Perturbed skeletal muscle insulin signaling in the adult female intrauterine growth-restricted rat

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Oak, Shilpa A., Cang Tran, Gerald Pan, Mannikkavasagar Thamotharan, and Sherin U. Devaskar. Perturbed skeletal muscle insulin signaling in the adult female intrauterine growth-restricted rat. Am J Physiol Endocrinol Metab 290: E1321–E1330, 2006. First published January 31, 2006; doi:10.1152/ajpendo.00437.2005.—To determine the molecular mechanism(s) linking fetal adaptations in intrauterine growth restriction (IUGR) to adult maladaptations of type 2 diabetes mellitus, we investigated the effect of prenatal seminutrient restriction, modified by early postnatal ad libitum access to nutrients (CM/SP) or seminutrient restriction (SM/SP), vs. early postnatal seminutrient restriction alone (SM/CP) or control nutrition (CM/CP) on the skeletal muscle postreceptor insulin-signaling pathway in the adult offspring. The altered in utero hormonal/metabolic milieu was associated with no change in basal total IRS-1, p85, and p110α subunits of PI 3-kinase, PKCζ, and PKCε concentrations but an increase in basal IRS-2 (P < 0.05) only in the CM/SP group and an increase in basal phospho (p)-PKCζ (P < 0.05), p-Akt (P < 0.05), and p-PKCε (P < 0.05) concentrations in the CM/SP and SM/SP groups. Insulin-stimulated increases in p-PDK-1 (P < 0.05) and p-Akt (P < 0.05), with no increase in p-PKCζ, were seen in both CM/SP and SM/SP groups. SHP2 (P < 0.03) and PTP1B (P < 0.03) increased only in SM/SP with no change in PTKEN in CM/SP and SM/SP groups. Aberrations in kinase and phosphatase moieties in the adult IUGR offspring were initiated in utero but further sculpted by the early postnatal nutritional state. Although the CM/SP group demonstrated enhanced kinase activation, the SM/SP group revealed an added increase in phosphatase concentrations with the net result of heightened basal insulin sensitivity in both groups. The inability to further respond to exogenous insulin was due to the key molecular distal roadblock consisting of resistance to phosphorylate and activate PKCζ necessary for GLUT4 translocation. This protective adaptation may become maladaptive and serve as a forerunner for gestational and type 2 diabetes mellitus.

protein kinase Cζ; metabolic programming; glucose transporters; insulin resistance

EPIDEMIOLOGICAL 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 (7, 29, 46, 51, 57, 60). Mimicking these human epidemiological observations, we developed an animal model consisting of prenatal nutrient restriction that caused intrauterine growth restriction (IUGR) in the rat (53). Using this animal model, we (53) previously demonstrated aberrant skeletal muscle expression and insulin-induced translocation of the insulin-responsive glucose transporter isoform (GLUT4) that persisted in the adult offspring as the mechanism responsible for the subsequent development of glucose intolerance and insulin resistance. In addition, various early postnatal nutrient intake regimens causing differing adult phenotypes were observed not to affect the skeletal muscle GLUT4 imprint acquired in utero. However, the mechanism(s) behind the posttranslational perturbations of GLUT4 translocation remains unknown.

Previous investigations in the adult type 2 diabetic patient have revealed aberrations in the insulin-induced translocation of GLUT4 related to changes in the postreceptor insulin-signaling pathway involving either the kinase [IRS, phosphatidylinositol (PI) 3-kinase, phosphoinositide-dependent protein kinase (PDK), Akt, atypical PKC] (34, 44) and/or phosphatase [SH2-containing protein tyrosine phosphatase 2 (SHP2), protein tyrosine phosphatase 1B (PTP1B), tumor suppressor protein (PTEN)] arms (15, 32, 49). By use of the isocaloric selective nutrient (protein)-restricted maternal rat model, changes have been observed in the activating arm of the skeletal muscle insulin-signaling pathway indicating increased insulin sensitivity (55), whereas studies in white adipose tissue demonstrated alterations supporting insulin resistance (45). We therefore hypothesized that intrauterine nutrient restriction alters the skeletal muscle insulin-signaling pathway with adaptive perturbations in the kinase and/or phosphatase arms that further modified by early postnatal nutrient intake. To test this hypothesis, we employed the mid- to late-gestation maternal semi-nutrient-restricted pregnant rat model with early postnatal nutrient restriction or ad libitum access to nutrients and examined the effect on the skeletal muscle insulin-signaling pathway in basal and insulin stimulated states. This was accomplished by cross-fostering of animals, which generated four experimental groups. Thus the control mother (CM) with ad libitum access to nutrients fed the intrauterine semi-nutrient-restricted progeny (SP) and represented intrauterine nutrient restriction with early postnatal ad libitum nutrition (CM/SP), whereas the semi-nutrient-restricted mother (SM) fed the IUGR progeny (SM/SP) and represented intrauterine and early postnatal nutrient restriction (SM/SP). These groups were compared with the control mother that fed control progeny (CM/CP), which served as the “gold standard”, and the semi-nutrient-restricted mother that fed the control progeny (SM/CP) represented early postnatal nutrient restriction in the absence of IUGR (Fig. 1).

EXPERIMENTAL PROCEDURES

Animals

Sprague-Dawley rats (60 days 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 Seminutrient Restriction Model

Pregnant rats received 50% of their daily food intake (11 g/day) beginning from day 11 through day 21 of gestation (SM), which constitutes mid- to late gestation, compared with their control counterparts (CM) that received ad libitum access to rat chow (e11 to e21). Thus four groups were obtained by cross-fostering postnatal rat pups. SM mothers received 50% of daily nutrient intake from mid- to late pregnancy [embryonic day (e)11 to e21] through lactation [postnatal (pn) day 1 to day 21]. At day 60, the female offspring in each group received vehicle (basal, INS–) or insulin (insulin stimulated, INS+).

Postnatal Animal Maintenance

At birth, the litter size was culled to six. In addition, the newborn rats born to the semi-nutrient-restricted mothers (SP) were cross-fostered to be reared by either a mother that continued to be seminutrient restricted through lactation (27) (SM/CP) or a control mother with ad libitum access to rat chow (CM/CP). This food restriction regime ensured that the semi-nutrient-restricted maternal rats received ~50% of the ad libitum food intake through mid- to late pregnancy and lactation (1, 27, 28). Thus four groups were created, with control mothers rearing control (CM/CP) or prenatal semi-nutrient-restricted pups (CM/SP), and pre- and postnatal semi-nutrient-restricted mothers rearing prenatal semi-nutrient-restricted pups (SM/SP) or control pups (SM/CP) (Fig. 1). At day 21, the pups were weaned from the mother and maintained in individual cages on a similar diet of standard rat chow (53).

In Vivo Insulin Administration

At day 60, female animals from all four experimental groups received either vehicle or insulin (8 U/kg ip; Fig. 1). After 20 min, the predetermined optimal time point (23, 53), the animals were deeply anesthetized with inhalational isoflurane to maintain organ blood flow, and skeletal muscle from the hindlimb was harvested.

Skeletal Muscle Preparation

Skeletal muscle was rapidly separated from surrounding tissues, quickly snap-frozen in liquid nitrogen, and stored at −70°C. Homogenates were prepared as previously described (53). Briefly, skeletal muscle was powdered under liquid nitrogen and suspended in three volumes of cell lysis buffer (Cell Signaling Technology, Beverly, MA). The samples were homogenized with a handheld homogenizer for 1–2 min at half speed followed by a 30-min incubation on ice. The samples were then subjected to further homogenization with 20 up-and-down strokes using a tight fitting Potter-Elvehjem homogenizer. These homogenates were then centrifuged at 10,000 rpm at 4°C for 10 min and stored at −70°C until Western blot analysis was undertaken.

Antibodies

Rabbit polyclonal anti-Akt, anti-p-Akt (Ser473), anti-PDK-1, anti-p-PDK-1, anti-p-IRS-1, anti-p-IRS-2 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-PI 3-kinase p85 (p85PS1), anti-p85, anti-IRS-1 and anti-IRS-2 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-PTEN, anti-p110β, and mouse monoclonal PKCδ antibodies were from Santa Cruz Biotecnology (Santa Cruz, CA). Anti-PTP1B antibody was purchased from BD Transduction Laboratories (Lexington, KY). Anti-vinculin antibody was from Sigma Chemical (St. Louis, MO). Rabbit polyclonal anti-Akt2 antibody was a generous gift from Dr. M. J. Birnbaum (University of Pennsylvania School of Medicine).

Western Blot Analysis

The homogenates 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 was subjected to Western blot analysis as previously described (53). Briefly, 50 µg of skeletal muscle homogenates were separated on SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were then blocked in 5% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween 20 (PBST) for 1 h, incubated with the specific primary antibody [1:1,000 dilution for Akt, p-Akt, PDK-1, p-PDK-1, p85, p110β, and SHP2; 1:100 for PKCδ and PKCζ; 1:200 for PTEN; 1:2,500 for PTP1B; 1:500 for IRS-2 and p-PKCζ (Thr410); 1:2,000 for IRS-1 and Akt2; and 1:4,000 for vinculin] overnight at 4°C with gentle agitation. The membranes were then washed with PBST three times for 15 min each. The membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. After the membranes were washed three times for 15 min each, protein bands were visualized using the enhanced chemiluminescence method (Amersham Biosciences, Piscataway, NJ). The quantification of protein bands was performed by densitometry using the Scion Image software. The presence of linearity between the time of X-ray film exposure and the optical density of the various protein bands was initially ensured (53).

PKCζ Activity Assay

PKCζ activity assay was performed as previously described (37, 41), with slight modifications. One milligram of skeletal muscle homogenate was precleared by incubation with 50 µl of 50% slurry of protein A/G-agarose (Santa Cruz Biotechnology) for 2 h at 4°C. Five micrograms of anti-PKCζ rabbit polyclonal antiserum raised against a peptide in the T-loop of PKCζ were incubated with 50 µl of 50% slurry of protein A/G-agarose for 2 h at 4°C on the rotor. The antibody-bound beads were washed three times with the cell lysis buffer and incubated with the precleared homogenate overnight at 4°C on the rotor. The precipitated agarse beads were then washed four times with the cell lysis buffer followed by two washes with the kinase buffer and resuspended in kinase buffer without myelin basic protein (MBP, substrate) and ATP. In vitro kinase assay was carried out for 30 min at 25°C in 50 µl of buffer containing 35 mM Tris, pH
7.4, 10 mM MgCl₂, 1 mM EGTA, 2 mM Na₃VO₄, 20 µg/ml leupeptin, 0.5 mM ATP, 4 µg MBP and 0.4 µCi [γ-³²P]ATP. After incubation, ³²P-labeled substrate was trapped on P81 filter papers. P81 filter papers were then washed four times with 0.75% phosphoric acid and once with acetone and counted in a liquid scintillation counter. Samples that contained no antibody or no substrate were used as controls to account for background and endogenous phosphorylation respectively.

Data Analysis

Data are expressed as means (SD). The analysis of variance (ANOVA) models were used to compare the various treatment groups and the F values obtained. Intergroup differences were determined by Fisher’s paired least significant difference test when ANOVA revealed significance. Significance was assigned when the P value was ≤0.05.

RESULTS

The insulin-signaling pathway as it pertains to GLUT4 translocation is schematically represented in Fig. 2.

Activating Arm

Upregulation of skeletal muscle total IRS-2 but not IRS-1, p85, and p110β subunits of PI 3-kinase. Figure 3A shows the expression levels of IRS-1 and IRS-2 in the three experimental groups compared with the control group. IRS-1 protein expression levels are unchanged in the CM/SP, SM/SP, and SM/CP groups compared with the CM/CP group. However, IRS-2 protein levels are significantly higher in CM/SP (P < 0.05 vs. CM/CP, P < 0.05 vs. SM/SP) and SM/CP (P < 0.05 vs. CM/CP, P < 0.05 vs. SM/SP).

Figure 3B shows the total protein expression of p85, the regulatory subunit, and p110β, the catalytic subunit, of PI 3-kinase. As can be seen, both proteins remain unaltered in the CM/SP, SM/SP, and SM/CP groups compared with the CM/CP group. The total protein expression of p50α and p55α was also analyzed and found to be no different from control (data not shown).

Increased phosphorylation of skeletal muscle PDK-1 in the basal state. Figure 4A demonstrates the phosphorylation status of PDK-1 in basal and insulin-stimulated states. CM/SP (P < 0.05), SM/SP (P = 0.004), and SM/CP (P = 0.015) groups show higher phosphorylation of PDK-1 in the basal state compared with the CM/CP control group. All of the four groups, namely CM/CP (P = 0.02), CM/SP (P = 0.0002), SM/SP (P = 0.005), and SM/CP (P = 0.0003), respond to insulin stimulation, resulting in a further increase of PDK-1 phosphorylation. The CM/SP group shows the highest extent of PDK-1 phosphorylation in response to insulin stimulation. The SM/SP and SM/CP groups exhibit a similar extent of insulin-induced PDK-1 phosphorylation compared with the CM/CP control group.

Increased phosphorylation of skeletal muscle Akt (Ser⁴⁷³) at basal state. Figure 5A shows the phosphorylation status of Akt at basal state and in response to insulin stimulation.
Increased phosphorylation and kinase activity of skeletal muscle PKCζ in basal state with no further response to insulin. Figure 6A shows that protein expression of PKCζ and PKCθ are unaltered in the CM/SP, SM/SP, and SM/CP groups regardless of prenatal and/or early postnatal nutrient restriction compared with the CM/CP group. Figure 6B shows the phosphorylation of PKCζ in the basal and insulin-stimulated states in the four groups. The CM/CP group shows an insignificant phosphorylation in the basal state that significantly increases after insulin stimulation \( (P = 0.002) \). In contrast, the CM/SP \((P = 0.04)\) and SM/SP groups \((P = 0.015)\) show significantly higher basal phosphorylation of PKCζ compared with the CM/CP group. However, both groups fail to show further phosphorylation of PKCζ in response to insulin stimulation, unlike the CM/CP control group. Interestingly, the SM/CP group does not show significantly higher phosphorylation of PKCζ in the basal state but still fails to show a significant insulin-induced increase in the phosphorylation of PKCζ. Figure 6C shows the kinase activity of PKCζ in the basal and insulin-stimulated states of the four groups. The CM/CP group shows minimal kinase activity in the basal state that significantly increases after insulin stimulation \((P < 0.0001)\). In contrast, the CM/SP and SM/SP groups show a tendency toward higher basal kinase activity of PKCζ compared with the CM/CP group, albeit not statistically significant. However, both groups fail to show a further increase in the kinase activity.

CM/SP \((P < 0.05)\) and SM/SP \((P = 0.004)\) groups show higher phosphorylation of Akt in the basal state compared with the CM/CP control group. The SM/CP group shows an overall tendency toward increased phosphorylation of Akt in the basal state, although not significantly higher than CM/CP. All four groups, namely CM/CP \((P = 0.0006)\), CM/SP \((P < 0.0001)\), SM/SP \((P = 0.0003)\), and SM/CP \((P = 0.002)\), respond to insulin stimulation with a further increase in phosphorylation of Akt. The CM/SP group shows the highest extent of phosphorylation of Akt in response to insulin stimulation. The SM/SP and SM/CP groups exhibit a similar extent of insulin-stimulated phosphorylation of Akt compared with the CM/CP group. Figure 5B demonstrates higher Akt2 expression in the SM/SP \((P = 0.011)\) and SM/CP \((P = 0.027)\) groups compared with the CM/CP group. The CM/SP group shows a trend toward increased Akt2 expression that is not significantly different from the CM/CP group. The combined expression of Akt1,2,3 in the three groups, namely CM/SP, SM/SP, and SM/CP, is no different from the CM/CP control group.
of PKC\(\theta\)/H9256 in response to insulin stimulation, unlike the control CM/CP group. In agreement with the phosphorylation of PKC\(\theta\)/H9256 in Fig. 6B, the SM/CP group does not show significantly higher activity of PKC\(\theta\) at the basal state but still fails to show an insulin-induced increase in the phosphorylation of PKC\(\theta\).

Deactivating Arm

Increased skeletal muscle PTP1B and SHP2. Figure 7 demonstrates total protein concentrations of phosphatases PTP1B, PTEN, and SHP2 in the four groups. Only the SM/SP group shows augmented protein expression of PTP1B \((P < 0.05\) vs. CM/CP, \(P < 0.05\) vs. SM/CP) and SHP2 \((P < 0.05\) vs. CM/CP, \(P < 0.05\) vs. SM/CP) compared with the CM/CP and SM/CP groups. Increased PTP1B protein concentrations in the CM/SP group were not consistently observed, thereby not achieving statistical significance. SHP2 protein expression in the CM/SP and SM/CP groups is no different from that of the CM/CP control group. PTEN levels remain unaltered in the four groups.

All changes in the insulin-signaling pathway detected in all three experimental groups (CM/SP, SM/SP, and SM/CP) vs. the CM/CP group have been summarized in Table 1 within the context of the phenotype and skeletal muscle GLUT4 changes (53).

DISCUSSION

Our present study focused on the female offspring, unlike other investigations that have predominantly described the male progeny and reported changes consistent with insulin resistance in adipose tissue (45) and skeletal muscle (47, 48). Unlike a single previous study that examined aging females (18), we examined the adult stage (~6–8 wk), when pregnancy is most likely to occur, to allow assessment of the preconceptional metabolic imprint in these IUGR adult animals. Other groups have previously demonstrated that the IUGR adult female offspring is prone to gestational diabetes mellitus (8); hence, information regarding the preconceptional state may provide hints regarding the predisposition towards gestational metabolic disturbances.

Previously, using the four experimental groups described in this report (Table 1), we delineated the phenotype of the adult offspring where the CM/SP group expressed a nearly normal pancreatic insulin response to a glucose load followed subsequently by abnormally enhanced growth. In contrast, the SM/SP and the SM/CP groups had a decreased insulin response to the same glucose load, with the former demonstrating persistent growth restriction but the latter subsequently attainment normal growth (53). In skeletal muscle, the rate-limiting
were reported that the regulatory isoforms p50 restricted IUGR rat model (47). Recently, McCurdy et al. (43) group, unlike previous observations of a decline in a protein-

Summary of changes in the skeletal muscle insulin-signaling pathway in CM/SP, SM/SP, and SM/CP vs. CM/CP groups

<table>
<thead>
<tr>
<th>Compared with CM/CP (control: ad libitum access to nutrients pre- and postnatally)</th>
<th>CM/SP (prenatal seminutrient restriction)</th>
<th>SM/SP (pre- and early postnatal seminutrient restriction)</th>
<th>SM/CP (early postnatal seminutrient restriction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype: body wt gain and GTT</td>
<td>Higher growth rate Glucose tolerant</td>
<td>Lower growth rate Glucose tolerant</td>
<td>Catch up to CM/CP Glucose tolerant</td>
</tr>
<tr>
<td>Insulin response to glucose load and ITT</td>
<td>↔</td>
<td>↓</td>
<td>↓</td>
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<tr>
<td>GLUT4: basal</td>
<td>↑</td>
<td>↑ from basal</td>
<td>↔</td>
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<tr>
<td>GLUT4: Insulin stimulated</td>
<td>↔ from basal</td>
<td>↑ from basal</td>
<td>↔</td>
</tr>
<tr>
<td>Kinase arm</td>
<td>↑ IRS-2 protein expression</td>
<td>↔</td>
<td>↑ IRS-2 protein expression</td>
</tr>
<tr>
<td>Basal condition</td>
<td>↑ p-Akt ↑ p-PDK-1 ↑ p-PKCζ and trend toward higher PKCζ activity</td>
<td>↑ p-Akt ↑ p-PDK-1 ↑ p-PKCζ and trend toward higher PKCζ activity</td>
<td>↑ p-Akt (same as CM/CP)</td>
</tr>
<tr>
<td>Insulin-stimulated condition (vs. corresponding basal condition and CM/CP).</td>
<td>↑ p-Akt (same as CM/CP)</td>
<td>↑ p-Akt (same as CM/CP)</td>
<td>↑ p-Akt (same as CM/CP)</td>
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<td></td>
<td>↑ p-PDK-1 (same as CM/CP)</td>
<td>↑ p-PDK-1 (same as CM/CP)</td>
<td>↑ p-PDK-1 (same as CM/CP)</td>
</tr>
<tr>
<td></td>
<td>↔ p-PKCζ and activity from basal (unlike CM/CP)</td>
<td>↔ p-PKCζ and activity from basal (unlike CM/CP)</td>
<td>↓</td>
</tr>
<tr>
<td>Total Akt, PDK-1, PKCζ, p-PKCζ, IRS-1, p10βs, and p85 protein expression</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Phosphatase arm</td>
<td>↔ in PTP1B, SHP2, PTEN</td>
<td>↑ PTP1B</td>
<td>↑ PTP1B</td>
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<tr>
<td></td>
<td></td>
<td>↑ SHP2</td>
<td>↑ SHP2</td>
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<td>↔ PTEN</td>
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GTT, glucose tolerance test; PDK, phosphoinositide-dependent protein kinase; PTP1B, protein tyrosine phosphatase 1B; SHP2, SH-containing protein tyrosine phosphatase 2; PTEN, a tumor suppressor protein; p-, phosphorylated; ↑, increase; ↓, decrease; ↔, no change.

step in insulin-induced glucose uptake is the insulin-responsive glucose transport. Despite intergroup differences in the adult phenotype, we previously determined that both the IUGR groups exhibited altered subcellular GLUT4 localization in skeletal muscle. Although there was heightened basal accumulation of GLUT4 in the plasma membrane in both the CM/SP and SM/SP groups, there was no further increase in plasma membrane-associated GLUT4 concentrations in response to exogenous insulin. Unlike the two IUGR groups, the early postnatal nutrient-restricted group SM/CP demonstrated an intermediate response with retention of some insulin responsiveness of the GLUT4 subcellular localization (53).

Because the postreceptor insulin-signaling pathway is the predominant mechanism by which GLUT4 translocation is regulated, we investigated this pathway to determine the point at which this pathway is altered in the IUGR offspring. Although total IRS-1 was unaltered, IRS-2, the isoform that mediates glucose metabolism, was increased in the CM/SP and SM/CP groups compared with the ad libitum-fed adult rats that were unmanipulated. However, in our model of the IUGR adult offspring we did not see any difference in the experimental groups compared with the control.

PDK-1 is a serine/threonine kinase, activated by a lipid product of PI 3-kinase, phosphatidylinositol 3,4,5-trisphosphate (PIP₃) (13, 38, 54, 58). PDK-1 directly phosphorylates and activates downstream effectors of PI 3-kinase, such as Akt and atypical PKCs that mediate physiological actions of insulin (2, 13, 14, 19, 38). Akt is activated in response to insulin in a variety of cell types (35). Previously, Akt² was shown to be the critical isoform in insulin-dependent glucose uptake and maintenance of glucose homeostasis (42). In our present study, although total Akt1,2,3 changes were imperceptible, increased Akt² expression in the SM/SP and SM/CP groups and a trend toward an increase in the CM/SP group may contribute toward heightened basal insulin sensitivity. We also observed a basal increase in p-PDK-1 and p-Akt in the CM/SP and SM/SP groups compared with the CM/CP group. These changes are consistent with programming toward increased endogenous insulin signaling resulting in heightened insulin sensitivity with increased basal GLUT4 concentrations in the plasma membrane. In the presence of exogenous insulin that mimics a physiological response (10.220.33.5 on October 14, 2017 http://ajpendo.physiology.org/ Downloaded from http://ajpendo.physiology.org/ on October 14, 2017)
Studies demonstrate that the atypical PKC isoforms \( \lambda \) and \( \zeta \) are downstream mediators of PI 3-kinase (52) and PDK-1 (3) (Fig. 2). Overexpression of constitutively active PKCa or PKC\(\zeta\) in adipocytes (36) or wild-type PKC\(\zeta\) in muscle in vivo (17) enhances both basal and insulin-stimulated glucose transport. Early growth restriction by maternal selective nutrient (protein) restriction led to a downregulation of total PKC\(\zeta\) that was associated with insulin resistance of skeletal muscle glucose uptake (48). Increased expression of PKC\(\theta\) in skeletal muscle of diabetic patients has also been reported previously (31). These studies cumulatively suggest that atypical PKCs play a vital role in mediating insulin’s effect on GLUT4 translocation, thereby constituting an alternate mediator to Akt. However, in contrast to previous reports, we observed no difference in total PKC\(\zeta\) and PKC\(\theta\) protein expression in CM/SP, SM/SP, and SM/CP groups compared with that of control, the CM/CP group. However, in the basal state, there was increased phosphorylation of PKC\(\zeta\) in the CM/SP and SM/SP groups compared with the CM/CP group. These changes are also in keeping with the activated kinase arm in the basal state and may contribute along with increased IRS-2, Akt2, p-PDK-1, and p-Akt to the basal heightened insulin sensitivity evident as the increase in plasma membrane-associated GLUT4 concentrations (53).

In addition to the components of the kinase arm, PTPs form a large family of enzymes that serve as key regulators in signal transduction pathways. Because the kinase (activating) arm of the insulin-signaling pathway is tempered by the phosphatase (deactivating) arm, our studies demonstrate that, although PTEN changes were imperceptible, SHP2 and PTP1B basal levels were increased in the SM/SP group alone. In agreement with our results, muscle-specific deletion of PTEN that regulates insulin signaling at the PI 3-kinase level did not result in increased insulin sensitivity when the mice were fed a normal chow diet (61). Recently, however, both PTEN mRNA and protein concentrations increased in soleus muscle of obese Zucker rats (\(Fa/Fa\)) vs. the age-matched lean group and fructose-fed lean Zucker rats (\(fa/\alpha\)) vs. the regular chow-fed group, supporting a role for PTEN gene expression in insulin resistance (39). SHP2 and PTP1B have been reported to regulate insulin signaling at the IRS level (16, 40). Overexpression of the dominant negative SHP2 lacking the PTP domain (\(\Delta\)PTP) in transgenic mice led to end-organ insulin resistance despite physiological insulin concentrations (40). Thus SHP2, a protein tyrosine phosphatase contrary to conventional expectations of a phosphatase, may act as a positive regulator of insulin signaling at the IRS level.

In contrast, PTP1B knockout mice are not only insulin sensitive but also maintain euglycemia in the fed state, with one-half the level of insulin observed in wild-type littersmates. On the other hand, overexpression of PTP1B inhibits insulin signaling in cultured cells (12, 16, 33, 50, 59). The expression and/or activity of skeletal muscle PTP1B are increased in insulin-resistant rodents and humans. In addition, transgenic overexpression of PTP1B in muscle causes insulin resistance (62). Overexpression of PTP1B in adipocytes inhibits insulin-stimulated PI 3-kinase activity without altering glucose transport or Akt/PKB activation (59). Recently, an increased association of PTP1B with insulin receptor despite enhanced insulin-stimulated glucose uptake and insulin signaling was reported in 10-day-old rat pups born to food-restricted mothers (20). This suggests that the increase in PTP1B in the IUGR offspring may reflect an adaptive response to exaggerated insulin sensitivity. In keeping with this concept, our observed increase in PTP1B expression in IUGR pups that continue to be exposed to early postnatal nutrient restriction (SM/SP) may represent an adaptation toward providing a regulatory check to the heightened insulin-activating pathway acquired in utero. The need for this PTP1B check is not consistently seen in the CM/SP group that is exposed to a shorter period of in utero nutrient restriction alone. Our previous report (53) supports this concept, as well demonstrating higher whole body insulin sensitivity based on a lower insulin response in maintaining glucose tolerance in the SM/SP vs. the CM/SP group. Adaptations in the basal insulin-signaling pathway, although individualized in each IUGR group, led to a common imprint of a higher basal accumulation of GLUT4 in plasma membrane in both CM/SP and SM/SP groups.

In addition to heightened basal insulin activation, PTP1B-overexpressing mice exhibit impaired insulin-stimulated skeletal muscle activation of PKC\(\zeta\)/PKC\(\theta\) (62). Furthermore, overexpression of a dominant negative mutant of PKC\(\zeta\) or PKC\(\alpha\) abrogates insulin-induced GLUT4 translocation and glucose transport in adipose (6, 36) and muscle cells (5). Defective insulin-induced GLUT4 translocation is associated with increased basal activity and insulin unresponsiveness of PKC\(\zeta\)/PKC\(\theta\) in skeletal muscle of high-fat-fed rats compared with controls (56). Insulin-stimulated PKC\(\zeta\)/PKC\(\theta\) activity is also impaired in skeletal muscle of humans with obesity and type 2 diabetes (34). Thus, along with p-Akt, activation of PKC\(\zeta\)/PKC\(\theta\) is required for GLUT4 translocation-mediated maximal insulin-stimulated glucose uptake (4, 36, 52). The mechanism underlying this process involves PKC\(\zeta\)-mediated serine phosphorylation of VAMP2 (vesicle-associated membrane protein-2) in GLUT4-containing intracellular vesicles, thereby increasing glucose transport in skeletal muscle (9). In addition, insulin induces PKC\(\zeta\) interaction with Munc18c, thereby regulating GLUT4 translocation to the plasma membrane, a process that can be abrogated by a PI 3-kinase inhibitor (LY-294002) (25). More recently, PKC\(\zeta\) has been observed to interact with 80K-H (26), which is expressed abundantly in major insulin-sensitive tissues, including fat and muscle cells (21), and is related to vacuolar system-associated protein-60 (VASAP-60), which is involved in vesicular trafficking (11). Additionally, 80K-H serves as a substrate for atypical PKC (24) and interacts with both PKC\(\zeta\) and Munc18c in an insulin-dependent manner. On the basis of this accumulated information, it is suggested that insulin triggers the formation of a PKC\(\zeta\)-80K-H-Munc18c complex, which then separates Munc18c from syntaxin-4 (t-SNARE protein component) (25, 26), freeing the latter to allow easy interaction with VAMP-2 (v-SNARE protein component) via the coil-coiled sequences called SNARE motifs toward formation of the SNARE (N-ethylmaleimide-sensitive factor attachment protein receptors) complex. The SNARE complex is responsible for insulin-induced vesicular trafficking by intracellular membrane fusion to the plasma membrane (22), a process that underlies GLUT4 translocation (30).

In our present investigation, insulin-responsive p-PKC\(\zeta\) concentrations and activity increased only in the CM/CP group but failed to show a similar response in the CM/SP, SM/SP, and SM/CP groups. This impaired insulin-induced PKC\(\zeta\) phosphorylation and activation may underlie the aberrant insulin-
induced GLUT4 translocation observed previously (53). Thus, although the basal insulin-signaling mechanisms involving the activating arm globally set the stage for enhanced basal GLUT4 concentrations in the plasma membrane (53), the insulin resistance of GLUT4 translocation can be explained only by the absence of increased phosphorylation and activation of PKCζ. This step forms a critical distal roadblock in insulin signaling that may in turn be responsible for compensatory increases in insulin-stimulated proximal steps consisting of phosphorylation of PDK-1 and Akt that respectively constitute the immediate upstream or alternate steps to PKCζ. The cellular adaptive mechanisms targeted at survival, consisting of heightened basal skeletal muscle insulin sensitivity, may raise the set point for glucose delivery and therefore become protective against further insulin action in response to either exogenous insulin or a postprandial insulin surge. Thus in utero changes in p-PKCζ may be a key adaptive mechanism that sets the stage for the subsequent development of insulin resistance of GLUT4 translocation in the adult IUGR offspring.

Insulin-induced GLUT4 translocation to the skeletal muscle plasma membrane translates directly into insulin-responsive glucose transport (10). In a protein-restricted IUGR model, alterations in skeletal muscle GLUT4 distribution have directly altered skeletal muscle glucose transport (47). On this basis, we expect an increased basal glucose transport into skeletal muscle with resistance to a further increase in response to exogenous insulin in both the CM/SP and SM/SP groups. These changes would support a dysregulation of skeletal muscle glucose transport. However, these skeletal muscle-specific changes need to be placed in the context of the whole body glucose/insulin kinetics to make physiological sense of tissue-specific aberrations. We (53) have previously demonstrated no difference in whole body peripheral insulin sensitivity based on insulin tolerance testing at day 60 in the female CM/SP and SM/SP offspring compared with CM/CP. In addition, others have demonstrated no change in total body glucose utilization in response to a hyperinsulinemic glucose clamp in day-100 females in groups comparable to CM/SP and SM/SP (28). Thus skeletal muscle-specific changes may serve as necessary adaptations targeted at normalizing the whole body peripheral glucose/insulin homeostasis, and thereby the physiology, i.e., skeletal muscle mechanisms adapt toward preserving insulin sensitivity and glucose delivery to other organs (e.g., adipose tissue, liver, myocardium, and brain) as a protective response to in utero nutrient restriction. Thus, although the IUGR adult offspring may present with nearly normal whole body insulin sensitivity during the preconceptional stage (53) due to fine tuning achieved by various cellular adaptive/protective mechanisms, certain circumstances, such as pregnancy and cryptic adiposity, can tip this precariously balanced metabolic homeostasis, causing a maladaptive predisposition toward the development of insulin resistance, a forerunner of gestational and type 2 diabetes mellitus.

In summary, we have described, in skeletal muscle of the adult female IUGR offspring, phenotype-specific adaptive alterations that enhance basal but resist insulin-responsive insulin signaling targeted at GLUT4 translocation to the plasma membrane. These changes consist of an increase in the activating kinase arm and, in certain cases the phosphatase arm, in the basal state. In response to insulin stimulation, although a further increase in kinase activation was observed proximal to PKCζ, the key step mediating insulin resistance of GLUT4 translocation was the phosphorylation and activation of PKCζ. Hence, therapeutic targets of p-PKCζ may help reverse the subsequent development of insulin resistance under maladaptive circumstances in the IUGR female adult offspring, thereby aborting the development of gestational diabetes and propagation of the epidemic of type 2 diabetes mellitus.

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REFERENCES

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