Changed mitochondrial function by pre- and/or postpartum diet alterations in sheep

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Jørgensen W, Gam C, Andersen JL, Schjerling P, Scheibye-Knudsen M, Mortensen OH, Grunnet N, Nielsen MO, Quistorff B. Changed mitochondrial function by pre- and/or postpartum diet alterations in sheep. Am J Physiol Endocrinol Metab 297: E1349–E1357, 2009. First published October 13, 2009; doi:10.1152/ajpendo.00505.2009.—In a sheep model, we investigated diet effects on skeletal muscle mitochondria to look for fetal programming. During pregnancy, ewes were fed normally (N) or were 50% food restricted (L) during the last trimester, and lambs born to these ewes received a normal (N) or a high-fat diet (H) for the first 6 mo of life. We examined mitochondrial function in permeabilized muscle fibers from the lambs at 6 mo of age (adolescence) and after 24 mo of age (adulthood). The postpartum H diet for the lambs induced an ∼30% increase (P < 0.05) of mitochondrial V\textsubscript{O\textsubscript{2max}} and an ∼50% increase (P < 0.05) of the respiratory coupling ratio (RCR) combined with lower levels of UCP3 and PGC-1α mRNA levels (P < 0.05). These effects proved to be reversible by a normal diet from 6 to 24 mo of age. However, at 24 mo, a long-term effect of the maternal gestational diet restriction (fetal programming) became evident as a lower V\textsubscript{O\textsubscript{2max}} (∼40%, P < 0.05), a lower state 4 respiration (∼40%, P < 0.05), and lower RCR (∼15%, P < 0.05). Both PGC-1α and UCP3 mRNA levels were increased (P < 0.05). Two analyzed muscles were affected differently, and muscle rich in type I fibers was more susceptible to fetal programming. We conclude that fetal programming, seen as a reduced V\textsubscript{O\textsubscript{2max}} in adulthood, results from gestational undernutrition. Postnatal high-fat diet results in a pronounced RCR and V\textsubscript{O\textsubscript{2max}} increase in adolescence. However, these effects are reversible by diet correction and are not maintained in adulthood.

metabolic syndrome; high-fat diet; nutrient restriction; maternal diet; respiratory coupling ratio

EPIDEMIOLOGICAL STUDIES IN HUMANS since the 1980s and subsequent clinical studies have revealed that small size or thinness at birth is associated with increased risk of developing metabolic dysfunctions like obesity, type 2 diabetes, and abnormal lipid and carbohydrate metabolism, leading to coronary heart disease and elevated blood pressure in adulthood (2, 21). This has spurred an increasing interest in the long-term consequences of maternal nutrition during pregnancy and early postnatal nutrition, a phenomenon first described by Hales and Barker (22) and termed metabolic programming. Although this concept is now widely accepted, the mechanisms behind it remain poorly understood. The observed effects reflect phenotypical alterations, probably established through epigenetic mechanisms, which occur as a result of fetal adaptations to intrauterine and probably early postnatal influences (23, 38). Maternal low-protein diet during pregnancy in rats may in adulthood result in reduced glucose tolerance and high blood pressure, evidently due to a prenatally programmed tendency to dysfunction of small arteries and abnormal pancreatic development (9, 17, 25). Likewise, maternal low-protein diets may result in very significantly shortened life span in mice offspring (44). Thus, both the type of malnutrition and the time of exposure during pregnancy appear to influence the programming effects observed later in life (21, 23). Furthermore, the potentially detrimental effects of fetal metabolic programming on health and life expectancy appear to be expressed when the postnatal diet does not match the conditions for which the individual was programmed during the fetal stage (45, 63).

Maternal mitochondrial dysfunction has been shown to concur with metabolic disorder. In skeletal muscle, this may appear as decreased capacity of oxidative phosphorylation (4, 48). One of the mechanisms behind this effect is thought to be an elevated formation of reactive oxygen species (ROS) resulting in damage to the mitochondria, nuclear DNA, proteins, and membrane lipids (40).

In the present paper, we tested the hypothesis that insufficient gestational caloric nutrition combined with early postnatal exposure to hypernutrition leads to mitochondrial dysfunction in skeletal muscle. Specifically, we tested whether these early-life adverse nutritional conditions would 1) increase respiratory coupling of mitochondrial energy production, 2) decrease the expression of mitochondrial uncoupling protein, 3) decrease the state 4 oxygen consumption, or 4) decrease the mitochondrial maximal oxygen uptake (V\textsubscript{O\textsubscript{2max}}) and 5) whether the phenotypical expression of these programming events would be age dependent.

MATERIALS AND METHODS

Chemicals and Solutions

Reagents. Unless otherwise stated, reagents were purchased from Sigma-Aldrich and were of analytical grade or better.

Incubation media for the mitochondrial experiments. Two different media were used in the experiments: 1) relaxing medium containing 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 2.77 mM CaCl\textsubscript{2} (free [Ca\textsuperscript{2+}] ~100 mM), 6.56 mM MgCl\textsubscript{2}, 0.5 mM dithiothreitol (DTT), 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) hydrate, 20 mM imidazol, 20 mM taurine, 5.3 mM Na\textsubscript{2}ATP, and 15 mM PCr, adding ~90 mM KOH for a pH of 7.1,
Table 1. \( \dot{V}O_{2\text{max}} \) (\( \mu \text{mol } O_2/\text{g wet wt} \)) in permeabilized fibers from BF and FHL muscles of 6- and 24-mo-old lambs

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<th>Progeny Groups</th>
<th>2-Way ANOVA</th>
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<tr>
<td>BF (6 mo)</td>
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<tr>
<td>FHL (24 mo)</td>
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Values are means ± SD; \( n = 4-5 \). \( \dot{V}O_{2\text{max}} \), maximal oxygen uptake; BF, biceps femoris; FHL, flexor hallucis longissimus; LGD, late-gestation diet; PD, progeny diet; LGD × PD, interaction between diets; NS, not significant. Progeny groups based on maternal [1st letter (N or L)] and maternal diet (2nd letter (N or H)), which refers to the progeny’s diet after birth are normal-normal (NN), normal-high (NH), low-normal (LN), and low-high (LH). Statistics are shown in the 2-way ANOVA columns.

Table 2. RCR in permeabilized fibers from BF and FHL muscles of 6- and 24-mo-old lambs

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<td>FHL (24 mo)</td>
<td>5.08±1.0</td>
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Values are means ± SD; \( n = 4-5 \). RCR, respiratory coupling ratio. Data show the ratio between state 4 and uncoupled state 3 respiration. Statistics are shown in the 2-way ANOVA columns.
The respiratory coupling ratio (RCR) was calculated as the state 3/state 4 ratio. The VO₂max was taken as the state 3 respiration with the additional effect of FCCP uncoupling.

**ATPase staining.** Fresh muscle samples were mounted with Tissue-Tek and frozen in isopentane cooled with liquid nitrogen and stored at −80°C until sectioning. Sections (10 μm) were processed for ATPase histochemistry, which was performed prior to preincubation at pH of 4.37 and 10.30, to enable distinguishing fiber types I (slow, red) and II (fast, white) (11).

**Protein Content and Citrate Synthase Activity**

Protein concentrations and citrate synthase (CS) activity in the biopsies were determined in 10% homogenates prepared in buffer (50 mM Tris·HCl, 0.6 mM MnCl₂, 2 mM citrate, 0.1% Triton X-100, pH = 7.4; Ultra Turaxhomogenizer). Protein concentrations were determined using the method of Lowry et al. (39). CS (EC 4.1.3.7) activity was assayed at 25°C (55).

**Aconitase Activity**

The activity of aconitase (EC 4.2.1.3) was used as a measure of formation of ROS in the mitochondria (41). Aconitase was assayed spectrophotometrically at 30°C and pH 7.4 (20). Ten percent muscle homogenates were prepared in assay stock buffer (50 mM Tris·HCl, 0.6 mM MnCl₂, 2 mM citrate, 0.1% Triton X-100, pH = 7.4). Assay mix was prepared fresh on the day: 0.2 mM NADP, 1 U/ml isocitric dehydrogenase (Sigma lot 073K7073), and 5 mM sodium citrate in assay stock buffer. The reaction was initiated when homogenate was added to 1,000 μl of assay mix.

**RT-PCR**

Uncoupling protein 3, PGC-1α, and PPARδ mRNA levels were assessed by real-time RT-PCR. Total RNA was isolated from the muscle samples by a phenol extraction (Tri Reagent; Sigma-Aldrich). RNA integrity was confirmed by Agilent Bioanalyzer measurements (RIN > 6.0), and concentrations were measured spectrophotometrically. Five-hundred nanograms of total RNA was converted into cDNA in 20-μl volume using the OmniScript reverse transcriptase (Qiagen, Valencia, CA) according to the manufacturer’s protocol. The cDNA was diluted 20 times, and 5 μl was amplified in a 25-μl SYBR Green PCR reaction using Quantitect SYBR Green Master Mix (Qiagen) and 100 nM primer. The reaction was initiated at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s, 63°C for 30 s, and 72°C for 1.5 min. The last step included 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. The amplification was monitored using the MX3000P real-time PCR machine (Stratagene, La Jolla, CA). The cycle threshold values were related to a standard curve made with cloned PCR products. The quantities were normalized to mRNA for the large ribosomal protein P0, which was not different between groups (data not shown). Primers were uncoupling protein 3 (UCP3): sense TGTCAACTGTGGTGAGATGGTG, antisense GCAGGGGAAGTTGTCGGTGAG; peroxisome proliferator-activated receptor (PPAR)γ coactivator-1α (PGC-1α): sense GGAGTAACAGGAAAGAGGAAGCGGCAAG, antisense TCTTGATGGAAGCAGGGTCAAAGT; PPARδ: sense AGGAGAAAGGAAGAGCGGCCAAG, antisense TCTGTGTA- GCTGCTGGAAGGAAG.

**Mitochondrial DNA.** Mitochondrial DNA (mtDNA) was measured as the ratio between mitochondrial and genomic DNA. Total DNA was isolated using DNAzol reagent (Molecular Research Center). Following the manufacturer’s protocol, DNA was precipitated with ethanol, and the final resolubilization mixture was diluted 100 times. Five microliters of this dilution was amplified in a 25-μl PCR reaction containing 1× Quantitect SYBR Green Master Mix (Qiagen, Hilden, Germany) and 100 nM of both sense and antisense primer. The amplification was monitored using the MX3000P real-time PCR machine (Stratagene), following the same thermal setup as above. Primers designed to target ST7 for genomic DNA (sense CCTGAGGACATCATTCCACCCAAG, antisense GCCAGGGAGGGACAAAA-GACAA) and cytochrome C oxidase-1 for mtDNA (sense ATCGGTGGATTCCGGCAACTGA, antisense GGAGTAACAGGAAAGGAAGCGGCCAAG).

**Table 3. State 4 respiration (μmol O₂ min/g wet wt) in permeabilized fibers from BF and FHL muscles of 6- and 24-mo-old lambs**

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<td></td>
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<td><strong>Progeny Groups</strong></td>
<td><strong>State 4</strong></td>
<td><strong>State 4</strong></td>
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<td>BF (24 mo)</td>
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<tr>
<td>FHL (24 mo)</td>
<td>0.15±0.02</td>
<td>0.16±0.02</td>
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Values are means ± SD; n = 4–5. Statistics are shown in the 2-way ANOVA columns. *Significant difference between the NH and LH groups.
TGGGGGAAG) were used. The cycle threshold values were related to a standard curve made with the cloned PCR products.

Statistical Analysis

Two-way analysis of variance (ANOVA) was performed on log-transformed data in the R programme (R 2.9.0, www.r-project.org) and shown in tables together with the data. The level of significance was set at $P < 0.05$. Where significant interactions appeared, post hoc Student’s $t$-test was applied and marked with an asterisk in case of statistical significance. Data in tables are given as means ± SD.

RESULTS

$\dot{V}O_{2\text{max}}$ and RCR

Mitochondrial $\dot{V}O_{2\text{max}}$ values, given as the FCCP-uncoupled state 3 respiration normalized to tissue wet weight, are shown in Table 1, and RCR, given as the state 3/state 4 ratio respiration, is shown in Table 2. In the 6- and the 24-mo groups, $\dot{V}O_{2\text{max}}$ was higher in the FHL compared with the BF muscle ($P < 0.05$), corroborating that FHL contained more type 1 fibers than BF (Fig. 1). In the 6-mo groups, the high-fat diet caused a $\dot{V}O_{2\text{max}}$ increase of 25–80% ($P < 0.05$) in both muscles (Table 1), and it also caused a pronounced increase in the RCR ($50\%$, $P < 0.05$) (Table 2). Furthermore, RCR differed with the fiber type and was highest in the FHL muscle ($P < 0.05$; Table 2). At 2 yr of age (adulthood), the highly significant effects on $\dot{V}O_{2\text{max}}$ and RCR seen at 6 mo as a result of the high-fat diet were not retained, indicating that full reversibility is possible by a normal diet from 6 to 24 mo (Tables 1 and 2).

Fetal programming (the effect of the late-gestation diet restriction) was not manifested in the 6-mo animals (adolescence), whereas at 2 yr of age (adulthood) fetal programming could be observed as a 15–20% decrease in $\dot{V}O_{2\text{max}}$ ($P = 0.05$; Table 1) and a drop of the RCR ($P < 0.05$) in the FHL muscle (Table 2).

A general increase in $\dot{V}O_{2\text{max}}$ was observed at 24 mo compared with 6 mo of age ($P < 0.05$; Table 1). The state 4 respiration (Table 3) was unaffected by the dietary regimes, except for a decrease at 6 mo in the BF muscles of lambs exposed to a late-gestational undernutrition (Table 3).

UCP3, PGC-1α, and PPARδ mRNA

There was an approximately threefold higher ($P < 0.05$) expression of UCP3 mRNA in BF compared with FHL (Fig. 2), and FHL displayed a decreased expression ($\sim 85\%$, $P < 0.05$) in response to the postnatal high-fat-diet. Fetal programming of
UCP3 expression was seen at 24 mo of age (adulthood) as an increase in the mRNA levels by ~60% for the BF (P < 0.05) and ~100% for the FHL (P < 0.05), and there was also an effect of the high-fat diet. Combining the 6-mo data from the two muscles, they showed a decreased expression of PGC-1α when the animals had been exposed to the high-fat diet (P < 0.05; Fig. 3). However, this effect was not visible when the muscles were analyzed separately. The effect of the maternal feed restriction (fetal programming) on the PGC-1α mRNA was seen as a 60% increase in the FHL muscle after 24 mo (P < 0.05; Fig. 3). Also, the PGC-1α mRNA level was strongly decreased from 6 to 24 mo, in particular in the BF muscle. Fetal programming of the PPARγ mRNA levels was observed as a decrease at 6 mo, but only when data from the two muscles were analyzed in combination (P < 0.05). However, a decreased expression appeared only in the FHL muscle after 24 mo (P < 0.05). There was no effect of the high-fat diet on PPARγ expression (Fig. 4).

**DISCUSSION**

The key findings of the present study were that skeletal muscle mitochondria of offspring are affected by both undernutrition during late gestation and a high-fat diet in the early postnatal life, but the timing of these nutritional effects differs. Thus, maternal undernutrition during the third trimester programs a reduction in mitochondrial VO2max in adult life, as seen in the 24-mo-old sheep, but not in the adolescent animals (6 mo). The postnatal high-fat diet, on the other hand, induces a pronounced increase in the RCR and VO2max, effects that were reversible by exposure to a normal diet from 6 mo to 2 yr of age.

**Maternal Late-Gestation Feed Restriction**

Intrauterine growth restriction (IUGR) caused by undernourishment of the mother is known to alter the metabolism of the fetus and is associated with highly increased risk of developing hypertension and/or diabetes in adult life in experimental animals and in man (fetal programming) (2, 21, 42, 59). In the sheep model used in the present study, pregnant dams were exposed to 50% undernutrition in the last trimester. This gave rise to fetal programming in the offspring lambs, manifested as a decreased VO2max in the FHL muscle at 2 yr of age (adulthood), as well as a decreased respiratory coupling. Thus, it appears that undernourishment in fetal life “programs” the mitochondria to a lower oxygen consumption primarily in type I muscle fibers. Such a decreased oxygen consumption was

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<td>LGD</td>
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<tr>
<td>BF (6 mo)</td>
<td>NS</td>
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<tr>
<td>FHL (6 mo)</td>
<td>NS</td>
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<td>BF (24 mo)</td>
<td>NS</td>
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<td>FHL (24 mo)</td>
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Values are means ± SD; n = 4–5. Statistics are shown in the 2-way ANOVA columns.
also observed in prediabetic and diabetic IUGR rats (54), in accordance with the concept of mitochondrial dysfunction as the key event in the development of insulin resistance and metabolic disorders in adult life (29, 33, 36, 43). Another study in sheep showed downregulation of mammalian target of rapamycin in fetal muscle due to a midgestation 50% maternal nutrient restriction (66). Mammalian target of rapamycin plays an important role in skeletal myogenesis by affecting the muscle fiber composition in fetal life (66). In the study by Fahey et al. (18), ewes were 50% calorie restricted at three different time points during gestation. A decrease was found in skeletal muscle weight when restriction was late in gestation, whereas a higher proportion of type I fibers was observed with midgestational restriction. Other studies support the notion that duration and timing of gestational nutrient restriction is crucial in fetal development (13, 56). There is ample evidence that protein restriction during fetal development in mice and rats results in low birth weight and obesity in the adult life (14, 45, 65), although some studies suggest that this requires additional postnatal dietary challenges such as a high-fat diet (5, 31). Also in rat and guinea pig, a low-protein diet during gestation combined with a postnatal high-fat challenge may lead to hyperinsulinemia and altered glucose homeostasis in adult life (34, 60). In skeletal muscle of IUGR rats, PGC-1α mRNA level was increased (35). This is interesting, since PGC-1α is involved in the control of mitochondrial biogenesis (37, 62), control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes (58), gene expression of key lipid-metabolizing enzymes such as carnitine palmitoyltransferase I (35), and expression of mitochondrial uncoupling proteins (62, 64). In the present study, late gestation undernutrition caused an increased PGC-1α mRNA level at 6 mo if both muscles were analyzed together and in the FHL muscle after 24 mo (Fig. 3). PPARβ mRNA level, on the other hand, was decreased after both 6 and 24 mo (Fig. 4). These observations suggest that late gestation undernutrition caused impaired and/or lowered β-oxidation capacity, as indicated by studies employing PPARβ agonists (16, 32). The apparent discrepancy for the FHL muscle of a decreased V̇O₂max but an increased PGC-1α mRNA level as a result of fetal programming is probably explained by the fact that the absolute PGC-1α levels at 24 mo are approximately threefold lower compared with the 6-mo values (Fig. 3).

Although it is well established that the intrauterine environment may affect the metabolism of the offspring on a life-long time scale, it is not clear whether a particular genetic background is required to promote such phenotypical programming. However, it may be noted that recent data in mice suggest that the tendency to develop obesity and impaired glucose tolerance resulting from fetal programming of first-generation offspring is transmitted to second-generation offspring despite normal gestational circumstances (30). Similarly, a study in rats shows that impaired glucose tolerance can be transmitted over three generations, (6, 7, 24). Those studies suggest that epigenetic information may be transmitted through generations.

Postnatal High-Fat Diet

The baseline value of RCR in the present study was ~4, similar to previously reported values with permeabilized fibers in man and rat (8, 51), albeit lower than what is usually found in isolated mitochondria from similar rat muscles (19). We
found a pronounced increase (~50%) in RCR after 6 mo of high-fat diet, and this increase showed a tendency to be higher in the groups that have also been exposed to maternal caloric restriction (Table 2). However, we found that the postnatal dietary effect on the RCR is reversible, since the effects may be corrected by 1.5 yr of normal diet, as observed in the 24-mo group (Tables 1 and 2). The higher RCR after the high-fat diet (Table 2) correlates with a significantly lower expression of UCP3 in FHL muscle (Fig. 2), corroborated also by the observation of higher respiratory coupling in UCP3-knockout mice (61). The fact that a high level of fatty acids significantly enhances UCP3 expression (52) has led to the hypothesis that the UCP3 protein may facilitate fatty acid transport out of the mitochondria (53). However, recent studies have shown that UCP3 expression is enhanced by superoxide radicals rather than by fatty acid (47, 57). Since we subjected the animals to a high fatty acid load for 6 mo, we would have expected an increased rather than a decreased UCP3 mRNA level according to that hypothesis. On the other hand, the fact that RCR increased significantly after the high-fat diet suggested a lowering of UCP3, which was actually observed (Fig. 2). The most likely explanation for this apparent discrepancy is that the studies observing a fatty acid-induced increase of UCP3 expression are on much shorter time scales than the present study. In fact, one study on the effects of a fatty acid load on oxygen consumption suggests a maximal effect initially followed by a downregulation after 40 days (15).

Another possibility is that UCP3 is first activated late in gestation or at birth and that the maternal undernutrition in the last trimester might have hindered this activation (12).

The magnitude of the RCR is obviously influenced by both state 4 and state 3 respiratory rates. In the context of the present study, it is of particular interest that the higher the coupling ratio, the higher the mitochondrial ROS formation may be (1, 46) and potentially the higher the risk of permanent damage to mitochondria and other organelles or pathways in the muscle cell. However, the fact that mitochondrial capacity for ATP synthesis in vivo is not significantly affected in adult human subjects of low birth weight as assessed by $^{31}$P-MRS suggests that mitochondrial damage may not be the initial step toward a diabetic phenotype (10). Taking the aconitase activity as a measurement of ROS formation (41), our data suggest decreased rather than increased ROS formation (Fig. 5), for which we at the present time do not have any explanation.

Our results (Table 1) show that intake of a high-fat diet in early life causes a significant increase (30–80%) in mitochondrial respiration. This is consistent with several previous studies on isolated mitochondria from rats (26–28). However, when normalized to the amount of mtDNA, this increase in oxygen consumption is no longer significant, suggesting that the increase is caused entirely by an increased amount and not by a qualitative change of the mitochondria.

A time course study, also in rats, has shown that the increase in oxygen consumption in response to high-fat feeding involves an initial increase followed by a downregulation visible after 40 days (15). However, in the present study we have only one time point with the high-fat diet, 6 mo. This time point would seem to correspond to 40–60 days in rats (rats get ≤3 yr old and sheep ~9 yr old). Thus, the transient effect of a high-fat diet on oxygen consumption reported in rats (15) does not seem to occur within the same time frame in sheep.

Nevertheless, we observed that, 1.5 yr after the discontinuation of the high-fat diet (the 24-mo time point), the enhanced respiratory capacity was no longer present (Table 1).

The high-fat diet induced a decrease of the PGC-1α mRNA levels (Fig. 3). A possible mechanism for this could be hypermethylation of the PGC-1α promoter region caused by free fatty acids, as suggested in a recent study (3).

Methodological Considerations

It is often assumed that CS activity reflects the amount of mitochondria, since CS is expressed only in the mitochondria (49). The CS activity in the different groups of the present study was similar, except for an ~20% higher value in FHL compared with the BF muscle (Table 4). When expressing the $V_{\text{O}_2}\text{max}$ data of Table 1 per unit CS rather than per gram wet weight, the effects of high-fat feeding as well as the muscle fiber type differences were no longer statistically significant, although the P values remained low. This suggests that the diet interventions caused a quantitative rather than a qualitative change of the mitochondria. On the other hand, it is possible that the activity of CS and other TCA enzymes vary as expressed per mitochondrion. Therefore, to investigate this issue further, we measured the amount of mtDNA (Table 5). Expressed per unit mtDNA, the $V_{\text{O}_2}\text{max}$ data of Table 1 showed fetal programming as a lowered $V_{\text{O}_2}\text{max}$ in both muscles (P < 0.05), supporting the data of Table 1. It should be noted that the mtDNA/nDNA ratio was not affected by any of the diet interventions (Table 5), although an age effect was manifested as an ~40% reduction from 6 (adolescence) to 24 mo (adult-hood), also very clearly corroborated by the decreased PGC-1α mRNA level at 24 mo (Fig. 3).

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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