Aberrant insulin-induced GLUT4 translocation predicts glucose intolerance in the offspring of a diabetic mother

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The offspring of a diabetic mother is hyperglycemic and hyperinsulinemic in utero (5, 22, 31). At birth, the offspring develops complications due to persistent hyperinsulinemia that include developing hypoglycemia and other related problems (5, 22, 31). These late-onset complications have been related to the presence of hyperinsulinemia at birth (9, 20, 21, 33). To decipher the pathophysiology of maternal diabetes and the impact on the offspring, various animal models, including genetic models, have been developed (7, 29, 30). Of these, the chemically induced diabetic rat model has been extensively examined in comparison with chronic glucose infusion models (2, 7, 30). Streptozotocin selectively destroys the pancreatic β-cell islets when it is given to pregnant rats in early gestation, resulting in maternal hypoinsulinemia and concomitant hyperglycemia (2, 29, 30). This maternal diabetic state is known to cause fetal hyperglycemia and hypoinsulinemia (2). Previous studies have demonstrated that the adult offspring of a streptozotocin-diabetic mother develops glucose intolerance (4, 12). The cellular and molecular mechanisms by which this occurs remain unknown.

In NIDDM, a postinsulin receptor mechanism has been observed to be defective, leading to insulin resistance. This mechanism has involved the predominant insulin-responsive facilitative glucose transporter isoform, namely GLUT4 (15). A defective insulin-responsive translocation of GLUT4 to the cell surface has been implicated as the mechanism by which insulin resistance develops in NIDDM (18, 28). Hence, we hypothesized that, similar to the adult with NIDDM, the offspring of a diabetic mother that is exposed to high concentrations of glucose and/or insulin in utero will develop defects in the insulin-responsive glucose-transporting mechanism that persist into adulthood and can be altered by postnatal nutrient modification(s). To test this hypothesis, we examined GLUT4 expression, concentrations, and insulin-responsive GLUT4 translocation in skeletal muscle and white adipose tissue in the progeny of a diabetic mother treated with streptozotocin selectively destroys the pancreatic β-cell islets when it is given to pregnant rats in early gestation, resulting in maternal hypoinsulinemia and concomitant hyperglycemia (2, 29, 30). This maternal diabetic state is known to cause fetal hyperglycemia and hypoinsulinemia (2). Previous studies have demonstrated that the adult offspring of a streptozotocin-diabetic mother develops glucose intolerance (4, 12). The cellular and molecular mechanisms by which this occurs remain unknown.

In NIDDM, a postinsulin receptor mechanism has been observed to be defective, leading to insulin resistance. This mechanism has involved the predominant insulin-responsive facilitative glucose transporter isoform, namely GLUT4 (15). A defective insulin-responsive translocation of GLUT4 to the cell surface has been implicated as the mechanism by which insulin resistance develops in NIDDM (18, 28). Hence, we hypothesized that, similar to the adult with NIDDM, the offspring of a diabetic mother that is exposed to high concentrations of glucose and/or insulin in utero will develop defects in the insulin-responsive glucose-transporting mechanism that persist into adulthood and can be altered by postnatal nutrient modification(s). To test this hypothesis, we examined GLUT4 expression, concentrations, and insulin-responsive GLUT4 translocation in skeletal muscle and white adipose tissue in the progeny of a diabetic mother subjected to two different postnatal nutrient modifications. Thus control mother-fed diabetic progeny (CM/DP) and diabetic mother-fed diabetic progeny (DM/DP) were compared with the control mother-fed control progeny (CM/CP).

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (−250 g) were housed in individual cages, exposed to 12:12-h light-dark cycles at 21–23°C, and

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allowed ad libitum access to standard rat chow (Purina, St. Louis, MO). As approved by the Animal Research Committee of the University of California, Los Angeles (UCLA), the guidelines of the National Institutes of Health were followed.

Diabetic Pregnancy

At 3 days of gestation, pregnant rats received, via the tail vein, varying doses of streptozotocin (Sigma, St. Louis, MO) ranging from 15 to 35 mg/kg mixed in 0.45 M citrate buffer. At gestational days 9 and 21, tail vein blood glucose levels were assessed to detect hyperglycemia. The control animals received an equal volume of the vehicle. The animals that became diabetic were defined as having a blood glucose of >150 mg/dl. All animals were allowed to deliver, and the newborn rats were weighed to determine the presence of macro- vs. normo- or microsomnia.

Postnatal Animal Maintenance

At birth, the litter size was culled to six. In addition, the newborn rats born to diabetic mothers alone were cross-fostered to be reared by either a diabetic mother or a control mother. Thus three groups were created, with control mothers rearing control (CM/CP) or diabetic (CM/DP) pups, and the diabetic mothers rearing diabetic (DM/DP) pups throughout the suckling phase. At day 21, the pups were weaned from the mother and maintained in individual cages until day 180 of life.

Glucose Tolerance Test

At days 60 and 180, adult male and at day 235, adult male awake animals in rat restrainers were subjected to a glucose tolerance test after a 16-h fast or in the fed state. The animals received 0.5 g of glucose via the tail vein, and blood glucose was obtained subsequently at 0, 15, 30, 60, and 120 min to measure glucose levels.

Insulin Tolerance Test

At days 60 and 180, adult awake animals in rat restrainers were subjected to an insulin tolerance test in the fed state. The animals received 0.75 U/kg of human insulin via the tail vein, and blood was obtained subsequently at 0, 15, 30, 60, and 120 min to measure glucose concentrations.

Food and Water Intake

At days 120, 150, and 180, food intake was measured over a 24-h period by weighing rat chow at the beginning and end of that period to account for spillage and evaporation. Similarly, water intake was assessed by measuring the volume of water at the beginning and end of the 24-h period to account for evaporation.

Anthropometric Measurements

Body/organ weights were assessed longitudinally at days 1, 21, 35, 60, 90, 120, 150, and 180. In addition, at days 60 and 180, some of the animals were euthanized with intraperitoneal pentobarbital sodium (100 mg/kg), and various organ weights were assessed with a scale of an accuracy of 0.001 g.

Plasma Assays

Animals were euthanized as described above, and blood was collected from the left ventricle. The plasma was separated and aliquoted for measurement of glucose by the glucose oxidase method (Sigma Diagnostics, St. Louis, MO; sensitivity = 0.1 mM with an intra-assay coefficient of variation = 1.2%). Insulin and leptin were quantified by double-antibody radioimmunoassays using rat standards and anti-rat insulin or leptin antibodies (Linco Research, St. Charles, MO; sensitivity: insulin = 0.1 ng/ml, leptin = 0.5 ng/ml). Corticosterone was quantified with the anti-rat corticosterone antibody (Amersham Life Science, Little Chalfont, Buckinghamshire, UK; sensitivity: corticosterone = 0.06 ng/ml). In addition, serum triacylglycerol, cholesterol, high-density lipoprotein (HDL), unesterified cholesterol, and free fatty acids were measured by colorimetric assays as previously described (38). Plasma glycerol concentrations were determined and used to correct the triacylglycerol values. The HDL cholesterol was derived from the measurement of the supernatant after the precipitation of apoB-containing lipoproteins with heparin and MnCl2 (25). Each lipid determination was measured in triplicate. An external control sample with known analyte concentration was run in each plate to ensure accuracy. All lipids were analyzed by the UCLA Lipid and Lipoprotein Laboratory, which is certified by the Centers for Disease Control and Prevention and the National Heart, Lung, and Blood Institute Lipid Standardization Program.

Hypothalamic Tissue Neuropeptide Y Concentrations

The hypothalamus was obtained as a frontal slide by vertical cuts 1 mm anterior to the body of the optic chiasm and 1 mm posterior to the mammary bodies. The tissue block was weighed and extracted in four volumes of 0.1 N HCl (wt/vol). The extract was sonicated for 10 s (60 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA) using 10 W of output power. The sonicated acid extracts were centrifuged at 10,000 rpm for 10 min to remove the tissue debris. The supernatant was freeze-dried. The freeze-dried extracts were reconstituted in 0.05 M Tris·HCl buffer containing 0.1% bovine serum albumin (pH 7.8) for neuropeptide Y (NPY) measurements by radioimmunoassay. NPY was assessed by a radioimmunoassay that employed a polyclonal rabbit anti-NPY antibody and rat NPY standards (Peninsula Laboratories, Belmont, CA). NPY was expressed as picograms per milligram hypothalamic wet weight and hypothalamic protein content (28).

Skeletal Muscle and White Adipose Tissue Studies

Tissue collection. To overcome the confounding influence of either contraction or hypoxia, skeletal muscle and adipose tissue were obtained from the anesthetized animal that continued to maintain good organ blood flow. The tissues were immediately snap-frozen in liquid nitrogen and stored at −70°C until analysis.

GLUT4 mRNA analysis. Total RNA was isolated from rat mixed skeletal muscle obtained from the hindlimb and peri-renal white adipose tissue by use of TRIzol reagent (Life Technologies, Gaithersburg, MD). RNA was quantified spectrophotometrically, and the purity of the samples was assessed as a ratio of 1.8–2.0 at a 260 to 280 nm wavelength. Total RNA (10 μg) was loaded on 1.5% agarose-2.2 M formaldehyde slab gels and electrophoresed overnight at 30 V for 16 h. The gels were stained with ethidium bromide and visualized under UV light to confirm the integrity of the RNA samples. The RNA from the gels was transferred to Hybond N (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK) membranes and cross-linked by UV light in a Stratalinker at 1,200 × 100 μJ for 32 s (Stratalinker 1800, Stratagene, La Jolla, CA). The rat GLUT4 cDNA probe was prepared by amplifying a rat GLUT4 cDNA (15) containing the rat...
GLUT4 coding region from the translational start to the translational stop sites, with the forward primer 5'-agtc-
ctgctggttcatggc-3' and the reverse primer 5'-tcagctttt-
catgctggc-3'. The PCR product was gel purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). The Decaprise II labeling kit (Ambion, Austin, TX) was used to radiolabel 25 ng of the gel-purified product to a specific activity of 2.25 × 10^9 dpm/µg of DNA. The full-length mouse ribosomal protein S2 cDNA (internal control) (6) was excised from the vector by digesting with PsI and XhoI restriction enzymes, and 10 ng were labeled in the same manner as the rat GLUT4 cDNA fragment.

The blots containing the RNA were prehybridized for 2 h at 42°C and subsequently hybridized overnight at 42°C in 50% formamide, 0.65 M NaCl, 50 mM Tris at pH 7.5, 2 mM Na pyrophosphate, 0.2% polyvinyl pyrrolidone, 0.2% BSA, 0.2% Ficoll, and 1% SDS containing 1 × 10^6 cpm/ml of the radiolabeled rat GLUT4 cDNA fragment. The blots were washed two times at room temperature in 2× SSC-0.5% SDS and then washed three times for 10 min, each at 65°C. The signals on blots were quantified with a Molecular Analyser Phosphorimager and then exposed to X-ray film. The blots were reprobed with a mouse ribosomal protein S2 cDNA (6) to correct for interlane loading variability. The results were expressed as a ratio between the GLUT4 mRNA and S2 mRNA phosphorimage values (6, 26).

Glucose Transporter Protein Analysis

Skeletal muscle preparation. Homogenates and subcellular fractions were prepared. Snap-frozen skeletal muscle (0.5–1.0 g) was transferred into 30 ml of ice-cold sucrose buffer (250 mM sucrose, 20 mM HEPES-Tris, 1 mM EDTA, and 100 µM PMSF, pH 7.4). The muscle was crushed on dry ice and then homogenized on ice with a Polytron homogenizer (30 mm probe, setting 3) for one 15-s burst. This homogenate was transferred to a 40-ml glass beaker. A 9 ml/10 ml of the suspension was transferred with a Te
on-coated magnet was added to each beaker, stirred at a setting of 3 for 2.5 h on a multiport magnetic stirring base (Lab Line Multiperm, Lab Line Instruments, Melrose, IL). The resulting suspension was centrifuged at 6,000 g for 10 min at 4°C. The supernatant that was saved for Western blot analysis, and the rest of this suspension was centrifuged at 16,000 g for 30 min at 4°C. The fat layer on top was discarded, and the supernatant was saved for preparation of the LDM fraction. The pellet was resuspended in 1–2 ml of the sucrose buffer and layered on a 1.12 M sucrose cushion containing 20 mM HEPES-Tris and 1 mM EDTA, pH 7.4, and was centrifuged for 1 h at 100,000 g at 4°C with an ultracentrifuge. The plasma membranes (PM), collected at the interface, were resuspended in 10 ml of the sucrose buffer and centrifuged at 48,000 g for 30 min. The pellet containing the PM subfraction was resuspended in 1–2 ml of the sucrose buffer and saved at −70°C until Western blot analysis. The initial supernatant that was saved for the preparation of the LDM subfraction was centrifuged at 48,000 g for 30 min, and the resulting supernatant was centrifuged again at 250,000 g at 4°C with an ultracentrifuge to yield the LDM subfraction, which was also stored at −70°C until Western blot analysis was undertaken (32).

White adipose tissue preparation. Briefly, 1 g of the snap-

frozen perirenal fat pad was crushed on dry ice and homog-

enized on ice in 30 ml of a sucrose buffer (see Skeletal muscle preparation) with a Polytron homogenizer (30 mm probe, setting 3) for one 15-s burst, followed by 10 strokes of homog-

enization with a Te
on pestle. The resuspended

suspension was transferred to a 40-ml glass beaker. A 9 ml/10 ml of the suspen-

sition was transferred with a Te
on-coated magnet was added to each beaker, stirred at a setting of 3 for 2.5 h on a multiport magnetic stirring base (Lab Line Multiperm, Lab Line Instruments, Melrose, IL). The resulting suspension was centrifuged at 6,000 g for 10 min at 4°C. The supernatant that was saved for Western blot analysis, and the rest of this suspension was centrifuged at 16,000 g for 30 min at 4°C. The fat layer on top was discarded, and the supernatant was saved for preparation of the LDM fraction. The pellet was resuspended in 1–2 ml of the sucrose buffer and layered on a 1.12 M sucrose cushion containing 20 mM HEPES-Tris and 1 mM EDTA, pH 7.4, and was centrifuged for 1 h at 100,000 g at 4°C with an ultracentrifuge. The plasma membranes (PM), collected at the interface, were resuspended in 10 ml of the sucrose buffer and centrifuged at 48,000 g for 30 min. The pellet containing the PM subfraction was resuspended in 1–2 ml of the sucrose buffer and saved at −70°C until Western blot analysis. The initial supernatant that was saved for the preparation of the LDM subfraction was centrifuged at 48,000 g for 30 min, and the resulting supernatant was centrifuged again at 250,000 g at 4°C with an ultracen-

trifuge to yield the LDM subfraction, which was also stored at −70°C until Western blot analysis was undertaken (32).

Western blot analysis. The fractionated sarcoplasmic (PM) and LDM samples were sonicated (60 Sonic Disembrator), using two 50-s cycles of 5–7 W. The resulting suspension was centrifuged at 10,000 g at 4°C for 10 min and the supernatant was saved for Western blot analysis. Predetermined optimal protein concentrations of the PM or LDM subfractions obtained from either the skeletal muscle or white adipose tissue were subjected to discontinuous 10% SDS-PAGE followed by elec-

troblot transfer to nitrocellulose (Nytran, Schleicher & Schuell, Keene, NH). The nitrocellulose filters were rinsed once in PBS-Tris (PBS-T) and blocked for 1 h in 5% nonfat dry milk at room temperature. The filters were washed three times (1× for 15 min and 2× for 10 min) in PBS-T, followed by incubation for 1 h at room temperature with an affinity-purified rabbit anti-rat GLUT4 antibody (1:2,500 dilution). After the filters were washed three times with PBS-T, they were treated with the peroxidase-linked goat anti-rabbit IgG and subsequently exposed to a chemiluminescence reagent (Amersham Life Science). The chemiluminescence was cap-
tured on X-ray film over an optimal period of time (1–5 min) to determine the optimal exposure time. Glucose transporter protein concentrations were assessed by quantification of the protein bands by densitometry. The presence of linearity between the time of X-ray film exposure and the optical

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density of the glucose transporter bands was initially ensured (1).

Data Analysis

Data are expressed as means ± SE. One-way analysis of variance was used to compare the various treatment groups at the same age. Intergroup differences were validated by the Newman-Keuls test. Significance levels were computed on the basis of exact methods that accounted for the small sample size.

RESULTS

Figure 1A demonstrates the effect of maternal streptozotocin dosing on neonatal body weight. The control animals were used as the gold standard for comparison. Doses of 15 and 20 mg/kg of streptozotocin led to no change in neonatal body weight and caused no overt diabetes in mothers. In contrast, 25 mg/kg of streptozotocin led to an increase in neonatal body weight, with inconsistency with respect to creating maternal diabetes. This relationship was difficult to reproduce consistently, because some of the animals developed hyperglycemia and some did not. At 30 mg/kg, although the production of maternal diabetes was also inconsistent, there was no change in neonatal body weight compared with the control weight. An increase to 35 mg/kg led to the consistent presence of overt maternal diabetes, with a neonatal body weight that remained comparable to that of the vehicle-treated controls. Plasma glucose concentrations were not different; however, plasma insulin concentrations were higher in newborns born to diabetic mothers (DP) than in those from controls (CP) (mean values of 2.47 ± 0.1 in CP vs. 16.44 ± 0.5 mg/ml in DP). Figure 1B depicts the maternal blood glucose concentrations, which demonstrate a doubling of these values in diabetic mothers (DM) vs. control mothers (CM). The diabetic mothers in an arbitrarily chosen subset were observed to gain only 15% of their prepregnancy body weight during gestation as opposed to the 48% increase noted in vehicle-treated control mothers (Fig. 1C).

The insets of Fig. 2, A and B, show the neonatal body weight in animals studied in response to 35 mg/kg of streptozotocin that was employed for the rest of the study. In the two groups (CP and DP), no difference is observed in the neonatal body weights. In addition, the body weights of females and males from days 1–180 in all three groups after the initiation of the cross-fostering protocol are depicted in Fig. 2, A and B. The CM/DP group demonstrated no difference from the CM/CP group until day 40 in males and females. At day 60, the males alone demonstrated an increase in body weight, whereas at days 120, 150, and 180, males and females demonstrated an increase in body weight compared with the CM/CP group. In contrast, the DM/DP group demonstrated a decline in body weight beginning as early as day 21 compared with the CM/CP group. This postnatal growth restriction persisted into adulthood predominantly in females but not in males. The DM/DP males demonstrated body weights comparable to those of the CM/CP group. On the basis of these observations, days 60 and 180 were chosen for further investigations, because day 60 denoted a time point before the development of obesity in the CM/DP group and day 180 a time point after the development of obesity.

Figure 3A depicts the results of glucose tolerance tests in female animals. At day 60, in a fed state, the CM/DP animals demonstrated glucose intolerance lasting from 30 to 120 min compared with the CM/CP group. In contrast, the DM/DP group demonstrated mild glucose intolerance only at the 60-min time point. This glucose intolerance is exaggerated at day 180, particularly in the fed state. Figure 3, B and C, dem-
onstrate glucose intolerance at day 180 in the fasted and fed states, respectively. Both states exhibit glucose intolerance in the CM/DP group compared with the CM/CP group from 30 to 120 min. In contrast, the DM/DP group revealed no similar glucose intolerance at any time point. Also in contrast, glucose tolerance tests in 235-day-old males revealed glucose intolerance in both the CM/DP and DM/DP groups compared with the age-matched CM/CP group (Fig. 3D).

The insulin tolerance test in females at day 60 revealed a trend toward greater insulin sensitivity in the DM/DP group than in either the CM/DP or the CM/CP group (Fig. 3E); however, these differences did not achieve statistical significance. However, at day 180, although greater insulin sensitivity was retained in the DM/DP group compared with the CM/CP group, the CM/DP group demonstrated lower insulin sensitivity (i.e., insulin resistance) compared with the CM/CP and DM/DP groups (Fig. 3F). In contrast, the 235-day-old males revealed decreased insulin sensitivity in both the CM/DP and the DM/DP groups compared with the age-matched CM/CP group (Fig. 3G).

Table 1 depicts the anthropometric measurements of adult female animals at days 60 and 180. At day 60, the liver, brain, and nose-tail length were diminished in the CM/DP group compared with the CM/CP group. However, in the DM/DP group, there was a generalized decline in all organ weights and the nose-tail length. By day 180, although no differences in either organ weights or the nose-tail length were observed in the CM/DP group compared with the age-matched CM/CP group, a marked increase in visceral fat mass was observed. In contrast, the DM/DP group demonstrated a continued decline in the liver and kidney weights and the nose-tail length. Brain and brown adipose tissue weights were not obtained at this age.

Table 2 demonstrates the food and water intake at days 120, 150, and 180. At day 120, the CM/DP group males demonstrated an increase in food intake, whereas the DM/DP group males and females were not different from those of the CM/CP group. No intergroup differences were observed at day 150. However, at day 180, whereas the CM/DP group males and females were not different from those of the CM/CP group, the DM/DP group females demonstrated a decline in food intake. No intergroup differences in water intake were observed at any of the three ages. Hypothalamic NPY concentrations were not different between the CM/CP and the CM/DP group females; however, the DM/DP group females demonstrated a decline in the total NPY concentrations (Fig. 4, A and B).

Table 3 demonstrates the plasma leptin, insulin, and corticosterone concentrations at day 60 and day 180 for female animals in the basal, vehicle-treated state. Plasma leptin, insulin, and corticosterone concentrations were assessed 20 min after exogenous insulin administration only at day 60. No change in leptin and insulin concentrations was noted in the CM/DP and DM/DP groups compared with the CM/CP group under basal conditions at day 60; however, at day 180, whereas no change was present in the DM/DP group, a trend toward relative hyperleptinemia and hyperinsulinemia was observed in the CM/DP group compared with the age-matched CM/CP group. Plasma corticosterone concentrations were not measured at this age. Insulin administration at day 60 led to no change in plasma leptin concentrations; however, as expected, a 10- to 15-fold increase in plasma insulin concentrations was noted in all three experimental groups compared with their counterparts in the control state. Similarly, an increase in plasma corticosterone concentration was observed after exogenous insulin administration in the CM/CP and the DM/DP groups. No such increase was noted in the CM/DP group at day 60.

Table 4 depicts the plasma metabolite profile at days 60 and 180 for females in the basal, vehicle-treated state. No interexperimental group differences were noted in the basal plasma glucose concentration at
either day 60 or day 180. Similarly, no intergroup differences were observed in the plasma triacylglycerol concentrations except in the day 60 DM/DP group, where a decline was observed compared with the age-matched CM/CP group. No interexperimental group differences were observed in the plasma total cholesterol concentrations at days 60 and 180 except for a decline in total cholesterol concentrations in the CM/DP group compared with the CM/CP group at day 60. Unesterified cholesterol and free fatty acids were also no different except for a decline in unesterified cholesterol concentrations in the day 60 CM/DP and DM/DP groups compared with the CM/CP group.
Table 1. Organ weights and nose-tail length in female adults

<table>
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<tr>
<th></th>
<th>Heart</th>
<th>Liver</th>
<th>Pancreas</th>
<th>Kidney</th>
<th>Brain</th>
<th>WAT</th>
<th>BAT</th>
<th>NT-L</th>
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<tr>
<td>CM/CP</td>
<td>8</td>
<td>9.2±0.36</td>
<td>0.54±0.04</td>
<td>1.95±0.06</td>
<td>1.74±0.024</td>
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<td>0.34±0.05</td>
<td>38±0</td>
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<td>CM/DP</td>
<td>8</td>
<td>7.7±0.36</td>
<td>0.54±0.03</td>
<td>1.75±0.08</td>
<td>1.61±0.02</td>
<td>5.26±0.29</td>
<td>0.24±0.02</td>
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<tr>
<td>DM/DP</td>
<td>8</td>
<td>7.23±0.28</td>
<td>0.55±0.04</td>
<td>1.67±0.053</td>
<td>1.59±0.014</td>
<td>4.05±0.43</td>
<td>0.27±0.019</td>
<td>36.75±0.16</td>
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</table>

Values are means ± SE: weights in g, lengths in cm; n, no./group. CM/CP, control mothers/control progeny; CM/DP, CM/diabetic progeny; DM/DP, diabetic mothers/DP; WAT, white adipose tissue; BAT, brown adipose tissue; NT-L, nose-tail length. *P < 0.05, †P < 0.02, ‡P < 0.001.

Insulin treatment of females at day 60, although not affecting the interexperimental group plasma glucose concentrations, led to an increase in the plasma total cholesterol concentration in the CM/DP and the DM/DP groups compared with the insulin-treated CM/CP group and an increase in the unesterified cholesterol content in the insulin-treated CM/DP group alone compared with the CM/CP group. Insulin administration in the 60-day-old animals revealed a dramatic decline in the plasma glucose concentrations in all three experimental groups compared with their corresponding basal vehicle-treated state (P < 0.001). In addition, a decline in the total cholesterol concentration in the CM/CP group was noted in response to insulin compared with the corresponding basal state. No such change was observed in the CM/DP and the DM/DP groups. Although insulin treatment at day 60 failed to affect the HDL and the unesterified cholesterol concentrations in all three groups, a decline in the free fatty acid level was observed in the CM/CP and the CM/DP groups alone.

Figure 5A demonstrates no difference in skeletal muscle total GLUT4 mRNA concentrations between the two newborn groups and the females from the three experimental groups at day 21, 60, and 180. Paralleling this observation, no difference in the day 1 newborn skeletal muscle homogenate GLUT4 protein concentrations was observed between the CP and DP groups (Fig. 5B) and in the three experimental groups at day 60 (Fig. 5C). Insulin-induced translocation to the sarcolemma-associated GLUT4 protein concentrations was also similar at both the 8 and 40 U/kg insulin doses. No further increase above that observed with 8 U/kg of insulin in sarcolemma-associated GLUT4 protein concentrations was observed when the 40 U/kg dose of insulin was administered. In the vehi-

Table 2. Food and water intake in female and male adults

<table>
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<tr>
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<th>CM/CP</th>
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<td>day 150</td>
<td>day 180</td>
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<tr>
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<tr>
<td>Food intake, g/day</td>
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<td>28.3±1.25*(9)</td>
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<td>Water intake, ml/day</td>
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<td>18.42±0.81(9)</td>
<td>14.27±0.8(11)</td>
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<tr>
<td>Male</td>
<td>37±4.2(5)</td>
<td>42±5(9)</td>
<td>31.3±4(6)</td>
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<td>21.5±2(7)</td>
<td>26.2±2.7(9)</td>
<td>23.9±1.64(11)</td>
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<td></td>
<td>day 150</td>
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<td>day 180</td>
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<td>Food intake, g/day</td>
<td>24.6±2.69(5)</td>
<td>28.66±1.06(9)</td>
<td>23.3±3(6)</td>
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<td>Water intake, ml/day</td>
<td>18±2.25(7)</td>
<td>18.14±0.63(9)</td>
<td>15.54±0.9(11)</td>
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<tr>
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<td>28.4±2.69(5)</td>
<td>35.44±2.29(9)</td>
<td>24.16±4.84(6)</td>
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<td>22.14±1.56(7)</td>
<td>24.85±1.59(9)</td>
<td>26.28±3.55(11)</td>
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<tr>
<td>Food intake, g/day</td>
<td>26.8±1.28(5)</td>
<td>26.9±0.69(9)</td>
<td>25.16±2.24(6)</td>
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<td>Water intake, ml/day</td>
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<td>19.85±1.12(11)</td>
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<tr>
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<td>28.4±3.38(5)</td>
<td>30.77±3.23(9)</td>
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<tr>
<td>Female</td>
<td>25.4±2.47(7)</td>
<td>21.8±2.16(9)</td>
<td>19.9±1.23(11)</td>
</tr>
</tbody>
</table>

Values are means ± SE of no. of adult rats in parentheses. *P < 0.05, †P < 0.01 vs. age-matched and sex-matched CM/CP group.
cle-treated DP group at day 1, a relative increase in sarcolemma-associated GLUT4 protein concentrations was observed in the basal vehicle-treated state compared with the corresponding age-matched CP group, which then failed to increase further after insulin (8 and 40 U/kg) treatment (Fig. 6C). The corresponding low-density microsomal fraction showed an opposing effect on GLUT4 protein concentrations after insulin treatment; thus, in the CM/CP group, insulin led to a decrease in LDM-associated GLUT4 protein concentrations, whereas in the CM/DP group, no similar postinsulin treatment decrease in LDM-associated GLUT4 protein was noted. In the DM/DP group, the degree of insulin-induced decrease in the LDM-associated GLUT4 protein concentration was observed to be between that of the CM/CP and the CM/DP groups (Fig. 6C).

The sarcolemma fraction isolated from the adult and newborn skeletal muscle and used in this study revealed an eightfold enrichment in the K+-sensitive p-nitrophenol phosphatase compared with the homogenate, and the EGTA-sensitive Ca2+-ATPase activity was not detectable. The LDM fraction did not reveal any K+-sensitive p-nitrophenol phosphatase or EGTA-sensitive Ca2+-ATPase activity.

White adipose tissue (WAT) GLUT4 mRNA concentrations did not show any intergroup differences (Fig. 7A). Paralleling this observation, WAT homogenate GLUT4 protein concentrations were not different in the three groups examined (Fig. 7B). However, when PM-associated GLUT4 protein concentrations were measured, in vivo insulin treatment led to a 10-fold increase in the PM-associated GLUT4 concentrations in the CM/CP group (Fig. 7C). Both the CM/DP and DM/DP groups revealed higher concentrations of PM-associated GLUT4 secondary to vehicle treatment (basal state). Insulin treatment led to a 50% increase in PM-associated GLUT4 amounts in the CM/DP group and a twofold increase in the DM/DP group, but these changes never reached the total amount noted after insulin treatment in the CM/CP group. The LDM-associated GLUT4 concentrations demonstrated a reciprocal change from that seen in the corresponding PM-associated GLUT4 amounts, namely a decrease in two groups, with the degree of decrease varying from the CM/CP group, demonstrating the greatest decrease.
Table 4. Plasma metabolite concentrations in female adults

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Glucose</th>
<th>TG</th>
<th>CHOL</th>
<th>HDL</th>
<th>UC</th>
<th>FFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM/CP 6</td>
<td>170 ± 9.9</td>
<td>135 ± 19</td>
<td>79 ± 4.4</td>
<td>62 ± 4.1</td>
<td>23.3 ± 2.18</td>
<td>10.38 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>CM/DP 4</td>
<td>157 ± 24</td>
<td>71 ± 26</td>
<td>64 ± 1.6††</td>
<td>57 ± 0.71</td>
<td>17 ± 0.91†</td>
<td>10.8 ± 0.85</td>
<td></td>
</tr>
<tr>
<td>DM/DP 4</td>
<td>144 ± 14</td>
<td>49 ± 19††</td>
<td>66 ± 5.4</td>
<td>52 ± 4.8</td>
<td>16 ± 1.8†</td>
<td>9 ± 0.48</td>
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</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM/CP 6</td>
<td>58 ± 4.7***</td>
<td>56 ± 15</td>
<td>60 ± 1.8**</td>
<td>54 ± 2.4</td>
<td>16 ± 1.3</td>
<td>6 ± 0.23**</td>
<td></td>
</tr>
<tr>
<td>CM/DP 4</td>
<td>45 ± 5.5***</td>
<td>32 ± 20</td>
<td>71 ± 4.4†</td>
<td>65 ± 3.6‡</td>
<td>17 ± 1.6</td>
<td>6.8 ± 0.75†</td>
<td></td>
</tr>
<tr>
<td>DM/DP 4</td>
<td>38 ± 11.7***</td>
<td>22 ± 10</td>
<td>70 ± 1.31†††</td>
<td>61 ± 2.53</td>
<td>15 ± 0.41</td>
<td>7.7 ± 1.35</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE in mg/dl; n, no. of rats/group; TG, triacylglycerol; CHOL, total cholesterol; HDL, high-density lipoprotein; UC, unesterified cholesterol; FFA, free fatty acids. *P < 0.05, **P < 0.02, ***P < 0.001 vs. control value from same age-matched treatment group. †P < 0.05, ††P < 0.02, †††P < 0.01 vs. age-matched CM/CP group.

DISCUSSION

Our study demonstrates that the dose and timing of streptozotocin given to pregnant rats are crucial in predetermining the outcome of the fetus/newborn. Previous studies have demonstrated that administration of streptozotocin at later stages of pregnancy causes fetal hyperglycemia but does not either change the fetal insulin concentrations or lead to fetal hypoinsulinemia (7, 30). This is related to an effect on the fetal pancreas that is formed by day 11 of gestation. Administration of streptozotocin before gestation negatively affects fertility and prevents a resultant successful pregnancy, but streptozotocin during early gestation before the development of a pancreas does not adversely affect the fetal pancreas. Hence, the offspring at birth were noted to be hyperinsulinemic, as described previously (2). Although 25 mg/kg of streptozotocin to the mother closely mimicked the human Class A diabetic pregnancy with respect to fetal macrosomia, this model was difficult to reproduce consistently and hence was not used in our current study. We chose to use 35 mg/kg of streptozotocin because of the consistency we observed in producing overt maternal hyperglycemia (100%) and the absence of adverse effects on neonatal body weight. Higher doses of streptozotocin have the propensity of leading to fetal microsomia (2, 7) and were therefore avoided.

Although the dose of 35 mg/kg of streptozotocin did not lead to macrosomia at birth, depending on how the pups were reared during the postnatal period, the newborn of the diabetic mother either continued to grow at the same rate as the control group or lost weight. When reared by diabetic mothers, there was a loss of body weight particularly in the females, resulting in postnatal growth restriction that manifested itself as early as day 21. Previous studies that analyzed the milk of diabetic rats failed to demonstrate differences in milk composition that could account for this observation. However, compared with the control mothers, the diabetic mothers were observed to have adequate nesting behavior but an overall diminution in milk secretion/ejection, leading to an inadequate milk supply (14, 17). This resulted in relative postnatal nutrient restriction (17). In contrast, the newborns of diabetic mothers that were reared by control mothers were no different initially from control pups fed by control mothers. However, during adult life, the diabetic pups reared by control mothers developed obesity, manifested as visceral adiposity, which was associated with a trend toward relative hyperinsulinemia, hyperleptinemia, and glucose intolerance only at day 180. In contrast, the DM/DP group revealed persistent growth restriction in females, with no changes in circulating insulin and leptin concentrations, and failed to demonstrate glucose intolerance at day 180. In contrast, the males in both the CM/DP and the DM/DP groups were glucose intolerant and insulin resistant when assessed beyond day 180 of age.

To determine the cause of obesity in the CM/DP group, food intake was assessed. The CM/DP group males demonstrated enhanced food intake only at day 120, which may have contributed toward the resultant obesity. However, at days 150 and 180, no further increase in food intake in the presence of obesity indirectly signifies a decline in energy expenditure. Thus it appears that the earlier presence of enhanced food intake, particularly in the males, has a role in the development of adult-onset obesity. The DM/DP group females, however, demonstrated a decline in food intake by day 180, which is in keeping with a decline in body weight and the absence of glucose intolerance. To decipher the mechanism responsible for the decline in food intake at day 180, hypothalamic NPY concentra-
tions were measured in females and revealed a parallel decline in the DM/DP group. Thus, in the females, it appears that the decrease in hypothalamic NPY concentrations paralleled the decline in food intake. These adult changes in hypothalamic NPY (DM/DP) are in keeping with the decline observed in the fetus of a streptozotocin-induced diabetic rat, as previously reported by us (35). However, an increase in hypothalamic NPY (arcuate nucleus) immunoreactivity at day 570 with an increase in food intake at day 330 in the adult male offspring of diabetic mothers has previously been reported (23). Thus it is plausible that the CM/DP adult males could present with higher hypothalamic NPY along with hyperphagia, as we observed at day 120. This may be due to the acquisition of insulin and/or leptin resistance that signifies adult-onset obesity in this experimental group.

Previous studies employing the hyperinsulinemic euglycemic clamp technique, differences in insulin sensitivity were revealed in females among the three experimental groups that were suggestive of the CM/DP group being relatively more insulin resistant than the CM/CP and DM/DP groups. The explanation for the DM/DP group being more insulin sensitive than the CM/CP group may stem from a concomitant decline in the pancreatic islet β-cell mass and function secondary to postnatal nutrient restriction (10, 11). However, the males in both groups were obese and insulin resistant by day 235. To tease the cellular difference between the CM/DP and the DM/DP group, we focused mainly on the females for our GLUT4 studies.

Our studies demonstrate that exposure to an intrauterine maternal diabetic environment causes no change in the total concentrations of skeletal muscle GLUT4 mRNA and protein levels. However, an increase in basal sarcolemmal GLUT4 concentrations in the newborn skeletal muscle, perhaps resulting in increased basal glucose transport, was observed. In vivo administration of insulin led to a decline in circulating glucose concentrations. However, no major change in the subcellular distribution of GLUT4 was noted in the
newborn pups born to diabetic mothers. This observation attests to the fact that in utero exposure to a diabetic metabolic milieu causes an aberrant subcellular distribution of GLUT4 that mimics the distribution seen with GLUT1 in fetal/neonatal skeletal muscle (1, 30). This change in the basal distribution of GLUT4 resulted in no further response to insulin. This defect persists into adulthood despite postnatal removal from an in utero diabetic milieu. In keeping with the defect in the skeletal muscle, a similar defect in insulin-induced GLUT4 translocation was observed in WAT. Although we could not examine WAT in the newborn because of a lack of adequate tissue at this stage, it is quite likely that this defect occurred in utero as well. The defects in both skeletal muscle and WAT were noted at 60 days, before the development of overt glucose intolerance, relative insulin resistance, and obesity with its associated hyperinsulinemic and hyperleptinemic trends.

This increase in basal concentrations of sarcolemmal/PM-associated GLUT4 is an in utero adaptation to the high circulating levels of fetal glucose and/or insulin. A similar observation was made in vitro when L6 myoblast cells were exposed to 24 h of high glucose and insulin. This increase in PM-associated GLUT4 was associated with an increase in basal glucose transport.

Fig. 6. Subfractionation studies in skeletal muscle. A: studies in the newborn: time course and dose response. Representative Western blots (top) depict GLUT4 in sarcolemmal/plasma membrane (PM) and low-density microsomal (LDM) subcellular fractions in 1-day-old rats born to control mothers that were treated with either vehicle (0 U/kg at 0 min) or insulin at 8 or 40 U/kg at 10, 20, and 40 min before analysis. Quantification is depicted (bottom); values are means ± SE; n = 25 per group, per dose, and per time point. B: studies in the newborn: pups born to control and diabetic mothers. Representative Western blots demonstrate GLUT4 in PM and LDM subcellular fractions in 1-day-old rats born to CP and DP mothers that were treated with vehicle (0 min) or with 8 U/kg (low dose) or 40 U/kg (high dose) of insulin 20 min before analysis. Values are means ± SE; n = 25 per group, per dose, and per time point. C: studies in the adult: representative Western blots (top) depict GLUT4 in the PM and LDM fractions in 60-day-old female rats from the 3 experimental groups that received either the vehicle (− insulin) or insulin (+ insulin at 8 U/kg) 20 min before analysis. Quantification is shown (bottom). Values are means ± SE; n = 4/experimental group. *P < 0.05 vs. corresponding vehicle-treated basal values.
This fetal adaptation to excessive substrate in utero, along with hyperinsulinemia, may be an attempt by the cells to handle the increased glucose load that is presented at the plasma membrane. However, after birth, when the circulating glucose and insulin concentrations have normalized, a persistence of increased sarcolemma/PM-associated GLUT4 concentrations suggests a permanency of this adaptation despite removal of the extracellular fetal metabolic stimulus that initiated this process. This change continues in the face of euglycemia and normoinsulinemia into adulthood. Further exposure to exogenous insulin during the postnatal or adult stages of development leads to no additional increase in sarcolemma/PM-associated GLUT4, setting the stage for development of insulin resistance. A suppression of insulin-induced glucose uptake by peripheral insulin-sensitive tissues is a forerunner and may serve as a marker for the future development of glucose intolerance (day 180), leading into NIDDM when islet cell failure sets in (24). These changes in GLUT4 precede the later manifestation of diabetes mellitus and/or obesity at day 180 and beyond. Hence, in the progeny of a diabetic mother, aberrant GLUT4 subcellular distribution may be one of the initial events noted as early as in the newborn, before the development of adult onset diabetes and obesity.

The mechanism(s) responsible for this aberrant distribution of GLUT4 remains unknown. The process of GLUT4 translocation in the adult is regulated by insulin (16), contraction in the skeletal muscle (8), and hypoxia in the myocardium (27). This process consists...
of exocytosis of the GLUT4-containing vesicular compartment from the Golgi to the plasma membrane (19, 36). This process is then followed by the recycling of GLUT4 internally, i.e., endocytosis (37). It is not clear which arm of this process, endocytosis or exocytosis, is altered by in utero exposure to a diabetic metabolic environment.

In contrast to the CM/DP group, relative nutrient restriction postnatally due to diminished milk production by diabetic mothers, as seen in the DM/DP group, led to a relative amelioration of the GLUT4 defect in skeletal muscle and WAT. This relative recovery of insulin-induced GLUT4 translocation in skeletal muscle and WAT suggests that some form of postnatal nutrient restriction, with extension beyond the suckling phase, may contribute toward reversing the later development of glucose intolerance and insulin resistance that occurs in response to exposure in utero to a maternal diabetic environment.

In summary, exposure in utero to a diabetic milieu causes a defect in GLUT4 subcellular distribution in both skeletal muscle and WAT. This translates into increased basal concentrations of GLUT4 on the cell surface and an associated defect in insulin-induced GLUT4 translocation. This defect persists into adulthood and is also associated with hyperphagia when there is liberal availability of milk/food postnatally, predicting the adult onset of glucose intolerance, relative insulin resistance, and obesity. In contrast, relative postnatal nutrient restriction that causes postnatal growth restriction results in a decline in hypothalamic NPY concentrations and food intake, which are associated with a partial amelioration of the defect in insulin-induced GLUT4 translocation in skeletal muscle and WAT, leading to glucose tolerance and adult growth restriction, which are more prominent in the female. Further investigations involving extension of the period of nutrient restriction beyond the suckling phase are required to demonstrate complete reversal of this defect. In addition, the mechanism responsible for increasing basal concentrations of PM-associated GLUT4 with no further response to exogenous insulin in the CM/DP group needs to be delineated.

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REFERENCES


