GLUT4 expression and subcellular localization in the intrauterine growth-restricted adult rat female offspring

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Thamotharan, Manikkavasagar, Bo-Chul Shin, Dilika T. Suddirikku, Shanthie Thamotharan, Meena Garg, and Sherin U. Devaskar. GLUT4 expression and subcellular localization in the intrauterine growth-restricted adult rat female offspring. Am J Physiol Endocrinol Metab 288: E935–E947, 2005. First published December 29, 2004; doi:10.1152/ajpendo.00342.2004.—Intrauterine growth restriction (IUGR) leads to obesity, glucose intolerance, and type 2 diabetes mellitus in the adult. To determine the mechanism(s) behind this “metabolic imprinting” phenomenon, we examined the effect of total calorie restriction during mid- to late gestation modified by postnatal ad libitum access to nutrients (CM/SP) or nutrient restriction (SM/SP) vs. postnatal nutrient restriction alone (SM/CP) on skeletal muscle and white adipose tissue (WAT) insulin-responsive glucose transporter isoform (GLUT4) expression and insulin-responsive translocation. A decline in skeletal muscle GLUT4 expression and protein concentrations was noted only in the SM/SP and SM/CP groups. In contrast, WAT demonstrated no change in GLUT4 expression and protein concentrations in all experimental groups. The altered in utero hormonal/metabolic milieu was associated with a compensatory adaptation that persisted in the adult and consisted of an increase in the skeletal muscle basal plasma membrane-associated GLUT4 concentrations. This perturbation led to no further exogenous insulin-induced GLUT4 translocation, thereby disabling the insulin responsiveness of the skeletal muscle but retaining it in WAT. These changes, which present at birth, collectively maximize basal glucose transport to the compromised skeletal muscle with a relative resistance to exogenous/ postprandial insulin. Preservation of insulin responsiveness in WAT may serve as a sink that absorbs postprandial nutrients that can no longer efficiently access skeletal muscle. We speculate that, in utero, GLUT4 aberrations may predict type 2 diabetes mellitus, whereas postnatal nutrient intake may predict obesity, thereby explaining the heterogeneous phenotype of the IUGR adult offspring.

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EPIEDEMOLOGICAL STUDIES HAVE LINKED prenatal nutrient restriction presenting with low birth weight to the metabolic syndrome, consisting of insulin resistance/type 2 diabetes mellitus, obesity, hypertension, and coronary artery disease, during adult life (4, 17, 35, 52, 55, 62). Mimicking these human epidemiological observations, various animal models have been developed to determine the mechanistic basis for this link between intrauterine events and the eventual adult phenotype. These animal models range from maternal hypoxia (11, 56), bilateral uterine artery ligation resulting in an acute hypoxic-ischemic injury to the fetus (33, 40, 48), prenatal calorie restriction (16, 58), or a low-protein diet throughout pregnancy (16, 31, 57). In all these animal models, the newborn offspring is growth restricted and develops glucose intolerance as an adult (16, 51, 58). Although a definite link between prenatal nutrient restriction and diabetes mellitus in the adult offspring has been established (16, 17, 35, 51, 58), the role of postnatal nutrient restriction in this cascade of events remains relatively unclear. This is of major clinical importance, because postnatal starvation is experienced by many infants in developing countries. In contrast, in the Western world, although certain critically ill or socially deprived infants may experience immediate postnatal nutrient restriction, generally the low birth weight and intrauterine growth-restricted (IUGR) infant therapeutically receives excessive calories, thereby experiencing “catch-up” growth. Despite these nutrient restitution efforts during infancy, many reports suggest that the small-for-gestational-age human infants continue to demonstrate growth restriction during childhood, resulting in the initiation of growth hormone clinical trials in this subset of children (26, 29). These clinical observations support heterogeneity in the subsequent phenotype. The differences between intrauterine and postnatal nutrient restriction and their separate contributions to the adult phenotype have not been systematically investigated.

Toward deciphering the mechanism(s) responsible for glucose intolerance that develops in later life, in vivo and in vitro studies in animals have focused on and discovered major changes in the β-islet cells and their vascularization (6, 20, 41). In addition, various investigators have examined the liver, skeletal muscle, and white adipose tissue (WAT), attempting to unravel the insulin receptor and its signaling system (10, 17, 34, 35, 37), the growth hormone-IGF system (59, 60), and the insulin-responsive glucose transporter (GLUT4) (1, 10, 13, 21, 38) as possible mechanisms underlying the development of type 2 diabetes mellitus. In the adult type 2 diabetic human and animals, aberrations in the expression and/or translocation of the GLUT4 glucose transporter isoform have been observed to cause insulin resistance that is characteristic of this condition (18, 30, 46). In contrast, neonatal and adult nutrient restriction in animals increases insulin sensitivity by increasing GLUT4 gene expression and protein concentrations (9, 10). The intrauterine nutrient-restricted adult offspring expresses heterogeneous phenotype in perturbations, lending to some controversy (1, 13, 34, 37). We therefore hypothesized that intrauterine nutrient restriction negatively affects GLUT4 gene expression and subcellular distribution in a tissue-specific manner, whereas post-
nontal nutrient restriction may have a relatively protective effect. To test this hypothesis, we employed the mid- to late gestation maternal calorie-restricted pregnant rat model with postnatal nutrient restriction or ad libitum access to nutrients and examined in a tissue-specific manner the effect on insulin-responsive GLUT4 gene expression and translocation in the basal and insulin-stimulated states. This was accomplished by cross-fostering of animals, which generated four experimental groups. Thus the control mother with ad libitum access to nutrients fed the intrauterine calorie-restricted semistarved progeny (CM/SP) and represented intrauterine nutrient restriction, and the calorie-restricted semistarved mother fed the IUGR progeny (SM/SP) and represented intrauterine and postnatal nutrient restriction. These groups were compared with the control mother fed control-progeny (CM/CP), which served as the “gold standard”, and the semistarved mother fed control-progeny (SM/CP), which represented postnatal nutrient restriction in the absence of IUGR.

**MATERIALS AND METHODS**

**Animals**

Sprague-Dawley rats (7–8 wk old, 200–250 g; Charles River Laboratories, Hollister, CA) were housed in individual cages, exposed to 12:12-h light-dark cycles at 21–23°C, and allowed ad libitum access to standard rat chow. The National Institutes of Health guidelines were followed, and the protocols were approved by the Animal Research Committee of the University of California, Los Angeles.

**Maternal Semistarvation Model**

Pregnant rats received 50% of their daily food intake beginning from day 11 through day 21 of gestation, causing caloric restriction during mid- to late gestation, compared with their control counterparts who received ad libitum access to rat chow. Both groups had ad libitum access to drinking water.

**Postnatal Animal Maintenance**

At birth, the litter size was culled to six. In addition, the newborn rats born to semistarved mothers were cross-fostered to be reared by either a mother that continued to be semistarved by receiving 50% of daily food intake through lactation (11 g/day) or a control mother with ad libitum access to rat chow (~20 g/day). Similarly, newborn pups born to control mothers were cross-fostered to be reared by either a mother that continued to be semistarved by receiving 50% of daily food intake through lactation (11 g/day) or a control mother with ad libitum access to rat chow (20 g/day). Thus four groups were created, with control mothers rearing control pups (CM/CP) or prenatally semistarved pups (CM/SP), and pre- and postnatally semistarved mothers rearing prenatally semistarved pups (SM/SP) or control pups (SM/CP). At day 21, the pups were weaned from the mother and maintained in individual cages on a similar diet of standard rat chow until ~day 240 of life.

**Glucose Tolerance Test**

The day 2 neonatal pups (under gentle restraint), day 60 and days 180–240 adult female animals (in plastic rat restrainers), were subjected to a glucose tolerance test (GTT) as described previously (53). The neonatal pups from several litters were pooled to perform a single GTT. All pups received 0.5 g to 1 mg/g body wt of glucose intraperitoneally, and blood from a pooled set of pups was collected from the jugular vein for each time point (0, 15, 30, 60, and 120 min). The adult awake female animals received 0.5 g of glucose via the tail vein, and blood was obtained at 0, 15, 30, 60, and 120 min subsequently to measure glucose concentrations. In certain day 60 adults, jugular vein catheters were placed surgically after the intraperitoneal administration of an anesthetic cocktail (1 ml/kg) containing ketamine (50 mg/ml), acepromazine (1 mg/ml), and xylazine (5 mg/ml). GTTs were performed (0, 5, 15, 30, 60, and 120 min) via these catheters after ≥24 h of recovery to allow assessment of plasma insulin in addition to the blood glucose concentrations.

**Insulin Tolerance Test**

At day 60, adult awake female animals in rat restrainers that had jugular vein catheters placed were subjected to an ITT. The animals received 0.75 U/kg human insulin via the jugular vein catheter, and blood was obtained at 0, 15, 30, and 60 min subsequently to measure glucose concentrations.

**Anthropometric Measurements**

Body weights were assessed longitudinally at days 1, 21, 35, 60, 90, 150, 180, and 240. In addition, at days 60 and 180, animals were deeply anesthetized with inhalational isoflurane to maintain organ blood flow, and various organs/tissues were harvested and weighed on a scale with an accuracy of 0.001 g.

**Plasma Assays**

The female animals were anesthetized as described above, blood was collected from the left ventricle, and the plasma was separated and aliquoted for measurement of glucose by the glucose oxidase method (Sigma Diagnostics, St. Louis, MO; sensitivity = 0.1 mM). Insulin and leptin were quantified by the 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 using the anti-rat corticosterone antibody (Amersham Life Science, Buckinghamshire, UK) (sensitivity: corticosterone = 0.06 ng/ml) (53). In addition, serum triacylglycerol, cholesterol, high-density lipoprotein (HDL), unesterified cholesterol (UC), and free fatty acids were measured by colorimetric assays as previously described (61). Plasma glycerol concentrations were determined and used to correct the triacylglycerol values. The HDL cholesterol was derived from the measurement of the supernatant following the precipitation of apolipoprotein B (apoB)-containing lipoproteins with heparin and MnCl2 (45). 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.

**Skeletal Muscle and WAT Studies**

For tissue collection, skeletal muscle and WAT were rapidly separated from surrounding tissues, quickly snap-frozen in liquid nitrogen, and stored at −70°C.

**GLUT4 mRNA Analysis**

Northern blot analysis. Total RNA was isolated from the newborn and adult female rat mixed skeletal muscle obtained from the hindlimb and the adult perirenal WAT by use of TRIzol reagent (Life Technologies, Gaithersburg, MD). Ten micrograms of total RNA were subjected to Northern blot analysis as previously described (53). The rat GLUT4 cDNA (18) containing the coding region from the translational start to the translational stop sites was used as the probe and the full-length mouse ribosomal protein S2 cDNA as the internal control (53), each with a specific activity of 2.25 × 10⁶ dpm/μg DNA.

Reverse transcription and real-time polymerase chain reaction. A second method was also undertaken to assess skeletal muscle and WAT GLUT4 mRNA. First-strand cDNA was synthesized from 1 μg...
of DNAse-pretreated skeletal muscle or WAT RNA with Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s recommendations. Real-time PCR primers were selected from exon 5 (forward: 5'-ccgaaaggtcatc-aagccct-3') and exon 6 (reverse: 5'-cctttcacttctgctgctc-3') of the rat GLUT4 sequence (GenBank no. G16678014), which were designed using the Primer Express computer software (Applied Biosystem, Foster City, CA). These primers amplified an 80-bp DNA fragment. The Taqman probe was the intervening sequence (5'-tgcatacgacatacgccagcct-3'), which was synthesized and labeled with fluorescent dyes, 6-carboxyfluorescein (FAM) on the 5' end and N,N,N,N-tetramethyl-6-carboxyhydroamine (TAMRA) on the 3' end (Applied Biosystems). Taqman PCR was carried out in triplicate using an ABI Prism 7700 sequence detector (PerkinElmer), and quantification of the amplified product was done against the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as the internal control. The cycling consisted of 12 min at 95°C, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s (5). Relative quantification of PCR amplification products was based on differences between the target and GAPDH control using the comparative critical threshold (CT) method according to the manufacturer’s recommendations. Crossing of CT values obtained for the GLUT4 gene were normalized against each individual GAPDH value, which was run in the same real-time RT-PCR run (42). Prevalidation of the real-time PCR was performed to ensure that the slope of the ΔCT against total RNA was not beyond the manufacturer’s recommendations.

Glucose Transporter Protein Analysis

In vivo insulin administration. At days 2 and 60, female animals from all four groups received either vehicle or insulin (8 U/kg) intraperitoneally. After 20 min, which was a predetermined optimal time point (14), the animals were anesthetized as described above, and skeletal muscle and WAT were obtained for subfractionation studies.

Skeletal muscle preparation. Homogenates and subcellular fractions were prepared from newborns and day 60 adult female animals. Plasma membrane (PM) and low-density microsomal (LDM) subfractions were isolated as previously described, and the relative purity was determined (53). The homogenate and sub-fractions were stored at −70°C until western blot analysis was undertaken.

WAT. One gram of the perirenal fat pad obtained from day 60 adult female animals was used to prepare homogenates, PM, and LDM subfractions, as previously reported, and their relative purity was determined (53). The homogenate, PM, and LDM were stored at −70°C until Western blot analysis was undertaked (53).

Western blot analysis. The homogenates and the fractionated sarcolemma/PM and LDM samples were sonicated (60 sonic, Dismembrator; Fisher Scientific, Pittsburgh, PA) 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 subjected to Western blot analysis as previously described (53). The affinity-purified rabbit anti-rat GLUT4 antibody (1:2,500 dilution) was used as the primary antibody. 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 density of the glucose transporter bands was initially ensured (53).

RESULTS

Body weights of animals from day 1 to day 240 are demonstrated in Fig. 1. The birth weight of pups born to SM mothers (SP) was ~25% lower than those born to CM mothers (CP), consistent with IUGR. There was no difference in the litter size or the male-to-female ratio between these two groups. The animals in the SM/CP and SM/SP groups, which were exposed to nutrient restriction either postnatally or both in utero and postnatally, respectively, demonstrate ongoing growth restriction until day 60, following which the SM/CP group demonstrates catch-up growth, achieving the body weight of the CM/CP group. In contrast, the SM/SP group, with a longer period of nutrient restriction, continues to be growth restricted until day 240. The CM/SP group exposed to nutrient restriction in utero alone mimics the CM/CP group from day 21 until day 90, following which the CM/SP group is heavier than the CM/CP group. The SM/CP group was similar to the SM/SP group at day 60 and similar to the CM/CP group at day 180 in body weight. Organ weights and the nose-to-tail length of the CM/SP and SM/SP groups at days 60 and 180, and the SM/CP group at day 180 were compared with those of the age-matched CM/CP group in Table 1. Various organ weights (heart, liver, pancreas, kidney, brain, WAT) are lower in both groups compared with the CM/CP group, with the SM/SP group demonstrating the maximal reduction at day 60. At day 180, most of the organ weights in the SM/SP group were restored to those of the CM/CP group, with the kidney overshooting and the WAT equaling the CM/CP values. In the SM/CP group, particularly the heart, kidney, and WAT remained decreased at day 180. In contrast to the WAT, there was no change in the brown adipose tissue (BAT) in the CM/SP and SM/SP groups compared with the CM/CP group at both ages. However, a decrease at day 180 was observed only in the SM/CP group. At day 60, the nose-to-tail length was decreased only in the SM/SP group compared with the CM/CP group. No change in the nose-to-tail length was observed in any of the groups at day 180.
Figure 2 demonstrates the GTTs in the day 2 newborns, at day 60, before an increase in body weight in the CM/SP group, and at days 180–240, after the increase in body weight in the CM/SP group. In the newborn, glucose intolerance was observed along with relatively lower basal insulin concentrations in the SP group compared with the CP group (Fig. 2A). At day 60, although no major differences in the intravenous GTTs were observed, as reflected by the area under the curve (AUC) values shown in Fig. 2A, inset, the experimental groups SM/SP and SM/CP demonstrated lower glucose values at 5 min compared with the CM/CP group (Fig. 2B). Assessment of circulating insulin concentrations during the GTT revealed lower insulin levels in the SM/SP and SM/CP groups compared with the CM/CP and CM/SP groups (AUC). The CM/SP group demonstrated insulin concentrations at 5 min to be in between that of the SM/SP and SM/CP groups on the one hand and the CM/CP group on the other hand. At 30, 60, and 120 min, the SM/SP and SM/CP groups continued to demonstrate lower insulin concentrations compared with the CM/CP and CM/SP groups, and the CM/SP group demonstrated a lower insulin level compared with the CM/CP group at 30 min alone (Fig. 2C). The glucose-to-insulin ratio reflective of glucose intolerance during the GTT at day 60 revealed an increase in the SM/SP and SM/CP groups compared with both the CM/CP and the CM/SP groups (AUC). These differences were evident at all time points, with no difference between the SM/SP and the CM/SP groups observed (data not shown). The insulin-to-glucose ratio reflective of an insulin response to the glucose load is significantly lower in the SM/SP and SM/CP groups compared with the CM/CP and CM/SP groups (AUC; data not shown). ITTs at day 60 revealed no significant differences among the four experimental groups, although at 60 min the
In response to exogenous insulin treatment, GLUT4 concentrations in the SP group were one-half those in the CP group. In contrast, the LDM-associated GLUT4 concentrations were double in the SP group compared with the CP group. Figure 3 demonstrates the results of the subcellular distribution experiments. A decline in skeletal muscle GLUT4 mRNA concentrations was observed, no change from basal levels in either the PM or LDM was observed in the SP group. In the day 60 female adult, a decline in the skeletal muscle GLUT4 mRNA amounts was noted in the SM/SP group compared with the CM/CP control group by Northern blot analysis (Fig. 4A). A similar trend was observed when mRNA was quantified by the real-time PCR method, with the SM/SP and SM/CP group being statistically different from the CM/SP group (Fig. 4B). However, paralleling the mRNA changes, decline in skeletal muscle total GLUT4 protein concentrations was observed in the SM/SP and SM/CP groups compared with the CM/CP group (Fig. 4C).

Examination of GLUT4 distribution in the day 60 adult skeletal muscle revealed a persistence of higher PM- and lower LDM-associated GLUT4 in the basal state in both the CM/SP and the SM/SP groups, whereas the SM/CP group revealed sensitivity to insulin evidenced by translocation of GLUT4 to the PM that was intermediate to that noted in the CM/CP group on the one hand and the CM/SP and SM/SP groups on the other hand (Fig. 4D).

Table 3. Plasma metabolite concentrations

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose</th>
<th>Triglycerides</th>
<th>Tot. Chol.</th>
<th>HDL</th>
<th>UC</th>
<th>FFA</th>
</tr>
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<tr>
<td>CP (2 day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>70.67</td>
<td>43.00</td>
<td>50.00</td>
<td>10.67</td>
<td>16.00</td>
<td>18.00</td>
</tr>
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<td>[3]</td>
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<td>5.70</td>
<td>1.53</td>
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<td>50.67</td>
<td>14.00</td>
<td>14.33</td>
<td>15.00</td>
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<tr>
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<td>1.77</td>
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<td>3.29</td>
<td>3.47</td>
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<td>1.53</td>
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<td></td>
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<td>35.00***</td>
<td>6.40</td>
<td>14.80</td>
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<td>36.00***</td>
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<tr>
<td>Control</td>
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<td>11.50</td>
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<td>56.78**</td>
<td>49.50</td>
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<td>9.00**</td>
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<td>66.00***</td>
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<tr>
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<td>69.40</td>
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<td>5.38</td>
<td>4.54</td>
<td>4.06</td>
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<td>0.31</td>
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Values are expressed in mg/dl; nos. in brackets, n. HDL, high-density lipoprotein; UC, unesterified cholesterol; FFA, free fatty acids. P < 0.02 for total cholesterol between CM/CP and SM/SP groups; P < 0.04 for HDL between CM/CP and SM/SP groups; P < 0.009 for FFA between CM/CP and SM/CP groups. *Significantly different from respective CM/CP group; #significant difference between basal and insulin-treated groups within the same experimental group: *, #P ≤ 0.05, **P ≤ 0.02, ###P ≤ 0.01, ####P ≤ 0.0001.

return to baseline was much slower in the SM/CP, next in the SM/SP, followed by the CM/SP, and last in the CM/CP group (Fig. 2D). At days 180–240, the GTTs carried out via the tail vein revealed the SM/CP group to be glucose tolerant, similar to the CM/CP group, whereas the CM/SP and SM/SP groups demonstrated glucose intolerance (Fig. 2E). This intolerance was evident at 15 and 30 min in the CM/SP and SM/SP groups, with the CM/SP group alone demonstrating glucose intolerance at 60 min.

Figure 3 demonstrates the day 2 newborn skeletal muscle results. A decline in skeletal muscle GLUT4 mRNA concentrations is observed at day 2 between the CP and SP groups by Northern blot analysis (Fig. 3A). In contrast, real-time PCR, which employed the GAPDH enzyme mRNA as the internal control, detected no statistical difference in GLUT4 mRNA between the CP and SP groups (Fig. 3B). Figure 3C demonstrates the total skeletal muscle homogenate GLUT4 protein amounts, with vinculin, a structural protein, serving as the internal control. A decline in total GLUT4 protein concentrations was observed, paralleling the decline in GLUT4 mRNA levels as assessed by Northern blot analysis. Figure 3D demonstrates the results of the subcellular distribution experiments. In the day 2 newborn skeletal muscle, the basal PM-associated GLUT4 concentrations were double in the SP group compared with the CP group. In contrast, the LDM-associated GLUT4 concentrations in the SP group were one-half those in the CP group (Fig. 3D). In response to exogenous insulin treatment, although an increase in the PM-associated GLUT4 and a
Because of a lack of WAT in the day 2 newborn, similar studies could not be undertaken during the early postnatal stage; however, WAT studies were undertaken at day 60. In contrast to skeletal muscle, although no major differences in WAT GLUT4 mRNA concentrations were noted in the CM/SP and SM/SP groups compared with the CM/CP group by Northern blot analysis (Fig. 5A; the SM/CP group was not analyzed), the real-time RT-PCR method (Fig. 5B) of mRNA detection

![Graphs showing glucose tolerance and insulin tolerance tests](image_url)

Fig. 2. Glucose tolerance (GTT) and insulin tolerance tests (ITTs). A: GTTs in day 2 newborn CP (n = 4; n = a pool of 6 pups) and SP (n = 4; n = a pool of 6 pups) groups are shown. Solid lines represent blood glucose concentrations; dotted lines represent plasma insulin concentrations. Inset: respective area under the curve calculations (AUC) for blood glucose concentrations. *P values for comparisons between CP and SP groups: at 15 min = 0.002, 30 min = 0.003, 120 min = 0.002, and 180 min = 0.0002 for both glucose and insulin concentrations. B: GTTs in day 60 female adults of the CM/CP (n = 6), CM/SP (n = 6), SM/SP (n = 6), and SM/CP (n = 6) groups are shown. Inset: respective AUC calculations. P ≤ 0.02 at 5 min when SM/SP and SM/CP are compared with the CM/CP group (*) and at 120 min when SM/SP and SM/CP groups are compared with the CM/SP group (#). C: plasma insulin concentrations of GTTs in day 60 female adults are shown in the 4 experimental groups (n = 6 per group). Inset: respective AUC calculations. P ≤ 0.05 compared with CM/CP (*) or CM/SP (#). D: ITTs in day 60 female adults in CM/CP (n = 6), CM/SP (n = 6), SM/SP (n = 6), and SM/CP (n = 6) groups. Inset: respective AUC calculations. No significant differences were observed. E: GTTs in day 180–240 female adults in CM/CP (n = 6), CM/SP (n = 6), SM/SP (n = 6), and SM/CP (n = 6) groups. Insets: respective AUC calculations. P ≤ 0.02 when CM/SP and SM/SP are compared with CM/CP (*) and SM/SP with SM/CP ($).
demonstrated a decrease in the CM/SP and SM/SP groups compared with the CM/CP group. However, the WAT total GLUT4 protein concentrations (Fig. 5C) were no different in all four experimental groups, reflecting the GLUT4 mRNA concentrations as assessed by Northern blot analysis (Fig. 5A).

Translocation investigations in WAT revealed a preservation of the insulin responsiveness of GLUT4 protein translocation to the PM in all four groups, thereby exhibiting characteristics similar to the CM/CP group (Fig. 5D). There were some relative differences observed, such as a decline in the PM-associated GLUT4 concentrations in the CM/SP and SM/SP groups, with the latter achieving statistical significance and an increase in the SM/CP group compared with the CM/CP group in the insulin-responsive GLUT4 translocation to the PM (Fig. 5D).

**DISCUSSION**

In the present study, we demonstrated that IUGR caused by prenatal nutrient restriction results in significant heterogeneity of the adult phenotype on the basis of postnatal calorie intake. Ongoing postnatal nutrient restriction leads to persistent growth failure in the adult with an aberrant hormonal and metabolic profile (SM/SP), whereas relative postnatal nutrient excess results in a propensity toward the subsequent development of obesity, as evidenced by a superseding “catch-up”...
growth profile (CM/SP), both conditions being associated with adult onset glucose intolerance. In contrast, in the absence of intrauterine nutrient restriction and IUGR, postnatal nutrient restriction alone led to an early recovery without persistent growth failure or a propensity toward developing obesity subsequently in the glucose tolerant adult (SM/CP). The adverse effect of a nutritionally perturbed intrauterine environment presents as early as day 2 of postnatal age, persisting into adulthood. In addition, we have separated the impact of prenatal vs. postnatal nutrient restriction on the adult phenotype.

A

B

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>GLUT4 Average C&lt;sub&gt;Τ&lt;/sub&gt;</th>
<th>GAPDH Average C&lt;sub&gt;Τ&lt;/sub&gt;</th>
<th>ΔC&lt;sub&gt;Τ&lt;/sub&gt; GLUT - GAPDH</th>
<th>ΔΔC&lt;sub&gt;Τ&lt;/sub&gt;</th>
<th>GLUT4 Relative to CM/CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM/CP</td>
<td>24.10±3.60</td>
<td>17.66±0.33</td>
<td>6.41±0.05</td>
<td>0.05±0.05</td>
<td>1.02±0.03</td>
</tr>
<tr>
<td>CM/SP</td>
<td>26.41±0.55</td>
<td>20.17±0.58</td>
<td>6.24±0.05</td>
<td>-0.17±0.05</td>
<td>1.14±0.05</td>
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<tr>
<td>SM/SP</td>
<td>23.71±0.13</td>
<td>17.26±0.11</td>
<td>6.47±0.06</td>
<td>0.03±0.06</td>
<td>0.98±0.04*</td>
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<tr>
<td>SM/CP</td>
<td>24.45±0.43</td>
<td>17.89±0.38</td>
<td>6.51±0.07</td>
<td>0.09±0.07</td>
<td>0.94±0.04*</td>
</tr>
</tbody>
</table>

C

D

* p < 0.05 vs CM/CP
# p < 0.01 vs CM/CP
Furthermore, we have demonstrated the mechanism(s) underlying the different postnatal nutrient states that modify the impact of the prenatal insult on the adult phenotype.

We employed the mid- to late gestation calorie-restricted-IUGR rat model for our studies, since nutrient restriction during the third trimester of human pregnancy is associated with the classical “disproportionate” IUGR, which is a fetal adaptation to survive in an adverse in utero nutrient environment (22, 23, 44). Furthermore, although models of maternal/fetal global hypoxia (11, 56) or the bilateral uterine artery-ligated hypoxic ischemia (33, 40, 48) produce IUGR (11, 56), there is an element of permanent cellular damage due to compromised cellular oxidative metabolism (11, 56) and ATP depletion (11, 24, 33, 40, 41, 43, 48, 49, 51, 56). In this latter model, the human situations of utero-placental insufficiency are mimicked rather than nutrient restriction alone (32). However, the acute reduction in blood flow in this model fails to reproduce the chronic evolution of the human disease process that gradually culminates in utero-placental insufficiency (24).

In the context of our present study, hypoxia is known to independently alter the subcellular distribution of GLUT4 (47). Hence, such models would offer limitations in the accurate assessment and distinction between the insulin-stimulated vs. hypoxia- or hypoxic ischemia-induced GLUT4 redistribution in the IUGR offspring.

On the other hand, chronic nutrient restriction models may not initially affect the oxygenation and acid-base status of the growing conceptus but may gradually alter utero-placental blood flow (2). Thus this model mimics the human condition of malnutrition existent worldwide and the chronic evolution of utero-placental insufficiency that typically occurs during mid-to late gestation and is the predominant cause of IUGR in the Western world. Although nutrient restriction during pregnancy increases fetal corticosterone levels, which in turn may explain the adult phenotype (27), our present investigations of nutrient restriction only during mid- to late gestation failed to alter the circulating corticosterone concentrations in the IUGR offspring. In addition, the effect of fetal or neonatal hypercorticoстерonism on glucose transporters is distinct and different (7, 15, 28) from what we have presently observed in the IUGR offspring.

Studies involving severe and prolonged nutrient restriction (calorie or protein restriction) that either spans the entire pregnancy or the postnatal period (8, 34–38) or the late intrauterine and postnatal life until adulthood (70) have demonstrated changes in total and subcellular levels of skeletal muscle and myocardial GLUT4 reflecting enhanced insulin sensitivity (1, 10, 13). These changes are similar to the increased insulin sensitivity of the nutrient-restricted adult (1, 8, 9). Thus the effect of postnatal and/or adult undernutrition overshadows or reverses any changes due to in utero nutrient restriction. Those studies along with others (19, 36) provided the basis for our present study, which investigated whether extrauterine nutrient restriction would be therapeutic in the adult IUGR offspring and prevent adult onset glucose intolerance, a forerunner of type 2 diabetes.

Calorie restriction-induced IUGR is associated with hyperinsulinemia due to aberrations in pancreatic β-islet cells (41), which in turn interfere with nutrient delivery to tissues/cells. Persistently low circulating insulin concentrations result in decreased total GLUT4 concentrations in skeletal muscle, perhaps due to a decline in transcription (39). As an adaptation to this altered and adverse in utero hormonal milieu, the diminished skeletal muscle GLUT4 redistributes to the PM to increase access of glucose to GLUT4. Thus an increase in PM-associated GLUT4 enhances glucose transport to meet the oxidative needs of a nutrient-deprived IUGR skeletal muscle, thereby reflecting “heightened” insulin sensitivity in the basal state. This adaptation serves to protect the fetal skeletal muscle metabolism as a survival mechanism, whereby GLUT4 functions like GLUT1 of the fetal skeletal muscle, enhancing glucose transport under basal conditions (15). Thus, although total GLUT4 content is decreased, the fractional amount available on the surface that is functional is increased.

When nutrient restriction is reversed postnatally (CM/SP) or continued (SM/SP), the in utero posttranslational GLUT4 adaptation of skeletal muscle persists in the adult regardless of the postnatal nutrient availability. Thus the intrauterine perturbation that is evident in the immediate postnatal stage persists as an “imprint” in the adult offspring despite removal of nutrient restriction (CM/SP). On the other hand, postnatal nutrient restriction in the IUGR (SM/SP) fails to further worsen or ameliorate this GLUT4 imprint in skeletal muscle. The persistence of this fetal adaptation causes a maladaptation in the adult.

Exogenous insulin administration fails to reverse this perturbed subcellular distribution of GLUT4 in skeletal muscle. There is no insulin responsiveness of skeletal muscle GLUT4 observed in the IUGR newborn and adult offspring. This insulin resistance of GLUT4 distribution suggests unresponsiveness to the intermittent postprandial insulin surges that are part of normal physiology, thereby interfering with insulin-induced glucose transport into skeletal muscle, the major site of insulin-induced glucose utilization (3). This insulin resistance of skeletal muscle GLUT4 translocation, which persists in the adult, does not culminate in major whole body glucose intolerance or insulin resistance at day 60 due to other compensatory mechanisms, which include various hormonal responses and other tissue-specific (e.g., liver) changes (12, 38). Subsequently, however, glucose intolerance emerges only in

Fig. 4. Day 60 female adult skeletal muscle studies. A: Northern blot analysis demonstrating GLUT4 mRNA. Top: representative Northern blots of the GLUT4 mRNA (above) and ribosomal S2 mRNA (below; internal control). Bottom: phosphorimager quantification of GLUT4/S2 mRNA concentrations in CM/CP (n = 6), CM/SP (n = 6), SM/SP (n = 6), and SM/CP (n = 6) groups. *P < 0.05 in SM/SP vs. CM/CP. B: real-time RT-PCR quantifying GLUT4 mRNA with GAPDH as internal control. Top: PCR amplification curves. Bottom: AttC; for GLUT4 mRNA-GAPDH mRNA concentrations relative to the CM/CP value in CM/CP (n = 6), CM/SP (n = 6), SM/SP (n = 6), SM/CP (n = 5) groups. Values are means ± SE; *P < 0.05 in SM/SP and SM/CP vs. CM/CP. C: Western blot analysis demonstrating total GLUT4 concentrations. Top: representative Western blots showing the GLUT4 (above) and vinculin (below; internal control) proteins. Bottom: densitometric quantification of GLUT4/vinculin protein concentrations in CM/CP (n = 6), CM/SP (n = 6), SM/SP (n = 6), and SM/CP (n = 5) groups. #P < 0.05 in SM/SP and SM/CP vs. CM/CP; *P < 0.05 in SM/SP vs. CM/SP. D: Western blot analysis demonstrating GLUT4 protein concentrations in PM and LDM subfractions of skeletal muscle. Top: representative Western blots of the GLUT4 protein. Bottom: densitometric assessment of GLUT4 protein concentrations in the absence (−) or presence (+) of insulin in CM/CP (n = 6), CM/SP (n = 6), SM/SP (n = 6), and SM/CP (n = 6) groups. *, #, $, P ≤ 0.01.
the IUGR offspring whether exposed postnatally to nutrient excess or restriction. Thus, contrary to previous suggestions (1, 36), postnatal nutrient restriction of an IUGR offspring may not serve as a therapeutic intervention targeted at reversing the subsequent development of type 2 diabetes. This is in stark contrast to its protective role against adult onset obesity (36).

This inability of GLUT4 to respond to exogenous insulin may relate to either a defective process of internalization or exocytosis, or both may be related to a saturation of the PM docking sites with GLUT4 in the basal state (54). In addition, the classical insulin-signaling pathway, which involves the insulin receptor, phosphatidylinositol-3-kinase, and Akt/pro-
tein kinase B, and other, nonclassical pathways that induce GLUT4 translocation (21) may be permanently altered, causing the imprint observed in our current investigations.

The WAT demonstrates no change in total GLUT4 protein concentrations and the insulin-induced subcellular localization of GLUT4 in the adult IUGR offspring. Access to ad libitum milk intake and absence of postnatal calorie restriction make no difference to the total GLUT4 amounts or the subcellular localization. In addition, insulin sensitivity of GLUT4 in WAT is retained across all experimental groups. Thus, in the IUGR offspring, the contribution by WAT toward retained insulin responsiveness must be sizeable to translate into relatively normal GTTs and ITTs at day 60. Later in life, at days 180–240, both the CM/SP and the SM/SP groups, one demonstrating catch-up growth that supersedes that of the control and the other growth retarded, are glucose intolerant. At this stage, the peripheral cellular changes consistent with insulin resistance outstrip the capacity of the altered and perhaps failing pancreatic β-islets to produce the required amounts of insulin. The IUGR offspring is born with insulin resistance of the skeletal muscle GLUT4 translocation, which in turn serves as a forerunner of type 2 diabetes mellitus regardless of postnatal nutrition. The circulating postprandial glucose not utilized by the insulin-resistant skeletal muscle is perhaps shunted to the insulin-sensitive WAT at day 60. Thus, in situations of ad libitum availability of nutrients, insulin-stimulated glucose transport into WAT may be enhanced, thereby setting the stage for the development of obesity, hyperinsulinemia, and hyperleptinemia in later adult life (58). Thus postnatal nutrient availability is incapable of altering the posttranslational GLUT4 imprint in skeletal muscle but may adversely affect the WAT causing obesity. As long as WAT demonstrates insulin sensitivity of GLUT4 translocation, the animal remains insulin sensitive and demonstrates enhanced insulin-induced glucose utilization in euglycemic hyperinsulinemic clamp experiments (1). Once obesity sets in, these animals begin expressing total body insulin resistance, as seen by glucose intolerance (on GTTs) and insulin intolerance (ITTs), with hyperinsulinemia and hyperleptinemia (58).

In contrast, although postnatal nutrient restriction fails to ameliorate the skeletal muscle insulin resistance of IUGR, it prevents the development of adult onset obesity and concomitant WAT insulin resistance. However, recent evidence in human studies reveals that, despite a small adult phenotype with a low body mass index, the persistently growth-restricted IUGR adult demonstrates visceral adiposity detected by magnetic resonance imaging and an increased incidence of diabetes mellitus (63). The visceral adiposity may be related to adequate nutrient intake during infancy, childhood, and adult life or to a sedentary life style. Once the skeletal muscle and other organs have been compromised in utero due to nutrient restriction, postnatal catch-up growth secondary to the availability of excess or even adequate nutrient intake is perhaps related to “cryptic” adiposity in various tissues.

Contrary to the prenatal nutrient restriction paradigm, postnatal nutrient restriction while reducing the insulin-induced translocation of GLUT4 in skeletal muscle and increasing the insulin-induced GLUT4 translocation in WAT maintains insulin sensitivity and glucose tolerance (days 180–240) despite a propensity toward hypoinsulinenia. This is similar to low protein exposure of postnatal rats during the first 10 days of life, where the overall insulin sensitivity in the day 60 adult was increased (10).

In summary, prenatal nutrient restriction led to an altered in utero hormonal/metabolic milieu, which caused aberrations in basal and insulin-induced skeletal muscle GLUT4 subcellular distribution that presented in the immediate neonatal period. This aberration persisted as an “imprint” in the adult female IUGR offspring regardless of the type of postnatal nutritional modification, setting the stage for heightened basal insulin sensitivity of GLUT4 translocation and the subsequent development of glucose intolerance and perhaps insulin resistance. This skeletal muscle GLUT4 aberration occurs as an adaptation toward survival in a nutritionally adverse uterine environment. However, persistence of this aberration in the presence of adequate nutrient availability causes a maladaptation predisposing toward adult onset diseases. In contrast, WAT GLUT4 retained its insulin sensitivity in the IUGR adult offspring, thereby serving as a sink that absorbs postprandial glucose that is perhaps not efficiently utilized by the skeletal muscle due to the insulin resistance of GLUT4 translocation. Postnatal ad libitum access to milk caused “catch-up” growth in the IUGR offspring, which superseded that of the normally grown offspring, suggesting that this phenotype is a forerunner of obesity in the IUGR adult offspring. In contrast, despite the persistence of skeletal muscle GLUT4 aberrations and glucose intolerance, postnatal nutrient restriction led to ongoing growth retardation and curbed the subsequent development of obesity. As opposed to the effects of prenatal nutrient restriction, postnatal nutrient restriction in the absence of IUGR failed to cause overt glucose intolerance or abnormally enhanced growth. In the IUGR adult female offspring, although postnatal nutrient restriction does not reverse the subsequent development of glucose intolerance, it protects against abnormal enhanced growth, which may serve as a forerunner of obesity. Thus the mechanism(s) responsible for heterogeneity of the adult IUGR phenotype (obese vs. lean) in the presence of skeletal muscle insulin resistance of GLUT4 translocation may be related to postnatal milk ingestion/caloric intake.
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