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Pancreastatin, a Chromogranin-A-Derived Peptide, Inhibits Insulin-Stimulated Glycogen Synthesis by Activating GSK-3 in Rat Adipocytes

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We have previously found that pancreastatin (PST) inhibits glucose uptake in rat adipocytes by preventing GLUT4 translocation to the plasma membrane. We have also described that this effect is mediated by the cross-talk with insulin signaling, inhibiting Tyr-phosphorylation and PI3-kinase (PI3K) activity, via protein kinase C (PKC) activation. In the present work, we have further investigated the effects of PST on glucose metabolism and the signaling pathways involved in its regulation. As expected, we found that PST inhibited insulin-stimulated PKB activity, since it depends on PI3-kinase activity. Next, we studied the activity of the target enzyme of PKB, glycogen synthase kinase-3 (GSK-3). PST not only prevented the insulin effect decreasing GSK-3 activity, but PST itself was able to activate GSK-3 activity in rat adipocytes. As previously described, phosphorylation level of GSK-3 was negatively correlated with the activity. Thus, insulin stimulated GSK-3 serine phosphorylation, whereas PST inhibited this effect, and even decreased basal phosphorylation. The PST stimulation of GSK-3 activity seems to be mediated by PKC since it can be prevented by a specific PKC inhibitor (bisindolylmaleimide). Finally, the PST effect on GSK-3 activity resulted in an inhibition on both basal and insulin stimulated glycogen synthesis in rat adipocytes. This effect of PST can also be prevented by using a PKC inhibitor. In conclusion, the chromogranin-A-derived peptide PST inhibits glycogen synthesis in rat adipocytes by activating GSK-3 activity through the activation of PKC. © 2001 Academic Press

Pancreastatin (PST) is a chromogranin-A-derived peptide (1, 2), which is widely distributed throughout the neuroendocrine system (3–6), and very abundant in chromaffin granules (7, 8). Many biological effects have been ascribed to PST which may act as an autocrine, paracrine, and endocrine peptide (9, 10). We have found that PST has metabolic effects in rat adipocytes and hepatocytes (9–11). Thus, PST inhibits basal and insulin-stimulated glucose transport and lactate production within a physiological range of concentrations in rat adipocytes (11). We have also characterized PST receptors and signaling in adipocyte membranes (12). 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 nM. Studies with blocking antibodies and GTP binding revealed that PST activates $G\alpha_{q/11}$ protein, and, to a lesser extent $G\alpha_{i1,2}$ protein in adipocyte membranes. The pertussis toxin-insensitive $G\alpha_{q/11}$ protein leads to the specific PST activation of PLC-β3 in the plasma membrane (12), which may mediate PST action in the adipocyte by increasing $[Ca^{2+}]_i$ and activating PKC. In this line, we have also found that PST promotes translocation of classical PKC isoforms to the plasma membrane in rat adipocytes (13). Moreover, PKC activation seems to mediate the inhibition of insulin-stimulated PI3K signaling pathway, by serine phosphorylation of insulin receptor and IRS-1 (13). PI3K plays a central role in regulating glucose transport and glycogen synthesis (14, 15), therefore, the PST inhibition of PI3K pathway may also lead to an inhibitory effect on glycogen synthesis.

One of the downstream kinases in this pathway is protein kinase B (PKB, also known as Akt, or Rac) (16), which has glycogen-synthase kinase-3 (GSK-3) as a target (17, 18). Two isoforms of GSK3, α and β are present in many tissues including adipocytes and play regulatory roles in metabolism and development (19). GSK3 is inhibited by insulin stimulation (18), through

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the activation of PI3K pathway, and is mediated by the downstream serine/threonine kinase PKB, which phosphorylates GSK3 on Ser-21 (the α-isoform) and Ser-9 (the β-isoform) (18). GSK3 then regulates glycogen synthesis via inhibitory phosphorylation of glycogen synthase (19, 20). Thus, insulin inhibition of GSK3 results in activation of glycogen synthesis.

In the present work we sought to further investigate the metabolic effects of PST in rat adipocytes by studying glycogen synthesis as well as the signaling pathway that regulates this metabolic action.

MATERIALS AND METHODS

Materials. PST was purchased from Peninsula Laboratories (Merseyside, UK). Polyclonal antibodies to phosphorylated GSK-3 that recognizes GSK-3α phosphorylated at serine 21 and GSK-3β phosphorylated at serine 9, were obtained from New England Biolabs (Beverly, MA). Monoclonal antibody that recognizes GSK-3α and GSK-3β, and polyclonal anti-PKB were from Santa Cruz Biotechnology (Santa Cruz, CA). [U-^{14}C]Glucose (350 Ci/mmol) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from AmershamPharmacia Biotech (Madrid, Spain). The PKC inhibitor (bisindolylmaleimide) was from Roche Molecular Biochemicals (Barcelona, Spain).

Adipocyte isolation. Adipocytes were prepared from the epididymal fat pads of *ad libitum*-fed 120–180-g male Wistar rats according to the method described by Rodbell (21) as previously described (21), with minor modifications (11, 13).

Glycogen synthesis. Adipocytes were incubated in KRB with 20 mM Hepes, supplemented with 1% BSA, and 10 mM glucose at 37°C (22). After a 30 min preincubation period with the stimuli, 2 μCi of [U-^{14}C]glucose were added and the incubation continued for 1 h (15, 22). Glycogen was isolated using KOH and ethanol extraction (15, 22) and counted in scintilliation counter (Wallac 1490, Turku, Finland).

Immunoprecipitation and Western blotting. Cells were solubilized for 1 h at 4°C in lysis buffer as previously described (13). Protein concentration was determined by the Bradford method using BSA as standard (23). The same amount of protein (0.5 mg) from cell lysates were employed for immunoprecipitation. Soluble lysates were precleared with 50 ml protein A or protein G-sepharose (AmershamPharmacia, Madrid, Spain). The precleared cellular lysates were incubated with appropriate antibodies for 3 h at 4°C, washed three times with lysis buffer or kinase buffer (24). Samples were denatured and resolved by SDS–PAGE, transferred onto nitrocellulose membranes and analysed by immunoblot using the appropriate antibodies as previously described (25) using a high sensitive chemiluminescence system (Supersignal, Pierce, Rockford, IL). The bands obtained were scanned and analyzed by the PCBAS2.0 program.

GSK-3 and PKB activity. Adipocytes were incubated in KRB with 20 mM Hepes, supplemented with 1% BSA, and 6 mM glucose at 37°C, for 5 min to measure PKB activity, or 15 min for GSK-3 activity. GSK-3 and PKB activity were assayed in the specific immunoprecipitates in 20 mM Mops, pH 7.0, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 mM MgAc, 100 μM ATP, and 1 μCi of [γ-^{32}P] using myelin basic protein (MBP, 0.5 mg/ml final concentration) as substrate (26). Reactions were carried out at 30°C for 20 min. After centrifugation, the supernatants were spotted onto Whatman phosphocellulose paper. Filters were washed in three changes of 0.75% phosphoric acid, dried and counted in a liquid scintillation counter (27).

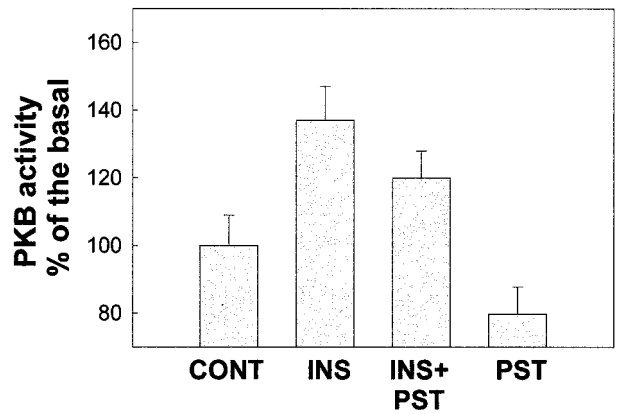

FIG. 1. Pancreastatin inhibits insulin-stimulated PKB activity in rat adipocytes. Adipocytes were incubated with or without 100 nM insulin and 100 nM pancreastatin for 5 min. Cells were solubilized and precleared lysates were immunoprecipitated with anti-PKB antibodies to measure PKB activity as described under Materials and Methods. Results are expressed as a percentage of the control value. Basal activity of PKB was 6 ± 0.5 pmol of phosphate incorporated/min/g of protein. The results are the means ± SEM of three independent experiments.

RESULTS

Pancreastatin inhibits basal and insulin-stimulated PKB activity in rat adipocytes. We have previously described that PST crosstalk with insulin receptor signaling results in the inhibition of PI3K activation (13). One of the known pathways downstream of PI3K is the serine/threonine kinase PKB (14). Therefore, we studied PKB activity in rat adipocytes in the absence and presence of 100 nM insulin and the effect of 100 nM PST. As shown in Fig. 1, 100 nM insulin induced a 60% increase in basal PKB activity. Basal PKB activity was 6 ± 0.5 pmol of phosphate incorporated/min/g of protein. One hundred nanomolarsof PST slightly inhibited basal PKB activity to 79%, whereas insulin-stimulated PKB activity was strongly blunted (down to 120% basal activity).

Pancreastatin stimulates GSK-3 activity and prevents the insulin-mediated inactivation. Since GSK-3 is one of the known targets of PKB (15, 16), GSK-3 activity was measured in anti-GSK-3 immunoprecipitates from adipocyte incubated in the presence or absence of insulin and PST. As shown in Fig. 2 (upper panel), insulin (100 nM) inhibited basal GSK-3 activity (down to 80%). Basal activity was 4 ± 0.5 pmol of phosphate incorporated/min/g of protein. PST (100 nM) stimulation induced a 52% increase in GSK-3 activity, and prevented the inhibitory effect of insulin.

The presence of the PKC inhibitor (50 nM bisindolylmaleimide) abolished the stimulatory effect of PST on GSK-3 activity (Fig. 2, lower panel).

To confirm these results of GSK-3 activity, we studied serine phosphorylation of GSK-3 by specific immunoblot with antibodies that recognize GSK-3 phosphor-

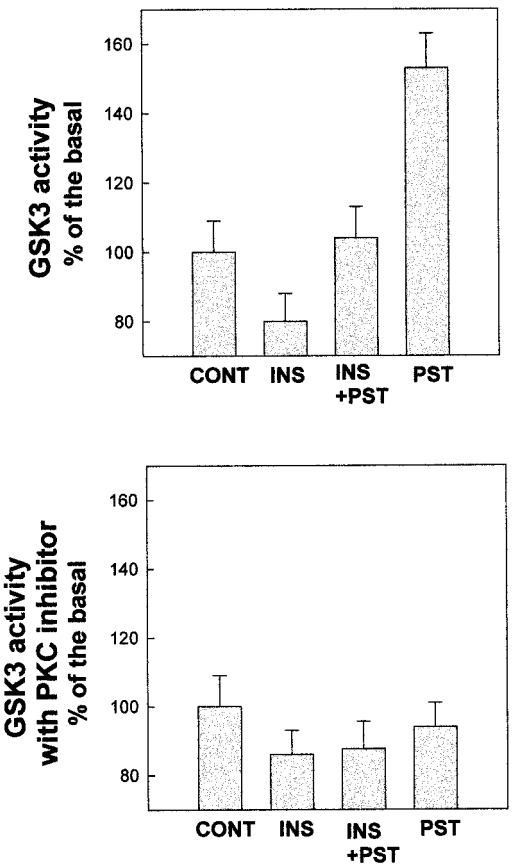

FIG. 2. Pancreastatin stimulates GSK-3 activity and prevents the insulin-mediated inhibition of GSK-3 activity in rat adipocytes by a PKC-mediated mechanism. Adipocytes were incubated with or without 100 nM insulin and 100 nM pancreastatin in the absence (upper panel) or the presence (lower panel) of the PKC inhibitor (50 nM bisindolylmaleimide) for 15 min. Cells were then solubilized and precleared lysates immunoprecipitated with anti-GSK-3 antibodies to measure GSK-3 activity as described under Materials and Methods. Results are expressed as a percentage of the control value. Basal activity of GSK-3 was 4 ± 0.5 pmol of phosphate incorporated/min/g of protein. The results are the means ± SEM of four independent experiments.

ylated in serine 21 (GSK-3α) and 9 (GSK-3β). As shown in Fig. 3 (upper panel), insulin (100 nM) increased the phosphorylation level of both isoforms of GSK-3. PST (100 nM) partially prevented the effect of insulin and also decreased basal phosphorylation level of GSK-3, specially the β isoform, which has higher basal phosphorylation level than the α isoform. The inhibition of PKC activity with 50 nM bisindolylmaleimide prevented this inhibitory effect of PST on basal and insulin-stimulated serine phosphorylation of GSK-3 (Fig. 3, lower panel).

Pancreastatin inhibits basal and insulin-stimulated glycogen synthesis in rat adipocytes. Finally, we studied the final physiological action of the pathway, i.e., the glycogen synthesis. We used a range of PST concentrations to assess its effect on glycogen synthesis in rat adipocytes. First, we investigated the counterregulatory effect on insulin-stimulated glycogen synthesis. Basal [U-^{14}C]glucose incorporation into glycogen was 18 ± 1 pmol/mg protein and rose if stimulated by insulin 100% over the basal. PST dose-dependently inhibited this effect of insulin to 60% over the control (Fig. 4, upper panel). Moreover, PST dose-dependently inhibited basal glycogen synthesis down to 50% of control value (Fig. 4, lower panel).

When the PKC inhibitor (50 nM bisindolylmaleimide) was included in the incubation of adipocytes the effect of PST on basal and insulin-stimulated glycogen synthesis was completely abrogated (Fig. 5).

DISCUSSION

We have previously found that PST inhibits glucose transport and utilization in rat adipocytes (11). We have also found that the inhibitory effect of PST on insulin-mediated glucose uptake is mediated by impairing GLUT4 translocation to the membranes (13). These effects of PST are exerted by the interaction with specific receptors coupled to $G\alpha_{q/11}$ proteins (12), which then activate PKC activity. PKC activation of classical isoforms then cross-talk with insulin receptor signaling by serine/threonine phosphorylation of the insulin receptor β-subunit and IRS-1 (13), inhibiting tyrosine kinase activity and phosphorylation, their association with p85 PI3K and the PI3K activity (13). Now, the aim of the present study was to further investigate the effect of PST on glucose metabolism in rat adipocytes, by measuring glycogen synthesis and studying the different stages of the pathway controlling glycogen synthesis, downstream of PI3K.

3′-Phosphorylated phospholipids generated by PI3K act as cofactors to a variety of cellular components (28).

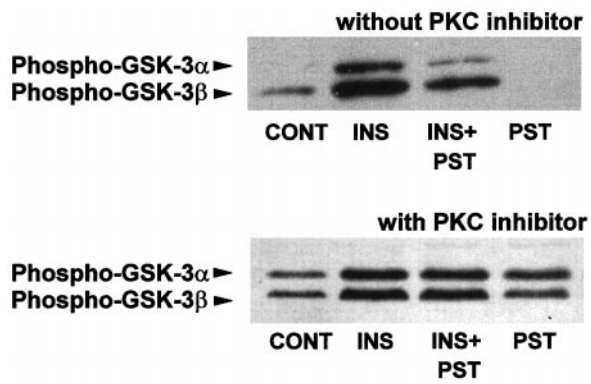

FIG. 3. Pancreastatin decreases basal and insulin-mediated serine phosphorylation of GSK-3 by activating PKC. Adipocytes were incubated as described in the legend to Fig. 2 in the presence (upper panel) and the absence (lower panel) of the PKC inhibitor (50 nM bisindolylmaleimide), but immunoprecipitates were analyzed by immunoblot with antibodies that recognize GSK-3 phosphorylated in serine 21 (GSK-3α) and 9 (GSK-3β). Each experiment shown is representative of three independent experiments.

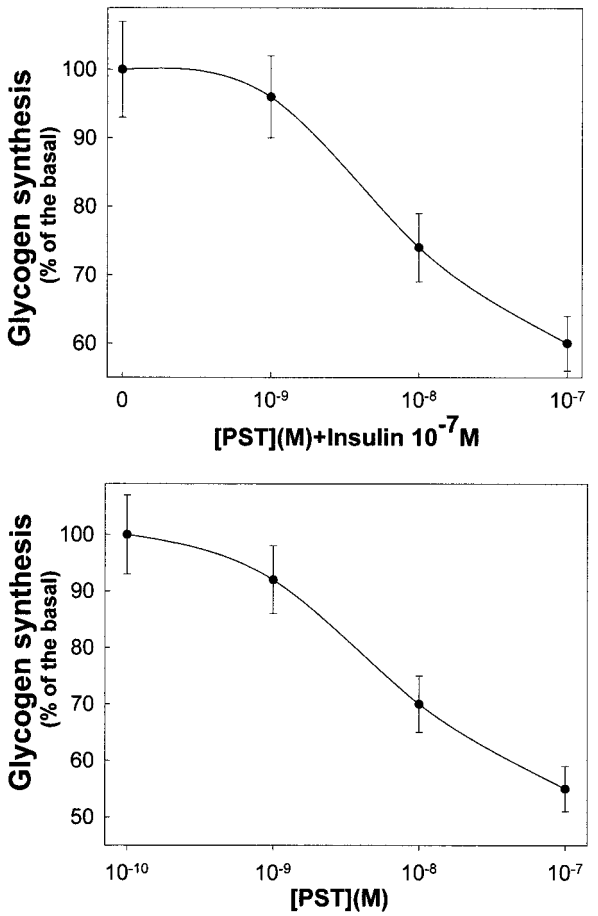

FIG. 4. Pancreastatin inhibits basal and insulin-stimulated glycogen synthesis in rat adipocytes. Cells were treated as described in the legend for Fig. 1, but stimulation with agonists was extended for 30 min. [U-^{14}C]glucose (1 μCi/well) was then added to the cells and the incubation further continued for 1 h to determine glucose incorporation into glycogen. Upper panel shows a dose-response of pancreastatin in the presence of 100 nM insulin, and lower panel a dose-response in the absence of insulin. Results are expressed as a percentage of the control value. Basal activity of glycogen synthesis was 18 ± 1 pmol/mg protein. Data are means ± SEM of four independent experiments.

One of such cellular target is PKB (16, 29). Thus, phosphatidylinositol (3,4,5)-triphosphate and phosphatidylinositol (3,4)-bisphosphate bind to the pleckstrin homology domain of PKB and lead to the translocation of the kinase to the plasma membrane (30), where 3′-phosphoinositide-dependent kinases (PDKs) phosphorylate PKB, increasing the activity of the kinase (31, 32).

We have found that PST inhibits insulin-stimulated PKB activity, which is not striking since we have previously observed that PST prevented insulin-stimulated PI3K activity in rat adipocytes (13). On the other hand, we had previously observed that PST had no effect on basal PI3K activity, whereas in the present study we have found that PST also inhibits basal PKB, suggesting that inhibition of PKB independently of PI3K may occur. Other groups have found that PKB activity can be regulated by heterotrimeric G proteins (26, 33–36). In fact, dual regulation of PKB by heterotrimeric G protein subunits has been shown (33). Thus, $G\alpha_q$ has been found to inhibit PKB activity, whereas $G\beta\gamma$ can activate the kinase, by stimulating PI3K (33, 37, 38). The inhibition of PKB by $G\alpha_q$ seems to be mediated by the activation of PLC-β, which then stimulates PKC activity, since phorbol ester activators of protein kinase C have been shown to inhibit insulin-stimulated PKB activation in 3T3-L1 adipocytes (39). In this line, our results are consistent with this hypothesis, and the inhibition of PKB activity by PST in rat adipocytes may be mediated by the $G\alpha_{q/11}$ protein activation (12).

PKB is the upstream regulator of GSK-3 by inhibitory phosphorylation in fat cells (18), therefore, some stimulation of GSK-3 activity in response to PST should not be striking. Thus, consistent with the inhibition by PST of basal and insulin-stimulated PKB activity, basal GSK-3 activity was increased and the inhibitory effect of insulin was prevented by PST. These data of GSK-3 activity were confirmed by studying the phosphorylation state of the enzyme by specific immunoblot that recognizes the serine 21 and 9 from GSK-3α and GSK-3β respectively, leading to enzyme deactivation (18, 40, 41). Thus, PST decreases insulin-mediated as well as basal serine phosphorylation level of GSK-3. Moreover, the fact that PKC activity inhibition prevents the PST-mediated dephosphorylation and activation of GSK-3 further support the hypothesis of the PST mechanism of action. Thus, these effects may be mediated by the triggering of the $G\alpha_{q/11}$-PLC-β-PKC pathway.

Finally, we have evaluated the relative contribution of these signaling activities to the regulation of the metabolic output, i.e., the glycogen synthesis (19, 20).

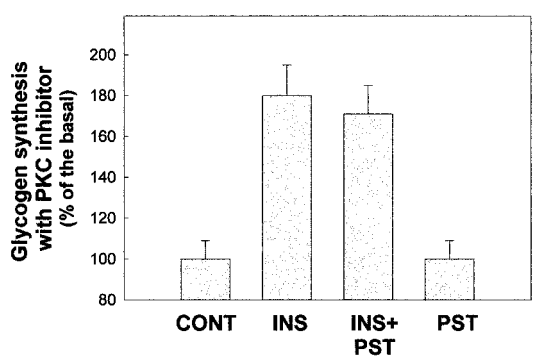

FIG. 5. Inhibition of PKC activity prevents the inhibitory effect of pancreastatin on basal and insulin-mediated glycogen synthesis. Cells were treated as described in the legend for Fig. 4, but a PKC inhibitor was included in the incubation (50 nM bisindolylmaleimide). Results are expressed as a percentage of the control value. Basal activity of glycogen synthesis was 20 ± 1 pmol/mg protein. Data are means ± SEM of four independent experiments.

Thus, PST inhibits the glycogen synthesis rate both in basal and insulin-stimulated conditions. However, these effects in glycogen synthesis were milder than those observed in the signaling. A possible explanation is that other mechanisms may account for the glycogen synthesis, in addition to GSK-3. In fact, insulin has been shown to stimulate glycogen synthase in other systems with no detectable GSK-3 (42), and alternative pathways to PI3K have been indicated, such as S6 kinase (15) or a protein phosphatase (20) which may partially mediate the stimulation of glycogen synthesis.

In conclusion, these results further demonstrate the role of the chromogranin A-derived peptide PST in the regulation of glucose metabolism, inhibiting glycogen synthesis by a mechanism that is dependent on PKC activity. Besides, these data provide some mechanisms thereby PST-stimulated PKC activity inhibits glycogen synthesis in rat adipocytes, i.e., the inhibition of PKB and the activation of GSK-3 activity.

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