

# Impaired Glucose Transport and Insulin Receptor Tyrosine Phosphorylation in Skeletal Muscle From Obese Women With Gestational Diabetes

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Women who develop gestational diabetes mellitus (GDM) have severe insulin resistance and markedly increased risk to develop subsequent type 2 diabetes. We investigated the effects of pregnancy and GDM on glucose transport activity and the expression and phosphorylation of the insulin receptor and insulin receptor substrate (IRS)-1 in human skeletal muscle fiber strips in vitro. Rectus abdominis muscle biopsies were obtained at the time of cesarean section from 11 pregnant women with normal glucose tolerance (pregnant control), 7 pregnant women with GDM, and 11 non-pregnant women undergoing elective surgery (non-pregnant control). Subjects were matched for age and similar degree of obesity. The rate of maximal insulin ( $10^{-7}$  mol/l)-stimulated 2-deoxyglucose transport was reduced by 32% ( $P < 0.05$ ) in muscle strips from the pregnant control group and even further in GDM subjects by 54% ( $P < 0.05$  vs. pregnant control). The maximal effect of insulin on tyrosine phosphorylation of the insulin receptor was 37% lower ( $P < 0.05$ ) in GDM subjects than in pregnant control subjects and was not related to changes in the abundance of the insulin receptor. Compared with nonpregnant control subjects, maximal insulin-stimulated IRS-1 tyrosine phosphorylation was significantly lower by  $59 \pm 24\%$  (mean  $\pm$  SD) ( $P < 0.05$ ) and  $62 \pm 28\%$  ( $P < 0.05$ ) in pregnant control and GDM subjects, respectively. This was reflected by a 23% ( $P < 0.05$ ) and 44% ( $P < 0.002$ ) reduction in IRS-1 protein levels in muscle from pregnant control and GDM subjects. Both pregnant control and GDM subjects exhibited a 1.5- to 2-fold increase in the levels of IRS-2 ( $P < 0.01$ ) and p85 $\alpha$  regulatory subunit of phosphatidylinositol (PI) 3-kinase ( $P < 0.05$ ), despite reduced glucose transport activity. These data indicate that insulin resistance to glucose transport during pregnancy is uniquely associated with a decrease in IRS-1 tyrosine phosphorylation, primarily due to decreased expression of IRS-1 protein. However, in GDM subjects, a decrease in tyrosine phosphorylation

of the insulin receptor  $\beta$ -subunit is associated with further decreases in glucose transport activity. Thus, impaired insulin receptor autophosphorylation is an important early distinction underlying muscle insulin resistance in young women with GDM, and it may underlie future risk for the development of type 2 diabetes. *Diabetes* 48:1807–1814, 1999

**N**ormal human pregnancy is associated with hyperinsulinemia and a progressive decline in insulin sensitivity. Studies using the hyperinsulinemic-euglycemic clamp technique demonstrate that a 40–60% reduction in whole body glucose disposal occurs from early to late pregnancy (1,2). In 3–5% of pregnant women, gestational diabetes mellitus (GDM), defined as abnormal glucose tolerance, develops, usually during the 3rd trimester, and is associated with an increase in neonatal morbidity and mortality (3). Women diagnosed with GDM appear to have abnormalities in insulin secretion that contribute to the development of GDM (4–6). Some investigators have shown more pronounced insulin resistance in GDM patients compared with women with normal glucose tolerance during pregnancy (7–9), which may contribute to hyperglycemia, in addition to defects in insulin secretion. Although diabetes usually remits after pregnancy, up to 50% of women diagnosed with GDM go on to develop type 2 diabetes later in life, particularly if obesity is present (10–12). GDM shares many of the characteristics of type 2 diabetes. Both are aggravated by increasing obesity and age, suggesting that the components of insulin resistance and decreased insulin secretion, which lead to GDM, may be common to type 2 diabetes. Thus, GDM may represent an unmasking of the genetic predisposition toward type 2 diabetes induced by the hormonal changes of pregnancy.

With regard to human skeletal muscle, there is no information on glucose transport or postreceptor defects in insulin signaling in pregnancy or GDM. The initial mechanism of insulin signaling involves insulin binding to its cell surface receptor, leading to autophosphorylation and activation of the  $\beta$ -subunit as a tyrosine kinase toward other protein substrates (13–15). Phosphorylation of insulin receptor substrate-1 (IRS-1) and IRS-2 on tyrosine residues after insulin treatment is important in coupling the insulin receptor signal to glucose uptake. For example, in mice with a gene knock-out of IRS-1, there is a 50% reduction in insulin-stimulated glucose transport in skeletal muscle and adipose tissue (16,17). IRS-1 phosphorylation results in the binding and phospho-

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BSA, bovine serum albumin; ECL, enhanced chemiluminescence; FFM, fat-free mass; GDM, gestational diabetes mellitus; IRS, insulin receptor substrate; KHB, Krebs-Henseleit buffer; OGTT, oral glucose tolerance test; PI, phosphatidylinositol; PLSD, protected least significant difference; TBS-T, 0.05% Tween 20.

rylation of the regulatory subunit (p85 $\alpha$ ) of phosphatidylinositol (PI) 3-kinase to IRS-1 (18,19). Binding of the p85 $\alpha$  isoform to tyrosine-phosphorylated IRS-1 results in increased catalytic activity of the PI 3-kinase complex (20) and appears to be necessary, although not sufficient, for stimulating glucose transport in 3T3-L1 adipocytes (21,22).

The levels of skeletal muscle GLUT4 protein are normal in obese subjects with GDM (23) and in the majority of subjects with type 2 diabetes (23–25). In type 2 diabetes, hyperglycemia is associated with reduced glucose transport and impaired insulin receptor tyrosine kinase activity in skeletal muscle (25–30). In morbidly obese subjects, this defect is accompanied by decreased expression of the insulin receptor, IRS-1, and p85 $\alpha$  subunit of PI 3-kinase (31). However, in skeletal muscle from lean type 2 diabetic subjects, these proteins were not significantly altered, despite reduced tyrosine phosphorylation by insulin (32). The purpose of the present study, therefore, was to examine the cellular mechanisms for insulin resistance in skeletal muscle from obese pregnant control and obese GDM subjects during late pregnancy. We hypothesized that pregnancy-induced insulin resistance might result from impaired activity or altered expression of one or more of the proteins in the insulin signaling cascade, while GDM might be associated with additional abnormalities that further exacerbate insulin resistance and unmask defects that are characteristic of type 2 diabetes.

In the present study, we investigated insulin receptor signaling in normal pregnancy and GDM, including glucose transport activity, using human skeletal muscle fibers *in vitro*. We also examined the levels of IR $\beta$ , IRS-1, IRS-2, and p85 $\alpha$ , together with the levels of insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1. Our results indicate that pregnancy is uniquely associated with impaired muscle glucose transport activity and decreased IRS-1 expression and phosphorylation. In GDM subjects, the decrease in muscle glucose transport is exacerbated and appears to be associated with a reduction in insulin receptor tyrosine phosphorylation.

## RESEARCH DESIGN AND METHODS

**Materials.** 2-[1,2-<sup>3</sup>H(N)]Deoxy-D-glucose (26.2 Ci/mmol) was obtained from DuPont-NEN (Boston, MA). [<sup>14</sup>C]D-Sorbitol (317 mCi/mmol) was obtained from Sigma (St. Louis, MO). Sodium vanadate was purchased from Fisher Scientific (Pittsburgh, PA). Unless otherwise stated, all other chemicals were obtained from Sigma. Protein A-Sepharose was from Pierce Chemical (Rockford, IL). Reagents for SDS-PAGE and protein assay kits were from Bio-Rad Laboratories (Richmond, CA). The Immobilion-P membranes were from Millipore (Bedford, MA). The antiphosphotyrosine antibody, IRS-2, and p-85 $\alpha$  antibodies were purchased from Upstate Biotechnology (Lake Placid, NY), and anti-insulin receptor  $\beta$  and IRS-1 antibodies were from Transduction Laboratories (Lexington, KY).

**Human subjects.** We studied 11 obese nonpregnant subjects (nonpregnant control), 11 obese pregnant patients with normal glucose tolerance (pregnant control), and 7 obese pregnant patients, diagnosed with GDM during the 3rd trimester (GDM). Subjects were recruited as outpatient volunteers. Pregnant subjects scheduled for cesarean delivery were classified as normal or GDM after a 100-g oral glucose tolerance test (OGTT) according to National Diabetes Data Group criteria (33). The OGTT was administered between 26–28 weeks of gestation. Subjects diagnosed with GDM were treated with diet therapy before delivery. None of the pregnant control subjects had a family history (first- or second-degree relative) of diabetes. Nonpregnant patients undergoing gynecological surgery were recruited as nonpregnant control subjects. None of the nonpregnant control subjects were classified as having diabetes based on a 75-g OGTT (33). Of the 11 nonpregnant subjects, 4 had a positive family history for diabetes. However, none of the nonpregnant control subjects had any diseases or had taken any medications known to alter carbohydrate metabolism for at least 3 months prior to the study. The experimental protocol was approved by the Institutional Review board at MetroHealth Medical Center and the Scientific Advisory Com-

mittee of the General Clinical Research Center at Case Western Reserve University. Written informed consent was obtained from all subjects before enrollment into the study.

**Experimental protocol.** Before surgery subjects were admitted to the General Clinical Research Center for estimation of body composition by underwater weighing ( $n = 17$ ) or anthropometric methods ( $n = 10$ ) as described previously (34,35). In order to investigate the relationship between physiological estimates of insulin resistance *in vivo* and muscle glucose transport *in vitro*, a 2-h hyperinsulinemic-euglycemic clamp ( $40 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ ) was performed in a subset of subjects either before cesarean section ( $n = 5$  pregnant control, 1 GDM) or after elective gynecological surgery ( $n = 7$  nonpregnant control). Pregnant subjects were studied between 36 and 40 weeks of gestation, and nonpregnant control subjects were studied at least 6–8 weeks postsurgery. The glucose infusion rate, corrected for fat-free mass, was used as the estimate of insulin sensitivity. The specific details of the insulin clamp procedure in pregnancy have been described previously (36).

**Muscle biopsies.** Each subject was given a standard weight-maintenance diet to be followed for 2 weeks before surgery. The composition of the diet was identical to that used in the treatment of GDM subjects. Subjects were admitted to the hospital on the morning of surgery, having been instructed to fast overnight. A blood sample was obtained just before surgery for measurement of glucose and insulin levels. The pregnant patients received a continuous lumbar epidural infusion of local anesthetic. In nonpregnant control patients, general anesthesia was induced with nitrous oxide–oxygen mixture, except for two patients who received epidural anesthesia. Only saline was given intravenously before the biopsy. The experimental protocol for muscle biopsy and preparation of muscle fiber strips was performed as described previously by Dohm et al. (25), with minor modifications. After opening the abdominal wall and fascia, prior to delivery of the fetus, two pairs of hemostats fixed together were placed on the lower rectus abdominis muscle 3 cm apart. A muscle biopsy ( $3 \times 2 \times 0.5 \text{ cm}$ ) weighing  $\sim 3.5 \text{ g}$  was excised on either side of the clamps. The muscle sample was immediately placed in 150 ml Krebs-Henseleit buffer (KHB) pre-gassed with 95:5 ( $\text{O}_2/\text{CO}_2$ ) at room temperature. The biopsy was transported directly back to the laboratory where up to 20 muscle fiber strips weighing  $\sim 25 \text{ mg}$  each were teased from the muscle sample. The muscle fiber strips were mounted on Lucite clips 12 mm wide, before being cut from the muscle sample. After preparation, the fibers were placed in a specially designed chamber containing 20 polycarbonate wells (16 mm width, 25 mm depth) each filled with 4 ml KHB, 1.0 mmol/l pyruvate, and 1% bovine serum albumin (BSA). The lid to the chamber was fitted with tubing so that each well received continuous gassing with 95:5 ( $\text{O}_2/\text{CO}_2$ ) in a shaking water bath maintained at 29°C. The remaining muscle tissue not used for incubation was frozen in liquid nitrogen.

**Glucose transport measurements and muscle phosphorylation protocol.** After 30 min of preincubation, basal and maximal insulin-stimulated glucose transport were measured in the absence or presence of  $10^{-7} \text{ mol/l}$  insulin as previously described (37). Muscle biopsies were large enough to accommodate treatments in triplicate. To measure glucose transport activity, the pre-incubation medium was removed and replaced with fresh KHB containing 5 mmol/l 2-deoxyglucose, 20 mmol/l sorbitol, 0.4  $\mu\text{Ci/mol}$  [<sup>3</sup>H]2-deoxyglucose, 0.005  $\mu\text{Ci/mmol}$  [<sup>14</sup>C]sorbitol, with or without  $10^{-7} \text{ mol/l}$  insulin. The media was gassed continuously using 95%  $\text{O}_2$ , 5%  $\text{CO}_2$ . Transport was measured at 29°C for 1 h. Afterwards, the samples were removed and washed twice by incubating for 5 min in ice cold KHB, blotted, weighed, and solubilized overnight in a 0.5-ml mixture of 0.32 mol/l hexaethyltrimethyl ammonium bromide, 0.29 mol/l potassium bromide, and 1:1 mixture of MeOH and  $\text{H}_2\text{O}$  at 30°C. After digestion, 5 ml of Cryoscient liquid scintillation fluid (ICN, Costa Mesa, CA) was added to the solubilized muscle sample and to 50  $\mu\text{l}$  samples obtained from each incubation well. All samples were counted for radioactivity in a Beckman LS 8100 Liquid Scintillation Counter (Fullerton, CA), with dual quench correction. The rate of <sup>3</sup>H-2-deoxyglucose transport was expressed in nanomoles per milligram wet weight per minute, after correction for extracellular <sup>3</sup>H-2-deoxyglucose.

Insulin-induced tyrosine phosphorylation of the insulin receptor and IRS-1 was measured according to the protocol of Goodyear et al. (31) with minor exceptions. We tested a series of time points of 5, 15, and 30 min for collection of the muscle sample after insulin stimulation in samples from pregnant and nonpregnant control subjects. We found a maximally effective stimulation of insulin receptor and IRS-1 phosphorylation occurred at 15 min using  $10^{-7} \text{ mol/l}$  insulin (J.E.F., T.L., unpublished data). Additional muscle strips from each subject were preincubated in KHB containing 1% BSA and 1.0 mmol/l pyruvate for 30 min at 29°C, followed by removal of the media and replacement with KHB, 1.0 mmol/l pyruvate, with and without insulin ( $10^{-7} \text{ mol/l}$ ) for up to 15 minutes (maximum stimulation). At the end of incubation, the strips were removed and immediately frozen between two blocks of dry ice. Three muscle strips from each treatment (basal, insulin) were pooled together and stored at  $-70^\circ\text{C}$  until analysis.

**Immunoprecipitation and tyrosine phosphorylation of the insulin receptor and IRS-1.** The frozen samples were pulverized in liquid nitrogen and homogenized immediately under denaturing conditions using a Polytron PTA

20S generator (Kinematica, Switzerland) at maximum speed for 30 s in 10× volume ice cold homogenization buffer. The samples were run in pairs with muscles from nonpregnant control subjects paired with either pregnant control subjects or GDM patients and homogenized and processed on the same gel. The homogenization buffer contained phosphatase and protease inhibitors (50 mmol/l HEPES, pH 7.5, 100 mmol/l Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 10 H<sub>2</sub>O, 100 mmol/l NaF, 10 mmol/l EDTA, and 10 mmol/l Na<sub>3</sub>VO<sub>4</sub> plus aprotinin (2 µg/ml), leupeptin (10 µg/ml), pepstatin (0.5 µg/ml) PMSF (34 µg/ml), and 1% Triton-X 100. The homogenate was allowed to sit on ice for 30 min at 4°C, followed by centrifugation at 38,000 rpm (150,000g) in a 70 Ti rotor (Beckman Instruments) at 4°C for 30 min to remove insoluble material. The supernatant was collected and assayed for protein concentration (Bradford dye assay; Bio-Rad Chemicals, Hercules, CA) using crystalline BSA and standard. For immunoprecipitation, 4 mg of muscle protein was incubated overnight at 4°C with an antiphosphotyrosine antibody (5 µg Ab/8 mg protein) in 1 ml immunoprecipitation buffer containing 2% Triton-X-100, 300 mmol/l NaCl, 20 mmol/l Tris-HCl, 2 mmol/l EDTA, 2 mmol/l EGTA, 0.4 mmol/l PMSF, 0.4 mmol/l sodium vanadate, and 1% NP-40. After immunoprecipitation, the samples were mixed with 50 µl of Protein-A Sepharose (10% solution) for 4 h at 4°C and the immunoprecipitate was washed in 1 ml immunoprecipitation buffer, followed by centrifugation at 500g for 1 min at 4°C, repeated four times. The washed precipitate was mixed with Laemmli sample buffer (50 µl) and boiled for 5 min, centrifuged for 5 min at 500g and the supernatant (20 µl) separated on a 7% Tris polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Mini-Protein gel apparatus. Proteins were electrotransferred from the gel to Immobilon-P membrane at 100 V (constant current) for 2 h using a minitransfer apparatus (Idea Scientific, Minneapolis, MN). Gels were stained with 2% Coomassie blue to ensure equal protein transfer. To reduce nonspecific protein binding, the membrane was blocked using 5% nonfat dry milk (1% BSA in the case of anti-PY antibody) in buffer containing 10 mmol/l Tris-HCl, 150 mmol/l NaCl, with 0.05% Tween 20 (TBS-T). The membranes were incubated with anti-insulin receptor β (0.4 µg/ml), or IRS-1 antibody (1.5 µg/ml) in blocking buffer for 4 h at 22°C, followed by extensive washing with TBS-T. At the end of the final wash, the membranes were incubated with secondary antibody linked to horseradish peroxidase in 10 ml of blocking buffer for 1 h at 22°C and washed again before exposing the blots to enhanced chemiluminescence (ECL) reagent according to the manufacturer's instructions (Amersham, Arlington Heights, IL). Autoradiography was carried out using Kodak XAR X-ray film. After treatment with the ECL reagent, the exposure time was varied from 1–3 min and each exposure was quantified by scanning densitometry. The specific band intensities were quantified using a Digiscan scanner (US Biochemical, Cleveland, OH). The data were expressed as arbitrary scanning units depicting the net effect of insulin on phosphorylation above basal, assigning a value of 100 to the average increase for the nonpregnant control subjects. The values for insulin-treated muscles from pregnant control and GDM subjects were expressed relative to the 100% value.

**Western blot of the insulin receptor, IRS-1, IRS-2, and p85α.** To determine the cellular content of insulin signaling proteins, muscle protein extracts were prepared from samples frozen in liquid nitrogen at the time of surgery without incubation. A total of 120 mg of frozen muscle was powdered in liquid nitrogen and homogenized for 1 min in 1.5 ml of homogenization buffer containing 25 mmol/l HEPES, 1 mmol/l EDTA, 150 mmol/l dithiothreitol, 10 mg/ml leupeptin, aprotinin, pepstatin, and PMSF (in ETOH), pH 7.4. The samples were centrifuged using a 70 Ti rotor at 150,000g (38,000 rpm) at 4°C for 75 min and the pellet was resuspended in 1.5 ml of homogenization buffer. The proteins were measured by using the Bradford assay, and 75 µg was resolved on 7% SDS-PAGE gel, transferred to Immobilon-P membrane, and detected with polyclonal antibodies against IRβ (COOH-terminus), IRS-1, IRS-2, and PI 3-kinase (p85α subunit), and secondary antibody horseradish peroxidase IgG using the ECL reagent as outlined above. Each gel contained an internal control standard—a muscle protein preparation from a single control patient that was run on every blot. Autoradiography was carried out using Kodak XAR X-ray film. After treatment with the ECL reagent, the exposure time was varied from 1–3 minutes and each exposure was quantified by densitometry. Each sample was run an average of 3 separate assays using different minigels containing the same internal control and the results normalized to the control value.

**Other assays and statistical analysis.** Plasma glucose was measured by the glucose oxidase method with a glucose analyzer (Yellow Springs, OH). Serum insulin was measured using a double antibody radioimmunoassay. The intra- and interassay coefficients of variation were 5 and 6%, respectively. Results are presented as means ± SD for the indicated number of patients. The data were analyzed by analysis of variance and Fisher's protected least significant difference (PLSD) testing for post hoc analysis between groups. A paired *t* test was used to test for significant differences between basal and insulin-stimulated glucose transport. Linear regression was used for correlation analysis using StatView 4.02 (Abacus Concepts, Berkeley, CA). Chi-square was used to analyze differences in parity. Statistical significance was set at *P* < 0.05.

TABLE 1  
Subject characteristics

	Nonpregnant control subjects	Pregnant control subjects	GDM subjects
<i>n</i>	11	11	7
Age (years)	36.4 ± 2.0	34.4 ± 1.4	33.6 ± 2.2
Body fat (%)	37.0 ± 2.3	31.7 ± 2.4	37.2 ± 2.3
Fasting glucose (mg%)	89 ± 4	78 ± 2*	91 ± 4
Fasting insulin (µU/ml)	10.8 ± 2.1	13.1 ± 2.1	32.1 ± 9.7†
Parity ( <i>n</i> )	1.6 ± 0.5	1.6 ± 0.3	1.3 ± 0.4

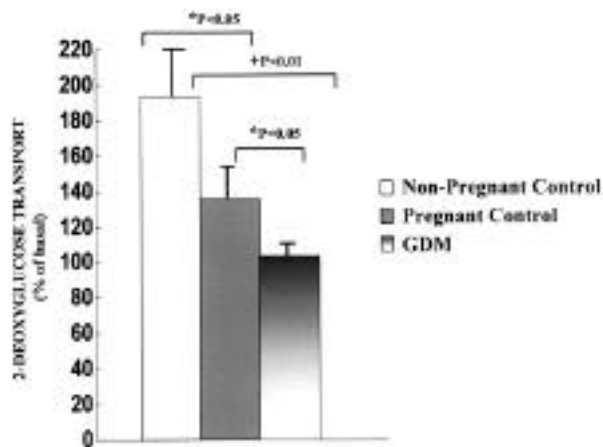
Data are means ± SE. \**P* < 0.05, significantly less than nonpregnant control and GDM subjects; †*P* < 0.05, significantly greater than nonpregnant and pregnant control subjects.

## RESULTS

**Subject characteristics and glucose transport studies.** Subjects were closely matched in age, parity, and degree of obesity (Table 1). All subjects were considered obese, inasmuch as the average percent fat for all three groups was >30%. Fasting glucose levels in pregnant control subjects were lower compared with nonpregnant control and GDM subjects (*P* < 0.05). GDM patients had normal fasting glucose levels similar to the nonpregnant control subjects. Basal insulin levels were significantly higher in GDM compared with pregnant and nonpregnant control subjects (*P* < 0.05).

Glucose transport was measured using 2-deoxyglucose in isolated strips of rectus abdominus muscle obtained at the time of surgery. The rate of maximal insulin-stimulated glucose transport activity increased by 193 ± 16% above basal in nonpregnant control subjects (*P* < 0.05). In pregnant control subjects maximal insulin-stimulated glucose transport was lower by 32% compared with nonpregnant control subjects (*P* < 0.05), and lower still further in GDM subjects by 54% (*P* < 0.05 vs. pregnant control) (Fig. 1). In the absence of insulin, the basal transport of 2-deoxyglucose was not different between groups.

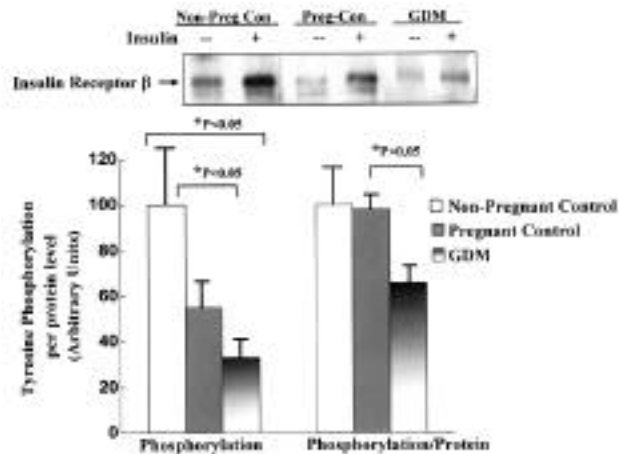
**Effect of pregnancy and GDM on skeletal muscle insulin receptor and IRS-1 tyrosine phosphorylation.** To determine whether impaired glucose transport in muscle from pregnant control and GDM subjects was associated with an abnormality in insulin receptor or IRS-1 signaling, the levels of tyrosine phosphorylation were studied in incubated skeletal muscle. Muscles were incubated in the absence and presence of a maximal insulin concentration, homogenized, and processed by immunoprecipitation followed by SDS-PAGE. A representative autoradiogram, seen in Fig. 2, shows levels of basal and insulin-stimulated insulin receptor tyrosine phosphorylation in a single nonpregnant control, pregnant control, and GDM subject. After insulin treatment, maximal insulin receptor tyrosine phosphorylation increased by ~5.2-fold (range 3.7–6.8 fold) over basal in nonpregnant control subjects. In pregnant control subjects, the net increase above basal was significantly lower by 45 ± 17% (mean ± SD) compared with nonpregnant control subjects (*P* < 0.05). In GDM subjects, the maximal insulin receptor tyrosine phosphorylation was also significantly lower by 67 ± 10% (*P* < 0.05) compared with nonpregnant control subjects. To



**FIG. 1.** Effect of insulin on maximal 2-deoxyglucose transport activity in skeletal muscle fiber strips from nonpregnant control, pregnant control, and GDM subjects. Rectus abdominus muscle biopsies were obtained during gynecological surgery or at the time of scheduled cesarean-section delivery as described in METHODS. Multiple muscle fiber strips from each subject were incubated in KHB with and without maximal insulin ( $10^{-7}$  mol/l) concentration for 1 h as outlined in METHODS. Basal and insulin-stimulated glucose transport was measured in triplicate samples from each patient, and the results were expressed as percent increase above basal transport. The basal rates were not significantly different between groups ( $2.8 \pm 0.3$ ,  $2.6 \pm 0.2$ , and  $3.0 \pm 0.3$  ng  $\cdot$  mg $^{-1}$   $\cdot$  min $^{-1}$  for nonpregnant control, pregnant control, and GDM subjects, respectively). The data were analyzed by analysis of variance and Fisher's PLSD testing for post hoc analysis between groups. Data are means  $\pm$  SE for nonpregnant control ( $n = 5$ ), pregnant control ( $n = 6$ ), and GDM ( $n = 4$ ) subjects.

examine the relationship between insulin receptor tyrosine phosphorylation and changes in protein content, we expressed the maximal tyrosine phosphorylation as a function of the amount of insulin receptor protein. The nonpregnant and pregnant control subjects had a similar level of IR $\beta$  phosphorylation per amount of receptor protein. However, in the GDM subjects, the insulin receptor phosphorylation was significantly lower by 37% ( $P < 0.05$ ) compared with either pregnant control or nonpregnant subjects, after adjustment for protein content. This suggests that the decreased tyrosine phosphorylation in GDM subjects may be related to an intrinsic defect in the receptor rather than to changes in receptor protein content.

Figure 3 is an autoradiogram example showing the effect of insulin on IRS-1 tyrosine phosphorylation in skeletal muscle from a single nonpregnant control, pregnant control, and GDM subject. In nonpregnant control subjects, acute insulin treatment led to a  $5.3 \pm 1.3$  fold (mean  $\pm$  SD) increase in IRS-1 tyrosine phosphorylation above basal levels (range from 3.6- to 7.2-fold). Compared with nonpregnant control subjects, maximal insulin-stimulated IRS-1 tyrosine phosphorylation was significantly lower by  $59 \pm 24\%$  ( $P < 0.05$ ) and  $62 \pm 28\%$  ( $P < 0.05$ ) in pregnant control and GDM subjects, respectively. To examine the relationship between IRS-1 tyrosine phosphorylation and changes in protein content, we expressed the tyrosine phosphorylation as a function of IRS-1 protein content. After normalizing for changes in IRS-1 protein content, there was no significant decrease in maximal tyrosine phosphorylation of IRS-1 in pregnant control or GDM subjects. **Effect of pregnancy and GDM on insulin receptor, IRS-1, IRS-2, and p85 $\alpha$  protein content.** To determine whether



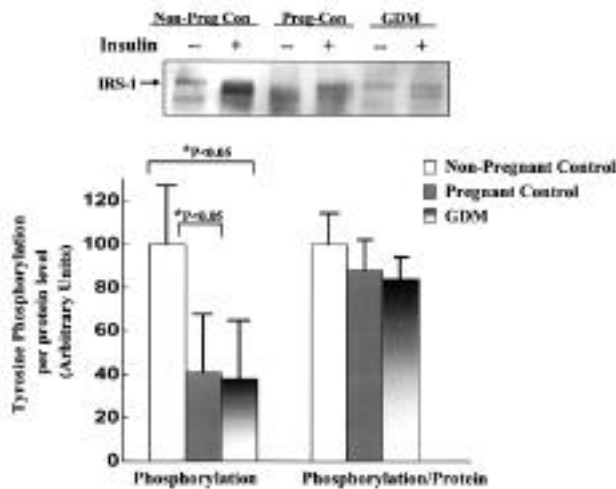
**FIG. 2.** Effect of insulin on maximal tyrosine phosphorylation of insulin receptor (IR $\beta$ ) in skeletal muscle in vitro from nonpregnant control, pregnant control, and GDM subjects. Rectus abdominus muscle biopsies were obtained as outlined in Fig. 1 and in METHODS. Muscle fiber strips were incubated in the absence (--) or presence (+) of maximal insulin ( $10^{-7}$  mol/l) concentration for 15 min. Triplicate muscle samples from each condition were frozen immediately on dry ice and later homogenized under denaturing conditions at 4°C. Equal aliquots of protein were immunoprecipitated with antiphosphotyrosine antibodies and protein A-Sepharose. The immunoprecipitated proteins were run on SDS-PAGE, transferred to membranes, and analyzed by Western blotting with anti-IR $\beta$  antibody as outlined. The image shown is a sample autoradiogram showing tyrosine phosphorylation from a single subject in each group. The bar shows quantification of the autoradiograms from multiple subjects. The data are expressed as arbitrary units relative to the net effect of insulin in nonpregnant control subjects, assigning a value of 100 to the result. The bars on the right show the net effect of insulin on IR $\beta$  tyrosine phosphorylation divided by the IR protein content for each of the subjects as determined by Western blot analysis. The data were analyzed by analysis of variance and Fisher's PLSD testing for post hoc analysis between groups. Data are means  $\pm$  SD for nonpregnant control ( $n = 5$ ), pregnant control ( $n = 6$ ), and GDM subjects ( $n = 4$ ).

the decrease in insulin receptor or IRS-1 phosphorylation in muscles from pregnant control and GDM subjects was associated with an abnormality in expression of these proteins, we measured the relative abundance of several proteins by Western blot analysis. A representative autoradiogram is shown in Fig. 4 for the insulin receptor and IRS-1. The cellular content of insulin receptor protein was highly variable in human muscle. When the results from all patients were tabulated by densitometry, the overall mean values were similar in all three groups. The levels of IRS-1 were more consistent. Compared with nonpregnant control subjects, the level of IRS-1 was significantly reduced by  $22 \pm 6\%$  ( $P < 0.05$ ) in pregnant control subjects and  $44 \pm 5\%$  in GDM subjects ( $P < 0.001$ ). There was no significant difference in IRS-1 content between pregnant control and GDM subjects ( $P = 0.08$ ). To determine whether pregnancy and GDM are associated with changes in the amount of PI 3-kinase protein, we immunoblotted skeletal muscle samples with an antibody that recognizes the p85 regulatory subunit of the PI 3-kinase enzyme. There was a large amount of variability in p85 $\alpha$  among the pregnant control and GDM subjects; however, the overall mean data showed a 1.5–2 fold increase ( $P < 0.05$ ) in levels of p85 $\alpha$  protein in muscles from pregnant control and GDM subjects compared with nonpregnant control subjects. The levels of

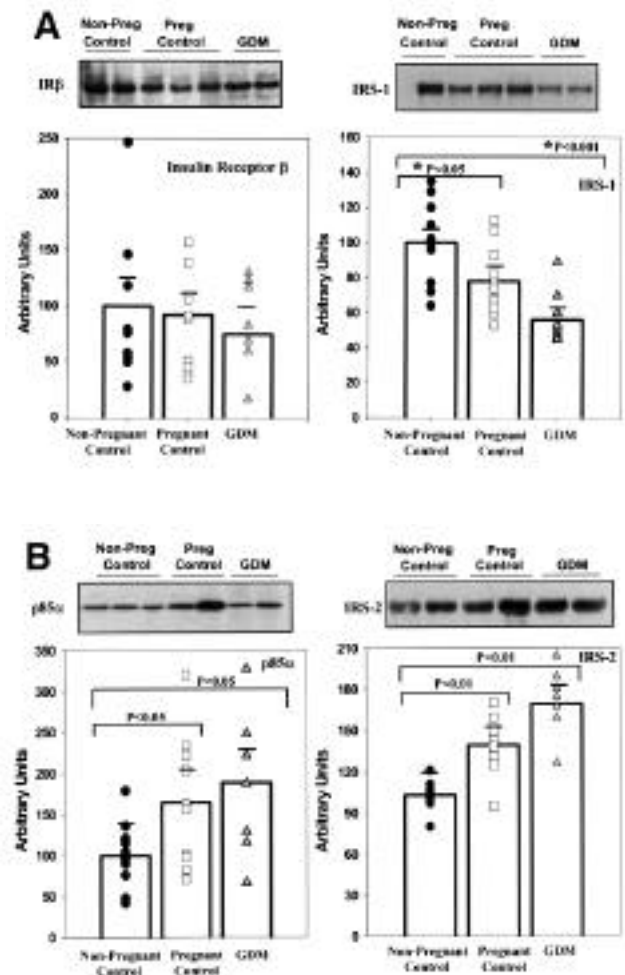
IRS-2 were immunoblotted and found to be increased in skeletal muscle from GDM subjects ( $P < 0.01$ ) (Fig. 4).

**Effect of pregnancy and GDM on glucose disposal.** Insulin-stimulated glucose disposal and estimates of endogenous (primarily hepatic) glucose production were made under basal conditions and during a hyperinsulinemic-euglycemic clamp in a subset of subjects: seven nonpregnant control subjects, five pregnant control subjects, and one GDM subject. Basal (mean  $\pm$  SD) endogenous glucose production was  $3.00 \pm 0.46 \text{ mg} \cdot \text{kg}^{-1} \text{ fat-free mass (FFM)} \cdot \text{min}^{-1}$  for pregnant control subjects;  $3.23 \pm 0.32 \text{ mg} \cdot \text{kg}^{-1} \text{ FFM} \cdot \text{min}^{-1}$  for nonpregnant control subjects; and  $3.47 \text{ mg} \cdot \text{kg}^{-1} \text{ FFM} \cdot \text{min}^{-1}$  in the GDM patient. There was essentially complete suppression ( $94 \pm 5\%$ ) of endogenous glucose production in all subjects with insulin infusion during the clamp: pregnant control subjects  $93 \pm 4\%$ , nonpregnant control subjects  $95 \pm 5\%$ , and GDM 85%. The glucose infusion rate during the clamp required to maintain euglycemia averaged  $6.20 \pm 2.86 \text{ mg} \cdot \text{kg}^{-1} \text{ FFM} \cdot \text{min}^{-1}$  for all subjects: nonpregnant control subjects  $6.6 \pm 3.7 \text{ mg} \cdot \text{kg}^{-1} \text{ FFM} \cdot \text{min}^{-1}$ , pregnant control subjects  $6.11 \pm 1.45 \text{ mg} \cdot \text{kg}^{-1} \text{ FFM} \cdot \text{min}^{-1}$ , and GDM  $3.97 \text{ mg} \cdot \text{kg}^{-1} \text{ FFM} \cdot \text{min}^{-1}$ . Statistical analysis among the three groups was not performed because of only one subject with GDM. However, we did assess the correlation between various estimates of insulin sensitivity and insulin signaling proteins using linear regression analysis. There was a positive trend between the M value, as measured during the hyperinsulinemic-euglycemic

clamp, and maximal insulin-stimulated 2-deoxyglucose uptake ( $r = 0.62$ ,  $P = 0.10$ ,  $n = 8$ ) and between the M value and IRS-1 phosphorylation ( $r = 0.67$ ,  $P = 0.10$ ,  $n = 7$ ). A power analysis suggests that nine more subjects would have been necessary to reach statistical significance. There was no significant correlation found between fasting insulin concentration and the levels of IRS-1 ( $r = -0.35$ ,  $P = 0.08$ ,  $n = 26$ ) or between percent fat and IRS-1 content ( $r = 0.15$ ,  $P = 0.49$ ,  $n = 23$ ). When IR $\beta$  phosphorylation was correlated with insulin levels, there was a trend but no significant relationship ( $r = -0.49$ ,  $P = 0.06$ ,  $n = 15$ ). An additional 15 subjects would be needed to reach statistical significance. The correlation between the rate of 2-deoxyglucose uptake versus IRS-1 was low ( $r = 0.46$ ,  $P = 0.07$ ,  $n = 16$ ). Power calculations indicated 18 additional subjects would be needed to reach statistical significance.



**FIG. 3.** Effect of insulin on maximal tyrosine phosphorylation of IRS-1 in skeletal muscle in vitro from nonpregnant control, pregnant control, and GDM subjects. Muscle biopsies were treated and processed as outlined in Fig. 2. The immunoprecipitated proteins were analyzed by Western blotting using anti-IRS-1 antibody and subjected to autoradiography. The image shows a representative autoradiogram of one subject from each group. The tyrosine-phosphorylated band corresponding to IRS-1 was analyzed by scanning densitometry. The bar graphs show quantification of the autoradiograms from multiple subjects in each group. The data are expressed as the fold-increase above basal (arbitrary units) relative to the value for nonpregnant control subjects, assigning a value of 100 to the result. The bars on the right show the net effect of insulin on IRS-1 tyrosine phosphorylation divided by the IRS-1 protein content for each of the subjects as determined by Western blot analysis. The data were analyzed by analysis of variance and Fisher's PLSD testing for post hoc analysis between groups. Data are means  $\pm$  SD for nonpregnant control ( $n = 5$ ), pregnant control ( $n = 6$ ), and GDM subjects ( $n = 4$ ).



**FIG. 4.** Effect of pregnancy and GDM on expression of insulin signaling proteins in human skeletal muscle. Rectus abdominus muscle biopsies were obtained during gynecological surgery or at the time of scheduled cesarean delivery. Muscles were frozen at the time of surgery and analyzed by SDS-PAGE as described in METHODS. The images in A show an example autoradiogram of proteins detected with anti-insulin receptor (COOH-terminal) antibody and anti-IRS-1; images in B show IRS-2 anti-PI 3-kinase (p85 $\alpha$  subunit) detection. The bar graphs show quantification of the autoradiograms from multiple patients analyzed by scanning densitometry. The data are expressed as arbitrary units relative to the basal value for nonpregnant control subjects, assigning a value of 100 to the result. Data are means  $\pm$  SE for nonpregnant control ( $n = 11$ ), pregnant control ( $n = 11$ ), and GDM subjects ( $n = 7$ ).

However, 2-deoxyglucose transport correlated very highly with IRS-1 phosphorylation ( $r = 0.75$ ,  $P = 0.008$ ,  $n = 15$ ).

## DISCUSSION

The purpose of the present study was to assess insulin regulation of glucose transport activity in skeletal muscle during pregnancy in women with normal glucose tolerance and in GDM, and to test the hypothesis that postreceptor defects in insulin signaling contribute to the pathogenesis of GDM in obese subjects. We have shown here that pregnancy is associated with a marked impairment in insulin-stimulated glucose transport in skeletal muscle and this impairment is worsened in obese women with GDM. These results are analogous to those of Garvey et al. (8), who measured glucose transport in isolated adipocytes and found a more severe decrease in glucose transport in obese GDM subjects compared with lean pregnant control subjects. However, the GDM patients in the study by Garvey et al. (8) were obese compared with control subjects, and obesity per se impairs glucose transport in isolated human adipocytes (41). Our findings that insulin-stimulated glucose transport activity was reduced further in skeletal muscle from GDM subjects are consistent with the recent *in vivo* studies of Catalano et al. (42). They reported decreases in whole-body insulin-stimulated glucose disposal in pre-, early, and late pregnancy in obese GDM subjects as compared with obese pregnant control subjects with normal glucose tolerance. Two other studies have reported more pronounced whole-body insulin resistance in GDM subjects (7,8); however, the latter studies utilized GDM patients with higher fasting plasma glucose concentrations that may have played a role in further worsening the insulin resistance.

Glucose transport in skeletal muscle is widely considered the rate-limiting step for whole-body glucose disposal under most conditions (38–40). A large body of evidence indicates that glucose transport and/or phosphorylation in muscle is impaired in obesity and type 2 diabetes. Dohm et al. (12), using freshly isolated human rectus abdominus muscle, found a decrease in insulin-stimulated glucose transport in subjects with morbid obesity and this impairment was similar in obese type 2 diabetic subjects. Likewise, Kelly et al. (43), using Positron emission tomography scanning, showed that insulin had no effect on glucose transport in skeletal muscle of obese and type 2 diabetic subjects, while phosphorylation was further decreased in type 2 diabetic patients. Bonadonna et al. (44) used a combination of limb balance technique and  $^3\text{H}$ -3-O-methylglucose infusion and reported defects in both glucose transport and phosphorylation in type 2 diabetic subjects. Finally, Rothman et al. (45), using  $^{13}\text{C}$ -NMR, detected lower glucose-6-phosphate (G-6-P) in muscle from type 2 diabetic subjects during insulin stimulation, which is indicative of either impaired glucose transport or phosphorylation. Our data do not address whether a potential greater decrease in insulin sensitivity to glucose transport exists at lower insulin concentrations in pregnancy. Likewise, we cannot rule out that a defect in glucose phosphorylation contributes to reduced maximal glucose transport in pregnancy. However, this seems unlikely given that hexokinase activity is rate-limiting only at high glucose flux rates, and insulin-stimulated glucose transport activity barely increased above basal in pregnant control and GDM subjects.

The decrease in insulin signaling could be a consequence of decreased insulin receptor, IRS-1, or distal proteins in the

insulin signaling cascade. It appears from our studies that IRS-1 downregulation may be a major mechanism for the insulin resistance of pregnancy. The downregulation of IRS-1 protein closely paralleled the decreased ability of insulin to induce tyrosine phosphorylation and this correlated highly significantly ( $r = 0.75$ ,  $P < 0.001$ ) with insulin-stimulated 2-deoxyglucose uptake. Although we did not measure IRS-2 phosphorylation, we did find increased expression of IRS-2 in pregnant subjects. Ours is the first study to report IRS-2 protein levels in human muscle. Rondinone et al. (46) reported that IRS-1 levels were greatly reduced in adipocytes from type 2 diabetic subjects, while IRS-2 levels remained unchanged but required a much higher insulin concentration than IRS-1 to increase tyrosine phosphorylation. In addition, the maximal levels of IRS-2 phosphorylation were decreased compared with nondiabetic subjects. In our studies we used a maximal ( $10^{-7}$  mol/l) concentration of insulin, suggesting that the IRS-2 pathway, if used as an alternative, was not sufficient to compensate for reduced IRS-1 expression and impaired glucose transport. Similar results have been reported in tissues from IRS-1 knockout mice (16,17). Our studies also revealed an unexpected increase in the levels of p85 $\alpha$  subunit of PI 3-kinase in pregnant subjects. The reasons for these increases are not known. Although p85 $\alpha$  is a major regulatory subunit of PI 3-kinase in muscle, there are at least seven known alternative forms of PI 3-kinase regulatory subunits expressed in human muscle with different PI 3-kinase activity elevating responses to insulin (47). The potential roles of the multiple regulatory subunits in regulating the p110 catalytic subunit and increasing glucose transport are unclear at the moment. The ability of the p85 subunit of PI 3-kinase to bind to IRS proteins generally parallels the level of tyrosine phosphorylation of the IRS (18,19,47). This suggests there could be reduced association of p85 $\alpha$  with IRS-1 in these subjects. Furthermore, there may be additional, as yet undefined, molecules that transduce signals from PI 3-kinase to mediate GLUT4 translocation that may be inhibited in our subjects. Further studies are currently underway to examine PI 3-kinase activity, and possibly other downstream defects in cellular trafficking of GLUT4 that may play a role in the insulin resistance to muscle glucose transport in pregnancy, in addition to decreased IRS-1 expression/phosphorylation.

The cellular mechanisms for pregnancy-associated decreased IRS-1 are unknown, but may be related to the hormonal milieu of pregnancy. Decreased IRS-1 expression has been reported previously in muscle of pregnant rats (48). An increase in maternal plasma corticosterone has been observed during early and late gestation, and there is evidence that treatment with excess glucocorticoids can reduce tyrosine phosphorylation of IR $\beta$  and IRS-1 as well as PI 3-kinase activity in skeletal muscle and liver (49,50). Morbidly obese subjects with extreme hyperinsulinemia have decreased IRS-1 in skeletal muscle (31); however, Bjornholm et al. (32) found IRS-1 tyrosine phosphorylation by insulin was decreased without changes in IRS-1 protein in lean to moderately obese hyperinsulinemic type 2 diabetic subjects. Our results suggest that pregnancy-specific effects on tyrosine phosphorylation of IRS-1 are mainly a reflection of the lower IRS-1 protein levels and may be a result of hypercortisolemia and/or other hormones of pregnancy.

One of the important new findings of the present study is that GDM subjects have an additional significant defect in the

maximal tyrosine phosphorylation of the insulin receptor above and beyond that in normal pregnancy. Further, this decrease in insulin receptor tyrosine phosphorylation in GDM subjects was not caused by a change in insulin receptor protein content. Insulin binding to skeletal muscle is unchanged in pregnant control and GDM subjects (51). Damm et al. (51) reported that insulin receptor kinase activity, as measured in isolated receptors *in vitro*, was normal in muscles from pregnant and GDM subjects compared with lean nonpregnant control subjects. In the present studies, the maximal tyrosine phosphorylation of the insulin receptor  $\beta$ -subunit was studied in the intact muscle, which allows for measurement of maximum receptor activation with minimal disruption of the receptor environment, and for measurement of endogenous (IRS-1) substrate phosphorylation without using an artificial substrate. It should be noted that the methods used in these studies do not allow us to analyze which domains in the insulin receptor account for reduced maximal tyrosine phosphorylation. The insulin receptor contains domains in the COOH-terminal region, for example, that can account for as much as 40% of insulin-stimulated tyrosine phosphorylation but are not binding sites for engaging signaling proteins via SH2 domains (52). Deletion of these domains can impair insulin stimulation of glucose transport (53), suggesting dephosphorylation in this region could affect insulin action at some other intracellular site downstream of IRS-1. Since the decreased IRS-1 phosphorylation was primarily attributable to decreased IRS-1 expression, the impaired insulin receptor tyrosine phosphorylation found in GDM subjects suggests decreased tyrosine kinase activity could affect postreceptor signaling processes other than IRS-1 phosphorylation. Thus, while it is clear that the defect in insulin receptor tyrosine phosphorylation in muscle from GDM subjects is associated with impaired insulin-stimulated glucose transport, in the future it will be important to assess signal events downstream from IRS-1.

There are good data to suggest that GDM might mark the development of type 2 diabetes later in life. Decreased insulin receptor autophosphorylation has been demonstrated in skeletal muscle from both lean (29,30) and obese subjects with type 2 diabetes (28–30,32). The GDM subjects studied here had impaired glucose tolerance without fasting hyperglycemia, suggesting that impaired insulin receptor tyrosine phosphorylation could be a primary cellular abnormality underlying the natural history of type 2 diabetes, and a causal factor for the decrease in maximal glucose transport activity. Examination of insulin action in women with previous GDM indicates that decreased insulin sensitivity and glucose disposal persists in the majority of patients after GDM reverts (54,55), suggesting that some genetic components of skeletal muscle insulin resistance may persist and predispose these women to a risk for developing type 2 diabetes. It will therefore be important in future investigations to determine whether one or both of the defects in IRS-1 and IR $\beta$  phosphorylation remit postpartum with the resolution of GDM. A persistent abnormality could represent a primary genetic defect that marks the risk for subsequent development of type 2 diabetes. It is also important to note that GDM subjects have a defect in first-phase insulin secretion (4–6), suggesting that women with GDM have  $\beta$ -cell defects in addition to chronic insulin resistance that may contribute to the pathogenesis of type 2 diabetes after pregnancy.

In summary, the impaired glucose transport in skeletal muscle during pregnancy is characterized by a defect in IRS-1 tyrosine phosphorylation, primarily due to decreased expression of IRS-1 protein. The decrease in IRS-1 is not due to obesity and may therefore be unique to pregnancy. Our data also indicate that GDM is associated with a further decrease in maximal insulin-stimulated glucose transport activity compared with obese pregnant subjects. The GDM subjects studied here have an additional defect in tyrosine phosphorylation of the insulin receptor  $\beta$ -subunit without overt fasting hyperglycemia, suggesting that this defect may be an early indicator for increased risk of type 2 diabetes. There is ample evidence for multiple genetic factors in the pathogenesis of type 2 diabetes; however, unlike type 2 diabetes it is clear in GDM that the environmental factors involved in provoking GDM are no longer present after delivery. Thus, GDM represents a unique opportunity to investigate whether the defects that occur in young women during pregnancy resolve after delivery or represent potential genetic factors for future risk of developing type 2 diabetes.

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