Short-Term Modulation of Extracellular Signal-Regulated Kinase 1/2 and Stress-Activated Protein Kinase/c-Jun NH2-Terminal Kinase in Pancreatic Islets by Glucose and Palmitate

Possible Involvement of Ceramide

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Objectives: The effect of glucose and palmitate on the phosphorylation of proteins associated with cell growth and survival (extracellular signal-regulated kinase 1/2 [ERK1/2] and stress-activated protein kinase/c-Jun NH2-terminal kinase [SAPK/JNK]) and on the expression of immediate early genes was investigated.

Methods: Groups of freshly isolated rat pancreatic islets were incubated in 10-mmol/L glucose with palmitate, LY294002, or fumonisin B1 for the measurement of the phosphorylation and the content of ERK1/2, JNK/SAPK, and v-akt murine thymoma viral oncogene (AKT) (serine 473) by immunoblotting. The expressions of the immediate early genes, *c-fos* and *c-jun*, were evaluated by reverse transcription-polymerase chain reaction.

Results: Glucose at 10 mmol/L induced ERK1/2 and AKT phosphorylations and decreased SAPK/JNK phosphorylation. Palmitate (0.1 mmol/L) abolished the glucose effect on ERK1/2, AKT, and SAPK/JNK phosphorylations. LY294002 caused a similar effect. The inhibitory effect of palmitate on glucose-induced ERK1/2 and AKT phosphorylation changes was not observed in the presence of fumonisin B1. Glucose increased *c-fos* and decreased *c-jun* expressions. Palmitate and LY294002 abolished these latter glucose effects. The presence of fumonisin B1 abolished the effect induced by palmitate on *c-jun* expression.

Conclusions: Our results suggest that short-term changes of mitogen-activated protein kinase and AKT signaling pathways and *c-fos* and *c-jun* expressions caused by glucose are abolished by palmitate through phosphatidylinositol 3-kinase inhibition via ceramide synthesis.

Key Words: pancreatic islets, glucose, palmitate, ERK1/2, SAPK/JNK, ceramide

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Impairments of the response to glucose and/or alterations in the survival of pancreatic β cell are implicated in the onset of type 2 diabetes (DM2). This condition is associated with peripheral

insulin resistance, but diabetes is clinically established only after the impairment of insulin secretory function and the decrease of β -cell mass.¹ Phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways regulate pancreatic β -cell insulin secretion, growth, and cell survival.^{2,3}

We have recently shown that short-term PI3K/AKT/protein kinase C ζ/λ modulation is involved in glucose- and palmitate-induced insulin secretion.⁴ Other proteins from the MAPK family, such as p42 and p44 (ERK1/2) and p46 and p54 (SAPK/JNK) MAPKs, also modulate the insulin secretion.^{5–7} However, the most important role of ERK1/2 and SAPK/JNK for pancreatic β cell is the regulation of proliferation, cell growth, and survival.^{8,9}

Extracellular signal-regulated kinase 1/2 regulates gene expression, mitosis, and cell differentiation,⁸ whereas activation of SAPK/JNK has been described to cause cell death through apoptosis.⁹ Nutrients such as glucose and free fatty acids (FFAs), anticipatory hormones, insulin, and growth factors raise the expression of ERK1/2 in pancreatic β cells.¹⁰ In turn, SAPK/JNK is stimulated by several exogenous and endogenous stress-inducing stimuli including reactive oxygen species, proinflammatory cytokines, and hyperglycemia and high FFA levels.¹¹ In addition, signaling events triggered by glucose and FFA also result in a rapid and transient MAPK-induced activation of immediate early genes (IEGs), including *c-fos* and *c-jun*.^{12,13}

The levels of IEGs in β cells react and rapidly change in response to metabolic signals and transcription factors and regulate, in turn, the transcription of downstream target genes. The importance of such indirect mechanism has recently been shown in genome-scaled studies.^{14,15} Both IEGs and target codes for protein are involved in a large spectrum of cellular functions and long-term cellular adaptations.¹⁵ An important actor in this scenario is the activator protein 1 transcription factor that binds to DNA as a dimer composed by *fos* and *jun* gene products and regulates the transcription of several secondary genes including insulin gene.^{16,17}

Prolonged exposure to elevated levels of palmitate affects glucose-stimulated insulin gene expression, long-term insulin secretion, and cellular replication by mechanisms that involve ceramide synthesis.^{18,19} This sphingolipid is classically reported as a molecule that controls antiproliferative responses such as apoptosis, growth arrest, cell differentiation, and senescence.²⁰

We have recently shown that short-term PI3K activation regulated by ceramide is involved in glucose- and palmitate-induced insulin secretion.⁴ Although the consequences of long-term administration of ceramide on MAPK cascade have been investigated,²¹ the short-time effect of ceramide on pancreatic islet MAPK-regulated functions remains to be investigated. In

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the present study, the effect of glucose and palmitate in up to a 1-hour exposure on the phosphorylation state of ERK1/2 and SAPK/JNK and the expressions of *c-fos* and *c-jun* was examined. The association of the effects induced by glucose and/or palmitate with ceramide synthesis was also investigated.

MATERIALS AND METHODS

Reagents

The reagents for protein assay (BioRad Protein Assay-Dye Reagent Concentrate), the apparatus for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the immunoblotting and nitrocellulose paper (0.45 μ m) were obtained from Bio-Rad (Melville, NY). Trizma, aprotinin, dithiothreitol, phenylmethylsulfonyl fluoride, Triton X-100, glycerol, Tween 20, sodium palmitate, collagenase type V, and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co (St Louis, Mo). Immunoblot-enhanced chemiluminescence detection kit was purchased from Amersham Biosciences/GE Healthcare (Buckinghamshire, UK).

The antibodies against phosphotyrosine; phospho-AKT1/2/3 (serine 473 [Ser473]); and total insulin receptor (IR), IR substrate 1 (IRS1), IRS2, AKT1, and AKT2 were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). The antibodies against phospho-ERK1/2, phospho-SAPK/JNK, and total ERK1/2 and the PI3K inhibitor LY294002 were acquired from Cell Signaling Technology, Inc (Beverly, Mass). Trizol, SuperScript II (200 U), MgCl₂, dNTPs, and *Taq* DNA polymerase were from Invitrogen (Carlsbad, Calif). The band intensity was measured in a UVP Bio-imaging System equipped with LabWork image acquisition and software analysis from UVP Inc (Upland, Calif).

Animals

Female albino rats weighing 250 to 350 g were obtained from the Institute of Biomedical Sciences (University of São Paulo). The animals were kept under standard lighting conditions (12-hour light-dark cycle, lights on at 7:00 A.M.) at 23 $\pm$ 1°C, in groups of 5, and were allowed free access to standard rodent chow and water. The study was approved by the Ethical Committee of the Institute of Biomedical Sciences, Sao Paulo University.

Isolation of Pancreatic Islets

Islets were isolated by collagenase digestion of the exocrine pancreas after a method previously described.²² Hanks buffer containing 137-mmol/L NaCl, 5-mmol/L KCl, 1-mmol/L CaCl₂, 1-mmol/L MgSO₄ 7H₂O, 0.3-mmol/L Na₂HPO₄, 0.4-mmol/L KH₂PO₄, and 4-mmol/L NaHCO₃ was used for isolation and pooling the islets.

Incubation of Pancreatic Islets

Groups of 200 to 300 freshly isolated islets were initially incubated for 30 minutes at 37°C in 0.5 mL of Krebs bicarbonate buffer (KB; 460-mmol/L NaCl, 96-mmol/L NaHCO₃, 20-mmol/L KCl, 4-mmol/L MgCl₂, and 4-mmol/L CaCl₂) with 0.2% albumin. The buffer was equilibrated in a mixture of 95/5% (O₂/CO₂), and pH was adjusted to 7.4. This solution was replaced, and islets were maintained for different periods in KB containing 5.6- or 10-mmol/L glucose, 10-mmol/L glucose plus 0.1-mmol/L palmitate, 10-mmol/L glucose plus 50- μ mol/L LY294002 (a PI3K inhibitor), or 10-mmol/L glucose plus 15- μ mol/L fumonisin B1 (a ceramide synthase inhibitor). Before addition, palmitate was dissolved in ethanol to form an emulsified fatty acid solution. The final ethanol concentration in the incubation

medium was always lower than 0.5%. Preliminary experiments established that this ethanol concentration has no effect on basal insulin release.²³ In experiments with LY294002 and fumonisin B1, the islets were preincubated and postincubated in the absence or presence of the inhibitors as described in the figure legends. The inhibitors were dissolved in dimethyl sulfoxide before addition to the incubation medium. The final dimethyl sulfoxide concentration in the medium was lower than 0.1%, and this concentration was also used in control conditions (5.6- and 10-mmol/L glucose).

At the end of the experiments, the KB was removed, and 80 to 100 μ L of extraction buffer (100-mmol/L Trizma, 10% SDS, 10-mmol/L sodium pyrophosphate, 100-mmol/L sodium fluoride, 10-mmol/L EDTA, and 10-mmol/L sodium vanadate) was added. The islets were then sonicated and boiled for 10 minutes. The extracts were then centrifuged at 12,000g at 4°C for 20 minutes to remove cellular debris. The protein of the supernatant was determined by the Bradford dye method using the Bio-Rad reagent.

Protein Electrophoresis and Immunoblotting

Equal amounts of proteins from the samples were added to Laemmli buffer (1:5) and boiled for 10 minutes before loading the SDS-polyacrylamide gel. The electrotransfer of proteins from the gel to nitrocellulose was performed for 1 to 2 hours at 120 V using a Bio-Rad miniature transfer apparatus. Nonspecific protein binding to nitrocellulose was reduced by preincubating the membrane for 2 hours at 22°C in blocking buffer (5% non-fat dry milk, 10-mmol/L Tris, 150-mmol/L NaCl, and 0.02% Tween 20). The nitrocellulose membranes were incubated for 4 hours at 22°C or overnight at 4°C, with antibodies against phosphotyrosine, IR, IRS1, IRS2, phospho-AKT1/2/3 (Ser473), phospho-ERK1/2, phospho-SAPK/JNK, AKT1, AKT2, or ERK1/2 diluted in blocking buffer replaced by 3% of non-fat dry milk, and then washed for 30 minutes in blocking buffer without milk. The blots were subsequently incubated with peroxidase-conjugated secondary antibody for 1 hour and processed for enhanced chemiluminescence to visualize the immunoreactive bands. When necessary, the membranes were stripped, washed, and reprobed with different primary antibodies. Band intensities were quantified by optical densitometry (Scion Image-Release Beta 3b; National Institutes of Health, Bethesda, Md) of the developed autoradiograph.

Isolation of Total RNA

Total RNA was extracted from fresh islets, using the Trizol reagent (Invitrogen) as described in the product protocol. RNA was spectrophotometrically determined by measuring absorbance at 260 nm. The integrity of the RNA samples was evaluated in 2% (wt/vol) agarose gel containing ethidium bromide and visualized with ultraviolet transillumination.

Reverse Transcriptase-Polymerase Chain Reaction

The RNA used for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was obtained from 3 independent experiments. The RT-PCR was performed with 1 to 2 μ g of total RNA of each experimental condition using Moloney murine leukemia virus reverse transcriptase (Superscript II) and random hexanucleotide primers. The complementary DNA was diluted in a 1:2 ratio, and 1 μ L was used for RT-PCR. The assays were performed in quadruplicate with recombinant *Taq* DNA polymerase and 10 pmol of each primer in a master mix of 100 μ L. Gene-specific primers were designed according to GenBank sequences encoding *c-fos* (X06769), *c-jun* (X17163), *rpl37a* (X14069), and β -actin (NM_031144). The primers used

for the RT-PCR analysis with their respective melting points and product lengths were as follows:

c-fos, 5'-CCCTGCCTCTTCTCAATGACCC-3' (sense) and 5'-CAGCTTGGTGTGTTTCACGCAC-3' (antisense), 60.4°C, 556 bp;
c-jun, 5'-TACGACGATGCCCTCAACGC-3' (sense) and 5'-GTGATGTGCCCATTGCTGGAC-3' (antisense), 59.9°C, 239 bp;
rpl37a, 5'-CAAGAAGGTCGGGATCGTCG-3' (sense) and 5'-ACCAGGCAAGTCTCAGGAGGTG-3' (antisense), 57.5°C, 290 bp; and
 β -actin, 5'-ATGAAGATCCTGACCGAGCGTG-3' (sense) and 5'-CTTGCTGATCCACATCTGCTGG-3' (antisense), 58.5°C, 510 bp.

For each gene, the number of cycles was within the logarithmic phase of amplification and defined through standard curves with 20 to 42 cycles. Polymerase chain reaction products were separated on 1.2% ethidium bromide-agarose gels, and the intensities of the bands were determined by digital scanning and quantified by using Scion Image analysis software (Scion Corp). The results were expressed as a ratio of target gene to *rpl37a* or β -actin signal.

Statistical Analysis

Results are presented as mean (SEM). Significant differences between groups were analyzed by using analysis of

variance followed by the Tukey post test. The level of significance was set at $P < 0.05$.

RESULTS

Temporal ERK1/2, SAPK/JNK, and AKT Phosphorylations Induced by Glucose

The temporal analysis of ERK1/2 (Fig. 1A) and SAPK/JNK (Fig. 1B) phosphorylations induced by glucose revealed a 40% increase and a 26% decrease, respectively, after a 5-minute incubation. This period of glucose exposure was then adopted there on to investigate the combined effect of palmitate with glucose (10 mmol/L). To investigate the mechanism by which glucose changed ERK1/2 and SAPK/JNK phosphorylations, temporal AKT phosphorylation induced by 10-mmol/L glucose was also analyzed. The temporal analysis showed that the maximum increasing effect of glucose on threonine AKT phosphorylation occurred after 1.5 minutes of the stimulus and returned to the unstimulated level at 5 minutes (data not shown). Serine AKT phosphorylation was detected 1.5 minutes after the stimulus and remained increased up to 5 minutes of the stimulation (Fig. 1C). Based on these results, the experiments performed to evaluate the effect of palmitate on glucose-induced phosphorylation of serine AKT were carried out in incubations for 5 minutes.

Palmitate Abolished the Effect of Glucose on AKT Phosphorylation

Palmitate abolished the effect of glucose (10 mmol/L) on ERK1/2 and SAPK/JNK phosphorylations. The values observed for the combination of palmitate with glucose (10 mmol/L) were

FIGURE 1. Time-course response of ERK1/2 (A), SAPK/JNK (B), and AKT (Ser473) phosphorylations (C). Groups of 200 to 300 freshly isolated islets were preincubated for 30 minutes at 37°C in Krebs-Henseleit buffer with 5.6-mmol/L glucose. This solution was then replaced, and islets were maintained for 1.5, 5, 15, 30, 45, or 60 minutes in Krebs-Henseleit buffer containing 10-mmol/L glucose. Protein (100 μ g) extracts from homogenized islets were resolved in SDS-PAGE, transferred to nitrocellulose membranes, and blotted (IB) with specific antibodies. Scanning densitometry was performed on autoradiograms from independent experiments. The results are expressed as mean (SEM). A: pERK1/2: $n = 6$; ERK1/2: $n = 3$; B: pSAPK/JNK: $n = 4$; SAPK/JNK: $n = 3$; C: pAKT (Ser473): $n = 4$; AKT1: $n = 3$; AKT2: $n = 3$; * $P < 0.05$ versus control islets (CTL). CTL indicates control; IB, immunoblotting; pAKT, phospho-AKT; pERK1/2, phospho-ERK1/2; pSAPK/JNK, phospho-SAPK/JNK. The same abbreviations are used in Figures 2–5.

similar to that reported for islets incubated with only 5.6-mmol/L glucose (Figs. 2A and B, respectively). Indeed, incubation of isolated pancreatic islets with palmitate completely abolished the effect of 10-mmol/L glucose on serine AKT phosphorylation (Fig. 2C).

Phosphatidylinositol 3-Kinase Inhibition Mimicked the Effect of Palmitate on Glucose-Induced Changes in AKT, ERK1/2, and SAPK/JNK Phosphorylations

The PI3K inhibitor LY294002 was used to confirm the participation of PI3K in the effect of palmitate on the changes of AKT, ERK1/2, and SAPK/JNK phosphorylations induced by glucose. As observed for palmitate, LY294002 abolished glucose-induced AKT and ERK1/2 phosphorylations (Figs. 3A and C, respectively). Glucose inhibition of SAPK/JNK phosphorylation was also abolished by LY294002 treatment (Fig. 3B).

Palmitate Modulated Glucose Effects on AKT, ERK1/2, and SAPK/JNK Phosphorylations Possibly via Ceramide Synthesis

Palmitate raises the intracellular levels of ceramide by increasing its synthesis.²⁴ Increased de novo synthesis of ceramide inhibits PI3K/AKT pathway in insulin-responsive cells.^{25–27} Fumonisin B1

(a ceramide synthase inhibitor) was used to evaluate whether the effect of palmitate on glucose-induced changes of ERK1/2, AKT, and SAPK/JNK pathways could be a result of ceramide generation resulting in PI3K inhibition. Fumonisin B1 (15 μ mol/L) completely inhibited the effect of palmitate on AKT (Ser473), ERK1/2, and SAPK/JNK phosphorylations (Figs. 4A–C). Under the blockade of ceramide synthesis, palmitate was no longer able to alter the glucose effect on the signaling pathway downstream to PI3K.

Palmitate Abolished Glucose-Induced Changes of *c-fos* and *c-jun* Expressions

The expressions of *c-fos* and *c-jun* genes are regulated by the MAPK signaling cascade.²⁸ Short-term exposure (30 minutes) of isolated pancreatic islets to 10-mmol/L glucose caused an increase of 40% in *c-fos* expression (Fig. 5A) and a decrease of 32% in *c-jun* expression (Fig. 5B). The addition of palmitate and LY294002 abolished the effect of glucose on *c-fos* and *c-jun* expressions. The treatment of pancreatic islets with fumonisin B1 abolished the effect induced by palmitate on *c-jun* but not on *c-fos* expression.

DISCUSSION

High plasma levels of glucose and fatty acids lead pancreatic β -cell to death and so the establishment of diabetes. Short-term

FIGURE 2. Effect of palmitate on glucose-induced phosphorylation of ERK1/2 (A), SAPK/JNK (B), and AKT (Ser473; C) in rat pancreatic islets. Groups of 200 to 300 freshly isolated islets were preincubated for 30 minutes at 37°C in Krebs-Henseleit 5.6-mmol/L glucose. This solution was then replaced, and islets were maintained for 5 minutes in Krebs-Henseleit containing 5.6-mmol/L and 10-mmol/L glucose or 10-mmol/L glucose plus 0.1-mmol/L palmitate. Protein (100 μ g) extracts from homogenized islets were resolved in SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with specific antibody. Scanning densitometry was performed on autoradiograms from independent experiments. The results are expressed as mean (SEM). A: pERK1/2: n = 6; ERK1/2: n = 3; B: pSAPK/JNK: n = 4; SAPK/JNK: n = 3; C: pAKT (Ser473): n = 7; AKT1: n = 3; AKT2: n = 3; * P < 0.05 versus islets exposed to G10 or ** P < 0.05 versus islets exposed to G10.

FIGURE 3. Effect of PI3K inhibitors (LY294002) on glucose-induced changes of ERK1/2, SAPK/JNK and AKT (Ser473) phosphorylation. Groups of 200 to 300 freshly islets were preincubated in the absence or presence of LY294002. The islets were then incubated for 5 minutes in Krebs-Henseleit buffer containing 5.6-mmol/L and 10-mmol/L glucose and 10-mmol/L glucose plus LY294002. Total protein extracts from homogenized islets were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted (IB) with pAKT (Ser473), pERK1/2, and pSAPK/JNK antibodies. Scanning densitometry was performed on autoradiograms from independent experiments. The results are expressed as mean (SEM). A: pERK1/2: $n = 3$; ERK1/2: $n = 3$; pSAPK/JNK: $n = 3$; SAPK/JNK: $n = 3$; pAKT (Ser473): $n = 3$; AKT1: $n = 3$; AKT2: $n = 3$; * $P < 0.05$ versus islets exposed to G5.6 or ** $P < 0.05$ versus islets exposed to G10.

exposure of the pancreatic β cells to high glucose and palmitate concentrations results in a substantial increase of insulin release. However, prolonged exposure of pancreatic β cells to high levels of FFA (>24 hours) increases insulin secretion at low glucose level and leads to desensitization and suppression of the secretory response stimulated by glucose.²⁹ Data obtained in rodent and human pancreatic islets and in β -cell lines indicate that FFAs, in particular, palmitic acid, also decrease β -cell number, essentially by accelerating cell death. Evidence has been accumulated indicating that loss of β cells may also occur in DM2. Studies report a decreased β -cell mass and increased apoptosis in autopsies of the pancreas from patients with DM2 compared with appropriate controls.³⁰

Stress-activated protein kinase/Jun N-terminal kinase cascade plays an important role in the initiation of apoptosis, whereas ERK cascade promotes cell proliferation and/or differentiation.³¹ Accordingly, the balance between SAPK and ERK activation has been proposed as the key determinant of cell survival.³² In this study, the short-term effect (up to 1 hour) of glucose and palmitate on ERK1/2 and SAPK/JNK phosphorylations was investigated.

In agreement with previous studies,^{33,34} high glucose levels increased ERK1/2 phosphorylation. However, in contrast to studies that evaluated the effect of palmitate on insulin-induced phosphorylation of ERK1/2,^{35,36} this fatty acid decreased glucose-induced ERK1/2 phosphorylation.³⁷

High glucose concentration may induce oxidative stress and activation of the proteins of the stress signaling pathway in several tissues, including the pancreas.^{38,39} However, in opposition to the effect on ERK1/2, glucose at 10 mmol/L decreased SAPK/JNK phosphorylation. As also observed by others,^{40,41} we showed that palmitate increased SAPK/JNK activity.

AKT (also known as serine/threonine protein kinase B) is a key mediator of signal transduction processes associated with cell survival.⁴² AKT may suppress the JNK pathway and apoptotic signal in pancreatic islets.⁴³ AKT can indeed regulate the acting of several proteins to suppress the JNK pathway, such as mixed-lineage kinase 3,⁴⁴ JNK-interacting protein 1,⁴⁵ and mitogen-activated protein kinase kinase 4.⁴⁶ In particular, AKT phosphorylates apoptosis signal-regulating kinase 1, leading to the suppression of JNK signaling.⁴⁵ We have recently shown that glucose and palmitate induced short-term regulation of PI3K/AKT phosphorylation.⁴ We then explored the possibility of an AKT-SAPK/JNK crosstalk to occur in islets after treatment with glucose and palmitate. In fact, some authors have demonstrated a temporal and inverse correlation between activation of AKT and SAPK after islet isolation.⁴³ Incubation with glucose significantly increased serine AKT phosphorylation. The curves of glucose effect on AKT and SAPK/JNK phosphorylations indicate an opposite correlation between these two proteins. Palmitate addition abolished the

FIGURE 4. Effect of ceramide synthesis blockage (by fumonisin B1) on changes of AKT (Ser473), ERK1/2, and SAPK/JNK phosphorylations in islets of rats. Groups of 200 to 300 fresh islets were preincubated in the absence or presence of fumonisin B1. The islets were then incubated for 5 minutes in Krebs-Henseleit buffer containing 5.6-mmol/L and 10-mmol/L glucose and 10-mmol/L glucose plus fumonisin B1. Total protein extracts from homogenized islets were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted (IB) with pAKT (Ser473), pERK1/2, and pSAPK/JNK antibodies. Scanning densitometry was performed on autoradiograms from independent experiments. The results are expressed as mean (SEM) [pERK1/2: $n = 4$, ERK1/2: $n = 3$; pSAPK/JNK: $n = 3$, SAPK/JNK: $n = 3$; pAKT (Ser473): $n = 4$; AKT1: $n = 3$, AKT2: $n = 3$; * $P < 0.05$ versus islets exposed to G5.6, ** $P < 0.05$ versus islets exposed to G10, or # $P < 0.05$ versus islets exposed to G10P].

glucose effects on AKT phosphorylation. A similar observation was reported by us and others in pancreatic islets and other tissues.^{4,26}

Glucose-induced changes of ERK1/2, SAPK/JNK, and AKT phosphorylations could be mediated by an autocrine/paracrine effect on insulin release. However, no alteration of IR and IRS1/2 phosphorylation was observed after incubation of pancreatic islets with glucose or glucose plus palmitate for 30 minutes or 1.5 hours (data not shown). Thus, we next evaluated if the effect of glucose on the phosphorylation state of ERK1/2, SAPK/JNK, and AKT was directly associated with PI3K activation, using an inhibitor of this enzyme. LY294002 significantly inhibited the increase of AKT (Ser473) and ERK1/2 phosphorylations induced by glucose. The decrease of SAPK/JNK phosphorylation induced by glucose was also abolished in the presence of LY294002. These observations suggest that activation or inhibition of these proteins is IR and IRS1/2 independent but PI3K dependent.

The mechanism involved in the effect of glucose on PI3K activation, leading to an increase of AKT and consequently a

decrease in SAPK/JNK activity, is not still known. On the other hand, ERK1/2 activation may be directly or indirectly dependent on PI3K.^{47,48}

Ceramides are natural membrane sphingolipids and have been regarded as important second messengers in signaling pathways that regulate several cell functions. Ceramide modulation of apoptosis involves hydrolysis of sphingomyelin or de novo synthesis. Serine/threonine protein kinases, serine/threonine protein phosphatases, and protein kinase C ζ are targets of ceramide. Ceramide formation regulates PI3K activation and, consequently, ERK1/2, SAPK/JNK, and AKT activity.⁴⁹

Palmitate is a substrate for de novo ceramide synthesis, which involves condensation of palmitoyl-CoA with L-serine by serine palmitoyltransferase.⁵⁰ In turn, high glucose levels increase palmitoyl-CoA formation in pancreatic β cell and lead to insulin secretion.⁵¹ We have shown that ceramide formation is involved in the modulation of insulin secretion by glucose and palmitate.⁴ Herein, the effect of glucose and palmitate on the activity of signal proteins under a blockage of ceramide synthesis was investigated. The augmentation of ERK1/2 and AKT (Ser473)

FIGURE 5. Reverse transcriptase-polymerase chain reaction of the expression of *c-fos* and *c-jun* genes. Groups of 200 to 300 fresh islets were preincubated in Krebs-Henseleit containing 5.6-mmol/L glucose. The islets were then incubated for 30 minutes in Krebs-Henseleit containing 5.6-mmol/L and 10-mmol/L glucose, 10-mmol/L glucose plus palmitate, 0.1-mmol/L and 10-mmol/L glucose plus LY294002, or 10-mmol/L glucose plus fumonisins B1. The Krebs-Henseleit was removed, and the RNA was extracted by the Trizol method. The data are shown as mean (SEM) [$n = 5$, $*P < 0.05$ versus islets exposed to G5.6 or $**P < 0.05$ versus islets exposed to G10].

phosphorylations and the reduction of SAPK/JNK phosphorylation induced by glucose were abolished by palmitate. Treatment with fumonisins B1 (an inhibitor of ceramide synthesis) abolished the effect induced by palmitate. Therefore, the effect of palmitate was possibly mediated by ceramide formation.

The expression of genes associated with metabolism, cell cycle, and apoptosis is also regulated by glucose and FFAs.^{52,53} These nutrients modulate the expression of IEGs that are involved in the regulation of cell growth and differentiation. Expression of the IEGs *c-fos* and *c-jun* is regulated by the MAPK signaling cascade.²⁸ In the present study, glucose caused a significant increase of *c-fos* expression but a decreased *c-jun* expression. Palmitate and LY294002 abolished these effects. Other studies, however, showed that glucose induces an increase in the expression of both *c-fos* and *c-jun* in pancreatic tumoral β cell and other cell types, but FFAs induce an increase in the expression of both genes.^{54,55} These discrepancies might occur because of differences between cell lines and pancreatic islets and concentrations of palmitate and/or glucose in the medium. Indeed, incubation with fumonisins B1 slightly abolished the effect induced by palmitate on *c-fos* possibly because of the period of incubation.¹⁵ However, fumonisins B1 was able to abolish the effect induced by palmitate on *c-jun* gene expression.

In summary, our results have shown the short-time effect (up to 30 minutes) of glucose on AKT and MAPK pathways and on *c-fos* and *c-jun* expressions in rat pancreatic islets. These effects were abolished by palmitate treatment and could be a

consequence of a PI3K inhibition through intracellular accumulation of ceramide. These findings suggest a crosstalk and a balance between the MAPK and PI3K/AKT signaling cascades in the regulation of pancreatic β -cell growth and survival. Glucose (at 10 mmol/L) raised pancreatic β -cell survival, whereas palmitate caused an opposite effect. Alterations in the synthesis of early genes such as *c-fos* and *c-jun*, which then regulate late genes such as *GLUT-2*, *PEPCK*, and insulin, may also represent an important mechanism involved in the effect of glucose and palmitate on pancreatic β -cell function.

Changes in the MAPK protein activity induced by short exposure to glucose and/or FFA, via ceramide synthesis, may compromise growth and survival of pancreatic β cells that may lead to failure of pancreatic islet function and DM2 later on.

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