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Uteroplacental insufficiency and reducing litter size alters skeletal muscle mitochondrial biogenesis in a sex-specific manner in the adult rat

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Wadley GD, Siebel AL, Cooney GJ, McConell GK, Wlodek ME, Owens JA. Uteroplacental insufficiency and reducing litter size alters skeletal muscle mitochondrial biogenesis in a sex-specific manner in the adult rat. *Am J Physiol Endocrinol Metab* 294: E861–E869, 2008. First published March 4, 2008; doi:10.1152/ajpendo.00037.2008.—Uteroplacental insufficiency has been shown to impair insulin action and glucose homeostasis in adult offspring and may act in part via altered mitochondrial biogenesis and lipid balance in skeletal muscle. Bilateral uterine vessel ligation to induce uteroplacental insufficiency in offspring (Restricted) or sham surgery was performed on day 18 of gestation in rats. To match the litter size of Restricted offspring, a separate cohort of sham litters had litter size reduced to five at birth (Reduced Litter), which also restricted postnatal growth. Remaining litters from sham mothers were unaltered (Control). Offspring were studied at 6 mo of age. In males, both Restricted and Reduced Litter offspring had reduced gastrocnemius PPARγ coactivator-1α (PGC-1α) mRNA and protein, and mitochondrial transcription factor A (mtTFA) and cytochrome oxidase (COX) III mRNA ($P < 0.05$), whereas only Restricted had reduced skeletal muscle COX IV mRNA and protein and glycogen ($P < 0.05$), despite unaltered glucose tolerance, homeostasis model assessment (HOMA) and intramuscular triglycerides. In females, only gastrocnemius mtTFA mRNA was lower in Reduced Litter offspring ($P < 0.05$). Furthermore, glucose tolerance was not altered in any female offspring, although HOMA and intramuscular triglycerides increased in Restricted offspring ($P < 0.05$). It is concluded that restriction of growth due to uteroplacental insufficiency alters skeletal muscle mitochondrial biogenesis and metabolic characteristics, such as glycogen and lipid levels, in a sex-specific manner in the adult rat in the absence of impaired glucose tolerance. Furthermore, an adverse postnatal environment induced by reducing litter size also restricts growth and alters skeletal muscle mitochondrial biogenesis and metabolic characteristics in the adult rat.

programming; glucose metabolism; intrauterine growth restriction

IN HUMANS, UTEROPLACENTAL INSUFFICIENCY is relatively common and a major cause of fetal growth restriction, which is characterized by increased perinatal morbidity and mortality and an increased predisposition to adult disease (3, 9, 19). In particular, low birth weight and fetal growth restriction in humans is associated with whole body, peripheral, and skeletal muscle insulin resistance in the young adult and the subsequent onset of diabetes (19). The mechanisms and defects underlying this prenatally induced insulin resistance are poorly understood, but candidates include impaired mitochondrial biogenesis and altered intramuscular lipid balance (17, 23, 26).

Mitochondria are the primary controllers of cellular metabolism, and several studies suggest that mitochondrial dysfunction and the associated impairment in the ability of key insulin-sensitive tissues, such as skeletal muscle, to produce ATP or to oxidize fats can ultimately lead to insulin resistance, impaired glucose tolerance, and type 2 diabetes (10, 17, 18, 23). Recently, Boushel et al. (4) have shown that the reduced mitochondrial function in skeletal muscle of people with type 2 diabetes can be attributed to the reduced mitochondrial volume. Some studies (11, 17, 23), but not all (18), report reduced mitochondrial biogenesis in insulin-resistant skeletal muscle, although it is still unclear whether it is a cause of insulin resistance or a consequence (2, 42). One consequence of this, accumulated intracellular lipids, is thought to inhibit insulin signaling via activation of insulin-receptor substrate (IRS)-1 serine phosphorylation (18, 26).

The role of such defects in insulin resistance and impaired glucose tolerance associated with uteroplacental insufficiency has been investigated by uterine vessel ligation in the rat (29, 30, 35) and carunclectomy in the sheep (7, 21). These models restrict placental delivery of substrates to the fetus and also impair mammary development and lactation (20), restricting substrate supply to offspring. This restriction of prenatal and postnatal substrate induces insulin resistance, impaired glucose tolerance, and subsequently diabetes in adult offspring (29, 30). However, the impact of uteroplacental insufficiency on skeletal muscle lipid accumulation and mitochondrial biogenesis and how this relates to changes in adult insulin action in animal models is limited and equivocal (12, 13, 29). Uteroplacental insufficiency increases skeletal muscle triglycerides in the juvenile rat, but this coincides with elevated enzyme activity and mRNA levels of several mitochondrial β-oxidation enzymes, consistent with increased fatty acid oxidation (12). Furthermore, uteroplacental insufficiency increases the protein expression of a major regulator of mitochondrial biogenesis, peroxisome proliferator-activated receptor (PPAR)γ coactivator-1α (PCG-1α) in hindlimb skeletal muscle from juvenile rats (13). This suggests improved mitochondrial biogenesis and fatty acid oxidation, inconsistent with elevated triglycerides and later onset of insulin resistance.

In the one study in the adult rat to date, uteroplacental insufficiency did lead to insulin resistance, diabetes, and reduced muscle glycogen levels but did not alter mitochondrial number, morphology, and distribution in skeletal muscle of

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adult offspring, suggesting that mitochondrial biogenesis was unaffected (29). However, uteroplacental insufficiency did impair the state 3 oxygen consumption of isolated mitochondria from skeletal muscle, although fatty acid metabolism was not altered (29).

A major aim of the present study was to therefore determine the effect of uteroplacental insufficiency on mitochondrial biogenesis markers and lipid and glycogen levels in a key insulin-sensitive tissue, skeletal muscle, and on glucose tolerance and circulating insulin in adult offspring. In previous studies examining these in the rat, control litters were typically culled to reduce the litter size to that of uteroplacental-insufficient litters (12, 13, 29). We have now shown, however, that reductions in litter size have an adverse impact on mammary function and on postnatal growth of the offspring (20) and may itself independently program later adverse outcomes. Thus, the present study aimed to compare offspring of sham-operated controls of normal litter size and a separate group of reduced litter size, which underwent sham surgery (Reduced Litter), to litters exposed to uteroplacental insufficiency (Restricted). To minimize stress associated with metabolic assessment in vivo, animals were chronically catheterized. Furthermore, male and female offspring were studied separately, as marked sex differences in a range of outcomes following uteroplacental insufficiency and other challenges have been reported, notably in insulin action and glucose tolerance and their molecular determinants (13, 21). On the basis of the association between reduced mitochondrial biogenesis, lipid accumulation, and insulin resistance in skeletal muscle (11, 17, 18, 23, 26), we hypothesized that mitochondrial biogenesis markers [i.e., PGC-1α, mitochondrial transcription factor A (mtTFA) and cytochrome oxidase (COX)] would be reduced, and triglyceride levels increased, in the skeletal muscle of adult (6-mo-old) rats that were exposed to uteroplacental insufficiency.

RESEARCH DESIGN AND METHODS

Animals. All experiments were approved by The University of Melbourne Animal Experimentation Sub-Committee. Wistar Kyoto rats (9–13 wk of age) were obtained from the Australian Resource Centre (Murdoch, Western Australia) at least 1 wk prior to mating. Animals were housed in an environmentally controlled room (temperature 22°C) with a 12:12-h light-dark cycle and had access to food and tap water ad libitum.

A vaginal impedence reader (model MK-10B; Mukomachi Kikai, Osaka, Japan) was used to determine whether females were in the appropriate stage of the estrous cycle for mating, as described previously (20, 40, 41). The following morning, the presence of sperm in vaginal smears was taken as day 1 of pregnancy. On day 18 of gestation, pregnant rats were randomly allocated into placentally restricted or sham-surgery groups. The placentally restricted group underwent bilateral uterine vessel (artery and vein) ligation to induce uteroplacental insufficiency, as described previously, and the sham surgery was identical except that the uterine vessels were not ligated (20, 40, 41). At birth, one-half of the litters from the sham-surgery group (litter size 10–14 pups) had their litter size randomly reduced to five pups (Reduced Litter) to match the litter size of pups born to uteroplacentally restricted mothers (Restricted) (20, 40). The remaining half of the litters from the sham surgery were left with a normal litter size of 10–14 pups (Control). All pups were handled at similar times and in the same manner and remained with their own mothers until weaning at 5 wk of age. For all groups, seven to eight offspring of each sex were studied, each arising from different mothers.

Catheterization surgery and intra-arterial glucose tolerance test. At 6 mo of age, offspring were weighed and given a single subcutaneous dose of analgesic (Temgesic, 0.0 5mg/kg body wt) and anesthetic (ketamine 50 mg/kg + xylazine 10 mg/kg). The carotid artery was then catheterized, and the catheter was exteriorized between the shoulder blades. Catheter patency was maintained by flushing daily with 0.5 ml of saline.

Intra-arterial glucose tolerance tests (IAGTT) were performed 2 days after the catheterization surgery following an overnight fast (12–16 h). Extension lines were attached to the polyethylene catheters, with animals remaining conscious and unrestrained in their cages throughout the experiment. Two blood samples were taken 10 and 5 min before administration of glucose. A bolus injection of glucose (0.5 g/kg body wt) was administered through the carotid artery catheter over a 2-min period, followed immediately by 0.2 ml of saline. Arterial blood samples (150–200 μl) were collected 1, 2, 3, 5, 10, 20, 30, 40, 60, and 90 min after injection of the intra-arterial bolus of glucose. Blood removed (maximum of 8% blood volume) was subsequently replaced with a similar volume of saline. Plasma was stored at −20°C until analysis. At completion of the IAGTT experiments, the animals were allowed access to food and water ad libitum.

Plasma analysis. Plasma glucose was measured using the Roche Glucose HK kit (Roche Diagnostics, NSW, Australia). Plasma nonesterified fatty acids (NEFA) were assessed by an enzymatic colorimetric procedure (NEFA-C test; Wako, Osaka, Japan). Plasma insulin was measured using a commercially available radioimmunoassay kit (Linco Research, St. Charles, MO). Homeostasis model assessment (HOMA) measurements of insulin action were calculated from fasting glucose and insulin values obtained prior to the glucose bolus: HOMA = fasting plasma insulin (μU/ml) × fasting plasma glucose (mmol/l) ÷ 22.5 (15). Area under the glucose and insulin curves were calculated using the trapezoidal model (1, 16), and the insulin-to-glucose ratio was calculated by dividing the areas under insulin and glucose curves, respectively.

Preparation of rat tissue. Three days after the IAGTT experiment, rats were killed with an intraperitoneal injection of xylazine (30 mg/kg) and ketamine (225 mg/kg). Dorsal, visceral, retroperitoneal, perirenal, and, for males epididymal fat was dissected, pooled, and weighed. The gastrocnemius muscle was excised, weighed, frozen in liquid N_2, and stored at −80°C. Total RNA was extracted from frozen rat gastrocnemius by use of the Micro-to-Midi Total RNA Purification System kit (Invitrogen, Carlsbad, CA). For immunoblotting and enzyme activity, frozen muscle (10 μl buffer/mg muscle) was homogenized as previously described (36) in freshly prepared ice-cold buffer [50 mM Tris at pH 7.5 containing 1 mM EDTA, 10% vol/vol glycerol, 1% vol/vol Triton X-100, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM PMSF, and 5 μl/ml protease inhibitor cocktail (P8340; Sigma, St. Louis, MO)]. Tissue lysates were incubated on ice for 20 min and then spun at 16,000 *g* for 20 min at 4°C. Protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL) with BSA as the standard.

Intramuscular triglycerides and glycogen. Intramuscular triglycerides were extracted and measured as previously described (8). Briefly, total neutral lipid was extracted in $CHCl_3$-MeOH, and the phases were separated with NaCl. The organic extracts were assayed by measuring the glycerol liberated after enzymatic hydrolysis of triglycerides (GPO-PAP kit; Boehringer Mannheim, Mannheim, Germany). Muscle glycogen was extracted by incubating the sample in HCl, then NaOH, and then analyzed for glucose units using an enzymatic fluorometric method (22).

Gene expression. RNA concentration was determined by spectrophotometric analysis. First-strand cDNA was generated from 0.5 μg of RNA using AMV Reverse Transcriptase (Promega, Madison, WI) (37). The primer sequences, as previously described (36), were obtained from gene sequences from GenBank: PGC-1α, AY237127; mtTFA, AB014089; COX III, AF504920; COX IV, J05425. Primer sequences (forward and reverse, respectively) for β-actin were 5′-

GACAGGATGCAGAAGGAGATTACT-3′ and 5′-TGATCCACATCTGCTGGAAGGT-3′; for PGC-1α were 5′-ACCCACAGGATCAGAACAAACC-3′ and 5′-GACAAATGCTCTTTGCTTTATTGC-3′; for mtTFA were 5′-AGCCATGTGGAGGGAGCTT-3′ and 5′-TTGTACACCTTCCACTCAGCTTTAA-3′; for COX III were 5′-GACGGAATTTACGGCTCAACAT-3′ and 5′-AATTAGGAAAGTTGAGCCAATAATTACG-3′; and for COX IV were 5′-GTGCTGATCTGGGAGAAGAGCTA-3′ and 5′-GGTTGACCTTCATGTCCAGCAT-3′. Real-time PCR using SYBR Green chemistry was performed as previously described (36), using the sequence detector software (Rotor-Gene v6; Corbett Research, Sydney, Australia). Samples were subjected to a heat dissociation protocol after the final cycle of PCR to ensure that only one product was detected. Relative quantification of gene expression was performed by the comparative C_T ($\Delta\Delta C_T$) method, with β-actin as the endogenous control.

Immunoblotting. Total lysates for determination of PGC-1α and COX IV were solubilized in Laemmli sample buffer. Equal amounts of total protein were separated by SDS-PAGE and electrotransfer of proteins from the gel to PVDF membranes. Blots were probed with anti-PGC-1α rabbit polyclonal (Chemicon, Temecula, CA) and anti-COX IV mouse monoclonal (Molecular Probes, Eugene, OR) antibodies as previously described (36). Binding was detected with IRDye 800-conjugated anti-rabbit IgG (Rockland, Gilbertsville, PA) or IRDye 680-conjugated anti-mouse IgG (Molecular Probes) secondary antibodies. All data are expressed as integrated intensity following infrared detection (Odyssey Imaging system; LI-COR Biosciences, Lincoln, NE).

Enzyme activities. All enzyme activities were measured spectrophotometrically at room temperature using the muscle homogenates and expressed in micromoles per minute per gram of total protein (36). β-Hydroxyacyl-CoA dehydrogenase (β-HAD; β-oxidation of fatty acids) activity was measured at 340 nm by following the disappearance of NADH. Citrate synthase (TCA cycle) activity was measured at 412 nm by following the increase in 5,5′-dithiobis-2-nitrobenzoate (DTNB) (31).

Statistical analyses. Glucose tolerance and insulin secretion data from the IAGTT were analyzed using two-way analysis of variance [ANOVA; time (within factor) × treatment (between factor)]. All other results were analyzed using one-way ANOVA with Newman-Keuls post hoc analysis where appropriate. All data are presented as means ± SE. The level of significance was set at $P < 0.05$.

RESULTS

Effect of uteroplacental insufficiency and reduced litter size on body, fat, and gastrocnemius muscle weights. Reducing the litter size in mothers exposed to sham surgery reduced body weight of the remaining male and female pups at 3 days of age (Reduced Litter) compared with pups born to sham-operated mothers with unchanged litter size (Control, $P < 0.05$; Table 1). The effect of reducing litter size on growth was still evident at 6 mo of age in the female (but not male) rats, which had reduced body weight compared with Controls ($P < 0.05$, Reduced Litter vs. Control; Table 1). Uteroplacental insufficiency reduced body weight of male and female offspring at 3 days and 6 mo of age compared with Controls ($P < 0.05$, Restricted vs. Control; Table 1). Uteroplacental insufficiency also reduced body weight of adult male (but not female) offspring compared with Reduced Litter ($P < 0.05$; Table 1). Despite the reduced body weights in the Restricted and Reduced Litter groups, total fat mass in absolute or relative terms was not different compared with Controls (Table 1). Both Restricted and Reduced Litter groups had reduced absolute gastrocnemius weight in male and female offspring at 6 mo of age compared with Controls, but not relative to body weight ($P < 0.05$; Table 1).

Table 1. *Effect of uteroplacental insufficiency (Restricted) and reducing litter size (Reduced Litter) on body, fat, and gastrocnemius weight*

	Control	Reduced Litter	Restricted
Male			
BWT: *day 3*, g	6.01±0.38	5.29±0.15*	4.41±0.24*†
BWT: 6 mo, g	366.6±7.1	352.4±5.0	329.0±8.5*†
Fat weight: 6 mo, g	17.6±1.0	16.8±0.8	14.5±1.3
Fat weight: 6 mo, %BWT	4.8±0.3	4.8±0.2	4.4±0.4
Gastrocnemius: 6 mo, g	1.77±0.05	1.60±0.08*	1.55±0.05*
Gastrocnemius: 6 mo, %BWT	0.48±0.01	0.41±0.04	0.47±0.01
Female			
BWT: *day 3*, g	5.57±0.23	4.97±0.19*	4.35±0.20*
BWT: 6 mo, g	231.2±3.9	214.5±5.8*	213.0±3.3*
Fat weight: 6 mo, g	8.2±0.6	7.3±0.6	8.0±0.6
Fat weight: 6 mo, %BWT	3.5±0.2	3.3±0.2	3.7±0.3
Gastrocnemius: 6 mo, g	1.18±0.03	1.04±0.03*	1.00±0.03*
Gastrocnemius: 6 mo, %BWT	0.51±0.02	0.49±0.01	0.47±0.01

Body weight (BWT) measured at postnatal *day 3* and at post mortem (6 mo of age) in male and female rats. Fat weight is the sum of dorsal, visceral, retroperitoneal, perirenal, and, for males, epididymal fat. Gastrocnemius muscle weight measured at post mortem in male and female rats. Values are means ± SE. *$P < 0.05$ vs. Control; †$P < 0.05$ vs. Reduced Litter (one-way ANOVA).

Plasma glucose, insulin, HOMA, and free fatty acids. Uteroplacental insufficiency or reductions in litter size did not alter fasting plasma glucose, insulin, and free fatty acids (Fig. 1 and Table 2, respectively) or glucose tolerance and insulin secretion following IAGTT in male (Fig. 1, *A* and *B*, respectively) or female offspring (Fig. 1, *C* and *D*, respectively) at 6 mo of age. Furthermore, no treatment altered the area under the glucose or insulin curves or the insulin-to-glucose ratio during IAGTT of male or female offspring (data not shown). In adult female offspring, uteroplacental insufficiency increased HOMA compared with Control and Reduced Litter rats ($P < 0.05$; Table 2). HOMA was not altered by any treatment in adult male offspring.

Muscle glycogen and triglyceride levels. In males, Reduced Litter offspring had lower skeletal muscle glycogen compared with Control and Restricted offspring ($P < 0.05$; Fig. 2*A*). In females, skeletal muscle glycogen was not altered by treatment (Fig. 2*C*). Reduced Litter male offspring tended to have increased skeletal muscle triglycerides compared with Control and Restricted offspring ($P = 0.06$; Fig. 2*B*). In Restricted female offspring, skeletal muscle triglyceride levels were increased compared with Control and Reduced Litter offspring ($P < 0.05$; Fig. 2*D*).

Muscle mitochondrial biogenesis markers. In males, skeletal muscle, PGC-1α protein, PGC-1α mRNA, and mtTFA mRNA levels were lower in both Restricted and Reduced Litter offspring compared with Controls ($P < 0.05$; Fig. 3 *A–C*, respectively). Similarly, skeletal muscle COX IV protein and mRNA levels were lower in Restricted male offspring compared with Controls ($P < 0.05$; Fig. 4, *A* and *B*, respectively), and COX III mRNA levels were lower in both Restricted and Reduced Litter offspring compared with Controls ($P < 0.05$; Fig. 4*C*).

In females, Reduced Litter offspring had lower skeletal muscle mtTFA levels ($P < 0.05$; Fig. 3*F*) and tended to have lower PGC-1α mRNA levels ($P = 0.11$; Fig. 3*E*). Treatment

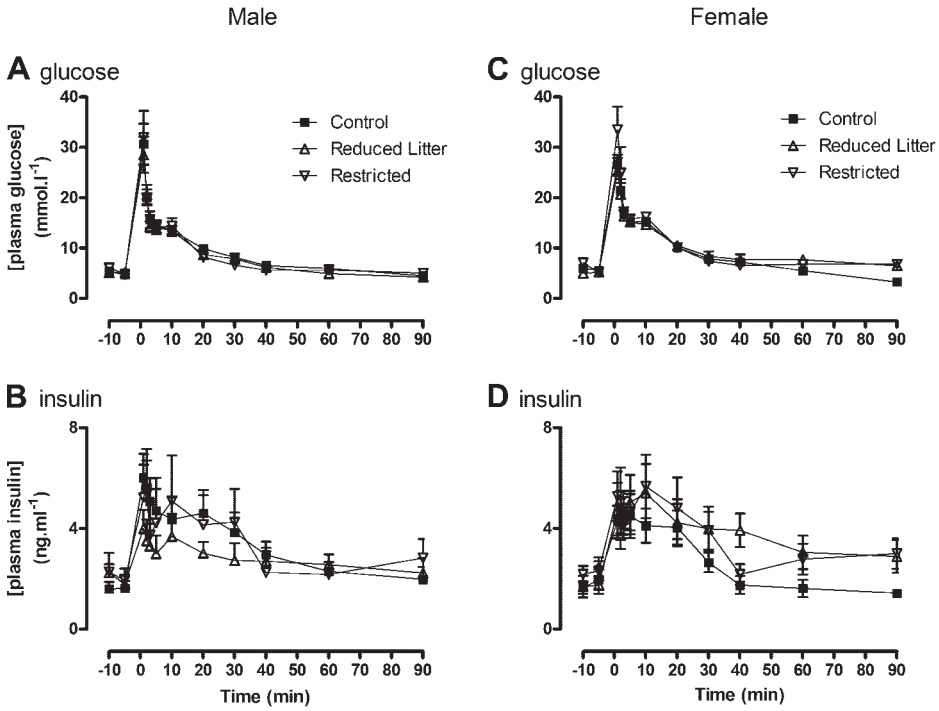

Fig. 1. Effect of uteroplacental insufficiency (Restricted) and reducing litter size (Reduced Litter) on plasma glucose and insulin measured during an intra-arterial glucose tolerance test in adult male and female offspring. Values are mean ± SE; $n = 7$ for all groups.

did not alter skeletal muscle PGC-1α protein (Fig. 3*D*), COX IV protein, mRNA and COX III mRNA in female offspring, however (Fig. 4, *D–F*, respectively).

Treatment did not alter skeletal muscle citrate synthase and β-HAD activities in male or female offspring (data not shown).

DISCUSSION

A major finding of the present study was that the early-life perturbations of both uteroplacental insufficiency and restriction of postnatal growth via reductions in litter size reduced the expression of skeletal muscle mitochondrial biogenesis markers in the adult male rat, whereas only reducing litter size, and therefore postnatal growth, induced similar trends in female offspring. One of the expected metabolic consequences of lower skeletal muscle mitochondrial protein levels would be increased lipid accumulation and impaired glucose utilization, resulting in insulin resistance (10, 18, 26). Therefore, a surprising finding from the present study was that these early-life interventions caused a dissociation between elevated intramuscular triglycerides (Fig. 2) and the reduced mitochondrial biogenesis markers (PGC-1α mRNA and protein, mtTFA mRNA) and mitochondrial protein (COX IV mRNA and protein and COX III mRNA) in male and female offspring (Figs. 3 and 4). Furthermore, in contrast to previous reports (30, 35), uteroplacental insufficiency did not alter glucose tolerance or adiposity in adult offspring.

Table 2. *Effect of uteroplacental insufficiency (Restricted) and reducing litter size (Reduced Litter) on HOMA and FFA levels in 6-mo-old male and female rats*

	Control	Reduced Litter	Restricted
Male			
HOMA	9.3±1.8	11.7±2.0	12.8±4.0
FFA, mmol/l	1.0±0.1	1.4±0.1	1.5±0.3
Female			
HOMA	10.5±1.7	8.9±1.7	17.1±3.1*†
FFA, mmol/l	1.2±0.1	0.9±0.2	1.3±0.1

FFA, free fatty acids, HOMA, homeostasis model assessment. Values are means ± SE. *$P < 0.05$ vs. Control; †$P < 0.05$ vs. Reduced Litter (one-way ANOVA).

We clearly show that our model of uteroplacental insufficiency in the Wistar Kyoto rat does not impair fasting glucose or insulin or glucose tolerance (or the insulin response). This contrasts with earlier reports that uteroplacental insufficiency in the Sprague-Dawley rat impairs glucose tolerance as early as 1 wk after birth in offspring, with a clear diabetic phenotype present at 6 mo of age (29, 30). One factor that may contribute to these contrasting findings may be strain differences between the Wistar Kyoto rat used in the present study, which has reduced body weight following uteroplacental insufficiency (Table 1), and the Sprague-Dawley rat used in previous studies, which instead gains body weight and fat and subsequently develops diabetes in adulthood (29, 30). Apart from differences in strain, experimental differences between the present and previous studies are also evident that may account for these very different findings in development of obesity and glucose intolerance. These potentially include fostering placentally restricted offspring onto unoperated mothers following birth (30) and minor reductions in litter size, which, in addition to placental restriction (29), may have induced additional stress or nutritional differences that exacerbated prenatal programming of obesity and glucose tolerance. Future studies utilizing different rat strains on the impact of uteroplacental insufficiency in the programming of insulin action and glucose control and body composition may be highly informative and may complement studies of gene and early-environment interactions in humans. Furthermore, future uteroplacental insufficiency experiments utilizing both rat strains under identical experimental conditions may provide novel insights into the relationship

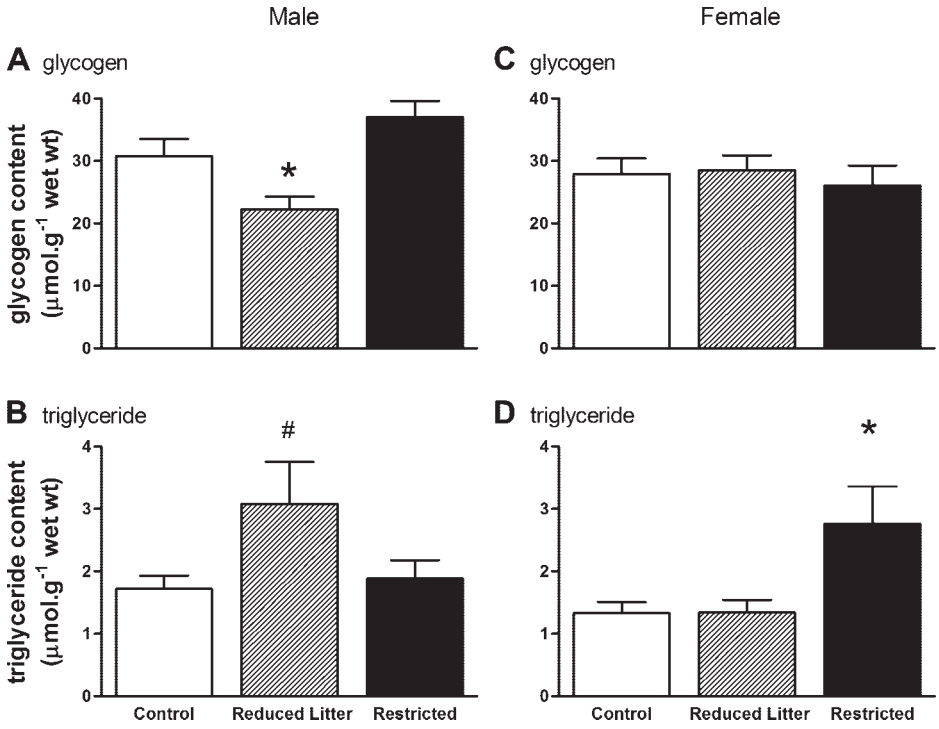

Fig. 2. Effect of uteroplacental insufficiency (Restricted) and reducing litter size (Reduced Litter) on gastrocnemius muscle glycogen (*A* and *C*) and triglyceride (*B* and *D*) content in adult male and female offspring. Values are mean ± SE; $n = 8$ for all groups, except $n = 7$ for female Restricted group. *$P < 0.05$ vs. Control, #$P = 0.06$ vs. Control (one-way ANOVA).

between the programming of insulin action and glucose control in response to body and fat weight gain. The present study also found that uteroplacental insufficiency in the rat increased HOMA in female, but not male, adult offspring. This could perhaps be indicative of onset of hepatic insulin resistance, as reported in a previous study (35), which has yet to affect whole body insulin resistance or glucose tolerance. Although the use of HOMA as a measurement of insulin resistance in rodent studies is quite common (14, 27), it is also controversial (38) and better suited to larger cohorts. Nevertheless, our finding of elevated HOMA in the Restricted female rats suggests that alterations in insulin sensitivity could be occurring in our model of restriction that might be more readily detected by more sensitive measures such as a euglycemic hyperinsulinemic clamp. Also, future studies incorporating additional groups of insulin-stimulated rats could examine skeletal muscle insulin signaling, thus clarifying the potential impact of growth restriction on tissue metabolism in our model of placental insufficiency.

The uncoupling observed in the present study between intramuscular triglyceride levels and skeletal muscle mitochondrial biogenesis markers and mitochondrial proteins suggests that this relationship is not necessarily causative. Several studies have reported a strong association between either reduced mitochondrial biogenesis, impaired mitochondrial function, or lower mitochondrial volume, and elevated lipid content in skeletal muscle that is insulin resistant or from people with type 2 diabetes (10, 17, 23–25). Recent studies in obese rodent models (34) and insulin infusion into humans (2) suggest, however, that mitochondrial dysfunction and reduced expression of mitochondrial proteins in skeletal muscle may be a secondary defect in response to insulin resistance and lipid accumulation. Genetic models of obesity and insulin resistance, such as the obese Zucker rat and *db/db* mice have elevated intramuscular triglycerides but higher PGC-1α protein and skeletal muscle mitochondrial enzyme activities and increased fatty acid oxidative capacity (34). High-fat feeding in rats and mice also induces a similar phenotype (34). Also, if mitochondrial dysfunction causes skeletal muscle insulin resistance, then reducing mitochondrial function should result in insulin resistance. However, skeletal muscle-specific mtTFA knockout mice have reduced oxidative capacity but increased insulin-stimulated glucose uptake (42).

Another major finding of the present study is the influence of reducing litter size on postnatal growth and its subsequent effects on skeletal muscle characteristics in adulthood. Modestly reducing the litter size of pups born to sham-operated mothers reduced the body weight of the remaining pups at 3 days of age. This resulted in changes to adult male skeletal muscle, including lower glycogen, a tendency for higher triglycerides, and reduced expression of markers of mitochondrial biogenesis and mitochondrial proteins. Restricting postnatal growth of female offspring by reducing litter size also reduced mtTFA mRNA and tended to reduce that of other mitochondrial biogenesis markers in skeletal muscle. These findings have important implications for the design and interpretation of studies of prenatal and postnatal perturbations in the rat and highlight the need to include controls with unaltered litter size (12, 13, 29). The use of a sham-operated group with reduced litter size as the only control in the present study would have obscured and changed a number of our findings. Indeed, if we had used only the Reduced Litter group as our control group we would have observed no alterations in mitochondrial biogenesis markers in the males and higher mtTFA mRNA in the females (compared with the Restricted group).

Although severe reductions in litter size have been used as a model of overfeeding and obesity (39), we have previously shown that more modest reductions in litter size, as a control for uteroplacentally restricted pups, reduces milk quality and quantity and impairs mammary function, possibly by a reduced

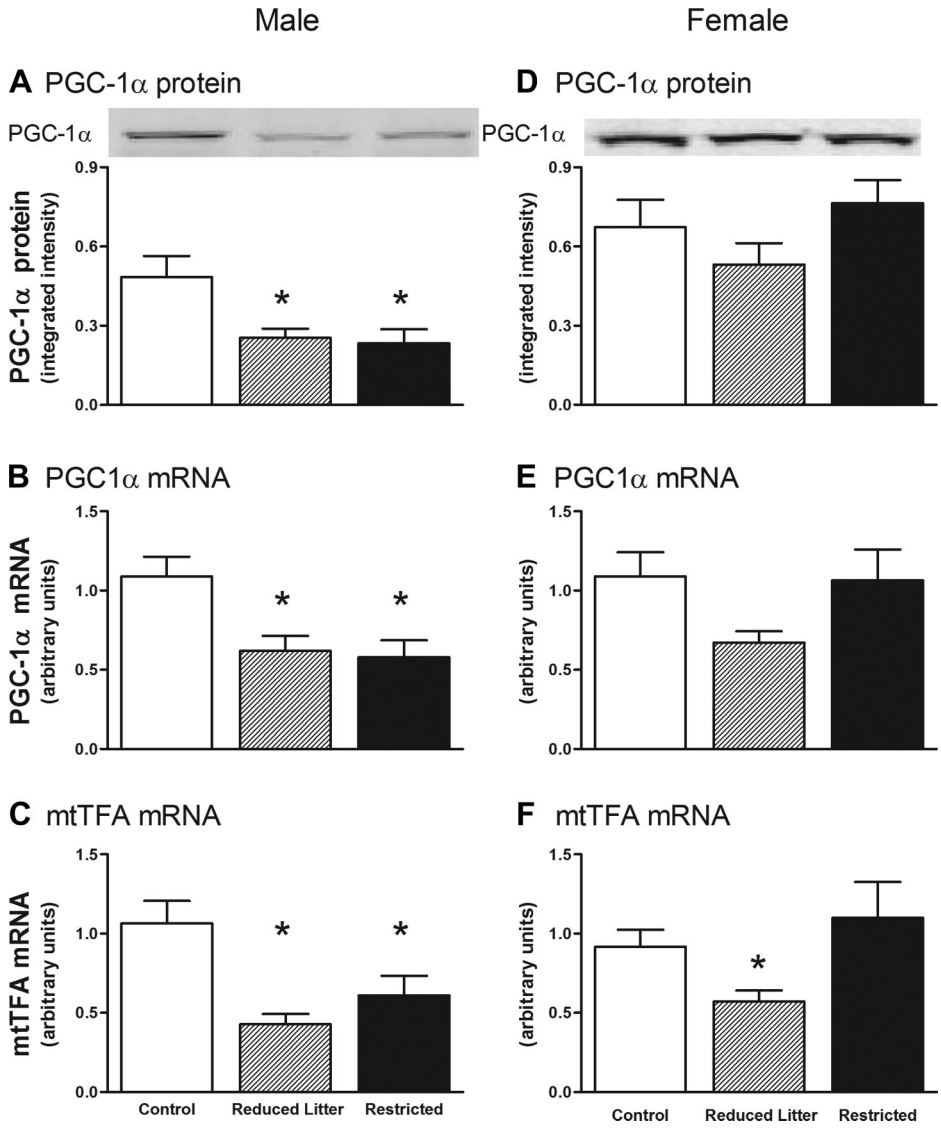

Fig. 3. Effect of uteroplacental insufficiency (Restricted) and reducing litter size (Reduced Litter) on gastrocnemius muscle on PPARγ co-activator-1α (PGC-1α) protein and mRNA and mitochondrial transcription factor A (mtTFA) mRNA in adult male and female offspring. Western blots are representative from one rat from each treatment group. Values are mean ± SE; $n = 8$ for all groups, except $n = 7$ for female and male Restricted groups. *$P < 0.05$ vs. Control (one-way ANOVA).

suckling stimulus, resulting in slowed postnatal growth (20). Also, the impact of reducing litter size on postnatal growth and other long-term outcomes observed here is in agreement with the outcomes of our recent cross-fostering studies related to hypertension (40). Furthermore, we observed differences in metabolic characteristics, such as skeletal muscle triglycerides and glycogen levels between the male Restricted and Reduced litter groups. This is not surprising given that these groups experienced nutrient perturbations at different stages of development. Indeed, the metabolic milieu of these animals is quite nutritionally different, with Restricted animals exposed to restriction of substrates and oxygen in utero and also postnatal lactational restraint (20), whereas the Reduced Litter animals were exposed to lactational restraint only in the postnatal period. Our data highlight that an adverse postnatal environment can program later metabolic function. Further studies may identify specific nutrient deficiencies in growth-restricted offspring to further define the mechanistic pathways leading to metabolic dysfunction. Thus, reduced postnatal growth as a consequence of a reduction in litter size appears to exert its own influence on the metabolic characteristics of adult skeletal muscle independently of uteroplacental insufficiency and warrants further study.

The present study also found that restriction of postnatal growth via reductions in litter size and uteroplacental insufficiency reduced mitochondrial biogenesis markers to a greater extent in adult male than in female rats. In support of our findings, Lane et al. (13) found that PGC-1α mRNA levels were altered to a greater extent in male compared with female juvenile rats following uteroplacental insufficiency. It is possible that estrogen may be playing a protective role in terms of minimizing the impact of restriction on skeletal muscle mitochondrial biogenesis. Estrogen treatment of female ovariectomized rats increases the mRNA and enzyme activity of lipid oxidative enzymes (5, 6) in skeletal muscle and mitochondrial biogenesis in blood vessels (32), and PGC-1α is a known activator of the estrogen receptor (33). However, our finding of lower mtTFA mRNA and a tendency for lower PGC-1α mRNA following reductions in litter size suggests that skeletal muscle mitochondria from adult females is vulnerable to particular insults, at least in early postnatal life, although the mechanisms for this remain unclear. Furthermore, it is not clear

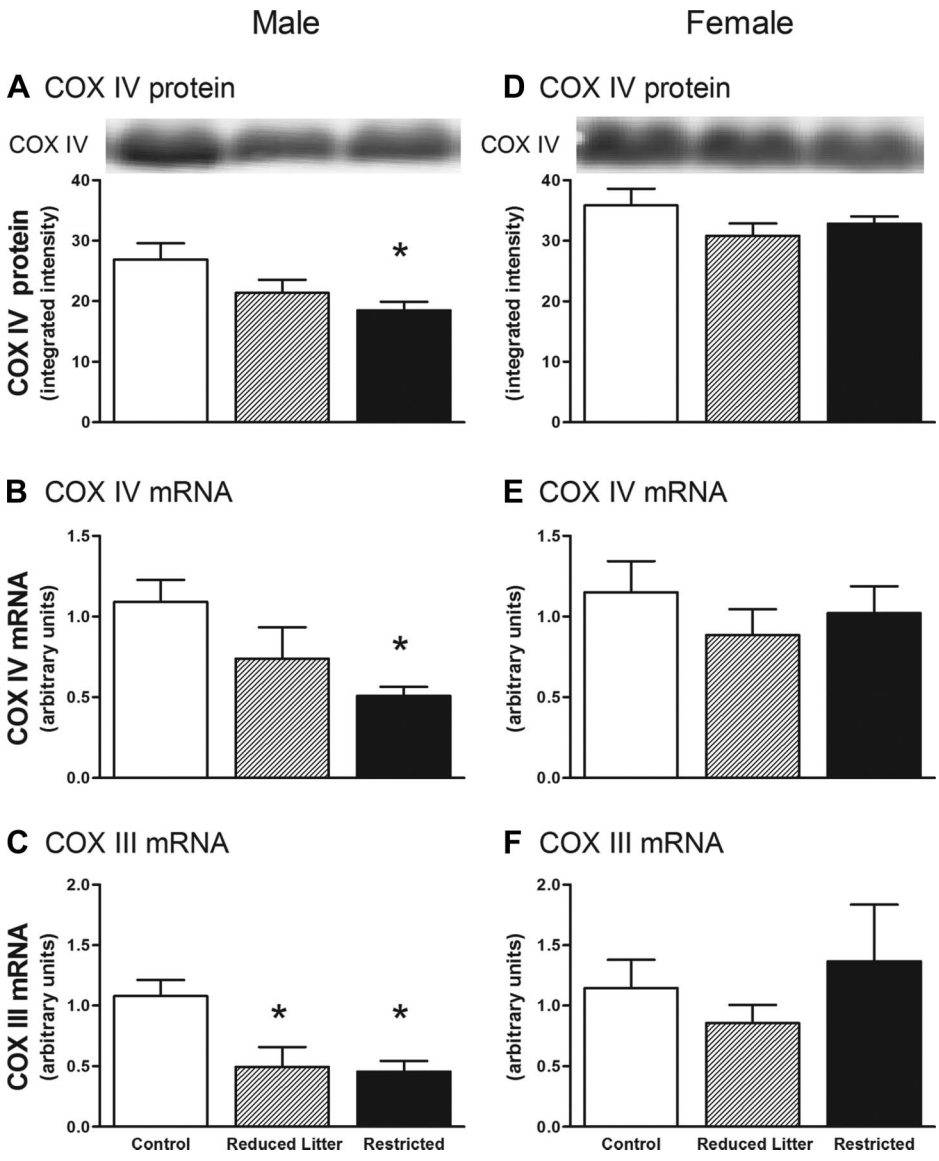

Fig. 4. Effect of uteroplacental insufficiency (Restricted) and reducing litter size (Reduced Litter) on gastrocnemius muscle cytochrome oxidase (COX) IV protein and mRNA and COX III mRNA in adult male and female offspring. Western blots are representative from one rat from each treatment group. Values are mean ± SE; $n = 8$ for all groups, except $n = 7$ for female and male Restricted groups. *$P < 0.05$ vs. Control (one-way ANOVA).

what other sex-specific factors could explain the differences in the present study, as the effects of sex hormones on the regulation of mitochondrial biogenesis in skeletal muscle have not been studied in detail. The effects of testosterone on mitochondrial biogenesis in skeletal muscle are not known, although it does inhibit mitochondrial biogenesis in brown fat cells in culture. However, in this model, estrogen has no effect on PGC-1α mRNA and downregulates other mitochondrial biogenesis markers (28). Thus, further study is required into the influence of sex hormones on, and the mechanistic pathways that may alter, mitochondrial biogenesis in a sex-specific manner. Nevertheless, it appears that the skeletal muscle mitochondria from males are more vulnerable to early-life nutrient restriction overall than from females.

Because of clear reductions in skeletal muscle PGC-1α mRNA and protein and mtTFA mRNA in male Restricted and Reduced Litter rats, it was somewhat surprising that the maximal citrate synthase and β-HAD enzyme activities were also not reduced. Since the maximal in vitro activity of mitochondrial enzymes largely reflects the abundance of the enzyme, these findings suggest that certain components of the mitochondrial respiratory chain (i.e., TCA cycle and β-oxidation) were not altered in these rats despite reduced mitochondrial biogenesis markers. However, other components of the respiratory chain, such as the electron transport chain, were altered, as evidenced by reduced COX III (mitochondrial encoded) mRNA and COX IV (nuclear encoded) mRNA and protein in the male Restricted and Reduced Litter rats. Future studies are now required to determine the functional consequences of altered mitochondrial biogenesis markers and proteins in the skeletal muscles of this model of uteroplacental insufficiency.

In conclusion, the present study shows that uteroplacental insufficiency and restriction of postnatal growth via reductions in litter size differentially alter skeletal muscle mitochondrial biogenesis and metabolic characteristics (i.e., intramuscular triglycerides and glycogen) in a sex-specific manner in the adult rat. Furthermore, these early-life interventions alter skeletal muscle characteristics in the absence of overt changes in plasma glucose, glucose tolerance, insulin secretion, or adiposity. Notably, reducing litter size exposes pups born of normal

birth weight to reduced postnatal growth and alterations in skeletal muscle markers of mitochondrial biogenesis in adult male offspring, which are less evident in female offspring. The longer-term impact of such alterations in mitochondrial biogenesis in peripheral tissues on adult health that are induced in early life and in other paradigms require further investigation.

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