

Pancreastatin Modulates Insulin Signaling in Rat Adipocytes

Mechanisms of Cross-Talk

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Pancreastatin (PST), a chromogranin A-derived peptide, has counterregulatory effects on insulin in the hepatocyte and the adipocyte, suggesting a possible role in insulin resistance. The mechanism of PST action on glucose and lipid metabolism is typical of a calcium-mobilizing hormone and involves a receptor $G_{q/11}$ protein-phospholipase C (PLC)- β pathway. In the rat adipocyte, PST inhibits insulin-mediated glucose transport, glucose utilization, and lipid synthesis, and it has a lipolytic effect but stimulates basal and insulin-stimulated protein synthesis. We have also recently studied the PST receptor-effector system in adipocyte membranes. To further investigate the mechanisms of PST effect on insulin action, we studied the cross-talk of PST with insulin signaling in the rat adipocyte. We found that PST inhibits insulin-stimulated GLUT4 translocation to the membrane, which may explain the reported inhibition of glucose transport. Tyrosine phosphorylation of the activated insulin receptor, insulin receptor substrate (IRS)-1, and p60-70 was also blunted, preventing their association with p85 phosphatidylinositol 3-kinase (PI3K) and their activity. The mechanism of this inhibition involves the activation of the "classical" protein kinase C isoforms and the serine phosphorylation of insulin receptor and IRS-1. On the other hand, PST activates the mitogen-activated protein kinase (MAPK) signaling module and enhances the effect of insulin. This pathway may account for the described effect of PST on protein synthesis. In conclusion, PST seems to inhibit the insulin-stimulated PI3K pathway in the adipocyte, whereas it activates the MAPK pathway. These data provide some clues to explain the PST effects on glucose metabolism and protein synthesis. *Diabetes* 49:1288-1294, 2000

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BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular calcium concentration; ERK, extracellular-regulated kinase; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PST, pancreastatin; PY, phosphotyrosine; SH, Src homology; TBST, Tris-buffered saline-0.05% Tween 20.

Pancreastatin (PST) is a chromogranin A-derived peptide (1,2) widely distributed throughout the neuroendocrine system (3-6). In islets, PST is present in β -, α -, and δ -cells (4-6). PST may be secreted from the neuroendocrine cells after the precursor glycoprotein chromogranin A is processed (7,8). Postsecretory processing of chromogranin A also occurs (9,10). A PST-like sequence has been found in different species, including the rat (11-13).

PST was named after its first described effect inhibiting insulin secretion (1). However, many other different biologic effects have been reported (14). The best characterized effect of PST was studied in the rat liver (15), in which it has a glycogenolytic effect (16-18) and a counterregulatory effect to insulin (19). PST action in the liver is mediated by a specific G protein-coupled receptor (20-22), activating $G_{q/11}$, which then activates PLC- β 3 in the plasma membrane (23). This signaling pathway leads to an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and activation of protein kinase C (PKC), which may finally mediate PST action (24,25). Recently, we found that PST also has metabolic effects in rat adipocytes (26). Thus, PST has a lipolytic effect and inhibits insulin action. PST dose-dependently blocks insulin-stimulated glucose transport and metabolism and inhibits insulin-dependent lipogenesis within a physiological range of concentrations. On the other hand, PST stimulates protein synthesis and enhances the effect of insulin, increasing protein synthesis in isolated adipocytes (26). We have also characterized PST receptors and signaling in adipocyte membranes (27). We have found a single class of binding sites, with a maximum binding capacity (B_{max}) of 5 fmol/mg protein and a binding affinity (K_d) of 1 nmol/l. Studies with blocking antibodies and GTP binding revealed that PST activates $G_{q/11}$ protein and, to a lesser extent, $G_{\alpha_{11,2}}$ protein in adipocyte membranes. The pertussis toxin-insensitive $G_{q/11}$ protein leads to the specific PST activation of PLC- β 3, which may finally mediate PST action in the adipocyte by increasing $[Ca^{2+}]_i$ and activating PKC.

The insulin receptor is a tyrosine kinase (28) that upon activation undergoes autophosphorylation and phosphorylates intracellular protein substrates such as insulin receptor substrate (IRS)-1 and other proteins of 60-70 kDa (29). IRS proteins then interact with other molecules such as Grb2 and p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) via their Src homology (SH)-2 domains (29). This interaction results in the activation of the Ras-mitogen-

activated protein kinase (MAPK) and PI3K pathways. PI3K activity is important for many insulin-sensitive metabolic processes, such as glucose transport (30,31) and glycogen synthesis (32). On the other hand, the MAPK pathway has been shown to mediate adipocyte protein synthesis in response to insulin by phosphorylation of PHAS-I (33–35).

To further elucidate the mechanisms involved in the PST counterregulatory effect of insulin in the rat adipocyte, we studied the cross-talk of PST and insulin receptor signaling.

RESEARCH DESIGN AND METHODS

Materials. Pancreastatin was purchased from Peninsula Laboratories (Merseyside, U.K.). Antibodies to phosphotyrosine (PY) and the p85 subunit of PI3K (α -p85) were purchased from Upstate Biotechnology (Lake Placid, NY); antibodies to insulin receptor β -subunit and IRS-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to phosphoserine were from Sigma (Alcobendas, Madrid, Spain) and to "classical" PKC isoforms (α , β I, and β II) from Calbiochem (AMS Biotecnología, Madrid, Spain). Protein A- and protein G-sepharose were from Amersham Pharmacia Biotech (Barcelona, Spain). The PKC inhibitor (bisindolylmaleimide) was purchased from Boehringer Mannheim (Barcelona, Spain). Other chemicals were from Sigma unless specified otherwise.

Adipocyte isolation, incubation, and solubilization. Adipocytes were prepared from the epididymal fat pads of ad libitum-fed 100- to 160-g male Wistar rats according to the method described by Rodbell (36) with minor modifications (26). Fat pads were minced and then digested with collagenase at 37°C for 1 h in Krebs-Ringer buffer (113 mmol/l NaCl, 2 mmol/l CaCl₂, 5 mmol/l KCl, 10 mmol/l NaH₂CO₃, 1.18 mmol/l KH₂PO₄, and 1.18 mmol/l MgCl₂), pH 7.4, supplemented with 20 mmol/l HEPES, 6 mmol/l glucose, and 1% bovine serum albumin (BSA). Aggregated material was removed by filtration through a mesh cloth. Isolated adipocytes were washed 3 times, and the packed cells were subsequently suspended in the final volume of the same buffer with 0.1% BSA for signaling experiments (3×10^5 cells/ml). In each condition, 2 ml cell suspension was used. Typically, cells were treated with insulin for 5 min at 37°C. When other agonists and agents were included in the experiment, cells were preincubated for 10 min at 37°C. Cells were then solubilized for 30 min at 4°C in lysis buffer (20 mmol/l Tris, pH 8, 1% Nonidet P-40, 137 mmol/l NaCl, 1 mmol/l MgCl₂, 1 mmol/l CaCl₂, 1 mmol/l dithiothreitol, 10% glycerol, 1 mmol/l phenylmethylsulfonyl fluoride, and 0.4 mmol/l sodium orthovanadate) (37,38). Protein concentration was determined by the Bradford method using BSA as standard (39).

GLUT4 translocation. Adipocytes were incubated at 37°C in the same buffer described above. Cells were treated for 20 min with insulin, after which the presence of GLUT4 in the plasma membrane was assessed by Western blotting using a specific rabbit anti-serum (OSCR6) (a gift from Dr. A. Zorzano, University of Barcelona, Barcelona, Spain) (40). Plasma and microsomal membrane fractions were prepared as previously described (41). Plasma membrane-enriched fractions were separated by SDS-PAGE and transferred onto nitrocellulose membranes for detection by immunoblotting.

Immunoprecipitation and Western blotting analysis. Soluble cellular lysates (0.5 mg protein) were precleared with 50 μ l protein A-sepharose (Pharmacia, Uppsala, Sweden) for 2 h at 4°C by end-over-end rotation. The pre-cleared cellular lysates were incubated with appropriate antibodies for 3 h at 4°C (37). Next, 50 μ l protein A-sepharose was added to immune complexes, and incubation was continued for 2 h at 4°C. The immunoprecipitates were washed 3 times with lysis buffer. We added 40 μ l SDS-stop buffer containing 100 mmol/l dithiothreitol to the immunoprecipitates and boiled for 5 min. The soluble supernatants were then resolved by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (37). The membranes were blocked with Tris-buffered saline-0.05% Tween 20 (TBST) containing 5% non-fat dry milk for 1 h at 23°C. The blots were then incubated with primary antibody for 1 h, washed in TBST, and further incubated with secondary antibodies linked to horseradish peroxidase. Bound horseradish peroxidase was visualized by a high sensitive chemiluminescence system (SuperSignal; Pierce, Rockford, IL) (42). The bands obtained in the Western blots were scanned and analyzed by the PCBAS2.0 program.

PI3K activity. PI3K activity was measured directly in immunoprecipitates in 50 μ l of a reaction mix containing 0.2 mg/ml phosphatidylinositol (Sigma), 20 mmol/l HEPES, pH 7.1, 0.4 mmol/l EGTA, 0.4 mmol/l sodium phosphate, 10 mmol/l MgCl₂, and [γ -³²P]ATP (40 μ mol/l and 0.1 μ Ci/ μ l) (37,43). After 5 min, the reaction was stopped by the addition of 15 μ l of 4 N HCl and 130 μ l chloroform/methanol (1:1). Then, 20 μ l of the lower organic layer was spotted on a silica gel-60 plate (Merck, Darmstadt, Germany), which was preactivated with 1%

potassium oxalate at 100°C and analyzed by thin-layer chromatography in chloroform/methanol/water/ammonia (60:27:11:2 [vol/vol]). Dried plates were then exposed to a film for 3–7 days, with intensifying screens for autoradiography.

Activation of PKC. Cells were treated for 10 min at 37°C in the incubation buffer described above with or without 10 nmol/l PST. Subsequently, cells were lysed in the same lysis buffer described above but without Triton X-100 for membrane preparation. Then, cells were centrifuged at 40,000g for 30 min, and the pellet was made soluble with lysis buffer containing 1% Triton X-100. Lysates were next centrifuged at 14,000g for 15 min, and the supernatant was taken and analyzed by Western blotting, as described previously (44), to detect PST-induced PKC translocation.

MAPK activation. Adipocytes were incubated in the same conditions described above and stimulated with or without 10 nmol/l insulin and 10 nmol/l PST for 10 min. Next, cells were solubilized as described above. Cell lysates were then denatured and separated by SDS-PAGE. MAPK activation was analyzed by Western blotting with the α -phospho-MAPK monoclonal antibody, which specifically recognizes the Tyr/Thr phosphorylated form of MAPK because mitogen-activated protein kinase kinase (MEK), the upstream kinase, is known to activate MAPK through phosphorylation of threonine and tyrosine residues (45).

RESULTS

PST impairs insulin-stimulated GLUT4 translocation. PST has been shown to inhibit insulin-stimulated glucose transport in isolated adipocytes (26). GLUT4 is the end point of insulin-induced glucose transport; therefore, we determined whether insulin stimulation of GLUT4 translocation to the plasma membrane was affected. PST (10 nmol/l) impaired GLUT4 translocation to the plasma membrane when adipocytes were stimulated with 10 nmol/l insulin (Fig. 1). Densitometric analyses demonstrated that PST produced a significant inhibition ($56 \pm 7\%$, $n = 4$) of insulin-stimulated GLUT4 translocation, whereas it decreased only slightly ($8 \pm 3\%$) the presence of GLUT4 in basal conditions. **PST inhibits insulin-stimulated tyrosine phosphorylation of insulin receptor and insulin receptor substrates associated with PI3K.** To examine the effect of PST on insulin-stimulated tyrosine phosphorylation, cells were preincubated with 10 nmol/l PST for 10 min before stimulation for 5 min with 10 nmol/l insulin. Cell soluble

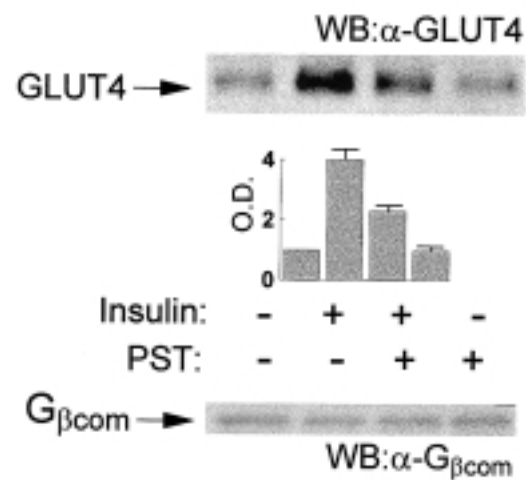


FIG. 1. PST inhibits insulin-stimulated GLUT4 translocation in isolated adipocytes. Rat adipocytes were treated with or without PST for 10 min. Cells were then treated with or without 10 nmol/l insulin for 20 min and fractionated to obtain plasma membranes. Plasma membranes were separated by SDS-PAGE and immunoblotted for GLUT4. Anti-G protein β subunit (α -G_{βcom}) was used for control of plasma membrane protein loading. An immunoblot representative of 4 separate experiments is shown. O.D., optical density; WB, Western blotting.

extracts were denatured and analyzed by Western blot with anti-PY antibodies to assess general tyrosine phosphorylation levels of adipocyte proteins (Fig. 2A). PST impaired insulin-stimulated tyrosine phosphorylation of different proteins, which according to their apparent molecular weight, may be the insulin receptor β -subunit, IRS-1, and p60-70 substrates. To further assess this issue, we next performed the same experiment, but the cell lysates were subjected to immunoprecipitation with insulin receptor-specific and anti-IRS-1 antibodies and analyzed by Western blot with anti-PY antibody (Fig. 2B and C). PST inhibited insulin-stimulated autophosphorylation of the insulin receptor β -subunit ($79 \pm 9\%$ inhibition, $n = 3$) (Fig. 2B). It had no effect on basal tyrosine phosphorylation of the insulin receptor (data not shown). Moreover, PST inhibited insulin-stimulated tyrosine phosphorylation of IRS-1 ($89 \pm 8\%$ inhibition, $n = 3$)

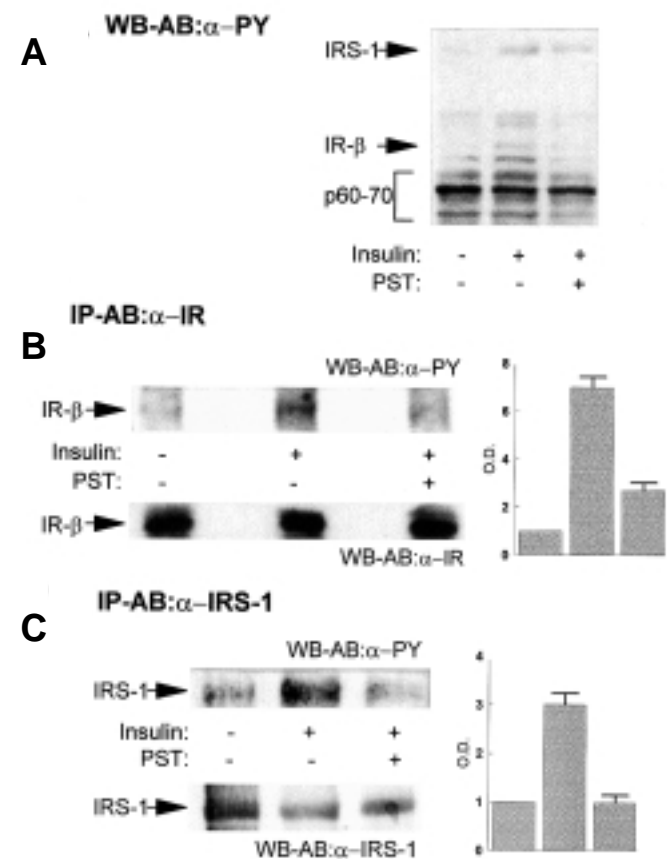


FIG. 2. PST inhibits insulin-mediated tyrosine phosphorylation of adipocyte proteins, the insulin receptor, and IRS-1. **A:** Isolated adipocytes were incubated with and without 10 nmol/l insulin for 5 min in the presence or absence of 10 nmol/l PST. Cells were then solubilized and denatured. Samples were next subjected to SDS-PAGE and analyzed by Western blotting with anti-PY antibodies (α -PY). **B:** Cells were treated as described above, but the soluble cell lysate was subjected to immunoprecipitation with anti-insulin receptor antibodies. Samples were then denatured and electrophoresed in SDS-PAGE and analyzed by Western blotting with anti-PY antibodies. **C:** IRS-1 was immunoprecipitated from cell lysates obtained in the conditions described above and analyzed by Western blotting with anti-PY (α -PY). The immunoblots shown are representative of at least 3 separate experiments. α -IR, anti-insulin receptor antibody; IP-AB, immunoprecipitating antibody; IR- β , insulin receptor β -subunit; WB-AB, Western blotting antibody.

(Fig. 2C). PST alone had no effect on basal tyrosine phosphorylation of IRS-1 (data not shown).

We also studied postreceptor signaling complexes. PST (10 nmol/l concentration) inhibited the tyrosine phosphorylation of insulin receptor substrates (IRS-1 and p60-70) associated with PI3K (IRS-1, $84 \pm 5\%$; p62, $30 \pm 6\%$, $n = 4$) (Fig. 3). PST alone had no effect on basal tyrosine phosphorylation of these substrates (data not shown).

PST inhibits insulin-stimulated PI3K activity. To check whether the PST inhibition of tyrosine phosphorylation of substrates and their association with p85-PI3K actually led to changes in the PI3K activity, we measured PI3K activity in anti-PY immunoprecipitates of adipocytes incubated in the absence and presence of insulin and PST (Fig. 4). As expected, insulin (10 nmol/l) stimulated the phosphorylation of phosphatidylinositol ~ 5 -fold ($386 \pm 23\%$ increase, $n = 3$), and this effect was significantly blunted by 10 nmol/l PST ($62 \pm 7\%$ inhibition, $n = 3$). PST alone had no effect on basal PI3K activity (data not shown).

PST stimulates serine phosphorylation of insulin receptor β -subunit and IRS-1. To further evaluate the possible mechanisms of cross-talk between PST and insulin receptor signaling, we studied the phosphorylation of insulin receptors in serine using Western blots with specific phosphoserine antibodies in insulin receptor immunoprecipitates. PST (10 nmol/l) increased the serine phosphorylation level of the insulin receptor β -subunit ~ 2 -fold ($120 \pm 9\%$ stimulation,

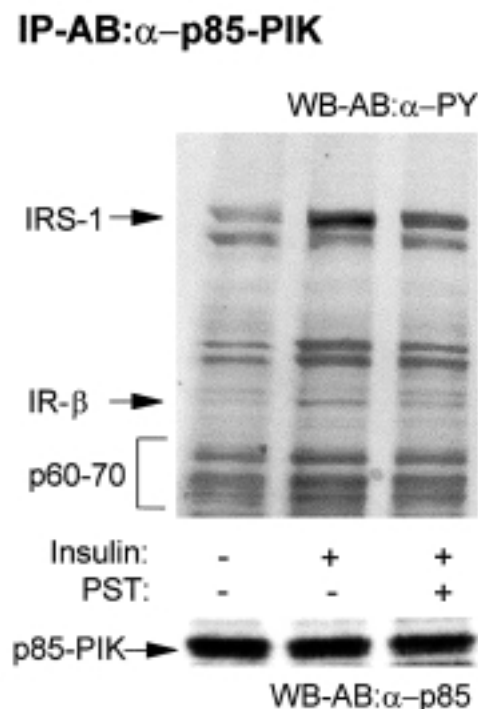


FIG. 3. PST inhibits the association of insulin receptor substrates with p85 PI3K (PIK). Isolated adipocytes were treated with and without 10 nmol/l insulin for 5 min in the presence or absence of 10 nmol/l PST. Cells were then solubilized, and soluble cell lysates were subjected to immunoprecipitation with anti-p85 PI3K antibodies. Then, samples were analyzed by Western blotting with anti-PY (α -PY). An immunoblot representative of 4 independent experiments is shown. IP-AB, immunoprecipitating antibody; IR- β , insulin receptor β -subunit; WB-AB, Western blotting antibody.

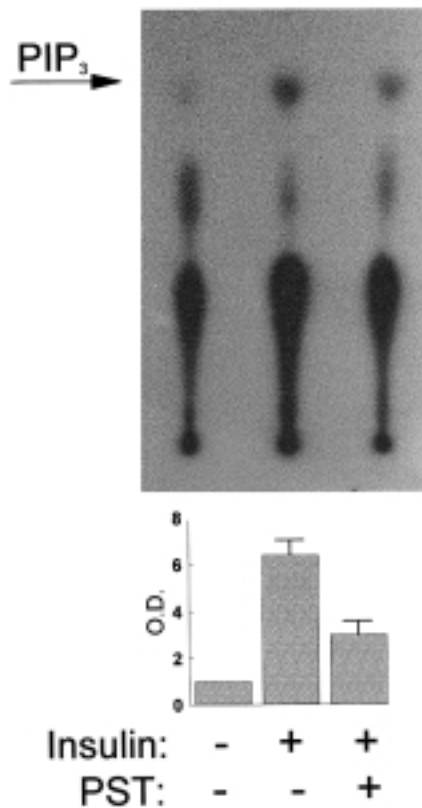


FIG. 4. PST inhibits insulin-stimulated PI3K activity. Cells were treated with and without 10 nmol/l insulin for 5 min in the presence or absence of 10 nmol/l PST. Anti-PY immunoprecipitates were then used for PI3K activity as described in RESEARCH DESIGN AND METHODS. Samples were chromatographed by thin-layer chromatography, and an autoradiography representative of 3 experiments is shown. O.D., optical density; PIP₃, phosphatidylinositol 3-phosphate.

$n = 3$) (Fig. 5A) after 10 min of incubation. Similar results were obtained when IRS-1 was immunoprecipitated and analyzed for serine phosphorylation (Fig. 5B). PST (10 nmol/l) increased the serine phosphorylation level of IRS-1 ~3-fold ($320 \pm 12\%$ stimulation, $n = 3$). These effects of PST increasing the serine phosphorylation of insulin receptor β -subunit and IRS-1 were prevented by the presence of a specific PKC inhibitor (50 nmol/l bisindolylmaleimide), with no significant differences in serine phosphorylation with controls.

PST stimulates classical PKC isoforms in adipocytes. PST (10 nmol/l) induced a 3-fold increase of PKC in the plasma membrane after 10 min of incubation ($215 \pm 8\%$ above control, $n = 3$), as expected from the previously described effect of PST on PLC- β by the activation of $G_{\alpha_{q/11}}$ in adipocyte membranes (27) (Fig. 6). Furthermore, the PKC isoform activated by PST belongs to the family of classical isoforms (α , β I, and β II), as assessed by a specific immunoblot (Fig. 6).

PST inhibition of insulin receptor signaling is blocked by the PKC inhibitor bisindolylmaleimide. Because PST serine phosphorylates insulin receptor and IRS-1 by activation of classical isoforms of PKC in rat adipocytes, we checked the casual link between these effects of PST and the inhibition of insulin receptor signaling. Thus, inhibition of PKC by 50 nmol/l bisindolylmaleimide prevented the PST inhibition on insulin-stimulated GLUT4 translocation (Fig. 7A). More-

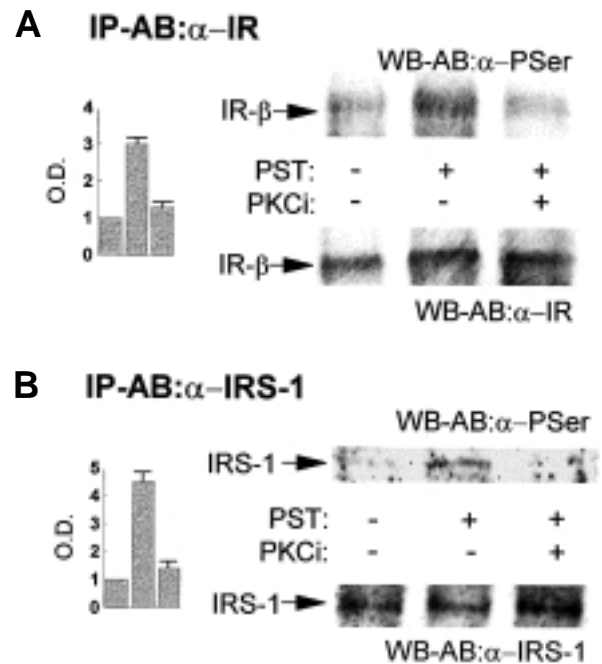


FIG. 5. PST stimulates serine phosphorylation of the insulin receptor and IRS-1. Cells were incubated for 10 min with and without 10 nmol/l PST in the presence or absence of the PKC inhibitor (50 nmol/l bisindolylmaleimide). Cells were then washed and solubilized. Cell lysates were next immunoprecipitated with anti-insulin receptor (A) or anti-IRS-1 (B), subjected to SDS-PAGE, and analyzed by Western blotting with antiphosphoserine. Representative immunoblots of 3 experiments are shown. α -IR, anti-insulin receptor antibody; α -PSer, anti-phosphoserine antibody; IP-AB, immunoprecipitating antibody; IR- β , insulin receptor β -subunit; O.D., optical density; PKCi, PKC inhibitor; WB-AB, Western blotting antibody.

over, inhibition of PKC also blocked the inhibiting effect of PST on insulin-stimulated tyrosine phosphorylation of the insulin receptor β -subunit (Fig. 7B) and IRS-1 (Fig. 7C). In a similar way, bisindolylmaleimide abrogated the effect of PST, blunting the insulin-stimulated PI3K activity (Fig. 7D).

Bisindolylmaleimide did not modify the basal levels of GLUT4 in plasma membranes, PI3K activity, or tyrosine phosphorylation in rat adipocytes (data not shown).

Effect of PST and insulin on MAPK activation. We finally checked the MAPK activation by PST in isolated adipocytes by studying its tyrosine/threonine phosphorylation level, which reflects the activation of MEK and, indirectly, all of the MAPK module pathway. As shown in Fig. 8, PST (10 nmol/l) stimulates tyrosine/threonine phosphorylation of MAPK (extracellular-regulated kinase [ERK]-1 and ERK-2), as assessed by a specific immunoblot. As expected, insulin (10 nmol/l) stimulated MAPK phosphorylation. This effect of insulin was potentiated by PST when adipocytes were challenged with both hormones at the same time (Fig. 8).

DISCUSSION

PST has been shown to inhibit both insulin secretion and insulin action (14), and this result has helped create the hypothesis that PST could have a role in insulin resistance (46). Moreover, PST has been found in high concentrations in subjects with type 2 diabetes, gestational diabetes, and hypertension—conditions that are characterized by abnormal insulin secretion and insulin resistance (47–51), i.e., like those

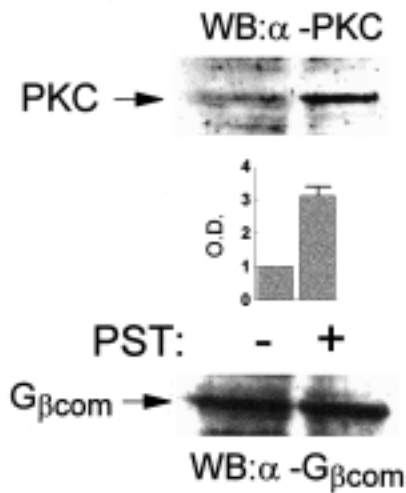


FIG. 6. PST induced translocation of classical PKC isoforms. Cells were incubated for 10 min with or without 10 nmol/l PST, and plasma membrane fraction was solubilized, subject to SDS-PAGE, and analyzed by Western blotting with specific antibodies against classical PKC isoforms (α , β I, and β II). Anti-G β common blot was used for control of sample loading. A Western blot representative of 3 experiments is shown. O.D., optical density; WB, Western blotting.

conditions in metabolic syndrome X (52). In this context, we studied the possible effects of PST on insulin signal transduction in rat adipocytes, where we previously found counterregulatory effects of PST on insulin action (26). Thus, PST dose-dependently inhibited insulin-stimulated glucose uptake, glucose utilization, and lipogenesis and had a lipolytic effect in isolated adipocytes. Moreover, we studied the signaling mechanisms of PST in adipocyte membranes (27) and found a specific receptor $G_{\alpha_{q11}}$ -PLC- β 3 pathway.

Because we found that PST produces a 50% inhibition of insulin-stimulated glucose transport (26), the important question arose as to how PST impairs glucose uptake in adipocytes. The final step of the signaling in the stimulation of glucose transport by insulin is the translocation of GLUT4 to the plasma membrane. Here, we found that PST impairs ~60% of the insulin-stimulated GLUT4 translocation to the adipocyte plasma membrane. These results are consistent with the observed inhibition of glucose uptake in isolated adipocytes, and therefore it may be the final cause of this PST effect. Next, we further investigated the mechanisms of PST cross-talk by dissecting the signaling pathway that leads to the insulin-stimulated GLUT4 translocation. The best known pathway to GLUT4 in insulin receptor signaling is PI3K (31). PI3K consists of 2 subunits: a p110 catalytic subunit (53) and a p85 regulatory subunit that contains 2 SH2 domains and 1 SH3 domain (54). In the case of the insulin receptor, the p110 catalytic subunit of PI3K is activated by interaction of SH2 domains of the p85 regulatory subunit with the tyrosine phosphorylated docking protein, IRS-1 (55), and the autophosphorylated insulin receptor β -subunit (56,57). In previous studies, we showed that blocking PI3K activity inhibits insulin-mediated stimulation of 2-deoxyglucose uptake and glycogen synthesis in 3T3 fibroblasts and HTC hepatoma cells transfected with insulin receptors, respectively (30,58). Moreover, the inhibition of PI3K has been reported to block the effect of insulin inhibiting the isoproterenol-induced lipolysis (31). In the present study, we have found that PST inhibits tyro-

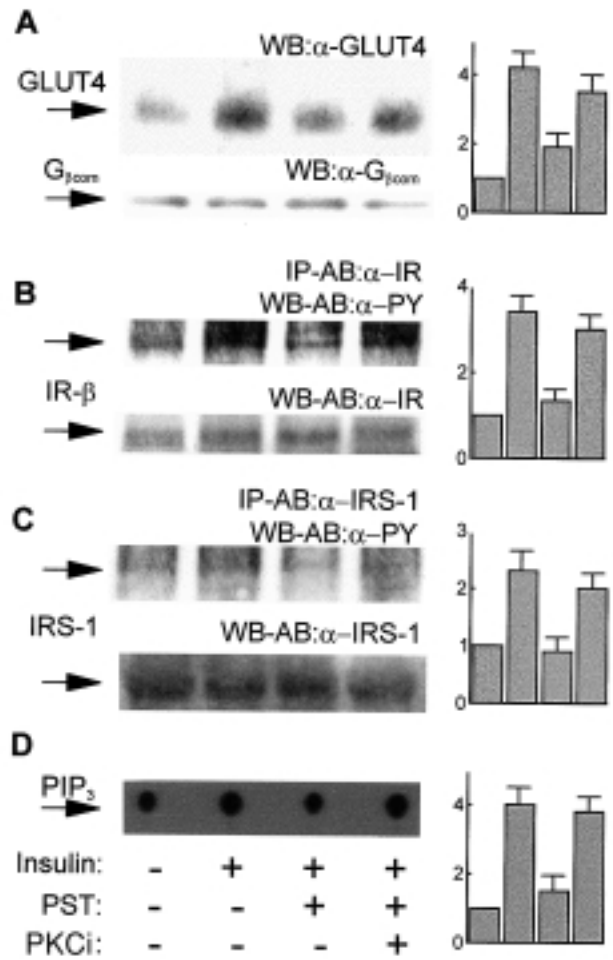


FIG. 7. PST inhibition of insulin receptor signaling is blocked by PKC inhibition. Cells were incubated with or without insulin in the presence or absence of a PST and PKC inhibitor (50 nmol/l bisindolylmaleimide). Samples were processed for analysis of GLUT4 translocation (A), insulin receptor Tyr-phosphorylation (B), IRS-1 Tyr-phosphorylation (C), and PI3K activity (D), as described in Figs. 1, 2, and 4. Representative experiments of 3 experiments are shown. α -G β_{com} , anti-G protein β subunit; α -IR, anti-insulin receptor antibody; IP-AB, immunoprecipitating antibody; IR- β , insulin receptor β -subunit; PIP₃, phosphatidylinositol 3-phosphate; PKCi, PKC inhibitor; WB, Western blotting; WB-AB, Western blotting antibody.

sine phosphorylation of the insulin receptor and IRS-1 and its association to p85 PI3K, which should result in impairing the activation of the PI3K pathway. In fact, as assessed in anti-PY immunoprecipitates, PST blocked the insulin-stimulated PI3K activity to a similar extent to that observed for tyrosine phosphorylation (~80%). Therefore, these results provide some evidence of the mechanisms of PST inhibition of insulin-stimulated glucose and lipid metabolism.

One of the possible mechanisms of inhibition of insulin receptor tyrosine kinase activity by counterregulatory hormones has been shown to be serine phosphorylation of the insulin receptor (59). Moreover, serine phosphorylation of IRS-1 may also suppress its tyrosine phosphorylation by the insulin receptor (60,61). In this context, the observed serine phosphorylation of both insulin receptor and IRS-1 on PST stimulation may cause the inhibition of tyrosine phosphorylation. In fact, this effect of PST seems to be mediated by PKC

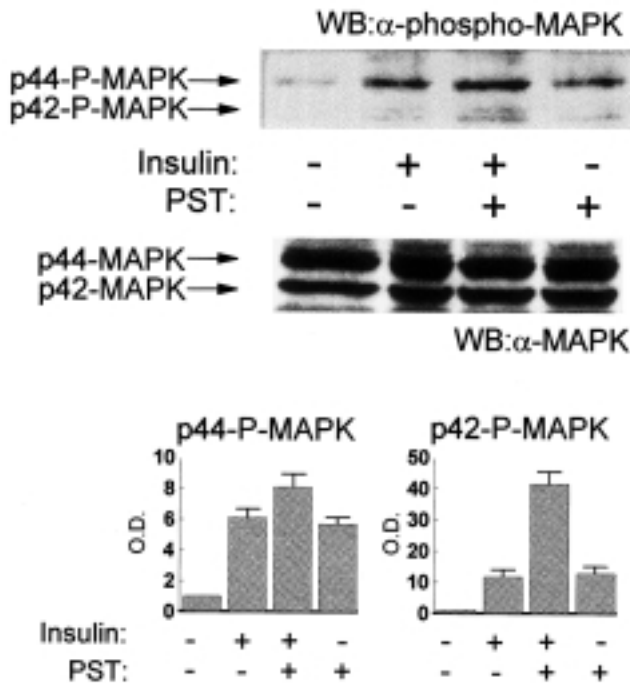


FIG. 8. PST activates the MAPK pathway and potentiates the effect of insulin. Cells were incubated for 10 min with 10 nmol/l insulin, 10 nmol/l PST, or both. Cells were then solubilized, and cell lysates were analyzed by Western blotting with α -phospho-MAPK. The immunoblot of 1 of 3 experiments is shown. O.D., optical density; P-MAPK, phospho-MAPK; WB, Western blotting.

activity, since it is prevented by the presence of a PKC inhibitor. We have previously described the PST activation of classical isoforms of PKC in HTC hepatoma cells (43). Here, we have confirmed this mechanism of action of PST in the adipocyte. This result should be expected because we have previously found the PST activation of PLC- β 3 in adipocyte membranes through the activation of $G_{\alpha_{q11}}$ (27). Classical isoforms of PKC are known to modulate insulin receptor tyrosine kinase activity by serine phosphorylation of the insulin receptor and IRS-1 (62,63). Thus, the activation of PKC by PST in adipocytes may account for the serine phosphorylation of the insulin receptor and IRS-1 produced by PST stimulation and therefore may also mediate the inhibition in tyrosine phosphorylation. This hypothesis was substantiated by the data on the effect of PKC inhibition abrogating the inhibitory action of PST on insulin-stimulated tyrosine phosphorylation, PI3K activity, and GLUT4 translocation. Enhanced serine kinase activity has been described in insulin resistance (64), and PST may contribute to this pathophysiological feature along with other counterregulatory hormones, such as catecholamines, whose circulating levels correlate with those of PST (49–51).

Even though PST has a counterregulatory effect on insulin in glucose and lipid metabolism in rat adipocytes (26), it has a dose-dependent stimulatory effect on protein synthesis (26). Moreover, PST enhances the effect of insulin on protein synthesis. Protein synthesis is started by the phosphorylation of PHAS-I, which then dissociates from the initiation factor 4E (eIF-4E), activating the translation process (65). In adipocytes, the major insulin-stimulated PHAS-I kinase is MAPK (33,65,66). Therefore, MAPK is a good candidate to be tested as a pathway that controls protein synthesis by PST. Thus, we found that PST

activates the MAPK pathway, as assessed by phosphorylation of ERK1/ERK2 (MAPK), and it enhances the activation of MAPK by insulin stimulation in rat adipocytes. G protein-coupled receptors are known to signal the MAPK pathway through the $G_{\beta\gamma}$ dimer of the heterotrimeric G protein (67). In addition, Gq-coupled receptors have an alternative pathway to connect with the MAPK module by activating PKC (67,68). Therefore, PST activation of the MAPK pathway and the potentiation of insulin effect is not striking, since PST signaling is mediated by $G_{\alpha_{q11}}$ -PLC- β PKC, and it may provide clues to the mechanism of the observed effect of PST on protein synthesis in rat adipocytes.

In conclusion, these results may help to explain the intracellular mechanisms through which PST may eventually lead to insulin resistance in vivo while still promoting protein synthesis in adipose tissue.

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