Resistance to Insulin-Stimulated Glucose Uptake in Adipocytes Isolated From Spontaneously Hypertensive Rats

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The ability of insulin to stimulate glucose uptake and inhibit catecholamine-induced lipolysis was measured in adipocytes of similar size isolated from SHR and WKY rats. The results indicate that glucose transport was decreased in adipocytes from SHR rats; both basal (19 ± 2 vs. 32 ± 2 fmol · cell^{-1} · s^{-1}, $P < .001$) and maximal (207 ± 30 vs. 373 ± 20 fmol · cell^{-1} · s^{-1}, $P < .01$) insulin-stimulated glucose transport were lower in SHR than in WKY rats. In addition, the EC_{50} of insulin-stimulated glucose uptake was higher (921 ± 82 vs. 557 ± 69 pM insulin, $P < .05$) in adipocytes from SHR rats than from WKY rats. The ability of phenylisopropyladenosine (PIA) to modulate basal and maximal insulin-stimulated glucose uptake was compared in adipocytes from SHR and WKY rats. These results also demonstrated that glucose uptake was decreased in adipocytes from SHR rats and that PIA similarly enhanced both basal and maximal insulin-stimulated glucose uptake in adipocytes from both groups. Although maximal isoproterenol-stimulated lipolysis was decreased in adipocytes from SHR rats, the ability of insulin to inhibit catecholamine-stimulated lipolysis was at least as great in adipocytes from SHR as from WKY rats. Despite the decrease in insulin-stimulated glucose transport in isolated adipocytes from SHR rats, total number of insulin receptors, their affinity for insulin, and the ability of insulin to stimulate receptor-associated tyrosine kinase activity were similar in adipocytes from SHR and WKY rats. These results demonstrate a defect in both basal and insulin-stimulated glucose uptake and a loss of sensitivity of the adipocyte glucose-transport system to insulin in adipocytes from SHR rats and suggest that the site of the abnormality is distal to the insulin receptor. *Diabetes* 38:1155–60, 1989

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Results of two recent studies have shown that resistance to insulin-stimulated glucose uptake exists in patients with both drug-treated and untreated hypertension (1,2). More recently, we have shown that the induction of insulin resistance and hyperinsulinemia secondary to eating a fructose-enriched diet is associated with the development of high blood pressure in healthy rats (3). Finally, we have noted the presence of insulin resistance and hyperinsulinemia in rats with spontaneous hypertension (SHR) (4).

To further explore the relationship between resistance to insulin-stimulated glucose uptake and high blood pressure, we thought it useful to see if a defect in insulin action at the tissue level could be demonstrated in rats with high blood pressure. To do this, we compared the ability of insulin to promote glucose uptake and inhibit catecholamine-induced lipolysis in adipocytes isolated from SHR and WKY rats. In addition, we quantified the total number of adipocyte insulin receptors in these two experimental groups and the ability of insulin to stimulate receptor-associated tyrosine kinase activity of solubilized insulin receptors.

RESEARCH DESIGN AND METHODS

Male SHR rats, originally derived from Wistar-Kyoto stock from the National Institutes of Health Animal Genetic Resource colony, and the Wistar-Kyoto (WKY) control models for the SHR rat were obtained from Taconic (Germantown, NY). They were given Purina Laboratory Chow (no. 5012, St. Louis, MO) and water ad libitum and maintained on a 12-h (0600–1800) light-dark cycle. Food was removed at 0800 on days that measurements were taken, and all procedures were initiated 5 h later as indicated.

Preparation of adipocytes. Adipocytes were prepared from epididymal fat pads according to the method of Rodbell (5) with minor modifications. The fat pads were minced with scissors and placed in plastic flasks in Krebs bicarbonate buffer with 4% bovine serum albumin (BSA), 3 mM glucose,

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and 1 mg collagenase/ml. Collagenase digestion was carried out at 37°C in a gyratory water-bath shaker for 75 min. Cells were washed three times in fresh Krebs buffer with 4% albumin and 2.5 mM glucose and allowed to separate from the infranatant by flotation. A 100-μl aliquot of diluted cells was fixed in a solution of 2% osmium tetroxide in collidine buffer and counted in a Coulter counter (Hialeah, FL) to determine cell number. Aliquots of cells were taken for measurement of glucose transport and catecholamine-stimulated lipolysis.

Measurement of glucose transport. Glucose transport was determined by a recently validated method based on the observation that glucose uptake is a measurement of glucose transport when studies are carried out at trace glucose concentration (6,7). Briefly, isolated adipocytes (2% lipocrit) were incubated in 500 μl 3.5% albumin buffer in the absence and presence of different concentrations of insulin (25–80,000 pM) and tracer (300 nM) amounts of D-[U-^{14}C]-glucose. Parallel studies were carried out in the absence and presence of adenosine deaminase (1 U/ml) in the incubation medium. In other studies, basal and maximal insulin-stimulated glucose uptake were measured in the absence or presence of 10^{-7} M phenylisopropyladenosine. The cell suspension was incubated at 37°C for 1 h with continuous shaking at 40 cpm. The incubation was terminated by centrifuging a 400-μl aliquot in a 500-μl microfuge tube and the amount of activity associated with the adipocytes (and the total radioactivity in the incubation medium) determined by liquid-scintillation counting. Values for EC_{50} (concentration of insulin required for half-maximal activation of tyrosine kinase activity) were calculated from an insulin dose-response curve as described previously (6,7). Earlier studies demonstrated that values for glucose transport are similar when either the conventional 3-*O*-methylglucose method or the one in this study was used (6,7).

Measurement of lipolysis. Adipocytes were diluted in Krebs buffer with 4% albumin and 2.5 mM glucose buffer, pH 7.4; aliquots of diluted cells were placed in plastic vials (1×10^5 cells/ml) and incubated for 1 h at 37°C in the presence of adenosine deaminase (1 U/ml) and isoproterenol (10^{-7} M) in an atmosphere of 95% O_2/5% CO_2. At the end of incubation, an aliquot (0.2 ml) of infranatant was removed from each incubation mixture for measurement of glycerol concentration by the enzymatic method of Wieland (8). Measurements were made in the absence and presence of increasing concentrations of insulin in the incubation medium.

Solubilization and partial purification of insulin receptors. Solubilization and partial purification of solubilized insulin receptors were carried out as described by Hedo et al. (9). In brief, adipocyte preparations (1 to 2×10^7 cells/10 ml) were homogenized in 5 ml of homogenization buffer (50 mM Tris-HCl, pH 7.4; 1 mM bacitracin; 1000 trypsin inhibitor U/ml of aprotinin; 1 mM paramethylsulfonylfluoride; and 1% Triton X-100), and homogenates were incubated at 4°C for 60 min. After incubation, samples were centrifuged at $100,000 \times g_{av}$ for 90 min to sediment particulate material.

Under these incubation conditions, ~90–92% insulin receptors were solubilized from adipocytes isolated from either WKY or SHR rats. In each case, the solubilized extract (supernatant) was diluted 6.6-fold with homogenization buffer minus Triton X-100 and was chromatographed on a column containing 1.5 ml of wheat-germ agglutinin (WGA)–agarose. Before application of the sample, the WGA-agarose column was preequilibrated with ~150 ml of column buffer (50 mM Tris-HCl, pH 7.4; 0.1% Triton X-100; 0.15 M NaCl). After application of the sample, the column was washed extensively with column buffer (~50 ml) to remove unbound proteins. Lectin-bound insulin-receptor activity was eluted with 2 ml of column buffer containing 0.3 M *N*-acetyl-D-glucosamine. The eluted material was used for the measurement of insulin binding and insulin-receptor–associated tyrosine kinase activities. This procedure resulted in an ~20-fold purification of insulin receptors and >90% recovery of applied receptors assessed by insulin binding. Finally, no difference in receptor yield after WGA-agarose column chromatography was observed between the WKY and SHR groups. Protein concentration of eluates was estimated by the method of Bradford (10).

Insulin binding to solubilized insulin receptors. Binding of A14-^{125}I-labeled monoiodoinsulin to WGA-agarose–purified receptor preparations was determined as described previously (11). Briefly, WGA-agarose eluates (3–5 μg protein) were incubated with ^{125}I-insulin (~25,000 counts/min) plus increasing amounts of unlabeled porcine insulin (total insulin concentrations in the assay varied from 0.05 to 17.0 nM) in a final volume of 200 μl buffer containing 50 mM Tris-HCl, pH 7.4; 0.05% Triton X-100; 75 mM *N*-acetyl-D-glucosamine; 1 mg/ml BSA; and 0.15 M NaCl. After incubation at 4°C for 18–24 h, receptor-bound ^{125}I-insulin was separated from free insulin by polyethylene glycol precipitation (12). Nonspecific binding was determined in the presence of 0.7 μM unlabeled insulin and was <10% of the total binding. This nonspecific binding was subtracted from the total radioactivity. All binding values reported represent specific insulin binding and are expressed as femtomoles of bound ^{125}I-insulin per 10^6 cells.

Data from the binding studies were analyzed by the method of Scatchard (13), with the LIGAND program of Munson and Rodbard (14) to obtain estimates of the binding sites and hormone affinity of the receptor.

Insulin-receptor–associated tyrosine kinase activity was assayed with the polyamino acid polymer $Glu^{80}NaTyr^{20}$ [poly($Glu^{80}Tyr^{20}$)] as exogenous substrate (15). Partially purified insulin-receptor preparations (2.0–3.5 μg protein) were preincubated without (basal) or with increasing concentrations of insulin (0.3–300 mM) in 100 μl of incubation buffer (50 mM Tris-HCl, pH 7.4; 0.12 M *N*-acetyl-D-glucosamine; 0.3 mM *p*-nitrophenyl phosphate; 0.15 M NaCl; 0.05% Triton X-100; and 1 mg/ml BSA) at room temperature (22–24°C) for 20 min. The phosphorylation of artificial (exogenous) substrate is initiated by the addition of 50 μM [γ-^{32}P]ATP (0.7 μCi/nmol), 20 mM $MgCl_2$, and 2 mg/ml poly($Glu^{80}Tyr^{20}$) (final concn). The reaction was terminated 20 min later by applying 50 μl of the reaction mixture to a phosphocellulose filter paper square. The papers were then precipitated and washed extensively with 75 mM phosphoric acid (16). The amount of ^{32}P incorporated into poly($Glu^{80}Tyr^{20}$) was determined by liquid-scintillation counting; background counts obtained in the absence of exogenous substrate were subtracted.

Tyrosine kinase activity was expressed as femtomoles of

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^{32}P incorporated into poly(Glu^{80}Tyr^{20}) per minute per 10^6 cells.

Chemicals. Chemicals were obtained from the following sources: collagenase from Worthington (Freehold, NJ); (−)-isoproterenol (+)-bitartrate, *N*-acetyl-D-glucosamine, poly(Glu^{80}Tyr^{20}), and adenosine deaminase from Sigma (St. Louis, MO); [γ-^{32}P]ATP, tetra (triethylammonium) salt (sp act 10–40 Ci/mmol), 0.37–1.48 TBq/mmol from New England Nuclear (Boston, MA); and WGA coupled to agarose from Vector (Burlingame, CA). All other chemicals were obtained from standard commercial sources. ^{125}I-insulin was kindly provided by B.H. Frank (Lilly, Indianapolis, IN).

Results are means ± SE. The statistical significance of differences were evaluated by Student's *t* test or two-way analysis of variance (ANOVA) (17,18).

RESULTS

Body weight, adipocyte size, and blood pressure of the two groups of rats are shown in Table 1. Values for body weight and cell size were similar in the two groups, but blood pressure was significantly elevated in the SHR rats.

Basal and insulin-stimulated glucose transport values by adipocytes isolated from SHR and WKY rats are seen in Fig. 1. Glucose uptake was significantly lower in adipocytes from SHR rats when incubated in the absence of insulin (19 ± 2 vs. 30 ± 2 fl · cell^{-1} · s^{-1}, $P < .001$) and at every insulin concentration (2-way ANOVA, $P < .001$). In addition, the EC_{50} of insulin-stimulated glucose transport was greater in adipocytes from SHR rats (921 ± 82 vs. 557 ± 69 pM, $P < .05$). The data shown in Fig. 1 were derived from experiments carried out in the presence of adenosine deaminase, which removes endogenous adenosine. The difference between the two groups persisted when adenosine deaminase was omitted from the incubation medium (data not shown), although the EC_{50} was approximately twice as high in both groups.

Additional experiments were carried out in which we evaluated the ability of the adenosine agonist phenylisopropyladenosine (PIA) to enhance basal and maximal insulin-stimulated glucose uptake by adipocytes isolated from WKY and SHR rats. The results again demonstrated that both basal ($P < .001$) and maximal insulin-stimulated ($P < .001$) glucose uptake were lower in adipocytes from SHR rats (Fig. 2, *left*). PIA increased glucose transport, both in the basal state and in response to insulin (Fig. 2, *right*). More relevant to this study is that the stimulatory effect of PIA was similar in adipocytes from both groups of rats. In other words, adipocytes from SHR and WKY rats responded comparably to the adenosine agonist, and the differences in glucose uptake between the two groups persisted.

The ability of insulin to inhibit isoproterenol-stimulated lipolysis is shown in Fig. 3. Catecholamine-induced lipolysis

TABLE 1
Mean (±SE) body weight, adipocyte size, and blood pressure of SHR and WKY rats

Rats	Body weight (*g*)	Adipocyte size (μg · lipid^{-1} · cell^{-1})	Blood pressure (mmHg)
SHR	304 ± 3	0.26 ± 0.01	203 ± 4
WKY	299 ± 2	0.28 ± 0.01	118 ± 3

n = 16 in each group.

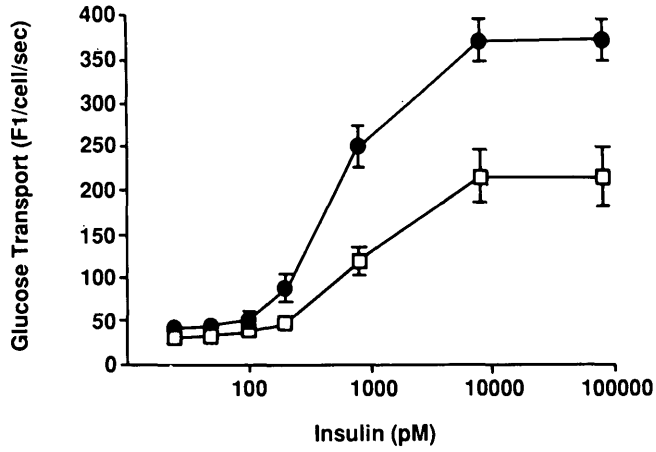

FIG. 1. Glucose transport in absence or presence of varying amounts of insulin by adipocytes isolated from WKY (●) or SHR (□) rats. Results are means ± SE of 8 experiments.

was greater in adipocytes isolated from WKY rats both in the absence of insulin and at every insulin concentration ($P < .001$; Fig. 3*A*). Because catecholamine-induced lipolysis was decreased in adipocytes from SHR compared with WKY rats in the absence of insulin, to demonstrate the ability of insulin to suppress lipolysis, the data in Fig. 3*A* were replotted as percent suppression of lipolysis. These results are seen in Fig. 3*B* and demonstrate that the relative ability of insulin to suppress lipolysis was, if anything, increased in adipocytes from SHR rats.

Insulin-binding activity was assessed by incubating

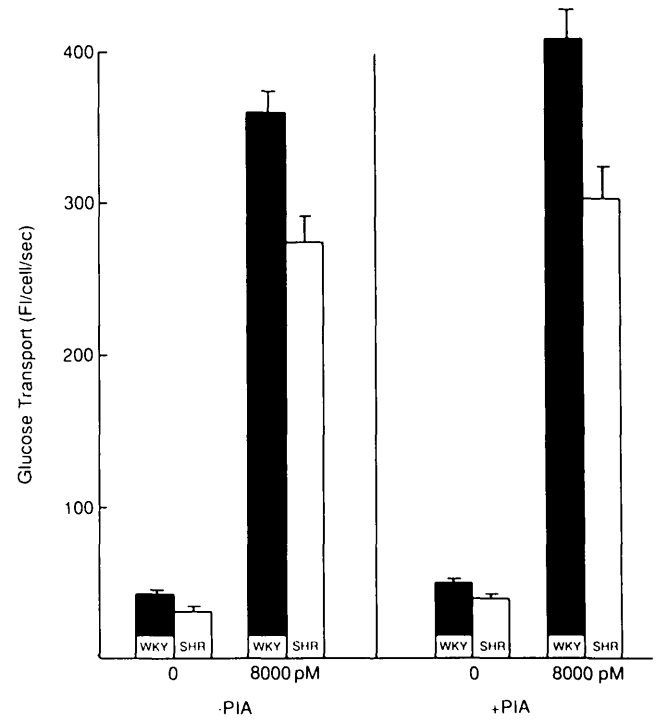

FIG. 2. Basal (0) and maximal (8000 pM) insulin-stimulated glucose transport by adipocytes isolated from either WKY (*solid bars*) or SHR (*open bars*) rats. Studies were conducted in absence or presence of adenosine agonist phenylisopropyladenosine (PIA). Results are means ± SE of 8 experiments.

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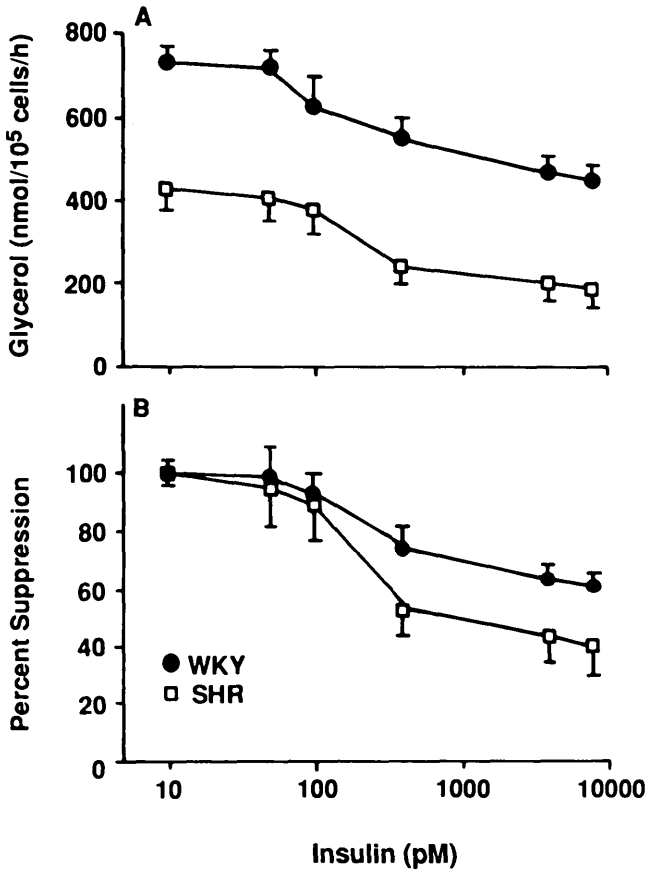

FIG. 3. Ability of varying amounts of insulin to inhibit isoproterenol-stimulated (10^{-7} M) lipolysis by adipocytes isolated from WKY (●) or SHR (□) rats. *A*: lipolytic rate estimated by appearance of glycerol in medium. *B*: percentage to which lipolysis was suppressed by addition of insulin. Results are means ± SE of 6 experiments.

partially purified adipocyte receptor preparations with ^{125}I-insulin in the presence of increasing concentrations of unlabeled insulin. These competition curves were then transformed into Scatchard plots (13). In each case, equilibrium insulin binding was characterized by a curvilinear Scatchard plot, thus suggesting the presence of high- and low-affinity–binding components (Fig. 4). Mean high-affinity–binding constants from nine individual plots for both groups are given in Table 2. It is clear that both total insulin binding and the affinity (K_d) of insulin for its receptor were similar in the two groups.

Additional studies were done to test the ability of different concentrations of insulin to stimulate phosphorylation of exogenous substrate [poly(Glu^{80}Tyr^{20})] in lectin-purified receptor preparations (Fig. 5). It is obvious that insulin produced a dose-dependent increase in tyrosine kinase activity that reached a maximum (~20-fold) at an insulin concn of ~30 nM. The numerical values for basal (in the absence of insulin) and insulin-stimulated tyrosine kinase activity are shown in Table 2. Both basal and insulin-stimulated maximal tyrosine kinase activity (i.e., maximally stimulated activity minus the basal level) and insulin-stimulated maximal tyrosine kinase activity normalized to insulin-binding activity were similar in adipocytes isolated from the two groups of rats. Furthermore, EC_{50} values were essentially identical in adipocytes from both WKY and SHR rats (Table 2).

DISCUSSION

Our results demonstrate that both basal and maximal insulin-stimulated glucose uptake is decreased in adipocytes isolated from SHR rats, associated with a significant decrease in insulin sensitivity of the glucose-transport system. These data provide evidence that the resistance to in vivo insulin-stimulated glucose uptake previously shown in SHR rats (4) can also be seen at the cellular level. Although not the goal of this paper, it seems worth pointing out that adipocytes from both WKY and SHR rats seemed less insulin sensitive than adipocytes isolated from Sprague-Dawley rats. For example, when we studied Sprague-Dawley rats weighing about half that of the rats used in these experiments, the EC_{50} for insulin-stimulated glucose uptake was ~100 pM versus an EC_{50} of ~500 pM in adipocytes from the WKY rats in this study. Furthermore, the EC_{50} of insulin-stimulated glucose transport of adipocytes isolated from 300-g Sprague-Dawley rats studied in our laboratory was still only ~200 pM. Thus, it appears that adipocytes from both SHR and WKY rats may be insulin resistant compared with adipocytes from Sprague-Dawley rats. This apparent species difference is certainly worthy of further study, but it should not detract from our observation that the ability of insulin to stimulate glucose uptake by adipocytes from SHR rats is decreased compared with cells isolated from control WKY rats.

The results of this study also provide some insight into the cellular basis of the insulin resistance. First, it does not seem likely that the defect in insulin action in adipocytes from SHR rats is secondary to a decrease in either the number of cellular insulin receptors or the affinity of the receptors for insulin. In addition, tyrosine kinase activity of insulin receptors from SHR and WKY rats was comparable, suggesting that the reduction in glucose uptake in adipocytes from SHR rats is distal to insulin binding and the insulin-receptor phosphorylation system. Finally, it appears that the ability of the adenosine agonist PIA to stimulate glucose transport in both the basal state and in response to a maximal dose of insulin was similar in the two groups. Because PIA appears to increase glucose uptake by virtue of its ability to inhibit intracellular accumulation of cyclic AMP (cAMP), it seems likely that the decrease in insulin-stimulated glucose transport in adipocytes from SHR rats is not mediated by modulation of cAMP metabolism.

Note that not all of the effects of insulin on adipocyte metabolism are abnormal in SHR rats, and the ability of insulin to inhibit catecholamine-induced lipolysis was not decreased in adipocytes isolated from SHR rats. Parenthetically, maximal catecholamine-induced lipolysis was significantly lower in adipocytes from SHR rats, a finding that has been noted in earlier studies (19). When this difference is taken into account, the relative ability of insulin to suppress catecholamine-induced lipolysis was, if anything, greater in adipocytes isolated from SHR rats (Fig. 3*B*). Disassociation between the ability of insulin to stimulate glucose transport and inhibit catecholamine-induced lipolysis is not unique to the SHR rats, and a similar finding was seen when adipocytes from patients with non-insulin-dependent diabetes mellitus were compared with adipocytes from healthy individuals (6). The second-messenger systems involved in insulin-receptor–mediated stimulation of glucose transport or inhibition of lipolysis are uncertain and probably involve dif-

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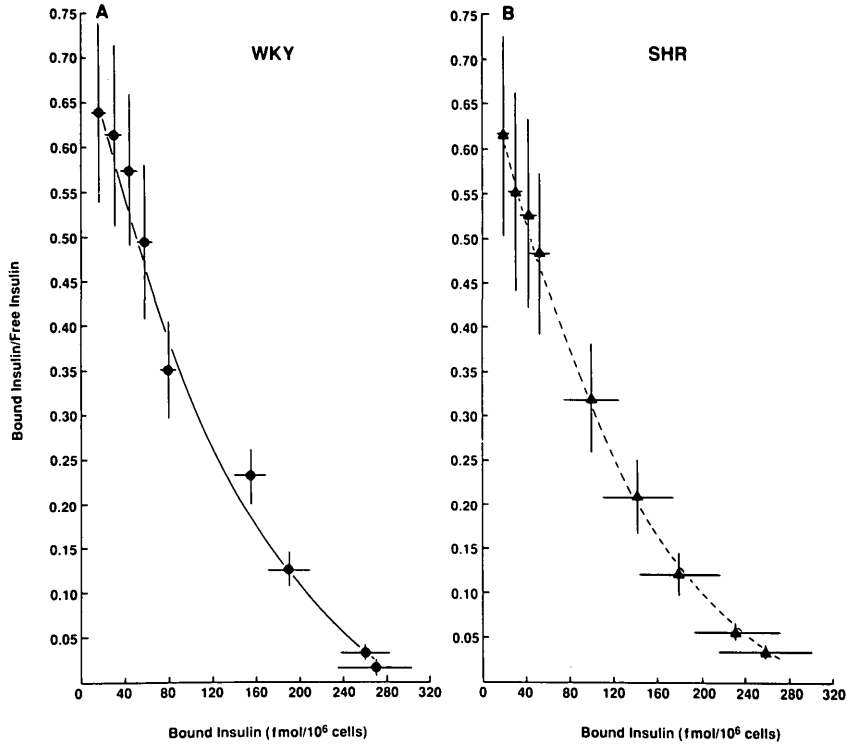

FIG. 4. Scatchard plot analysis of A14-^{125}I-labeled monoiodoinsulin binding to partially purified insulin-receptor preparations from adipocytes of WKY and SHR rats. Wheat-germ agglutinin–agarose eluates in 200 μl of buffer were incubated with ^{125}I-insulin (~25,000 counts/min, 0.3 ng/ml) and increasing concentrations of unlabeled insulin (total insulin concentrations in assay varied from 0.05 to 17 nM) for 18–24 h at 4°C. Hormone-receptor complexes were precipitated with polyethylene glycol (10% final concn) with γ-globulin as carrier protein. Nonspecific binding in presence of 0.78 μM insulin was subtracted from total binding to compute "specific" binding. Binding data derived from competition curves were analyzed according to method of Scatchard (13). Values are means ± SE of 8 separate experiments. Protein concentrations used were 12.5–22.5 and 7.5–17.5 μg/ml for adipocyte receptor preparations from WKY and SHR rats, respectively.

ferent molecular mechanisms. Consequently, the selective impairment in the ability of insulin to stimulate glucose transport in adipocytes from SHR rats could involve a specific alteration in one component of action of the insulin receptor that was not involved in the regulation of lipolysis. Alternatively, the results are compatible with the hypothesis that there may be a defect in the function or number of the glucose transporters themselves in the adipocytes from SHR rats. This possibility is consistent with the observation that adipocytes from SHR and WKY rats responded similarly to PIA, with the difference persisting between the two groups in terms of both basal and maximal insulin-stimulated glucose uptake. On the other hand, it is clear that further experiments will be required to provide the explanation for this interesting discrepancy in the relative ability of insulin to regulate adipocyte metabolism. In any case, the data presented seem most consistent with the view that the defect in adipocyte glucose transport activity in SHR rats is distal to the insulin-receptor phosphorylation cascade.

It has become increasing clear that patients with hypertension are insulin resistant and hyperinsulinemic (1,2,20) and that these defects do not necessarily disappear when blood pressure is reduced with conventional antihypertensive agents (1). Furthermore, insulin resistance and hyperinsulinemia have now been documented in two models of experimental hypertension, one that is produced in healthy rats (fructose induced) and the other that develops spontaneously in a genetic model of hypertension (SHR rats). In

TABLE 2
Mean (±SE) insulin-binding and insulin-receptor tyrosine kinase activity

	Insulin binding		Insulin-receptor–associated tyrosine kinase activity			
Rats	Maximal insulin-binding activity*	K_d (nM)	Basal tyrosine kinase activity†	Maximal insulin-stimulated tyrosine kinase activity†‡	Maximal insulin-stimulated tyrosine kinase activity/pmol of insulin-binding activity§	EC_{50} (nM)‖
WKY	238 ± 25	0.340 ± 0.015	380 ± 76	9519 ± 1823	40.3 ± 5.6	1.74 ± 0.14
SHR	238 ± 44	0.360 ± 0.028	408 ± 79	8260 ± 2224	33.1 ± 6.2	2.17 ± 0.15

n = 8 in each group.
*Insulin-binding activity is expressed as fmol A14-^{125}I-labeled monoiodoinsulin bound/10^6 cells. Estimates of binding sites (receptor concn; B_{max}) and hormone affinity of the receptor (K_d) were derived from Scatchard plots with the LIGAND program (14).
†Tyrosine kinase activity is expressed as fmol ^{32}P incorporated into poly(Glu^{80}Tyr^{20}) · min^{-1} · 10^{-6} cells.
‡Total insulin-stimulated (30 nM) tyrosine kinase activity minus basal activity. Basal activity is expressed as activity observed in absence of added insulin.
§Tyrosine kinase activity is expressed as fmol ^{32}P incorporated into poly(Glu^{80}Tyr^{20}) · min^{-1} · $fmol^{-1}$ insulin-binding activity. Insulin-binding (capacity) activity was derived from Scatchard plots via LIGAND program.
‖Defined as concentration of insulin required for half-maximal activation of tyrosine kinase activity.

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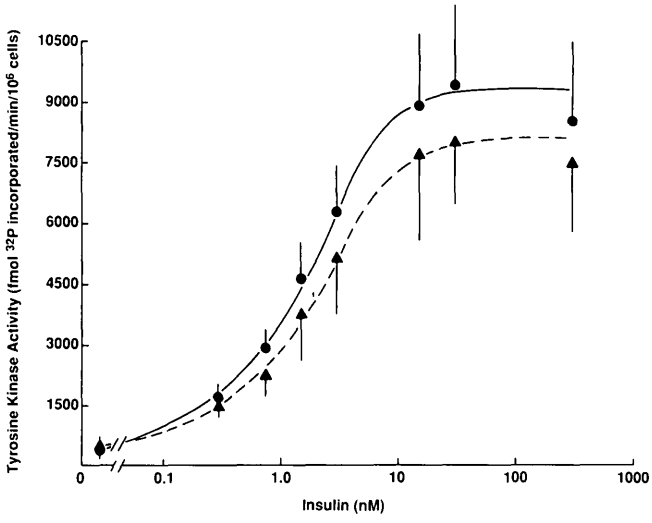

FIG. 5. Dose response of insulin-stimulated phosphorylation of exogenous substrate [poly(Glu^{80}Tyr^{20})] by insulin-receptor kinase. Partially purified receptor preparations were first preincubated for 20 min at room temperature in absence or presence of increasing concentrations of insulin. Phosphorylation was initiated by addition of 50 μM of [γ-^{32}P]ATP, 20 mM $MgCl_2$, and 2 mg/ml poly(Glu^{80}Tyr^{20}) and allowed to proceed at room temperature for 20 min. Reactions were terminated by applying aliquots to phosphocellulose filter papers, drying them, and then counting by liquid-scintillation spectrometry. Results are means ± SE of 8 separate experiments. Results were also analyzed as described previously (21) to compute K_a (half-maximal concentration of insulin) and V_{max} (maximal insulin-stimulated tyrosine kinase activity). ●, WKY rats; ▲, SHR rats.

this context, the current demonstration of cellular resistance to glucose uptake in adipocytes from SHR rats adds further experimental evidence to the view that insulin resistance and hyperinsulinemia may play a role in the etiology of hypertension.

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