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Glucose stimulation of protein acylation in the pancreatic β -cell

Mossaad Abdel-Ghany, Geoffrey W. G. Sharp, and Susanne G. Straub*

The Department of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853 USA

Abstract

Aims—To determine whether protein acylation plays a role in the effects of glucose on the insulin secreting β -cell.

Main methods—The measurement of ^3H -palmitate incorporation into protein in the INS 832/13 cell that has a robust and well-characterized biphasic insulin secretory response to stimulation with glucose.

Key findings—Stimulating the cells with glucose increased the incorporation of ^3H -palmitic acid into protein by up to 90%. Similarly, 2-aminobicyclo [2.2.1] heptane-2-carboxylic acid (BCH) the non-metabolizable analog of leucine that mimics the stimulatory effect of glucose on insulin secretion also increased the incorporation of ^3H -palmitic acid into protein. Treatment of cell lysates with hydroxylamine substantially reduced the incorporation indicating that most of the incorporation was due to enzymatic palmitoylation of proteins. Cerulenin, a classical inhibitor of protein acylation also substantially reduced the incorporation. Using PAGE and autoradiography a glucose-induced increase in protein palmitoylation and specific glucose-induced increases in the palmitoylation of proteins of 30, 44, 48 and 76 kD were identified.

Significance—The data suggest that protein acylation plays multiple roles in β -cell function.

Keywords

Glucose; biphasic insulin secretion; signaling pathways; protein acylation

Introduction

The hypothesis that protein acylation could be involved in stimulus-secretion coupling for glucose-stimulated insulin secretion was first suggested in 1989 (Corkey et al. 1989). Subsequent studies proposed that protein acylation might be involved in the KATP channel-independent (amplification) pathway responsible for time-dependent potentiation (TDP) and the second phase of glucose-stimulated insulin secretion (Yamada et al. 2002; Straub et al. 2002). Circumstantial evidence in support of the idea has been based primarily on the malonyl CoA long chain acyl CoA hypothesis (Corkey et al. 1989; Prentki et al. 1992) and on the use of cerulenin, a fungal antibiotic and well-known inhibitor of protein acylation (Straub et al. 2002; Yajima et al. 2000). Unfortunately, cerulenin has a profound inhibitory

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*Corresponding author, Address correspondence to: Dr. Susanne G. Straub, The Department of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853 USA, Telephone: 1-607-253-3650; facsimile 1-607-253-3659. sgs4@cornell.edu.

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effect on metabolism in the β -cell (Straub and Sharp 2006) and this alone could be responsible for the inhibition of glucose-stimulated secretion. Consequently, direct evidence of a role for protein acylation in stimulus-secretion coupling rather than indirect evidence is required. The first attempt to seek such evidence was by Yamada et al (Yamada et al. 2003). In their paper they identified ^3H -palmitoylated proteins by SDS-PAGE and two-dimensional gel electrophoresis after labeling rat pancreatic islets with ^3H -palmitate. Among several palmitoylated bands detected, the palmitoylation in four major bands was reduced when the labeling was performed in the presence of high glucose or other nutrients such as the combination of leucine and glutamine, and α -ketoisocaproic acid. This reduction was ascribed to the effect of glucose and mitochondrial fuels to decrease fatty acid oxidation, increase long chain acyl-CoA levels and thereby decrease the specific activity of the ^3H -palmitic acid in the cell. As the rat pancreatic islet only contains $\sim 70\%$ β -cells, we decided to study a pure β -cell population in our current work. Thus we measured ^3H -palmitate incorporation by a method that is still regarded “as the gold standard” (Magee 2009) and used the INS 832/13 cell (Hohmeier et al. 2000) that has a robust and well-characterized biphasic response to stimulation with glucose (Straub and Sharp 2000).

Methods

Cell Culture

INS 832/13 cells at passages 61 to 70 were grown in 6-well plates. They were cultured in RPMI containing 11.1 mM glucose with 10% FBS, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM β -mercaptoethanol, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in a 95% air plus 5% CO_2 atmosphere.

Labeling INS 832/13 cells with [$9,10\text{-}^3\text{H}$] Palmitic acid and the determination of protein palmitoylation

18 hrs prior to the experiments the glucose concentration in the culture medium was reduced to 5 mM. For the experiments cells were preincubated for 30 minutes at 37°C in KRBH buffer with 10 mM HEPES at pH 7.4 supplemented with 0.1 % bovine serum albumin and 2.8 mM glucose (basal conditions). They were then incubated under the same conditions for 60 minutes in the presence of 30 $\mu\text{Ci}/\text{ml}$ ^3H -palmitic acid before application of the test and control conditions for a further 30 min still in the presence of ^3H -palmitic acid. At the end of the incubation period, the cells were washed twice with KRBH buffer, harvested and solubilized in an extraction buffer of 50 mM Tris, 1% Triton X-100, 0.5% DOC, 0.1% SDS and 1 mM of the protease inhibitor PMSF at pH 7.4 for 1 hour at 4°C . The extracts were centrifuged for 20 minutes at 15,000 g, supernatants collected and protein measured by the BCA method. Lysate samples containing 20–50 μg protein in 50 μl of the extraction buffer were mixed with 150 μl of 1% SDS + 2 mg/ml BSA. 500 μl of 10% TCA was then added to precipitate the protein and the precipitates were collected on Whatman GF/A glass fiber filters. Where hydroxylamine was used the lysates were treated with 1 M hydroxylamine or 1 M Tris (as control) at 37°C for 1 – 2 hours before protein precipitation and collection on filters as already described. The filters were washed five times each with 1 ml of 5% TCA, five times each with 1 ml of 50% ethanol and 3% TCA, and three times each with 1 ml of chloroform/methanol (2:1). The filters were then dried before scintillation spectrometry to determine the amount of incorporated ^3H -palmitic acid. When cerulenin was used an additional preincubation with or without 30 or 100 μM Cerulenin was made prior to the addition of the ^3H -palmitic acid. The results are expressed as $\text{cpm} \times 10^{-4}/\text{mg}$ protein. The method is a modified version of that of Duncan and Gilman (Duncan and Gilman 1996).

Polyacrylamide gel electrophoresis and autoradiography

After incorporation of ^3H -palmitic acid as described above, lysates were subjected to SDS-PAGE on 15% gels. Subsequently, the gels were fixed in a solution of 10% acetic acid and 30% methanol (v/v) for 30 minutes, followed by a wash in 15–20 volumes of water for 30 minutes. The gels were soaked in Fluoro-Hance (Research Products International Corp., Mt. Prospect, IL) and agitated gently at room temperature for 30 minutes. They were then dried and exposed to Kodak X-OMAT film for periods ranging from 10 – 60 days at -80°C .

Chemicals

Cerulenin, hydroxylamine and BCH were obtained from Sigma. $[9,10\text{-}^3\text{H}]$ palmitic acid was from Perkin Elmer, Boston MA.

Statistics

Statistical significance was determined by ANOVA and *post hoc* Fisher's least significance difference test.

Results

In the two left hand bars of figure 1A is shown the effect of 16.7 mM glucose on ^3H -palmitate incorporation into protein compared with the control condition in which the cells are exposed to 2.8 mM glucose. These two concentrations were chosen because they are the most frequently used concentrations in studies of glucose-stimulated insulin secretion *in vitro* where basal conditions and maximal stimulation is required. 16.7 mM glucose increased the incorporation into protein by 68% from 47 ± 2.1 to $78 \pm 3.8 \text{ cpm} \times 10^{-4}/\text{mg}$ protein ($P < 0.05$, $n = 6$).

To determine whether the incorporation was due to protein acylation, experiments were performed in which the lysates were incubated for 1 – 2 hours at 37°C with either 1 M Tris buffer (control) or 1 M hydroxylamine which cleaves the ^3H -palmitate from the protein (Bizzozero 1995) (figure 1A). Under control conditions in which the lysates were incubated in Tris buffer, 16.7 mM glucose increased the incorporation of ^3H -palmitate into protein by 89 % from 40 ± 2.7 to $77 \pm 6.1 \text{ cpm} \times 10^{-4}/\text{mg}$ protein ($P < 0.05$). These values are similar to those obtained without treatment with Tris. Incorporation in the presence of 2.8 mM glucose followed by hydroxylamine treatment was reduced by 67% from 40 ± 2.7 to $16 \pm 7.0 \text{ cpm} \times 10^{-4}/\text{mg}$ protein ($P < 0.05$). Incorporation in the presence of 16.7 mM glucose followed by hydroxylamine treatment was reduced by 55% from 77 ± 6.1 to $30 \pm 9.6 \text{ cpm} \times 10^{-4}/\text{mg}$ protein ($P < 0.05$). Thus much of the incorporation of ^3H -palmitate into protein under control and glucose-stimulated conditions is due to protein palmitoylation. Studies were also performed with cerulenin, a well-known inhibitor of protein acylation. Cerulenin, at 30 and 100 μM , decreased the incorporation under both basal and stimulated conditions (figure 1B). In the presence of high glucose a reduction in the ^3H -palmitic acid incorporation of 42% (78 ± 3.8 to $45 \pm 5.1 \text{ cpm} \times 10^{-4}/\text{mg}$ protein) and 62% (78 ± 3.8 to $30 \pm 4.0 \text{ cpm} \times 10^{-4}/\text{mg}$ protein) respectively was observed. The results of the experiments with hydroxylamine and cerulenin show that protein acylation is occurring under basal conditions in the INS 832/13 cell and that it is stimulated by high glucose.

As it has been proposed that glucose increases malonyl CoA levels, thereby inhibiting CPT-1 and fatty acid oxidation, and increasing cytosolic acyl CoA levels in the β -cell (Corkey et al. 1989; Prentki et al. 1992; Corkey et al. 2000), the possibility exists that the specific activity of the ^3H -palmitoyl CoA in the cell would be reduced by the putative increase in acyl CoA's. This would have the effect of reducing the incorporation of ^3H -palmitate into protein. To determine whether this was the case we studied the effect of 2-

aminobicyclo [2.2.1] heptane-2-carboxylic acid (BCH), which is a non-metabolizable analog of leucine on the incorporation of ^3H -palmitate into protein. BCH mimics the effect of high glucose on insulin secretion without having any effect on palmitate oxidation and, therefore, without any effect on the concentration of long chain acyl CoA's in the cytosol (Liu et al. 2003). Therefore, the possibility exists that BCH would give an even larger increase in the incorporation of ^3H -palmitate into protein than glucose. The results are shown in figure 2. BCH, used at 20 mM, the concentration at which it best mimics the effect of 16.7 mM glucose on insulin secretion (Liu et al. 2003), stimulated the incorporation of ^3H -palmitate into protein by 75% (29 ± 4.0 to $51 \pm 7.8 \text{ cpm} \times 10^{-4}/\text{mg protein}$, $P < 0.05$) an effect not dissimilar from that of 16.7 mM glucose but certainly not larger. 30 and 100 μM cerulenin reduced the incorporation of ^3H -palmitate into protein under BCH-stimulated conditions by 65% and 85% to 18 ± 4.8 and $8 \pm 2.1 \text{ cpm} \times 10^{-4}/\text{mg protein}$ respectively ($P < 0.05$ for both). Experiments with hydroxylamine confirmed that most of the ^3H -palmitate incorporation into protein under control and BCH-stimulated conditions was in fact due to protein palmitoylation. Hydroxylamine treatment reduced protein acylation under BCH-stimulated conditions by 68% from 55 ± 4.9 to $17 \pm 1.7 \text{ cpm} \times 10^{-4}/\text{mg protein}$ ($P < 0.05$). These results are similar to the effects of hydroxylamine and cerulenin treatment on ^3H -palmitate incorporation in the presence of 2.8 and 16.7 mM glucose that are shown in figure 1.

In the final series of experiments, polyacrylamide gel electrophoresis and autoradiography was employed to determine whether the effect of 16.7 mM glucose to increase protein acylation was due to an overall increase in the acylation of many proteins of a wide range of molecular weights, whether there was increased acylation in a few proteins that accounted for the 90% increase in overall incorporation of ^3H -palmitate, or whether both phenomena could be detected. In eight such experiments in which the effect of 16.7 mM glucose was compared to 2.8 mM glucose, increased incorporation of ^3H -palmitate was observed in proteins of 30, 44, 48, and 76 kD (figure 3, left hand bars). The increased incorporation of ^3H -palmitate into protein of 30 kD occurred in 7 out of the 8 gels; into proteins of 44 and 48 kD in 6 out of the 8 gels; and into protein of 76 kD in all 8 of the gels. In paired experiments, where the cells were incubated with 2.8 and 16.7 mM glucose plus 100 μM cerulenin, the incorporation under both control and stimulated conditions was markedly reduced (figure 3, right hand bars). These data confirm the effects of cerulenin to decrease the palmitoylation of proteins under both basal and stimulated conditions as shown in figure 1b. In an additional experiment, incorporation of ^3H -palmitate was determined in three paired sets of cells incubated with 2.8 and 16.7 mM glucose and subjected to polyacrylamide gel electrophoresis. After cutting the gels, one of the three was treated with 1M hydroxylamine, one with 1M Tris (control) and the other was untreated (control for any possible effects of Tris). Subsequent autoradiography showed that the hydroxylamine treatment reduced the incorporation of ^3H -palmitate under both control conditions with 2.8 mM glucose and under stimulated conditions with 16.7 mM glucose (data not shown). This result is similar to that of the experiments shown in figure 3 in which cells were treated with cerulenin. In view of the cerulenin and hydroxylamine data presented in figure 1, and the fact that cerulenin blocks the palmitoylation of proteins and that hydroxylamine cleaves off palmitate from palmitoylated proteins, this result was expected.

Discussion

Glucose stimulus-secretion coupling in the β -cell consists of at least two pathways (Straub and Sharp 2000). The triggering pathway (also called the KATP channel-dependent pathway) acts to close KATP channels, depolarize the cell and activate voltage-dependent Ca^{2+} channels to increase Ca^{2+} influx and $[\text{Ca}^{2+}]_i$. This triggers the release of insulin-containing granules. The second pathway, the amplification pathway synergizes with the triggering pathway to augment the release induced by elevated $[\text{Ca}^{2+}]_i$. Glucose metabolism

is essential for the operation of both pathways. An increase in the ATP/ADP ratio in the cell is certainly responsible for activation of the triggering pathway. However, the signaling mechanisms responsible for the amplification pathway are currently unknown. Several hypotheses have been advanced to explain the mechanisms but none have gained general acceptance (Straub and Sharp 2000). Surprisingly, despite the widespread recognition of the importance of protein acylation in cell signaling generally (Linder and Deschenes 2007; Charollais and Van Der Groot 2009; Baekkeskov and Kanaani 2009), little work has been done in this area on the β -cell. Two studies with cerulenin suggested that protein acylation could be involved in the amplification pathway (Straub et al. 2002; Yajima et al. 2000). For example, cerulenin blocked glucose-stimulated insulin secretion without affecting the response to a depolarizing concentration of KCl (Yajima et al. 2000) implying that Ca^{2+} -signaling and exocytosis were unaffected by cerulenin but that glucose-signaling was blocked. The idea was supported by a study in HIT-T15 cells in which Ca^{2+} -induced exocytosis (measured under patch clamp conditions as whole cell capacitance change) was enhanced by the addition of long chain acyl CoA and the enhancement reversed by cerulenin (Deeney et al. 2000). These inhibitor studies indicate the need for direct evidence of a role for protein acylation in stimulus-secretion coupling.

In the current study we used a classical method to measure the incorporation of ^3H -palmitate into protein. Incorporation of ^3H -palmitate into protein occurred under both basal and stimulated conditions. A stimulatory concentration of glucose (16.7 mM) increased the incorporation of ^3H -palmitate into protein by $\sim 90\%$. That most of the incorporated ^3H -palmitate under basal and stimulated conditions was due to acylation of proteins was shown by the marked reduction in incorporation seen after treatment of the lysates with hydroxylamine that cleaves the palmitate from the protein. The incorporation was also reduced to a similar extent by cerulenin. These experiments indicate that protein acylation is occurring under basal conditions in the INS 832/13 cell and that it is stimulated by high glucose.

A major hypothesis underlying the mechanisms responsible for the second phase of glucose stimulated insulin secretion was that glucose increases malonyl CoA levels, inhibits CPT-1 and fatty acid oxidation, and increases cytosolic acyl CoA levels in the β -cell to increase the rate of exocytosis (Corkey et al. 1989; Prentki et al. 1992). In accord with this hypothesis, the specific activity of the ^3H -palmitoyl CoA in the cell should be reduced by a glucose-induced increase in acyl CoA's. This would result in a reduction of the incorporation of ^3H -palmitate into protein even though the palmitate incorporation was unchanged. It was for this reason that we examined the effect of BCH a non-metabolizable analog of leucine on the incorporation of ^3H -palmitate into protein. BCH stimulates insulin secretion without affecting palmitate oxidation or lipid incorporation (Liu et al. 2003). It should therefore have no effect on the concentration of long chain acyl CoA's or the specific activity of ^3H -palmitate. BCH also mimics the stimulatory effect of glucose on insulin secretion in the INS 832/13 cell (data not shown). In our current experiments, it stimulated the incorporation of ^3H -palmitate into protein by $\sim 70\%$, similar to, and certainly no greater than the effect of glucose. Control experiments with hydroxylamine and cerulenin confirmed again that most of the ^3H -palmitate incorporation into protein under control and the BCH-stimulated conditions was in fact due to protein palmitoylation. Thus, from these data, there is no evidence to suggest that stimulation with glucose is decreasing the specific activity of ^3H -palmitate in the cell. These data are in contrast to those of Yamada *et al* (2003) who suggested a functional role for a rapidly turning over palmitoylated protein of 24 kD in nutrient stimulation of insulin secretion, primarily on the basis that in islets its palmitoylation was decreased by glucose and mitochondrial fuels. The decrease was explained on the hypothesis, as already mentioned, that the specific activity of the ^3H -palmitoyl CoA in the cell could be reduced by a nutrient-induced increase in acyl CoA's and

reduction of the specific activity of the intracellular ^3H -palmitate. We see no evidence of this in a pure clonal β -cell line. Furthermore, our results from PAGE show increased acylation in several protein bands in response to glucose. This could suggest that the decreased palmitoylation detected by Yamada *et al* occurred in the ~30% of non- β -cells in the pancreatic islets and not in the β -cells.

Conclusions

Our results, showing that protein acylation is occurring under basal conditions and is stimulated by secretagogues are in accord with the idea that protein palmitoylation, consisting of cycles of acylation and deacylation is involved in signal transduction by glucose and other insulin secretagogues. Among numerous possible protein targets for acylation in the β -cell are ion channels (Gubitosi-Klug *et al.* 2005; Hurley *et al.* 2000), G-proteins (Cao and Huang 2005), receptors (Tobin and Wheatley 2004) and SNARE proteins (Roth *et al.* 2006) that are implicated in stimulus secretion coupling. In this regard we found that two inhibitors of protein acylation, cerulenin and tunicamycin, both increase Ca^{2+} currents through L-type voltage-dependent Ca^{2+} channels in the INS 832/13 cells (Zhao *et al.* 2007) showing that protein acylation is attenuating the responsiveness of these channels. Additionally, we have provided evidence that protein acylation may be involved in the mechanisms of inhibition of insulin secretion by the physiological inhibitors norepinephrine, somatostatin, galanin and PGE_2 (Cheng *et al.* 2003). This suggests that in the same way that signaling mechanisms involving protein phosphorylation can have stimulatory or inhibitory effects within the cell depending upon their targets, so protein acylation can do likewise. It is important now to identify the targets of protein acylation in the β -cell signal transduction pathways and to determine the effects and physiological control of protein acylation on their function.

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Figure 1.

Incorporation of ³H-palmitate into protein in the presence of 2.8 mM and 16.7 mM glucose, and the effects of hydroxylamine and cerulenin. ³H-palmitate incorporation is expressed as cpm × 10⁻⁴/mg protein. **A.** The results shown in the left hand bars are the incorporation that occurred in the presence of 2.8 mM and 16.7 mM glucose. The results shown in the middle and right hand bars are the incorporation that occurred in the presence of 2.8 mM and 16.7 mM glucose after the cell lysates were treated with either 1 M Tris or 1 M hydroxylamine. **B.** The effects of treating the cells with 30 and 100 μM cerulenin on the incorporation of ³H-palmitate into protein in the presence of 2.8 mM and 16.7 mM glucose.

Figure 2.

Incorporation of ³H-palmitate into protein in the presence of 2.8 mM glucose and 2.8 mM glucose plus 20 mM BCH, and the effects of hydroxylamine and cerulenin. ³H-palmitate incorporation is expressed as cpm × 10⁻⁴/mg protein. **A.** The results shown in the left hand bars are the incorporation that occurred in the presence of 2.8 mM glucose and 2.8 mM glucose plus 20 mM BCH. The results shown in the middle and right hand bars are the incorporation that occurred in the presence of 2.8 mM glucose and 2.8 mM glucose plus 20 mM BCH after the cell lysates were treated with either 1 M Tris or 1 M hydroxylamine. **B.** The effects of treating the cells with 30 and 100 μM cerulenin on the incorporation of ³H-palmitate into protein in the presence of 2.8 mM glucose and 2.8 mM glucose plus 20 mM BCH.

Figure 3.

Autoradiograph of a polyacrylamide gel following electrophoresis of cell lysates showing the incorporation of ^3H -palmitate into protein under control, glucose-stimulated and cerulenin-treated conditions. In the two left hand gels INS 832/13 cells were incubated in the presence of ^3H -palmitate and 2.8 mM glucose and 16.7 mM glucose respectively before lysis and electrophoresis; in the two right hand gels the cells were again incubated in the presence of ^3H -palmitate and 2.8 mM glucose and 16.7 mM glucose respectively but also in the presence of 100 μM cerulenin. The numbers and arrows indicate the molecular weights of bands of interest that were increased by 16.7 mM glucose (30, 44, 48, and 76 kD). These

gels are representative of 8 such gels for 2.8 and 16.7 mM glucose, and 5 such gels for cerulenin.