Reduced insulin receptor signaling in the obese spontaneously hypertensive Koletsky rat

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Friedman, Jacob E., Tatsuya Ishizuka, Sha Liu, Craig J. Farrell, David Bedol, Richard J. Koletsky, Hue-Lee Kaung, and Paul Ernsberger. Reduced insulin receptor signaling in the obese spontaneously hypertensive Koletsky rat. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E1014–E1023, 1997.—Insulin resistance is associated with both obesity and hypertension. However, the cellular mechanisms of insulin resistance in genetic models of obese-hypertension have not been identified. The objective of the present study was to investigate the effects of genetic obesity on a background of inherited hypertension on initial components of the insulin signal transduction pathway and glucose transport in skeletal muscle and liver. Oral glucose tolerance testing in SHROB demonstrated a sustained postchallenge elevation in plasma glucose at 180 and 240 min compared with lean spontaneously hypertensive rat (SHR) littersmates, which is suggestive of glucose intolerance. Fasting plasma insulin levels were elevated 18-fold in SHROB. The rate of insulin-stimulated 3-O-methylglucose transport was reduced 68% in isolated epimysialar muscles from the SHROB compared with SHR. Insulin-stimulated tyrosine phosphorylation of the insulin receptor β-subunit and insulin receptor substrate-1 (IRS-1) in intact skeletal muscle of SHROB was reduced by 36 and 23%, respectively, compared with SHR, due primarily to 32 and 60% decreases in insulin receptor and IRS-1 protein expression, respectively. The amounts of p85 regulatory subunit of phosphatidylinositol-3-kinase and GLUT-4 protein were reduced by 28 and 25% in SHROB muscle compared with SHR. In the liver of SHROB, the effect of insulin on tyrosine phosphorylation of IRS-1 was not changed, but insulin receptor phosphorylation was decreased by 41%, compared with SHR, due to a 30% reduction in insulin receptor levels. Our observations suggest that the leptin receptor mutation fa° imposed on a hypertensive background results in extreme hyperinsulinemia, glucose intolerance, and decreased expression of postreceptor insulin signaling proteins in skeletal muscle. Despite these changes, hypertension is not exacerbated in SHROB compared with SHR, suggesting these metabolic abnormalities may not contribute to hypertension in this model of Syndrome X.

Syndrome X; tyrosine phosphorylation, leptin receptor

METABOLIC SYNDROME X describes a cluster of atherogenic risk factors in hypertensive patients, including insulin resistance, reduced glucose disposal, hyperinsulinemia, and dyslipidemia (33). Although obesity and non-insulin-dependent diabetes mellitus (NIDDM) are known to be linked to insulin resistance, when these coexist with hypertension, whole body insulin sensitivity is further impaired in humans (28). Although many epidemiological studies have confirmed that hyperinsulinemia and insulin resistance are common features in obese and diabetic hypertensive subjects, the mechanism of insulin resistance in hypertension is unknown. Whole body insulin resistance has been documented in some (14, 20, 32) but not all (4, 6) studies of spontaneously hypertensive rats (SHR) compared with Wistar-Kyoto (WKY) controls. Reaven et al. (35) showed that glucose uptake in adipocytes from SHR was resistant to insulin, whereas another group (6) found glucose transport in skeletal muscle from the SHR was not insulin resistant. Similarly, Bader et al. (4) found normal insulin receptor tyrosine kinase activity and glucose transporter GLUT-4 levels in skeletal muscle of SHR. These data imply that the SHR background may not lead to insulin resistance in skeletal muscle, the primary target for glucose uptake, suggesting that tissue-specific defects may account for the whole body insulin resistance.

The genetically obese hypertensive strain (SHROB or Koletsky rat) was originally established in 1970 after a genetic mutation spontaneously appeared, causing obesity in offspring of a cross between a female Kyoto-Wistar SHR and a normotensive Sprague-Dawley male (26). The SHROB is a unique strain with genetic obesity, hyperlipidemia (type IV), hyperinsulinemia, glomerulopathy with proteinuria, and spontaneous hypertension, characteristics paralleling human obese hypertension and Syndrome X (24–26). In the SHROB, obesity exists as a recessive trait on a hypertensive background. The obese phenotype results from a single homozygous recessive trait, originally designated fa°, and is allelic with the Zucker fatty trait (fa) but of distinct origin (39). Takaya et al. (40) recently identified a nonsense point mutation (T→A) in the SHROB at position +2289 in the extracellular domain of the leptin receptor common to all isoforms so far identified. The fa° mutation in the SHROB is a null mutation, resulting from a premature stop codon, and differs from the Zucker fatty (fa) rat, which has a missense mutation at position +269 coding for a different exon in the extracellular domain of the leptin receptor (40), resulting in decreased sensitivity to leptin in Zucker rats (9). There is considerable evidence that attenuated insulin sensitivity associated with obesity and NIDDM is caused by postreceptor defects in intracellular signaling, primarily in skeletal muscle. However, the cellular mechanisms for insulin resistance in genetic models of obese-hypertension have not been identified. The intracellular events that couple the stimulation of insulin receptors to the movement of glucose across the muscle membrane are partially understood. The initial events include binding of insulin to the α-subunit of the insulin receptor on the extracellular surface of the cell, activation of the insulin receptor tyrosine kinase, result-
ing in autophosphorylation, the subsequent phosphorylation of insulin receptor substrates, and the interaction of these substrates with several downstream signaling molecules that stimulate the translocation of GLUT-4-containing vesicles to the cell surface and to tubules (21). Insulin stimulates the receptor to undergo autophosphorylation, thereby enhancing the tyrosine kinase activity of the receptor toward other protein substrates (23, 43, 46). The phosphorylation of insulin receptor substrate-1 (IRS-1) (and IRS-2) on multiple tyrosine residues after insulin treatment has been shown to be important in coupling the insulin receptor to glucose uptake. For example, in mice with a gene knockout of IRS-1, there is growth retardation and a mild form of glucose intolerance, including a 50% reduction in insulin-stimulated glucose transport in skeletal muscle and adipose tissue (2, 41), confirming that the IRS-1 pathway plays an important role in the postreceptor signaling of growth and glucose metabolism. The phosphorylation of IRS-1 results in the binding of the regulatory subunit (p85α) of phosphatidylinositol-3-kinase (PI-3-kinase) to IRS-1 (44). Binding of the p85α isoform to tyrosine-phosphorylated IRS-1 results in increased catalytic activity of the PI-3-kinase complex (3). Formation of this protein complex appears to be necessary, although not sufficient, for stimulating glucose transport in 3T3-L1 adipocytes (19, 45). The purpose of the present study, therefore, was to determine the contribution of proximal insulin signaling defects in skeletal muscle and liver in vivo to the complex metabolic abnormalities of the SHROB rat, a model of Syndrome X.

METHODS
Reagents. Human insulin (Humulin R) was purchased from Eli Lilly (Indianapolis, IN). Affinity-purified polyclonal antibodies to IRS-1 and p85α were obtained from Upstate Biotechnology (Saranac Lake, NY). Monoclonal antiphosphotyrosine antibody and rabbit polyclonal antiserum to the insulin receptor (α-subunit) were obtained from Transduction Laboratories (Lexington, KY). Rabbit antiserum raised against the COOH-terminal 12 amino acids of rat GLUT-4 was kindly obtained by brother-sister mating for the last 23 years and at 1970 at Case Western Reserve University (CWRU) from SHR and SHROB at 12–18 wk of age. All rats were fasted for 18 h and administered a 50% glucose solution by a feeding tube at a dose of 6 g/kg body weight. Blood samples (0.2 ml) were obtained from the tail vein of unrestrained, conscious animals at 0, 30, 60, 90, 120, 180, and 240 min, and glucose was measured in whole blood by colorimetric glucose oxidase assay (One-Touch, Lifestan, Milipitas, CA). The remaining blood sample was allowed to clot on ice and was centrifuged for 20 min at 13,000 revolutions/min (rpm) at 4°C, and the serum was frozen at −70°C until assayed for insulin. An insulin radioimmunoassay kit was used with rat insulin standards and antibodies directed against rat insulin (Linco Research). Assays were conducted in duplicate, and the intra-assay coefficient of variation was <5%. Blood cholesterol and triglyceride levels were assayed in blood obtained at the time of euthanasia, after an overnight fast, using a colorimetric assay run on a Kodak Ektachem 700 Clinical Chemistry Autoanalyzer (Johnson & Johnson, Rochester, NY).

3-O-MG transport. The epitrochlearis muscle was used to study glucose transport activity as described previously (27). The advantage of the epitrochlearis is that it is a thin muscle and, therefore, there are no diffusion limitations for oxygen or substrates, even in muscles from larger obese rats (17). After an overnight fast, rats were anesthetized with ketamine (150 mg/kg) and acepromazine (5 mg/kg) and the epitrochlearis muscle, with tendon attached, was isolated and removed from both forelimbs. The muscles were then preincubated at 29°C for 30 min in 2 ml of Krebs-Henseleit bicarbonate buffer containing 1% BSA, 32 mM mannitol, 8 mM d-glucose, and either 0 or 20 mM bovine insulin and were gassed continuously with 95% O2-5% CO2. After preincubation, muscles were rinsed for 10 min in fresh buffer containing 1% BSA, 40 mM mannitol, and the appropriate insulin concentration. The muscles were then transferred to fresh buffer containing 8 mM 3-O-MG, 250 µCi/mmol 3-O-[3H]MG, 30 mM mannitol, 10 µCi/mmol [1-14C]mannitol, and 2 mM sodium pyruvate, with or without insulin for 10 min. After incubation, muscles were removed, trimmed of connective tissue, quickly blotted on gauze, and immediately freeze-clamped. Frozen muscles were weighed and digested in 0.5 ml of 1 M KOH for 30 min at 70°C and neutralized with 0.5 ml of 1 M HCl. A 0.3-ml aliquot of the supernatant was added to 5 ml of Cysoscent liquid scintillation fluid (ICN, Costa Mesa, CA). The specific activity of the incubation media was obtained using 50-µl samples obtained from each well. The incubation media samples were added to 950 µl of 1 M KOH-HCl solution similar to the muscle digest, and all samples were counted for radioactivity in a Beckman LS 8100 liquid scintillation counter with dual quench correction. The rate of 3-O-[3H]MG transport was expressed in nanomoles per milligram wet weight per 10 min, after correction for extracellular 3-O-[3H]MG, and the results were analyzed by analysis of variance.

Insulin receptor and IRS-1 tyrosine phosphorylation in vivo. Insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1 in liver and muscle of intact rats was assayed by the method originally described by Saad et al.
(36), with minor modifications. Rats were fasted for at least 12 h and were anesthetized, and the abdominal cavity was opened and the portal vein exposed. For studies of skeletal muscle, the skin from one hindlimb was removed and a 200-mg sample of the gastrocnemius was taken and frozen immediately in liquid nitrogen. We tested a combination of time points of 30 s, 1, 3, 5, and 10 min and insulin dosages of 1, 10, and 200 U/kg. We chose 10 U/kg body wt and time points of 30 s in liver and 5 min in muscle on the basis of preliminary experiments that showed a maximally effective stimulation of insulin receptor and substrate phosphorylation in liver and gastrocnemius muscle from control animals using these conditions (15, 36, and unpublished data). A 1-ml bolus of normal saline (0.9% NaCl) with or without insulin (10 U/kg body wt) was injected into the portal vein, and within 30 s a liver sample was obtained, and within 5 min a sample from the opposite gastrocnemius muscle was quickly excised and frozen immediately in liquid nitrogen. The frozen samples were pulverized in liquid nitrogen and homogenized immediately under denaturing conditions using a Polytron PTA 205 generator at maximum speed for 30 s in ice-cold 10× volume of homogenization buffer [50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.5, 100 mM Na2PO4, 100 mM EDTA, 10 mM Na3VO4, plus aprotinin (0.1 mg/ml), leupeptin (10 µg/ml), phenylmethylsulfon- 

fyl fluoride (PMSF; 34 µg/ml), and 1% Triton X-100]. The homogenate was allowed to sit on ice for 30 min at 4°C, followed by centrifugation at 38,000 rpm in a 70 Ti rotor (Beckman Instruments, Fullerton, CA) at 4°C for 30 min to remove insoluble material. The supernatant was collected and assayed for protein concentration (Bradford dye assay, Bio-Rad Chemicals, Hercules, CA).

Immunoprecipitation and immunoblotting. Equal amounts of protein from the liver or muscle of SHR and SHROB rats were immunoprecipitated overnight at 4°C with an antiphosphotyrosine antibody (5 µg Ab/8 mg protein) in 1 ml of immunoprecipitation buffer containing 2% Triton X-100, 300 mM NaCl, 200 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 2 mM EDTA, 2 mM ethylene glycol-bis(s- aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 0.4 mM PMSF, 0.4 mM sodium vanadate, and 1% Nonidet P-40 (NP-40). After immunoprecipitation, the sample was centrifuged, and with 10 µl of protein A-Sepharose (10% solution) for 4 h at 4°C, the immunoprecipitate was washed in 1 ml of immunoprecipitation buffer, followed by centrifugation at 500 g for 1 min at 4°C, repeated four times. The washed precipitate was mixed with Laemml sample buffer (50 µl), boiled for 5 min, and centrifuged for 5 min at 500 g, and the supernatant (30 µl) was separated on a 7% Tris polyacrylamide gel electrophoresis (PAGE) using the Bradford procedure (Bio-Rad Mini-Protein gel apparatus). Proteins were then electrotransferred from the gel to polyvinylidene difluoride (PVDF) membrane at 100 V (constant current) for 2 h using a mini transfer apparatus (Idea Scientific, Minneapolis, MN). Gels were stained with Coomassie blue to ensure equal protein transfer. To reduce nonspecific protein binding, the membrane was blocked using 5% nonfat dry milk (1% BSA in the case of anti-PY antibody) in buffer containing 10 mM Tris-HCl, 150 mM NaCl, with 0.02% Tween 20 (TBS-T). The PVDF membranes were incubated with antiphosphotyrosine antibodies (p-Tyr, 0.3 µg/ml) or with anti-insulin receptor β (0.4 µg/ml) or IRS-1 antibody (1.5 µg/ml) in blocking buffer for 4 h at 22°C, followed by extensive washing with TBS-T. At the end of the final wash, the blots were incubated with secondary antibody linked to horseradish peroxidase in 10 ml of blocking buffer for 1 h at 22°C and washed again before the membranes were exposed to enhanced chemiluminescence (ECL) reagent according to the manufacturer’s instructions (Amersham, Arlington Heights, IL). Autoradiography was carried out using Kodak XAR X-ray film. After treatment with the ECL reagent, the exposure time was varied from 1 to 3 min, and each exposure was quantified by densitometry. In preliminary experiments, the PTyr antibody was found to immunoprecipitate >95% of the tyrosine-phosphorylated insulin receptor and IRS-1, based on immunoblotting an aliquot of the muscle protein extract remaining after immunoprecipitation (data not shown). The specific band intensities were quantitated by optical densitometry using a Digiscan scanner (US Biochemical, Cleveland, OH) for integrating the autoradiographic signals. The results shown are expressed as the average signal intensity (arbitrary units) expressed relative to the effect of insulin on phosphorylation of insulin receptor and IRS-1 in lean animals.

Western blot analysis of insulin receptor β, IRS-1, p85α, and GLUT-4 protein. To quantify the levels of insulin receptor β, IRS-1, p85α, and GLUT-4, analysis was carried out in samples of gastrocnemius muscle and a portion of the liver. For GLUT-4 determination, total muscle membranes were prepared by homogenization of a portion of the muscle, as described previously (13). Each muscle or liver sample was homogenized, aliquoted, and run in an average of three separate assays involving different minigels. Each gel contained the same internal standard: a rat heart protein preparation (20-µg aliquot) prepared similarly to skeletal muscle that was run on every blot. The muscle was homog-

enized in 10× solubilization buffer containing 25 mM HEPES, pH 7.5, 1 mM EDTA, 0.8 µg/ml aprotinin, 0.6 µg/ml leupeptin, 1 µg/ml pepstatin, and 50 µg/ml PMSF, and the sample was centrifuged at 38,000 g for 60 min. The pellet was resus-

pended in solubilization buffer, and 40 µg of protein were treated with Laemmli sample buffer, boiled for 5 min, and resolved on 8% sodium dodecyl sulfate (SDS)-PAGE gel. For insulin receptor β, IRS-1, and p85α analyses, frozen samples were homogenized in 10 volumes of solubilization buffer A (50 mM HEPES, pH 7.5, 175 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM Na2VO4, 10 mM Na2PO4, 10 mM NaF, 2 mM EDTA, 1% NP-40, 10% glycerol, 2 µg/ml aprotinin, 10 µg/ml antipain, 0.5 µg/ml leupeptin, 1.5 mg/ml benzamidine, and 34 µg/ml PMSF) using a Polytron PTA 205 generator at maximum speed for 30 s. The homogenate was then centrifuged at 65,000 rpm at 4°C in a model 70 Ti rotor for 60 min to remove insoluble material, and the supernatant was used for analysis. Protein was measured using the Bradford procedure (Bio-Rad Biochemical). For insulin receptor β, IRS-1, and p85α, 100 µg of homogenate protein were treated with Laemmli sample buffer containing 100 mM dithiothreitol and heated in a boiling water bath for 4 min and subjected to electrophoresis on a 7% SDS-Tris acrylamide gel using a Bio-Rad Mini-Protein gel apparatus at 100 V for 1 h. Proteins were electrotransferred from the gel to nitrocellulose at 90 V (constant) for 1 h, using a mini transfer apparatus. Nonspecific protein binding to the filter was blocked with the use of 5% milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20. The PVDF filter was incubated with antibodies to insulin receptor β (1.5 µg/ml), IRS-1 (1.5 µg/ml), p-85α (1.5 µg/ml), or GLUT-4 (1.5 µg/ml) diluted in blocking buffer for 4 h at 22°C, followed by extensive washing with Tris-buffered saline (150 mM NaCl, 10 mM Tris + Tween 20). At the end of the final wash, the blots were incubated with secondary antibody linked to horseradish peroxidase in 10 ml of blocking buffer for 1 h at 22°C and washed again before the membranes were exposed to ECL reagent, according to the manufacturer’s instructions (Amersham, Arlington Heights, IL). Autoradiography was carried out using Kodak XAR X-ray film.
film, with exposure time varied from 30 s to 3 min, and the average specific band intensities from each exposure were quantified by optical density using a Digiscan scanner (US Biochemical) for integrating the autoradiographic signals. The results were expressed as arbitrary units relative to an internal standard sample (rat heart membrane) run together with each blot, and the value for the lean animals was set at 100.

Statistical analysis. Results are presented as means ± SE for the indicated number of rats. Comparisons between groups were made using Student’s unpaired t-test, except for glucose tolerance data, which were analyzed by analysis of variance for repeated measures using Prism (Graph Pad Software, San Diego, CA). Statistical significance was set at P < 0.05.

RESULTS

Animal characteristics. Table 1 summarizes the body weight, blood pressure, and metabolic characteristics of animals used in these studies. The SHROB rats used in this study were 200% heavier (P < 0.01) than their lean SHR littermates. Nonetheless, the level of hypertension was similar in the SHROB compared with lean SHR littermates. The SHROB rat displayed marked hypertriglyceridemia and a moderate increase in plasma cholesterol (P < 0.01) compared with aged-matched lean littermates. Fasting serum insulin levels were significantly elevated up to 18-fold in the SHROB (P < 0.01) compared with lean SHR, indicative of insulin resistance, but SHROB did not show fasting hyperglycemia consuming regular rat chow ad libitum.

Oral glucose tolerance test. Figure 1 shows the results of the oral glucose tolerance test in SHR and SHROB. In response to glucose challenge, the SHROB had a more sustained increase in plasma glucose, with significantly higher glucose values at 180 and 240 min compared with SHR, suggestive of relative glucose intolerance. Large differences were seen in the plasma insulin response during the oral glucose tolerance test. Plasma insulin levels in the SHROB demonstrated a 273% increase by 60 min and remained elevated at 4 h, whereas in SHR insulin increased by a similar proportion, 320% during the first 60 min, but declined toward fasting levels between 60 and 240 min. At 240 min, insulin levels were 30 times greater in SHROB compared with SHR.

Glucose transport in skeletal muscle of SHROB rats. To assess whether impaired glucose tolerance is associated with impaired glucose transport in skeletal muscle, 3-O-MG transport was measured in the epitrochlearis muscle obtained from lean SHR and obese SHROB rats (Table 2). Basal 3-O-MG transport was not altered in muscles from the obese animals. Muscles were incu-

Table 1. Metabolic characteristics of SHR and SHROB

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<th>SHR</th>
<th>SHROB</th>
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<tr>
<td>Body weight, g</td>
<td>310 ± 13</td>
<td>621 ± 13*</td>
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<td>Mean arterial pressure, mmHg</td>
<td>178 ± 3</td>
<td>168 ± 4</td>
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<tr>
<td>Glucose, mg/dl</td>
<td>53 ± 6</td>
<td>50 ± 2</td>
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<tr>
<td>Insulin, µU/ml</td>
<td>0.4 ± 0.1</td>
<td>8.5 ± 2.0*</td>
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<tr>
<td>Triglyceride, mg%</td>
<td>60 ± 2</td>
<td>488 ± 48*</td>
</tr>
<tr>
<td>Cholesterol, mg%</td>
<td>53 ± 5</td>
<td>104 ± 13*</td>
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Values are means ± SE. Metabolic data were obtained in 12- to 18-wk-old male animals after an overnight fast in spontaneously hypertensive rats (SHR) (n = 9) and SHROB (n = 16). Mean arterial pressure was measured by direct carotid cannula under urethan anesthesia. *Significant difference between phenotypes (obese relative to lean): P < 0.01 by analysis of variance.

Table 2. 3-O-methylglucose transport activity in epitrochlearis muscle of SHR and SHROB

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<th>SHR</th>
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<tr>
<td>Basal</td>
<td>0.06 ± 0.05</td>
<td>0.06 ± 0.04</td>
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<tr>
<td>Insulin stimulated</td>
<td>0.69 ± 0.20*</td>
<td>0.22 ± 0.10</td>
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Values are means ± SE in nmol·mg muscle⁻¹·10 min⁻¹; n = 6–8 muscles/treatment. *Significantly increased in SHR compared with SHROB: P < 0.05 by analysis of variance.
bated in the presence of a maximally effective dose of $10^{-7}$ M insulin, and glucose uptake was measured over 10 min. The ability of insulin to stimulate glucose transport was reduced by 68% in muscle from the SHROB compared with its age-matched lean littermate.

p85α and GLUT-4 expression. To determine whether decreased insulin-stimulated glucose transport activity was reflected by a decrease in total cellular content of p85α and GLUT-4 proteins, the expression of these proteins was determined in a sample of gastrocnemius muscle from SHR and SHROB. Figure 2 shows a representative autoradiogram of the level of GLUT-4 protein measured in total membranes of both SHR and SHROB. The levels of GLUT-4 protein from eight animals were quantified and found to be reduced by 25% (P < 0.05) in skeletal muscle from SHROB compared with SHR. As shown in Fig. 2C and quantified in Fig. 2D, the content of p85α was similarly decreased by 28% (P < 0.05) in gastrocnemius of SHROB compared with SHR.

Insulin receptor and IRS-1 tyrosine phosphorylation in skeletal muscle of SHROB rats. Factors other than reduced p85α and GLUT-4 protein that may contribute to the decreased glucose disposal and insulin-stimulated glucose transport in SHROB include decreased tyrosine phosphorylation and/or cellular content of the insulin receptor β-subunit and IRS-1 or impaired association of the p85α-subunit to tyrosine-phosphorylated residues of the substrate IRS-1. To investigate these possibilities, rats were injected with 10 U/kg body weight of insulin or saline via the portal vein, and hindlimb muscles were rapidly harvested after 5 min and frozen in liquid nitrogen. Relative to saline-injected controls, insulin injection induced tyrosine phosphorylation of two major proteins with molecular weight corresponding to the insulin receptor β-subunit (−95 kDa) and IRS-1 (−165–185 kDa) in both SHR and SHROB (Fig. 3A). To quantify the phosphorylation of the insulin receptor and IRS-1, muscle protein extracts from both SHR and SHROB were immunoprecipitated with PTyr antibodies and blotted using specific antibodies to the insulin receptor β-subunit and IRS-1, as shown in Fig. 3, B and C (representative autoradiograms). The exposure to insulin resulted in a three- to fourfold increase in insulin receptor and IRS-1 tyrosine phosphorylation. Figure 4A shows the results of eight experiments analyzed by densitometry and the results quantified and expressed relative to lean SHR control rats. The level of insulin-stimulated insulin receptor phosphorylation in skeletal muscle of SHROB was decreased by 36% compared with SHR, P < 0.01. The level of IRS-1-phosphorylation was modestly but significantly reduced by 23% compared with SHR, P < 0.01. To determine whether the reduction in insulin receptor and IRS-1 phosphorylation could be accounted for by a decrease in the total quantity of insulin receptors or IRS-1 or possibly a decrease in the amount of tyrosine residues phosphorylated per receptor, the levels of insulin receptor and IRS-1 protein were measured by Western blotting using muscle protein extracts from eight animals from each group. A representative autoradiogram is shown in Fig. 3, D and E, and the quantification in Fig. 4B. There was a 32% decrease (P < 0.01) in insulin receptor protein in skeletal muscle of SHROB compared with SHR, and the level of IRS-1 protein was decreased by 60% in SHROB compared with SHR (P < 0.01). When the decrease in phosphorylation was expressed relative to the amount of insulin receptor or IRS-1 (Fig. 4C), there was no difference in the insulin receptor phosphorylation per receptor protein in SHROB compared with SHR, suggesting that the decrease in insulin-receptor tyrosine phosphorylation per receptor protein in SHROB compared with SHR, despite decreases in protein and overall phosphorylation.

Insulin receptor and IRS-1 tyrosine phosphorylation in liver of SHROB rats. The liver is an additional important locus of insulin resistance. Therefore, we isolated tyrosine-phosphorylated proteins by immunoprecipitation from the intact liver with and without insulin treatment in the SHROB and SHR rats (Fig. 5A, representative autoradiogram). The antiphospho-tirosine antibody reacted with proteins of apparent mo-
lecular mass of IRS-1 between 165 and 185 kDa, and the insulin receptor β-subunit of ~95 kDa. To quantify the level of phosphorylation of IRS-1 and insulin receptor in liver from insulin-treated animals, the immunoprecipitated proteins from liver extracts were separated by SDS-PAGE and immunoblotted with either anti-PTyr antibodies (A), anti-insulin receptor (β-subunit) antibody (B), or anti-IRS-1 antibodies (C) and subjected to autoradiography. A representative autoradiogram of each is shown. Note: large bands below insulin receptor and IRS-1 are washed immunoglobulin (Ig) G proteins detected by chemiluminescence reagent. To measure total quantity of insulin receptors or IRS-1 present in muscle, equal amounts of solubilized muscle proteins from SHR and SHROB rats were resolved by SDS-PAGE, transferred to PVDF membranes, and detected with anti-insulin receptor (β-subunit) antibodies (D) or anti-IRS-1 (E) antibody.

Fig. 3. Effect of insulin on maximal tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1 (IRS-1) in intact skeletal muscle from SHR and SHROB rats. A sample of mixed gastrocnemius muscle was obtained before and after insulin injection, as described in METHODS. Muscles were homogenized with extraction buffer under denaturing conditions at 4°C, and aliquots containing the same amount of protein were incubated at 4°C with antiphosphotyrosine (PTyr) antibodies (Ab) and protein A-Sepharose. Immunoprecipitated proteins were run on SDS-PAGE and analyzed by Western blotting with either anti-PTyr antibodies (A), anti-insulin receptor (β-subunit) antibody (B), or anti-IRS-1 antibodies (C) and subjected to autoradiography. A representative autoradiogram of each is shown. Note: large bands below insulin receptor and IRS-1 are washed immunoglobulin (Ig) G proteins detected by chemiluminescence reagent. To measure total quantity of insulin receptors or IRS-1 present in muscle, equal amounts of solubilized muscle proteins from SHR and SHROB rats were resolved by SDS-PAGE, transferred to PVDF membranes, and detected with anti-insulin receptor (β-subunit) antibodies (D) or anti-IRS-1 (E) antibody.

Fig. 4. Quantification of tyrosine phosphorylation and protein expression of insulin receptor and IRS-1 in muscle of SHR and SHROB rats after maximal insulin stimulation. Scanning densitometry of multiple autoradiograms performed according to procedures outlined in METHODS and shown in Fig. 3 were obtained from experiments on 8 different animals/group. A: level of insulin receptor and IRS-1 tyrosine phosphorylation in multiple experiments. B: level of insulin receptor β-subunit and IRS-1 protein expression. C: ratio between in vivo phosphorylated insulin receptors and IRS-1 and total quantity of receptors and IRS-1 in skeletal muscle from same SHR and SHROB rats. Data are expressed as means ± SE. *P < 0.001 vs. SHR.
amounts of liver protein from the experimental animals were resolved by SDS-PAGE and immunoblotted with anti-insulin receptor antibody (Fig. 5D) or IRS-1 antibody (Fig. 5E). Figure 6A quantifies autoradiograms from six separate experiments by scanning densitometry. The level of insulin receptor was reduced by 42% (P < 0.01) in SHROB compared with SHR, and the level of IRS-1 was not changed. When the phosphorylation data were expressed relative to the level of insulin receptor and IRS-1, there was no change in the insulin receptor tyrosine phosphorylation per level of receptor protein in the liver of the SHROB. Similarly, there was no change in IRS-1 phosphorylation (Fig. 6C). Thus the reduced insulin receptor phosphorylation was due to a reduction in the level of insulin receptor protein in the liver of SHROB rats. An increased basal phosphorylation in SHROB may lower the maximal response to insulin. However, after scanning densitometry, there

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**Fig. 5.** Effect of insulin on tyrosine phosphorylation in intact liver from SHR and SHROB rats. Rats were anesthetized, the abdominal cavity was opened, and a biopsy of the liver was obtained and frozen in liquid nitrogen. One milliliter of insulin (10 U/kg body wt) was then infused into portal vein as a bolus injection over a 1-min period. After 30 s, a portion of liver was excised, and proteins were isolated as described in METHODS and in Fig. 3. Extracts from rats were immunoprecipitated with antiphosphotyrosine antibody and protein A-Sepharose as described in METHODS, and aliquots of proteins were run on SDS-PAGE and analyzed by Western blotting with either antiphosphotyrosine antibodies (A), anti-insulin receptor (β-subunit) antibody (B), or anti-IRS-1 antibodies (C) and subjected to autoradiography. A representative autoradiogram of each is shown. Note: shadow in Fig. 5B, lanes 2 and 4, above the insulin receptor (IR) β-subunit is a chemiluminescence-related artifact, whereas larger bands below are IgG proteins. To measure total quantity of insulin receptors or IRS-1 present in muscle, equal amounts of solubilized muscle proteins from SHR and SHROB rats were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected with anti-insulin receptor (β-subunit) antibodies (D) or anti-IRS-1 (E) antibody.

**Fig. 6.** Quantification of tyrosine phosphorylation and protein expression of insulin receptor and IRS-1 in liver of SHR and SHROB rats after maximal insulin stimulation. Scanning densitometry of multiple autoradiograms from 8 experiments performed according to procedures outlined in METHODS and shown in Fig. 5. A: level of insulin receptor and IRS-1 tyrosine phosphorylation. B: level of insulin receptor β-subunit and IRS-1 protein expression. C: ratio between in vivo phosphorylated insulin receptors and IRS-1 and total quantity of receptors or IRS-1 in liver from same SHR and SHROB rats. Data are expressed as means ± SE. *P < 0.001 vs. SHR.
was no difference in basal phosphorylation in skeletal muscle or liver insulin receptor and IRS-1.

**DISCUSSION**

Available evidence suggests that genes responsible for insulin resistance, hypertension, and obesity may be inherited as part of a common syndrome of metabolic abnormalities termed Syndrome X in humans (33). The SHROB (Koletsky rat) is a unique highly inbred animal model with genetic obesity, hypertension, hyperlipidemia, and hyperinsulinemia, making it a potential model of human Syndrome X. Various other strains have been developed from the original Koletsky rat (fa<sup>+</sup>). The SHROB strain differs, however, from other obese rat models such as the SHR/N<sup>0</sup> rat, which is obese and diabetic but which has lost the hypertensive trait (42), and a colony of obese spontaneous heart failure rats SHHF/Mcc-fa<sup>0</sup>-<sup>0</sup> (30). The obese Zucker (fa) rat is obese and glucose intolerant but does not develop spontaneous genetic hypertension (31), and the Wistar fatty rat, derived from crosses between obese Zucker and Wistar-Kyoto rats, develops obesity, mild hyperglycemia, and mild hypertension (47). However, the SHROB is the only model that has retained the genetic traits for obesity and hypertension, making it a unique genetically stable rat strain for exploring the mechanisms and interactions of obesity, hypertension, hyperlipidemia, and salt sensitivity in the absence of heart failure (23a).

The recent identification of a null mutation in the leptin receptor gene in the SHROB Koletsky rat suggests that these animals should exhibit extreme insulin resistance resulting from the combined effects of the absence of leptin receptors and chronic hypertension. As shown here for the first time, the SHROB exhibits extreme hyperinsulinemia and demonstrates glucose intolerance in response to an oral glucose load but is not overtly diabetic. The SHROB demonstrated a 15- to 20-fold greater fasting insulin level than the SHR and an increased insulin secretion in response to an oral glucose load. The impaired glucose tolerance in SHROB suggests that severe insulin resistance may underlie the chronic hyperinsulinemia. The absence, however, of fasting hyperglycemia suggests that the fa<sup>+</sup> mutation expressed on this genetic background is not sufficient to trigger diabetes. The only other strains of rats in which the fa<sup>+</sup> receptor mutation results in obesity-induced diabetes is the SHR/N<sup>0</sup> rat and SHHF/Mcc-fa<sup>0</sup>-<sup>0</sup> rat. These obese animals, derived from the original Koletsky rat colony, suggest that diabetes is not an intrinsic function of the fa<sup>+</sup> mutation itself but likely requires polygenic interaction with other diabetogenic modifier genes present in these other strains. It is noteworthy that the SHROB also retain fasting normoglycemia when stressed by high sucrose feeding (P. Ernsberger, D. Bedol, R. J. Koletsy, and J. E. Friedman, unpublished observations), suggesting that genes controlling the ability of the pancreatic B cells to compensate for increasing levels of peripheral insulin resistance may be an important factor contributing to diabetic resistance in the SHROB.

In the postprandial state, skeletal muscle is the major site of glucose disposal, and, under hyperinsulinemic clamp conditions, insulin-mediated glucose uptake into skeletal muscle represents 75% of total glucose utilization at euglycemia and 95% during hyperglycemia (5). Thus it is widely believed that the cause of insulin-resistant states resides in skeletal muscle. This has been confirmed by ex vivo studies demonstrating severe resistance to insulin-stimulated glucose transport in human skeletal muscle fiber strips of obese and NIDDM patients (11). Furthermore, in vivo euglycemic hyperinsulinemic clamp studies of patients with NIDDM demonstrated that leg glucose transport was reduced by 45% compared with controls and indicated a strong positive correlation exists between leg glucose transport and reduced total body glucose uptake (10). In the present study, we used the isolated epitrochlearis muscle to study skeletal muscle glucose transport activity independent of blood flow. We found a marked impairment in insulin-stimulated glucose transport activity in SHROB, suggesting that the insulin resistance to glucose transport in skeletal muscle may contribute to whole body insulin resistance and hyperinsulinemia, independent of hemodynamic abnormalities. These data are consistent with previous studies demonstrating an impairment in maximal insulin-stimulated 2-deoxyglucose transport in muscle from the obese Zucker rat compared with lean controls (18).

We compared SHROB rats to their lean hypertensive SHR littermates, which may be insulin resistant relative to normotensive controls. The causes of hypertension in the SHR are considered to be polygenic and multifactorial. One of the components of hypertension in SHR might be related to insulin resistance, although severe exacerbation of insulin resistance, as in the SHROB, does not further increase blood pressure. Hulman et al. (20) reported that 2-deoxyglucose transport is not impaired in the SHR compared with nonhypertensive controls. These findings would suggest that the obesity and hypertension genes in the SHROB do not synergize with respect to insulin resistance in skeletal muscle. Furthermore, because the level of hypertension, if anything, may be slightly lower in the SHROB compared with SHR, these findings argue that insulin resistance and hypertension may segregate as independent phenotypes and do not show synergism in the pathogenesis of either insulin resistance or hypertension.

The mechanism(s) associated with decreased glucose transport in obesity and type II diabetes may involve defects in the expression, translocation, and/or function (intrinsic activity) of the GLUT-4 glucose transporter (23). However, insulin action involves a complex cascade of many gene products, and thus the insulin resistance in the SHROB could be multifactorial, involving several defects in insulin-signaling pathways upstream of GLUT-4 glucose transporter translocation. Our observation of a modest 23% reduction in GLUT-4 levels in skeletal muscle of SHROB compared with the 68% reduction in insulin-stimulated glucose transport implicate additional factors in the insulin resistance of...
glucose transport. A major cytosolic protein involved in insulin signaling, IRS-1 (38), has a molecular weight of 165–185 kDa on SDS-PAGE and has up to 22 potential tyrosine phosphorylation sites. Tyrosine-phosphorylated IRS-1 binds to the insulin receptor, and both proteins can be detected in their phosphorylated form in skeletal muscle protein extracts separated by SDS-PAGE and immunoblotted using antiphosphotyrosine antibodies (15, 36). Our results show that the skeletal muscle from the SHROB rat has 23–36% less insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1. Similar changes were recently reported in the skeletal muscle of normoglycemic morbidly obese patients (16). The mechanism for these impairments in insulin signal transduction are unknown. However, in SHROB decreased phosphorylation mainly reflected decreased expression of insulin receptor and IRS-1 protein. Kahn and Saad (22) reported that insulin receptor and IRS-1 phosphorylation in liver and skeletal muscle of SHR was decreased by 20% compared with WKY rats, despite similar levels of insulin receptor and IRS-1. Thus it is possible that the combination of genetic obesity and the SHR genetic background results in additive effects. However, the stoichiometry between the phosphorylation of the insulin receptor and IRS-1 and protein levels suggests that the average amount of tyrosine phosphorylation per IRS-1 protein may actually be increased in the muscle of the SHROB rat compared with SHR, suggesting perhaps a compensatory mechanism for reduced insulin receptor and IRS-1 protein expression.

Given the marked fasting hyperinsulinemia in the SHROB, it is likely there is insulin resistance in the liver as well as in skeletal muscle. The current study shows a large decrease in insulin receptor autophosphorylation in liver, whereas IRS-1 protein and phosphorylation appear to be normal. A divergence between tyrosine phosphorylation of insulin receptor vs. IRS-1 has also been observed previously in dexamethasone-treated rats, streptozotocin diabetic rats, and the ob/ob mouse (15, 36). In the present study, this divergence in SHROB liver relative to skeletal muscle may be due to an increase in IRS-1 protein relative to insulin receptor protein, resulting in an increase in the efficiency of coupling between the insulin receptor and its substrate. The importance of these different sites of insulin resistance to the etiology of cardiovascular diseases has not been studied. Insulin resistance, hyperinsulinemia, or hypertriglyceridemia might play a role in regulating blood pressure (34). Despite severe hyperinsulinemia, insulin resistance to glucose transport, and hypertriglyceridemia, the level of hypertension in SHROB is similar to or even lower than in lean SHR littersmates, suggesting that factors other than obesity are responsible for the hypertension in SHROB. In the SHR/Nob rat, a rat model with obesity and diabetes, but which no longer expresses hypertensive traits, there is a severely reduced level of GLUT-4 expression in skeletal muscle (29). A modest decrease in GLUT-4 expression, together with reduced insulin stimulation of postreceptor signaling in SHROB, could account for the insulin resistance to glucose transport. However, it is possible that changes at the molecular level leading to states of insulin resistance are different in the case of obesity, hypertension, and NIDDM. The association between insulin sensitivity and skeletal muscle blood flow in SHR (32) suggests that glucose and insulin delivery could contribute to insulin resistance in vivo. The present findings suggest that the obesity mutation fa results in decreased expression of insulin receptor, IRS-1, p85α, GLUT-4, and hyperinsulinemia in the SHROB rat. These defects contribute to impaired glucose tolerance and insulin resistance in this animal model of Syndrome X, but these metabolic abnormalities appear to be distinct from those involved in provoking hypertension.

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