

Autophosphorylation and kinase activity of insulin receptor in diabetic rats

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OKAMOTO, MOTOZUMI, MORRIS F. WHITE, RUTH MARON, AND C. RONALD KAHN. *Autophosphorylation and kinase activity of insulin receptor in diabetic rats*. *Am. J. Physiol.* 251 (Endocrinol. Metab. 14): E542-E550, 1986.—Insulin resistance is observed in insulin-deficient diabetic states in spite of an increase in insulin binding to its target cells. To characterize this type of insulin resistance, autophosphorylation and kinase activity of the insulin receptor on liver was studied with streptozotocin (STZ)-induced and BB diabetic rats. Insulin binding capacity was increased in proportion to the severity of the diabetic state in the STZ rat. In the diabetic BB rat, the insulin binding capacity was also increased, and this was partially normalized by insulin treatment. By contrast, insulin-stimulated autophosphorylation of the β -subunit of the insulin receptor was decreased in proportion to the severity of the diabetic state in the STZ rat. Peptide mapping by reverse-phase high-performance liquid chromatography revealed a decrease in labeling at all sites of autophosphorylation. Kinase activity of the insulin receptor to exogenous substrates was also decreased in proportion to the diabetic state. In the BB rat, autophosphorylation and kinase activity of the insulin receptor were both decreased in the diabetic state and partially normalized by insulin treatment. In addition to the β -subunit of insulin receptor, a 170 kdalton phosphotyrosine-containing protein was also identified in the glycoprotein fraction of liver. Although the phosphorylation of this protein was not insulin dependent, it was decreased markedly in the diabetic state. This protein is immunologically distinct from the insulin receptor, but is rich in phosphotyrosine. Based on its size and phosphotyrosine content, this protein may be the epidermal growth factor receptor. These results suggest that in insulin-dependent diabetic states there is decreased autophosphorylation and kinase activity of insulin receptor from rat liver as well as changes in other phosphotyrosine-containing proteins. These changes may be important in the altered metabolic state that is observed.

tyrosine kinase; hypoinsulinemia; liver

THE REGULATORY ACTION of insulin on cellular metabolism and growth is initiated by the binding of the hormone to receptors on the surface of cells (15). Over the past few years, it has become clear that the insulin receptor consists of two subunits (21). The α -subunit ($M_r = 135,000$) is an extracellularly oriented protein that contains the insulin binding site and is labeled upon covalent attachment of ^{125}I -insulin to the receptor. The β -subunit ($M_r = 95,000$), on the other hand, is a transmembrane protein (40) that undergoes an autophospho-

rylation reaction at tyrosine residues upon insulin stimulation (3, 17, 19, 23, 33, 34). This autophosphorylation is catalyzed by a protein kinase activity present in the receptor itself (19, 33, 35, 38, 41). Although the significance of receptor phosphorylation is uncertain, it appears highly likely that this is involved in signal transmission by the receptor, perhaps culminating in a cascade of phosphorylation/dephosphorylation reactions (7, 26, 36). To test this hypothesis, it is helpful to study receptor phosphorylation in states of altered insulin action.

Streptozotocin (STZ)-induced diabetes in rats is characterized by insulin deficiency. In this rat, impaired insulin-mediated glucose utilization is observed (16, 18, 25, 28) in spite of an increase in insulin binding to its target cells (6, 18, 25, 31). To understand the mechanisms of this paradox, three groups have studied the autophosphorylation of the insulin receptor from the liver of STZ-induced diabetic rat (1, 4, 14), however, there has been considerable disagreement in the results.

In the present study, we have explored this question by studying autophosphorylation and kinase activity of insulin receptor using two different animal models of insulin deficiency: STZ diabetic rats in which insulin deficiency is the result of chemical destruction of pancreatic β -cells and the BB rat in which insulin-deficient diabetes occurs due to an autoimmune destruction of islet cells. In addition, tryptic peptide mapping was performed to further characterize the decrease in autophosphorylation. During these studies, we have not only defined a defect in receptor kinase activity and autophosphorylation in insulin-deficient diabetes but have also identified a protein of $M_r = 170,000$, which is phosphorylated on tyrosine and which is decreased in diabetic states.

MATERIALS AND METHODS

Materials. [^{125}I]monoiodo-A 14 -insulin was purchased from Amersham. ^{32}P -ATP and Triton X-100 were from New England Nuclear; wheat germ agglutinin agarose was from Vector Laboratories (Burlingame, CA); reagents for NaDodSO $_4$ /polyacrylamide gel electrophoresis and the Bradford protein assay were purchased from Bio-Rad Laboratories (Richmond, CA); STZ, HEPES, phenylmethylsulfonyl fluoride (PMSF), aprotinin, *N*-acetyl-D-glucosamine, polyethylene glycol (PEG 7000), bovine serum albumin, and bovine gamma globulin were from Sigma Chemical, St. Louis, MO); porcine insulin

was from Elanco (Indianapolis, IN); and protein A was from Calbiochem (La Jolla, CA).

Animals. Mild and severe STZ-induced diabetic rats were produced using male Sprague-Dawley rats weighing ~200 g by injecting 50 (mild) or 60 (severe) mg/kg of STZ dissolved in citrate buffer (pH 4.5) via the tail vein of rats fasted overnight. The rats were used for experiments 5 wk after injection. BB rats were initially provided by Dr. Pierre Thibert (Dept. of National Health and Welfare, Ottawa, Canada) and bred from this stock in the animal facility at the Joslin Diabetes Center. Diabetes was diagnosed on the basis of glucosuria using TES-TAPE (Eli Lilly Indianapolis, IN) and a plasma glucose in excess of 250 mg/dl. Diabetic BB rats were divided into two groups. One was untreated, and the other was insulin treated (3 wk, NPH insulin). The dose of insulin was determined on the basis of daily urine test for glucose at 5:00–7:00 P.M. The primary dose was 5 U. For those animals that had positive urine glucose the dose was increased by 0.5 U and if negative the dose was decreased by 1 U. Nondiabetic BB rats were also studied as the control. All animals are fed ad libitum until the experiment.

Preparation of solubilized insulin receptor. After withdrawing the blood from the inferior vena cava and portal vein to measure plasma glucose and insulin concentration, the liver was washed with ice-cold saline, removed, weighed, and minced with scissors. Ten volumes of 50 mM HEPES (pH 7.4), 0.25 M sucrose supplemented with aprotinin (1 mg/dl), and 2 mM PMSF were added to 4 g of liver, and this was homogenized with a loose Dounce homogenizer. The homogenate was centrifuged (1,200 rpm), and Triton X-100 was added to the supernatant to give the final concentration of 1%. The resulting supernate was slowly stirred at 4°C for 60 min, centrifuged (4°C 100,000 g for 60 min), and was applied to 1 ml of agarose-bound wheat germ agglutinin column. The column was extensively washed with 50 mM HEPES buffer (pH 7.4) containing 0.1% Triton X-100, and the receptor was eluted with this buffer supplemented with 0.3 M *N*-acetylglucosamine and fractionated into 0.5-ml aliquots.

¹²⁵I-insulin binding to solubilized insulin receptor. Aliquots (2 µg protein) of wheat germ agglutinin eluate were incubated with ¹²⁵I-insulin (0.1 ng/ml), specific activity of 2,000 Ci/mmol, and various concentrations of unlabeled insulin at 15°C for 4 h in a medium of 150 mM NaCl and 50 mM HEPES at pH 7.4 containing 0.1% bovine serum albumin. Separation of free and receptor-bound insulin was then performed by the polyethylene glycol method (8) using bovine gamma globulin as carrier protein.

Phosphorylation with solubilized receptors. Phosphorylation was studied with slight modification of the method of Kasuga et al. (22). Solubilized, lectin-purified insulin receptor (200 fmol binding capacity and 3 µg protein for the control group) was diluted to 50 µl, having a final concentration of 50 mM HEPES (pH 7.4), 5 mM Mn²⁺, 0.1% Triton X-100, and with or without insulin (1,000 ng/ml). These mixtures were incubated at 22°C for 30 min. The phosphorylation reaction was initiated

by adding γ-[³²P]ATP and terminated by adding fivefold concentrated Laemmli sample buffer. The mixture was heated immediately in boiling water for 3 min. The proteins were separated in 7.5% polyacrylamide gel electrophoresis according to Laemmli (27). The gels were stained with Coomassie blue in 50% trichloroacetic acid, destained in 7% acetic acid, dried, and autoradiographed with Kodak X-Omat film. Molecular weights of proteins were calculated by using standard proteins (Bio-Rad). The incorporation of ³²P into corresponding bands was quantitated by scanning densitometry of the film or by counting the corresponding bands of the gel in a scintillation counter.

Phosphorylation of histone. Phosphorylation was performed with the same conditions described above except for the following condition. Histone H2B (0.1 mg/ml in the final concentration) was added to the mixture 10 min before the initiation of phosphorylation reaction. The proteins were separated in 15% polyacrylamide gel electrophoresis.

Tryptic peptide mapping. Tryptic phosphopeptides were obtained from the protein bands in polyacrylamide gel fragments as previously described (22, 42). After the position of the phosphorylated proteins separated by polyacrylamide gel electrophoresis were determined by autoradiography, the corresponding bands were excised, washed for 12 h at 37°C in 20% methanol, dried at 80°C for 2 h, and dissolved with 2 ml of 50 mM NH₄HCO₃ containing 50 µg/ml of trypsin, pH 8. After 6 h incubation at 37°C, another 50 µg/ml of trypsin was added and the enzymatic digestion was continued for 16 h. The supernatant was lyophilized, and the phosphopeptides were separated with a Waters high-performance liquid chromatography system equipped with a µBondapak C-18 reverse-phase column (Waters Associates, Milford, MA). Phosphopeptides applied to the column were eluted at a flow rate of 1 ml/min with a mobile phase composed of water with 0.05% trifluoroacetic acid and a nonlinear, concave upward gradient (curve type 7) of acetonitrile increasing between 0 and 40% during 85 min. Fractions were collected in 1 ml, and the radioactivity in each tube was measured as Cherenkov radiation using an LKB scintillation counter.

Phospho-amino acid analysis. Phospho-amino acids were identified by a modified version of the method of Hunter and Sefton (11, 13). Each peak of fractions after tryptic peptide mapping was lyophilized and dissolved in 100 µl of 6 N HCl (Pierce Chemical, Rockford, IL) and hydrolyzed for 2 h at 110°C. The phospho-amino acids were separated by high-voltage electrophoresis on thin-layer plates (Avidil, Analtech, Newark, DE; 250 µm) using a solution of H₂O:acetic acid:pyridine (89:10:1). Phosphoserine, phosphothreonine, and phosphotyrosine (Sigma Chemical) were added to all samples immediately before electrophoresis. These standards were identified by reaction with ninhydrin, and the radioactivity was located by autoradiography.

Others. Glucose was measured with glucose analyzer 2 (Beckman, Fullerton, CA). Plasma immunoreactive insulin concentration was determined by polyethylene gly-

col method using rat insulin as a standard (8). Statistical analyses were performed by Student's *t* test.

RESULTS

Physiology of diabetic animal. Data concerning body weight, weight gain, liver weight, blood glucose, and plasma insulin concentration in STZ and BB diabetic rats are summarized in Tables 1 and 2. In the STZ rats, the body weight, liver weight, and body weight gain were decreased in proportion to the state of diabetes. Blood glucose levels were markedly increased, while the plasma insulin concentration was decreased in both the portal vein and inferior vena cava (IVC). Similar changes were observed in body weight, blood glucose, and liver weight when nontreated diabetic BB rats were compared with nondiabetic BB rats. Serum insulin concentrations in diabetic BB rats were distributed over a wide range (<0.1–0.7 ng/ml in IVC and 0.1–2.4 ng/ml in portal vein). The BB rats whose insulin concentration in portal vein was <0.5 ng/ml were selected to represent the noninsulin-treated diabetic BB rat. The high plasma insulin concentration in insulin-treated BB rat was due to the insulin given on the day before the experiment.

Insulin binding. Insulin binding to insulin receptors solubilized and partially purified from the liver membranes was increased in STZ-treated rat in proportion to the state of diabetes (Fig. 1A). Specific insulin binding at a tracer concentration of insulin (0.1 ng/ml) was $6.4 \pm 1.1\%$ /2 μ g protein of wheat germ agglutinin purified fraction for control rats and was increased to 8.2 ± 1.0 and $13.8 \pm 1.4\%$ for mild and severe STZ-diabetic rats, respectively. A proportional increase in binding in STZ diabetes was observed over the entire range of insulin concentrations, and there was no change in the concentration of insulin producing 50% inhibition of binding,

TABLE 1. Characteristics of STZ-induced diabetic rats

	Control Rats	Mild Diabetic Rats	Severe Diabetic Rats
Body weight, g	356±8	313±15*	228±18†
Body weight gain, g/day	4.1±0.2	2.9±0.1†	1.0±0.4†
Blood glucose, mg/dl	128±9	292±18†	391±33†
Plasma insulin, ng/ml			
Portal vein	9.3±2.1	0.8±0.2†	0.5±0.2†
IVC	2.7±0.9	0.5±0.1	0.2±0.1*
Liver weight, g	14.5±0.7	11.8±0.6*	7.2±1.1†

Values are means ± SE. STZ, streptozotocin; IVC, inferior vena cava. * *P* < 0.05; † *P* < 0.01.

TABLE 2. Characteristics of BB rats

	Nondiabetic	Insulin-Treated Diabetic	Nontreated Diabetic
Body weight, g	302±9	288±10	265±12*
Blood glucose, mg/dl	138±8	142±16	397±58†
Plasma insulin ng/ml			
Portal vein	6.8±1.0	5.3±0.4	1.3±0.4†
IVC	2.1±0.8	7.5±2.5	0.4±0.1
Liver weight, g	11.9±0.8	10.8±0.7	9.6±1.0

Values are means ± SE. IVC, inferior vena cava. * *P* < 0.05; † *P* < 0.01.

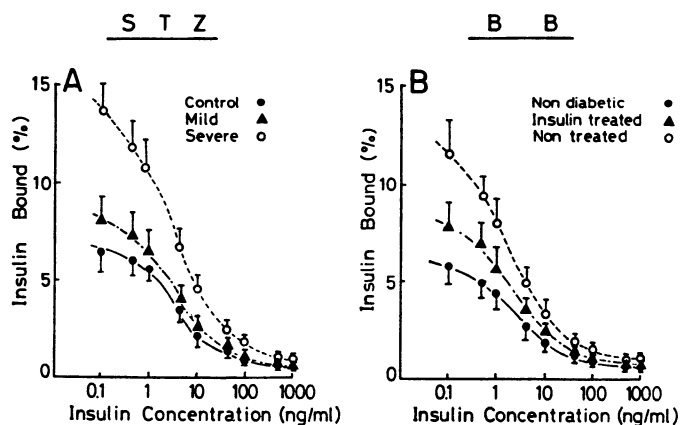


FIG. 1. A: insulin binding to solubilized and wheat germ agglutinin purified receptor from liver of streptozotocin (STZ)-induced diabetic rat. Two μ g of wheat germ agglutinin eluate in 200 μ l of buffer were incubated with 125 I-insulin (0.1 ng/ml) and various concentrations of unlabeled insulin (from 0 to 1,000 ng/ml) for 4 h at 15°C. Hormone-receptor complexes were precipitated with PEG (12.5% final concentration) using bovine γ -globulin as carrier protein (0.1% final concentration). Nonspecific binding obtained in presence of 100 μ g/ml of unlabeled insulin was subtracted from total binding. Data for control rat (●), mild diabetic rat (▲), and severe diabetic rat (○) represent means ± SE. B: insulin binding to solubilized and wheat germ agglutinin purified receptor from liver of BB rat. Insulin binding was measured as described in MATERIALS AND METHODS. Data for nondiabetic BB rat (●), insulin-treated diabetic BB rat (▲), and noninsulin-treated diabetic BB rat (○) represent means ± SE.

suggesting that the increase in binding was due to an increase in receptor concentration. A similar change was observed in diabetic BB rats compared with nondiabetic animals (Fig. 1B). This increase in insulin binding in BB rats was partly reversed by insulin treatment. Thus insulin-treated diabetic BB rats exhibited insulin binding that was higher than that in nondiabetic rats and lower than that in nontreated diabetic rats. Scatchard analysis revealed that the increase in insulin binding to solubilized insulin receptors in both STZ and BB rats was due primarily to an increase in receptor concentrations (not shown).

Effect of STZ diabetes on receptor and kinase activity. When the eluate from wheat germ agglutinin affinity column from the liver were incubated with γ -[32 P]ATP in the presence of 5 mM $MnCl_2$ and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography, two major [32 P]labeled proteins were detected (Fig. 2A, left 2 lanes). These have $M_r = 95,000$ and 170,000. Insulin stimulated the ^{32}P incorporation into the 95 kdalton protein but not into 170 kdalton protein. Only 95 kdalton protein was immunoprecipitated with anti-insulin receptor antibody (B-9) (22), and both proteins were immunoprecipitated with antiphosphotyrosine antibody (32) (data not shown).

These findings suggest that 95 kdalton protein is the β -subunit of the insulin receptor which is known to undergo insulin-stimulated tyrosine phosphorylation in vitro (19), whereas the 170 kdalton protein is another phosphotyrosine containing protein that is immunologically distinct from the insulin receptor.

An autoradiogram showing in vitro phosphorylation of wheat germ agglutinin purified extracts derived from the livers of STZ diabetic and control rats is shown in Fig.

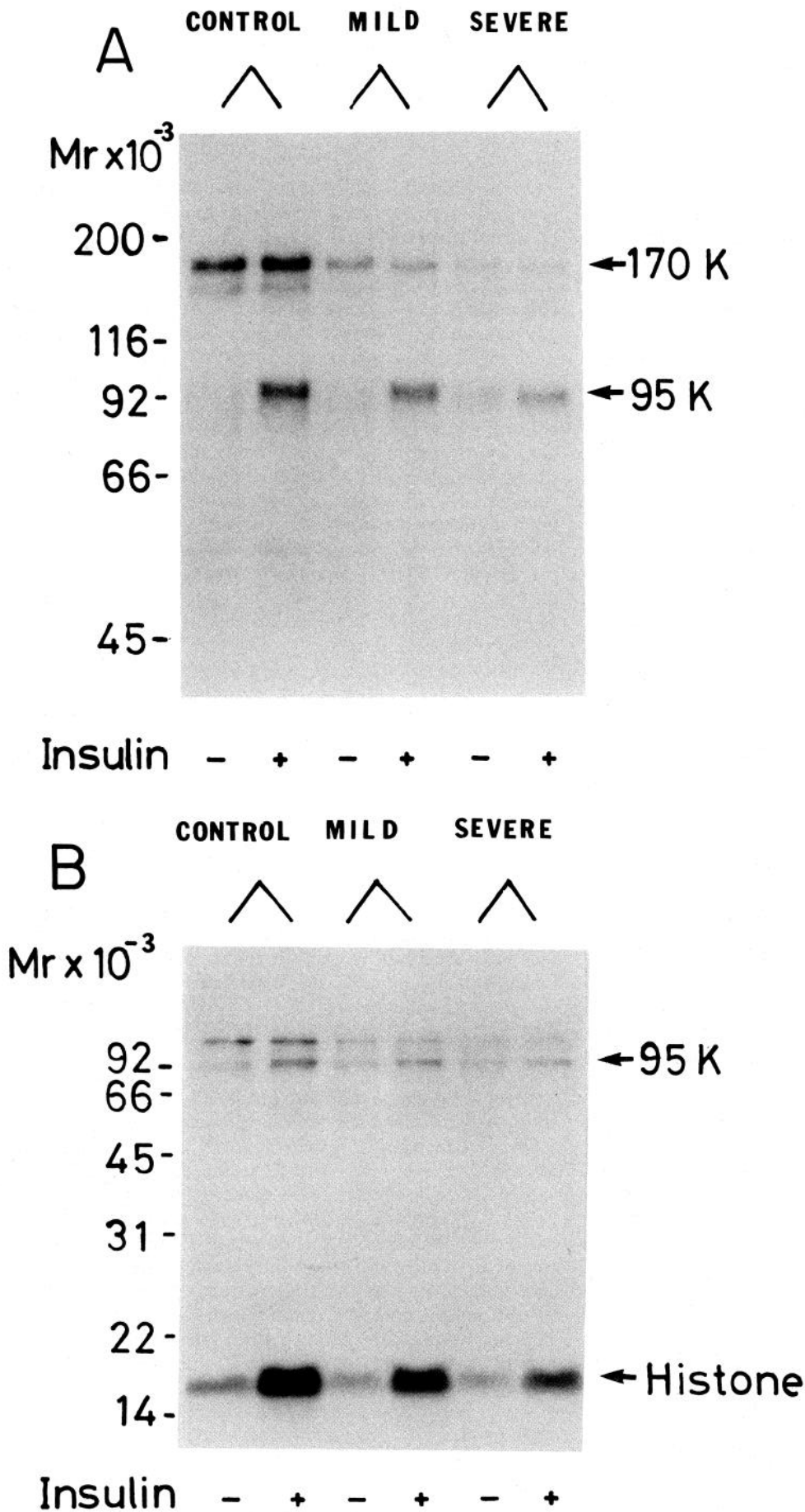


FIG. 2. A: autoradiogram showing phosphorylation of 95 kdalton β -subunit of insulin receptor and 170 kdalton protein from liver of streptozotocin-induced diabetic rat (50 mg/kg for mild diabetic rat and 60 mg/kg for severe diabetic rat). Aliquots of wheat germ agglutinin eluate in 50 μ l of buffer was incubated with (1 μ g/ml) or without insulin for 30 min at 22°C. Phosphorylation reaction was initiated by adding 20 μ M ATP and 20 μ Ci/tube of γ -[³²P]ATP. After stopping reaction, mixture was reduced with dithiothreitol, separated by SDS-PAGE and subjected to autoradiography. In each pair, *left lane* is without insulin and *right lane* is insulin stimulated. B: autoradiogram showing kinase activity of insulin receptor from liver of streptozotocin-induced diabetic rat. Histone H2B (5 μ g in 50 μ l) was used as a substrate, and phosphorylation was performed as described in MATERIALS AND METHODS. In each pair *left lane* is without insulin and *right lane* is insulin stimulated (1 μ g/ml).

2A. In this experiment, the amount of extract used was adjusted such that equal amounts of insulin receptor as determined by Scatchard analysis (200 fmol of the insulin binding capacity) were present. For each group, both basal and insulin-stimulated incorporation of ^{32}P from $\gamma\text{-}[^{32}\text{P}]\text{ATP}$ into 95 kdalton subunit of the insulin receptor and into the 170 kdalton protein were examined. Insulin stimulated phosphorylation of the β -subunit of the solubilized receptor was decreased in the preparation from STZ rats in proportion to the state of diabetes. Likewise, there was a decrease in the tyrosine phosphorylation of the 170 kdalton protein in proportion to the severity of diabetes, although this phosphorylation was not insulin stimulated.

To estimate the kinase activity of the insulin receptor on exogenous protein substrates, in a parallel experiment histone H2B was incubated with insulin receptor for 10 min in the presence of Mn^{2+} followed by the addition of $\gamma\text{-}[^{32}\text{P}]\text{ATP}$ (Fig. 2B). Histone H2B acts as a substrate and is tyrosine phosphorylated in an insulin-dependent manner in this incubation (20). Histone H2B also partially inhibited the phosphorylation of β -subunit of insulin receptor kinase under these conditions. Like receptor autophosphorylation, insulin-stimulated phosphorylation of histone H2B was decreased in proportion to the severity of diabetes in the STZ-treated rats. When these autoradiograms were subjected to densitometry scanning to quantitate the changes in autophosphorylation of the β -subunit of insulin receptor, 170 kdalton protein, and kinase activity of β -subunit of insulin receptor (Fig. 3), all three decreased in parallel, although the change in the 170 kdalton protein was slightly greater. It is also important to note there was a 40–60% decrease in all of

these parameters even in the mild STZ diabetic rat in which insulin binding was only minimally increased.

Autophosphorylation and kinase activity of insulin receptor from BB rat. Similar experiments were performed with insulin-treated and nontreated diabetic BB rats and nondiabetic BB rats (Fig. 4, A and B). The result of densitometry scanning is also shown in Fig. 3. A mild decrease in both insulin-stimulated β -subunit autophosphorylation and kinase activity of insulin receptor were observed in nontreated diabetic BB rats compared with nondiabetic BB rats. Insulin treatment partially normalized these abnormalities. Again, when compared with the nondiabetic control rats, the decrease in ^{32}P incorporation into 170 kdalton protein was most pronounced.

Tryptic peptide mapping and phospho-amino acid analysis. To examine the mechanism underlying the decrease in phosphorylation of the 170 kdalton protein and the β -subunit of the insulin receptor and the decrease in kinase activity of insulin receptor, tryptic peptide mapping and phospho-amino acid analysis of the β -subunit were performed (Fig. 5). By using reverse-phase high-performance liquid chromatography, at least six phosphorylated peptides were separated from insulin-stimulated 95 kdalton protein. The major peaks between 40 and 55 min elution time correspond with the major peaks previously reported (43). On phospho-amino acid analysis, most peptides contained only phosphotyrosine (labeled with a Y), although some of the late eluting peaks also contained phosphoserine and phosphothreonine (labeled S and T, respectively). Each peak was reduced in receptors from STZ rats when compared with control rats, and no change in the pattern was observed.

The peptide maps of the insulin receptor of liver from control rats (Sprague-Dawley) and nondiabetic BB rats (derived from Wistar strain) were similar (data not shown). Diabetic BB rats, like the STZ diabetic rat, showed a decrease in all sites of autophosphorylation.

There was no similarity in the pattern of tryptic peptide mapping and phospho-amino acid analysis between 170 and 95 kdalton protein, confirming that the 170 kdalton protein is unlikely to be a form of insulin receptor (data not shown).

DISCUSSION

Insulin-deficient diabetes produced by alloxan or STZ in rodents has been shown to be associated with impaired glucose utilization in muscle (10, 30), adipose tissue (18, 24, 37), and liver (12), despite an increased insulin binding to its receptor on these tissues (6, 18, 24, 28, 37). Similar findings have been reported using the BB diabetic rat, which is another insulin-deficient diabetic model in which no potentially toxic drugs have been given (2). This paradox suggests that some steps in the insulin action pathway distal to insulin binding are defective. Some attempts have been made to elucidate the nature of this defect. By using STZ-induced diabetic rat, Karnieli et al. (16) have shown a decrease in the number of glucose transporters in the intracellular pool. Kadowaki et al. (14) showed the decreased autophosphorylation of the insulin receptor kinase in STZ rat, but this has not been confirmed in other studies (1, 4). Further-

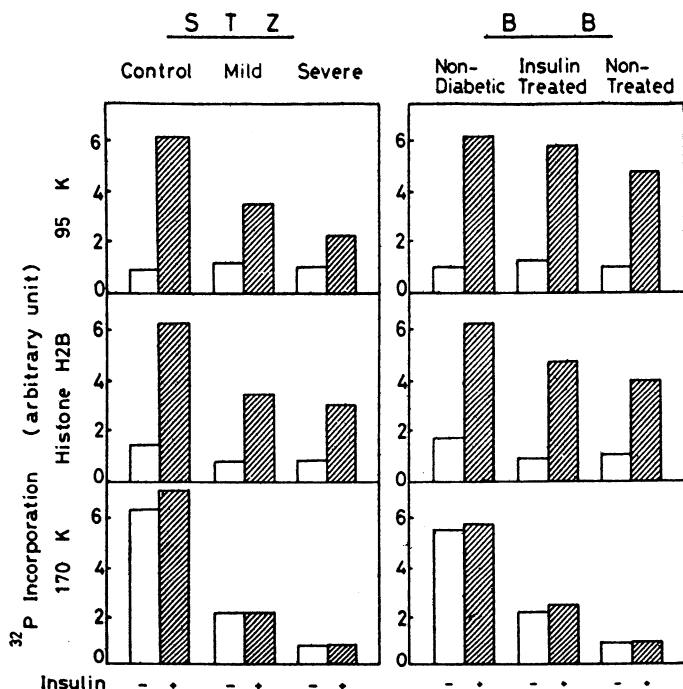


FIG. 3. ^{32}P incorporation into the 95 k protein, histone H2B, and 170 k protein from streptozotocin-induced (STZ) rat and BB rat. These data were derived from Figs. 2, A and B, and 4, A and B by densitometry scanning and represented by arbitrary unit. In each pair, open column is without insulin and shaded column is insulin stimulated (1 $\mu\text{g}/\text{ml}$).

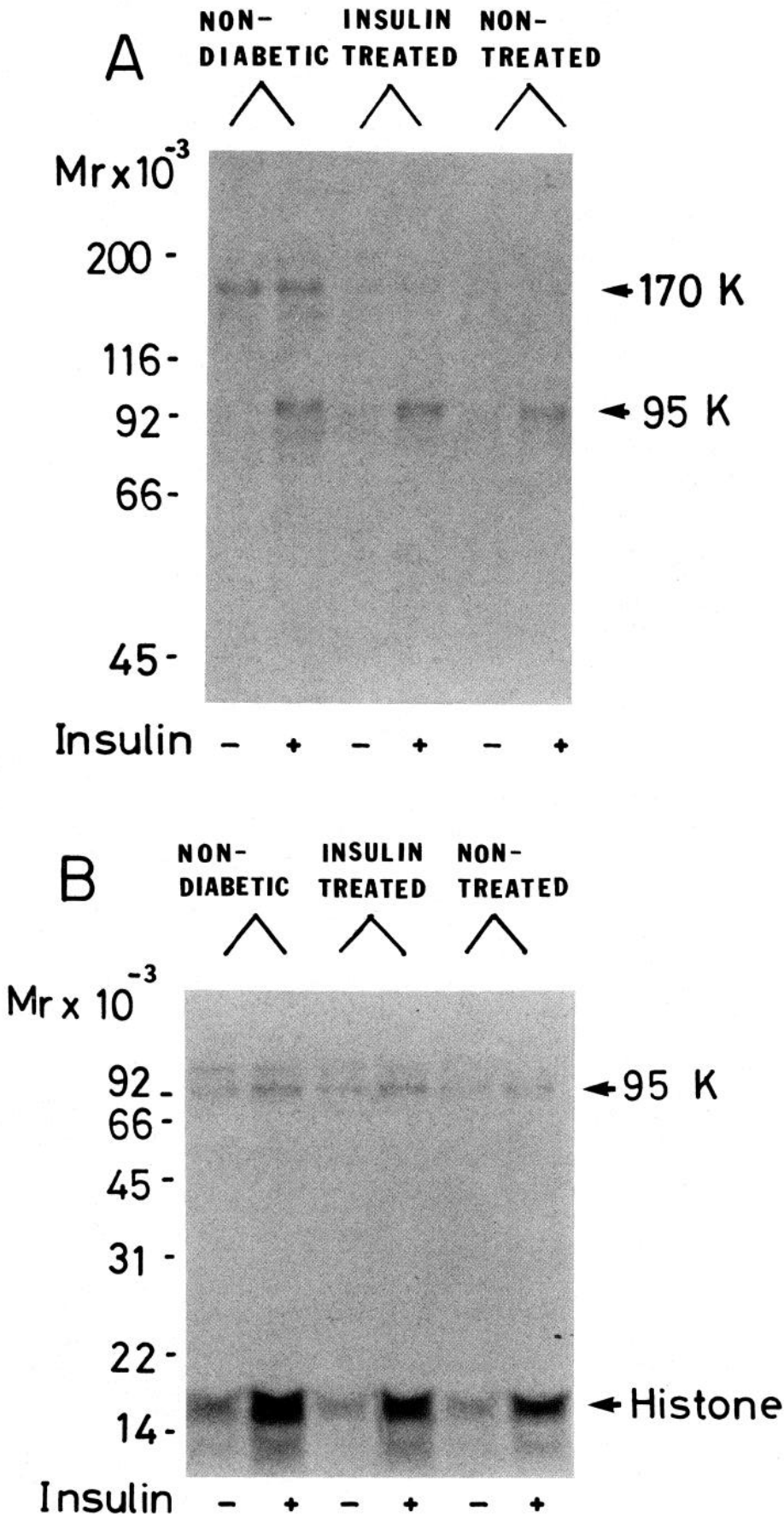


FIG. 4. A: autoradiogram showing phosphorylation of 95 kdalton β -subunit of insulin receptor and 170 kdalton protein from liver of nondiabetic BB rat, insulin-treated diabetic BB rat, and nontreated diabetic BB rat. Others are same as Fig. 2A. B: autoradiogram showing kinase activity of insulin receptor from liver of nondiabetic BB rat, insulin-treated diabetic BB rat, and nontreated diabetic BB rat. Others are same as Fig. 2B.

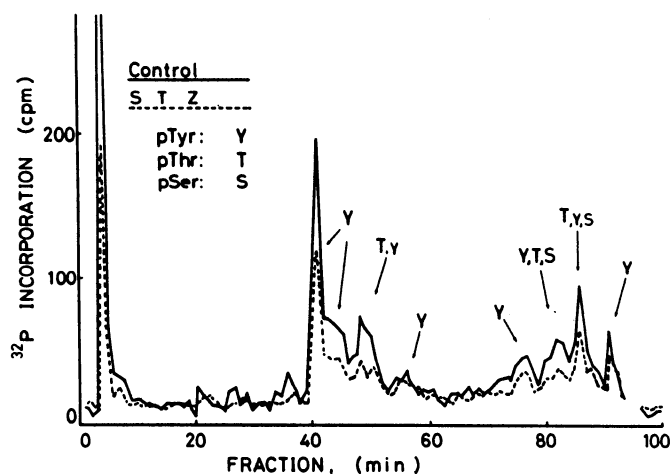


FIG. 5. Tryptic peptide mapping and phospho-amino acid analysis of 95 k β -subunit of insulin receptor from control rat (—) and STZ rat (---). Y, phosphotyrosine; T, phosphothreonine; S, phosphoserine. Wheat germ agglutinin agarose eluate (400 fmol insulin binding capacity) was incubated with insulin (1 μ g/ml) and Mn^{2+} (5 mM) for 30 min, and then γ - ^{32}P ATP (100 μ Ci/point) and ATP (20 μ M) were added for 10 min. After stopping reaction with Laemmli buffer, sample was reduced with dithiothreitol and separated by SDS-PAGE. β -Subunit of insulin receptor was identified by autoradiography, excised from the gel, and digested with trypsin, and phosphopeptides were separated by reverse-phase high-performance liquid chromatography. Data represent Cerenkov radiation measured in each fraction. Phosphopeptides in indicated fraction were partially hydrolyzed with 6 N HCl. ^{32}P -phosphoamino acids were separated by high-voltage electrophoresis and identified by the migration of known standards. Small characters represent relatively small amounts than others in the same peak.

more, since the latter studies were done only in STZ-treated rats, the potential effects of this drug on liver cells can not be neglected.

In attempt to determine the mechanisms underlying the insulin resistance of insulin deficiency, in this study we have characterized in detail changes in insulin receptor autophosphorylation and kinase activity. We have used two independent models of diabetes, i.e., STZ-induced diabetes rat and BB rats, and have characterized the reactive products immunologically as well as by peptide mapping.

Our data show that there is a decrease in insulin receptor autophosphorylation in STZ diabetic rats. Kinase activity of insulin receptor to exogenous substrates was also decreased in the diabetic rats. These results in STZ rats are comparable with those by Kadowaki et al. (14). Furthermore, in diabetic BB rats in whom insulin treatment was withheld, a similar decrease in autophosphorylation and kinase activity was observed. In BB rats, insulin treatment partially normalized the decrease in autophosphorylation and kinase activity of the insulin receptor. Therefore our data and that of Kadowaki et al. suggests that in insulin-deficient diabetic states, autophosphorylation, and kinase activity of insulin receptor is decreased, and this decrease may be partially normalized by insulin treatment. Furthermore, this change can be observed in animals in whom changes in insulin binding are minimal. In addition, we found that the decrease in autophosphorylation and kinase activity of insulin receptor in insulin-deficient diabetic state is associated with a decrease in phosphorylation of all *in vitro* sites, which can be detected by tryptic peptide mapping

and phospho-amino acid analysis. No specific peak was specifically suppressed in diabetic animals.

Our results, however, are in disagreement with those of Amatruda et al. (1) and Blackshear et al. (4) who found no change in receptor phosphorylation in STZ diabetes. This may be due to a number of methodological differences. In our study, the animals were insulinopenic for a longer period (5 wk) than other studies (7–21 days and 48 h, respectively). Also, the dosage of streptozotocin in our experiments (50–60 mg/kg) was lower than that used by others (85 and 150 mg/kg). Also, we used wheat germ agglutinin-agarose purified insulin receptor of total liver where Amatruda (1) used a detergent extract of plasma membranes and Blackshear (4) used a crude detergent extract of microsomes. In severe diabetic rats, care was also taken to use similar amounts of receptor, since there is a major increase in receptor concentration that would obscure changes if the same total protein were used (25, 31). Recently, decreased insulin receptor kinase activity has been observed using different insulin-resistant models (9, 29). Insulin receptors from skeletal muscles of obese mice have been shown to have an altered kinase activity for phosphorylation of both the receptor itself and exogenous substrate (29). The alteration of insulin receptor kinase induced by fasting and carbohydrate feeding shows substrate specificity (9). These data, along with ours, suggest that several insulin-resistant states may be due, at least in part, to changes in insulin receptor kinase activity.

The exact mechanism of decreased autophosphorylation and kinase activity of insulin receptor in insulin-deficient diabetic rat is unclear. Several factors should be considered as possible reasons for this change. Alterations in the metabolic state could result in changes in the maturation or glycosylation of insulin receptor; however, these were not apparent in the sodium dodecyl sulfate gels of the β -subunit after phosphorylation, and there was no change in recovery on lectin affinity chromatography. Conformational changes in insulin receptor or small molecules that affect the kinase activity of the insulin receptor might also exist in diabetic animals. Also, changes in degradation or internalization of insulin receptor in insulin-deficient rats may result in secondary changes in the receptors that remain longer on the cell surface than those in the control rat. One of the most attractive hypotheses is that the difference in endogenous phosphorylation state of the insulin receptor in the insulin-deficient rat and the control rat produces changes in kinase activity. In hepatoma cells in culture, factors that alter the serine phosphorylation of the receptor alter its tyrosine kinase activity (39). This serine phosphorylation occurs only in the intact cell and is believed to be the result of activation of C-kinase (32, 43). Studies of C-kinase activity and receptor serine phosphorylation in diabetic animals may help clarify this finding.

In these experiments, a ^{32}P -labeled protein with a $M_r = 170,000$ was also observed in the glycoprotein fraction of cells, which was decreased in diabetic animals. Interestingly, the decrease in ^{32}P incorporation into this protein was even more prominent than that of the β -subunit or exogenous substrate, and this decrease was observed

even when the same amount of protein was subjected for phosphorylation study (data not shown). The phosphorylation of this phosphotyrosine-containing protein was not stimulated by insulin. Furthermore, it was immunologically different from insulin receptor and had a distinctly different tryptic peptide map, suggesting that this protein is unlikely to be the precursor or some other form of insulin receptor. This protein has the same migration on gels as the receptor for epidermal growth factor, and results using anti-epidermal factor receptor antibodies suggest that this is the case (unpublished observation). Further studies will be necessary to determine the nature of this modification and its relationship to the alteration in the insulin receptor kinase.

We have hypothesized that insulin-stimulated autophosphorylation of β -subunit plays an important role in transmembrane signaling through the insulin receptor. Therefore it is reasonable to suppose that the decrease in insulin-stimulated autophosphorylation can play a role, at least in part, for the insulin resistance observed in the insulin-deficient diabetic state. Although insulin binding capacity per microgram protein was almost double in the diabetic rats, the liver weight was reduced to 50–60% of normal, suggesting that total receptor concentration per liver changes very little. Thus, the decrease in receptor kinase activity per receptor indicates a decrease in overall insulin-stimulated autophosphorylation and tyrosine-specific protein kinase activity of insulin receptor. Also, it is important to note that decreased kinase activity occurs even in mildly diabetic animals in whom insulin binding is only minimally increased. Thus the decreased autophosphorylation and kinase activity of insulin receptor, as well as change in other phosphotyrosine-containing proteins from liver of diabetic rats, suggest that these changes could be important in the altered metabolic state that is observed.

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