Insulin Mediates Glucose-stimulated Phosphorylation of PHAS-I by Pancreatic Beta Cells

AN INSULIN-RECEPTOR MECHANISM FOR AUTOREGULATION OF PROTEIN SYNTHESIS BY TRANSLATION*

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Guang Xu‡, Connie A. Marshall‡, Tai-An Lin‡, Guim Kwon‡, Raghava B. Munivenkatappa‡, Jeanette R. Hill‡, John C. Lawrence, Jr.§, and Michael L. McDaniel¶

From the ‡Department of Pathology, Washington University School of Medicine, St. Louis Missouri 63110 and §Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Although glucose regulates the biosynthesis of a variety of beta cell proteins at the level of translation, the mechanism responsible for this effect is unknown. We demonstrate that incubation of pancreatic islets with elevated glucose levels results in rapid and concentration-dependent phosphorylation of PHAS-I, an inhibitor of mRNA cap-binding protein, eukaryotic initiation factor (eIF)-4E. Our initial approach was to determine if this effect is mediated by the metabolism of glucose and activation of islet cell protein kinases, or whether insulin secreted from the beta cell stimulates phosphorylation of PHAS-I via an insulin-receptor mechanism as described for insulin-sensitive cells. In support of the latter mechanism, inhibitors of islet cell protein kinases A and C exert no effect on glucose-stimulated phosphorylation of PHAS-I, whereas the phosphatidylinositol 3-kinase inhibitor, wortmannin, the immunosuppressant, rapamycin, and theophylline, a phosphodiesterase inhibitor, promote marked dephosphorylation of PHAS-I. In addition, exogenous insulin and endogenous insulin secreted by the beta cell line, βTC6-F7, increase phosphorylation of PHAS-I, suggesting that beta cells of the islet, in part, mediate this effect. Studies with beta cell lines and islets indicate that amino acids are required for glucose or exogenous insulin to stimulate the phosphorylation of PHAS-I, and amino acids alone dose-dependently stimulate the phosphorylation of PHAS-I, which is further enhanced by insulin. Furthermore, rapamycin inhibits by ~62% the increase in total protein synthesis stimulated by high glucose concentrations. These results indicate that glucose stimulates PHAS-I phosphorylation via its own receptor on the beta cell which may serve as an important mechanism for autoregulation of protein synthesis by translation.

The metabolism of glucose regulates a variety of physiological processes by beta cells of the islets of Langerhans. These processes include insulin biosynthesis and secretion, beta cell replication, and the synthesis of a number of beta cell proteins. Glucose exerts a specific stimulatory effect on insulin gene expression over time intervals of hours (1, 2), whereas the biosynthesis of insulin is significantly controlled within minutes at the level of protein translation (3–5). Although the enhancement of insulin synthesis by glucose at the translational level occurs rapidly and does not require synthesis of new mRNA, limited information is available on processes that regulate translation. More recent studies indicate that insulin is only one of a number of beta cell proteins whose synthesis is regulated by glucose at the level of translation (6). Glucose has been shown to increase the biosynthesis of a secretory granule membrane protein, SGM 110 (7), and also chromogranin A (8) to a similar extent as insulin, suggesting that translational control may extend to a variety of other beta cell proteins.

The initiation phase of mRNA translation is generally rate-limiting for protein synthesis. Initiation is mediated in part by the eIF1–4F complex, which is composed of three subunits, eIF-4γ, eIF-4A, and eIF-4E (9). eIF-4γ is a large subunit (Mr 220,000) that binds eIF-4A (Mr 45,000) and eIF-4E (Mr 25,000). eIF-4A is an ATP-dependent helicase, and eIF-4E is the mRNA cap-binding protein. Thus, eIF-4F is involved in both the recognition of the capped mRNA and melting of secondary structure in the 5′-untranslated region. eIF-4E is the least abundant of the eIF-4F subunits, and it is generally believed that the amount of eIF-4E is limiting for translation initiation.

The availability of eIF-4E is regulated by PHAS-I, a heat- and acid-stable eIF-4E binding protein first identified in rat adipocytes (10, 11). The nonphosphorylated form of PHAS-I binds tightly to eIF-4E, and prevents eIF-4E from binding to eIF-4γ. When phosphorylated in the appropriate site(s), PHAS-I dissociates from eIF-4E, allowing the factor to participate in translation initiation.

PHAS-I is phosphorylated both in vitro and in vivo by a variety of protein kinases. Casein kinase II, protein kinase C, and mitogen-activated protein kinase have been reported to phosphorylate recombinant PHAS-I (12). Insulin-like growth factor-1 and platelet-derived growth factor in smooth muscle cells (13) and insulin in 3T3 L1 adipocytes (14) increase phosphorylation of PHAS-I and p70s6k phosphorylation by a rapamycin-sensitive pathway. This mitogen-induced phosphorylation is also sensitive to the phosphatidylinositol 3-kinase inhibitor, wortmannin (15). Recent studies on PHAS-I have implicated a signaling pathway involving the mammalian target of rapamycin.

* The abbreviations used are: eIF, eukaryotic initiation factor; mTOR, mammalian target of rapamycin; MRM, minimum essential medium; DMEM, Dulbecco’s modified Eagle’s medium; KRB, Krebs-Binger bicarbonate buffer; BSA, bovine serum albumin; RIA, radioimmunoassay; PAGE, polyacrylamide gel electrophoresis; CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate.

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(mTOR). Thus, mTOR appears to be a site by which the rapamycin-FKBP12 complex mediates the dephosphorylation of PHAS-I (15–18). The phosphorylation of PHAS-I and p70S6K appears to be regulated by mTOR in parallel (18). These studies suggest that PHAS-I has an important role in regulating translation in a variety of cells by signal transduction pathways that involve protein phosphorylation.

Stimulus secretion of insulin secretion from pancreatic beta cells is a complex process involving the transport and metabolism of glucose with the generation of metabolic intermediates including ATP. It is proposed that increases in ATP are associated with the closure of ATP-dependent K+ channels, cell depolarization, and Ca2+ entry through voltage-dependent Ca2+ channels (19). There is also strong evidence that protein kinases play a key role in modulating the insulin secretory process by beta cells. The major serine/threonine kinases that have been studied in insulin secretion include Ca2+- and calmodulin protein kinase (20), Ca2+ and phospholipid protein kinase C (21, 22), cAMP protein kinase A (23), and more recently mitogen-activated protein kinases (24, 25).

Recent evidence also indicates that glucose-stimulated insulin secretion activates a beta cell surface insulin receptor kinase and the intracellular effector substrate, insulin receptor substrate-1, from βTC3 cells (26). These results and others (27) have suggested that insulin released from the beta cell of the islet may bind to its own cell-surface receptor and serve as an autocrine mechanism for the regulation of beta cell function. We have therefore examined these aspects of stimulus secretion coupling of insulin secretion by beta cells on PHAS-I activation as reflected by its phosphorylation state, signaling pathways, and proposed functional role to regulate protein translation by the beta cell of the pancreatic islet.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats were purchased from Sasco (O’Fallon, MO) and Harlan Sprague-Dawley (Indianapolis, IN). Collagenase type P was obtained from Boehringer Mannheim. CMRL-1066 tissue culture medium, penicillin, streptomycin, Hanks’ balanced salt solution, t-glutamine, MEM amino acids solution, and MEM nonessential amino acids solution were obtained from Life Technologies, Inc. BSA was from Hyclone (Logan, UT). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Life Technologies, Inc. and the tissue was isolated, minced, and digested with 7 mg of collagenase/pancreas for 7 min at 37 °C. Islets were separated on a gradient of Ficoll-Paque and subjected to SDS-PAGE and immunoblotting as described above.

Insulin was obtained from Lilly and ICN Biomedicals (O’Fallon, MO) and Harlan Sprague-Dawley (Indianapolis, IN). Collagenase/pancreas for 7 min at 39 °C. Islets were separated on a density-matched islets were treated with Beckman Microfuge B for 10 s, the supernatant was removed for insulin RIA, and the islets were resuspended in 100 mM of homogenization buffer (50 mM β-glucosidase, pH 3.2, 100 mM NaCl, 2 mM EDTA, 0.1 mM Na3VO4, 1 mM benzamidine, 10 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride). The islets were homogenized and centrifuged at 10,000 × g for 20 min, and the supernatants were heated at 100 °C for 10 min. Heated supernatants were then centrifuged at 10,000 × g for 30 min, and centrifuged at 100,000 × g for 10 min. The supernatants were withdrawn, and 30 μl total protein from each reaction were used for PHAS-I immunoblotting analysis.

PHAS-I Fluorescent Antibody—For experiments to determine the binding of PHAS-I to eIF-4E, islets were incubated with an affinity resin of the cap homolog 7-methylguanosine triphosphate, proteins were eluted and subjected to SDS-PAGE and immunoblotting (11).

Measurement of Total Protein Synthesis—Groups of 100 islets were used for each reaction were used for PHAS-I immunoblotting analysis. Islets were washed twice with ice-cold phosphate-buffered saline, lyzed in 300 μl of Laemmli sample buffer, boiled for 5 min, and centrifuged at 10,000 × g for 10 min. The supernatants were withdrawn, and 30 μl total protein from each reaction were used for PHAS-I immunoblotting analysis.
were performed to determine both the effects of glucose concentration and the time dependence of glucose exposure on phosphorylation of PHAS-I. As shown in Fig. 1, exposure of pancreatic islets to 20 mM glucose in CMRL for 10, 30, or 180 min resulted in enhanced phosphorylation of PHAS-I (β) compared with a basal glucose concentration of 5.5 mM (lanes 1, 3, and 5). The increased phosphorylation of PHAS-I in response to 20 mM glucose occurred rapidly within 10 min or less and was stable for at least a 3-h period. As a control, PHAS-Iα, β, and γ obtained from isolated rat adipocytes are shown in lane 7.

Identification of Mediators in Glucose-induced Phosphorylation of PHAS-I—To determine if glucose-induced phosphorylation of PHAS-I is mediated by the metabolism of glucose and activation of major islet cell protein kinases, inhibitors of protein kinases A and C were initially evaluated. The selective protein kinase C inhibitor chelerythrine (10 μM) and phorbol 12-myristate 13-acetate (100 nM), an agonist of protein kinase C, exerted no effects on glucose-induced phosphorylation of PHAS-I. The cAMP analogues, 3′-5′-cAMP (0.2 mM) and dibutyryl-cAMP (1 mM) as well as staurosporine (100 nM), a potent and nonselective inhibitor of protein kinase A and protein kinase C were also ineffective in modulating glucose-induced phosphorylation of PHAS-I, suggesting that protein kinase A and most likely mitogen-activating protein kinase were not involved (30) (data not shown).

Recent studies have indicated a role for the insulin receptor, phosphatidylinositol 3-kinase and mTOR in the signaling pathway involved in phosphorylation of PHAS-I by insulin-sensitive cells (15–18). To determine if insulin secreted from the beta cell stimulates phosphorylation of PHAS-I via an insulin-receptor mechanism, we evaluated the phosphatidylinositol 3-kinase inhibitor, wortmannin, the immunosuppressant, rapamycin, and theophylline, a phosphodiesterase inhibitor that also blocks PDE-Ⅱb (31, 32), in this signaling pathway by pancreatic islets. As shown in Fig. 2, incubation of islets in CMRL in the presence of 20 mM glucose (lane 2) resulted in increased phosphorylation of PHAS-Iγ in comparison to 3 mM glucose (lane 1). Co-incubation of islets with wortmannin, rapamycin, and theophylline resulted in the dephosphorylation of PHAS-I to a level similar to that observed at basal glucose levels. PHAS-Iα, which is not normally visible at basal glucose concentrations, becomes apparent with these inhibitors. These findings are consistent with insulin-mediated phosphorylation of PHAS-I in insulin-sensitive cells.

Experiments were next performed to determine if insulin secretion from beta cells of the islet may be responsible for enhanced phosphorylation of PHAS-I. As illustrated in Fig. 3A (lane 2), exposure of islets incubated in CMRL to 20 mM glucose for 30 min resulted in enhanced phosphorylation of PHAS-Iγ in comparison to low glucose levels of 1 mM (lane 1). Furthermore, exposure of islets to exogenous insulin at 2, 20, and 200 nM (lanes 3, 4, and 5) under low glucose levels of 1 mM markedly stimulated in a dose-dependent manner phosphorylation of PHAS-I, similar to that observed with 20 mM glucose. These results suggested that glucose-stimulated phosphorylation of PHAS-I is mediated by insulin secreted into the incubation medium.

To further support this possibility, islets were incubated at 24 °C, a condition that blocks almost completely insulin exocytosis from beta cells. Under these conditions of reduced temperature, ion fluxes are minimally affected (33, 34) and glucose utilization ([3H]glucose) and glucose oxidation ([14C]glucose) are reduced by only 50–60% (35). As shown in Fig. 3B, blocking endogenous insulin secretion by reducing the incubation temperature from 37 °C (lanes 3, 4, 7, and 8) to 24 °C (lanes 1, 2, 5, and 6) significantly attenuated glucose-stimulated phosphorylation of PHAS-Iγ. Insulin secretion levels at basal 3 mM glucose were...
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Amino Acid Dependence of PHAS-I Phosphorylation—Our previous studies were performed with islets incubated in CMRL culture medium. The ability of glucose to stimulate phosphorylation of PHAS-I was also evaluated in KRBB. Unexpectedly, it was found that glucose did not stimulate the phosphorylation of PHAS-I in KRBB in the complete absence of amino acids, even though islets secrete insulin normally in the absence of these secretagogues (10 and 50 μM, conditions that inhibit insulin exocytosis (data not shown). Taken together, these findings support the concept that glucose-induced phosphorylation of PHAS-I is mediated by insulin secreted from the beta cell.

To further define this requirement for amino acids in PHAS-I phosphorylation, the effects of amino acids alone and in the presence of exogenous insulin were evaluated with the beta cell line, RINm5F. In this experimental design, KRBB was supplemented with amino acids over the range of 0.1–10 mM (see Material and Methods for concentrations defined as 1×). As shown in Fig. 4A, amino acids dose-dependently stimulated phosphorylation of PHAS-I by islets incubated in KRBB in the complete absence of amino acids. However, amino acids present in CMRL produced similar effects from islets incubated in KRBB containing 1 mM glucose (lane 8), and exogenous insulin (200 nM) induced PHAS-I phosphorylation by islets incubated in KRBB in the absence of amino acids.

In this experimental design, supernatants were saved for insulin RIA, and islets were solubilized in 30 μl of Laemmli sample buffer and processed for immunoblotting of PHAS-I as described under “Experimental Procedures.” Results are representative of three separate experiments.

FIG. 4. Amino acids are essential for phosphorylation of PHAS-I in rat islets. Rat islets (200) were serum- and glucose-depleted in 1 ml of CMRL (0.1% BSA) or KRBB (0.1% BSA) for 2 h at 37 °C. Islets were then stimulated for 30 min in 1 ml of CMRL or KRBB, 1 mM glucose, 20 mM glucose, or 1 mM glucose + 200 mM insulin. Supernatants were saved for insulin RIA, and islets were solubilized in 30 μl of Laemmli sample buffer and processed for immunoblotting of PHAS-I as described under “Experimental Procedures.” Results are representative of four separate experiments.

Amino acids are essential for increased phosphorylation of PHAS-I. Insulin secretion observed in the absence of exogenous glucose was 1.2 ± 0.2 nM at 37 °C, and 20 mM glucose increased insulin secretion to 2.4 ± 0.5 nM at 37 °C. These results strongly suggest that secreted insulin mediates phosphorylation of PHAS-I by βTC6-F7 cells.

In Fig. 6B, the lowering of the incubation temperature from 37 to 24 °C significantly reduced both insulin secretion and phosphorylation of PHAS-I by βTC6-F7 cells in comparison to that of 37 °C (lane 4 versus lane 2). Basal insulin secretion values (0.1× amino acids, without glucose or carbachol) were 0.5 ± 0.1 nM at 24 °C and 0.4 ± 0.1 nM at 37 °C, and 20 mM glucose + 0.5 mM carbachol increased insulin secretion to 2.4 ± 0.6 nM at 24 °C and 8.1 ± 1.0 nM at 37 °C. These results strongly suggest that secreted insulin mediates phosphorylation of PHAS-I by βTC6-F7 cells.

Binding of PHAS-I to eIF-4E—We further explored the ability of theophylline, a methylxanthine phosphodiesterase inhibitor which attenuates PHAS-I phosphorylation (Figs. 2 and 5C), to modulate the binding of PHAS-I to eIF-4E. In this...
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Experimental design, islets were exposed to 3 or 20 mM glucose in DMEM for 30 min, and extracts were prepared as described under “Experimental Procedures.” Islet extracts were then passed over an affinity resin of the cap homolog 7-methyl-guanosine triphosphate cross-linked to eIF-4E. Highly phosphorylated PHAS-I and to a lesser extent PHAS-I (β) do not bind to cross-linked eIF-4E and elute into the “bound” fraction. Conversely, unphosphorylated PHAS-I (α) (and to some degree PHAS-Iβ) binds to cross-linked eIF-4E and are designated as “bound.”

As shown in Fig. 7, exposure of islets to 20 mM glucose resulted in a shift of PHAS-Iβ to PHAS-Iγ (lane 2) compared with 3 mM glucose (lane 1) in the islet extract. Theophylline (5 mM), regardless of glucose concentration, caused a dramatic shift in phosphorylation of PHAS-Iγ to β (and α (lanes 3 and 4). PHAS-I that is not bound to eIF-4E, i.e. phosphorylated PHAS-I, is indicated in lanes 5–8. In lane 6, 20 mM glucose induced a small shift in the amount of PHAS-Iβ to γ compared with 3 mM glucose (lane 5). These results as anticipated mirrored those in the islet extract (lanes 1 and 2) and correspond to more free eIF-4E available for translation initiation. PHAS-I that is bound to eIF-4E is shown in lanes 9–12. We anticipated that 3 mM glucose more eIF-4E would be bound to PHAS-I than at 20 mM glucose (lanes 9 and 10), which was not detected. In a repeat experiment, increasing glucose from 3 to 20 mM produced the expected results in the bound fraction and demonstrated a greater shift in the extract blot from PHAS-Iβ to γ.

In the second part of Fig. 7, theophylline (lanes 7, 8, 11, and 12) caused a dramatic increase in PHAS-I binding to eIF-4E, regardless of the glucose concentration. Almost all of PHAS-I present was bound to eIF-4E due to theophylline (lanes 11 and 12), which was mirrored by the lack of unbound PHAS-I (lanes 7 and 8). These results suggest the following: 1) glucose stimulation of islets increases phosphorylation of PHAS-I from the β to the γ form and is associated with more eIF-4E available to initiate protein translation, and 2) theophylline results in dephosphorylation of PHAS-I, which remains bound to eIF-4E and would be predicted to inhibit initiation of protein translation.

Effects of Rapamycin on Total Protein Synthesis—To evaluate the role of the phosphorylation state of PHAS-I on beta cell function, rapamycin was evaluated for its effects on glucose stimulated protein synthesis by islets. Fig. 8 demonstrates that islets incubated in CMRL in the presence of 20 mM glucose for 60 min increases by 50% the amount of [35S]methionine incorporated into total protein compared with basal conditions of 3 mM glucose. Under these same conditions, rapamycin at 25, 50, and 100 nM significantly inhibited by ~62% glucose stimulated incorporation of [35S]methionine into total protein. Rapamycin at 20 and 100 nM had no effect on glucose-stimulated insulin secretion determined over a 1-h incubation (data not shown). These results suggest that the biosynthesis of a number of proteins may be regulated at the level of translation by the beta cell in an autocrine loop through glucose-induced insulin secretion.

DISCUSSION

This study demonstrates that glucose stimulates in a time- and concentration-dependent manner the rapid phosphorylation of PHAS-I, an inhibitor of mRNA cap-binding protein, eIF-4E, by isolated pancreatic islets. Our initial approach was to determine if this effect was mediated by the metabolism of glucose and subsequent activation of islet cell protein kinases, or whether insulin secreted from the beta cell may stimulate phosphorylation of PHAS-I in a manner analogous to that described for insulin-sensitive cells. Evidence in support of the latter mechanism was that conditions that block insulin exocytosis from beta cells prevented glucose-stimulated phosphorylation of PHAS-I, and exogenous insulin mimicked the effects of elevated glucose concentrations.

During this investigation, in vitro incubations were routinely performed in CMRL and DMEM tissue culture media. To more easily manipulate the components of this tissue culture system, parallel studies were also performed in KRBB in the absence of amino acids, co-factors, and vitamins. Insulin secretion studies are routinely performed in KRBB, and islets secrete insulin normally under these conditions. Unexpectedly, neither elevated glucose concentrations that stimulated insulin secretion from beta cells nor exogenous insulin altered the basal patterns of PHAS-I, β, or γ when islets were incubated in KRBB in the absence of amino acids. However, addition of a normal complement of amino acids to KRBB restored the ability of both endogenous and exogenous insulin to stimulate the phosphorylation of PHAS-I by islets (data not shown). Additional studies with the insulinoma-derived cell line, RINm5F, also indicated that this normal complement of amino acids alone induced in a concentration-dependent manner phosphorylation.
Recent studies in smooth muscle cells and 3T3-L1 adipocytes (13, 14) indicated a role for p70s6k in the phosphorylation of PHAS-I. Previous studies in smooth muscle cells (9) and PHAS-I in parallel and that rapamycin is acting on mTOR to prevent phosphorylation of both kinases (17, 18). Phosphorylation of p70s6k and PHAS-I has also been reported with the phosphatidylinositol 3-kinase inhibitor, wortmannin (15, 38), and the phosphodiesterase inhibitor, SQ20006 (31, 32). Wortmannin and rapamycin promoted significant dephosphorylation of PHAS-I by islets exposed to elevated glucose concentrations. These same inhibitors also induced dephosphorylation of PHAS-I by RINm5F cells exposed to amino acids, further indicating that beta cells of the islet mediate this effect in a similar manner as insulin sensitive cells.

The ability of cAMP to regulate PHAS-I phosphorylation was also evaluated in pancreatic islets. Studies in 3T3-L1 adipocytes and aortic smooth muscle cells suggested that increasing cAMP levels may promote dephosphorylation of PHAS-I (13, 14, 39). Phosphodiesterase inhibitors such as theophylline and isobutylmethylxanthine are commonly used to modulate cAMP levels in islets. These phosphodiesterase inhibitors significantly enhance glucose-stimulated proinsulin biosynthesis and insulin secretion from beta cells by increasing cAMP levels. Our studies indicated that theophylline results in marked dephosphorylation of PHAS-I, but unexpectedly, CPT-cAMP and dibutyryl-cAMP exerted no effects on the phosphorylation state of PHAS-I (data not shown). These paradoxical results were explained by recent studies indicating that methylxanthines including theophylline block activation of p70s6k, similar to rapamycin, by a mechanism independent of cAMP and cGMP production (31, 32). Our studies demonstrated that rapamycin mimics the ability of theophylline to block glucose-stimulated phosphorylation of PHAS-I. Although theophylline increases cAMP levels and greatly enhances glucose-induced insulin secretion by islets, its effect on mTOR presumably causes significant dephosphorylation of PHAS-I in a similar fashion as rapamycin. In addition, cAMP analogues, CPT-cAMP and dibutyryl-cAMP, had no effect on the phosphorylation level of PHAS-I by glucose-stimulated islets. Although insulin secretion increased further in the presence of these cAMP analogues as anticipated, glucose-induced phosphorylation of PHAS-I was already maximal, and this additional insulin secretion caused no further increase in phosphorylation. Overall, our findings suggest that cAMP independent of insulin secretion does not appear to affect glucose-stimulated phosphorylation of PHAS-I by islets.

Previous studies on the regulation of the biosynthesis of insulin secretory granule proteins by glucose have indicated that a subset of proteins exist whose synthesis is stimulated to a similar extent as insulin (7). These proteins include granule matrix and membrane constituents and possibly other proteins not associated with beta cell granules. Although rapamycin did not affect the acute phase of glucose-stimulated insulin secretion, it inhibited both glucose-stimulated phosphorylation of PHAS-I and total protein synthesis. Even though exogenous insulin stimulated phosphorylation of PHAS-I at basal glucose levels, these conditions did not result in enhanced protein synthesis, suggesting that both elevated glucose concentrations necessary for increased metabolism and the release of insulin are required to mediate increased protein translation by beta cells (data not shown).

Our findings suggest that glucose-stimulated phosphorylation of PHAS-I by beta cells of the islet is mediated via insulin receptor activation. The possibility that the insulin receptor activation may target downstream insulin signaling proteins such as insulin receptor substrate-1 and -2 and may...
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