Palmitate modulates the early steps of insulin signalling pathway in pancreatic islets

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Abstract **Insulin stimulates its own secretion and synthesis by pancreatic β-cells. Although the exact molecular mechanism involved is unknown, changes in β-cell insulin signalling have been recognized as a potential link between insulin resistance and its impaired release, as observed in non-insulin-dependent diabetes. However, insulin resistance is also associated with elevated plasma levels of free fatty acids (FFA) that are well known modulators of insulin secretion by pancreatic islets. This information led us to investigate the effect of FFA on insulin receptor signalling in pancreatic islets. Exposure of pancreatic islets to palmitate caused up-regulation of several insulin-induced activities including tyrosine phosphorylation of insulin receptor and pp185. This is the first evidence that short exposure of these cells to 100 μM palmitate activates the early steps of insulin receptor signalling. 2-Bromopalmitate, a carnitine palmitoyl-CoA transferase-1 inhibitor, did not affect the effect of the fatty acid. Cerulenin, an acylation inhibitor, abolished the palmitate effect on protein levels and phosphorylation of insulin receptor. This result supports the proposition that protein acylation may be an important mechanism by which palmitate exerts its modulating effect on the intracellular insulin signalling pathway in rat pancreatic islets.**

Key words: Insulin action; Insulin receptor signalling; Pancreatic β-cell; Palmitate

1. Introduction

The process of glucose-induced insulin secretion in the pancreatic β-cell is dependent on the two signalling pathways. The K_{ATP} channel-dependent pathway acts via elevation of ATP or the ATP/ADP ratio, which closes the ATP potassium channel (K_{ATP}), increasing the intracellular potassium concentration that leads to membrane depolarization [1]. This phenomenon induces the opening of the voltage-sensitive calcium channels provoking a rapid increase in calcium influx and in the intracellular calcium concentration that triggers insulin release [2]. The glucose-induced insulin secretion also activates a K_{ATP} channel-independent pathway, which involves tyrosine protein kinases that control the activities of diverse enzymes and proteins in islet cells [3,4]. Insulin has been demonstrated to activate tyrosine kinase-linked cell surface receptors in β-cells and its intracellular signalling transduction pathway [5]. The insulin receptor (IR) β-subunit, which contains an intrinsic tyrosine kinase activity, undergoes tyrosyl autophosphorylation and is activated after insulin binding. The major early steps of the insulin signalling pathway involve tyrosine phosphorylation of IR substrates 1 and 2 (IRS-1 and IRS-2) [6,7]. Tyrosine phosphorylation of IRS proteins transduces signalling pathways that regulate essential cellular processes including intermediary metabolism, gene expression, growth and differentiation of pancreatic islets [8,9]. Recent studies have shown that activation of the IR in β-cells mediates not only insulin secretion but also insulin gene transcription [10–13]. In insulin-resistant tissues or cells, deficiencies have been shown in several steps of the signalling, including autophosphorylation of the IR in response to insulin binding [14,15].

Effects of free fatty acids (FFA) on pancreatic β-cells have long been recognized. Acute exposure of the pancreatic β-cell to FFA results in an increase of insulin release, whereas a chronic exposure results in desensitization and suppression of secretion [16,17]. These observations raise the possibility that fatty acids may directly modulate insulin secretion in vivo and thus contribute to the coexistence of hyperlipidemia and hyperinsulinemia that is seen in insulin resistance states [18–20]. Although fatty acid oxidation is necessary [17,21], the complete mechanism by which these metabolites modulate insulin secretion remains largely unclear.

We examined if the increase of insulin secretion induced by acute administration of FFA is related to modulation of the early steps of insulin action. The phosphorylation status and expression of IR and IRS-1 in freshly isolated rat pancreatic islets incubated with palmitate were determined. The effect of 2-bromopalmitate (a carnitine palmitoyl transferase-1 (CPT-1) inhibitor) and cerulenin (an inhibitor of protein acylation) was tested to establish the possible mechanism involved.

2. Materials and methods

2.1. Materials

Reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting were obtained from Bio-Rad (Richmond, CA, USA). Trizma, aprotinin, dithiothreitol, Triton X-100, glycerol, Tween 20, sodium palmitate, cerulenin and bovine serum albumin (BSA, fraction V) were obtained from Sigma

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Abbreviations: FFA, free fatty acids; IR, insulin receptor; IRS-1/-2, insulin receptor substrates-1 and -2

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(St. Louis, MO, USA). 2-Bromopalmitate was from Aldrich (Steinheim, Germany). Protein A-Sepharose 6 MB was obtained from Pharmacia (Uppsala, Sweden). Immunoblot ECL chemiluminescence detection kit was from Amersham Pharmacia Biotech (Buckinghamshire, UK) and nitrocellulose paper (0.45 μm) was from Bio-Rad (Hercules, CA, USA). Antibodies against phosphotyrosine, IR, and IRS-1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animals

Female albino rats weighing 250–400 g were kept under standard lighting conditions (12 h light/dark cycle) at a temperature of 23 ± 1°C, in groups of five, and were allowed access to standard rodent chow and water ad libitum until studied. The study was approved by the Ethical Committee of the Institute of Biomedical Sciences, University of São Paulo.

2.3. Isolation of pancreatic islets

The islets were isolated by collagenase digestion of the pancreas following a method previously described [22]. Krebs buffer containing 115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ was used for isolation and pooling of the islets.

2.4. Effect of palmitate, 2-bromopalmitate and cerulenin on insulin signalling proteins

Groups of 200–300 islets were incubated at 37°C in Krebs–bicarbonate buffer containing 5.6 mM glucose equilibrated with a mixture of 95% O_2/5% CO_2, pH 7.4, in the absence or presence of palmitate in the concentrations described in the figure legends and/or 2-bromopalmitate (100 μM) for 30 min and/or preincubation with cerulenin (30 μg/ml). Palmitate and 2-bromopalmitate were dissolved in ethanol before addition into the incubation medium to form an emulsified fatty acid solution. The final ethanol concentration in this fatty acid solution was 0.5%. Preliminary experiments established that 0.5% ethanol has no effect on basal insulin release [23]. At the end of the experiments, the islets were transferred to an Eppendorf tube containing 80 μl extraction buffer (100 mM Trizma, 1% SDS, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA and 10 mM sodium vanadate), homogenized using a Polytron (Brinkmann Instruments, Westbury, NY, USA), and boiled for 5 min. The extracts were then centrifuged at 12 000 rpm at 4°C for 20 min to remove insoluble material. Protein determination in the supernatants was performed by the Bradford dye method using the Bio-Rad reagent [24].

2.5. Electrophoresis and immunoblotting

The proteins were treated with Laemmli sample buffer [25] containing dithiothreitol and boiled for 5 min before loading onto 8% SDS–PAGE in a Bio-Rad miniature slab gel apparatus. Similar-sized aliquots (60 μg) were subjected to SDS–PAGE. The electrotransfer of proteins from the gel to nitrocellulose was performed for 1 h at 120 V (constant) in a Bio-Rad miniature transfer apparatus. Non-specific protein binding to the nitrocellulose was reduced by preincubation for 1 h at 22°C in blocking buffer (5% non-fat dry milk, 10 mM Trizma, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose membranes were incubated for 4 h at 22°C with antibodies against phosphotyrosine, IR, and IRS-1 diluted in blocking buffer with 3% non-fat dry milk, and then washed for 60 min in blocking buffer without milk. The blots were subsequently incubated with peroxidase-conjugated secondary antibody for 1 h, and processed for enhanced chemiluminescence to visualize the immunoreactive bands. For reprobing, membranes were stripped, washed, and incubated with either anti-IR polyclonal antibody or anti-IRS-1 polyclonal antibody. Band intensities were quantified by optical densitometry (Scion Image-Release Beta 3b, NIH, Bethesda, MD, USA) of the developed autoradiographs.

2.6. Statistical analysis

Data are presented as the mean ± S.E.M. Statistical significant differences between groups were analyzed using Student's unpaired *t*-test. The level of significance was set at $P < 0.05$.

3. Results

After the incubation of the islets with Krebs–bicarbonate buffer containing 5.6 mM glucose, in the absence or presence of different concentrations of palmitate (1–100 μM) for 30 min, the islets were homogenized and the total islet extracts were analyzed by immunoblotting using an anti-phosphotyrosine antibody. An increase in tyrosine phosphorylation of IR and pp185 (IRS-1/-2) even at low palmitate concentrations such as 1 μM was detected. The densitometry of three distinct experiments is represented in the graph bars (Fig. 1A–C).

The same membranes were reprobed with anti-IR and anti-IRS-1. We detected an augmentation in IR protein level to 148 ± 12% ($P < 0.05$) in palmitate-treated islets (Fig. 2A). The same approach was used to identify IRS-1 protein level. Similar to IR, the IRS-1 protein level increased to 194 ± 26% ($P < 0.05$) in the palmitate group (Fig. 2B).

In order to investigate if the palmitate-induced tyrosine phosphorylation and increased protein levels of IR and IRS-1 were due to palmitate oxidation, the islets were incubated with 2-bromopalmitate, an inhibitor of CPT-1. The exposure of the islets to palmitate plus 2-bromopalmitate resulted in a similar increase of tyrosine phosphorylation status in IR (by 40 ± 14%, $P < 0.05$) and in pp185 (by 82 ± 34%, $P < 0.05$) to that observed when palmitate was added alone. The incubation of pancreatic islets with 2-bromopalmitate alone did not change the phosphorylation status of IR and pp185 when compared with the control (without fatty acid) (Fig. 3A,B).

Total islet extracts were also analyzed by immunoblotting

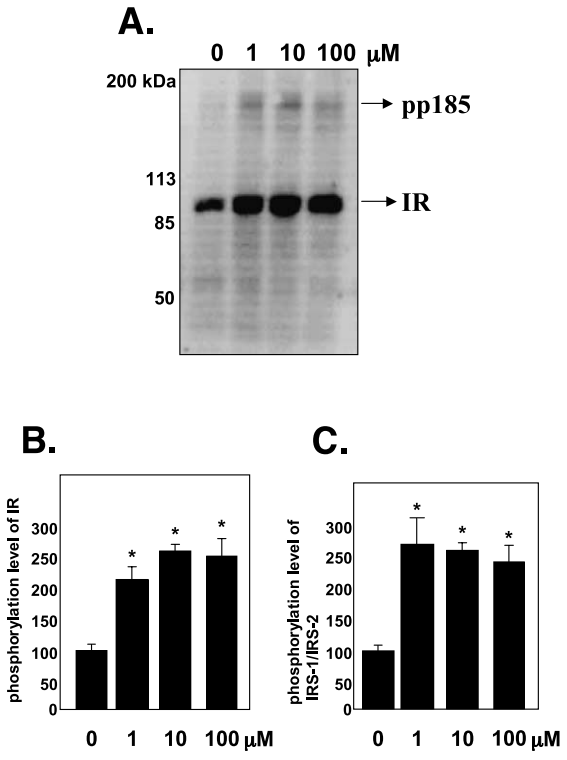

Fig. 1. Palmitate-induced tyrosine phosphorylation of IR and pp185 in isolated pancreatic islets of normal rats. Groups of 200–300 islets were incubated with 5.6 mM glucose in the presence of 1, 10 and 100 μM palmitate for 30 min. Afterwards, the islets were homogenized in extraction buffer at 100°C for 5 min. After centrifugation, aliquots with the same amount of proteins were resolved on 8% SDS–PAGE, transferred to nitrocellulose, and detected with anti-phosphotyrosine antibody (A). Scanning densitometry was performed on autoradiograms from three distinct experiments to determine IR (B) and pp185 (C) tyrosine phosphorylation. The values are expressed as mean ± S.E.M. *$P < 0.05$ compared with 5.6 mM glucose.

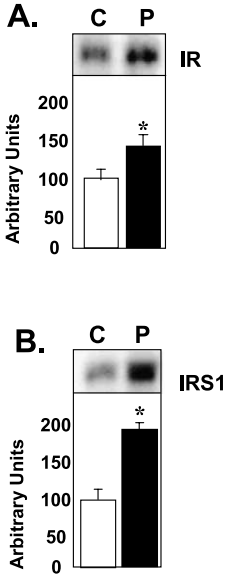

Fig. 2. Palmitate effects on protein levels of IR and IRS-1 in isolated pancreatic islets of normal rats. Groups of 200–300 islets were incubated with 5.6 mM glucose in the absence (C) or presence of 100 μM palmitate (P) for 30 min. The islets were treated as described in the legend of Fig. 1, except that the immunoblot was done using anti-IR (A) and anti-IRS-1 (B) antibodies. Scanning densitometry was performed on autoradiograms from three distinct experiments to determine IR (A) and IRS-1 (B) protein levels. The values are expressed as mean ± S.E.M. $*P < 0.05$.

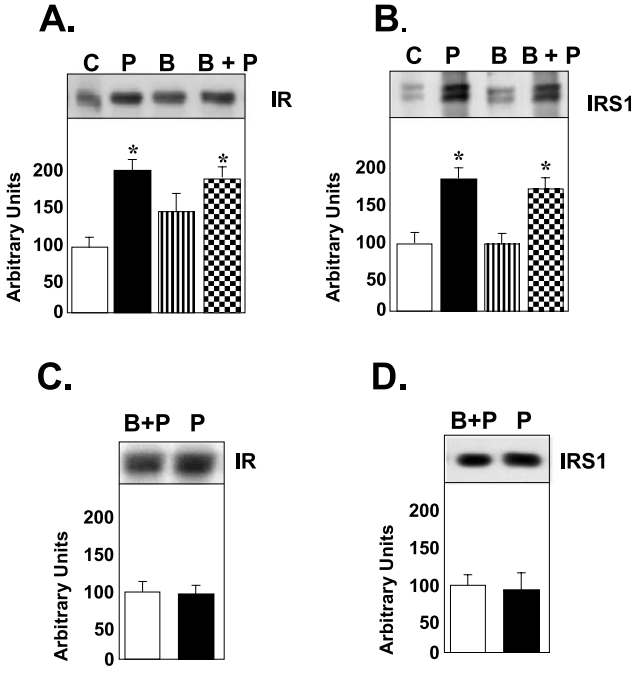

Fig. 3. Effects of inhibition of palmitate oxidation on tyrosine phosphorylation and protein levels of IR and IRS-1. In order to analyze if the effects of palmitate-induced tyrosine phosphorylation and increased protein levels of IR and IRS-1 were due to palmitate oxidation, 2-bromopalmitate, an inhibitor of CPT-1, was used. 100 μM 2-bromopalmitate was incubated in the absence (B) or presence of 100 μM palmitate (B+P) for 30 min. The islets were homogenized and subjected to SDS–PAGE as described in Section 2. A and B are representative of immunoblots using anti-phosphotyrosine antibody. C is a representative blot with anti-IR antibody and D is a representative blot with anti-IRS-1 antibody. Values are expressed as means ± S.E.M. of nine experiments. $*P < 0.05$.

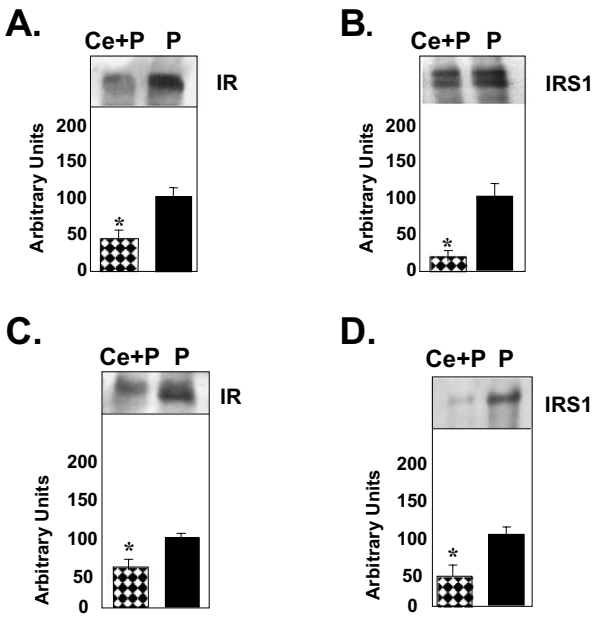

Fig. 4. Effects of inhibition of palmitate acylation on tyrosine phosphorylation and protein levels of IR and IRS-1. In order to analyze if the effects of palmitate-induced tyrosine phosphorylation and increased protein levels of IR and IRS-1 were due to protein acylation, the islets were treated with cerulenin (Ce), an acylation inhibitor, for 30 min prior to palmitate (P) incubation. The islet proteins were isolated as described in Section 2 and in the legends of the previous figures. A and B are representative of immunoblots using anti-phosphotyrosine antibody. C is a representative blot with anti-IR antibody and D is a representative blot with anti-IRS-1 antibody. Values are expressed as means ± S.E.M. of five experiments. $*P < 0.05$.

using anti-IR and anti-IRS-1 antibodies (Fig. 3C,D). The exposure of pancreatic islets to palmitate plus 2-bromopalmitate had the same effect as palmitate alone. Similar to what was observed for tyrosine phosphorylation, the protein levels of IR and IRS-1 remained unchanged in the pancreatic islets incubated with 2-bromopalmitate alone when compared to control.

To determine if protein acylation could be involved in the palmitate-induced IR and IRS-1 tyrosine phosphorylation and increased protein levels, the effect of cerulenin, an acylation inhibitor, was tested. Preincubation with cerulenin at 30 μg/ml for 30 min abolished the IR and pp185 tyrosine phosphorylation induced by 100 μM palmitate to 48 ± 5% ($P < 0.05$; Fig. 4A) and to 78 ± 5% ($P < 0.05$; Fig. 4B) respectively. The increased protein levels of IR and IRS-1 induced by palmitate were also abolished by preincubation of pancreatic islets with cerulenin (Fig. 4C,D). Incubation with cerulenin alone had no effect either on tyrosine phosphorylation or on protein levels of IR and IRS-1 when compared to control (data not shown).

4. Discussion

Evidence is presented herein that palmitate induces tyrosine phosphorylation of some insulin signalling molecules in incubated rat pancreatic islets and also increases their protein levels. Short-term incubation with palmitate in medium containing 5.6 mM glucose induced an increase in tyrosine phosphorylation status and in protein levels of IR and IRS-1 in

isolated rat pancreatic islets. These effects may have a biological significance, since IR autophosphorylation has been correlated with insulin secretion [26].

In β-cells, the cytoplasmic fatty acids are converted to Long-chain acyl-CoA (LC-CoA) by acyl-CoA synthase. In basal conditions, the LC-CoA molecules are transported into the mitochondria via CPT-1, where β-oxidation takes place. High levels of glucose inhibit this process and raise the cytoplasmic content of LC-CoA [27,28]. This action occurs due to the elevated malonyl-CoA concentration formed from glucose metabolism. The activity of CPT-1 is inhibited by high levels of malonyl-CoA, leading to decreased oxidation of fatty acids and an increase in LC-CoA in the cytosol [29,30]. It has been suggested that LC-CoA may act as an effector molecule in the K_{ATP} channel-independent pathway of glucose-induced insulin secretion [2]. LC-CoA controls several β-cell functions, including activation of certain types of protein kinase C, modulation of ion channels, and gene expression [2,20,27]. Fatty acids also affect the late stages of the secretory cascade probably at the level of Ca^{2+} entry into the β-cell or at a point distal to this step (perhaps at the stage of fusion of the insulin granule with the plasma membrane or exocytosis). These actions may result in an efficient β-cell functioning and insulin release.

In spite of the information above, the precise mechanism involved in the effect of FFA on insulin secretion remains unknown. An effect of fatty acids on signalling molecules such as IR, IRS-1 and protein kinase C has been proposed in skeletal muscle cells but little is known about this molecular mechanism in pancreatic islets [31]. We showed herein for the first time that palmitate stimulates the early steps of the insulin signalling pathway in pancreatic islets. Exposure of these cells to palmitate for 30 min showed up-regulation of some insulin-induced activities including tyrosine phosphorylation of IR. These findings are consistent with reports describing that FFA improve insulin sensitivity [16,32] and play a critical role to modulate the stimulatory effect of glucose on insulin release [17,33]. The increased IR phosphorylation in isolated pancreatic islets at 5.6 mM glucose by palmitate may play a role in the cellular mechanism by which this fatty acid modulates glucose-induced insulin secretion. Using the CPT-1 inhibitor 2-bromopalmitate, we could detect that palmitate-induced tyrosine phosphorylation and increased protein levels of IR and IRS-1 were not a result of fatty acid oxidation.

Several Src family members can be acylated and palmitate regulates their membrane binding and subcellular distribution [34–37]. The group of Src family tyrosine kinases is modified by saturated fatty acyl chains, i.e. myristoyl and palmitoyl moieties. Palmitoyl-CoA can attach posttranslationally to protein cysteine residues through a thioester linkage in a reversible modification with dynamically regulated cycles of acylation and deacylation. The role of protein acylation includes direction of proteins to appropriate membrane sites, stabilizing protein–protein interactions, and regulating enzyme activities in mitochondria [38]. Palmitoylation of receptor tyrosine kinases is of particular importance since these proteins are proximal components that transduce diverse receptor-mediated signalling pathways. Modulation of their functional activity by palmitate may affect the downstream signalling pathway and target gene expression [39]. Cerulenin abolished the palmitate effect on protein levels and phosphorylation of IR and IRS-1. This result supports the proposition that protein acylation is involved in the activation of the early steps of insulin signalling in isolated pancreatic islets induced by palmitate.

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