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Insulin Stimulated Protein Phosphorylation in Human Plasma Liver Membranes: Detection of Endogenous or Plasma Membrane Associated Substrates for Insulin Receptor Kinase

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SUMMARY: The present work discloses a procedure for preparation of human liver plasma membranes containing catalytically competent insulin receptor kinase. In addition to insulin promoted phosphorylation of the β-subunit of insulin receptor kinase, insulin promoted phosphorylation of pp120 and two other new proteins was demonstrated. The new proteins with molecular weights of 50,000 and 120,000 do not bind to WGA, pp120 antibody or insulin receptor antibody, but bind to the antiphosphotyrosyl antibody. The identity and physiological significance of these putative substrates for insulin receptor kinase remains to be established. © 1987 Academic Press, Inc.

Insulin promotes autophosphorylation of the β-subunit of its receptor (1-11), and concomitantly activates the receptor as a tyrosyl kinase (12,13). In addition to catalyzing phosphorylation of exogenous substrates in cell-free systems (14-19), insulin receptor kinase appears to catalyze phosphorylation of several endogenous substrates with molecular weights in the range of 15,000-185,00 in several animal models and cell lines (20-26). The fact that liver is a major insulin target together with the possibility that endogenous substrates for insulin receptor kinase may function as second messengers, makes it important to characterize the reactions of insulin receptor with other membrane proteins. Such studies have been hampered, however, by the difficulty of obtaining liver plasma membranes with catalytically competent insulin receptor. This problem may be due in part to the high concentration of proteinases in liver cells. Previous studies have demonstrated that insulin receptor is sensitive to proteolytic nicking at the C-terminal end of its β-

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subunits (27), that a large fraction of the insulin receptor in human placental membrane preparations, although competent to bind insulin, is proteolytically nicked in one or both of its β-subunits (28,29), and that this proteolytically nicked receptor is incompetent to undergo insulin promoted autophosphorylation and activation as a tyrosyl kinase (6,8). The present study discloses a procedure for preparing human liver plasma membranes containing catalytically competent insulin receptor kinase. Additionally, this work reports insulin promoted phosphorylation of endogenous or plasma membrane associated substrates, in liver plasma membranes from normal individuals, a morbidly obese individual with type II diabetes and a nondiabetic morbidly obese individual.

MATERIALS AND METHODS

Liver plasma membranes preparation: Liver biopsies (1-2g) were obtained from 2 patients undergoing elective cholecystectomy, and 3 brain-dead organ donors without any known metabolic diseases, and from 2 morbidly obese patients, one with type II diabetes and the other with normal glucose tolerance test, undergoing an elective gastric bypass procedure. Written consent was obtained from all patients after they were informed about the nature and potential risks of the study.

The liver tissue was homogenized in 50 ml of 1 mM $NaHCO_3$ buffer, containing 0.5 mM $CaCl_2$ pH 7.5 as described by Ray (30). The plasma membranes were isolated by the aqueous two-phase polymer method of Lesko *et al.* (31) as we have previously described (32). The membranes were suspended in 50 mM Tris buffer, pH 7.4 and kept at -70°C until they were used. All the buffers contained 2 μM leupeptin, 2 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml bacitracin, 1000 U/ml aprotinin and 1 mM vanadate. The addition of these proteinase and phosphatase inhibitors was found to be essential for the activation of insulin receptor kinase by insulin.

Phosphorylation of liver plasma membranes: Liver plasma membranes (~ 50 μg protein) were preincubated in 500 μl of Krebs-Ringer bicarbonate buffer pH 7.4 containing 3% bovine serum albumin and the above mentioned proteinase and phosphatase inhibitors in the presence and absence of insulin 1 x 10^{-7}M for 16 hours or 1 hour at 4°C. Then [γ-^{32}P] ATP (100 μM) was added in the presence of 5 mM $MnCl_2$ and 5 mM $MgCl_2$ and the reaction continued for an additional one hour at 4°C. The reaction was stopped with an equal volume of 50 mM HEPES buffer pH 7.4 containing 10 mM EDTA, 100 mM NaF, 20 mM pyrophosphate and 4 mM ATP. The membranes were pelleted by centrifugation at 10,000 x g for 30 minutes and then solubilized for 1 hour at 4°C in 50 mM HEPES pH 7.4, containing 1% Triton X-100 and above-mentioned inhibitors. The mixture was then centrifuged at 100,000 x g for 60 minutes and the crude solubilized extract was partially purified by wheat germ agglutinin affinity chromatography (WGA) as previously reported (33). Then, both the material absorbed and that nonabsorbed by WGA were immunoprecipitated by our insulin receptor antibodies (33), antiphosphotyrosine antibodies (29) and by pp120 antibodies (20). The immunoprecipitates were subjected to polyacrylamide gel electrophoresis after reduction (34) followed by autoradiography. In some experiments, comparison of phosphoamino acid analysis of β-subunit of insulin receptors in both the human liver plasma membrane preparation and the solubilized receptor preparation was determined by a described method (35). Also, for comparison between different patients, the ^{125}I insulin binding activity of plasma membrane and solubilized receptors was equalized before performing phosphorylation reaction (33).

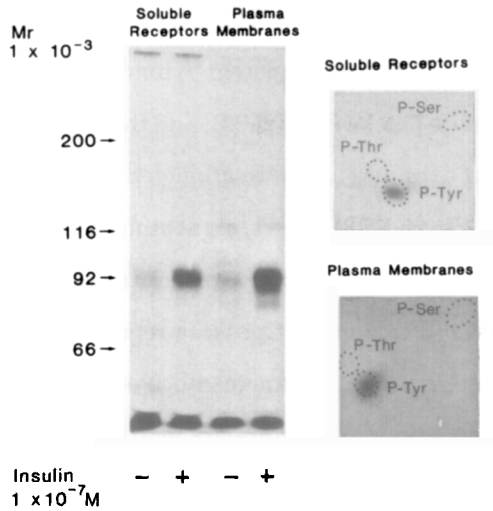

Figure 1. Autoradiogram of insulin-stimulated autophosphorylation of the β-subunit of solubilized insulin receptors or plasma membranes and phosphoamino acid analysis of the β-subunit of insulin receptors from human liver.

RESULTS

Studies of insulin promoted phosphorylation of insulin receptor were performed using insulin receptor antibodies to precipitate insulin receptor selectively. The observations depicted in Fig. 1 indicate that the human liver plasma membranes obtained by the procedure described under "MATERIALS AND METHODS" contains insulin receptor that is competent to undergo insulin promoted tyrosyl phosphorylation. The success of the procedure was dependent on the presence of the indicated proteinase and phosphatase inhibitors. Omission of one or more of these inhibitors resulted in a marked decrease in the yield of catalytically competent insulin receptor (data not shown). The observations depicted in Fig. 1 also demonstrate that the extent of autophosphorylation relative to insulin binding was the same for insulin receptor in plasma membranes and insulin receptor partially purified from solubilized membranes by lectin chromatography. This finding suggests that these procedures did not result in selective loss of kinase activity as might be expected if β-chain nicking by membrane-bound proteinases occurred during solubilization and lectin chromatography. Phosphoamino acid analysis (Fig. 1) revealed that (as expected) autophosphorylation of insulin receptor kinase occurred predominantly at tyrosyl residues.

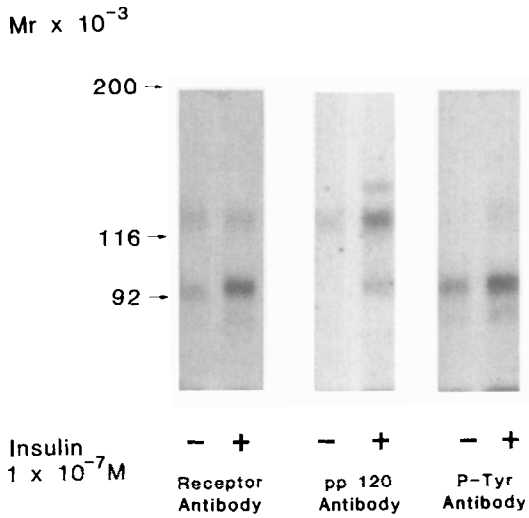

Figure 2. Autoradiogram of insulin-stimulated phosphorylated human liver plasma membranes absorbed by WGA-Sepharose and immunoprecipitated by insulin receptor antibodies, pp120 antibodies and phosphotyrosine antibodies.

Studies of insulin promoted phosphorylation of insulin receptor kinase and endogenous proteins in plasma membranes proteins are illustrated in Fig. 2 and 3. In these studies plasma membranes were exposed to [γ-^{32}P]ATP in the presence and absence of insulin for one hour, after which time the reaction was quenched, the membranes solubilized and subjected to lectin chromatography on immobilized WGA. Figure 2 depicts an autoradiogram of SDS-PAGE gels of material that was absorbed and eluted from WGA and immunoprecipitated by either insulin receptor antibody, phosphotyrosyl binding antibodies or an antibody to pp120, and endogenous substrate present in preparations of insulin receptor partially purified from solubilized rat microsomes by affinity chromatography on WGA (20). The appearance of ^{32}P-labeled material in the left and right panels of Fig. 2 having a mobility similar to that expected for the β-subunit of insulin receptor kinase is consistent with the fact that insulin receptor is a WGA-binding glycoprotein which, after undergoing insulin promoted tyrosyl phosphorylation, binds to phosphotyrosyl binding antibodies.

The center panel in Fig. 2 shows that insulin receptor in human liver membranes promotes phosphorylation of pp120, a protein recently shown to be present in liver plasma membranes from rat, monkey and rabbit (20,36). Although pp120 present in solubilized insulin receptor preparation from rat liver microsomes

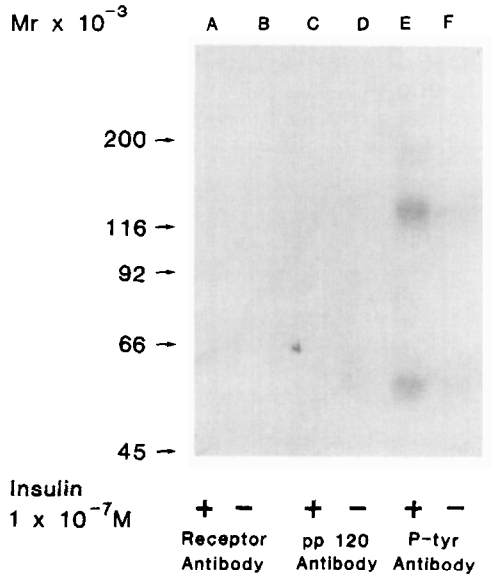

Figure 3. Autoradiogram of insulin-stimulated phosphorylated human liver plasma membranes not absorbed by WGA-Sepharose and immunoprecipitated by insulin receptor antibodies, pp120 antibodies and phosphotyrosine antibodies.

undergoes insulin promoted tyrosyl phosphorylation, it is not adsorbed by the phosphotyrosyl binding antibodies (36). The right panel in Fig. 2 indicates that human liver pp120 that had been phosphorylated in intact plasma membranes is also not adsorbed by the phosphotyrosyl binding antibodies.

Figure 3 depicts an analysis of phosphorylation in the material that did not bind to immobilized WGA. This analysis indicates the existence of two new endogenous or plasma membrane associated substrates for insulin receptor kinase that migrate on SDS-PAGE (after reduction) with molecular weights of 50,000 and 120,000. The fact that these proteins do not bind to WGA, pp120 antibody or insulin receptor antibody, but bind to the antiphosphotyrosyl antibody, suggests that they are tyrosyl phosphorylated and distinct from insulin receptor, pp120 and degradation products thereof.

In light of the report that preparations of insulin receptor partially purified from solubilized hepatic receptors from morbidly obese patients with type II diabetes exhibit depressed activity toward an artificial exogenous substrate (32), we were prompted to determine the behavior of insulin receptor in intact plasma membranes from a morbidly obese diabetic and a morbidly obese nondiabetic. The observations

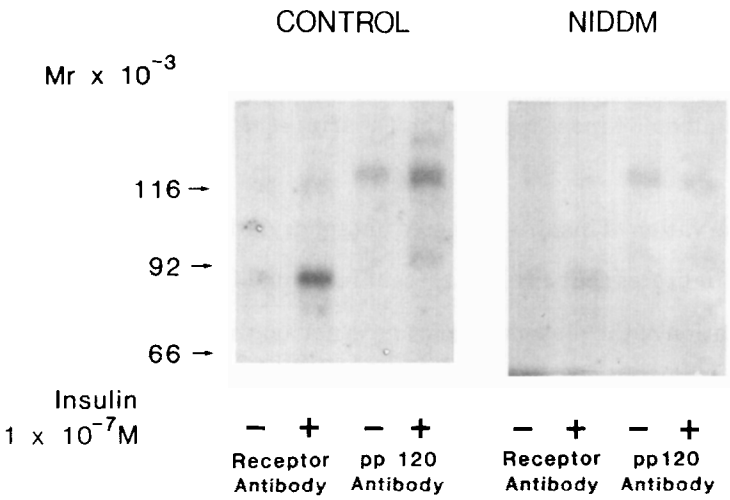

Figure 4. Autoradiogram of insulin-stimulated phosphorylated human liver plasma membranes absorbed by WGA-Sepharose and immunoprecipitated by insulin receptor antibodies and pp120 antibodies.

depicted in Fig. 4 indicate that liver plasma membranes from the morbidly obese diabetic exhibit markedly less insulin promoted phosphorylation of insulin receptor kinase and pp120 relative to that from a morbidly obese nondiabetic control.

DISCUSSION

Determination of the molecular basis for the dysfunctions associated with type II diabetes is likely to require more information concerning the reactions of insulin and insulin receptor in liver, a major insulin target. Although we have developed methodologies to isolate insulin responsive human hepatocytes (32), studies of insulin receptor kinase in these cells are difficult, mainly because of the relatively small number of cells available from intraoperative biopsy material (1-2 g). Moreover, studies of insulin receptor partially purified from solubilized human plasma membranes while informative (32), are limited, since proteins that interact with the receptor in the plasma membrane may be lost during solubilization and purification. Thus, the studies of insulin receptor in human liver plasma membranes reported in the present work revealed the presence of two previously undetected endogenous or plasma membrane associated potential substrates for insulin receptor kinase. The identity of these putative substrates for insulin receptor kinase and the physiological significance (if any) of their insulin promoted

phosphorylation remains to be established. In this regard it is interesting to note that one of the putative substrates exhibits a similar molecular weight to that of the insulin stimulated S6 kinase reported by Tabarini *et al.* (25) to be present in 3T3-L1 cells.

The observation of insulin promoted phosphorylation of pp120, reported in the present work indicates that pp120 is present in human liver plasma membranes, and that solubilization of the plasma membrane is not a prerequisite for phosphorylation of pp120. This finding is consistent with the notion that pp120 is accessible to insulin receptor kinase in intact cells. The fact that pp120 was detected in liver from several species, but could not be detected in several other tissues including rat brain, skeletal muscle, heart, kidney, and adipocytes suggested that pp120 is a liver specific protein (36). This finding together with the observation that pp120 undergoes insulin promoted phosphorylation in intact plasma membranes suggests the possibility that pp120 is a determinant of the unique effects of insulin on hepatocyte metabolism. It remains to be established whether pp120 in human liver membranes undergoes tyrosyl phosphorylation as does the pp120 present in preparations of insulin receptor partially purified from solubilized rat liver microsomes. The failure of the phosphotyrosyl binding antibodies to adsorb tyrosyl phosphorylated pp120 from rat liver microsomes is consistent with our unpublished observations that although these antibodies bind most phosphotyrosyl containing proteins, certain tyrosyl phosphorylated proteins and peptides bind poorly to the phosphotyrosyl binding antibodies.

Since depressed autophosphorylation of insulin receptor kinase is correlated with incomplete activation of the kinase activity of insulin receptor, the reduced insulin promoted phosphorylation of insulin receptor and pp120 in plasma membranes from a diabetic morbidly obese individual is consistent with the previously reported observation that solubilized partially purified insulin receptor from adipose (37) and hepatic tissue (32) from this individual (and other morbidly obese type II diabetics) exhibited reduced insulin stimulated kinase activity toward an exogenous substrate. It is interesting to note, however, that in contrast to the behavior of insulin receptor partially purified from adipocytes and hepatocytes, solubilized insulin receptor partially purified from skeletal muscle of the type II

diabetic studied in this report (as well as other morbidly obese type II diabetics) appears to undergo insulin promoted autophosphorylation in a manner indistinguishable from that of insulin receptor from normal skeletal muscle tissue (33). This finding suggests the possibility that the altered insulin responsiveness of insulin receptor characteristic of certain types of type II diabetes may be dependent on receptor locale.

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