Effects of Diabetes and Sex Steroid Hormones on Insulin Receptor Tyrosine Kinase Activity in R3230AC Mammary Adenocarcinomas

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ABSTRACT

Insulin binding and receptor tyrosine kinase activity were investigated in the insulin-responsive R3230AC mammary adenocarcinoma. Insulin receptors, partially purified by wheat germ agglutinin-agarose chromatography, displayed electrophoretic properties similar to those of normal tissues and demonstrated autophosphorylation of the β subunit. Tyrosine kinase activity of tumor preparations was measured by incorporation of \( ^{32}P \) from ATP into the synthetic polypeptide substrate glutamic acid-tyrosine. The \( K_m \) (app) for ATP, 15 to 30 \( \mu \)M in tumors from ovariectomized or intact rats, appeared to be increased by 10\(^{-7}\) M insulin in vitro, with the calculated \( V_{max} \) increased by 3- to 5-fold; the \( K_c \) (app) for glutamic acid-tyrosine was 2 to 3 \( \mu \)M and insulin increased the \( V_{max} \) by 25 to 50%. The effects of diabetes and insulin treatment and of various doses of estradiol, progesterone, estradiol plus progesterone, or tamoxifen on insulin binding, basal tyrosine kinase activity, and insulin- and estradiol-inducible tyrosine kinase activity in vitro were studied in tumors from treated animals. Preparations from diabetic rats had elevated insulin binding and basal tyrosine kinase activity and displayed a striking dose-related increase in the ability for insulin induction of tyrosine kinase activity in vitro compared to intact animals; these effects of diabetes were prevented by administration of insulin. Over comparable doses, insulin growth factor 1 added in vitro induced tyrosine kinase activity minimally versus that seen for insulin. Treatment of rats with pharmacological doses of sex steroid hormones produced changes in insulin binding capacity and/or basal tyrosine kinase activity and, depending on dose, usually resulted in increased basal kinase activity relative to insulin binding. The insulin-inducible increase in tyrosine kinase activity in vitro was not altered by treatment with estradiol or estradiol plus progesterone in vivo, whereas high doses of progesterone attenuated the response. A consistent finding with increasing doses of sex steroids was an increase in the half-maximum dose or 50% maximum induction dose for insulin, implying reduced responsiveness. Tamoxifen administered to intact rats increased insulin binding and blunted the insulin-induced increase in tyrosine kinase in vitro; these effects were not seen in ovariectomized rats. We conclude that: (a) insulin receptors from R3230AC mammary tumor preparations display properties similar to those of nonneoplastic tissues, including induction of tyrosine kinase activity by insulin in vitro; (b) the effects of diabetes and insulin treatment on insulin receptor, and its induction by insulin in vitro, since this is a potential site of action for modulation of insulin receptors.

INTRODUCTION

There is growing evidence for the role of insulin as a hormonal factor in mammary cancer, based on the alterations of growth of rodent and human tumor cells subsequent to perturbations of the insulin milieu (1–8). Furthermore, data have been obtained that implicate insulin as a regulatory hormone for estrogen receptors (9–11), and reports have appeared indicating that estrogens and progesterone affect insulin receptors (12–14). On the basis of these findings, we have proposed that interrelationships between sex steroids and insulin action should be considered when defining mechanisms for regulation of mammary cancer growth (15, 16).

The action of insulin, as with other polypeptide hormones, is initiated by binding of the hormonal ligand to its plasma membrane receptor. The insulin receptor is a glycoprotein heterotetramer, each dimer consisting of an α subunit, \( M_r \), 130,000, which contains the ligand recognition site, and a β subunit, \( M_r \), 95,000, which contains the kinase catalytic domain (reviewed in Refs. 17 and 18). The observed insulin-induced autophosphorylation of the β subunit is considered to be a critical event for expression of its TyrK activity, which is postulated to be a mechanism for mediation of the insulin signal (19, 20). The fact that other growth factor receptors, such as epidermal growth factor receptor and IGF-1, also possess TyrK activity has given further credence to the importance of this inherent property of these receptors (21).

Previous studies with the R3230AC hormone-responsive transplantable mammary adenocarcinoma have shown that the characteristics of the insulin receptor in these tumors are comparable to those reported for normal tissues, e.g., binding affinity, specificity for ligand, down-regulation by insulin, etc. (22–24). The suppression that estradiol regulates insulin receptors in this tumor was based on increased insulin binding after ovariectomy and decreased binding after estradiol administration in vivo (9) and demonstration of estradiol down-regulation of insulin receptors in cultured R3230AC cells in vitro (13). In the present experiments, we examined the effects of various hormonal perturbations in vivo on the TyrK activity of the insulin receptor, and its induction by insulin in vitro, which has given further credence to the importance of this inherent property of these receptors (21).

MATERIALS AND METHODS

Tumor Transplantation and Animal Injections. Thirty-five-day-old Fischer rats from Charles River Breeding Laboratory (Wilmington, MA) were implanted s.c. with the R3230AC mammary adenocarcinoma by a sterile trocar technique as described by Hilf et al. (25). Animals were ovariectomized 1 week prior to tumor implantation. Some OVEX animals were given injections of either estradiol valerate (0.01 to 1 mg) or hydroxyprogesterone caproate (0.2 to 50 mg) twice weekly for 25 weeks, beginning 4 days after tumor implantation. Diabetes was induced with streptozotocin as described previously (26).

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2 The abbreviations used are: Glu4-Tyr1, glutamic acid-tyrosine; OXEV, ovariectomized; TyrK, tyrosine kinase; IGF, insulin growth factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; HAB buffer, HEPES buffer, pH 7.4, containing 2 mg/ml BSA and 1 mg/ml bacitracin; SDS, sodium dodecyl sulfate; ED\(_{50}\), amount of insulin producing 50% of the maximum induction.
consisted of twice daily injections of 3 IU protamine zinc insulin for 3 days, a regimen that normalized glucose levels in blood and urine (26). Animals were sacrificed 24 h after the last injection. Control animals (intact or OVEX) were used at the same stage of tumor growth, 17 to 21 days after implantation. The tumor was excised and a piece was removed for the receptor preparation.

Preparation and Purification of Insulin Receptors. A piece of tumor (0.3 to 0.6 g) was homogenized in a solubilizing buffer containing 50 mM HEPES, 5 mM sodium vanadate (ortho), 100 mM sodium fluoride, 5 mM EDTA, 2 mM p-nitrophenylphosphate, 1 mg/ml bacitracin, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, and 2% Triton X-100. The homogenate was solubilized (on ice) for 1 h and centrifuged for 30 min at 50,000 g (13). The supernatant was applied to a 2-ml wheat germ agglutinin-agarose column that had been washed extensively with alternating acid (1 M acetic acid containing 0.1% Triton X-100) and alkaline (50 mM HEPES containing 0.1% Triton X-100, pH 7.4) solutions. After the receptor preparation was recycled 4 times, the column was washed with a solution containing 50 mM HEPES, 0.1% Triton X-100, and 150 mM NaCl. The receptor was eluted with 2.5 ml of a solution of 50 mM HEPES and 0.1% Triton X-100 containing 0.3 M N-acetylglucosamine (27, 28); receptor was obtained in the eluant subsequent to the first ml, which was discarded. The resulting receptor preparation contained approximately 3 mg/ml protein as measured by the method of Lowry et al. (29). This eluant containing partially purified insulin receptors was used for all further studies.

Samples obtained from each treatment group showed similar recoveries of insulin binding (>85%). The recycling procedure on the column resulted in an average 50-fold purification; initial binding ranged from 0.06 to 0.54 fmol insulin bound/100 g protein versus 4 to 15 fmol insulin bound/100 g protein in the column eluates. No differences in recovery or fold purification could be attributed to any particular treatment regimen in vivo.

Insulin Binding. Insulin binding was performed as reported previously (13). Briefly, crystalline porcine insulin was labeled stoichiometrically with Na125I using chloramine-T, according to the method of Freychet et al. (30). Binding to the partially purified receptor was measured using 2 × 10^-10 M 125I-labeled insulin, plus or minus 1000-fold excess unlabeled insulin, for 18 h at 4°C in HAB buffer. Under these conditions, degradation of 125I-labeled insulin was less than 3% based on recovery of radioactivity in the trichloroacetic acid precipitate. The 125I-labeled insulin bound to the receptor was separated from the free 125I-insulin by the addition of 25% polyethylene glycol and 1 mg/ml bovine y-globulin. After centrifugation and washing, the resulting pellet was counted in a Beckman 8000 gamma counter (38). Under the conditions stated, 32P incorporated into Glu4:Tyrl; this modification did not affect the results. The phospho-tyrosine kinase was separated from the immunoprecipitate and subjected to sodium dodecyl sulfate electrophoresis under either reducing or nonreducing conditions. Protein A was then added and incubated for 1 h at 4°C. The immunoprecipitate was collected and washed according to the method of Fujita-Yamaguchi et al. (33). The receptor was extracted from the immunoprecipitate and subjected to sodium dodecyl sulfate electrophoresis under either reducing (34) or nonreducing conditions (35). For the former, the pellet was boiled for 3 min in 3.8% SDS-80 mM Tris pH 6.8-5% b-mercaptoethanol for extraction. For the nonreducing conditions, the pellet was boiled for 3 min in 1% SDS-10 mM phosphate buffer, pH 7.0. Autoradiography was carried out on the dried gel as described by Lerea and Livingston (35).

Assay of Tyrosine Kinase Activity with the Artificial Substrate Glu4:Tyrl. The ability of the partially purified insulin receptor to phosphorylate an artificial substrate was assessed, using the methods of Braun et al. (36) and Stadtmueller and Rosen (37) with some modifications. Thirty µl (about 100 µg protein) of the partially purified receptor were reincubated for 20 min at 24°C in the presence or absence of 10 µM of insulin (10^-10 to 5 x 10^-7 M) in a solution containing 50 mM HEPES, 0.1% Triton X-100 and 0.2% BSA, pH 7.4, to which 10 µl of the ion-substrate mixture were added. The final concentration of components in the ion-substrate mixture in the assay was 10% glycerol, 10 mM MgCl2, 2 mM MnCl2, 10 µg/ml BSA, 2 mM p-nitrophenylphosphate, and 1 mg/ml of the artificial substrate, Glu4:Tyrl. In some experiments, the final concentration of glycerol was reduced to 1% because of solubility problems with certain lots of Glu4:Tyrl; this modification did not affect the results. The phosphorylation reaction was initiated by the addition of 10 µCi [γ-32P]ATP and unlabeled ATP to a final concentration of 50 µM. After 20 min of incubation at 24°C, 20 µl of a solution containing 20 mM EDTA, 200 mM NaF, 40 mM sodium pyrophosphate, 40 mM sodium phosphate, 40 mM ATP, 40 mM HEPES, and 0.4% Triton X-100 were added to stop the enzyme reaction. Incorporation of [γ-32P]ATP into the artificial substrate was determined by applying 30-µl aliquots of the reaction mixture to Whatman No. 3MM filter paper discs. The discs were placed in 500 ml 10% trichloracetic acid containing 1 mM sodium phosphate for 15 min. The solution was decanted and this wash procedure was repeated 4 times. The filter paper disc remained in the last wash overnight to help reduce the background radioactivity. The next day, the filter papers were transferred to 95% ethanol for 15 min, washed in ethyl ether, and allowed to air-dry. The discs were placed in minivials, ACS was added, and they were counted in a liquid scintillation counter (38). Under the conditions stated, [32P]ATP incorporated into Glu4:Tyrl displayed a linear time course for 20 to 30 min and over a range of 50 to 300 µg protein in the receptor preparations.

Reagents. Bricitracin, BSA (radioimmunoassay grade), polyethylene glycol (M, 8000), bovine y-globulin, iodoacetamide, phenylmethylsulfonyl fluoride, p-nitrophenylphosphate, N-acetylgalactosamine, Glu4:Tyrl, and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO). Protein A came from Calbiochem-Behring (Los Angeles, CA). The next day, separate tubes containing a pellet of protein A were prepared. The actual preparation of protein A was Staphylococcus aureus (Pansorbitin; Boehringer Mannheim, Indianapolis, IN). The fixed bacterial cell suspension was washed in 1% Triton X-100-0.05% SDS-50 mM Tris-HCl, pH 7.4, followed by a wash in the HAB buffer before adding the receptor preparation. After the overnight incubation, the receptor-containing samples were added to the protein A pellet in the microtube. After a 1-h incubation at 4°C, each tube was centrifuged, and the pellet was washed twice with HAB buffer, and the radioactivity in the resulting pellet was counted in a Beckman gamma 8000 (31).

Insulin-induced Autophosphorylation. Insulin-induced receptor autophosphorylation was measured as detailed by Zick et al. (32). Briefly, 300 µl of the partially purified receptor, containing approximately 1 mg of protein, were incubated in the presence or absence of 10^-7 M insulin for 20 min at 24°C. The mixture was then placed on ice and 500 µl [γ-32P]ATP was added to start the reaction. After a 10-min incubation, the reaction was stopped. A 1:25 dilution of the antisera was added and the mixture was incubated overnight at 4°C (31). Protein A was then added and incubated for 1 h at 4°C. The immunoprecipitate was collected and washed according to the method of Fujita-Yamaguchi et al. (33). The receptor was extracted from the immunoprecipitate and subjected to sodium dodecyl sulfate electrophoresis under either reducing (34) or nonreducing conditions (35). For the former, the pellet was boiled for 3 min in 3.8% SDS-80 mM Tris pH 6.8-5% b-mercaptoethanol for extraction. For the nonreducing conditions, the pellet was boiled for 3 min in 1% SDS-10 mM phosphate buffer, pH 7.0. Autoradiography was carried out on the dried gel as described by Lerea and Livingston (35).

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was immunoprecipitated as described previously (31) and subjected to SDS-gel electrophoresis. Estrogens and 17-hydroxyprogesterone caproate (Delalutin) were from E. R. Squibb and Sons (Princeton, NJ). Wheat germ agglutinin-agarose came from either EY Laboratories (San Mateo, CA) or Pharmacia PL Biochemicals (Piscataway, NJ). All other chemicals were reagent grade.

RESULTS

Characterization of Insulin Receptor Autophosphorylation. At the outset, insulin receptors from R3230AC mammary tumors from either intact or OVEX rats were solubilized and partially purified by wheat germ agglutinin-agarose column chromatography; autophosphorylation was examined by gel electrophoretic techniques (Fig. 1). Based on the autoradiographic evidence, insulin receptors from R3230AC tumors behaved similarly to receptors from other tissues, displaying radioactivity in species with approximate molecular weights of about 398,000 and 338,000 (39). When these labeled species were reduced and then electrophoresed in SDS-acrylamide gels, radioactivity was seen in one species with an estimated molecular weight of 95,000, implying that $^32$P was labeling the $\beta$ subunit (19, 20). In other experiments, 80% of the $^{125}$I-insulin bound to partially purified insulin receptor preparations was precipitated by the antibody to the receptor; less than 1% of the counts were found when aliquots were treated with IgG.

Also shown in Fig. 1 is the effect of insulin stimulation on autophosphorylation. This was investigated in partially purified receptors prepared from tumors of animals given various doses of estradiol and incubated with or without insulin in the presence of $[^{32}]$P]ATP. Autoradiographs of the gels after electrophoresis revealed the presence of the same two species with approximate molecular weights of 398,000 and 338,000.

Tyrosine Kinase Activity Assay. Experiments were performed to establish a quantitative assay in vitro for measurement of insulin receptor TyrK activity, using the artificial substrate Glu4:Tyrl, as originally described by Braun et al. (36), and more recently by Arsenis and Livingston (31) to study adipocyte TyrK activity. A time course for substrate phosphorylation was linear to a maximum at 20 to 30 min, declining thereafter (data not shown). In the absence of Glu4:Tyrl substrate, radioactivity retained on the Whatman filter paper after thorough washing (see "Materials and Methods") was approximately 10% of that found when substrate was included. When the solution used to terminate the reaction (quench solution) was added prior to initiation of the assay, radioactivity measured on the filter paper was $\pm$20% of that measured in samples under optimum conditions. Both of these "blanks" were measured routinely for each receptor preparation, and the counts obtained were subtracted as representative of non-enzyme-catalyzed phosphorylation.

TyrK activity was examined in solubilized insulin receptors obtained from tumors of intact or OVEX rats under conditions where ATP concentration was varied while the concentration of the Glu4:Tyrl substrate was held constant. A typical Hofstee plot ($V$ versus $V/\left[S\right]$) is shown in Fig. 2. The results of several experiments gave an estimated $K_m$ (app) for ATP of 29 and 13 $\mu$M for receptors prepared from intact and OVEX rats, respectively (Table 1). These data gave a higher $V_{max}$ calculated for preparations from intact versus OVEX rats, expressed either on an insulin binding basis or protein basis. In these preparations subsequent to stimulation with $10^{-7}$ M insulin in vitro, the

![Figure 1](image1.png)

![Figure 2](image2.png)
K<sub>m</sub> (app) values were increased for both intact and OVEX animals, respectively. The calculated V<sub>max</sub> under these conditions was increased after insulin (10<sup>-7</sup> M) stimulation in vitro, the more dramatic increase occurring in preparations from tumors of OVEX rats (about 5-fold). The observed effects of insulin on the K<sub>m</sub> (app) for ATP differ from those for liver or adipocytes, in which either no change or a decrease in K<sub>m</sub> was observed (31, 40).

Insulin, 6 IU/day for 3 days was not constant in these 3 regimens, the ratios being 0.28, 0.42, and 0.12 for intact, diabetic, and insulin-treated animals, respectively.

The ability of insulin in vitro to stimulate TyrK activity, i.e., insulin-inducible activity, was examined and the data presented as the Δ TyrK activity in the presence of added insulin, an approach accounting for variations in basal TyrK activity (Fig. 3). The data clearly demonstrate different magnitudes of response over the range of insulin used (10<sup>-10</sup> to 5 × 10<sup>-7</sup> M), depending on the perturbation of the host animal. Preparations from diabetic rats were most responsive, with insulin-induced kinase activity consistently and significantly higher than that observed in preparations of tumors from intact or insulin-treated rats. In fact, insulin treatment of diabetic rats appeared to reduce the response to below that seen in the intact animal.

Thus, despite the higher basal TyrK activity in tumors from diabetic rats, the increment induced by insulin in vitro was greater than that observed for the other preparations. The estimated ED<sub>50</sub> was 2 × 10<sup>-9</sup> M, 4.75 × 10<sup>-9</sup> M, and 4.75 × 10<sup>-9</sup> M for intact, diabetic, and insulin-treated rats, respectively, indicating only a modest shift in dose responsiveness in the diabetic rats. Thus, unlike normal tissues studied by others, the response of TyrK to insulin in vitro was not diminished in R3230AC mammary carcinomas from diabetic rats.

Since insulin binds with weak affinity to IGF-1 receptors, which are similar to insulin receptors in subunit organization and autophosphorylation by receptor TyrK (41), some of the observed increase in kinase activity could have been due to the presence of IGF-1 receptors in these partially purified tissue preparations. We measured the effects of IGF-1 in vitro on preparations from intact and diabetic rats (range comparable

### Table 1 Kinetic parameters for ATP in phosphorylation of Glu4:Tyr1 by R3230AC mammary carcinomas

<table>
<thead>
<tr>
<th>Host status (no. of animals)</th>
<th>Treatment</th>
<th>No insulin</th>
<th>Plus insulin in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact (16)</td>
<td>None</td>
<td>29.3 ± 3.7</td>
<td>79.8 ± 17.8</td>
</tr>
<tr>
<td>Diabetic (6)</td>
<td>None</td>
<td>13.3 ± 1.6</td>
<td>50.8 ± 6.5</td>
</tr>
<tr>
<td>Diabetic (4)</td>
<td>Insulin, 6 IU/day for 3 days</td>
<td>11.84 ± 1.20</td>
<td>3.36 ± 0.41</td>
</tr>
</tbody>
</table>

* Mean ± SEM from 4 separate experiments.

### Table 2 Effects of diabetes and insulin treatment on insulin binding and basal tyrosine kinase activity of R3230AC mammary tumors

<table>
<thead>
<tr>
<th>Host status (no. of animals)</th>
<th>Treatment</th>
<th>Insulin binding (pmol/100 μg protein)</th>
<th>Tyrrosine kinase activity (pmol [32P]ATP incorporated/100 μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact (16)</td>
<td>None</td>
<td>11.84 ± 1.20</td>
<td>3.36 ± 0.41</td>
</tr>
<tr>
<td>Diabetic (6)</td>
<td>None</td>
<td>18.98 ± 2.36</td>
<td>8.12 ± 1.96</td>
</tr>
<tr>
<td>Diabetic (4)</td>
<td>Insulin, 6 IU/day for 3 days</td>
<td>9.46 ± 3.31</td>
<td>1.12 ± 0.33</td>
</tr>
</tbody>
</table>

* Mean ± SEM.  
* P < 0.02 versus intact host.  
* P < 0.025 versus intact host.  
* P < 0.001 versus diabetic host.  
* P < 0.01 versus intact host.  
* P < 0.001 versus diabetic host.

Effect of Diabetes and Insulin Treatment on Receptor Tyrosine Kinase Activity. Experiments were performed to assess perturbations of the insulin milieu of the host on insulin binding and receptor kinase activity in R3230AC tumors. It is evident (Table 2) that insulin binding was elevated in tumors from diabetic rats and returned to control (intact rats) levels after administration of insulin, confirming our earlier reports using whole cells from these tumors (9). The partially purified insulin receptor preparations displayed TyrK activity, referred to as basal TyrK activity, i.e., activity demonstrated in the absence of insulin added in vitro, an activity that reflects the presence of other kinases in addition to the insulin receptors in these partially purified preparations. Interestingly, this basal activity was elevated in tumors from diabetic rats and was significantly lower in preparations of tumors from insulin-treated animals. This pattern of changes resembled that seen for insulin binding, although the ratio of basal kinase activity to insulin binding was greater than that observed for the other preparations. The estimated ED<sub>50</sub> was 2 × 10<sup>-9</sup> M, 4.75 × 10<sup>-9</sup> M, and 4.75 × 10<sup>-9</sup> M for intact, diabetic, and insulin-treated rats, respectively, indicating only a modest shift in dose responsiveness in the diabetic rats. Thus, unlike normal tissues studied by others, the response of TyrK to insulin in vitro was not diminished in R3230AC mammary carcinomas from diabetic rats.

Since insulin binds with weak affinity to IGF-1 receptors, which are similar to insulin receptors in subunit organization and autophosphorylation by receptor TyrK (41), some of the observed increase in kinase activity could have been due to the presence of IGF-1 receptors in these partially purified tissue preparations. We measured the effects of IGF-1 in vitro on preparations from intact and diabetic rats (range comparable

![Graph](image-url)
to that used for insulin) under the same assay conditions. Results shown in Fig. 3 demonstrate that the increase in TyrK resulting from IGF-1 is markedly less than that induced by insulin, representing 0 to 15% of the magnitude of response induced by insulin. Thus, while some portion of the insulin-induced increase in TyrK might be attributed to binding of insulin to IGF-1 receptors, insulin stimulation of TyrK via IGF-1 receptors contributed only in a minor way to the enzyme activity measured.

Effect of Ovariectomy and Estradiol or Progesterone Administration on Insulin-induced Tyrosine Kinase Activity. Having demonstrated that ovarian steroid hormones altered insulin binding, i.e., receptor number with no change in affinity, to R3230AC tumors in vivo and in primary cultured tumor cells in vitro (9, 13), we investigated whether such perturbations in vivo would alter insulin receptor TyrK activity. The results are summarized in Table 3. Treatment of OVEX or intact rats with 1.0 mg estradiol significantly reduced insulin binding to partially purified receptor preparations. The basal TyrK activity appeared to be inversely related to the dose of estradiol administered. The ratio of basal TyrK activity to fmol insulin binding of preparations from estrogen-treated animals was elevated (0.62 to 0.75) compared to that in untreated intact or OVEX rats, implying that estradiol treatment probably altered the level of components other than insulin receptors, components capable of catalyzing [32P]ATP incorporation into the Glu4:Tyr1 substrate or, conversely, reduced the activity of substances in the preparation capable of dephosphorylating the phosphorylated substrate, i.e., phosphatase activity. Although the high dose of estradiol significantly reduced insulin binding, basal TyrK activity was not elevated. Progesterone treatment had only modest effects, with the lowest dose of this steroid causing a small decrease in insulin binding and the highest dose producing a modest decrease in basal kinase activity. As with estradiol treatment, an apparent but more modest increase in insulin induction of TyrK activity in vitro, the response being (0.44 to 0.54) in preparations of tumors from rats receiving the two middle doses of progesterone.

Because of differences in basal TyrK activity, the effects of insulin were evaluated by the increment in TyrK induced by each dose of insulin in vivo. When examined in this manner, no dose of estrogen administered in vivo altered the magnitude of insulin induction of TyrK in vitro, the response being similar to that in receptor preparations from OVEX untreated animals (data not shown). However, the estimated ED50 for insulin-induced kinase activity was increased in preparations from estrogen-treated animals, ranging from 7 to 9 x 10^{-9} M insulin, compared to 2.5 x 10^{-9} M for preparations from OVEX untreated rats. Thus, although basal kinase activity was elevated by estrogen treatment, the extent of insulin-inducible enzyme response was unchanged by estradiol treatment in vivo, but the dose-response curve was shifted to the right.

A different pattern was observed for the effects of progesterone treatment, in which the insulin-induced increase in TyrK activity revealed a complex relationship between the progesterone dose administered and the incremental response in vitro (Fig. 4). Receptor preparations from tumors of animals treated with either 2 or 10 mg progesterone showed similar responses to insulin in vitro as those in control (untreated) animals. However, preparations from animals treated with 50 mg progesterone displayed a blunted response in the magnitude of insulin-induced TyrK at each dose of insulin in vivo. Curiously, a blunted response in kinase activity at the highest levels of insulin in vitro (5 x 10^{-9} to 5 x 10^{-7} M) was also observed in preparations from animals treated with the lowest dose of progesterone (0.2 mg). The ED50 for the insulin-induced response showed a progesterone dose-related shift to the right, with preparations from animals receiving the highest two doses of progesterone yielding values of 6.5 x 10^{-9} M and 8.5 x 10^{-9} M compared to 2.5 x 10^{-9} M for untreated animals.

Effect of Selected Combination Treatments with Estradiol plus Progesterone on Insulin-induced Tyrosine Kinase Activity. Since ovariectomy removes estrogens and progesterone, we examined the effects of simultaneous administration of both hormones on insulin induction of TyrK in vitro. For these experiments, we used a constant dose of estradiol valerate (0.1 mg) and varied the dose of progesterone. The dose of estrogen selected had little effect by itself on the insulin-induced TyrK activity in vitro (compared to OVEX untreated animals). These results, pre-
sented in Table 4, indicate that all combinations significantly reduced insulin binding. However, basal TyrK activity was significantly reduced only in those combination treatments containing the lower doses of progesterone, 0.2 and 2.0 mg. The ratio of basal TyrK activity to insulin binding was essentially unchanged, except in the combination treatment containing the highest dose of progesterone, where the ratio was elevated. Despite these treatment-induced changes in insulin binding and/or basal kinase activity, the insulin-induced increase in TyrK activity was similar to that observed in preparations from untreated animals (data not shown). The E_max for insulin in vitro was the same as controls in preparations from animals given the low progesterone dose combination, but the E_max was shifted to the right as the dose of progesterone was raised. Thus, although these combination treatments significantly altered insulin binding and basal TyrK activity in these tumors, the response to insulin in vitro was similar to preparations from untreated animals.

Effect of Tamoxifen on Insulin-induced Tyrosine Kinase Activity. Since antiestrogens, such as tamoxifen, altered insulin-binding capacity of cultured R3230AC tumor cells in vitro (13), a study was conducted to assess the effects of tamoxifen administered to intact or OVEX R3230AC tumor-bearing animals. Tumors from tamoxifen-treated intact animals displayed higher insulin binding without a concomitant change in basal TyrK activity whereas neither parameter was altered in OVEX rats (Table 5). The insulin-induced kinase activity in vitro was blunted at the higher levels of insulin added to preparations of tumors from intact, tamoxifen-treated animals (Fig. 5). Thus, although insulin binding of these preparations was elevated, the responsiveness of TyrK activity was reduced by tamoxifen treatment in vivo. Since this was not manifested in OVEX rats, the results imply that tamoxifen treatment acted via effects on endogenous estrogens rather than directly on the tumor.

**DISCUSSION**

Since the molecular events that mediate the action of insulin are only partially understood, the finding by Kasuga et al. (19, 20) that the insulin receptor possessed TyrK activity offers a potential mechanism for signal transduction. A scheme for the action of insulin envisages binding of the hormone to the α subunit, a glycoprotein that contains the ligand binding site at or extended from the outer surface of the plasma membrane, which in turn induces phosphorylation of the β subunit, buried in the membrane, resulting in activation of the receptor-associated TyrK activity (18, 39). Hence, biological responses to insulin could be modified by alterations in a postbinding event such as TyrK activity, which has been reported to occur in insulin-induced insulin resistance in rat adipocytes, in adipocytes from morbidly obese patients, and in diabetic animals and humans (31, 40, 42–46). Since previously the effects of sex steroids and insulin on R3230AC tumors in vivo and in vitro were investigated on insulin receptor binding properties, we undertook the experiments presented here on insulin receptor TyrK activities.

Partially purified insulin receptors from R3230AC tumors displayed molecular properties similar to those of normal tissues (17, 39). Insulin stimulated 32P incorporation into a species with a molecular weight of approximately 375,000 and subsequent to reduction with mercaptoethanol, the 32P incorporated was seen in a Mr 95,000 species, the size of the β subunit. Under nonreducing conditions, there appeared to be another phosphorylated species with a molecular weight of approximately 120,000 stimulated by insulin, which may be a degradation product of the receptor that can serve as a substrate for the activated intact receptor. Whether this is unique to

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**Table 4** Effects of simultaneous administration of estradiol (0.1 mg) with different levels of progesterone on insulin binding and basal tyrosine kinase activity in R3230AC mammary carcinomas from ovariectomized rats. Administration of steroids was twice weekly for 3 weeks.

<table>
<thead>
<tr>
<th>Treatment (no. of animals)</th>
<th>Insulin binding (fmol/100 μg protein)</th>
<th>Tyrosine kinase activity (pmol 32P incorporated/100 μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (28)</td>
<td>14.18 ± 0.01*</td>
<td>4.81 ± 0.66</td>
</tr>
<tr>
<td>Estradiol, 0.1 mg, plus progesterone, 0.2 mg (5)</td>
<td>8.66 ± 1.53*</td>
<td>2.27 ± 0.47</td>
</tr>
<tr>
<td>Estradiol, 0.1 mg, plus progesterone, 2.0 mg (4)</td>
<td>7.09 ± 1.59*</td>
<td>2.41 ± 0.52</td>
</tr>
<tr>
<td>Estradiol, 0.1 mg, plus progesterone, 10 mg (5)</td>
<td>5.51 ± 1.43*</td>
<td>4.20 ± 1.10</td>
</tr>
</tbody>
</table>

* Mean ± SEM.
* P < 0.01 versus untreated animals.
* P < 0.001 versus untreated animals.

**Table 5** Effects of tamoxifen administration on insulin binding and basal tyrosine activity in R3230AC mammary carcinomas.

<table>
<thead>
<tr>
<th>Host (no. of animals)</th>
<th>Insulin binding (fmol/100 μg protein)</th>
<th>Tyrosine kinase activity (pmol 32P incorporated/100 μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact (16)</td>
<td>11.84 ± 1.20*</td>
<td>3.36 ± 0.41</td>
</tr>
<tr>
<td>Plus tamoxifen (3)</td>
<td>18.72 ± 0.11*</td>
<td>4.46 ± 0.79</td>
</tr>
<tr>
<td>Ovariectomized (25)</td>
<td>14.18 ± 1.01*</td>
<td>4.81 ± 0.66</td>
</tr>
<tr>
<td>Plus tamoxifen (4)</td>
<td>17.88 ± 1.93*</td>
<td>3.69 ± 0.42</td>
</tr>
</tbody>
</table>

* Mean ± SEM.
* P < 0.01 versus untreated hosts.
R3230AC tumors is not known. Since we encountered variability when quantitating phosphorylation by gel scanning we utilized an artificial substrate as a phosphate acceptor to quantitate TyrK activity (36, 37). In insulin receptor preparations obtained by wheat germ agglutinin-agarose chromatography, the estimated $K_m$ for ATP using the Glu4:Tyr1 substrate was 13 to 30 $\mu$M, values in the range of 30 to 150 $\mu$M reported for other tissues (18, 31). Although the estimated $K_m$ appeared to be increased by $10^{-7}$ M insulin in preparations from intact and OVEX rats, insulin in vitro caused a 3.5- to 5-fold increase in $V_{\text{max}}$. While the physiological consequences of these observations are unknown, changes in the phosphorylation state of the receptor were reported to modify kinase activity (47, 48) and receptor degradation rates (49). The estimated $K_m$ for the Glu4:Tyr1 substrate was 2 to 3 $\mu$M (molecular weight provided by the supplier), values in general agreement with those of Freidenberg et al. (40) for rat liver preparations. Insulin in vitro increased the $V_{\text{max}}$ by 25 to 50%, similar to that reported by Freidenberg et al. (40) for control animals. However, these findings on the partially purified tumor receptor preparations should be confirmed with study of highly purified receptor preparations.

The data demonstrate that certain hormonal perturbations of the host altered insulin binding capacity of the partially purified preparations from R3230AC mammary tumors. These alterations were frequent but not invariably accompanied by changes in basal TyrK activity, i.e., $^{32}$P incorporation into Glu4:Tyr1 in the absence of added insulin. The physiological significance of these changes is unknown. In these tissue preparations, the presence of other glycoprotein receptors, such as IGF-1 and epidermal growth factor (EGF), known to possess TyrK activity, may contribute to basal enzyme activity and such receptors may also be altered by hormonal perturbations. For example, Mukku and Stancel (51) reported that administration of estradiol increased epidermal growth factor receptors in the uterus. However, induction of TyrK activity by IGF-1 in these preparations from intact and diabetic rats was of much lower magnitude than for insulin over comparable levels (see Fig. 3). Assuming that IGF-1 receptors are present and that insulin can bind weakly to such receptors, the contribution to the hormone-induced kinase activity by IGF-1 receptor kinase would appear to be minor even when activated by the preferred ligand. These findings, along with the dose-related plateau in activity, are taken as indicating that we are measuring primarily insulin-induced TyrK in these preparations.

The ratio of TyrK activity to insulin binding, measured on the same preparations and protein basis, was not constant, an anticipated outcome if a coordinate relationship exists between insulin receptors and inherent kinase activity. Since this was not the case, e.g., elevated ratios after certain treatments with sex steroid hormones, we suggest that this may: (a) reflect effects of steroid hormones to reduce the binding capacity of $\alpha$ subunits and/or increase the activity of $\beta$ subunits of the insulin receptors in the tumors; (b) enhance autophosphorylation, leading to activation of TyrK; or (c) increase the presence of other glycoprotein components that would copurify and contribute to assayable kinase activity. In cultured R3230AC tumors, we found that estradiol in vitro altered the distribution of insulin receptors between the cell surface and the intracellular pool, decreasing the former while concomitantly increasing the latter, whereas insulin-induced down-regulation resulted in a decrease in both (13). It is possible that a redistribution could alter the relationship between binding and kinase activity, and study of both receptor pools is needed.

Despite the changes in basal TyrK, insulin induced an increase in TyrK activity, which was taken as evidence for response insulin receptors in tumors, i.e., increase in TyrK activity relative to the dose of insulin added in vitro. The most striking observation was the enhanced responsiveness of receptors from tumors of diabetic versus intact animals. This finding differs from reports of reduced or unaltered responsiveness of liver, muscle, or adipocytes from diabetic rats or humans (31, 40, 42-46). Furthermore, tumors from insulin-treated diabetic rats displayed a pattern similar to intact animals, indicating that insulin replacement counteracted the effects resulting from diabetes. This finding is opposite to that of Kadokawa et al. (42). Thus, the behavior of insulin-inducible TyrK activity seen in R3230AC mammary tumors versus normal tissues may not be attributable to differences in physical properties of insulin receptors in transformed cells, as suggested for IM-9 lymphocytes (52), since another human neoplastic cell line, HepG-2, displayed a desensitization of the kinase after insulin-induced down-regulation (46). Whether the R3230AC mammary adenocarcinoma is unique in its behavior is unknown and studies of other hormone-responsive tumors would seem warranted. It should be noted that the R3230AC carcinoma grows faster in diabetic rats and growth is retarded by insulin administration (4), implying that if insulin-inducible TyrK activity is related to tumor growth processes, the enhanced sensitivity in diabetic rats would be consistent with more rapid growth.

In contrast, perturbations that involved administration of therapeutic levels of estradiol or progesterone, or a combination, had modest effects on the magnitude of insulin induction of TyrK. Thus, despite changes in basal TyrK activity and/or insulin-binding capacity of tumor preparations, the dose-related induction of kinase activity by insulin in vitro was essentially unchanged in animals treated with different doses of estradiol, although the ED$_{50}$ for insulin was shifted to the right. Furthermore, a similar outcome was observed for animals that received the combined treatment of estradiol plus different doses of progesterone. A somewhat different result occurred when progesterone was administered to OVEX tumor-bearing rats, with the highest pharmacological dose of progesterone (50 mg) causing a significant reduction in the extent of insulin-induced TyrK activity. Curiously, a blunted response to insulin was also observed in tumors from animals that received the combined treatment of estradiol plus different doses of progesterone. A somewhat different result occurred when progesterone was administered to OVEX tumor-bearing rats, with the highest pharmacological dose of progesterone causing a significant reduction in the extent of insulin-induced TyrK activity. The findings with tamoxifen treatment are of interest since...
this agent may display antiestrogenic or estrogen activity, depending on dose, target tissue, and species studied (54). Tamoxifen administered to intact animals elevated insulin binding, a response seen for ovarectomy, and since this effect was not observed in OVEX rats, it suggests that tamoxifen was acting on this parameter as an antiestrogen. Interestingly, insulin induction of TyrK activity was attenuated in tumors from intact rats treated with tamoxifen but not in those from OVEX, treated rats (except at one dose level of insulin), results implying that the actions of tamoxifen were indirect, i.e., antiestrogenic, on the tumor. The dose of tamoxifen used would inhibit growth of the R3230AC tumor (55), although inhibition of tumor growth by pharmacological doses of estradiol used here (56) was not accompanied by similar changes in responsiveness of TyrK.

In conclusion, data are presented to demonstrate that certain hormonal perturbations of the R3230AC tumor-bearing host led to alterations in insulin binding, basal TyrK, and insulin-induced TyrK activities of this transplantable mammary carcinoma. The neoplasm from diabetic rats displayed increased responsiveness of insulin receptor kinase activity to insulin in vitro compared to tumors from intact or insulin-treated diabetic rats. Therapeutic doses of sex steroid hormones and tamoxifen consistently decreased the sensitivity of the insulin induction of TyrK in vitro, seen as a shift of the dose curve to the right and, at certain treatment doses, decreased the magnitude of the insulin-induced enzyme response. Together with previous demonstrations of altered numbers of insulin receptors resulting from these agents (9, 13, 15, 16), the proposed interaction between female sex steroids and insulin receptors may now be extended to their modulating actions on TyrK activity, an inherent property of insulin receptors. Although the exact role of receptor TyrK remains unknown, the possibility is raised that hormonal treatments altering growth of this mammary tumor may be correlated to altered responsiveness of TyrK, offering a discrete regulatory site for steroids to influence the actions of insulin.

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Effects of Diabetes and Sex Steroid Hormones on Insulin Receptor Tyrosine Kinase Activity in R3230AC Mammary Adenocarcinomas

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