Insulin Resistance in Rats Harboring Growth Hormone–Secreting Tumors: Decreased Receptor Number But Increased Kinase Activity in Liver

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Growth hormone (GH) is a potent antagonist of insulin action, and this resistance occurs primarily at a post-binding step(s). To elucidate the underlying mechanisms, the effects of chronic GH excess on the structure and function of insulin receptors partially purified from the liver were examined in rats harboring GH-secreting tumors. Insulin resistance was established in this animal model of GH hypersecretion by a hyperinsulinemic euglycemic clamp. Specific binding of ^{125}I-insulin and receptor number were reduced in tumor animals by 40% and 62%, respectively, reflecting downregulation of the insulin receptor by hyperinsulinemia in these animals. Receptors from tumor animals showed a 50% increase in β-subunit phosphorylation and in the kinase activity toward the synthetic polypeptide Glu4:Tyr1 when measured in vitro in the absence of insulin; however, the incremental stimulation by insulin (170 nmol/L) of the phosphorylation of either the β-subunit or Glu4:Tyr1 was not different between control and experimental animals. There was no difference between the two groups in Glu4:Tyr1 phosphorylation measured after immunodepletion of receptors by antibodies to the insulin receptor, indicating that the observed alteration in the kinase activity of tumor animals was intrinsic to the insulin receptor. Exposure to chronic GH excess did not alter insulin receptor structure, as evidenced by electrophoretic mobility under reducing and nonreducing conditions. The enhanced basal kinase activity of the receptor from tumor animals may reflect a more highly phosphorylated state of the receptor (and hence elevated enzyme activity) in these animals due to elevated serum insulin levels. These results demonstrate that the hepatic insulin resistance in rats chronically exposed to GH excess is not due to impaired insulin receptor kinase activity.

GROWTH HORMONE (GH) is a potent antagonist of insulin, as demonstrated by its effects on carbohydrate metabolism in acromegaly, when injected into normal animals or humans and when secreted physiologically.[1] Several studies suggest that this resistance to insulin action occurs primarily at post-binding steps. Thus, a rightward shift of the dose-response curves between insulin action on glucose utilization and inhibition of hepatic glucose production and the actual amount of bound insulin was observed in humans infused with GH.[2] Similarly, the ability of insulin to suppress glucose production and stimulate glucose disposal was attenuated in acromegalic patients in the absence of changes in insulin binding.[3] The action of insulin on glucose utilization was impaired in adipose tissue of patients with acromegaly because of postreceptor defects.[4] GH infusion has also been shown to stimulate hepatic glucose production and impair peripheral glucose uptake in type I diabetic patients.[5]

To study the interaction between insulin and GH, we have used a rat model with implanted GH-producing tumors.[6,7] In an earlier study,[6] we observed both binding and post-binding defects in insulin action on glucose incorporation into glycogen in hepatocytes isolated from tumor-bearing animals. It is now well established that one of the earliest events following insulin's binding to its receptor is the activation of tyrosine kinase activity of the β-subunit, and that the activated tyrosine kinase is important in coupling insulin binding to insulin action.[8-10] Impairment of autophosphorylation of the β-subunit of the insulin receptor and subsequent phosphorylations by the activated receptor have been implicated in the insulin resistance associated with obesity,[11] type II diabetes,[11] and high-fat diets.[12] Accordingly, we investigated the role of insulin receptor function in the development of hepatic insulin resistance in rats chronically exposed to GH excess.

MATERIALS AND METHODS

Materials

The following items were obtained from the indicated sources: wheat germ agglutinin-agarose from Vector Laboratories, Burlingame, CA; protein A (Pansorbin) from Calbiochem, La Jolla, CA; reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) from Bio-Rad, Richmond, CA; M_r markers and Glu4:Tyr1 from Sigma, St Louis, MO; and [γ-^{32}P]adenosine triphosphate (ATP) from ICN Radiochemicals, Irvine, CA. All other chemicals were of reagent grade and were obtained from standard suppliers. Porcine monocomponent insulin was a gift from Eli Lilly & Co, Indianapolis, IN, and serum containing antireceptor antibodies (B9 serum) was a kind gift from Dr C. Ronald Kahn, Joslin Diabetes Center, Boston, MA.

Experimental Animals

Animal studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female Wistar-Furth rats obtained from Harlan Sprague-Dawley, Indianapolis, IN, were fed standard laboratory chow until being killed. GH_3 cells, an established clone of rat pituitary tumor cells that secrete GH,[13] were maintained in monolayer culture in Dulbecco's modified Eagle's medium supplemented with horse serum (15%) and fetal calf serum (2.5%). For tumor generation, cells were harvested and suspended in Dulbecco's modified Eagle's

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medium and 1 mL containing 10^6 cells was injected subcutaneously into animals aged 6 to 7 weeks. Tumors were usually palpable by the end of 3 weeks. Control animals received only the vehicle. The animals were killed 5 weeks later, and the livers were rapidly frozen in liquid N_2 and stored at −70°C. Blood was collected for the determination of serum glucose by a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA) and insulin[14] and GH[15] by the appropriate radioimmunoassays.

Hyperinsulinemic Euglycemic Clamp

Hyperinsulinemic euglycemic clamps were performed as previously described.[16] The primed insulin infusion was administered as 15 mU/kg during the first minute, 10 during the second minute, and 7.5 during the third minute, followed by a continuous infusion of 5 mU/kg/min for the remainder of the 2-hour period. Glucose concentrations were clamped at 7.5 mmol/L, and samples were taken at 10-minute intervals between 60 and 90 minutes after the start of the infusion for measurement of glucose specific activity. The rate of total glucose appearance was calculated from the non–steady-state derivation of Steele's equations[17] and is equal to the rate of glucose disposal under equilibrium clamp conditions. The difference between glucose disposal and the exogenous glucose infusion rate represents the residual endogenous hepatic glucose production.

Partial Purification of Insulin Receptors

Microsomal membranes were prepared by a modification of the method of Blackshear et al.[18] Frozen livers were quickly thawed, minced, and homogenized with a Teflon-glass homogenizer in 4 vol ice-cold buffer containing 25 mmol/L HEPES (pH 7.6), 0.25 mol/L sucrose, 10% (vol/vol) glycerol, 5 mmol/L EDTA, 10 mmol/L each of benzamidine HCl and ε-amino-caproic acid, 1 mg/mL bacitracin, 10 μmol/L each of pepstatin and leupeptin, and 10^3 kallikrein-inhibiting units/mL aprotinin. The homogenate was centrifuged at 4°C for 20 minutes at 12,000 × *g* followed by centrifugation of the supernatant at 100,000 × *g* for 1 hour. The resulting microsomal pellet was resuspended in ice-cold buffer containing 0.25 mol/L sucrose and 50 mmol/L HEPES (pH 7.6), quick-frozen, and stored at −70°C until use. To obtain partially purified receptors, aliquots of membranes were thawed on ice in the presence of (final concentration) 10^3 kallikrein-inhibiting units/mL aprotinin and 10 μmol/L of pepstatin and leupeptin and solubilized by 1% (wt/vol) Triton X-100 at 4°C for 1 hour. The extract was centrifuged at 100,000 × *g* for 1 hour, and the supernatant was immediately applied to a 2-mL wheat germ agglutinin-agarose column at 4°C. After washing the column with 50 mL of a buffer containing 50 mmol/L HEPES (pH 7.6), 0.15 mol/L NaCl, and 0.1% Triton X-100, the bound glycoproteins were desorbed with washing buffer containing 0.3 mol/L *N*-acetyl-D-glucosamine. The fractions containing insulin receptors were combined and used for binding and phosphorylation studies. The protein content of microsomes and receptor preparations was determined by the Bradford dye method[19] using bovine serum albumin as the standard.

Insulin-Binding Assay

Aliquots of receptors (10 μg protein) were incubated with 10,000 cpm ^{125}I-insulin (0.3 ng/mL) and various concentrations of unlabeled insulin at 4°C for 18 hours in 50 mmol/L HEPES (pH 7.6), 0.1% Triton X-100, 0.15 mol/L NaCl, 0.1% bovine serum albumin, 1 mg/mL bacitracin, 10 μmol/L pepstatin, and 10 μmol/L leupeptin (buffer A). Insulin binding to microsomes (160 μg protein) was performed similarly, but in the absence of Triton X-100. Separation of free and receptor-bound insulin was achieved by the polyethylene glycol precipitation method[20] using bovine γ-globulin as carrier protein. Nonspecific binding obtained in the presence of 5 μg/mL unlabeled insulin was subtracted from total binding. Scatchard analysis of the binding data was used to determine the binding capacity.[21]

Cross-Linking of Receptors With ^{125}I-insulin

Aliquots of receptors were incubated with 4 ng ^{125}I-insulin (3×10^5 cpm) with or without 10 μg unlabeled insulin at 22°C for 2 hours in 60 μL buffer A, chilled on ice, and reacted with 2 mmol/L disuccinimidyl suberate in dimethyl sulfoxide at 0°C for 30 minutes. The reaction was terminated by the addition of threefold-concentrated Laemmli's sample buffer[22] containing 0.3 mol/L dithiothreitol (DTT). The mixture was heated in a boiling water bath for 5 minutes and analyzed by SDS-PAGE as described below.

Autophosphorylation of Insulin Receptors

Aliquots of receptors containing identical amounts of insulin-binding activity were preincubated with and without 170 nmol/L porcine insulin in buffer A supplemented with 10 mmol/L $MgCl_2$ and 5 mmol/L $MnCl_2$ at 22°C for 1 hour and chilled on ice. The phosphorylation reaction was initiated by the addition of a mixture yielding final concentrations of 10 μmol/L [γ-^{32}P]ATP, 1 mmol/L cytidine triphosphate (CTP), and 1 mmol/L sodium orthovanadate. The incubation was continued at 0°C for 30 minutes, and the reaction was terminated by the addition of (final concentrations) 20 mmol/L each of EDTA, sodium pyrophosphate, and NaF and 10 mmol/L ATP.

An aliquot of the terminated reaction mixture was boiled for 5 minutes in Laemmli's buffer containing 0.1 mol/L DTT, while another aliquot was denatured in the absence of DTT, and the phosphorylated proteins were separated by SDS-PAGE.

To analyze the phosphorylation of the insulin receptor–specific β-subunit, another aliquot of the terminated reaction mixture was incubated with a 1:40 dilution of serum containing antibodies to insulin receptor at 4°C overnight. The immune complex was precipitated with protein A, washed three times in 50 mmol/L HEPES (pH 7.6)–0.1% Triton X-100, and denatured as above in Laemmli's buffer supplemented with 0.1 mol/L DTT.

SDS-PAGE and Fluorography

Reaction samples or immunoprecipitates were applied to a 7.5% polyacrylamide gel or a 3% to 10% gradient gel along with M_r standards. After electrophoresis, gels were fixed, stained with Coomassie blue, destained, and dried, and autoradiography was performed with Kodak AR film (Eastman Kodak, Rochester, NY). The incorporation of ^{32}P into 95-kd protein and of ^{125}I into 135-kd protein was quantified by counting corresponding bands of the gel. The background was determined by counting a portion of a nonradioactive lane of the gel with the same surface as the band.

Phosphorylation of an Exogenous Substrate

Aliquots of receptors were preincubated with or without 170 nmol/L insulin at 22°C for 1 hour as described for the autophosphorylation reaction. Either the copolymer Glu4:Tyr1 (to a concentration of 1.5 mg/mL) or 50 mmol/L HEPES (pH 7.6) was then added, followed by [γ-^{32}P]ATP (10 to 20 μmol/L) and 1 mmol/L each of CTP and orthovanadate. After 30 minutes at 22°C, the reaction was terminated as before. Aliquots of the terminated reaction mixture were spotted onto Whatman 3MM paper squares, washed sequentially with ice-cold 10% trichloroacetic acid containing 10 mmol/L pyrophosphate, 5% trichloroacetic acid, and ethyl alcohol, dried, and counted in 3a70B scintillation fluid (RPI, Elk Grove Village, IL). The amount of radioactivity incorporated in the

presence of the copolymer minus that incorporated in its absence represented Glu4:Tyr1 phosphorylation.

In some experiments, insulin receptors were immunodepleted before the Glu4:Tyr1 kinase assay. The receptors were incubated with a 1:10 dilution of nonimmune serum (obtained from a control subject) or a 1:20 dilution of antireceptor serum for 16 hours at 4°C in the presence of protease inhibitors, precipitated by incubating with protein A for 2 hours at 4°C, and centrifuged. Aliquots of the supernatants were used as a source of tyrosine kinase for the phosphorylation of Glu4:Tyr1.

Measurement of ATPase Activity

Aliquots of receptor preparations or buffer without receptors (background) were preincubated in the absence or presence of insulin under conditions identical to those described for the autophosphorylation reaction and chilled on ice, and the phosphorylation reaction was initiated by the addition of 10 μmol/L [γ-^{32}P]ATP and 1 mmol/L each of CTP and orthovanadate. After 30 minutes at 0°C, 10-μL aliquots of the reaction mixtures were added to 2 mL 50-mmol/L KH_2PO_4 containing 0.1 g Norit A charcoal (Fisher, Pittsburgh, PA). The reaction mixture was vortexed vigorously, and the charcoal was pelleted by centrifuging at 4°C at 1,000 × *g* for 10 minutes. The percent of total radioactivity remaining in the charcoal-free supernatant represented the ^{32}P hydrolyzed from ATP.[23]

Data Analysis

Data are presented as the mean ± SEM. Statistical comparisons were made using a two-tailed nonpaired *t* test, and significance was accepted at a level of .05 or less.

RESULTS

Metabolic Characteristics

Metabolic characteristics of the two groups are shown in Table 1. The tumor-bearing animals were heavier and had lower glucose and higher insulin concentrations than the control group. GH levels were markedly elevated in the experimental animals as expected. The increase in liver weight seen here is in accordance with the larger hepatocyte size reported previously.[6] The increased weight of these animals is lean body mass, since they have very little fat as compared with control rats.[24] The hypoglycemic hyperinsulinemic status of the tumor animals noted here has been consistently observed by us[6,7] and others.[25,26] Although these tumor-bearing animals have lower glucose disappearance rates after an intravenous glucose load (in the face of enhanced insulin secretion) and following a glucose-insulin challenge, showing that these animals are insulin-resistant,[7] insulin resistance has been doubtful because of the fasting hypoglycemia.

To prove more definitively that insulin resistance characterizes rats with GH-secreting tumors, hyperinsulinemic euglycemic clamps were performed. In these experiments, weight gain was significantly higher in 14 tumor-bearing animals as compared with 11 controls (2.7 ± 0.2 *v* 1.0 ± 0.1 g/d, $P < .001$). After anesthesia and surgery, glucose concentrations remained significantly lower and insulin levels significantly higher in tumor-bearing animals (Table 2). Steady-state glucose and insulin levels were similar during the clamp. Tracer-derived glucose disposal and exogenous glucose infusion rates were significantly decreased in rats harboring tumors. Endogenous hepatic glucose production during the clamp period was not significantly different between the two groups at these relatively high insulin concentrations.

Table 1. Characteristics of Control and Tumor-Bearing Rats

	Control (n = 8)	Tumor-Bearing (n = 8)	*P*
Body weight (g)	190 ± 5	235 + 5	<.001
Serum glucose (mmol/L)	7.8 ± 0.1	6.4 ± 0.3	<.005
Serum insulin (pmol/L)	150 ± 30	267 ± 40	<.05
Serum GH (μg/L)	64 ± 14	6,040 ± 806	<.001
Liver weight (g)	6.6 ± 0.2	12.7 ± 4	<.001

NOTE. Values are the mean ± SEM.

Table 2. Effect of Chronic GH Excess on Glucose Metabolism in Rats

	Control (n = 14)	Tumor-Bearing (n = 11)	*P*
Basal			
Glucose (mmol/L)	8.1 ± 0.15	7.4 ± 0.14	<.01
Insulin (pmol/L)	212 ± 13	443 ± 28	<.001
Clamp			
Glucose (mmol/L)	7.4 ± 0.2	7.7 ± 0.1	NS
Insulin (pmol/L)	654 ± 38	658 ± 31	NS
Glucose disposal (mg/kg/min)	20.5 ± 0.9	15.1 ± 0.5	<.001
Glucose infusion (mg/kg/min)	14.5 ± 0.9	10.2 ± 0.6	<.001
Glucose production (mg/kg/min)	6.0 ± 0.5	4.9 ± 0.4	NS

NOTE. Values are the mean ± SEM.

Insulin Binding

Insulin binding to receptors that were solubilized and partially purified from the liver membranes was decreased in the tumor rats as shown by the competitive-binding curves (Fig 1). The data were subjected to Scatchard analysis, and the binding parameters are presented in Table 3. Liver receptors from tumor-bearing animals showed a significant reduction in tracer binding and receptor number

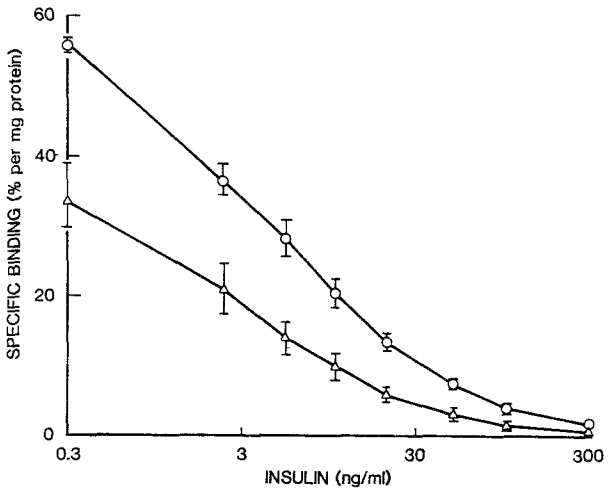

Fig 1. Insulin binding to receptors partially purified from the liver of (○) control and (△) tumor rats. Data points are the mean ± SEM for eight animals.

with no significant alteration in affinity. A similar reduction in tracer binding and receptor number was observed with microsomal membranes isolated from the tumor group (Table 3). However, the affinity of the membrane-bound receptor for insulin was higher (lower ED_{50}) in the tumor group, and this affinity change was not apparent after membrane solubilization and partial purification of the receptor; this may account for the less-pronounced decrease in tracer binding of the microsomal fraction as compared with that of purified receptors for the tumor group. Likewise, a decrease in receptor concentration and an increase in receptor affinity has been reported with liver membranes from GH-treated rats[27] and monocytes isolated from acromegalic patients.[28] The alterations in insulin binding observed here were not due to differences in ^{125}I-insulin degradation, since the reduction in trichloroacetic acid precipitability during the binding assay was not different between the two groups (Table 3).

Structure of the Receptor α-Subunit

Incubation of receptors from control and experimental animals with ^{125}I-insulin followed by cross-linking with disuccinimidyl suberate specifically labeled a protein of approximately 135 kd (Fig 2), which corresponds to the approximate M_r of the α-subunit of the insulin receptor. No differences were observed in the electrophoretic migration of the α-subunit between receptor populations from control and tumor-bearing animals.

For the purpose of kinase assays, receptor preparations are usually adjusted to achieve equal numbers of insulin receptors so that kinase activity of different groups can be compared on the basis of equal binding. In the present study, receptor preparations were adjusted to obtain equal amounts of binding potency as determined by the amount of specific ^{125}I-insulin cross-linked to the receptor (Fig 2). The amount of ^{125}I-insulin cross-linked (counts per minute) per nanogram of insulin bound (derived from Scatchard analysis) was similar for the two groups (1,038 ± 23 for control and 1,047 ± 80 for tumor animals, n = 8).

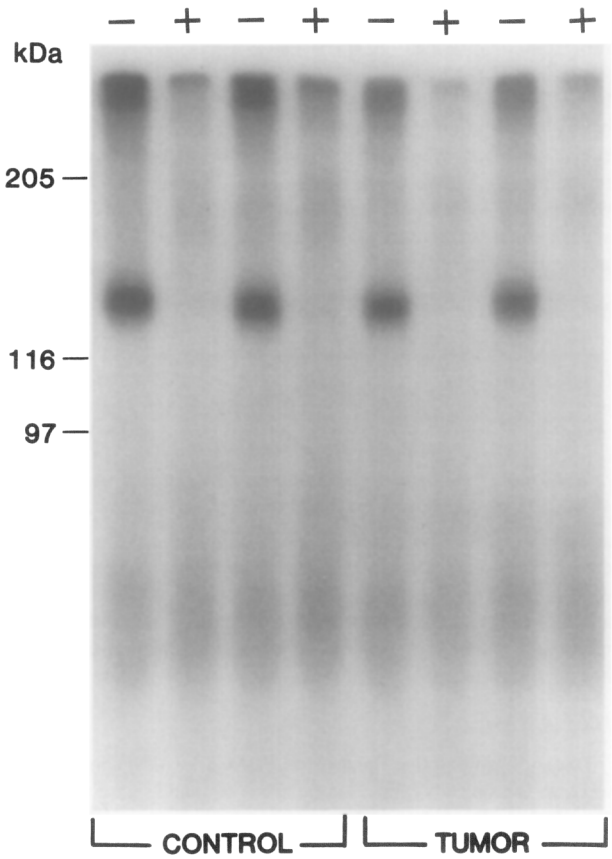

Fig 2. Structural analysis of the insulin receptor α-subunit. Control and tumor animal-derived receptors were incubated with 4 ng ^{125}I-insulin for 2 hours at 22°C in the (−) absence or (+) presence of 10 μg unlabeled insulin, cooled to 0°C, and reacted with 2 mmol/L disuccinimidyl suberate for 30 minutes. Samples were denatured in Laemmli's buffer containing 0.1 mol/L DTT and separated on 7.5% SDS-PAGE. Radioactivity in the 135-kd band in the (+) presence of unlabeled insulin was subtracted from that incorporated in the (−) absence of unlabeled insulin to determine the specific ^{125}I-insulin cross-linked to the receptor. The data represent results from two animals for each group.

Table 3. Insulin-Binding Parameters in Liver Receptors From Control and Tumor-Bearing Rats

	Control	Tumor-Bearing	*P*
Partially purified receptor (n = 8)			
Tracer binding (% per 10 μg protein)	55.5 ± 1.0	33.5 ± 3.5	<.025
Binding capacity (pmol/mg protein)	18.7 ± 1.8	7.1 ± 1.4	<.005
ED_{50} (pmol/L)	780 ± 135	495 ± 60	NS
Tracer degradation (%)	4.2 ± 0.1	4.4 ± 0.2	NS
Microsomal membrane fraction (n = 4)			
Tracer binding (% per 160 μg protein)	24.9 ± 0.8	21.7 ± 0.7	<.05
Binding capacity (pmol/mg protein)	0.33 ± 0.2	0.21 ± 0.04	<.05
ED_{50} (pmol/L)	2,685 ± 120	1,935 ± 270	<.05
Tracer degradation (%)	13.7 ± 0.2	13.7 ± 0.2	NS

NOTE: Values are the mean ± SEM.

Autophosphorylation of Insulin Receptor

When lectin-purified receptors from the two groups of animals were preincubated in the absence (basal) and presence of insulin and then exposed to [γ-^{32}P]ATP, there was an insulin-dependent increase in the amount of ^{32}P in the 95-kd protein as shown in Fig 3A. The phosphorylated protein was identified as the β-subunit of the insulin receptor by virtue of its M_r, dose-response to insulin in terms of ^{32}P labeling (data not shown), and the fact that it is immunoprecipitable by antibodies to insulin receptor (Fig 3B) but not with control serum (data not shown). It should also be noted from Fig 3 that there is no apparent difference in the electrophoretic mobility of the phosphorylated β-subunit of insulin receptors from control and tumor-bearing animals. The other major protein phosphory-

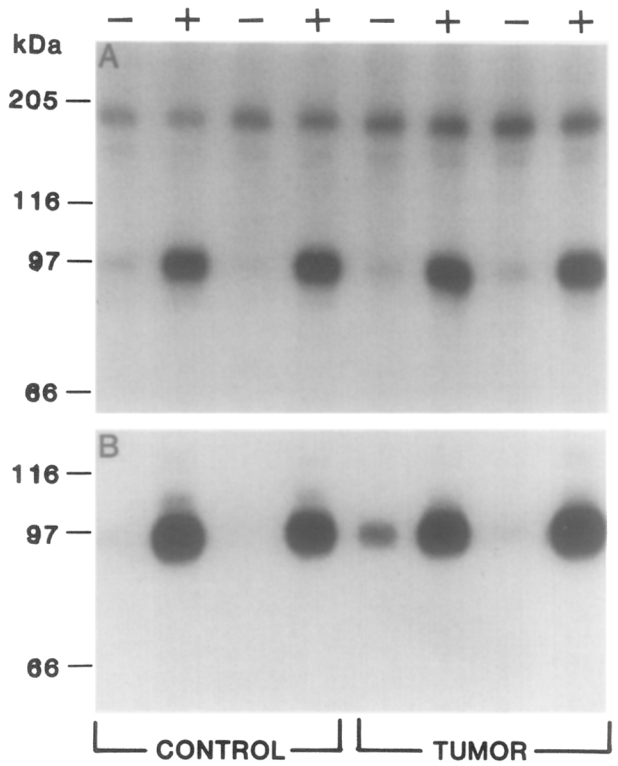

Fig 3. Autoradiogram showing receptor autophosphorylation in vitro. Aliquots of wheat germ agglutinin eluate were incubated (+) with 170 nmol/L or (−) without insulin at 22°C for 1 hour, chilled, phosphorylated in the presence of 10 mmol/L $MgCl_2$, 5 mmol/L $MnCl_2$, 10 μmol/L [γ-^{32}P]ATP, and 1 mmol/L each of CTP and vanadate at 0°C for 30 minutes, and the reaction was then stopped. (A) One aliquot was denatured and subjected to SDS-PAGE under reducing conditions. (B) Another aliquot of the terminated reaction mixture was incubated with a 1:40 dilution of serum containing antibodies to the receptor for 16 hours at 4°C, the immune complex was precipitated with protein A, and the immunoprecipitate was analyzed by SDS-PAGE under reducing conditions. Data are representative of two animals per group.

lated under these conditions has a M_r of 170 kd, and has been identified as the receptor for epidermal growth factor[18,29]; phosphorylation of this protein is not insulin-dependent (Fig 3A).

Quantitative data on the phosphorylation of the 95-kd protein are presented in Fig 4A. Autophosphorylation of receptors from tumor-bearing animals was enhanced by 48% in the absence of insulin and by 15% in the presence of a maximally stimulating concentration (170 nmol/L) of insulin in comparison to that for control liver receptors; however, the incremental stimulation by insulin was identical in the two groups. Sodium orthovanadate, a potent phosphatase inhibitor, and CTP, a competitive antagonist of ATPases but not a substrate for insulin receptor kinase, were present in all phosphorylation assays. The amount of ATP hydrolyzed was 9.5% in the absence of receptors (background) and 10.5% in the presence of receptors from either control or tumor-bearing animals. Thus, ATPase activity of isolated receptors was minimal under the conditions of phosphorylation, and the observed difference in the

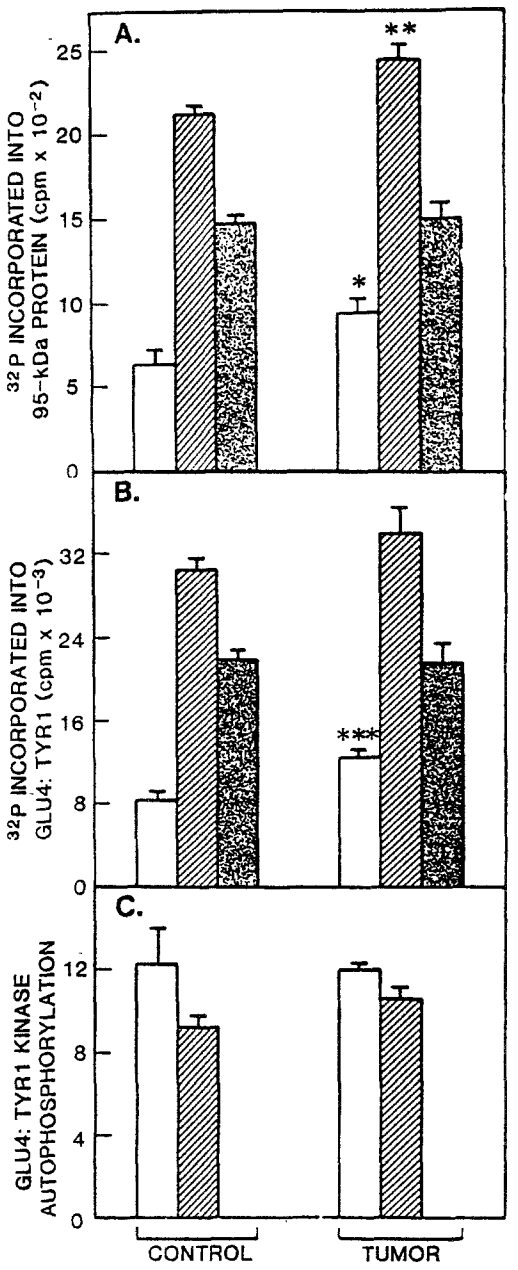

Fig 4. Phosphorylation of 95-kd protein and Glu4:Tyr1 in vitro. (A) Receptors containing equal amounts of binding activity from control and tumor animals were allowed to autophosphorylate in the (□) absence or (▨) presence of 170 nmol/L insulin as in Fig 3A, and ^{32}P incorporation into the 95-kd protein was quantified in the absence of immunoprecipitation. (■) Dark bars refer Insulin-stimulated incremental phosphorylation. Results represent the mean ± SEM of six receptor preparations per group. (B) Receptors containing equal amounts of insulin-binding activity were incubated for 1 hour at 22°C in the (□) absence or (▨) presence of 170 nmol/L insulin and then assayed for Glu4:Tyr1 kinase activity as described. (■) Net stimulation by insulin. Data represent the mean ± SE of eight receptor preparations for each group. (C) The ratio of Glu4:Tyr1 kinase activity to β-subunit phosphorylation (six receptor preparations per group). *$P < .05$, **$P < .02$, ***$P < .001$: compared with control under similar incubation conditions.

basal kinase activity of the two groups of liver receptors was not a result of differing phosphatase and ATPase activities.

Exogenous Substrate Phosphorylation

The insulin receptor serves as both enzyme and substrate in the autophosphorylation reaction. To determine the kinase activity of the receptor activated by the autophosphorylation event, phosphorylation of an exogenous substrate was measured. For this purpose, the synthetic polypeptide Glu4:Tyr1, whose phosphorylation is only minimally stimulated by epidermal growth factor receptors,[29] was chosen as the substrate, and the results are presented in Fig 4B. The basal kinase activity of the tumor animal receptors toward Glu4:Tyr1 was increased by 49%, whereas there was no difference between the two groups in maximally insulin-stimulated phosphorylation. The ratio of Glu4:Tyr1 kinase activity to autophosphorylation, measured in the presence or absence of insulin, was identical (Fig 4C) in receptors derived from control and tumor-bearing animals. Thus, a similar magnitude of kinase activity was expressed by receptors from the two groups following autophosphorylation in vitro.

Specificity of Enhanced Basal Kinase Activity in Tumor Rats

The absence of differences in insulin-stimulated incremental phosphorylation of either the β-subunit (Fig 4A) or Glu4:Tyr1 (Fig 4B) between the two groups raised the possibility that the observed increases in basal autophosphorylation (Fig 4A) and Glu4:Tyr1 phosphorylation (Fig 4B) could be due to the presence of free β-subunits or a tyrosine kinase different from the insulin receptor in tumor animal receptor preparations. Since free β-subunits would not respond to the signal generated by insulin's binding to the α-subunit of the receptor, they could presumably express basal but not insulin-stimulated kinase activity, thus accounting for the enhanced basal kinase activity of the receptors derived from these animals. To test this possibility, receptors from the two groups of animals were autophosphorylated in the absence and presence of insulin and analyzed on SDS-PAGE under nonreducing conditions. As seen in Fig 5, no phosphoproteins with a M_r of 95,000 were detected in either group, confirming the absence of free β-subunits in the receptor preparations. An insulin-stimulated phosphoprotein was observed in both groups at a M_r of 370,000 corresponding to the intact insulin receptor, the electrophoretic mobility of which was not different between the two groups of animals.

To assess the contribution of tyrosine kinase(s) other than the insulin receptor, we immunoprecipitated the insulin receptor from the preparations and used the supernatants as a source of kinase for Glu4:Tyr1 phosphorylation. Equal amounts of insulin-binding activity from the two groups were exposed to either control serum or serum containing anti–insulin receptor antibodies. Glu4:Tyr1 kinase activity was then measured in the supernatants obtained after precipitating receptor-antibody complexes with protein A. The activity remaining in the supernatant after precipitation with control serum represents "total" kinase, ie, insulin receptor kinase and other kinases that copurified

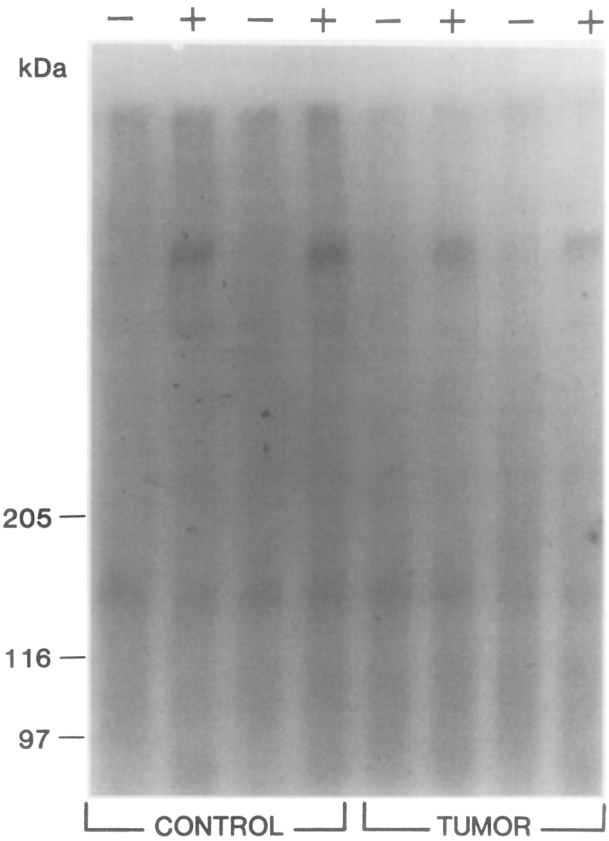

Fig 5. Autoradiograph showing the absence of free β-subunits in receptor preparations. Receptors were preincubated in the (−) absence or (+) presence of 170 nmol/L insulin and then autophosphorylated as in Fig 3. The reaction was terminated, and the phosphoproteins were separated by SDS-PAGE under nonreducing conditions on a 3% to 10% gradient gel. Results of two animals per group are shown.

with the insulin receptor, whereas the activity in the supernatant from precipitation with immune serum is due to tyrosine kinases other than the insulin receptor. Thus, the difference in the activities of the two supernatants gives a measure of insulin receptor–specific kinase. Such an approach has been used by Freidenberg et al[29] to demonstrate that the differences in basal kinase activity brought about by fasting and carbohydrate feeding are intrinsic to the insulin receptor. The results of these experiments are depicted in Fig 6. The total Glu4:Tyr1 kinase activity is shown in Fig 6A, and the results are similar to those displayed in Fig 4B in that the basal kinase activity is elevated in the tumor animals (by 64%) while there was no difference between control and tumor animals in net stimulation by insulin. However, the responsiveness to insulin in these experiments was much lower than in the absence of immunoprecipitation (Fig 6A *v* Fig 4A), and this experimental artifact may arise either from the particular control serum used or from the additional 16-hour incubation at 4°C. The tyrosine kinase activity of supernatants from immune serum precipitation was not further stimulated by insulin in vitro (data not shown). Thus, insulin receptors were quantitatively precipitated by the immune

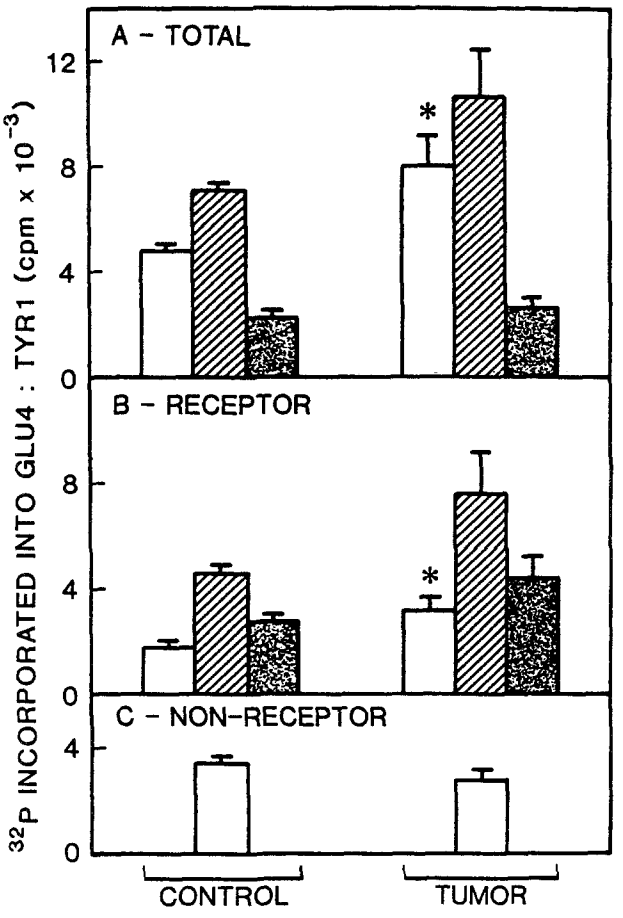

Fig 6. Specificity of insulin receptor kinase activity toward Glu4:Tyr1. Equal amounts of insulin-binding activity from control and tumor animals were incubated with a 1:20 dilution of antireceptor serum or a 1:10 dilution of control serum for 16 hours at 4°C followed by incubation with protein A for 2 hours. Immune complexes were sedimented by centrifuging at 10,000 × *g* for 3 minutes at 4°C. Aliquots of the supernatant were incubated for 1 hour at 22°C in the (□) absence or (▨) presence of 170 nmol/L insulin and assayed for Glu4:Tyr1 kinase activity. (■) Insulin-stimulated incremental phosphorylation. Kinase activity remaining in supernatants from receptors incubated with control serum and antireceptor serum represents (A) total Glu4:Tyr1 phosphorylating activity and (C) Glu4:Tyr1 activity of kinase(s) other than the insulin receptor, respectively. The difference between (A) total and (C) nonreceptor kinase activities is (B) the insulin receptor–specific kinase activity. Kinase activities are expressed as the amount of ^{32}P incorporated into Glu4:Tyr1 per equal amount of insulin-binding activity (A and B) or protein (C). Results represent the mean ± SEM of four receptor preparations for each group. *Significantly different from control, $P < .05$.

serum, and the activity of the supernatants represented non–receptor-associated kinase. The non–receptor-associated kinase activity, best expressed on the basis of eluate protein concentration, remained relatively constant in the two groups in the basal state (Fig 6C). The results of the kinase activity directly linked to insulin receptors (Fig 6B) show that the basal kinase activity of tumor animal receptors was 82% higher than that of control receptors, and that the magnitude of the increase in insulin-stimulated phosphorylation was fully accounted for by the increase in basal activity. Thus, the observed differences in Glu4:Tyr1 phosphorylation (Figs 4B and 6A) are due to intrinsic differences in insulin receptor kinase activity between control and experimental animals.

DISCUSSION

In an attempt to elucidate the mechanism(s) underlying GH-induced insulin resistance, we investigated the effects of chronic GH excess on the structure and function of hepatic insulin receptors in somatomammotrophic pituitary tumor–bearing rats. These animals gained weight more rapidly, had lower glucose and higher insulin and GH levels in the serum, and manifested insulin resistance during a hyperinsulinemic, euglycemic clamp (Table 2). Partially purified receptor preparations showed a significant decrease in both tracer insulin binding and receptor number in the tumor animals. These alterations in insulin binding were not a result of either increased insulin degradation or a direct action of GH, since GH by itself does not affect insulin binding.[28,30,31] Moreover, these changes were not due to a differential recovery of glycoproteins during receptor purification, since similar results were obtained with microsomal membranes in the present investigation and previously with freshly isolated liver cells.[6] The diminished insulin binding most likely reflects downregulation of the insulin receptor[32] by the high levels of circulating insulin in these animals.

An insulin-induced change in the proportion of the oligomeric forms of the insulin receptor has been observed in Fao hepatoma cells during downregulation.[33] The electrophoretic mobility of β-subunits derived from muscle[34] but not from liver[35] of streptozocin-induced diabetic rats was found to be altered possibly due to excess sialidation, and this was normalized by insulin treatment.[34] In the present study, the electrophoretic mobilities (and by inference, the apparent molecular size) of the tetramer, ^{125}I-insulin cross-linked α-subunit and autophosphorylated β-subunit were not altered in the downregulated liver receptors of tumor animals. Similarly, molecular weights of the nonreduced receptor and its β-subunit were not altered in fat cells downregulated by exposure to insulin for 16 hours.[36]

Autophosphorylation of the 95-kd protein in vitro was elevated in receptors isolated from tumor animals, both in the presence and absence of insulin. Furthermore, the difference in insulin-stimulated autophosphorylation between the two groups was fully accounted for by the difference in basal activity. It could be argued that the receptors that are more phosphorylated in vivo would have fewer sites for [γ-^{32}P]ATP in vitro, in which case the maximum autophosphorylation elicited by insulin should be less for receptors from tumor-bearing animals; however, evidence in the literature does not support this contention. Thus, receptors maximally activated by insulin in intact adipocytes[37] and in vivo in rat muscle[38] can be further phosphorylated by ATP in vitro, raising the possibility that some tyrosine-containing sites on the receptor are only phosphorylated in vitro and may not be accessible to phosphorylation in vivo. Moreover, kinetic studies[29] have shown that the maximum basal phosphorylation attained in vitro was less than 20% of the maximal level achieved with

insulin, indicating that insulin not only increases the rate of autophosphorylation but also increases its extent. In agreement with the autophosphorylation data, receptors from tumor animals exhibited higher basal but similar insulin-stimulated activity toward Glu4:Tyr1. The kinase activity toward Glu4:Tyr1 closely paralleled receptor autophosphorylation for both groups, suggesting a similar relationship between the activation state of the receptor and its ability to phosphorylate other proteins. The possibility that free β-subunits contributed to the enhanced basal kinase activity of the tumor animal receptors was ruled out, since they were not detectable by SDS-PAGE under nonreducing conditions. There was no difference in the non–receptor-associated kinase activity (toward Glu4:Tyr1) of the preparations from the two groups of animals, confirming that the observed alterations in the kinase activity of animals bearing GH-secreting tumors were intrinsic to the insulin receptor. Likewise, the basal kinase activity toward Glu4:Tyr1 has been found to be enhanced in carbohydrate-fed rats and decreased in starved rats, although concomitant changes in receptor autophosphorylation were not found.[29] An elevation in the basal kinase activity of the insulin receptor was also noted in fat cells rendered hyperinsulinemic by in vitro exposure to 0.1 μmol/L insulin for 16 hours.[36]

The mechanism by which the basal kinase activity of the insulin receptor is elevated in the liver of tumor animals is not clear. Alterations in the metabolic state associated with chronic GH excess could result in changes in the maturation or glycosylation of the insulin receptor; however, these were not apparent by electrophoretic analysis. Modifications in the kinase activity of the insulin receptor are known to be brought about by changes in the phosphorylation state of the receptor.[39,40] Thus, the receptor from downregulated liver tissue of tumor-bearing animals would be expected to be more highly phosphorylated than the control due to chronic hyperinsulinemia, which in turn would be reflected in higher basal kinase activity. This possibility is consistent with the finding that even a modest increase in serum insulin (comparable to the levels of tumor-bearing animals in this study) can activate the tyrosine kinase activity of muscle-derived receptors.[38] Recent evidence[41-43] suggests that either the tyrosine kinase activity of the insulin receptor or its phosphorylation state is essential for ligand-mediated receptor downregulation. Thus, the enhanced basal kinase activity of the receptor may be linked to its downregulation in tumor animals. Alternatively, it is possible that serine phosphorylation of the insulin receptor regulates its tyrosine kinase activity in these animals. Phosphorylation of the insulin receptor at serine residues has been shown to impair its tyrosine kinase activity both with purified enzymes in vitro[44,45] and in cultured cells.[46,47] Additional studies will be needed to determine whether a reduction in the endogenous serine phosphorylation of the insulin receptor is responsible for the observed increase in its tyrosine kinase activity in tumor-bearing rats.

In a previous investigation from this laboratory,[6] we reported a decrease in the sensitivity and maximal responsiveness to the action of insulin on glucose incorporation into glycogen in hepatocytes isolated from tumor-bearing rats, which suggested that the liver from these animals exhibited both binding and post-binding defects in insulin action. However, for a given amount of insulin bound, the effect of insulin was comparable for control and tumor animals, suggesting a normal functioning of the downregulated receptors in animals harboring GH-secreting tumors.[6] Although the insulin-binding capacity per unit protein of tumor rat liver was 38% to 64% of control values (Table 3), the liver weight of tumor rats was nearly twice that of normal rats (Table 1), indicating little change in total receptor concentration per liver. Thus, the enhanced kinase activity per insulin receptor in tumor animals reflects a net increase in the kinase activity of liver in tumor animals, and in animals rendered insulin-resistant by chronic exposure to GH, the post-binding defect is not reflected in an impairment of tyrosine kinase activity of the insulin receptor measured in vitro. Consistent with these results, there was no decrease in basal or insulin-stimulated phosphorylation of Glu4:Tyr1 in purified insulin receptors of adipocytes removed from GH-treated pigs.[48] In fact, there was an increase at higher insulin concentrations. Since there may be important differences in the autophosphorylation and protein kinase activity of the isolated insulin receptor and its function in intact cells,[49] it is possible that the kinase activity measured in vitro does not adequately reflect the alterations in insulin receptor in vivo brought about by GH excess. However, this appears unlikely, since muscle insulin receptor kinase activity was unaffected by 5-hour infusions of GH in human subjects that resulted in insulin resistance.[50] Thus, decreased insulin receptor function in liver (present report), fat,[48] or muscle[50] does not explain GH-induced insulin resistance.

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