Fetal and Early Postnatal Protein Malnutrition Cause Long-Term Changes in Rat Liver and Muscle Mitochondria1

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ABSTRACT Epidemiologic data suggest a strong association between low birth weight and increased risk of metabolic syndrome in adult life. However, the underlying mechanisms are largely unknown. To test the hypothesis that mitochondrial changes may serve as a link between poor nutrition in early life and insulin resistance in later life, we investigated the effect of protein malnutrition during gestation and lactation on mitochondria of the liver and skeletal muscle. Female offspring of Sprague-Dawley rats fed a low protein diet (casein, 80 g/kg) were randomly divided into two groups and weaned onto either the low protein diet or a control diet (casein, 180 g/kg). As a control group, offspring of rats fed the control diet were weaned onto the control diet. The rats in each group were randomly divided into four groups that were killed at 5, 10, 15 and 20 wk of age. Both mitochondrial DNA content and the expression of mitochondrial DNA-encoded genes in liver and muscle were measured. Mitochondrial transcription factor A and antioxidant enzyme activities were also determined. The mitochondrial DNA content of the liver and skeletal muscle were reduced in fetal and early postnatal malnourished rats even when proper nutrition was supplied after weaning. These changes were accompanied by a decrease in mitochondrial DNA-encoded gene expression; however, they were not dependent on mitochondrial transcriptional factor A. Our findings indicate that poor nutrition in early life causes long-lasting changes in mitochondria that may contribute to the development of insulin resistance in later life.


KEY WORDS: • fetal protein malnutrition • insulin resistance • mitochondrial DNA • mitochondrial transcription factors

Metabolic syndrome, a clustering of cardiovascular risk factors such as diabetes, hypertension, dyslipidemia and obesity, is now recognized as a major health problem in Westernized countries (1). Insulin resistance is a common underlying feature, but the mechanisms at the cellular and biochemical levels are not completely understood. In the early 1990s, Barker and Hales suggested that low birth weight was associated with increased risk of the metabolic syndrome in adulthood (2,3). This was subsequently confirmed by epidemiologic studies conducted in other countries (4–6). On the basis of these observations, the “thrifty phenotype” hypothesis was proposed: poor nutritional condition in early life programs a phenotype in later life, in a way that is beneficial to survival under poor nutritional conditions but detrimental when nutrition is abundant (7).

Mitochondria, intracellular powerhouses generating the cellular energy source ATP, have their own genome, the mitochondrial DNA (mtDNA).4 Mutations or deletions of mtDNA have been implicated in multisystemic disorders because the mtDNA encodes 13 polypeptide subunits of the mitochondrial oxidative phosphorylation complex, 22 tRNAs and 2 rRNAs (8). Mutations in mtDNA are also associated with diabetes mellitus (9,10).

In addition to qualitative changes, quantitative changes in mtDNA seem to be associated with type 2 diabetes and insulin resistance. In our previous study, a decrease in the mtDNA level of peripheral blood preceded the development of diabetes (11). mtDNA content was associated with insulin sensitivity in the offspring of type 2 diabetic patients (12) and was inversely correlated with the components of metabolic syndrome such as blood pressure and waist/hip ratio (11). In addition, mtDNA depletion resulted in dysfunctional insulin secretion in pancreatic β-cells (13) and disturbed glucose metabolism in liver cells (14). mtDNA is transmitted exclu-

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sively from the mother and easily influenced by environment because of its location outside the nucleus. Therefore, it was hypothesized that changes in the mitochondria may mediate the effect of fetal programming on glucose/insulin metabolism in later life.

To explore whether poor nutrition in early life decreases mtDNA content in adulthood, we examined changes in mtDNA content in rats exposed to protein restriction during gestation and lactation, which was shown to induce insulin resistance in later life (15). In the present study, we investigated whether protein malnutrition during gestation and lactation alters both mtDNA content and the expression of mtDNA-encoded genes of liver and muscle in adulthood. Mitochondrial transcription factor A (mtTFA or Tfam) and antioxidant enzyme activities were also measured because they are involved in mtDNA replication and mtDNA change, respectively (16, 17).

MATERIALS AND METHODS

Animals and materials. Male and female Sprague-Dawley rats were obtained from Daehan Laboratory Animal Research (Seoul, Korea) and maintained under conditions of controlled temperature (21–23°C) and light (12-h light:dark cycle, lights on at 0700 h), with free access to food and water. Diets were purchased from Harlan-Teklad (Madison, WI). The control diet and low protein diet were isoenergetic, differing only in protein content (Table 1). All of the experimental procedures were approved by the ethics committee for animal experimentation at the Seoul National University Hospital.

Study design. Female rats (8 wk old) were divided into two groups and fed either a control diet (18 g/100 g casein diet, TD 97120, Harlan-Teklad, Madison, WI) or a low protein diet (8 g/100 g casein diet, TD 97119, Harlan-Teklad, n = 20) for 15 d before mating. They were mated with 10-wk-old male Sprague-Dawley rats fed the control diet. Female offspring of dams fed low protein were randomly divided into two groups at 4 wk after birth and weaned onto either the low protein (low protein group, n = 48) or control diet (resuscitated group, n = 48). As a control group, offspring of the dams fed the control diet were weaned onto the control diet (n = 49). Rats in each group were again randomly divided into four groups and killed at 5, 10, 15, and 20 wk of age (n = 12 per group). Body weight was measured before rats were killed. Liver and quadriceps muscle were quickly collected under ether anesthesia, immediately frozen in liquid nitrogen and stored at −70°C until analysis. Venous blood from the inferior vena cava was collected into EDTA-coated tubes and centrifuged at 2000 × g for 5 min. The buffy coat was collected and stored at −70°C until DNA was extracted.

mtDNA levels. Total DNA was extracted using a QIAmp tissue kit (QIAGEN, Chatsworth, CA). Determination of mtDNA content was performed by competitive PCR (11). The mtDNA was amplified using a set of primers: forward 5'-AGGACTTAAACGACC-CAAAACCG-3' (nucleotides 4396–4418), backward 5'-CCTCTTTTCTGTAGCCGCGG-3' (nucleotides 5145–5164) yielding a 770-bp product under the following conditions: one cycle of 5 min at 94°C, 30 cycles of 30 s at 94°C, 40 s at 57°C and 40 s at 72°C and a final extension of 7 min at 72°C. The internal standard was designed to competitively use the same set of primers with the target gene as a competitor DNA but to yield a different-sized PCR product (770 bp for the target gene vs. 694 bp for the internal standard). The PCR products were analyzed on a 1.2% agarose gel by electrophoresis. The relative intensity ratio of the target mtDNA product vs. the internal standard was plotted against log (internal standard) to yield the equivalence point between the internal standard and the target mtDNA. The calibration curve of the standard curve was between 0.95 and 1.00. The interassay CV of mtDNA measurement was 12.2%. The mtDNA content was expressed as the relative amount (amol) mtDNA/250 ng total DNA.

Table 1

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control diet g/kg</th>
<th>Low protein diet g/kg</th>
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<tr>
<td>Casein</td>
<td>180.0</td>
<td>80.0</td>
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<tr>
<td>α-Methionine</td>
<td>0.0</td>
<td>2.0</td>
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<tr>
<td>Cornstarch</td>
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<td>774.99</td>
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<tr>
<td>Peanut oil</td>
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<tr>
<td>Calcium phosphate</td>
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<td>19.51</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>2.25</td>
<td>0.1</td>
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1 Vitamin mix (Harlan Teklad 40060, g/kg, Harlan Teklad, Madison, WI) p-aminobenzoic acid, 11.0132; ascorbic acid, coated (97.5%) 101.6604; biotin, 0.0441; vitamin B-12 (0.1% trituration in mannitol), 2.9736; calcium pantothenate, 6.6079; choline hydrochloride, 349.6916; folic acid, 0.1982; inositol, 11.0132; menadione, 4.9559; niacin, 9.1191; pyridoxine-HCl, 2.2026; riboflavin, 2.2026; thiamin-HCl, 2.2026; dry retinyl palmitate (500,000 U/g), 0.4405, dry dl-α-tocopherol acetate (500 U/g), 24.2291, cornstarch, 466.6878. Designed for use at 1% (10 g/kg of diet).

2 Mineral mix (Harlan Teklad TD 79055, g/kg) NaCl, 193.7325; potassium chloride, monohydrate, 575.9615; potassium sulfate, K2SO4, 136.1363; magnesium oxide, MgO, 62.8322; manganous carbonate, 9.163; ferric citrate, 5.1638; zinc carbonate, 4.1888; cupric carbonate, 0.7854; potassium iodate, KIO3, 0.0262; sodium selenite, Na2SeO3, 5H2O, 0.0262; chromium potassium sulfate, CrK(PO4)2 · 12H2O, 1.4399. This is a modification of the AIN-76 mineral mix (170915).
RESULTS

Body weights. Body weights were lower in the low protein group than in the control group at all ages examined (Table 2). In the resuscitated group, body weight was not different from that of the low protein group at weaning. At subsequent ages, the body weight of the resuscitated group was between that of the other two groups and different from both (Table 2).

Tissue and blood mtDNA content. The differences in mtDNA content paralleled those in body weight. The mtDNA levels of the liver and muscle in the low protein group were consistently lower than in the control group (Fig. 1A and B). At 5 wk of age, liver and muscle mtDNA contents were reduced in the resuscitated group to the levels of the low protein group. Liver and muscle mtDNA contents in the resuscitated group were partially restored after 10 wk of age, but were significantly lower than those in the control group at 20 wk of age (Fig. 1A, B).

Peripheral blood leukocyte mtDNA levels were lower in the low protein group than in controls throughout the study (Fig. 1C). In the resuscitated group, a partial recovery occurred at 10 wk of age, but mtDNA levels were again reduced to the level of the low protein group at 15 and 20 wk of age (Fig. 1C).

There were significant correlations between tissue and blood mtDNA contents at 20 wk of age (liver vs. blood $r^2 = 0.41, P < 0.01$; muscle vs. blood $r^2 = 0.64, P < 0.01$).

mtDNA-encoded gene expression. In the low protein group, COX III mRNA was higher than in the controls at 10 wk of age and lower at 20 wk of age (Fig. 2B). In the resuscitated group, COX III gene expression tended to be higher ($P = 0.088$) than in the control group at 10 wk. COX III and ND4 gene expression were lower and COX I gene expression tended to be lower ($P = 0.096$) in the resuscitated group than in control group at 20 wk of age (Fig. 2A–C).

### TABLE 2

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<td>47.7 ± 2.7b</td>
<td>43.3 ± 1.2b</td>
<td>47.7 ± 2.7b</td>
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<tr>
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<td>74.3 ± 3.2b</td>
<td>53.9 ± 1.6c</td>
<td>74.3 ± 3.2b</td>
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<tr>
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<td>109.2 ± 5.5c</td>
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<td>264.1 ± 5.7b</td>
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<td>171.6 ± 7.5c</td>
<td>264.1 ± 5.7b</td>
<td>171.6 ± 7.5c</td>
<td>264.1 ± 5.7b</td>
</tr>
<tr>
<td>20</td>
<td>416.8 ± 4.2a</td>
<td>321.5 ± 7.5b</td>
<td>231.8 ± 7.9c</td>
<td>321.5 ± 7.5b</td>
<td>231.8 ± 7.9c</td>
<td>321.5 ± 7.5b</td>
<td>231.8 ± 7.9c</td>
<td>321.5 ± 7.5b</td>
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1 Values are means ± SEM, $n = 12$. Means in a row with a different superscript letter differ, $P < 0.05$. 
**mtTFA expression.** The mtTFA expression in liver was lower than in controls in both the low protein and resuscitated groups at 10 wk of age \((P < 0.05, \text{Fig. 3})\). However, mtTFA expression increased in the low protein group and was greater than in the other two groups at 20 wk of age. The mtTFA expression in the resuscitated group was not different from that in controls but was lower than that in the low protein group at both 15 and 20 wk of age (Fig. 3).

**Antioxidant enzyme activities and lipid peroxidation products.** In both the low protein and resuscitated groups, liver SOD activity was significantly lower than in controls at 15 wk: control \(7.6 \pm 0.7\), resuscitated \(5.7 \pm 0.7\), low protein \(4.1 \pm 0.6\) U/mg protein. There was an inverse correlation between liver SOD activity and liver MDA content \((r = -0.359, P < 0.01)\). In contrast, muscle SOD activity and MDA level and liver and muscle GPx activities did not differ among the three groups.

**DISCUSSION**

We demonstrated that protein malnutrition in early life causes long-lasting changes in mitochondria that are present in later life. The mitochondrial changes are evident in the liver and skeletal muscle, the main sites of insulin action.

Poor nutrition in early life is associated with increased risk of type 2 diabetes and the insulin resistance syndrome in later life (2–6). Maternal nutrition may affect fetal growth and development directly through the availability of nutrients for transfer to the fetus and also permanently alter glucose/insulin metabolism (22,23). Several metabolic abnormalities that lead to insulin resistance have been suggested in the offspring of rat dams fed a protein-restricted diet. The organ weights of muscle and liver were reduced (22). The activities and gene expression of insulin-sensitive hepatic enzymes were changed. In addition, the glucokinase activity was reduced and phosphoenolpyruvate carboxykinase increased, both resulting in increased hepatic glucose production (24,25). Recently, it was

**FIGURE 2** mRNA expression of cytochrome oxidase I (COX I) (A), cytochrome oxidase III (COX III) (B) and NADPH dehydrogenase 4 (ND4) (C) in offspring of dams fed the control diet and weaned onto the control diet (control) and offspring of dams fed the low protein diet and weaned onto the control diet (resuscitated) or low protein diet (low protein). Values are mean ± SEM, \(n = 9\). Means at an age without a common letter differ, \(P < 0.05\).

**FIGURE 3** Expression of mtTFA in offspring of dams fed the control diet and weaned onto the control diet (control) and offspring of dams fed the low protein diet and weaned onto the control diet (resuscitated) or low protein diet (low protein). Values are mean ± SEM, \(n = 9\). Means at an age without a common letter differ, \(P < 0.05\).
reported that phosphatidylinositol-3 kinase activation in response to insulin was impaired in adipocytes from 15-mo-old offspring of rat dams fed a protein-restricted diet (26).

However, whether a link exists between poor nutrition in early life and insulin resistance in later life is unknown. On the basis of several previous observations, we hypothesized that mitochondrial dysfunction might be a possible link between poor nutrition in early life and insulin resistance. We found that mtDNA content was decreased in the peripheral blood of diabetic subjects, and also in those subjects who developed diabetes mellitus within 2 y. In this population, peripheral blood mtDNA content was inversely correlated with blood pressure and waist/hip ratio. Furthermore, mtDNA copy number is decreased in the muscle of diabetic subjects (27). Recently, decreased mitochondrial size in muscle was reported to be associated with insulin resistance (28). These findings suggest that low mtDNA content is associated with both insulin resistance and diabetes mellitus. We also found that peripheral blood mtDNA levels of the dams correlated with those of the offspring (r = 0.55, P < 0.01) and birth weight (r = 0.63, P < 0.01) in another study, which suggests the thrifty phenotype phenomena might be due to the low mitochondrial status (unpublished observation).

In the present study, we demonstrated for the first time that protein malnutrition during gestation and lactation decreased mtDNA levels of the liver and skeletal muscle in adult life, which were not completely recovered by good postnatal nutrition. The changes in mtDNA content paralleled those in body weight. These findings indicate that the nutritional state in early life determines mtDNA level as well as body weight in adulthood. They also support our hypothesis that protein malnutrition during gestation and lactation decreases mtDNA level, which may affect insulin sensitivity in later life. It has been suggested that mitochondrial gene expression is regulated mainly by mtDNA copy number, whereas nuclear gene expression is regulated by transcriptional factors (29). However, we found discordance between mtDNA content and mtDNA-encoded gene expression. It is of interest that mtDNA encoded-gene expression at 20 wk was greater in the resuscitated group than in the low protein group. This may imply that the exposure to a changing nutritional environment rather than to poor nutrition itself is more important in modulating mitochondrial gene expression. Indeed, epidemiologic studies have demonstrated that an association between impaired fetal growth and insulin resistance in later life becomes stronger in obese people (4,6). These findings agree with the “thrifty phenotype” hypothesis that adverse effects of poor nutrition in early life would be aggravated in those exposed to overnutrition in later life.

Although both mtDNA content and mitochondrial gene expression were decreased in the rats exposed to protein malnutrition during gestation and lactation, the mechanism mediating these changes remains to be determined. Transcription and replication of mtDNA is regulated by coordination of cis- and trans-acting factors (30). The cis elements responsible for the regulation of this process are located mainly in a small non-coding mtDNA fragment, the D-loop region, whereas all the known trans-acting factors are nuclear encoded. The mtTFA (or Tfam) is an important regulator of both mtDNA transcription and replication in mammalian cells (16). In our study, changes in mtTFA expression did not parallel those in mtDNA levels. The mtTFA expression decreased at 10 wk of age, but increased at 20 wk of age in the low protein group, which had the lowest tissue mtDNA content among the three groups. Thus, the reduction of mtTFA at an earlier age may be involved in the depletion of mtDNA, whereas the increase of mtTFA at a later age may be part of a compensatory process in response to mtDNA depletion. Because mtTFA was not decreased at 20 wk of age, when the reduced levels of mtDNA and mtDNA-encoded gene expression were prominent, the mtTFA-independent regulation of mtDNA might be involved in the mitochondrial changes. It is possible that mtDNA depletion might be caused by defects in genes not directly related to the replication machinery.

Protein malnutrition is associated with a depressed antioxidant defense system and increased oxidative stress (31). Because the cumulative data suggest a role for oxidative stress in mtDNA damage (17), we speculate that oxidative stress might be involved in malnutrition-associated mitochondrial changes. In our study, liver SOD activity was reduced in malnourished fetal rats and was inversely correlated with liver MDA concentration. However, muscle SOD activity as well as liver and muscle GPX activities were not changed. Thus, changes in the antioxidant system seem to occur in an organ- and enzyme-specific manner in fetal and early postnatal protein-restricted rats. Therefore, the mitochondrial changes in muscle and liver as a whole could not be explained by enhanced oxidative stress.

It has been suggested that maternal diet could affect gene expression in offspring by epigenetic mechanisms such as DNA methylation in developing embryos (32). Thus, it will be interesting to investigate the methylation state of the genes that are involved in mtDNA transcription and replication in these animals. Further studies will be required to elucidate the mechanisms by which protein malnutrition in early life causes the mitochondrial changes in later life.

LITERATURE CITED