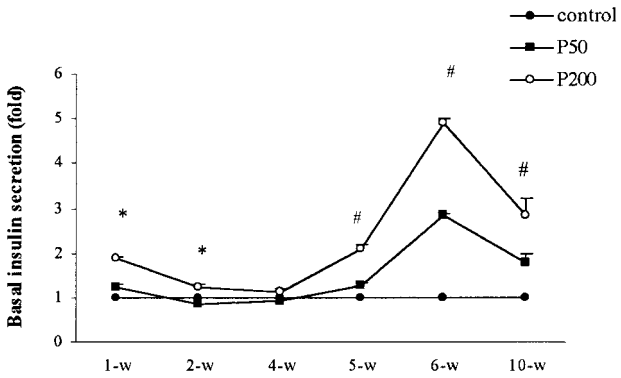

Fig 2. Insulin secretion (given as fold of control) in the presence of 3.3 mmol/L glucose after a 60-minute incubation of INS-1 cells precultured in 0, 50, or 200 μmol/L palmitate and 6.6 mmol/L glucose for 1 to 10 weeks (n = 12 for each column). * *P* < .05 for P200 *v* controls, #*P* < .05 for P50 and P200 *v* controls.

Fatty acid–stimulated insulin secretion. Only cells precultured in 1,000 μmol/L palmitate showed a decrease in insulin secretion in response to 16.7 mmol/L glucose plus 400 μmol/L palmitate (Fig 1A).

The Effect of Chronic Exposure (10 Weeks)

BIS. After adjustment for protein concentration, the BIS at 3.3 mmol/L glucose was expressed as the ratio to controls (without palmitate). BIS increased significantly in cells cultured with 200 μmol/L palmitate, except in week 4. It started to increase after 5 weeks in cells cultured with 50 μmol/L palmitate (Fig 2).

Glucose and fatty acid–stimulated insulin secretion. The difficulty inherent in determination of cell growth rates and appropriate allowance for such growth when calculating the insulin secretion at different time points made it necessary to express the stimulated insulin secretion data in relative terms, giving the insulin secretion of the control group to different stimulation the value of 1. This procedure gave us a relative measure of the effect of the fatty acid on insulin secretion at each time point and allowed comparison with the control group. Cells precultured with 50 μmol/L and 200 μmol/L palmitate showed enhanced insulin secretion in response to 16.7 mmol/L glucose at weeks 1, 2, and 4. After 5 weeks, it declined in cells precultured in 200 μmol/L palmitate (Fig 3A). The secretion pattern in response to 16.7 mmol/L glucose plus a 200-μmol/L palmitate challenge was quite similar to the response to 16.7 mmol/L glucose alone (Fig 3B). However, we did not detect increased insulin secretion at week 4 or reduced insulin secretion at week 5 in cells precultured in 200 μmol/L palmitate. In cells cultured in P50, we only observed suppression of insulin secretion in week 5 as a response to 16.7 mmol/L glucose (Fig

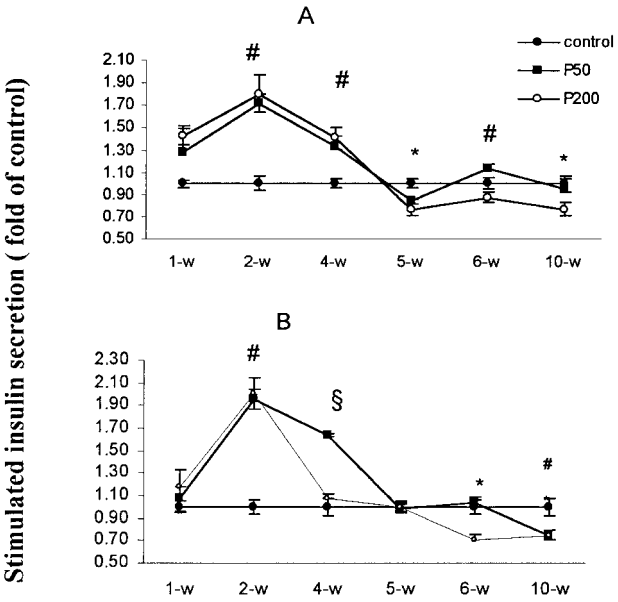

Fig 3. Insulin secretion (given as fold of control) in response to 16.7 mmol/L glucose (A) and 16.7 mmol/L glucose plus 200 μmol/L palmitate (B) during a 60-minute incubation of INS-1 cells precultured in 0, 50, or 200 μmol/L palmitate in the presence of 6.6 mmol/L glucose for 1 to 10 weeks (n = 12 for each column). #$P < .05$ for P50 and P200 *v* control. *$P < .05$ for P200 *v* controls. §$P < .05$ for P50 *v* control.

3A) and in week 10 in response to 16.7 mmol/L glucose plus 200 μmol/L palmitate (Fig 3B).

Insulin Concentration in the Culture Medium (Insulin Output)

In the long-term studies, palmitate increased insulin output during a 48-hour subculture. The insulin concentration was 91.1 ± 5.2 ng/mL with 0 μmol/L palmitate, 122.5 ± 7.6 ng/mL with 50 μmol/L palmitate, and 187.2 ± 14.9 ng/mL with 200 μmol/L palmitate (n = 36 for each group, $P < .05$).

Gene Expression

Acute Lipotoxicity Study

RNA was extracted after 2 days of culture. Palmitate caused a dose-dependent increase in expression of the CPT-1 gene (Fig 4). The expression of *IRS-2* was suppressed by 49% with 400 μmol/L palmitate and 48% with 1,000 μmol/L. IRS-1 expression decreased by 15% with 400 μmol/L palmitate and 17% with 1,000 μmol/L ($P < .05$). No changes in the expression of insulin 1, IR, HK1, and GLUT2 genes were identified.

Chronic Lipotoxicity Study

The CPT-1 gene expression to a 10-week palmitate culture was time and dose-dependent (Fig 5). Using the high palmitate (P200), CPT-1 expression was already increased after 2 weeks, whereas, it was increased after 6 weeks and 10 weeks of incubation with low palmitate (P50). An increased expression of the GLUT2 gene was found after 4 weeks of culture in 200 μmol/L palmitate. In contrast, palmitate significantly inhibited the expression of the HK1 gene during the entire culture period (reducing it by 58% to 99%). A suppression of the IR gene expression was found at weeks 6 and 10 in cells cultured in 200 μmol/L palmitate (21% at 6 weeks, 47% at 10 weeks; $P < .05$); in week 10, IR gene expression had decreased to 44% in cells cultured in 50 μmol/L palmitate. No changes in the expression of IRS-1 gene were found at any time point. Only at week 1, a suppression of IRS-2 expression was observed in cells cultured in 200 μmol/L palmitate (Fig 5).

Correlation Between Insulin Content and the Expression of Insulin 1 Gene

In acute lipotoxicity, the cellular insulin content decreased significantly at P1,000 (824 ± 200 ng/mg, $P < .05$), but did not change at P400 (2,274 ± 230 ng/mg protein) compared with the cellular insulin content at 0 μmol/L palmitate (1,790 ± 185 ng/mg) (n = 6 for each group).

The chronic lipotoxicity studies showed a cellular insulin content of 1,950 ± 112 ng/mg protein with 0 μmol/L palmitate, 2,183 ± 130 ng/mg with 50 μmol/L, and 2,367 ± 114 ng/mg with 200 μmol/L (n = 36 for each group). Only 200 μmol/L palmitate increased the insulin content significantly ($P < .05$). Correlation analysis showed a positive correlation between

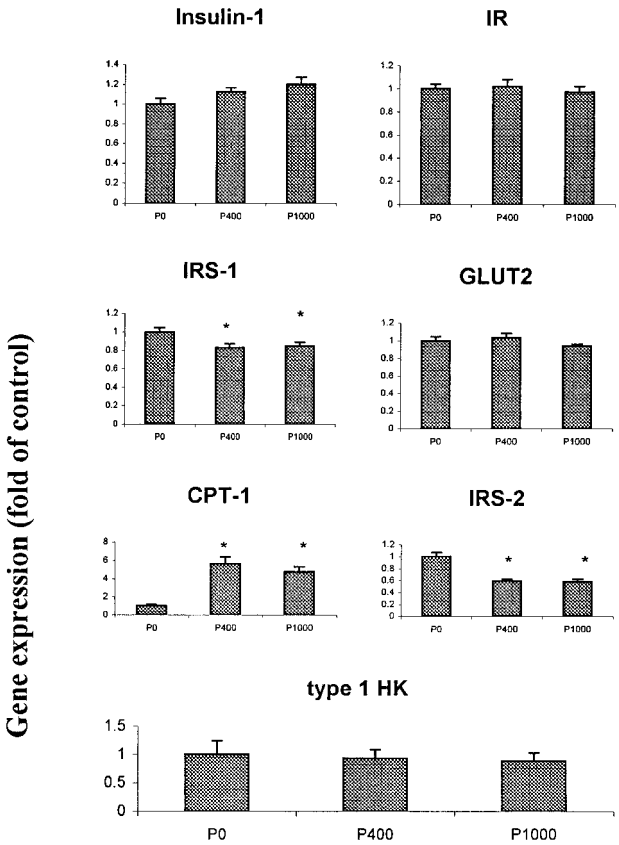

Fig 4. Gene expression (ratio compared with β actin) in INS-1 cells cultured for 48 hours (n = 12 for each column) with 0, 400, or 1,000 μmol/L palmitate in the presence of 6.6 mmol/L glucose. All data are expressed relative to controls. *$P < .05$ *v* corresponding controls.

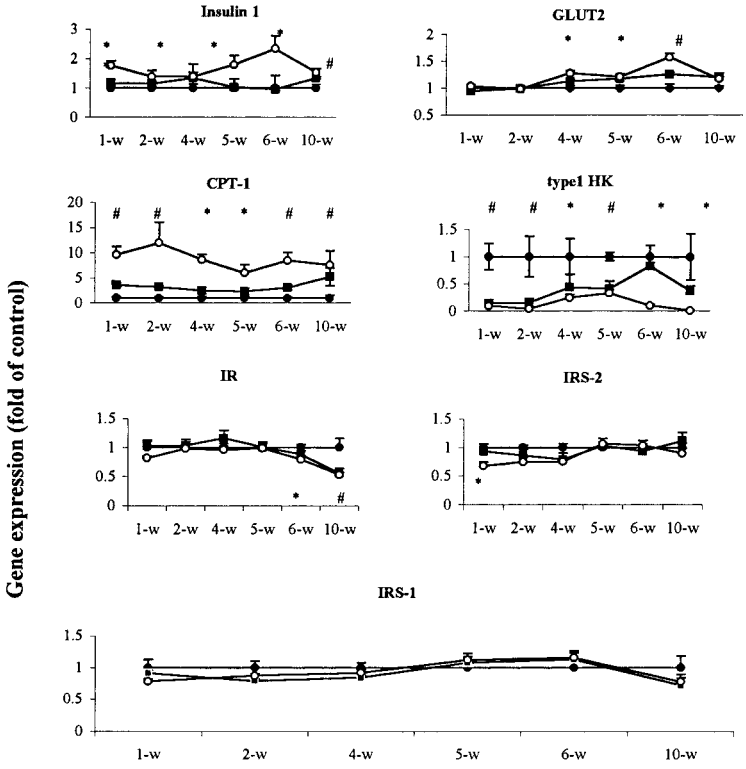

Fig 5. Gene expression (ratio by β actin) in INS-1 cells cultured for 1 to 10 weeks (n = 6 for each) with 0, 50, or 200 μmol/L palmitate in the presence of 6.6 mmol/L glucose (●, control; ■, P50; ○, P200). All data were expressed after normalization by control at the different time points. *$P < .05$ for P200 *v* controls. #$P < .05$ for P50 and P200 *v* controls.

insulin 1 gene expression and insulin content (n = 18, $r = .52$, $P = .026$, Fig 6).

^{3}H-Thymidine Incorporation

INS-1 cell mitogensis was glucose-dependent (Fig 7). The DNA synthesis of cells in the presence of 6.6 and 11 mmol/L glucose was about 2 times higher than in the presence of 3.3 mmol/L glucose. A total of 50 to 200 μmol/L palmitate increased cell mitogenesis with 3.3 mmol/L glucose, but the increment disappeared with 6.6 mmol/L. Cell mitogenesis was inhibited by 400 μmol/L palmitate, and the inhibition was more significant with 11 than with 6.6 mmol/L glucose. The cell number decreased after 48 hours of culture in P1,000 (60% ± 8% of control, n = 8, $P < .05$), while it did not change significantly after culture in P400 (96% ± 8% of control, n = 8, not significant [NS]).

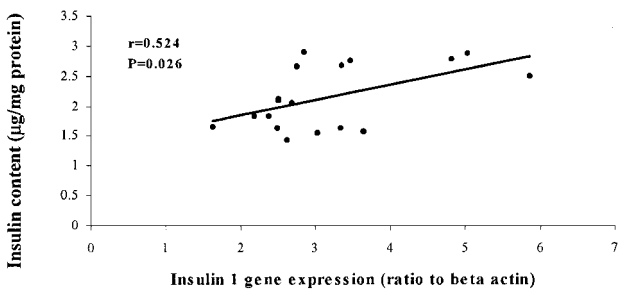

Fig 6. Correlation between average level of insulin 1 mRNA and corresponding average concentration of insulin content (n = 18).

DISCUSSION

In lipotoxicity, in vivo and in vitro experiments show different insulin secretion profiles. Thus, increased BIS and lost responsiveness to glucose stimulation (with increased absolute levels) represent the early stage of lipotoxicity in male Zucker fatty diabetic rats in vivo,[12,13] whereas the absolute insulin deficiency does not appear until later. In vitro, fatty acids elicit increased BIS, but decreased insulin secretion to glucose in absolute values.[7,15] This study was designed to determine how acute and chronic exposure to fatty acids influence the gene expression of the insulin-secreting β-cell line, INS-1. We found that culturing INS-1 cells in the presence of 400 and 1,000 μmol/L palmitate for 2 days inhibited insulin secretion in

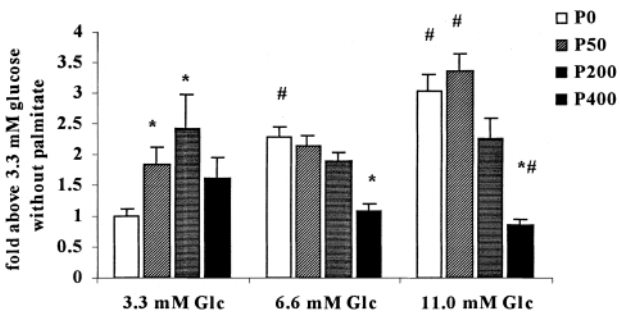

Fig 7. ^{3}H-thymidine incorporation of INS-1 cells in various concentrations of glucose during 24 hours exposure to 0, 50, 200, and 400 μmol/L palmitate (P0, P50, P200, and P400). *$P < .05$ *v* without palmitate, #$P < .05$ *v* same palmitate concentration and with 3.3 mmol/L glucose.

absolute terms. During this acute lipotoxicity, the decreased total cell protein and ^{3}H-thymidine incorporation indicated that a high concentration of palmitate inhibited cell proliferation. Glucose-stimulated insulin secretion peaked in the presence of 6.6 rather than 16.7 mmol/L glucose, suggesting that the cells are more sensitive to low- than to high-glucose levels. In long-term studies, insulin secretion was enhanced for a period of up to 4 weeks in cells cultured with 50 μmol/L and 200 μmol/L palmitate. Interestingly, signs of chronic lipotoxicity occurred after 5 weeks in cells cultured in 200 μmol/L palmitate. An increased insulin secretion was observed at a basal condition with 3.3 mmol/L glucose, while inhibition of glucose and fatty acid–stimulated insulin secretion occurred only in relative, not in absolute terms. The latter seems to resemble the insulin secretion pattern seen in type 2 diabetes[18] and in the prediabetic state,[19] in which pancreatic β cells secrete more insulin than healthy subjects in the basal (fasting) state, whereas the response to glucose is blunted.[20,21]

The mechanism behind the different secretion patterns in acute and chronic lipotoxicity were explored by investigating the expression of specific important genes in INS-1 cells using RT-PCR. As expected, culturing cells with fatty acids increased the expression of CPT-1, the key enzyme in fatty acid oxidation, both after short- and long-term exposure. Increased expression of genes involved in fatty acid oxidation reflects the adaptation to the changes of the external environment.[22] The increased CPT-1 gene expression was accompanied by a 2- to 3-fold higher fatty acid oxidation at low-glucose levels in cells cultured with fatty acids.[22] Fatty acids are the major substrate for INS-1 cell metabolism at low-glucose levels, and we may therefore hypothesize that increased fatty acid oxidation is associated with increased BIS.[22] Increased CPT-1 may limit lipid accumulation within cells, which is associated with lipid-induced apoptosis.[7,15,23] In the present study, CPT-1 gene expression did not increase proportionally to the elevation of fatty acid concentration during acute lipotoxicity (Fig 4), and it decreased relatively over time during chronic lipotoxicity (Fig 5). We have not determined the cellular triglyceride concentration. Further studies are therefore required to elucidate whether these disproportional expressions impact the triglyceride accumulation in β cells. The high fatty acid concentrations did not reduce the expression of GLUT2 and insulin 1 gene. The cellular insulin content decreased in cells cultured in 1,000 μmol/L palmitate. In contrast, palmitate enhanced their expression to some extent in the long-term studies. We also found that this increased gene-expression of insulin 1 correlated with the β-cell insulin content (Fig 6). Separate studies have observed a downregulation of insulin 1 and GLUT2 in INS-1 cells cultured for 2 days with $\geq$ 10 mmol/L glucose with and without 200 μmol/L palmitate; a downregulation that was accompanied by an inhibition of insulin secretion (unpublished observation). Taken together, these data suggest that INS-1 cells may be more vulnerable to glucose toxicity than to lipid toxicity in the downregulation of insulin 1 and GLUT2 gene expression and in the inhibition of insulin secretion. These results are consistent with results in Zucker diabetic fatty (ZDF) rats. In ZDF rats, a reduction of insulin and GLUT2 was observed at both mRNA and protein levels[6]; a reduction that was considered the cause rather than the consequence of hyperglycemia.[24,25] By transplanting islets from ZDF rats to normal rats and transplanting islets from normal rats to ZDF rats, it became obvious that the internal diabetic environment plays a major role in the change of the expression of GLUT2 and insulin 1 gene.[26] Furthermore, the fatty acid inhibition of the expression of insulin and GLUT2 genes was only found in the presence of stimulatory glucose concentrations (16.7 or 30 mmol/L) in some studies,[27,28] suggesting that fatty acids per se may not be sufficient to suppress the expression of GLUT2 and insulin. The relatively low glucose level (6.6 mmol/L) may account for the difference between our data and those reported by others. The discrepancies may, of course, also be attributed to the different experimental conditions of in vivo and in vitro studies, differences between islets and clonal β cells, and/or different concentrations of fatty acids.[6,26,29]

Interestingly, our study showed that the expression of the IR gene was downregulated in cells exposed to 200 μmol/L palmitate for 6 weeks or longer and a corresponding suppression of both glucose and fatty acid–stimulated insulin secretion was observed. However, we did not observe any change in the expression of IR during acute exposure. The INS-1 cells cultured with 200 μmol/L palmitate released more insulin and gave rise to relatively higher levels of insulin in the culture medium (50% and 100% higher than in controls and P50), which may have contributed to the downregulation of the insulin receptor gene expression.[30] Palmitate concentrations are obviously playing an important role in the regulation in β-cell mitogenesis (Fig 7). The mitogenesis was enhanced by 50 to 200 μmol/L palmitate in the presence of low-glucose concentration. This effect disappeared when glucose and palmitate concentrations increased. In contrast, 400 μmol/L palmitate suppressed mitogenesis with 6.6 and 11 mmol/L glucose, indicating that high-palmitate concentrations exert an inhibitory action on cell proliferation. Concomitantly, the suppression of IRS-1 expression was found in cells cultured with 400 and 1,000 μmol/L palmitate, which was accompanied by suppressed expression of the IRS-2 gene. Interestingly, the decreased expression of the IRS-2 gene found after 2 days and 1 week normalized during prolonged palmitate exposure. The insulin receptor and the insulin receptor substrate molecules are playing pleiotropic roles in cell growth, differentiation, and metabolism. Since the discovery of the IR in insulin-secreting β cells,[31,32] a rapidly growing body of evidence indicates that the insulin signal pathway is active and important in the development, replication, and function of β cells.[33-35] This suggests that the changes in the mRNA expression of key enzymes in the insulin signal transduction (IR, IRS-1, and -2) may have contributed to the suppressed insulin secretion in lipotoxicity in the present study. However, we did not observe that the suppression of IRS-2 alone correlated with a decline in insulin secretion. Although this suppression may therefore contribute to the growth suppression of cells in acute lipotoxicity, only the combined suppression of IRS-1 and IRS-2 gene may result in impairment in both insulin and growth factor signal transduction and related functions during acute lipotoxicity. In a study on mice with combined heterozygous null mutations in IR, IRS-1, and IRS-2, it was reported that diabetes developed in 40% of the IR/IRS-1/IRS-2$^{+/-}$, 20% of the IR/IRS-1$^{+/-}$, 17% of the IR/IRS-2$^{+/-}$, and 5% of the IR$^{+/-}$ genotypes.[36] The obser-

vation that the incidence of diabetes decreased when the defects were reduced agrees with our results, ie, the more defects present in the signal pathway, the greater the deterioration of the β-cell function. A concomitant suppression of IRS-1 and IRS-2 gene expressions may play an important role for the impaired cell growth and insulin secretion in acute lipotoxicity.

It has been reported that low K_m hexokinase increases in islets exposed to fatty acids, and that this may account for the hypersensitivity to low-glucose concentrations.[37-39] In the present study, we found a decreased expression of HK1 to P200 at weeks 1, 2, 4, 5, 6, and 10. Forty cycles were needed in PCR for amplification of HK1 cDNA, suggesting that the expression of HK1 gene was low in INS-1 cells. This seems to indicate that HK1 may not be very important for the palmitate-induced increased BIS in INS-1 cells. However, it does not exclude the importance of HK1 in other preparations, because INS-1 cells are a clonal cell line and not normal β cells.

In summary, this study is the first to report that fatty acids can change mRNA expression of genes encoding key proteins in the insulin signaling cascade, ie, IR, IRS-1, and IRS-2. Long-term exposure to lower, more physiologically relevant levels of fatty acid than previously applied increases BIS and reduces the response to glucose. The insulin secretion and gene expression patterns induced by fatty acids depend on the fatty acid concentration and the exposure time.

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