Maternal Hypoxia Increases the Susceptibility of Adult Rat Male Offspring to High-Fat Diet-Induced Nonalcoholic Fatty Liver Disease

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Exposure to an adverse intrauterine environment increases the risk for adult metabolic syndrome. However, the influence of prenatal hypoxia on the risk of fatty liver disease in offspring is unclear. The purpose of the present study was to evaluate the role of reduced fetal oxygen on the development and severity of high-fat (HF) diet-induced nonalcoholic fatty liver disease (NAFLD). Based on design implicating 2 factors, ie, maternal hypoxia (MH) and postnatal HF diet, blood lipid and insulin levels, hepatic histology, and potential molecular targets were evaluated in male Sprague Dawley rat offspring. MH associated with postnatal HF diet caused a significant increase in plasma concentration of triglycerides, free fatty acids, low-density lipoprotein cholesterol, and insulin. Histologically, a more severe form of NAFLD with hepatic inflammation, hepatic resident macrophage infiltration, and progression toward nonalcoholic steatohepatitis was observed. The lipid homeostasis changes and insulin resistance caused by MH plus HF were accompanied by a significant down-regulation of insulin receptor substrate 2 (IRS-2), phosphoinositide-3 kinase p110 catalytic subunit, and protein kinase B. In MH rats, insulin-stimulated IRS-2 and protein kinase B (AKT) phosphorylation were significantly blunted as well as insulin suppression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. Meanwhile, a significant up-regulation of lipogenic pathways was noticed, including sterol- regulatory element-binding protein-1 and fatty acid synthase in liver. Our results indicate that maternal hypoxia enhances dysmetabolic liver injury in response to an HF diet. Therefore, the offspring born in the context of maternal hypoxia may require special attention and follow-up to prevent the early development of NAFLD.

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ence of more than 15% in the United States (13). In addition, IUGR remains a leading contributor to perinatal mortality and morbidity and to most components of MetS in later life. A series of epidemiologic studies have demonstrated that poor growth in utero is associated with increased risk of developing obesity and other MetS (14, 15). Experimentally, a number of animal models resorting to maternal malnutrition during pregnancy (16, 17), fetal exposure to elevated glucocorticoid levels (18, 19), or surgical ligation of the uterine arteries (20) have been used. It has been reported that uteroplacental insufficiency in rats caused impaired glucose tolerance and hyperinsulinemia as well as elevated plasma triglycerides and leptin levels in adult offspring (21). Interestingly, another recent study, in which IUGR was induced in rats by prenatal hypoxia, indicated that prenatal reduction of oxygen supply might induce permanent metabolic changes that predispose to HF diet-induced MetS in adult offspring (22). Fetal hypoxia is a major clinical cause of IUGR, in the context, for instance, of preeclampsia, maternal anemia, asthma, smoking, and placental insufficiency, and it could have fundamentally different programming mechanisms and effects.

Previously, we have used a well-characterized experimental rat model of hypoxia during pregnancy that mimics complications commonly existing in human pregnancy. We have shown that maternal hypoxia during pregnancy leads to several structural and functional changes in thoracic aortas and periventricular white matter in adults (11, 23), which were amplified by an HF diet. Several similar experiments also demonstrated that adult offspring born with prenatal hypoxia have impaired vascular function (24), increased myogenic tone (25), and also increased cardiac susceptibility to ischemia/reperfusion injury (26). It has been reported in a number of studies, in humans as well as experimental animals, that maternal hypoxia results in a significant increase in the ductus venosus (DV) shunting rate (27–29), which may reduce the umbilical blood supply to the fetal liver. This, in turn, may influence the programming of hepatic carbohydrate and lipid metabolism, which would persist at adulthood (30–32). However, the specific effects of fetal hypoxia on the HF-induced fatty liver in mammalian offspring, and how fetal hypoxia affects the fetus and potentially programs hepatic lipid metabolism during adulthood remain unclear.

Taken together, these studies confirmed that impaired fetal oxygen supply constitutes a stressor that affects offspring with higher severity, particularly when nourished with an HF diet. To study the potential long-term effects of prenatal fetal hypoxia, we used animal models with 2 mechanisms of insult, intrauterine hypoxia, and then postnatal HF diet, a 2 × 2 full factorial design, to determine the impact of prenatal hypoxia on the plasma lipid concentrations, insulin and morphological injury of the liver, and further to investigate their susceptibility to dyslipidemia and insulin resistance in adult male offspring exposed to an HF diet. Moreover, in a mechanistic perspective, we explored alterations of insulin signaling and the functional relevance of insulin and adipogenic-signaling pathways that may be implicated in the development and progression of NAFLD.

Materials and Methods

Animals

Virgin female Sprague Dawley (SD) rats (Shanghai Experimental Animal Center, Shanghai, China) were mated at 3 months of age (rate 2:1), and vaginal smears obtained the following morning was examined for the presence of sperm, which signified day 0 of pregnancy (term = 21 days). On day 7, rats were randomly assigned to the control or maternal hypoxia group. During pregnancy and lactation, all rats were fed standard laboratory rat chow ad libitum. All procedures in this study were approved by the Standing Committee on Ethics and Animal Experimentation at the Fujian Medical University (China).

Maternal hypoxia

To reduce maternal oxygen supply, as we have previously described (11, 23), 8 pregnant female rats were housed inside a Plexiglas chamber from day 7 to day 21 of pregnancy, which was maintained at 10 ± 1% oxygen by continuous infusion of a mixture of nitrogen and compressed air. The dyed carbon dioxide was eliminated by circulating the atmosphere through soda lime, and the water contained in the dyed gas was trapped in a chilled glass tank. The oxygen concentration of the chamber was monitored throughout treatment using a portable oxygen analyzer, which was calibrated daily (5-450; IST-AIM Co). The chamber could house a maximum of 3 pregnant rats, maintained individually in separate standard rat cages at any time. After 3 hours of hypoxia, pregnant rats were removed from the chamber and housed in room air. Eight control pregnant rats were submitted to the same procedures but with continuous infusion of compressed air.

Offspring and experimental groups

All pups were weighed within 3–12 hours after birth, and the litter size was randomly reduced to 8 pups to ensure equal nutrient access for all the offspring. According to a 2 × 2 full factorial design, consisting of 2 factors (maternal hypoxia [MH], postnatal HF diet [HF]), each with 2 levels, offspring from 8 control pregnant and 8 hypoxia pregnant litters were randomly assigned into 4 groups (each group comprised 8 male animals) as follows: C/C group, offspring with no MH and no postnatal HF diet; MH/C group, offspring with MH and no postnatal HF diet; C/HF group, offspring with no MH and postnatal HF diet; MH/HF group, offspring with MH and postnatal HF diet.

After the age of 8 weeks, male offspring rats were assigned to HF diet (10% lard, 2% cholesterol, 0.5% sodium
A tolerance test was initiated at 8:00 AM by the ip injection of a 50% ethanol solution at room temperature. After washing in PBS, sections were incubated in 3% H2O2 for 10 minutes to quench endogenous peroxidase activity. Sections were then incubated in 3% H2O2 for 10 minutes to quench endogenous peroxidase activity. Sections were then incubated with the appropriate primary antibody overnight at 4°C and washed 3 times for 10 minutes each with Tris-buffered saline-Tween 20 at room temperature. The polyvinylidene fluoride membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Protein expression was detected with an enhanced potassium ferrocyanide solution for 1.5 hours, dehydrated progressively by ethanol, and embedded by Epon 618. The epoxy blocks were sliced on a LKB-V ultratome at 70–80 mm thickness, dyed with uranyl acetate and lead citrate, and observed through a Philips EM208 transmission electron microscope (EM) (Philips).

**TNF-α and F4/80 immunohistochemistry staining**

The paraffin-embedded sections (5 μm thick) were deparaffinized and rehydrated by passage through a graded series of ethanol and distilled water. Then, endogenous peroxidase activity was quenched by incubation in 3% H2O2 for 10 minutes at room temperature. After washing in PBS, sections were incubated overnight at 4°C with a goat polyclonal antibody against TNF (1:50, Santa Cruz Biotechnology, Inc) and F4/80 (Serotec). Biotinylated rabbit antidiot IgG (Zhongshan Biotechnology Co Ltd) was used as a secondary antibody. Diaminobenzidine was used as a color substrate. The sections were counterstained with hematoxylin, dehydrated, and mounted. Negative controls included the substitution of the primary antibodies with PBS. TNF-α-positive expression corresponded to cytoplasmic brown-yellow whereas negative cells were blue. For the quantitative analysis, the TNF-α-positive cells were counted under ×100 magnification in 10 randomly chosen fields. The results were expressed as the mean percentage of TNF-α-positive cells. F4/80-positive cells (brown) were counted on 4 random high-power (×200) field/slide using BZ-2 software (KEYENCE).

**Statistical analysis**

All data are expressed as mean ± SD. Student’s unpaired two-tailed t test was used to compare differences between MH...
and control groups. A general linear model of univariate process was performed to analyze the main effects and the interaction effect of MH and postnatal HF diet on the morphologic injury of NAFLD in a completely randomized factorial design experiment. Values of \( P < .05 \) were considered to be statistically significant. Statistical analysis was performed using SPSS statistical software (SPSS for Windows 11.5).

**Results**

**Baseline characteristics and serum biochemistry**

Consistent with our previous findings (11, 23), MH with an oxygen concentration of 10 ± 1% and a hypoxic duration of 3 hours/d resulted in reducing blood PaO\(_2\) and SaO\(_2\), but not PaCO\(_2\) and pH values in gestational rats compared with the control (\( P < .05 \)). Using this model, MH reduced neonatal size and perturbation of neonatal organ weight and proportion (asymmetric IUGR). However, MH insult had no effect on food intake daily, weight gain every other day, or maternal pregnancy length, which indicated that it did not result in maternal undernutrition. No mother died throughout the experiment period. MH caused a significant decrease in birth weight. However, at weaning and the start of HF diet (≈ 28 days of age), MH pups had comparable weights to control pups. After 12 weeks of HF diet, offspring gained more weight than those on the control diet (Figure 1a). Exposure to MH had no effect on body weight gain (Figure 1a).

As expected, HF diet increased plasma TG, FFA and low-density lipoprotein-C, and decreased plasma high density lipoprotein-C concentrations in both control and MH rats (\( ^{\circ}, P < .05 \)) (Table 1). Importantly, MH resulted in a further significant increase of these parameters as compared with the control group (\( ^{\circ\circ}, P < .05 \)). Moreover, there was a synergistic effect between MH and HF as shown by the results obtained in the MH/HF group (\( ^{\circ}, P < .05 \)). Although HF diet did not result in fasting hyperglycemia for any group, offspring receiving an HF diet had higher fasted plasma insulin concentrations and HOMA-IR than rats fed a control diet (Table 1). It is interesting to note that the plasma insulin concentrations and HOMA-IR were significantly higher in rat offspring of the MH/HF group than in the C/HF group (\( P < .05 \)), indicating a synergistic effect between MH and HF (\( ^{\circ}, P < .05 \)). The glucose tolerance test showed that MH and HF diet reduced glucose tolerance in adult offspring; moreover, consistent with the results of plasma insulin concentrations and HOMA-IR, glucose disposal impairment was significantly more pronounced in the MH/HF group rats than in the C/C group (Figure 1b). MH and HF diet reduced total area under the curve (AUC) between 0 and 120 minutes in adult offspring (\( ^{\circ}, P < .05 \)); moreover, with an additive effect between MH and HF diets (C/C = 755.2 ± 135 mM; MH/C = 744.8 ± 231 mM; C/HF = 1019.3 ± 253 mM; MH/HF = 1402.8 ± 185 mM; †, \( P < .05 \); Figure 1b).

**General observations of liver specimens**

No liver abnormalities were found in groups C/C and MH/C. In contrast, the liver in group C/HF was enlarged and appeared yellowish and greasy. Moreover, the livers of rats in group MH/HF appeared khaki and smooth in surface without any nodules, and the edge of the livers seems obtuse.

**Hepatic histologic findings**

Sections of liver showed no significant fibrosis in either treated animals or controls. No lipid accumulation and normal hepatic architecture were observed in livers from the C/C group (Figure 2a). In livers from MH/C offspring, few lipid droplets were observed (Figure 2b). C/HF offspring livers showed microvesicular steatosis, with steatosis grade 2, and hepatocellular swelling at grade 1—2, with occasional inflammatory cell infiltration but no perisinusoidal fibrosis (Figure 2c). However, in MH/HF livers, extensive fat accumulation was observed (Figure 2, d–f). The steatosis was grade 3, with microvesicular and macrovesicular steatosis, hepatocellular swelling at grade 2, and inflammatory cell infiltration (focal necrosis) at grade 1 (Figure 2e, arrow), without perisinusoidal fibrosis observed.

Whereas both the C/C and MH/C group offspring has a null NAFLD score, the C/HF offspring achieved a score...
of TG, FFA, LDL-C. The main effect of MH and HF diet resulted in an increase of plasma TG, FFA, LDL-C, insulin, and HOMA-IR. There was an additive effect between the MH/C and the C/HF group for plasma TG, FFA, LDL-C, insulin, and HOMA-IR.

Histologic Section of Offspring Rat Livers

A, The group C/C offspring liver generated a necro-inflammatory score of 5–6, which can be diagnosed as nonalcoholic steatohepatitis (NASH) (Table 2).

Hepatic ultrastructural findings

No abnormal liver tissue ultrastructure was found in group C/C (Figure 3a). The livers from MH/C offspring were almost normal, with only occasional small cytoplasm lipid droplets (Figure 3b). In group C/HF, the amount of liver cell cytoplasm lipid droplets was clearly increased (Figure 3c), part of them being fused, with mitochondrial swelling and decreased cytoplasmic organelles; liver sinusoid and the Disse space were enlarged, and endothelial cells degeneration and edema were noted. In group MH/HF, liver steatosis was more severe than that in group C/HF. A large amount of lipid droplets were seen, with irregular big lipid droplets squeezing nuclei of irregular shapes, and small and few cytoplasmic organelles (Figure 3d). Mitochondrial swelling with broken cristae was present. There were also increased myelin figures, serious endothelial cell injury, cytoplasmic aggregation, liver sinusoid, and Disse space enlargement. Globally, liver damage was significantly more severe than in the C/HF group.

TNF-α and F4/80 immunohistochemistry staining

At the monitoring time, the TNF-α-positive expression in the liver tissue of group C/C, MH/C, C/HF, and MH/HF was 18.41 ± 5.21, 21.42 ± 6.65, 61.37 ± 10.23, and 89.32 ± 12.08, respectively (Figure 4, a–e). There was no significant effect of MH on the expression of TNF-α in offspring liver. The main effect of HF diet was an increased expression of the protein TNF-α. Moreover, MH and postnatal HF diet exerted synergistic effects on the expression of TNF-α (Figure 4e). These data suggest that offspring from dams exposed to MH may be predisposed to early onset of inflammation and fibrosis. Furthermore, tissue inflammation was investigated by the assessment of macrophages in the liver using F4/80 immunohistochemistry staining (Figure 4, f–i). Whereas administration of the HF diet augmented the number of macrophage clusters preferentially distributed to cell pericentral region, MH

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Table 1. Serum Biochemistry of Control and MH Offspring Rats Fed Normal and HF Diets (n = 6)

<table>
<thead>
<tr>
<th>Group</th>
<th>C/C</th>
<th>MH/C</th>
<th>C/HF</th>
<th>MH/HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>3.80 ± 0.32</td>
<td>3.62 ± 0.25</td>
<td>3.90 ± 0.40</td>
<td>6.50 ± 0.38</td>
</tr>
<tr>
<td>Insulin (mIU/L)</td>
<td>38.32 ± 6.51</td>
<td>42.7 ± 4.82</td>
<td>56.7 ± 5.96</td>
<td>84.50 ± 12.6</td>
</tr>
<tr>
<td>HOMA-IR index</td>
<td>1.86 ± 0.15</td>
<td>1.92 ± 0.16</td>
<td>2.31 ± 0.12</td>
<td>3.21 ± 0.21</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>1.92 ± 0.26</td>
<td>1.95 ± 0.20</td>
<td>2.21 ± 0.16</td>
<td>2.88 ± 0.23</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>0.90 ± 0.41</td>
<td>0.32 ± 0.24</td>
<td>0.49 ± 0.19</td>
<td>0.50 ± 0.29</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>3.32 ± 7.50</td>
<td>386.40 ± 96.80</td>
<td>582.30 ± 217.70</td>
<td>1036.70 ± 127.70</td>
</tr>
<tr>
<td>FFA (µEq/L)</td>
<td>0.58 ± 0.05</td>
<td>0.59 ± 0.01</td>
<td>0.45 ± 0.54</td>
<td>0.41 ± 0.08</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>0.29 ± 0.11</td>
<td>0.34 ± 0.23</td>
<td>2.47 ± 1.40</td>
<td>5.97 ± 1.57</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>0.25 ± 0.11</td>
<td>0.34 ± 0.23</td>
<td>2.47 ± 1.40</td>
<td>5.97 ± 1.57</td>
</tr>
</tbody>
</table>

Data are mean ± SD, a and b P < .05, compared with the C/C group. The main effect of HF diet resulted in a decrease of HDL-C and an increase of TG, FFA, LDL-C. The main effect of MH and HF diet resulted in an increase of plasma TG, FFA, LDL-C, insulin, and HOMA-IR. c P < .05; there was an additive effect between the MH/C and the C/HF group for plasma TG, FFA, LDL-C, insulin, and HOMA-IR.

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Table 2. Assessment of NAFLD Severity in Offspring’s Liver Using the Kleiner Scoring System

<table>
<thead>
<tr>
<th>Group</th>
<th>C/C</th>
<th>MH/C</th>
<th>C/HF</th>
<th>MH/HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Ballooning</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1–2</td>
</tr>
<tr>
<td>Lobular inflammation</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Activity score</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>5–6</td>
</tr>
<tr>
<td>Indication</td>
<td>Normal</td>
<td>Normal</td>
<td>NAFLD</td>
<td>NASH</td>
</tr>
</tbody>
</table>

Kleiner scoring system described by Kleiner et al. (33). For each group (n = 6), the mean score for each item is given.
enhanced this infiltration. In fact, MH and postnatal HF diet exerted synergistic effects on the number of F4/80-positive cells, the number of positive cells in MH/HF rats being significantly higher than in C/C and C/HF rats ($P < .05$, Figure 4j).

**Assessments of hepatic levels of TNF-α mRNA and protein**

In accordance with immunohistochemistry data, HF diet increased TNF-α mRNA and protein expression ($\ast$, $P < .05$), with a synergistic effect of MH and HF diet (Figure 5, a, b, and d; †, $P < .05$).

**Effects of MH and HF diet on hepatic insulin signaling and relevant functional protein expressions**

In agreement with the data obtained from fasted plasma insulin concentrations, HOMA-IR, and glucose tolerance test experiments, the main effect of MH or HF diet was a significantly reduced expression of insulin receptor substrate (IRS)-2, phosphatidylinositol 3-kinase p110 subunit, and protein kinase B (PKB) (all $P < .05$). Moreover, there was a synergistic effect between MH and postnatal HF diet ($P < .05$) as shown by a significant decrease in the expression of IRS-2, p110, and PKB in the offspring of the MH/HF group as compared with the C/HF group ($P < .05$) (Figure 5, a and b). Furthermore, after the

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**Figure 3.** Ultrastructure of Rat Livers a, Group C/C (the control group) rat normal liver cell ultrastucture (electron microscopy [EM], $\times 4000$). b, In group MH/C rat liver, lipid droplets were occasionally seen (EM, $\times 4000$). c, Group C/HF, some lipid droplets in the liver cell cytoplasm and part of lipid droplets fused into clusters (EM, $\times 4000$). D, Group MH/HF; a large amount of lipid droplets in liver cell cytoplasm, lipid droplets fused into big bubble and cluster, and small and few cytoplasmic organelles were observed.

**Figure 4.** TNF-α and F4/80 Expression in Offspring Rat Livers TNF-α immunohistochemical staining: a, Group C/C (the control group), (b) group MH/C, (c) group C/HF, and (d) group MH/HF. e, TNF-α-positive expression rates in the liver tissue of the 4 groups. F4/80 immunohistochemical staining (panels f–j). Panel j represents the F4/80-positive expression rates. The main effect of HF diet resulted in an increases expression of TNF-α and F4/80 ($\ast$, $P < .05$). Moreover, there was an additive effect between the MH group and HF for the expression of TNF-α and F4/80 (†, $P < .05$).
administration of insulin, although phosphorylated IRS-2 was significantly increased in both control and MH group, however, it was blunted in MH group offspring. The same held true for the effect of insulin on phosphorylation at Ser473 of AKT. When studying the insulin suppression effect on phosphoenolpyruvate carboxykinase protein levels, this effect was significantly less pronounced in MH liver than in control offspring (18.9 / H11006 2.2% vs 42.3 / H11006 4.2% [P < .05]). Similarly, insulin suppression of glucose-6-phosphatase protein levels was blunted in MH vs control rats (36.8 / H11006 4.2 vs 65.2 / H11006 5.4%; P < .05) (Figure 5, c and e).

Effects of MH and HF diet on hepatic adipogenic signaling protein expressions

Corresponding to the increased plasma lipid concentrations in the HF-fed groups, protein expression of adipogenic indices (sterol regulatory element-binding protein [SREBP]-1 and fatty acid synthase [FAS]) was also significantly elevated in offspring males of C/HF and MH/HF group compared with those of C/C and MH/C group. Moreover, the increase in SREBP-1 and FAS expression was more pronounced in male offspring of the MH/HF group compared with the C/HF group. There was an additive effect between MH and postnatal HF diet. G6Pase, Glucose 6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase.

Discussion

To the best of our knowledge, this is the first study showing that fetal exposure to MH resulting in IUGR can promote the progression of postnatal HF diet-induced NAFLD in adult male offspring. The results showed herein that MH: 1) led to a significant increase in plasma lipid concentrations in response to HF diet in MH/HF group
compared with the C/HF group; 2) contributed to an increased susceptibility to HF diet-induced insulin resistance and impaired glucose tolerance; 3) exacerbated the effect of a HF diet in later life, leading to a more severe form of NAFLD, even progressing to NASH; and 4) increased susceptibility to HF diet-induced NAFLD by down-regulating liver insulin-signaling pathways and relevant functional proteins, and by up-regulating adipogenic signaling pathways. It should be noted that the present study was confined to males, because females would have required assessment of estrus, given the known estrogen impact on lipid metabolism (34).

In agreement with previous studies (22, 35), we showed that MH rat offspring with a hypercaloric HF diet developed much higher circulating concentrations of TG, FFA, and low-density lipoprotein-C, but not total cholesterol and high density lipoprotein-C. We also confirmed the synergistic effects of both MH and postnatal HF diet for an early onset of dyslipidemia and NAFLD (36–38). Because hepatic lipids have such a dramatic rate of increase during postnatal stages, it is possible that the catch-up growth of IUGR pups after their birth, as reported in our previous study (11), may contribute to greater accumulation of liver lipids and favor a later-stage NAFLD in MH-induced IUGR rats. Interestingly, this phenomenon concurs with a recent study showing that hypoxia-induced IUGR leads to a notable increase in the proportion of fat located in the intraabdominal cavity (22).

It has been proposed that a “two-hit” theory recapitulates the pathogenesis and progression of NAFLD (39). The insulin resistance associated with hyperlipidemia causes simple hepatic steatosis, which is the “first hit.” Insulin resistance has been well associated with IUGR (20, 21). We, therefore investigated the mechanism by which MH-induced IUGR may manifest this first hit and increase susceptibility to NAFLD and progression in adult offspring. To our knowledge, this is the first study showing that MH down-regulates the key molecules of insulin-signal cascades, including IRS-2, phosphatidylinositol 3-kinase p110 subunit, and PKB in offspring liver. On the other hand, the changes of AKT phosphorylation, IRS, and increased susceptibility to HF diet-induced NAFLD, that resulted from the HF diet. Our results are consistent with a study showing that hypoxia-induced IUGR could increase phosphorylation of protein kinase C with inhibition of insulin receptor substrate 1 and PKB in liver and skeletal muscle (22). Moreover, our observations indicate that alterations of protein expression, which persist at adulthood, may also contribute to insulin-signaling defect, and thus to the onset and progression of NAFLD, when HF diet exposure occurs in adulthood.

Hepatic lipogenic transcription factor (especially SREBPs) and lipid enzymes play a key role in modulating lipid metabolism and plasma lipid concentrations. SREBP-1 specifically regulates hepatic lipogenesis (41, 42). Its activation induces the genes for enzymes that are involved in the biosynthesis of fatty acids and triglycerides (43). Lipogenic FAS, which is the downstream target of SREBP-1, is the key enzyme of de novo fatty acid synthesis. Its expression is stimulated by insulin and glucose (44). Therefore, we hypothesized that programmed up-regulation of adipogenic signaling pathways may contribute to NAFLD development in adult offspring. In support of this hypothesis, we first showed the relative up-regulation of hepatic SREBP-1 and FAS in MH male offspring, by favoring the increased lipogenic pathways, increased plasma fat levels, and hepatic lipid synthesis and deposition. Moreover, the programmed enhanced expression of lipid profiles persisted at adulthood, resulting in severe steatosis with a NASH-like phenotype (45).

Several studies in humans and animals have demonstrated the effects of adverse conditions in utero on lipid metabolism (38, 46). Barker et al., (47) suggested that impaired body weight growth, especially at the liver level, might lead to permanent alterations in cholesterol metab-
olism during adulthood. Similarly, IUGR has been associated with up-regulation of SREBP-1 and lipid enzymes, and thus with enhanced hepatic lipogenesis and plasma lipid concentration (35, 48), which are closely related to fatty liver (42, 43).

Progression from steatosis to steatohepatitis is thought to depend on the existence of the second hit (49) (hepatocyte inflammation and liver fibrosis), which is closely related to the increase of reactive oxygen species (ROS) especially at the mitochondria level. In our study, MH increased hepatic TNF-α level in the HF diet-induced-NAFLD rat. Additionally, TNF-α mRNA and protein expression in the liver was enhanced by MH in the HF diet-fed rat. It is well known that the HF diet leads to the production of TNF-α and induces hepatic inflammation with high expression of hepatic TNF-α mRNA (50). Therefore, MH may exacerbate hepatic inflammation by enhancing the HF diet-induced TNF-α mRNA and protein expression. TNF-α plays a critical role in the development of NASH by inducing hepatic injury and fibrosis. Crespo et al. (51) reported that increased TNF-α expression and its type 1 receptor in NASH patients were significantly correlated with the degree of fibrosis. Elevated TNF-α could increase the production of ROS in liver mitochondria and trigger the release of inflammatory cytokines, thus aggravating liver inflammation (52).

Interestingly, it has been shown that Kupffer cells are associated with NASH development. Recent studies showed enhanced infiltration of F4/80+ macrophages in HF diet-induced obese mice (53). Kupffer cells produce inflammatory cytokines such as TNF-α. Kawanishi et al. (54) suggested that increasing macrophage infiltration within the liver enhances activation of hepatic stellate cells and fibrosis. Therefore, the induction of TNF-α production by hepatic resident macrophages may play a vital role in the pathogenesis of hepatic inflammation and fibrosis (NASH). Indeed, our study showed that the distribution pattern of F4/80-positive cells in the liver was similar to that of TNF-α. Therefore, the ability of MH to increase TNF-α levels and hepatic inflammation might be related to enhanced macrophage infiltration. Therefore, MH would constitute the second hit, leading the MH offspring to early onset of inflammation and subsequent fibrosis development. It should be noted, however, that the two-hit concept remains debated even in humans and that our two hits, hypoxia in utero and HF feeding after birth, are not comparable to the human disease.

It should be mentioned that, in contrast with our data, Rueda-Clausen et al. (22) found no differences in circulating concentrations of inflammatory markers, including TNF-α, IL-1β, and IL-6 between the hypoxia-induced IUGR and the control groups. Fetal DV shunting, which may have been induced by MH in our experiment, would ensure an adequate supply of oxygen and glucose to vitally important organs such as the brain and heart in fetuses in order to survive and adapt to distress conditions (27–29). Meanwhile, fetal DV shunting may influence cell proliferation in fetal organs. Consequently, we speculated that fetal hepatic perfusion may be a potential mechanism of hepatic insulin-signaling defect and lipid dysmetabolism in adult offspring (31, 32). The precise mechanisms whereby hepatic perfusion potentially programs hepatic lipid metabolism and leads to susceptibility to HF diet in adult offspring warrants further investigation.

At this point, we cannot yet definitely define the intimate mechanism that leads to this programmed priming of both insulin resistance and increased de novo fatty acid synthesis. However, our data highlight that hypoxia during the developmental period may serve as one of the regulators of this process, subsequently leading to persistent modulations in gene expression and increasing the susceptibility of male rats to HF diet-induced NAFLD during adulthood. One limitation in our MH rat model is that the hypoxic insult affected both mothers and fetuses in utero. Therefore, the potential effects of maternal hypoxic stress exerted on the fetuses should be considered. Moreover, the effect of oxygen deprivation and reperfusion injury to fetuses should also be kept in mind. Despite these limitations, the fetal hypoxic animal model remains a widely used noninvasive technique (11, 22, 23, 25). Another limitation of this study is that plasma FFA levels may not be the consequence of liver injury. Specifically, the markedly high concentration of FFA in plasma would suggest either a high rate of lipolysis, most likely in the adipose tissue, or an attenuated triglyceride synthesis. Future studies are needed to solve this issue.

In summary, our study highlighted the impact of a first insult caused by MH during pregnancy, modifying plasma concentrations of lipids and insulin, and a second insult of postnatal HF diet in adult offspring, leading to hepatic morphologic changes. It also provides evidence that maternal exposure to hypoxia acts through changes of insulin-signaling and adipogenic-signaling pathways. Therefore, our overall results suggest that the impact of prenatal hypoxic insults causing IUGR could persist postnatally and emphasize that individuals who underwent hypoxia during pregnancy may require closer clinical monitoring and could obtain major benefits from nutritional interventions designed to reduce the development of NAFLD.

Acknowledgments

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