Original Articles

Evidence for Glucose/Hexosamine In Vivo Regulation of Insulin/IGF-I Hybrid Receptor Assembly

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Hybrid receptors composed of an insulin αβ-hemireceptor and a type 1 IGF αβ-hemireceptor are formed in tissues expressing both molecules. We recently reported an increased hybrid receptor expression in skeletal muscle of type 2 diabetic patients that is inversely correlated with in vivo insulin sensitivity. It is unclear whether these changes were due to primary abnormalities or to secondary derangements acting in vivo, such as hyperglycemia. To address this, we determined abundance of hybrids in skeletal muscle from three groups of rats: controls, diabetic (90% pancreatectomy), and diabetic treated with phlorizin to normalize plasma glucose levels. We found that the abundance of hybrid receptors was higher in diabetic rats compared with control and phlorizin-treated diabetic rats (percentage of ^{125}I-insulin bound versus total added radioactivity [B/T] = 1.8 ± 0.11, 0.4 ± 0.01, and 0.32 ± 0.04, respectively; $P < 0.0001$). Fasting plasma glucose levels were positively correlated with hybrids abundance ($r = 0.77$, $P < 0.002$). Hybrid receptor protein content, assessed by immunoblotting, was 2.4-fold higher in diabetic rats as compared with control and phlorizin-treated diabetic rats. Because it has been shown that some of the regulatory effects of glucose may be mediated by the glucosamine pathway, we subsequently determined the effect of an in vivo glucosamine infusion on hybrid receptor formation. We found that abundance of hybrids was significantly higher in muscle from glucosamine-treated rats compared with control rats (B/T = 0.17 ± 0.02 and 0.11 ± 0.01, respectively; $P < 0.009$). Quantitation of hybrid content by immunoblotting revealed that their abundance was 1.9-fold higher in glucosamine-treated rats. The results demonstrate that *1*) elevated glucose levels in diabetic rats are associated with increased expression of hybrid receptors in muscle, *2*) correction of hyperglycemia with phlorizin completely reverses increased expression of hybrids, and *3*) glucosamine infused into control rats mimics the effects of hyperglycemia on hybrid receptor formation. Thus, the results support the hypothesis that glucose acting, at least in part, through the glucosamine pathway may play an important role in regulating hybrid receptor assembly in vivo. *Diabetes* 48:2277–2285, 1999

The insulin receptor shares structural and functional similarities with the type 1 IGF receptor (1–3). As a consequence of the high homology, hybrid receptors composed of an insulin αβ-hemireceptor and a type 1 IGF αβ-hemireceptor are formed in tissues expressing both molecules (4–8). It has been demonstrated that hybrid receptors function more like a type 1 IGF receptor rather than a typical insulin receptor or some intermediate version of the two receptors with respect to ligand binding affinity, receptor autophosphorylation, hormone internalization, and biological actions (7,9–13). Recently, we have reported that abundance of hybrid receptors is increased in skeletal muscle of patients with type 2 diabetes and is inversely correlated with in vivo insulin sensitivity (14). The question of how hybrid receptor assembly is regulated in vivo is still unsettled. Furthermore, it is still unclear whether changes in hybrid receptor content observed in type 2 diabetic patients subjects are due to primary cellular abnormalities or to secondary derangements acting in vivo, such as hyperglycemia or hyperinsulinemia. Chronic hyperglycemia adversely affects both insulin secretion and insulin action, a phenomenon referred to as glucose toxicity (15). Recent in vitro (16–18) and in vivo (19–21) studies suggest that hyperglycemia may contribute to insulin resistance by increasing routing of glucose through the glucosamine pathway, in which fructose-6-phosphate is converted to glucosamine-6-phosphate by glutamine:fructose-6-phosphate amidotransferase, with glutamine acting as the donor of the amido group. Partially (90%) pancreatectomized rats are an experimental model of diabetes that has been widely used to study the adverse effects of hyperglycemia on insulin action (15,22) and the role of the glucosamine pathway in glucose toxicity

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BSA, bovine serum albumin; B/T, percentage of ^{125}I-insulin bound versus total added radioactivity; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline.

(19). Since glucose is known to regulate the expression of several genes including type 1 IGF receptor and insulin receptor genes (23–25) at both the transcriptional and translational level, in this study, we investigated the role of the glucose and glucosamine pathway in the in vivo regulation of hybrid receptor assembly. In the present series of experiments, we wished *1*) to study, in a rat model characterized by chronic hyperglycemia (but not severe hypoinsulinemia), hybrid receptor abundance in skeletal muscle; *2*) to determine the possible deleterious role of glucose by the correction of hyperglycemia with phlorizin, a potent competitive inhibitor of renal tubular glucose transport; and *3*) to investigate the possible role in vivo of the glucosamine pathway in augmenting the generation of hybrid receptors.

RESEARCH DESIGN AND METHODS

Materials. Human ^{125}I-A14-monoiodoinsulin (290–320 µCi/µg) and ^{125}I-IGF-I (280–310 µCi/µg) were purchased from Amersham Life Science (Buckinghamshire, U.K.). Recombinant human insulin was purchased from Sigma (St. Louis, MO), and recombinant human IGF-I was purchased from Boehringer Mannheim (Mannheim, Germany). The following antibodies were used to detect receptors in microwell immunoassay and immunoprecipitation: anti-IGFR antibody, a rabbit polyclonal antibody against the type 1 IGF receptor α-subunit that does not cross-react with insulin receptors (14,26); anti-INSR antibody, a rabbit polyclonal antibody against the insulin receptor α-subunit that does not cross-react with type 1 IGF receptors (27); and anti-INSRβ antibody, a rabbit polyclonal antibody raised against a peptide corresponding to residues 1326–1343 of the insulin receptor β-subunit (27). Crystalline D-glucosamine and all other chemicals were purchased from Sigma-Aldrich (Milan, Italy).

Animals. Two groups of male Sprague-Dawley rats (Charles River Laboratories, Lecco, Italy) were studied: 10 partially pancreatectomized diabetic rats and 17 control rats. At 3–4 weeks of age, the diabetic rats (80–100 g) were anesthetized with phenobarbital (50 mg/kg body wt i.p.), and 90% of their pancreas was removed according to previously described method (22,28–32). Immediately after the pancreatectomy, the rats were housed in individual cages in an air-controlled room and were subjected to a standard 12-h light/dark cycle. The rats received rat food and water in an amount that sustained normal growth in all animals as previously reported (22,28–32). The rats were weighed twice weekly, and blood was obtained simultaneously from the tail vein for determination of nonfasting plasma glucose and insulin concentrations (0900). At 5–6 weeks after pancreatectomy or sham operation, the rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body wt), and indwelling catheters were inserted into the right jugular vein and the left carotid artery. The venous catheter was extended to the level of the right atrium, and the arterial catheter was advanced to the level of the aortic arch. Both catheters were filled with heparin/polyvinilpyrrolidone solution, sealed, tunneled subcutaneously around the side of the neck, and exteriorized through a skin incision (21,22,28–32).

Studies. All studies were conducted in the afternoon after a 6-h fast. Throughout the studies, the rats were awake and allowed to move freely within the confines of a large cage, with the connecting tubing suspended overhead. The venous catheters were used for blood withdrawal, while the arterial catheters were used for the infusion of the test substances. To prevent intravascular volume depletion and anemia, fresh whole blood obtained by heart puncture from fasting littermates of the test animal was administered at a constant rate, designed to quantitatively replace the total blood loss during the study (21,22,28–32). Five out of the ten diabetic animals received an acute intravenous treatment with phlorizin for 2–3 h until euglycemia was reached, and euglycemia was then kept for at least 2 h before the rats were killed. The remaining five diabetic rats were infused with saline instead of phlorizin to keep them in spontaneous hyperglycemia. In the glucosamine studies, a priming injection (180 µmol/kg over a 2-min period) followed by a constant (5 µmol · kg^{-1} · min^{-1}) glucosamine infusion was started 10 min before the clamp studies and continued throughout the study. Crystalline D-glucosamine was diluted in saline with a final concentration of ~40 nmol/l (according to rat body weight). Equal volumes of saline were infused during the saline control studies. The rate of glucosamine infusion was chosen on the basis of previous experience (21) as well as on the present estimates of the rate of fructose-6-phosphate conversion to glucosamine-6-phosphate (~0.8–1% of overall glucose metabolism, i.e., ~2–2.7 µmol · kg^{-1} · min^{-1} during the insulin clamp studies) to increase the rate of glucosamine-6-phosphate generation by two- to threefold. Both the glucosamine-infused rats (n = 6) and the saline-infused rats (n = 6) received a prime/continuous infusion of insulin (Bio-Insulin R, Laboratori Guidotti, Pisa, Italy) at 20 mU · kg^{-1} · min^{-1} for 2 h. A variable infusion of 25% glucose solution was started at time 0 and adjusted to clamp the plasma glucose concentration at ~6.7 mmol/l. [3-^{3}H]glucose was also infused for other purposes. Plasma samples for determination of glucose were obtained at 5- to 10-min intervals throughout the insulin clamp study. Plasma samples for determination of plasma insulin concentration were obtained at time –20, 0, 90, and 120 min during the study. At the end of the 120-min infusions, all the rats were injected intravenously with pentobarbital (60 mg/kg body wt), and the hind limb muscles were freeze-clamped with aluminum tongs precooled in liquid nitrogen. All tissue samples were kept frozen at –80°C for subsequent analysis.

Microwell immunoassay. The assay was performed according to a previously validated method (14,33,34). Briefly, skeletal muscle samples were solubilized in 50 mmol/l HEPES buffer, pH 7.6, containing 150 mmol/l NaCl, 1% Triton X-100, 1 mg/ml bacitracin, 2 mmol/l phenylmethylsulfonyl fluoride (PMSF), and 1,000 U/ml aprotinin for 60 min at 4°C. Insoluble material was removed by centrifugation at 100,000*g* for 60 min at 4°C, and soluble fractions were diluted to 0.2% Triton X-100 and immediately assayed. Protein content of tissue extracts was determined by the Bradford method. The 96-well microwells, coated with anti-INSR antibody or anti-IGFR antibody in 20 mmol/l $NaHCO_3$, pH 9.6, were incubated in buffer A containing 50 mmol/l HEPES buffer, pH 7.6, 150 mmol/l NaCl, 0.1% Triton X-100, 1 mg/ml bacitracin, 2 mmol/l PMSF, 1,000 U/ml aprotinin, and 0.1% bovine serum albumin (BSA), with skeletal muscle extracts (500 µg) for 16 h at 4°C. The wells were washed three times with buffer A, and immunoadsorbed receptors were incubated with ^{125}I-IGF-I or ^{125}I-insulin (60 pmol/l) for 16 h at 4°C in the presence or absence of multiple concentrations of unlabeled ligands. Thereafter, the wells were washed three times to remove unbound ligands. Radioactivity bound to immunoadsorbed receptors was collected by adding 2% SDS for 30 min at 24°C to the wells and counted.

Western blotting. Equal amount of tissue extracts (500 µg) were incubated for 16 h at 4°C with 1 µg of either anti-IGFR antibody or anti-INSR antibody, each bound to protein A-Sepharose. The immunoprecipitates were subjected to SDS-PAGE under reducing conditions. Proteins (500 µg/lane) resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose filters. The nonspecific binding sites of membranes were blocked by a 2-h incubation of Tris-buffered saline (TBS) (pH 7.5) containing 5% BSA. The filters were then incubated for 16 h at 4°C with either anti-IGFRα, raised against a peptide corresponding to residues 31–50 of the type 1 IGF receptor α-subunit, or anti-INSRα antibody, raised against a peptide corresponding to residues 29–48 of the insulin receptor α-subunit, according to the recommendations of the manufacturer (Santa Cruz Laboratories). After extensive washings with TBS, the filters were blotted with a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody, and bound antibodies were visualized by enhanced chemiluminescence (Amersham Life Science). Densitometric scanning was performed to quantitate the intensity of the bands. In experiments with deglycosylated receptors, tissue extracts (500 µg) were incubated for 60 min at 37°C in the presence or absence of a pool of glycosydases (Enzymatic Deglycosylation Kit, Bio-Rad Laboratories, Milan, Italy) before immunoprecipitation.

Purification of insulin, type 1 IGF, and hybrid receptors. Insulin, type 1 IGF, and hybrid receptors were purified according to previously described methods (7,9). Briefly, skeletal muscle extracts from control rats were applied to an anti–IGFR-Sepharose column. The flow through fractions containing insulin receptors was collected and applied to an anti–INSR-Sepharose column. The eluates of the anti–IGFR-Sepharose chromatography containing both type 1 IGF holoreceptors and hybrid receptors were applied to an anti–INSR-Sepharose column. Both the flow through fractions containing type 1 IGF holoreceptors and the eluates containing hybrid receptors were collected and concentrated by Centricon 10 (Amicon, Beverly, MA). The receptors bound to the columns were eluted with 50 mmol/l Tris-HCl buffer, pH 7.4, containing 1 mol/l NaCl and 2.5 mmol/l $MgCl_2$, and concentrated. Purified receptor preparations corresponding to insulin receptors, type 1 IGF receptors, and hybrid receptors were characterized for their binding properties using the above described microwell-based immunoassay. As expected, the insulin receptor preparation was free of hybrid receptors as deduced by the inability to bind ^{125}I-IGF-I when incubated in anti-IGFR–coated wells. The type 1 IGF receptor preparation was free of hybrid receptors, as deduced by the inability to bind ^{125}I-IGF-I when incubated in anti-INSR–coated wells. In contrast, the hybrid receptor preparation was able to bind ^{125}I-IGF-I with high affinity when incubated with either anti-IGFR– or anti-INSR–coated wells.

Quantitation of insulin and hybrid receptors glycosylation. Equal amount of tissue extracts (500 µg) were incubated for 16 h at 4°C with 1 µg of anti-INSR antibody, each bound to protein A-Sepharose. The immunoprecipitates were subjected to SDS-PAGE under reducing conditions. Proteins (500 µg/lane) resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose filters. The Immun-Blot kit for glycoprotein detection (Bio-Rad Laboratories) was used to detect the glycosylation state of the insulin receptor, according to the recommendations of the manufacturer.

Analytical procedures. Unpaired Student's t test was used to compare mean values. Linear correlations between variables were tested by calculating Pearson's correlation coefficient.

TABLE 1
General characteristics of the animals

	Control rats	Diabetic rats
Body weight (g)	248 ± 6	251 ± 13
Fasting plasma glucose (mmol/l)	6.1 ± 0.3	14.2 ± 1.8*
Fasting plasma insulin (pmol/l)	68 ± 7	59 ± 10
Fed plasma glucose (mmol/l)	8.4 ± 0.5	19.2 ± 1.6*
Fed plasma insulin (pmol/l)	165 ± 12	87 ± 9*

Data are means ± SE. *$P < 0.05$ or less, as compared with the control animals.

RESULTS

General characteristics of the animals and clamp data. As shown in Tables 1 and 2, there were no differences in the mean body weight between control and diabetic rats. Both the fasting and postmeal plasma glucose concentrations during the 2-week period before the studies were significantly higher in the diabetic group compared with the control group. The fasting plasma insulin concentrations were similar, whereas the postmeal plasma insulin concentrations were significantly diminished in diabetic rats compared with controls. Plasma glucose and insulin concentrations during the studies were similar between saline-infused control and phlorizin-treated diabetic animals; as expected, and as previously reported (22), saline-infused diabetic rats were severely hyperglycemic (Table 2). In the glucosamine clamp studies, steady-state conditions for plasma glucose concentration was reached within ~30 min. The coefficient of variation in the plasma glucose level was <5% in all studies. The glucosamine infusion during the hyperinsulinemic glucose clamp in control rats determined a significant decrease in glucose uptake (201 ± 12 vs. 281 ± 16 μmol · kg^{-1} · min^{-1}, respectively, $P < 0.003$), according to previously reported data (21).

Quantitation of insulin receptors, IGF-I receptors, and insulin/IGF-I hybrid receptors in skeletal muscle from control, diabetic, and phlorizin-treated diabetic rats by microwell immunoassay. A previously validated microwell-based immunoassay was used to measure insulin and IGF-I binding to receptors (14,33,34). Muscle extracts were incubated in microwell plates coated with anti-INSR antibody or anti-IGFR antibody to capture insulin or IGF-I receptors, respectively. After washing, ligand binding to immunoadsorbed receptors was assessed by incubating the wells with ^{125}I-insulin or ^{125}I-IGF-I in the presence or absence of varying concentrations of unlabeled ligands. Maximal specific insulin binding to immunoadsorbed insulin receptors, expressed as the percentage of ^{125}I-insulin bound versus total added radioactivity (B/T), was significantly higher in muscle from diabetic rats as compared with control and phlorizin-treated diabetic rats (Table 2). Maximal specific IGF-I binding to immunoadsorbed IGF receptors was significantly higher in muscle from diabetic rats as compared with control and phlorizin-treated diabetic rats (Table 2).

Quantitation of insulin/IGF-I hybrid receptors was based on the ability to bind with high affinity IGF-I, but not insulin, and to react with specific anti–insulin receptor antibodies (7,9,11,14,33,34). Microwells coated with anti-INSR antibody were incubated with muscle extracts from control, diabetic, and phlorizin-treated diabetic rats. After washing, immunoadsorbed receptors were incubated with ^{125}I-IGF-I in the presence or absence of unlabeled IGF-I or insulin. To determine whether the anti-IGFR or anti-INSR antibodies were able to react as well with hybrid receptors as with their own receptors, immunodepletion experiments were carried out using purified insulin, type 1 IGF, and hybrid receptors isolated by immunoaffinity chromatography according to previously described methods (7,9). Microwells were coated with increasing concentrations of either anti-INSR antibody or anti-IGFR antibody and incubated with a fixed amount of purified receptors. After removal of supernatants, insulin or IGF-I binding to immobilized receptors was determined by incubating the wells with ^{125}I-insulin or ^{125}IGF-I in the presence or absence of unlabeled ligands. Residual insulin or IGF-I binding activity in the supernatants were assayed by immunoprecipitation with anti-INSR or anti-IGFR antibody bound to protein A-Sepharose beads. As shown in Fig. 1, anti-INSR antibody was equally able to remove both insulin and hybrid receptors in a dose-dependent manner, with half-maximal effect and maximal effect at the same concentration for both receptors (4 μg/well for half-maximal effect and 10 μg/well for maximal effect for insulin and hybrid receptors, respectively). Similarly, anti-IGFR antibody removed both type 1 IGF and hybrid receptors in a dose-dependent manner, with half-maximal effect and maximal effect at the same concentration for

TABLE 2
Steady-state plasma glucose and insulin during the clamp studies, and quantitation of insulin and IGF-I binding to immunoadsorbed receptors

Study group	Glucose (mmol/l)	Insulin (pmol/l)	Insulin binding to immunoadsorbed receptors (B/T)	IGF-I binding to type 1 IGFR (B/T)	IGF-I binding to hybrids (B/T)
Control (saline)	6.2 ± 0.4	75 ± 8	1.15 ± 0.2	0.63 ± 0.1	0.4 ± 0.01
Diabetic (saline)	17.2 ± 1.6	62 ± 10	1.85 ± 0.15	1.97 ± 0.1	1.8 ± 0.11
Diabetic (phlorizin)	6.3 ± 0.3	65 ± 8	1.08 ± 0.3	0.57 ± 0.15	0.32 ± 0.04
Control (insulin plus saline)	5.72 ± 0.11	2841 ± 184	0.27 ± 0.1	0.23 ± 0.2	0.11 ± 0.01
Control (insulin plus glucosamine)	5.74 ± 0.10	2683 ± 194	0.25 ± 0.1	0.21 ± 0.2	0.17 ± 0.02

Data are means ± SE for five animals in each group. Tissue extracts from the five groups of rats were added to microwells coated with either anti-INSR antibody to capture insulin and hybrid receptors or anti-IGFR antibody to capture IGF-I receptors. After washing, ligand binding to immunoadsorbed receptors was assessed by incubating the wells with ^{125}I-insulin or ^{125}I-IGF-I in the presence or absence of varying concentrations of unlabeled ligands (100 nmol/l). Results are expressed as the percentage of ^{125}I-insulin or ^{125}I-IGF-I bound versus total added radioactivity (B/T).

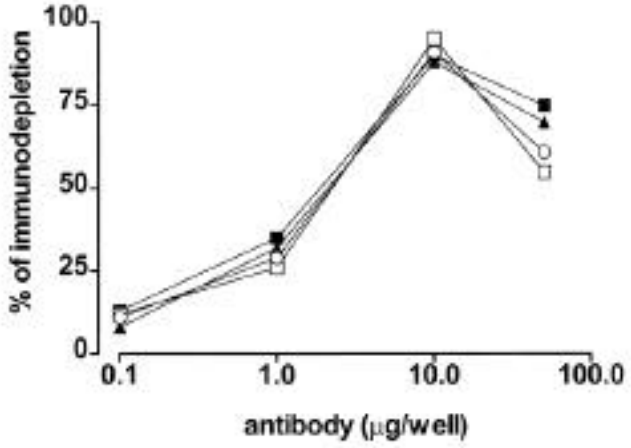

FIG. 1. Immunodepletion of insulin, IGF-I, and hybrid receptors. Microwells were coated with increasing concentrations of either anti-INSR antibody (○, ■) or anti-IGFR antibody (□, ▲) and incubated with a fixed amount of purified insulin (○), type 1 IGF (▲), and hybrid receptors (□, ■) isolated by immunoaffinity chromatography. After removal of supernatants, insulin or IGF-I binding to immobilized receptors was determined by incubating the wells with ^{125}I-insulin (○) or ^{125}IGF-I (□, ■, ▲) in the presence or absence of unlabeled ligands. Residual insulin or IGF-I binding activity in the supernatants was assayed by immunoprecipitation with anti-INSR (○, ■) or anti-IGFR (□, ▲) antibody bound to protein A-Sepharose beads. A representative experiment of three experiments, each performed in triplicate, is shown. Irrelevant polyclonal antibody did not deplete any receptor.

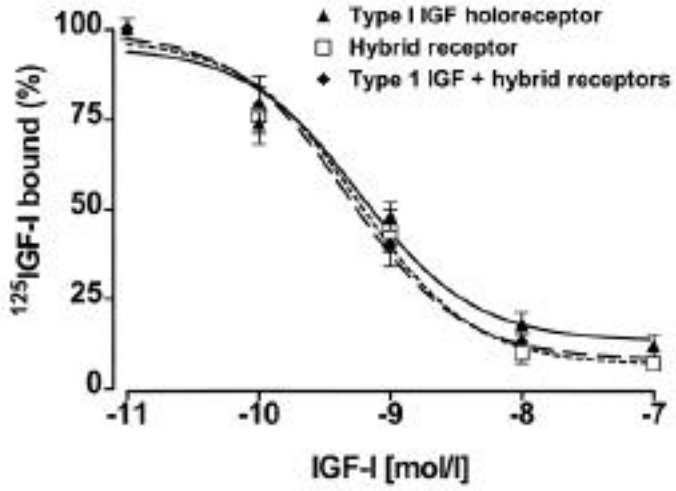

FIG. 2. Inhibition of ^{125}I-IGF-I binding to immobilized type 1 IGF and hybrid receptors. Muscle extracts from control rats were added directly (□, ▲) or after immunodepletion of hybrid receptors with anti-INSR antibody (◆) to microwells coated with anti-INSR antibody (□) or anti-IGFR antibody (▲, ◆). After washing, ligand binding to immobilized receptors was assessed by incubating the wells with ^{125}I-IGF-I in the presence or absence of increasing concentrations of unlabeled IGF-I. Results of IGF-I binding competition expressed as the percentage of maximal specific binding are presented as the mean ± SE of three experiments carried out in triplicate.

both receptors (4 μg/well for half-maximal effect and 10 μg/well for maximal effect for type 1 IGF and hybrid receptors, respectively). These results indicate that under the experimental conditions used, the antibodies react equally with both hybrid receptors and their respective holoreceptors. Therefore, subsequent assays to determine insulin, IGF-I, and hybrid receptor abundance were performed by using microwells coated with 10 μg/well of anti-INSR or anti-IGFR antibody. Figure 2 shows representative competition-inhibition curves of ^{125}I-IGF-I binding to hybrid receptors immobilized with anti-INSR antibody, hybrid plus type 1 IGF receptors immobilized with anti-IGFR antibody, or type 1 IGF holoreceptors immobilized with anti-IGFR antibody after immunodepletion of hybrid receptors with anti-INSR antibody. Receptor affinity for ^{125}I-IGF-I binding to hybrid receptors was similar to those observed with type 1 IGF receptors or with hybrid plus type 1 IGF receptors (concentration of unlabeled ligand required to inhibit 50% of maximal binding [ED_{50}] = 0.58, 0.50, and 0.52 nmol/l IGF-I for hybrid receptors, type 1 IGF holoreceptors, and hybrid plus type 1 IGF receptors, respectively). In contrast, low concentrations of unlabeled insulin were unable to compete for ^{125}I-IGF-I binding to hybrid receptors (data not shown), thus indicating that no significant ^{125}I-IGF-I binding to the insulin receptor occurred at the concentration of tracer used. Maximal specific ^{125}I-IGF-I binding to immunoadsorbed hybrid receptors was significantly higher in muscle from diabetic rats as compared with control and phlorizin-treated diabetic rats (B/T = 1.8 ± 0.11, 0.4 ± 0.01, and 0.32 ± 0.04; $P < 0.0001$, respectively) (Table 2). Hybrid receptor affinity for IGF-I binding was similar in the three groups of rats (ED_{50} = 0.18 ± 0.05 and 0.11 ± 0.10 nmol/l IGF-I for control, diabetic, and phlorizin-treated diabetic rats, respectively). Fasting plasma glucose levels were positively correlated with hybrid receptor abundance ($r = 0.77$; $P < 0.002$).

Quantitation of insulin receptors, IGF-I receptors, and insulin/IGF-I hybrid receptors in skeletal muscle from control, diabetic, and phlorizin-treated diabetic rats by Western blotting. Abundance of hybrid receptors was also assessed by immunoblotting with a specific anti–type 1 IGF receptor antibody (anti-IGFRα) after precipitation with anti-IGFR antibody or anti-INSR antibody. The anti-IGFRα antibody detected a single band of 130 kDa corresponding to the α-subunit of the type 1 IGF receptor (Fig. 3). Total type 1 IGF receptor protein content was 1.4-fold higher in skeletal muscle from diabetic rats (Fig. 3, *lane 3*) as compared with control and phlorizin-treated diabetic rats (Fig. 2, *lanes 1* and *5*, respectively; $P < 0.03$). Hybrid receptor protein content was 2.4-fold higher in diabetic rats (Fig. 3, *lane 4*) as compared with control and phlorizin-treated diabetic rats (Fig. 3, *lanes 2* and *6*, respectively; $P < 0.01$). These results were consistent with those obtained with the microwell-based immunoassay.

We then attempted to determine the fraction of total insulin receptors assembled as hybrids. Because it is not possible to assess the fraction of insulin receptors present in hybrids by measuring ^{125}I-insulin binding to immunoadsorbed hybrid receptors because of their low affinity for insulin (9,10,33), total and hybrid insulin receptors were compared by immunoblotting with a specific anti–insulin receptor antibody (anti-INSRα) after precipitation with anti-IGFR antibody or anti-INSR antibody. As shown in Fig. 4, both anti-IGFR antibody and anti-INSR antibody precipitate a single molecular species of ~130 kDa, corresponding to the α-subunit of the insulin receptor. The percentage of insulin receptors assembled as hybrid receptors, estimated as the ratio of insulin receptors precipitated by anti-IGFR antibody versus the total pool of insulin receptors precipitated by anti-INSR antibody, was increased in diabetic rats (65 ± 7%) (Fig. 4, *lane 4*) as compared with control and phlorizin-

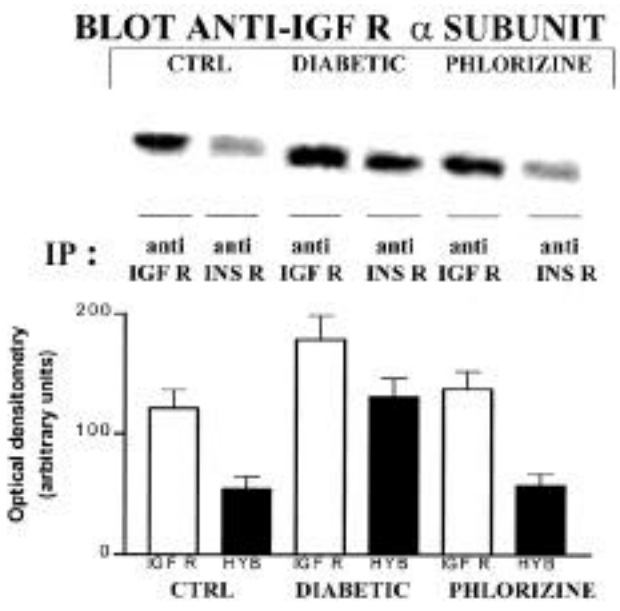

FIG. 3. Quantitation of type 1 IGF receptor and hybrid receptor protein content by Western blotting. Equal amounts of skeletal muscle extracts (500 µg) from control rats (*lanes 1* and *2*), partially pancreatectomized diabetic rats (*lanes 3* and *4*), and partially pancreatectomized diabetic rats treated with phlorizin (*lanes 5* and *6*) were immunoprecipitated with anti-IGFR antibody or anti-INSR antibody, resolved by SDS-PAGE, and Western immunoblotted with a specific anti-type 1 IGF receptor antibody (anti-IGFRα). The upper panel shows a representative experiment. The bar graph in the lower panel summarizes the densitometric data from five control rats, five diabetic rats, and five phlorizin-treated diabetic rats. Data are presented as means ± SE. HYB, hybrid receptors; IP, immunoprecipitation.

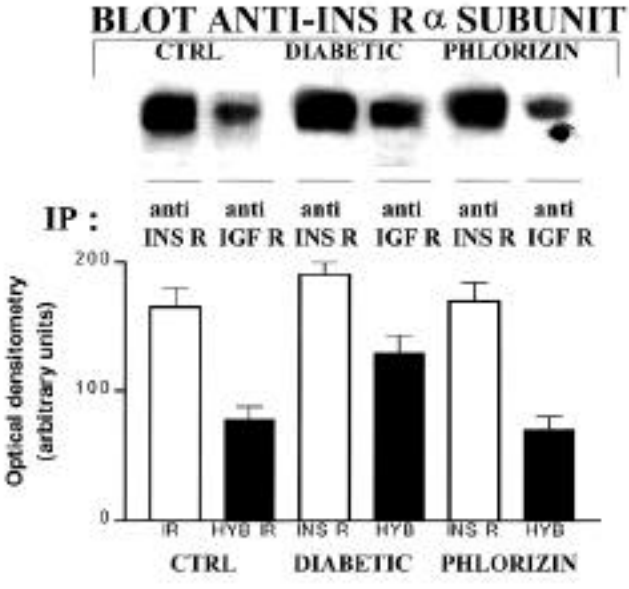

FIG. 4. Quantitation of insulin receptor and hybrid receptor protein content by Western blotting. Equal amounts of skeletal muscle extracts (500 µg) from control rats (*lanes 1* and *2*), partially pancreatectomized diabetic rats (*lanes 3* and *4*), and partially pancreatectomized diabetic rats treated with phlorizin (*lanes 5* and *6*) were immunoprecipitated with anti-INSR antibody or anti-IGFR antibody, resolved by SDS-PAGE, and Western immunoblotted with a specific anti–insulin receptor antibody (anti-INSRα). The upper panel shows a representative experiment. The bar graph in the lower panel summarizes the densitometric data from five control rats, five diabetic rats, and five phlorizin-treated diabetic rats. Data are presented as means ± SE. HYB, hybrid receptors; IP, immunoprecipitation.

treated diabetic rats (47 ± 4 and 41 ± 4%; $P < 0.04$, respectively) (Fig. 4, *lanes 2* and *6*, respectively). To address the question of whether increased abundance of hybrid receptors observed in diabetic rats causes a decrease in insulin holoreceptors content, skeletal muscle lysates were subjected to four rounds of immunoprecipitation with anti-IGFR antibody to deplete both type 1 IGF and hybrid receptors followed by immunoprecipitation with anti-INSR antibody and immunoblotting with anti-INSR or anti-IGFR antibody. As shown in Fig. 5, the amount of insulin holoreceptors was decreased by 34% in diabetic rats as compared with control rats. Immunoblotting with anti-IGFR antibody did not detect any band, thus implying that immunodepletion of both type 1 IGF and hybrid receptors was effective. To determine whether increased abundance of hybrid receptors in diabetic rats affects type 1 IGF holoreceptor content, muscle lysates were subjected to four rounds of immunoprecipitation with anti-INSR antibody to deplete both insulin holoreceptors and hybrid receptors followed by immunoprecipitation with anti-IGFR antibody and immunoblotting with anti-INSR or anti-IGFR antibody. As shown in Fig. 6, the amount of type 1 IGF holoreceptors was increased by 39% in diabetic rats as compared with control rats. Notably, immunoblotting with anti-INSR antibody did not detect any band. The lack of reactivity of either anti-IGFR antibody with anti-INSR immunoprecipitates or anti-INSR antibody with anti-IGFR immunoprecipitates indicates that the antibodies are specific and react exclusively with the corresponding receptors, according to previously reported data (26,27).

Quantitation of insulin/IGF-I hybrid receptors in skeletal muscle from control and glucosamine-treated rats. Skeletal muscles were obtained from glucosamine- or saline-infused rats at the end of a hyperinsulinemic glucose clamp. Plasma insulin levels did not differ between the two groups of rats (Table 2). However, plasma insulin levels in glucosamine- or saline-infused rats were ~40-fold higher as compared with control, diabetic, and phlorizin-treated diabetic rats described in the previous section (Table 2). Maximal specific insulin binding to immunoadsorbed insulin receptors was

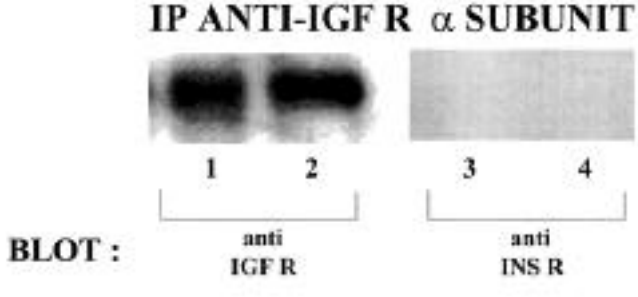

FIG. 5. Quantitation of insulin holoreceptor protein content by Western blotting after immunodepletion of type 1 IGF and hybrid receptors. Equal amounts of skeletal muscle extracts (500 µg) from control rats (*lanes 1* and *3*) and partially pancreatectomized diabetic rats (*lanes 2* and *4*) were immunoprecipitated four times with anti-IGFR antibody to deplete both type 1 IGF and hybrid receptors followed by immunoprecipitation with anti-INSR antibody. Immunoprecipitates were resolved by SDS-PAGE and Western immunoblotted with anti-INSR (*lanes 1* and *2*) or anti-IGFR antibody (*lanes 3* and *4*). A representative experiment of three experiments is shown. IP, immunoprecipitation.

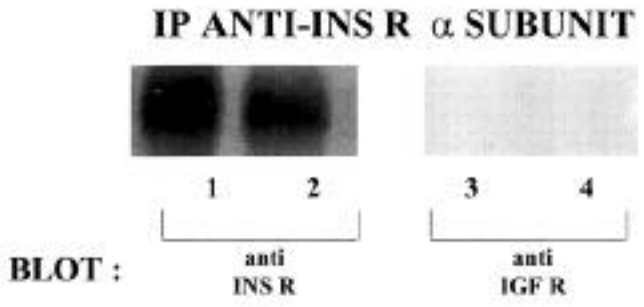

FIG. 6. Quantitation of type 1 IGF holoreceptor protein content by Western blotting after immunodepletion of insulin and hybrid receptors. Equal amounts of skeletal muscle extracts (500 µg) from control rats (*lanes 1* and *3*) and partially pancreatectomized diabetic rats (*lanes 2* and *4*) were immunoprecipitated four times with anti-INSR antibody to deplete both insulin and hybrid receptors followed by immunoprecipitation with anti-IGFR antibody. Immunoprecipitates were resolved by SDS-PAGE and Western immunoblotted with anti-IGFR (*lanes 1* and *2*) or anti-INSR antibody (*lanes 3* and *4*). A representative experiment of three experiments is shown. IP, immunoprecipitation.

similar in muscle from glucosamine-treated rats as compared with control rats (Table 2). Maximal specific IGF-I binding to immunoadsorbed IGF receptors was similar in muscle from glucosamine-treated rats as compared with control rats (Table 2). However, both insulin and IGF-I binding were significantly reduced in either glucosamine- or saline-infused rats compared with control, diabetic, and phlorizin-treated diabetic rats as a consequence of downregulation of the two receptor species induced by the high plasma insulin levels (Table 2). Maximal specific ^{125}I-IGF-I binding to immunoadsorbed hybrid receptors was significantly higher in muscle from glucosamine-treated rats compared with control rats (B/T = 0.17 ± 0.02 and 0.11 ± 0.01, respectively; $P < 0.009$) (Table 2). Hybrid receptors were also estimated as the fraction of ^{125}I-IGF-I binding to hybrids versus the total pool of ^{125}I-IGF-I binding sites (hybrids plus type 1 receptors). The percentage of hybrid receptors was significantly increased in muscle from glucosamine-treated rats as compared with control rats (74 ± 10 and 52 ± 10%, respectively; $P < 0.01$). Quantitation of hybrid receptor content by immunoblot analysis revealed that abundance of hybrids was 1.9-fold higher in skeletal muscle from glucosamine-treated rats as compared with control rats (Fig. 7, *lanes 2* and *4*, respectively; $P < 0.01$). The percentage of the total pool of insulin receptors assembled as hybrid receptors was significantly higher in skeletal muscle from glucosamine-treated rats (Fig. 8, *lane 2*) as compared with control rats (Fig. 8, *lane 4*) (66 ± 6 and 47 ± 5%, respectively; $P < 0.02$).

Quantitation of insulin and hybrid receptor glycosylation. To investigate the possibility that changes in hybrid assembly observed in diabetic and glucosamine-treated rats could stem from an identifiable alteration in insulin receptor glycosylation, we determined the glycosylation state of both insulin and hybrid receptors. To this aim, carbohydrate moieties of nitrocellulose membrane–immobilized α- and β-insulin receptor subunits, derived from control, diabetic, glucosamine-treated, and -untreated rats, were labeled with biotin and detected with streptavidin-alkaline phosphatase. As shown in Fig. 9, both insulin receptor subunits derived from

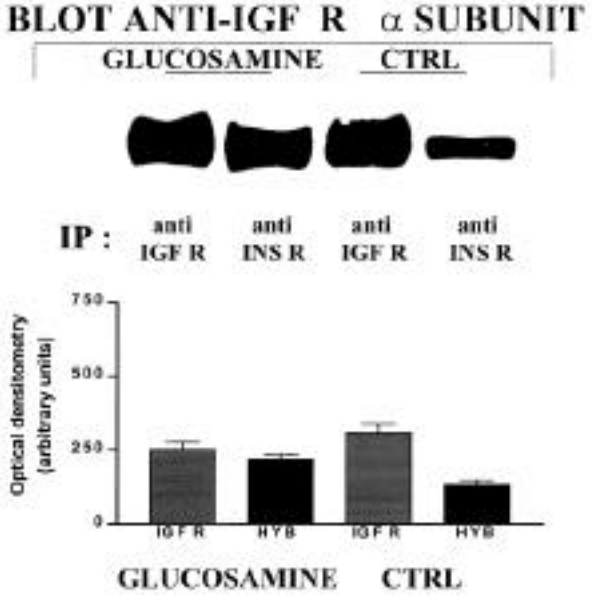

FIG. 7. Quantitation of type 1 IGF receptor and hybrid receptor protein content by Western blotting. Equal amounts of skeletal muscle extracts (500 µg) from rats infused with glucosamine (*lanes 1* and *2*) and control rats infused with saline (*lanes 3* and *4*) were immunoprecipitated with anti-IGFR antibody or anti-INSR antibody, resolved by SDS-PAGE, and Western immunoblotted with a specific anti–type 1 IGF receptor antibody (anti-IGFRα). The upper panel shows a representative experiment. The bar graph in the lower panel summarizes the densitometric data from six control rats and six glucosamine-treated rats. Data are presented as means ± SE. HYB, hybrid receptors; IP, immunoprecipitation.

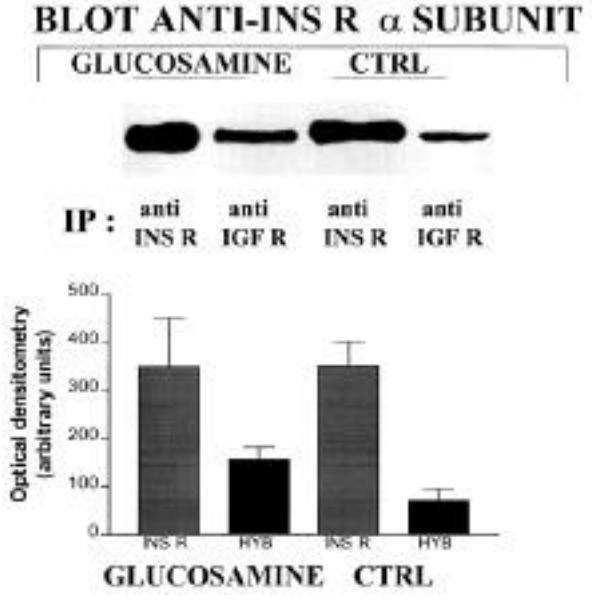

FIG. 8. Quantitation of insulin receptor and hybrid receptor protein content by Western blotting. Equal amounts of skeletal muscle extracts (500 µg) from rats infused with glucosamine (*lanes 1* and *2*) and control rats infused with saline (*lanes 3* and *4*) were immunoprecipitated with anti-INSR antibody or anti-IGFR antibody, resolved by SDS-PAGE, and Western immunoblotted with a specific anti–insulin receptor antibody (anti-INSRα). The upper panel shows a representative experiment. The bar graph in the lower panel summarizes the densitometric data from six control rats and six glucosamine-treated rats. Data are presented as means ± SE. HYB, hybrid receptors; IP, immunoprecipitation.

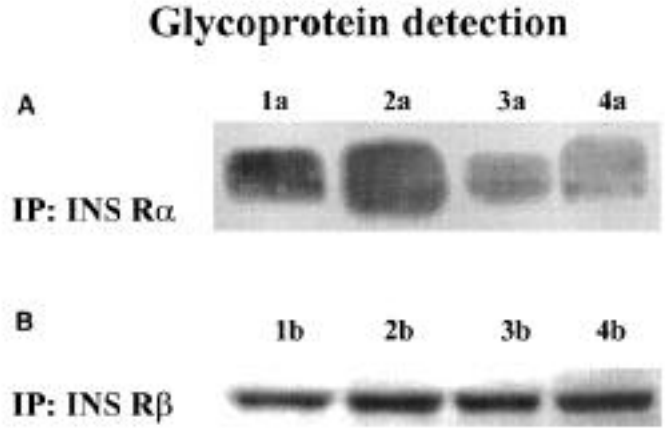

FIG. 9. Quantitation of insulin and hybrid receptor glycosylation. Equal amounts of skeletal muscle extracts (500 µg) from control rats (*lane 1*), partially pancreatectomized diabetic rats (*lane 2*), control rats infused with saline (*lane 3*), and rats infused with glucosamine (*lane 4*) were immunoprecipitated with either anti-INSR antibody (*A*) or anti-INSRβ antibody (*B*), resolved by SDS-PAGE, and electrophoretically transferred to nitrocellulose filters. Carbohydrate moieties of both α-insulin receptor (*A*) and β-insulin receptor (*B*) subunits were labeled with biotin, and biotinylated glycoproteins were detected with streptavidin-alkaline phosphatase. Band densities were quantified by scanning densitometry. The intensities expressed as arbitrary densitometric units were as follows: 2.73 (*lane 1a*), 4.02 (*lane 2a*), 0.92 (*lane 3a*), 2.14 (*lane 4a*), 0.94 (*lane 1b*), 1.18 (*lane 2b*), 0.97 (*lane 3b*), and 1.43 (*lane 4b*). A representative experiment is shown. IP, immunoprecipitation.

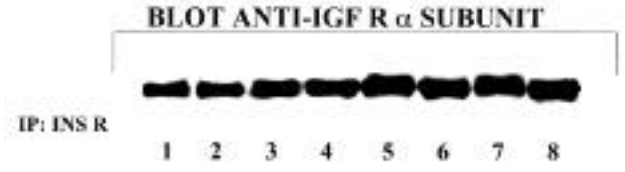

FIG. 10. Quantitation of insulin/IGF-I hybrid receptors in skeletal muscle from partially pancreatectomized diabetic rats, glucosamine-treated rats, and their respective control rats. Equal amounts of skeletal muscle extracts (500 µg) from control rats (*lanes 1* and *2*), control rats infused with saline (*lanes 3* and *4*), partially pancreatectomized diabetic rats (*lanes 4* and *5*), and rats infused with glucosamine (*lanes 7* and *8*) were incubated for 60 min at 37°C in the presence (*lanes 2*, *4*, *6*, and *8*) or absence (*lanes 1*, *3*, *5*, and *7*) of a pool of glycosydases. The skeletal muscle extracts were immunoprecipitated with anti-INSR antibody, resolved by SDS-PAGE, transferred to nitrocellulose filters, and Western immunoblotted with a specific anti-type 1 IGF receptor antibody (anti-IGFRα). Band densities were quantified by scanning densitometry. A representative experiment is shown. IP, immunoprecipitation.

either diabetic or glucosamine-treated rats showed increased glycosylation as compared with their respective controls. Furthermore, to determine whether increased receptor glycosylation could alter the detectability of the hybrid receptors derived from diabetic or glucosamine-treated rats, we measured abundance of hybrid receptors after treatment of solubilized receptors with a pool of glycosydases that enzymatically cleaves both N- and O-linked oligosaccharides from glycoproteins. As shown in Fig. 10, treatment with glycosydases did not affect the capability of antibodies to immunoprecipitate and immunoblot hybrid receptors, thus ruling out the possibility that increased hybrid abundance observed in diabetic or glucosamine-treated rats was caused by an artifact due to hyperglycosylation.

DISCUSSION

Our recent observation that expression of insulin/IGF-I hybrid receptors is increased in skeletal muscle of type 2 diabetic patients (14) coupled with the accessibility of skeletal muscle from a carefully characterized animal model of diabetes has provided the rationale for studying the role of hyperglycemia on the formation of hybrid receptors. Several experimental models have been used to investigate the role of hyperglycemia in contributing to insulin resistance. In theory, the ideal study protocol for assessing the alterations induced by hyperglycemia per se would be an infusion of glucose in control animals. Hyperglycemia, however, promptly stimulates insulin secretion, thus leading to hyperinsulinemia that, in turn, may induce changes in intracellular glucose metabolism that are not directly related to the increased availability of the substrate. Therefore, in the present study, we used a rat model of diabetes characterized by moderate-to-severe hyperglycemia (obtained by surgical reduction of the β-cell mass), but not severe insulinopenia, as in the streptozotocin-induced model of diabetes (22). We found that in diabetic rats, the abundance of hybrid receptors is increased in skeletal muscle and is positively correlated with plasma glucose levels. If hyperglycemia contributes to increased hybrid receptor formation, one would expect that normalization of the plasma glucose, regardless of the means, would lead to a reduction in hybrid receptor abundance. To test this possibility, we determined hybrid receptor abundance in diabetic rats treated with phlorizin, a potent inhibitor of renal tubular glucose transport that blocks tubular glucose reabsorption when plasma glucose levels are increased above basal (21). In diabetic rats, phlorizin treatment leads to both normalization of plasma glucose levels and marked reduction in hybrid receptor abundance. These data suggest that glucose may play a major role in regulating hybrid receptor assembly in vivo.

The mechanism(s) by which hyperglycemia causes the observed increase in hybrid receptor formation is not understood, but several hypotheses can be proposed. One possibility is that enzymatic glycation of insulin and type 1 IGF receptors induced by the high concentrations of glucose may impair homologous receptor formation while increasing the rate of hybrid receptor synthesis. It is known that insulin and type 1 IGF receptor glycosylation precedes the formation of intra- and intersubunit disulfide bonds in the endoplasmic reticulum. Because it is likely that hybrid receptor assembly occurs in the endoplasmic reticulum where insulin and type 1 IGF receptor precursors become disulfide-linked to one another rather than to a homologous precursor, it is possible that hyperglycemia may facilitate hybrid receptor formation by affecting the spatial or temporal separation during the synthesis of the two receptor homodimers. Another possibility is that modifications in insulin receptor or type 1 IGF receptor number due to high glucose levels would result in an increase in hybrid receptor abundance. We found that both insulin receptor and type 1 IGF receptor content is increased in diabetic rats as compared with control rats. These results are consistent with previous studies showing that expression of insulin receptor and type 1 IGF

receptor is upregulated in experimental diabetes (23,24,35). Assuming that hybrid receptor assembly is proportional to the relative amount of insulin and type 1 IGF receptors expressed, it is possible that increased expression of type 1 IGF receptors may lead to the formation of a higher proportion of hybrid receptors that, in turn, might affect insulin sensitivity in skeletal muscle by sequestering insulin receptors in a less insulin-responsive form.

Several studies have shown that at least some of the regulatory effects of glucose are mediated by the glucosamine biosynthetic pathway (36,37). The ultimate metabolic products derived from this pathway are obligatory intermediates in the glycosylation and processing of glycoproteins in both the endoplasmic reticulum and the Golgi system and may play a significant role in regulating the assembly of multimeric proteins. Thus, we sought to address the role of the glucosamine pathway in hybrid receptor formation. We reasoned that if activation of the glucosamine pathway plays a role in hybrid receptor formation, hybrid receptor abundance should be modified in rats infused with glucosamine as compared with control rats. According to this line of reasoning, we found that hybrid receptor content was increased in glucosamine-treated rats, although to a lesser extent, compared with that found in diabetic rats. These observations suggest that the effects of hyperglycemia on hybrid receptor formation are mediated, at least in part, by the glucosamine pathway. How the increased routing of glucose through the glucosamine-biosynthetic pathway may regulate hybrid receptor assembly is unknown. The effects of glucosamine infusion on hybrid receptor abundance occur very rapidly, suggesting post-translational mechanisms. One possibility is that a rapid interconversion occurs between insulin and IGF-I receptors leading to the formation of hybrid receptors after their initial biosynthesis. However, previous studies have shown that subunit exchange did not occur among the fully assembled insulin holoreceptors (38), thus making the possibility that the process of hybrid receptor assembly is reversible unlikely. Alternatively, it is possible to speculate that glucosamine affects hybrid receptor formation by altering the glycosylation pattern of intracellular proteins. It is now clear that the oligosaccharide moieties of glycoproteins have a variety of functions including regulation of folding of nascent protein in the endoplasmic reticulum, protection of the underlying proteins from the action of proteases, and participation as specific recognition molecules in the intracellular targeting of proteins (39). Recently, it has been shown that glucosamine increases O-linked glycosylation of skeletal muscle proteins in vivo (20). Because proteins modified by O-linked addition of glucosamine are multimeric, it is possible that these modifications may have an important role in regulating processing and assembly of large protein complexes such as insulin and type 1 IGF receptors. Previous in vitro studies have shown that short-term treatment of cells with glucosamine affects insulin proreceptor processing and alters the electrophoretic mobility of both the proreceptor and the processed α-subunit (17). Consistent with these observations, we found that insulin and hybrid receptors derived from both diabetic and glucosamine-treated rats are more glycosylated as compared with their respective controls. Our results strongly suggest that the glucose/glucosamine pathway is capable of posttranslationally affecting insulin and the type 1 IGF receptor, thus confirming the role of glycosylation (possibly O-linked enzymatic glycosylation) as a regulative mechanism of glucose metabolism.

The pathophysiological role of hybrid receptors is still unclear. Because hybrid receptors display functional properties similar to those of the type 1 IGF receptor, it is possible to speculate that the upregulation of hybrid receptors induced by hyperglycemia through the glucosamine pathway may be adaptive, perhaps protecting muscle cells from excessive insulin-induced glucose entry. On the other hand, the upregulation of hybrid receptors in tissues such as vascular smooth muscle cells may increase the sensitivity to the mitogenic effects of IGF-I, thus contributing to diabetic vascular complications.

In conclusion, we found that in partially pancreatectomized diabetic rats, elevated glucose levels are associated with an increased abundance of hybrid receptors in skeletal muscle. Correction of hyperglycemia with phlorizin completely reverses changes in hybrid receptor content. Glucosamine infused into control rats mimics the effects of hyperglycemia on hybrid receptor formation. Taken together, the present and previous results support the hypothesis that glucose regulates, possibly through a posttranslational mechanism mediated by the glucosamine pathway, hybrid receptor assembly.

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