

Original Article

Metabolic programming from neonate to adulthood in rats with maternal hypoxia

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Abstract: Background: Maternal hypoxia related programming has been linked to lots of chronic diseases in adulthood, while the potential pathogenesis is unclear. In this study we investigated the programming effects in maternal hypoxia offspring neonates and adults on glycolipid metabolism and related molecular mechanism. Methods: Sprague-Dawley rats were assigned to maternal hypoxia exposure from middle to last pregnancy. Offspring neonates and adults were sacrificed for physical characteristics and metabolic status. And the expression of main proteins in liver glycolipid metabolic regulation was evaluated by Western blot analysis. Results: Maternal hypoxia resulted in a low birth weight with an increase of blood lipid and insulin in offspring neonates. Difference in weight does not exist after breast-feeding. In maternal hypoxia adults, dyslipidemia and insulin resistance were detected. Then, maternal hypoxia would enhance the expression of proteins in gluconeogenesis, insulin action and lipogenesis and down-regulate carnitine palmitoyl transferase 1 in newborns. When maternal hypoxia ones full grown, gluconeogenesis, lipogenesis and lipid oxidation were enhanced, but insulin signaling pathway was significantly down-regulated. Additionally, uncoupling protein 2, a mitochondrial biogenesis marker, was found only increased in hypoxia neonates. While pro-inflammatory factors remained unchanged. Conclusion: Maternal hypoxia enhanced gluconeogenesis and lipogenesis in new borns which lasted and interacted with postnatal factors contributing to maladjustment of glycolipid metabolism and insulin resistance in adults.

Keywords: Fetal programming, maternal hypoxia, glucose metabolism, lipids, insulin resistance

Introduction

Both epidemiological and experimental studies have revealed adverse intrauterine environment would predispose offspring to chronic diseases including diabetes [1, 2], obesity [3], atherosclerosis [4] and nonalcoholic fatty liver disease [5-7]. Intrauterine hypoxia was the most common condition for placental pathology and fetal programming [8]. To date, however, previous studies over the effect of maternal hypoxia mostly focused on metabolic regulation in adulthood. Molecular mechanism involved in maternal hypoxia-induced programming that contributes to dysbolism phenotype leaves unclear. Thus, a further understanding is in urgent need.

It is widely accepted that fetal programming was involved in the pathogenesis of metabo-

lism related chronic diseases. Common lesions include placental insufficiency, cord compression, anemia, preeclampsia and maternal smoking are consistent with chronic hypoxia which is one of the leading causes of poor perinatal outcome and even adult diseases [9]. Reported in previous studies [10, 11] focused on fetal programming, offspring intervened by maternal hypoxia exhibited increases in triglyceride (TG) and free fatty acids (FFA) and a decrease in insulin. Su *et al.* [10] also noticed the proinflammatory factor, tumor necrosis factor α (TNF- α) climbed after hypoxia. Moreover, the maladjustment of hypothalamic-pituitary-adrenal axis and of adipokines (leptin, adiponectin, etc.) was also reported in adult offspring with maternal hypoxia [12, 13]. Taken the above into consideration, the first insult to metabolic disturbance might have a fetal origin. With maternal hypoxia models, Al-Hasan *et al.* [14,

15] reported maternal hypoxia could trigger mitochondrial dysfunction and obstruction of oxidative phosphorylation. State of hepatic oxidative stress in nonhuman primates was detected in the third trimester of pregnancy with adverse environment exposure [16]. And a further study [5] evinced fetal programming of hepatic oxidative stress were activated by maternal over-nutrition consumption and maintained even in adolescence. Hence, we speculated FFA metabolism in neonates was programmed, then, early lesions was aggravated by the vicious circle effect of abnormal deposition of lipids, developing into impaired glycolipid homeostasis and insulin action.

So far, the specific mechanism of fetal programming is indeterminacy. What have been mostly reported were researches predominantly focused on glycolipid homeostasis regulation in adult offspring, seldom on the relationship of molecular mechanism. Study the link of metabolic status and main proteins between offspring newborns and adults may provide information on the “fetal origins of adult disease” induced by maternal hypoxia. Hence, in this study, we tested pathologic changes of glycolipid homeostasis and key modulators in offspring newborns and linked with those in adults. It was aimed to investigate maternal hypoxia induced metabolic programming for progressive glycolipid dysbolism and molecular mechanism in offspring.

Materials and methods

Animal score

All experimental procedures were in accordance with National Institutes of Health guidelines and approval by the Animal Experimentation Ethics Committee of Quanzhou Medical College (Permit Number: 2012001). Sprague-Dawley rats (10 male, 20 female) weighed 170 g-220 g were purchased from Shanghai slack laboratory animals limited liability company (Shanghai, China). Rats were housed at constantly standard temperature and humidity with a 12 hours light/dark cycle and access to standard rat chow and water ad libitum. After two-week acclimatization, rats were mated (rate 2:1) and successful fertilization was confirmed by vaginal smear. Throughout pregnancy rats were housed individually in standard rat cages.

Maternal hypoxia model

According to the procedure we have previously reported [9, 11], from day 7 to 21 of pregnancy, rats were placed inside a plexiglas eight hours per day in light cycle with an infusion of nitrogen gas and compressed air mixture. Oxygen concentration was continuously monitored by an oxygen analyzer (S-450; IST-AIM) to maintain at $(10 \pm 1)\%$. Normal controls underwent the same procedures with only compressed air infused. After delivery, litter size of each mother was sexed depend on anogenital distance, counted and then randomly culled to four males for further nursing and weaned to ad libitum laboratory chow at 28 days of age. The surplus ones were sacrificed for birth weight and biomarkers detection.

Specimens collection and preparation

Upon completion of the interventions, rats were fasted overnight and anesthetized with 2% sodium pen to barbital intraperitoneal injection. Blood specimens were collected by intracardiac puncture (decapitation for neonates) then centrifugalized to obtain supernatant and stored at -20°C . Liver tissue samples were snap freezing in liquid nitrogen and kept at -80°C . Specimens of neonates mixed three into one.

Blood-borne biomarkers detection

Fasting glucose was detected by glucometer (ACCU-CHEK Active, Germany) via tail-snip blood samples. TG and FFA were assayed by standard enzymatic colorimetric method with an auto-biochemical analyzer (RapidlabTM850, Beckham). The concentrations of serum insulin, insulin-like growth factor 1 (IGF-1) and interleukin 6 (IL-6) were quantified by commercially available RIA kits (Beijing North Institute of Biotechnology, China). Insulin resistance (IR) index was evaluated by the homeostasis model assessment (HOMA) in light of Cao *et al.* [17].

Western blotting

All the primary antibodies were pursued from Santa Cruz Limited of Biotechnology and Sigma-Aldrich Company, including phosphoenolpyruvate carboxykinase (PEPCK), sterol-regulatory element-binding protein 1 (SREBP-1), fatty acid synthase (FASN), carnitine palmitoyl transferase 1 (CPT-1), uncoupling protein 2

Metabolic programming in maternal hypoxia offspring

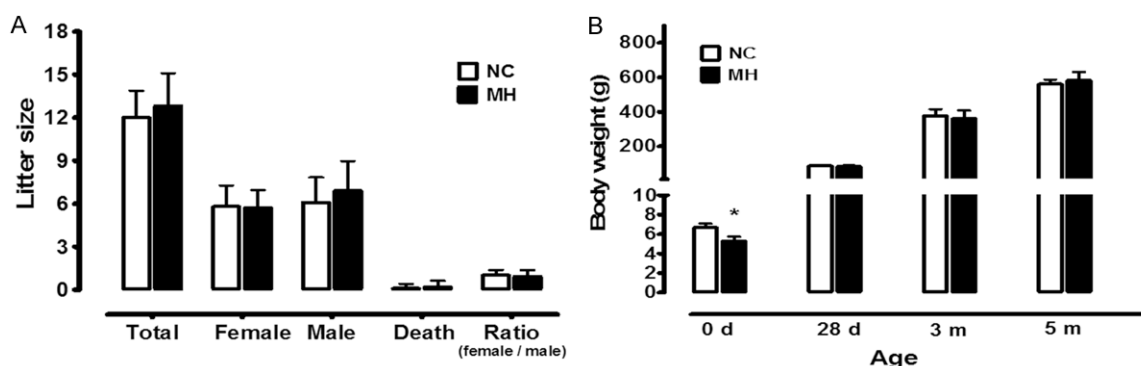


Figure 1. Litter size and weight gain of offspring rats. A. Litter size and structure of neonates. B. Birth weight and weight gain. NC, normal controls; MH, maternal hypoxia; * $P < 0.05$, vs normal controls.

Table 1. Blood-borne biomarkers of offspring neonates (n=6)

	Normoxia	Hypoxia
Glucose (mmol/L)	4.7±0.7	4.9±1.0
Lg Insulin (mIU/L)	2.1±0.0	2.2±0.0*
IGF-1 (µg/L)	342.6±50.7	514.3±66.4*
TG (mmol/L)	0.2±0.0	0.4±0.1*
FFA (µmol/L)	176±39.3	314±52.9*

Values are means ± SD. Lg, the base 10 logarithmic transformation. * $P < 0.05$, vs maternal normoxia.

Table 2. Blood-borne biomarkers of offspring adults (n=10)

	Normoxia	Hypoxia
Glucose (mmol/L)	4.8±0.4	5.2±0.6
Lg Insulin (mIU/L)	1.6±0.5	1.8±0.7
TG (mmol/L)	0.4±0.0	0.6±0.1*
FFA (µmol/L)	367.7±34.3	552.9±82.6*
IL-6 (ng/L)	79.9±21.0	84.1±34.0
Lg HOMA-IR	1.0±0.0	1.2±0.0*

Values are means ± SD. Lg, the base 10 logarithmic transformation. * $P < 0.05$, vs maternal normoxia.

(UCP-2) and so on. According to the manufacturer's protocol, tissue specimens were lysed on ice by protein extraction reagent (Beyotime institute of biotechnology, Co.), and harvested by differential centrifugation. Boiled with loading buffer, target proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred to 0.5 µm nitrocellulose membranes. Then target protein was successively incubated with a rabbit polyclonal antibody (1:500 in skim milk) and goat anti-rabbit IgG antibody (1:3000 in skim milk, EarthOx, USA). Colored by ECL method, relative protein levels were counted by density of target protein corrected by β-actin using Image-Pro Plus (6.0) software.

Statistical analysis

All data were statistically analyzed by the SPSS statistical software version 17.0 (SPSS Inc., Chicago, IL, USA). Serum insulin and HOMA-IR were logarithmically transformed to normally distribution. Results were expressed as means ± SD. Comparisons between two groups were performed using the unpaired student t-test. Significance was accepted at $P < 0.05$.

Results

Physical characteristics

No differences were found in litter size, sexual structure and death number between normoxic and hypoxic dams (all $P > 0.05$, **Figure 1A**). Pups exposed to maternal hypoxia exhibited low birth weight, while perturbation of neonatal weight was restored after 28 days breast-feeding ($P < 0.05$, **Figure 1B**).

Blood-borne biomarkers in offspring neonates and adults

In neonates, period of maternal hypoxia resulted in a significant increase in serum lipid, IGF-1 and insulin ($P < 0.05$) while serum glucose remained normal ($P > 0.05$, **Table 1**).

In adults, with maternal hypoxia exposure, serum concentration of TG and FFA, significantly increased, when compared with that in normal controls ($P < 0.05$). While, circulating IL-6, an inflammatory factor, remained unchanged in both groups, as well as serum glucose and insulin ($P > 0.05$). Additionally, maternal hypoxia

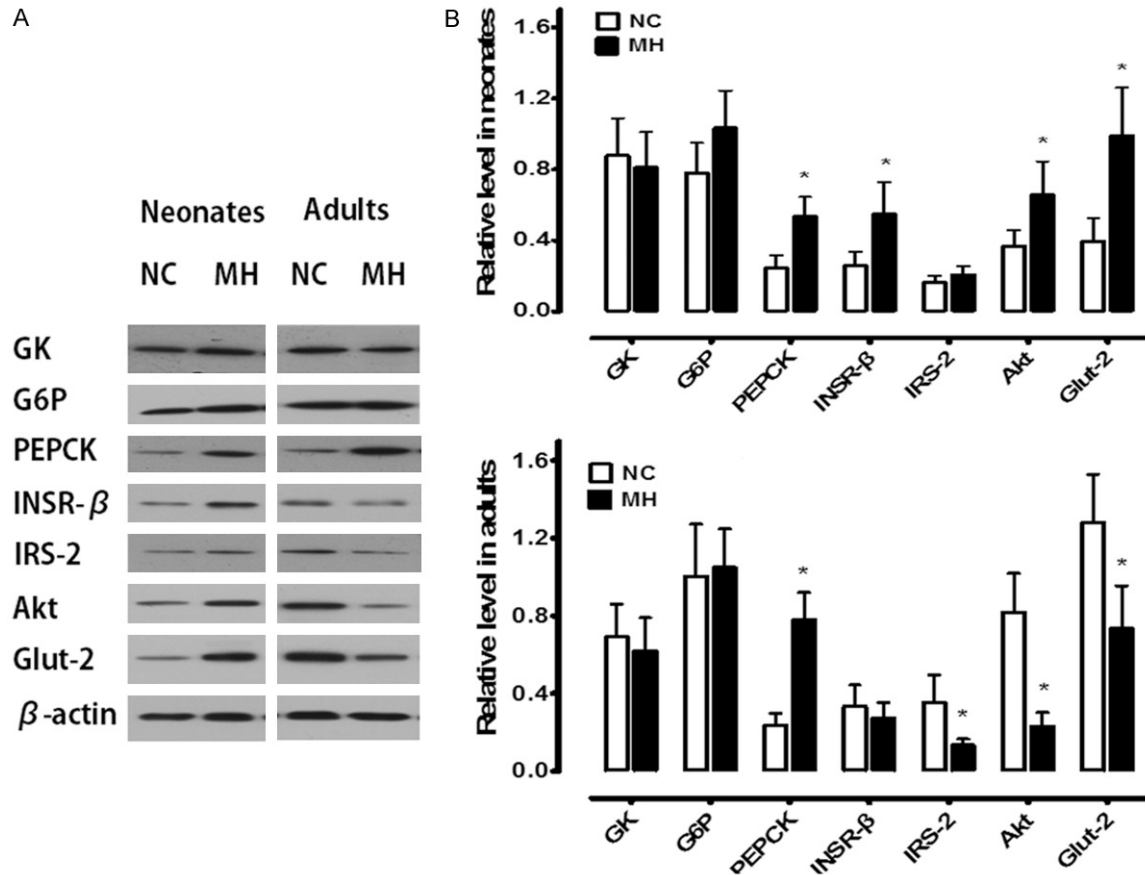


Figure 2. Western blot analysis of relevant active proteins in glucose metabolism and insulin signaling pathway in liver tissues. A. Bands in neonates (n=6) and adults (n=10). B. Relative quantitative analysis. NC, normal controls; MH, maternal hypoxia; * $P < 0.05$, vs normal controls.

caused IR (Lg HOMA-IR 1.2 ± 0.0 vs 1.0 ± 0.0 , $P < 0.05$, **Table 2**).

Main proteins in glycolipid metabolic regulation in offspring neonates and adults

As we expected, maternal hypoxia resulted in a significant increase of PEPCK, a gluconeogenesis-related protein, which lasted to adulthood ($P < 0.05$). When compared with that in the corresponding normoxic ones, insulin signaling pathway changed oppositely, respectively, up-regulation of insulin receptor β (INSR- β), protein kinase B (Akt) and glucose transporter 2 (Glut-2) expression in neonates and down-regulation of insulin receptor substrate 2 (IRS-2), Akt and Glut-2 expression in adults (**Figure 2**, all $P < 0.05$).

Under the influence of maternal hypoxia, no difference was found in TNF- α expression ($P > 0.05$). The expression of FANS and SCD-1 were enhanced with their upstream regulator

peroxisome proliferator-activated receptor γ (PPAR- γ), while CPT-1, a fatty acid oxidative hallmark, was inhibited (all $P < 0.05$) in neonates. In offspring adults, a comprehensive effect of both high-level lipogenesis (SREBP-1, FANS and SCD-1) and fatty acid oxidation (CPT-1) was detected (all $P < 0.05$). Surprisingly, in maternal hypoxic newborns, the expression of UCP-2, a hallmark of mitochondrial bioactivity, decreased significantly ($P < 0.05$). When full grown, UCP-2 expression was slightly reversed and stay equivalent to normal controls ($P > 0.05$, **Figure 3**).

Discussion

To the best of our knowledge, this is the first comprehensive study focused on the metabolic programming from neonate to adulthood induced by maternal hypoxia. Specifically, our findings demonstrated that maternal hypoxia resulted in a low birth weight and a catch-up growth; increased the susceptibility to hyperli-

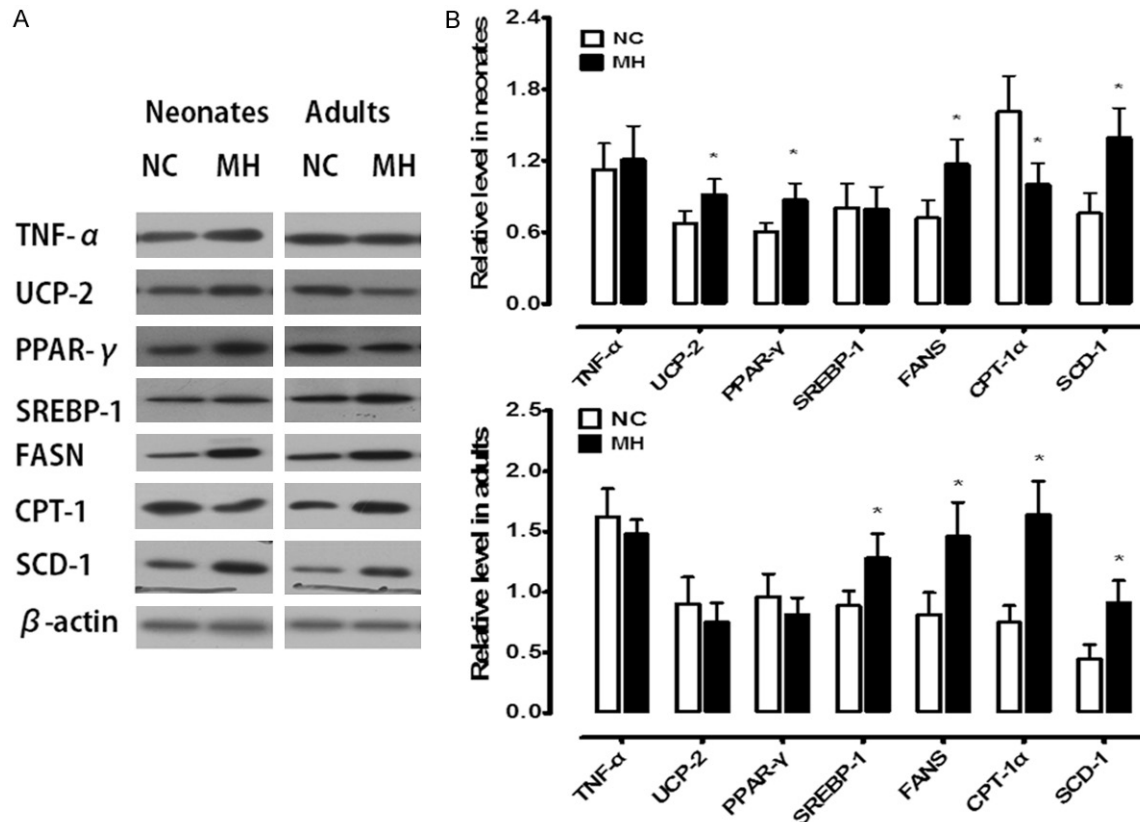


Figure 3. Western blot analysis of TNF- α and relevant active proteins in lipid metabolism. A. Bands in neonates (n=6) and adults (n=10). B. Relative quantitative analysis. NC, normal controls; MH, maternal hypoxia; * $P < 0.05$, vs normal controls.

pemia, abnormally high gluconeogenesis and IR. In addition, a series of proteins changed as potential molecular mechanism of programming these phenotypic changes.

Evidenced by Zamudio *et al.* [18], the etiology of IUGR induced by reduced fetoplacental oxygen supply was preferable limits of glucose availability rather than reduced oxygen availability, delivery or consumption. To complete the adaptation to chronic glucose deficiency, hepatic glucose production, glucose utilization rate and insulin sensitivity would with resultant be underpinned [2, 19, 20]. As we detected, in offspring neonates, insulin signaling pathway and gluconeogenesis was up-regulated. Theoretically, placental insufficiency-induced IUGR fetus was characterized by the shortage base line and glucose-stimulated insulin secretion [21]. Interestingly, in this study, no difference was found in glucose concentration, but an unexpected climbing of insulin in hypoxic neonates. Our finding was in accordance with the fluctuation of blood glucose and insulin in

hypoxic fetus depicted by Lueder *et al.* [22]. They reported that by day 20 of pregnancy, insulin increased as a compensatory regulation to balance the adaptively increased glucose coping with placenta insufficiency.

Compared with normoxia, a significant increase in serum TG and FFA in neonates of maternal hypoxia were detected. Concerning materials transportation from mother to infant, only in the late pregnancy (a main stage of adipose tissue development), an exponentially increase of maternal serum TG presents as a driving force in lipid flux owing to physiological IR accompanied a notable maternal lipolysis [23]. While stresses (hypoxia or low energy) would trigger glycolysis and then reduced glucose may stimulate compensatory increases of lipolytic hormones (e.g. cortisol) both in pregnant and fetus [24]. Hence, we reasoned that in our models, maternal hypoxia disposal lasted from mid to late trimester of pregnancy would give rise to a high level of catabolics which could create a concentration gradient driving lipid flux to the

fetus. Additionally, mass lipid metathesis deposition in fetal organs and lipid de novo synthesis surpassed utilization would accordingly somewhat predispose liver to lipotoxicity, a local and systemic pathophysiological basis.

Our finding is consistent with previous data [10, 11, 25-27], suggesting maternal hypoxia contributed to hyperlipemia, abnormally enhanced gluconeogenesis and IR in offspring adults. In our study, PEPCK expression was enhanced dramatically by maternal hypoxia and somewhat maintained to adulthood. Such adaptations in uterus would generate maladaptation to relatively over-nutrition environment after birth. Persistently advanced gluconeogenesis without suppression by insulin in adult offspring was certificated to contribute to impaired glucose tolerance and IR [28]. Furthermore, with excess lipid flux, lipid metathesis deposited driving body to local and systematic lipotoxicity damages including impaired insulin action [29]. Additionally, catch-up growth after born small was revealed conducive to preferential catch-up fat rather than muscle tissue, which was sustained intimately linked with IR state of catch-up growth [30]. Disproportionately accelerated fat recovery could lead to redistribution of glucose from skeletal muscle to white adipose tissue, therefore resulted in a reduction in insulin-mediated glucose disposal and hyperinsulinemia. This in turn, would serve to redirect the spared glucose towards de novo lipogenesis and fat storage [31]. Thus, all these would interplay and form a vicious cycle contributing to disturbance of glycolipid metabolism and insulin action.

UCP-2 is known as a mitochondrial respiratory chain-related protein with its tissue-specific function referring response to oxidative stress in liver, regulation of energy availability, fatty acid metabolism and thermoregulation [32]. What's particularly intriguing was that UCP-2 decreased gently in response to maternal hypoxia while recovered to normal when full grown. While this result differed from what Hyatt *et al.* [33] have reported. As they found, half energy supply in mid-pregnancy would not affect UCP-2 expression in juvenile fat sheep. The object of their study underwent maternal half-diet and postnatal sedentariness with over-nutrition diet. We supposed fetal programming could take place both in uterus and outside uterus when experiencing some maladapt-

tive environment [34]. These might modify epigenetics. Additionally, according to Schrauwen *et al.* [35] the effect of UCP-2 on metabolism could be compensated for by controlling spontaneous physical activity and food consumption. Although the role of UCP-2 down-regulated after maternal hypoxia in our study was not clear, recently, its role in lipid metabolic regulation was emphasized [32, 35, 36]. Since liver is an important organ in glucose homeostasis regulation, it could improve the glucose production to make up for reducing the use of fatty acids as fuel [35]. Therefore, we speculated UCP-2 reduction might be an indicator of fuel switch (from FFA to glucose) and it would, in turn, cause more glucose production. In view of this, we measured UCP-2 and gluconeogenesis-related protein expression and found after maternal hypoxia UCP-2 reduced while PEPCK increased.

There still some limitations in our study. Although, IUGR induced by maternal hypoxia was widely applied by previous researches [9-11], an ignorance that repetitive ischemia-reperfusion may put additional threats into fetal development worth attention. Besides, more biomarkers for mitochondrial biogenesis evaluation were still needed. Last but not least, we shed the important sight into evaluation the relationship between physical activities affected by maternal hypoxia and glycolipid metabolic programming. Monitored by Rueda-Clausen *et al.* [37], offspring rats intervened with intra-uterine low energy environment were physical inactive, suggesting there may be an energy thrifty adaptation. Future studies are needed to address specific mechanism in mitochondrial biogenesis and neuroendocrine system programming. Additionally, whether a more health-conscious environment where infants are raised could help to reverse these pathological changes is a pending problem.

Conclusions

In conclusion, this study examined the hypotheses that “fetal origins of adult disease [38]” and “thrifty phenotype [39]” with maternal hypoxia models. Our findings suggested that fatty acid metabolism, gluconeogenesis, insulin signaling pathway and mitochondrial biogenesis were responsible for fetal metabolic programming. With time, disorders lasted and interacted with postnatal factors, ultimately

leading to hyperlipidemia and IR in adults. Further studies are needed to address whether maternal hypoxia programs mitochondrial biogenesis and neuroendocrine system and physical activity reverses these pathological changes.

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Disclosure of conflict of interest

None.

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