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NORMAL HEPATIC INSULIN RECEPTOR AUTOPHOSPHORYLATION IN NONKETOTIC DIABETES MELLITUS

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ABSTRACT. Insulin receptor autophosphorylation is the earliest recognizable event in insulin action subsequent to insulin binding. To determine if the postbinding hepatic insulin resistance of nonketotic diabetes mellitus could reside in an inability of insulin to stimulate insulin receptor autophosphorylation, we evaluated the ability of insulin to stimulate ^{32}P incorporation into the beta subunit of lectin-purified rat liver plasma membrane insulin receptors. The data indicate that both the absolute plasma membrane insulin receptor autophosphorylation in response to insulin as well as the insulin dose-response relationship for autophosphorylation are normal in diabetic animals when expressed per μg of protein or per unit of binding activity. The previous data from our laboratory indicates that hepatic insulin resistance in non-ketotic streptozotocin-induced diabetes mellitus is present despite normal to increased insulin binding, is selective, is reversible with insulin treatment and involves an inability of insulin to stimulate the release of the putative mediator of insulin action. We conclude, therefore, that the hepatic insulin resistance of nonketotic diabetes mellitus resides distal to insulin receptor binding and autophosphorylation and is reflected in metabolic events at or near the plasma membrane which may include the generation or release of the putative mediator of insulin action.

INTRODUCTION. Hepatic insulin resistance is a common feature of Type II diabetes mellitus in animals (1-3) and humans (4). Over the past few years, we have defined and evaluated this insulin resistance in an animal model of Type II diabetes mellitus, the non-ketotic, streptozotocin diabetic rat. These studies have indicated that insulin binding is normal to increased depending on the preparation used i.e., freshly isolated hepatocytes (5), purified plasma membranes (6), primary cultures of hepatocytes (2) and solubilized plasma membranes (6). Despite this normal to increased insulin binding, the liver is profoundly resistant to insulin with regard to lipogenesis (1,2), glycogenesis (unpublished observation), and the ability of insulin to stimulate the release of the putative mediator of insulin action (3). In contrast, however, the liver is only relatively resistant to the ability of insulin to stimulate aminoisobutyric acid uptake, i.e. the percent response due to insulin is decreased while the absolute response is normal (5). Both basal and insulin

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responsive hepatic lipogenesis (1) as well as the ability of insulin to stimulate the putative mediator of insulin action (3) are restored to normal following the treatment of animals with insulin. These previous studies support the concept that the first identifiable defect in pathways of insulin action in the liver of nonketotic diabetic animals may lie distal to insulin receptor binding but proximal to or at the level of the generation of the putative mediator of insulin action. Since insulin induced stimulation of insulin receptor kinase activity may be the first identifiable postbinding step in insulin action (7), we have evaluated the ability of insulin to stimulate receptor autophosphorylation in a partially purified hepatic plasma membrane insulin receptor preparation from control and streptozotocin diabetic rats.

MATERIALS AND METHODS

MATERIALS. [^{32}P] ATP (specific activity, 5000-6000 Ci/mol) was obtained from Amersham, (Arlington Heights, California) and wheat germ agglutinin coupled to agarose was obtained from Miles Laboratories (Elkart, Indiana). Phenylmethylsulfonyl flouride, (PMSF), iodoacetamide, aprotinin and standards used to calculate subunit molecular weights were obtained from Sigma Chemical Co. (St. Louis, Missouri). Pansorbin was from Calbiochemical-Behring (La Jolla, California). Carrier free [^{125}I] Na was from New England Nuclear (Boston, Massachusetts). Crystalline porcine insulin was kindly provided by Dr. Ronald Chance of Eli Lilly and Co. (Indianapolis, Indiana) and streptozotocin was a gift from Upjohn Co. (Kalamazoo, Michigan).

METHODS. Male Sprague-Dawley rats (207-277 g) fed ad libitum were used in all studies. Nonketotic diabetes was induced by femoral vein injection of streptozotocin (85 mg/kg) as previously reported (1). The morning plasma glucose levels at the time of sacrifice (7-21 days after injection) were 619 $\pm$ 27 mg/dl (control = 148 $\pm$ 3 mg/dl), and serum insulin levels were 8.8 $\pm$ 1 uU/ml (Control = 31.5 $\pm$ 3.1 uU/ml).

Isolation and preparation of the partially purified insulin receptor. The livers were removed from control and diabetic rats and homongenized at 4°C in the presence of lmM PMSF and I TIU/ml aprotinin. In some experiments 5mM iodoacetamide was also present. Plasma membranes were isolated by the two-phase polymer method of Lesko et al (8). The remaining steps were a modification of the method of Zick et al (9). Plasma membranes were solubilized 30 min at 4°C in 50mM (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), HEPES, buffer pH 7.6, containing 1% Triton X-100, and lmM PMSF. The solubilized plasma membranes were centrifuged at 100,000 xg for 45 min at 4°C. The supernatant containing the solubilized insulin receptors were recycled four times through a wheat germ agglutinin - agarose affinity column at 4°C. The column was then washed with 50mM HEPES buffer, pH 7.6, containing 0.15 M NaCl, 0.1% Triton X-100 and 1 mM PMSF. The insulin receptor was eluted with 0.3 M N-acetyl-D-glucosamine in the above buffer. The protein content of the solubilized purified receptor was determined by the Coomassie Blue method of Bradford (10) (Bio Rad Protein Assay) using bovine γ globulin as standard.

Phosphorylation and immunoprecipitation of the lectin purified insulin receptor. Phosphorylation and immunoprecipitation of the insulin receptor were performed according to a modification of the method of Zick et al (9). The receptor was suspended to a concentration of 83 ug/ml in an incubation buffer containing final concentrations of 60 mM HEPES, 90 mM NaCl, 0.06% Triton X-100, 6.3 mM Mg Cl_2, 85 mM N-acetyl-D-glucosamine and 40 ug/ml BSA, pH 7.6. The mixture was incubated for 16 h at 4°C in

the absence and presence of varying concentrations of insulin from 10^{-10} to 10^{-8}M. The phosphorylation was initiated by the addition of a solution containing 25 uM [γ-^{32}P]-ATP (~30 Ci/nmole), 10 mM Mn $(CH_3COOH)_2$, and 1 mM p-nitrophenol phosphate. After 10 min at 4°C the reaction was terminated by the addition of an ice cold solution composed of 20 mM EDTA, 5 mM p-nitrophenol phosphate, 20 mM NaF, 40 mM sodium pyrophosphate, 40 mM sodium phosphate, 0.4% Triton X-100, 40 mM ATP in 40 mM HEPES buffer, pH 7.6. The insulin receptor was immunoprecipitated with antibody A410, kindly supplied by Dr. Stephen Jacobs, or antibody B-9 kindly supplied by Dr. Ronald Kahn at a final serum dilution of 1/225. There was no difference in the data from experiments using both antibodies. After 16 h at 4°C, Pansorbin was addded and the incubation was allowed to proceed for one hour at 4°C. The immunoprecipitates were centrifuged and washed twice with 1% Triton X-100, 0.1% SDS in 50 mM HEPES, pH 7.6, followed by one wash with 0.1% Triton X-100 in 50 mM HEPES, pH 7.6. The washed precipitates were solubilized by boiling for five minutes in a solution containing 2% (w/v) SDS, 10% (w/v) glycerol, 0.05% bromophenol blue (w/v), 5% mercaptoethanol (v/v), 0.1 M dithiothereitol in 40 mM HEPES, pH 7.6.

Electrophoresis. An aliquot of the supernatants was applied to a 4% stacking gel and a 7.5% resolving gel. Electrophoresis was performed according to the method of Laemmli (11). The gels were fixed with 10% trichloroacetic acid, stained with 0.1% Coomassie Blue in 7% acetic acid and 30% methanol, and destained in 7.5% acetic acid. The dried gels were autoradiographed with Kodak X-Omat AR5 film with an intensifying screen for four days at -80°C. Quantitation of the radioactivity in the bands was obtained by excising the bands and measuring the radioactivity in a β counter, and by scanning the film in a laser ultrascan densitometer and measuring the peak area in arbitrary units. Subunit molecular weight were calculated by using as standards (Mr) myosin (205,000), β galactosidase (116,000), phosphorylase b (97,400) bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000).

Insulin binding of the partially purified insulin receptor. Insulin binding was measured with the same receptor preparations used for the phosphorylation studies by a modification of the method described above. The data is expressed as specific binding, i.e. the [^{125}I]-labeled insulin bound after the subtraction of insulin that remains bound in the presence of a large excess of unlabeled hormone. Aliquots of eluates from the wheat germ agglutinin-agarose columns (10 ug of protein) were incubated overnight at 4° C with [^{125}I]-labeled insulin at a concentration of 6 x 10^{-10}M in the presence or absence of 5 ug/ml of unlabeled insulin. Receptor bound insulin was then immunoprecipitated at 4°C with antibody A410 (final serum dilution 1/227). After 6 hours, Pansorbin was added and the incubation allowed to continue one hour at 4°C. The immunoprecipitates were centrifuged and the pellet washed twice with 50 mM HEPES pH 7.6, 1% Triton and 0.1% SDS. The precipitates were counted for radioactivity in a Packard gamma counter. [^{125}I]-labeled insulin was purified as previously reported (12).

RESULTS. Figure 1 illustrates a radioautograph of an SDS-polyacrylamide gel performed under reducing conditions from a typical experiment with partially purified receptor from a control animal and a diabetic animal. The Figure illustrates that the insulin dose-response characteristics with regard to phosphorylation of the beta subunit of the insulin receptor from the two preparations are similar. The absolute phosphorylation of the beta subunit of the hepatic plasma membrane insulin receptor in the presence of insulin is slightly greater in the preparation from the diabetic animal even though the amount of insulin binding activity in the two preparations is the same.

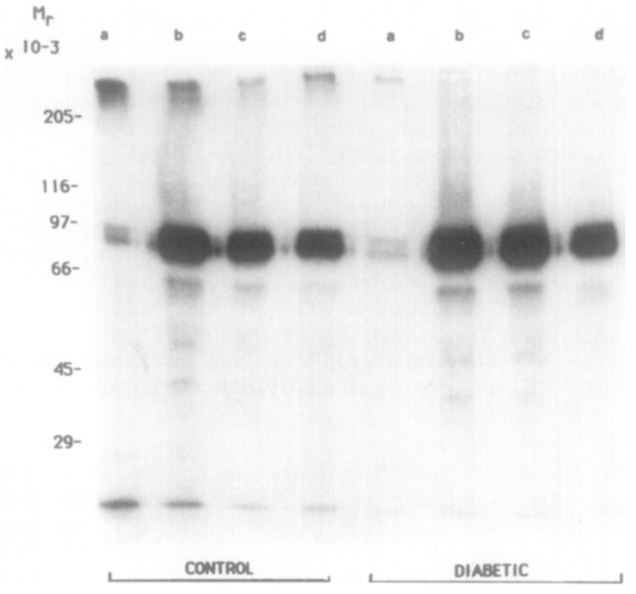

Figure 1. Autophosphorylation of the beta subunit of the hepatic plasma membrane insulin receptor from normal and nonketotic diabetic animals.

Aliquots of lectin-purified insulin receptor (50 µg protein) from control (left panel) and diabetic (right panel) animals containing equal binding activity were incubated without insulin (lanes a) and with insulin (lanes b, 10^{-8}M; lanes c, 10^{-9}M; lanes d, 10^{-10}M) at 4°C for 16 h. Phosphorylation, immunoprecipitation, electrophoresis and autoradiography were carried out as described in the Methods Section.

Figure 2 illustrates mean insulin dose-response relationships for phosphorylation of the beta subunit of the insulin receptor from 5 control and 5 diabetic animals. The mean maximal insulin response of the receptor from diabetic animals is slightly but

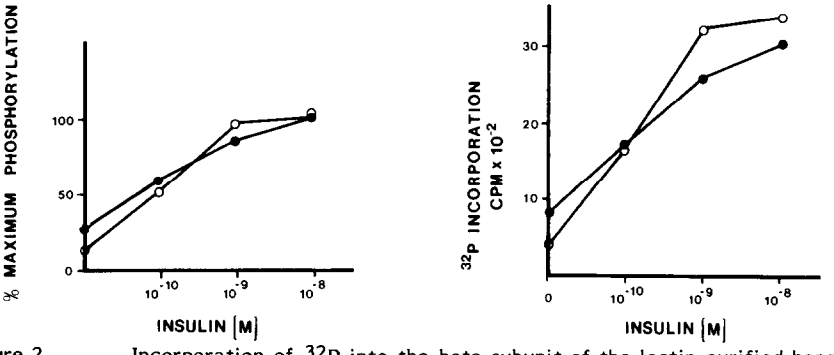

Figure 2. Incorporation of ^{32}P into the beta subunit of the lectin-purified hepatic plasma membrane insulin receptor from normal control (●) and nonketotic diabetic (○) animals. The methods are as described in the Methods Section. The left panel illustrates the percent maximal phosphorylation while the right panel illustrates the absolute ^{32}P incorporated into the beta subunit of the insulin receptor. The data represent the mean results from 5 control and 5 diabetic animals.

insignificantly greater than that from control animals (right panel), while the concentration of insulin eliciting a half maximal response is the same (left panel) and is 0.7 x 10^{-10}M insulin. Similar data were observed when the density of the beta subunit was determined from radioautographs. At an insulin concentration of 6 x 10^{-10}M, specific insulin binding was 37.8 $\pm$ 2.5% and 39.8 $\pm$ 2.9% in the receptor preparations from control and diabetic animals, respectively.

DISCUSSION. Several recent studies have demonstrated insulin stimulated phosphorylation of the insulin receptor in intact adipocytes (13), hepatocytes (14), hepatoma cells (15), cultured human lymphocytes (16) and human mononuclear cells (17) as well as in partially purified insulin receptor preparations from liver (15), brain (18), and placenta (19). In intact cells and in nonimmunoprecipitated receptor preparations, some or most of the insulin stimulated phosphorylation is on serine residues (14,15). In contrast, in partially purified preparations most or all the phosphorylation of the receptor and artificial substrates is on tyrosine residues (7,14,19). Whether this tyrosine kinase activity which is associated with the insulin receptor is involved in insulin action is presently unknown. A few reports have addressed this issue. For example, Zick et al. (20), and Simpson and Hedo (13) have demonstrated that some insulin receptor antibodies have insulin like effects in adipose cells but fail to stimulate hepatic receptor kinase activity and phosphorylation of the insulin receptor in intact adipocytes. Thus, insulin action was induced without receptor phosphorylation. As the authors' point out, however, this could be due to actions of the insulin receptor antibodies which differ mechanistically from those of insulin. Similarly, as proposed by Gazzano et al. (14), tyrosine kinase activity may be relatled to cell growth promoting actions of insulin whereas the metabolic functions stimulated by insulin may be through stimulation of serine kinase activity seen only in intact cells and nonimmunoprecipitated receptor preparations. In favor of receptor kinase activity being involved in insulin action is the study of Grunberger et al. (17), who demonstrated that in a patient with Type A insulin resistance and normal insulin binding, receptor phosphorylation and the ability of the mononuclear cell receptor to phosphorylate exogenous substrates are impaired. The authors' suggested a defect in receptor kinase activity as being the first biochemical defect of the receptor distal to ligand binding.

In my laboratory, we have demonstrated that hepatocytes from nonketotic streptozotocin diabetic rats are resistant to the ability of insulin to stimulate lipogenesis despite normal to increased insulin binding (1,5). This resistance is reversed by the in vivo treatment of animals with insulin. Preliminary studies also indicate that net glycogen accumulation as well as glycogen synthesis are resistant to insulin (unpublished observations). In contrast, aminoisobutyric acid uptake is relatively but not absolutely resistant to insulin (5). To further explore the mechanisms of insulin resistance in the liver of diabetic animals, we evaluated the ability of insulin to release the putative mediator of insulin action from a liver particulate fraction. These data again demonstrated marked resistance to insulin in the liver of diabetic animals and restoration of insulin responsiveness after insulin treatment in vivo (3). These data further suggested that alterations at or near the plasma membrane may be responsible for or accompany the hepatic insulin resistance of nonketotic streptozotocin-induced diabetes mellitus. Furthermore, such a defect must lie distal to insulin receptor binding but include generation or release of the putative mediator of insulin action. Since insulin stimulation of receptor kinase activity may be the first event in insulin action following insulin binding, and to further explore the site of hepatic insulin resistance associated with nonketotic diabetes mellitus, we evaluated the ability of insulin to stimulate 32-P incorporation into the beta subunit of lectin purified rat liver insulin receptors. The data indicate that receptor autophosphorylation in response to insulin is normal in diabetic animals.

To date, two papers have been published evaluating insulin receptor autophosphorylation in hepatic microsomal preparations from diabetic animals (21,22). Blackshear et al (21) have reported increased insulin (10^{-6}M) stimulated autophosphorylation of the beta subunit of non-lectin purified microsomal insulin receptors from diabetic animals. In contrast to these data, Kadowaki et al (22) recently reported decreased autophosphorylation of the beta subunit of the lectin purified microsomal insulin receptor from diabetic rats. In contrast to our data using receptors from hepatic plasma membranes (Fig. 2), the major increases in receptor phosphorylation seen by these authors were at insulin concentrations of 10^{-8} to 10^{-6}M. Interestingly, Kadowaki et al found (6) increased insulin binding in lectin purified microsomal receptor preparations from diabetic animals.

This is also in contrast to our data (23) utilizing non-lectin purified, but solubilized total cellular insulin receptors. In this preparation, we find that the total number of cellular receptors in livers from diabetic animals is the same as that from control animals. Whether lectin purification increases the recovery of receptors from diabetic animals or whether the receptor properties are different is unknown. Regardless, our data evaluating insulin receptor autophosphorylation differs substantially from that of Blackshear et al (21) and Kadowaki et al (22) perhaps because we used a lectin purified receptor preparation from purified plasma membranes. We feel that this may be a more physiologically relevant receptor preparation to make comparisons to insulin action.

We conclude, therefore, that the hepatic insulin resistance of nonketotic streptozotocin induced diabetes mellitus lies distal to insulin receptor binding and phosphorylation and is reflected in alterations in metabolic pathways which may include generation or release of the putative mediators of insulin action. The etiology and mechanisms of this post-receptor insulin resistance remain to be defined. Further studies will concentrate on insulin receptor autophosphorylation in intact hepatocytes from control and diabetic animals since this phosphorylation represents primarily serine kinase activity. Since we have found that the responsiveness of amino acid uptake to insulin is normal in hepatocytes from diabetic animals, it is possible that the growth promoting, protein synthetic actions of insulin are associated with those actions regulating amino acid uptake which may be mediated by receptor tyrosine kinase activity. All of these responses may be unaltered in the diabetic liver. If the metabolic effects which include lipogenesis and glycogenesis are mediated by the serine kinase activity closely associated with the receptor, it is possible that an alteration at this step may be the first demonstrable step in insulin action which is altered and may point to specific membrane defects associated with hepatic insulin resistance in diabetes.

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