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Dietary protein restriction induces steatohepatitis and alters leptin/signal transducers and activators of transcription 3 signaling in lactating rats☆

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Abstract

Dietary protein restriction during lactation affects lipid metabolism and food intake in rats. The goals of this study were to determine the effect of a low-protein diet on a liver damage in lactating rats, to determine whether dietary protein restriction of lactating dams affects the liver health of their offspring and to elucidate the molecular mechanisms underlying the development of hepatic damage. Lactating Sprague-Dawley rats were fed either a control 20% protein diet or an 8% low-protein diet for 11 or 23 days, respectively. After weaning, the offspring were continuously fed either the same control diet or the low-protein diet for an additional 22 days. Feeding a low-protein diet during lactation caused steatohepatitis with severe steatosis, lobular inflammation, ballooning degeneration and fibrosis. Offspring nourished by dams fed a low-protein diet showed simple hepatic steatosis. Combined effects of increased lipogenesis, decreased fatty acid oxidation and impaired very-low-density lipoprotein secretion were responsible for the development of hepatic steatosis. Hepatic up-regulation of genes linked to oxidative stress including nicotinamide adenine dinucleotide phosphate oxidase, inflammation and fibrogenesis supports the development of steatohepatitis in protein-restricted lactating rats. Furthermore, protein-restricted lactating rats showed activation of the leptin/signal transducers and activators of the transcription 3 signaling pathway. Taken together, oxidative stress induced by up-regulation of nicotinamide adenine dinucleotide phosphate oxidase with activation of leptin/signal transducers and activators of the transcription 3 signaling was responsible for development of steatohepatitis in protein-restricted lactating rats. Our findings suggest that protein malnutrition has a potential to induce steatohepatitis/hepatic steatosis in lactating mothers and infants during breast-feeding.
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Keywords: Protein restriction; Steatohepatitis; Fatty liver; Oxidative stress; Leptin

1. Introduction

In young growing rats, dietary protein restriction (PR) alters lipid metabolism and causes a fatty liver [1,2], and it increases food intake [3] or shows no change in food intake [4]. In contrast, feeding a low-protein diet during lactation decreased food intake in rats [5]. We have assumed that decrease in food intake may cause more severe protein deficiency in lactating rats fed a low-protein diet. Furthermore, feeding a low-protein diet during lactation increased circulating leptin levels in rats [5]. A recent study demonstrates that leptin is involved in oxidative stress, inflammatory cell infiltration and fibrosis in the liver [6-8]. Considering the role of leptin on liver pathophysiology, elevated leptin by low-protein diet may cause more severe hepatic damage in lactating rats compared with that in growing rats.

Metabolic syndrome, which includes fatty liver, has become a major health concern worldwide [9]. Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of pathological conditions, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis [10]. A combination of environmental and genetic factors determines individual risk of NAFLD development and progression, with a clear role for nutrition as a modifiable environmental risk factor. The typical Western diet, high in saturated fats and fructose, plays a significant role in the development of NAFLD [11]. In addition to in individuals who overconsume fats and fructose, fatty liver is seen in those who are in a state of protein malnutrition. Kwashiorkor is a form of severe infant protein–calorie malnutrition, and it has long been recognized that fatty liver is one of most striking characteristics

Abbreviations: PR, protein restriction; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; AMPK, 5′-adenosine monophosphate-activated protein kinase; VLDL, very-low-density lipoprotein; HSCs, hepatic stellate cells; NADPH, nicotinamide adenine dinucleotide phosphate; TNFα, tumor necrosis factor α; STAT3, signal transducers and activators of transcription 3.

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of kwashiorkor [12]. However, no experimental evidence on whether protein malnutrition affects the liver health of lactating mothers or dams and their infants or offspring has been reported.

In this study, we have examined whether dietary PR during lactation causes more severe hepatic damage compared with that in growing rats and whether this effect of dietary PR on lactating dam could be transmitted to their offspring. We found that a low-protein diet during lactation induced steatohepatitis. We have attempted to elucidate molecular mechanisms underlying the development of fatty liver induced by a low protein.

2. Materials and methods

2.1. Animals and diets

All experimental procedures involving animals were approved by the Chonnam National University Institutional Animal Use and Care Committee. All procedures for animal management followed the standard operation protocols of Chonnam National University. Pregnant female Sprague-Dawley rats (68 days old; average weight, 300 g) were purchased from Orient Bio, Gyonggi-Do, Korea, and maintained in a temperature- and humidity-controlled room on a 12:12-h light/dark cycle and allowed access to food and water *ad libitum*. At lactation day 5, the animals were divided into two groups [a control 20% protein (20P) diet and an 8% low-protein diet], and the number of pups per dam was adjusted to 8 (four males, four females). Two diets were isocaloric and contained an adequate supply of choline and cysteine (Table 1). The formulation of the control diet was based on a modified AIN-93G diet (Dytes, Bethlehem, PA, USA). Dyetrose (dextrinized corn starch or depolymerized corn starch) was added instead of some of corn starch in order to make better pellet diets as previously recommended [13]. We performed feeding trials with lactating and female growing rats for two feeding durations as shown in Fig. 1. Briefly, lactating rats received either a control 20P diet or an 8% low-protein (8P) diet. They were killed on the 11th and 23rd days of feeding. Their offspring were also killed. After weaning, the offspring were continuously fed either the same control 20P diet or the low-protein 8P diet for an additional 22 days, and growing rats were killed at total feeding duration of 45 days. After weaning, portion of offspring from control dams were fed the low-protein 8P diet for 22 days, and they were killed at 50 days of age.

The 8% low-protein diet has contained 23% sucrose, while the control diet contains 10% sucrose. In a separate experiment, we compared degree of fatty liver between rats fed 10% sucrose diet and 23% sucrose diet in order to check whether different sucrose levels in low-protein diet s affect the development of NAFLD.

2.2. Blood analyses

Serum glucose was analyzed using glucose II (HK) reagents (Bayer, Seoul, Korea). Serum triglyceride (TG) was analyzed by enzymatic colorimetry using a TG reagent (Bayer). Serum-free fatty acid (FFA) was analyzed using a NEFA kit (Wako, Osaka, Japan). Serum insulin and leptin levels were determined using a radioimmunoassay kit (Linco's Research, St. Charles, MO, USA).

2.3. Milk parameters

Milk yield was estimated using a stepwise forward multiple regression equation [14]. The milk was collected on the 16th day of lactation by gentle hand stripping of the teats after 4-h separation of the litter and the administration of oxytocin (4 IU/kg body weight; Sigma, St. Louis, MO, USA). Milk protein was determined using Bradford reagent (Sigma). Milk fat was determined by extraction with a chloroform–methanol mixture [15]. Milk lactose was determined by an enzyme-coupled reaction (ELAC-100; Bioassay Systems).

Table 1
Composition of diet (g/kg)

Ingredients	Control protein diet (20%)	Low-protein diet (8%)
Casein, 87% protein	230	92
L-Cystine	3	3
Sucrose	100	230
Corn starch	355.751	362.901
Dyetrose, dextrinized corn starch	121.9	121.6
Soybean oil	70	70
tert-Butylhydroquinone	0.014	0.014
Cellulose	50	50
Mineral mix no. 213272	35	35
Vitamin mix no. 310025	10	10
Calcium carbonate	12.495	12.495
Potassium phsophate·H_2O	6.86	10.49
Potassium citrate·H_2O	2.48	0
Choline bitartrate	2.5	2.5
Total	1,000	1,000
kcal/kg	3757	3807

2.4. Liver histology and Kleiner scores

Liver specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E), oil red O or Masson's trichrome. The severity of NAFLD was assessed by four parameters [16]: steatosis, ballooning of hepatocyte, fibrosis and lobular inflammation. An activity score was generated by adding the three parameters (steatosis, ballooning, inflammation). An activity score ≥5 correlated with NASH, and scores ≤4 were deemed to indicate NAFLD.

2.5. Hepatic lipid and protein carbonyl contents

Hepatic lipid was analyzed by extraction with chloroform–methanol [15]. Liver TG was measured by enzymatic colorimetric methods after the total lipids were dissolved in isopropanol. Liver FFA was measured using a NEFA kit (Wako). Hepatic proteins containing carbonyl groups were determined using a protein carbonyl assay kit (Cayman Chemical, Ann Arbor, MI, USA).

2.6. Real-time polymerase chain reaction (PCR)

Total RNA isolated using TRIzol Reagent (Invitrogen, Grand Island, NY, USA) was reverse transcribed into complement DNA using AccuPower RT Premix (Bioneer, Daejeon, Korea). Real-time PCR was performed using QuantiTect SYBR Green RT-PCR Master Mix (QIAGEN, Valencia, CA, USA). Primer information is shown in Supporting Table 1. The $\Delta\Delta C_t$ method was used to determine the relative fold changes, and messenger RNA (mRNA) levels were normalized with β-actin.

2.7. Western blotting

Tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor. Samples were centrifuged, and protein content in the supernatant was determined using the Bradford method. Protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by immunoblotting, using antibodies purchased from Cell Signaling (Danvers, MA, USA). Blots were developed with secondary antirabbit or antimouse antibodies conjugated to horseradish peroxidase (Invitrogen) and the luminal chemiluminescence reagent (ECL; Amersham Biosciences, Uppsala, Sweden). The processed blots were then exposed to X-ray film.

2.8. Statistical analyses

Data from the two experimental groups were compared using Student's *t* test. Differences between various physiological stages were compared using multiple analysis of variance followed by generalized linear model analysis.

3. Results

3.1. Growth and serum parameters

We fed 20P diet and 8% protein diet for control and protein-restricted rats, respectively. In protein-restricted lactating rat, food intake was decreased by 86% of control rats (Table 2). We calculated actual intake of protein, carbohydrate and energy based on food intake and protein, carbohydrate and energy content in the diet (Table 2). Actual consumption of protein, carbohydrate and energy in protein-restricted lactating rats was 34.4%, 106% and 87% of control rats, respectively. In growing rats fed low-protein diet, actual consumption of protein, carbohydrate and energy was 41%, 133% and 109% of control rats, respectively.

During lactation, control rats maintained body weight, but protein-restricted rats lost weight. Protein-restricted lactating rats displayed a reduced ($P<.05$) milk yield and milk protein content, with an increased ($P=.02$) milk fat content. Growth was retarded in offspring nourished by dams fed the low-protein diet.

Protein-restricted lactating rats showed higher ($P<.05$) total liver and total adipose tissue weights (sum of abdominal, perirenal and ovarian fat) after 23 days of feeding. These changes were not observed in the offspring or growing rats. PR increased serum TG levels in lactating rats, but this change was not observed in growing rats.

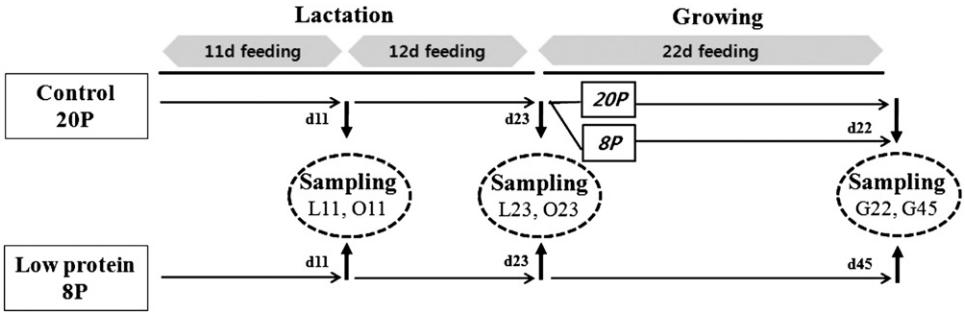

Fig. 1. Scheme of experimental design. We performed feeding trials with lactating and female growing rats for two feeding durations. Lactating rats received either a control 20P diet or an 8P diet. They were killed on the 11th day (L11, lactating days 5 through days 16) and 23rd day of feeding (L23, lactating days 5 through days 28). Their offspring (O11 or O23) were also killed. After weaning, the offspring were continuously fed either the same control 20P diet or the low-protein 8P diet for an additional 22 days, and growing rats (G45) were killed at total feeding duration of 45 days. After weaning, portion of offspring from control dams were fed the low-protein 8P diet for 22 days, and they (G22) were killed at 50 days of age.

3.2. Dietary PR develops steatohepatitis in lactating rats

We found that dietary PR in lactating rats induced severe hepatic steatosis after 11 days of feeding with H&E and oil red O staining (Fig. 2A). In addition to massive steatosis, mild infiltration of mononuclear cells was detected in hepatic lobules, indicating that PR induced lobular inflammation in the livers of lactating rats. We observed ballooning changes of hepatocytes in livers of protein-restricted lactating rats and detected mild hepatic fibrosis in perisinusoidal or periportal areas by Masson's trichrome staining. Human NASH is characterized by steatosis, inflammation and ballooning degeneration. Thus, our results demonstrate that PR during lactation developed NASH. The severity of NAFLD or NASH was assessed using the Kleiner scoring system [16]. The severity score of NASH at 23 days of feeding was similar to that at 11 days in lactating rats (Table 3). In our study, the 8% low-protein diet contained 23% of sucrose, while the control diet had 10% of sucrose. In a separate experiment, we have examined NASH in lactating rats fed an 8% low-protein diet with 10% sucrose. Both low-protein diets with 10% and 23% sucrose induced same degree of severe steatosis with mild lobular inflammation, ballooning degeneration and fibrosis (Table 3).

Offspring nourished by protein-restricted dams for both 11 and 23 days showed NAFLD, but the degree of steatosis (score 1) was lower than that of the lactating rats (score 3). In growing rats, PR for both 22 and 45 days induced NAFLD, but the severity of steatosis (score 2) was lower than that in lactating rats. In offspring and growing rats, there was no indication of inflammation, ballooning degeneration or fibrosis (Table 3).

Correspondingly, PR increased ($P<.05$) hepatic TG contents in lactating rats, their offspring and growing rats (Fig. 2B). PR increased ($P<.05$) hepatic FFA content in lactating rats, but not in either offspring or growing rats (Fig. 2C).

3.3. PR alters hepatic gene expression for lipid metabolism

We examined whether PR affected expression of genes for hepatic lipogenesis. In lactating rats, PR increased mRNA levels of X-box DNA binding protein, a recently identified lipogenic transcription factor

Table 2
Effect of dietary PR on growth, body, serum and milk parameters

Parameter	Lactating (23 days[a]; n=4–11)			Offspring (23 days[a]; n=5–7)			Growing (22 days[a]; n=4–8)		
	20%	8%	P	20%	8%	P	20%	8%	P
Growth and body parameters									
Food intake[b] (g/day)	58.7±1.4	50.5±2.5	.01	NA	NA	–	13.3±0.6	14.3±0.7	.41
Protein intake[b] (g/day)	11.7±0.3	4.0±0.2	<.001	NA	NA	–	2.7±0.1	1.1±0.0	.002
Carbohydrate intake[b] g/day)	34.5±0.8	36.5±1.8	.22	NA	NA	–	7.8±0.4	10.4±0.5	.04
Energy intake[b] (kcal/day)	219.2±5.3	192.1±9.6	.008	NA	NA	–	49.9±2.4	54.6±2.2	.34
Body weight (g) at									
Feeding day 1	277.5±5.7	277.5±7.8	1.0	14.0±0.3	14.8±0.3	.08	90.1±3.0	89.6±0.7	.85
Feeding day 9	279.9±5.9	263.6±6.8	.02	36.5±0.9	28.6±1.0	<.001	142.2±4.3	129.0±3.4	.04
Feeding day 19	272.5±7.0	259.0±8.0	.01	73.0±2.3	53.9±2.8	.002	199.8±6.6	175.0±6.2	.05
Total liver weight (g)	13.4±0.4	17.5±0.4	<.001	3.9±0.1	3.6±0.1	.13	10±0.4	10.4±1.0	.76
Total adipose weight (g)	3.8±0.5	5.0±0.4	.05	ND	ND	–	4.8±1.3	4.8±0.8	.96
Serum parameters									
TG (mg/dL)	55.9±5.4	82.8±7.1	.01	134.3±14.2	156.5±22.3	.39	131.3±20.8	135±20.2	.86
FFA (μEq/L)	368.6±30.3	441.2±49.3	.21	863.7±49.6	809.5±67.5	.51	510.5±100.3	439±84.8	.47
Milk parameters									
Milk yield (g/day) at									
Lactation day 5	16.9±0.7	17.2±0.9	.68	NA	NA	–	NA	NA	–
Lactation day 10	33.7±1.1	23.7±0.9	<.001	NA	NA	–	NA	NA	–
Lactation day 15	40.5±2.6	27.5±1.5	.004	NA	NA	–	NA	NA	–
Milk protein at day 16 (mg/mL)	64.9±1.0	55.2±2.3	.002	NA	NA	–	NA	NA	–
Milk fat at day 16 (mg/mL)	118.1±4.8	150.6±11.6	.002	NA	NA	–	NA	NA	–
Milk lactose at day 16 (mg/mL)	57.0±3.3	56.4±3.3	.89	NA	NA	–	NA	NA	–

Values are means±S.E.M. ND, not determined; NA, not available.
[a] Sampled at indicated feeding date if not specified.
[b] Average of entire experimental period; protein intake = food intake × protein content; carbohydrate intake = food intake × carbohydrate content [sucrose + corn starch + dyetrose + sucrose (9.7 g/kg) in vitamin mix]; energy intake = food intake × energy content.

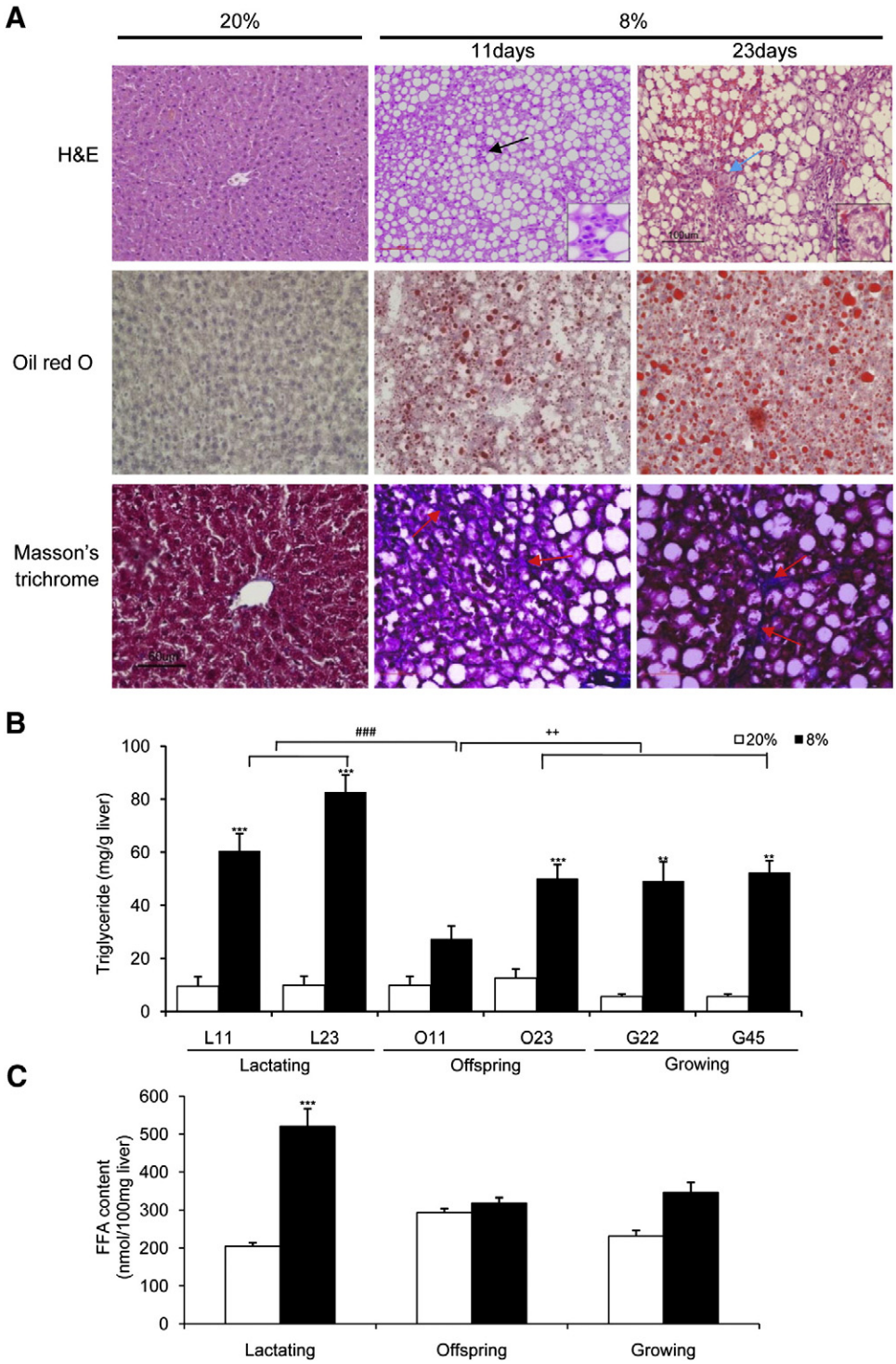

Fig. 2. Evidence of steatohepatitis and hepatic TG and FFA contents. (A) Liver sections were stained with H&E, oil red O and Masson's trichrome at 11 and 23 days of feeding in lactating rats fed with a 20P diet (20%) or an 8P diet (8%). The black arrow indicates infiltration of inflammatory cells in the hepatic parenchyma (also shown in the insert at 11 days). The blue arrow indicates ballooning degeneration in the hepatocytes (shown in the insert at 23 days). The red arrows indicate characteristic initial pattern of perisinusoidal fibrosis identified by Masson's trichrome staining. The magnification for H&E and oil red O was ×200, and that for Masson's trichrome was ×400. The scale bars represent 100 μm (×200) and 60 μm (×400), respectively. (B) The hepatic TG contents were measured in lactating (L11 and L23), offspring (O11 and O23) and growing rats (G22 and G45) as shown in Fig. 1. (C) Hepatic FFA was measured in the liver of lactating (23 days), offspring (23 days and growing rats (22 days). Values are mean±S.E.M (n=4–6); *P<.05, **P<.01, ***P<.001 versus control diet, $^{###}P$<.001 low-protein groups of lactating versus offspring rats fed for 11 days, ^{++}P<.01 low-protein groups of offspring fed for 11 days versus offspring fed for 23 days and growing rats.

(Fig. 3A) [17]. PR in lactating rats increased mRNA levels of diacylglycerol acyltransferase 1, an enzyme involved in the final and committed step of TG biosynthesis. Transcription of stearoyl-CoA desaturase 1, a central lipogenic enzyme catalyzing the synthesis of monounsaturated fatty acids, was also up-regulated in lactating rats fed the low-protein diet. In contrast, expression of X-box DNA binding protein, diacylglycerol acyltransferase 1 and stearoyl-CoA desaturase 1 genes was not altered by dietary PR in either offspring or growing rats. PR in lactating rats did not affect hepatic mRNA levels of lipogenic transcription factors, such as sterol regulatory element-binding

Table 3
Assessment of NAFLD severity in the liver of female rats fed a low-protein diet[a]

Stage, diet	Group[b]	Steatosis	Ballooning	Lobular inflammation	Activity score	Fibrosis	Indication
All, normal	Control	0	0	0	0	0	Normal
Lactating, low protein	L11[c]	3	1	1	5	1	NASH
	L23[c]	3	1	1	5	1	NASH
	L23[d]	3	1	1	5	1	NASH
Offspring, low protein	O11[c]	1	0	0	1	0	NAFLD
	O23[c]	1	0	0	1	0	NAFLD
Growing, low protein	G22[c]	2	0	0	2	0	NAFLD
	G45[c]	2	0	0	2	0	NAFLD

[a] NAFLD severity was assessed by the Kleiner scoring system as described in "Materials and methods" [16] (n=4–6).
[b] Feeding strategy of each group was shown in Fig. 1.
[c] Rats were fed a low-protein diet with 23% sucrose.
[d] Rats were fed a low-protein diet with 10% sucrose.

protein-1c, carbohydrate responsive element-binding protein, liver × receptor, and peroxisome proliferator-activated receptor γ genes, or mRNA levels of other lipogenic genes, such as fatty acid synthase, acetyl-CoA carboxylase-1, glycerol-3-phosphate acyltransferase and glyceraldehyde-3-phosphate dehydrogenase (Supporting Fig. 1A). PR also did not affect protein levels of ACC, phospho-ACC (inactive form), carbohydrate responsive element-binding protein or sterol regulatory element-binding protein-1c (Supporting Fig. 1B). Our results suggest that transcription of lipogenic genes was partially up-regulated.

Next, we analyzed hepatic gene expression for fatty acid oxidation (FAO). PR in lactating rats decreased phospho-5′-adenosine monophosphate-activated protein kinase (phospho-AMPK) levels (Fig. 3B). AMPK is considered a cellular energy sensor, and phospho-AMPK is an active form that can promote mitochondrial β oxidation of fatty acids [18]. AMPK positively regulates peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) activity in skeletal muscle [19]. PR in lactating rats reduced mRNA levels of PGC-1α, and it correspondingly decreased the expression of the FAO gene, medium-chain acyl CoA dehydrogenase (MCAD). In offspring, PR also down-regulated expression of PGC-1α and MCAD genes, but these changes were not found in growing rats. Our data demonstrate that PR down-regulated AMPK/PGC-1α signaling and caused a concomitant decrease in expression of the FAO gene, MCAD, in lactating rats and offspring.

We examined hepatic gene expression for very-low-density lipoprotein (VLDL) secretion. PR in lactating rats showed down-regulation (P<.05) of hepatic expression of both a membrane-associated apolipoprotein B and microsomal TG transfer protein (MTP) genes (Fig. 3C). In offspring and growing rats, transcription levels of apolipoprotein B and MTP genes also tended to decrease, but the differences were not statistically significant. MTP protein levels also decreased in lactating rats and their growing offspring (P=.1), demonstrating that impairment of VLDL secretion is involved in hepatic steatosis. Previous studies have also demonstrated that a low-protein diet impairs transport of TG s by VLDL in rat livers [20]. Recently, dysfunctional VLDL synthesis and release were suggested to be a key factor in NASH pathogenesis in human subjects [21].

3.4. PR induces oxidative stress with activation of hepatic stellate cells (HSCs)

We analyzed gene expression for oxidative stress and reactive oxygen species (ROS) generation, including cytochrome p450 2E1 (Cyp2E1), lipoxygenase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. We did not find any change in the expression of either lipoxygenase or Cyp2E1 genes in the protein-restricted group (Supporting Fig. 2). Interestingly, PR up-regulated hepatic transcript expression of NADPH oxidase subunits, including $p22^{phox}$, $gp91^{phox}$ and $p47^{phox}$, in lactating rats but not in either offspring or growing rats (Fig. 4A). The NADPH oxidase complex is an important source of the ROS generation responsible for oxidative stress in HSCs [22]. We detected elevated gene expression of α-smooth muscle actin, an activated HSC marker, in the livers of protein-restricted lactating rats. We also found increased proteins modified with 4-hydroxy-2-nonenal (4-HNE), which is a major aldehyde end-product of membrane lipid peroxidation due to oxidative stress, in the liver of protein-restricted lactating rats. Hepatic protein carbonyls, another marker of oxidative stress, also increased with a low-protein diet in lactating rats but not in females of either offspring or growing rats. Thus, our results demonstrate that PR induced oxidative stress by up-regulation of NADPH oxidase through activation of HSCs.

3.5. PR up-regulates hepatic gene expression for inflammation and fibrogenesis

In lactating rats, PR up-regulated expression of inflammatory genes, such as cyclooxygenase-2, hepcidin, tumor necrosis factor α (TNFα), TNF receptor (tumor necrosis factor receptor superfamily, member 12a) and lipocalin 2 genes (Figs. 4B and 5B). We examined expression of fibrogenic genes (Fig. 4C). PR in lactating rats up-regulated hepatic mRNA expression of transforming growth factor β (TGFβ), a master regulator of fibrogenesis, and its target genes, procollagen type 1 alpha 1 and procollagen type 4 alpha 1 genes. In addition, mRNA expression of plasminogen activator inhibitor-1, a key inducer of fibrogenesis, was up-regulated in protein-restricted lactating rats. We also detected up-regulation of mRNA expression of tissue inhibitor of metalloproteinase 1 (TIMP-1), a key regulator of hepatic fibrogenesis [23], in protein-restricted lactating rats (Fig. 5B). In contrast, expression of genes for inflammation and fibrogenesis was not changed by PR in offspring or growing rats. Thus, up-regulation of molecular markers supports the occurrence of hepatic inflammation and fibrosis in protein-restricted lactating rats.

3.6. PR alters leptin/signal transducers and activators of the transcription 3 (STAT3) signaling

Dietary PR increased (P<.01) serum leptin levels in lactating rats but not in either offspring or growing rats (Fig. 5A). Leptin signaling is mediated by activation of STAT3 phosphorylation. PR in lactating rats increased (P<.05) STAT3 tyrosine phosphorylation levels. This increase in phosphorylated STAT3 levels was not significant (P=.08) in both offspring and growing rats. Dietary PR in lactating rats up-regulated expression of both hepcidin and TIMP-1, which are target genes of STAT3 (Fig. 5B) [7,23]. These changes were not detected in both offspring and growing rats.

4. Discussion

Using a lactating rat model, we observed the novel findings that feeding a low-protein diet developed NASH with massive steatosis

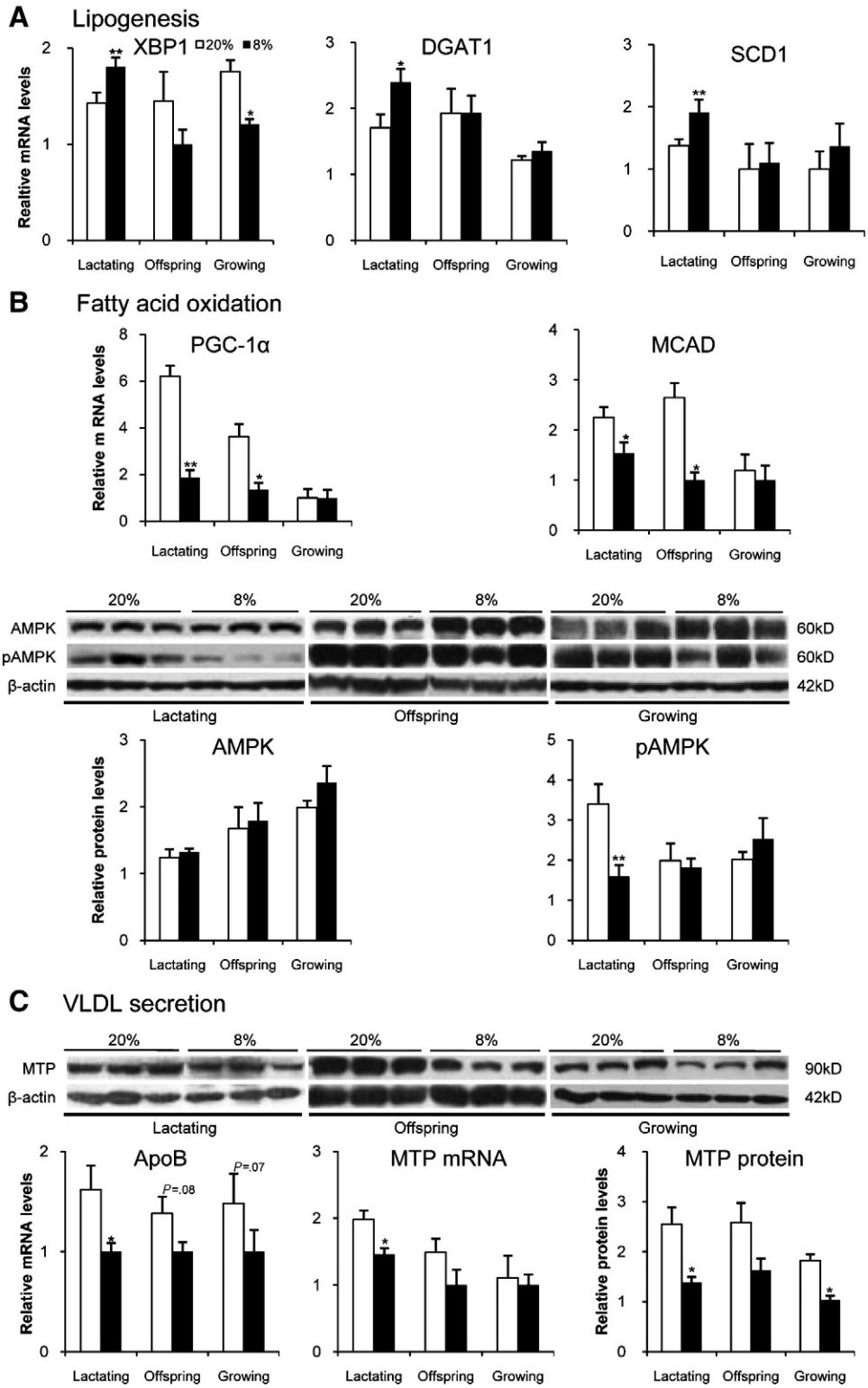

Fig. 3. Expression of genes for lipogenesis (A), FAO (B) and VLDL secretion (C) in the liver of lactating (23 days), offspring (23 days) and growing rats (22 days) fed or nourished with a 20P diet (20%) or an 8P diet (8%). mRNA levels were determined by real-time PCR and normalized with β-actin (n=4–6). Protein levels were determined by Western blotting and normalized with β-actin. Values are the mean±S.E.M. (n=3-4); *P<.05, **P<.01. XBP1, X-box DNA binding protein; DGAT1, diacylglycerol O-acyltransferase 1; SCD1, stearoyl-coenzyme A desaturase 1; pAMPK, phosphorylated AMPK; ApoB, apolipoprotein B.

and mild inflammation, ballooning degeneration and fibrosis. Correspondingly, we observed up-regulation of genes involved in inflammation (TNFα, TNF receptor, lipocalin 2, hepcidin) and fibrogenesis (procollagen type 1 alpha 1, procollagen type 4 alpha 1, TIMP-1). To our knowledge, this is the first report showing that dietary PR causes NASH in lactating rats.

In protein-restricted lactating rat, food intake was decreased by 86% of control rats. Actual consumption of protein, carbohydrate and

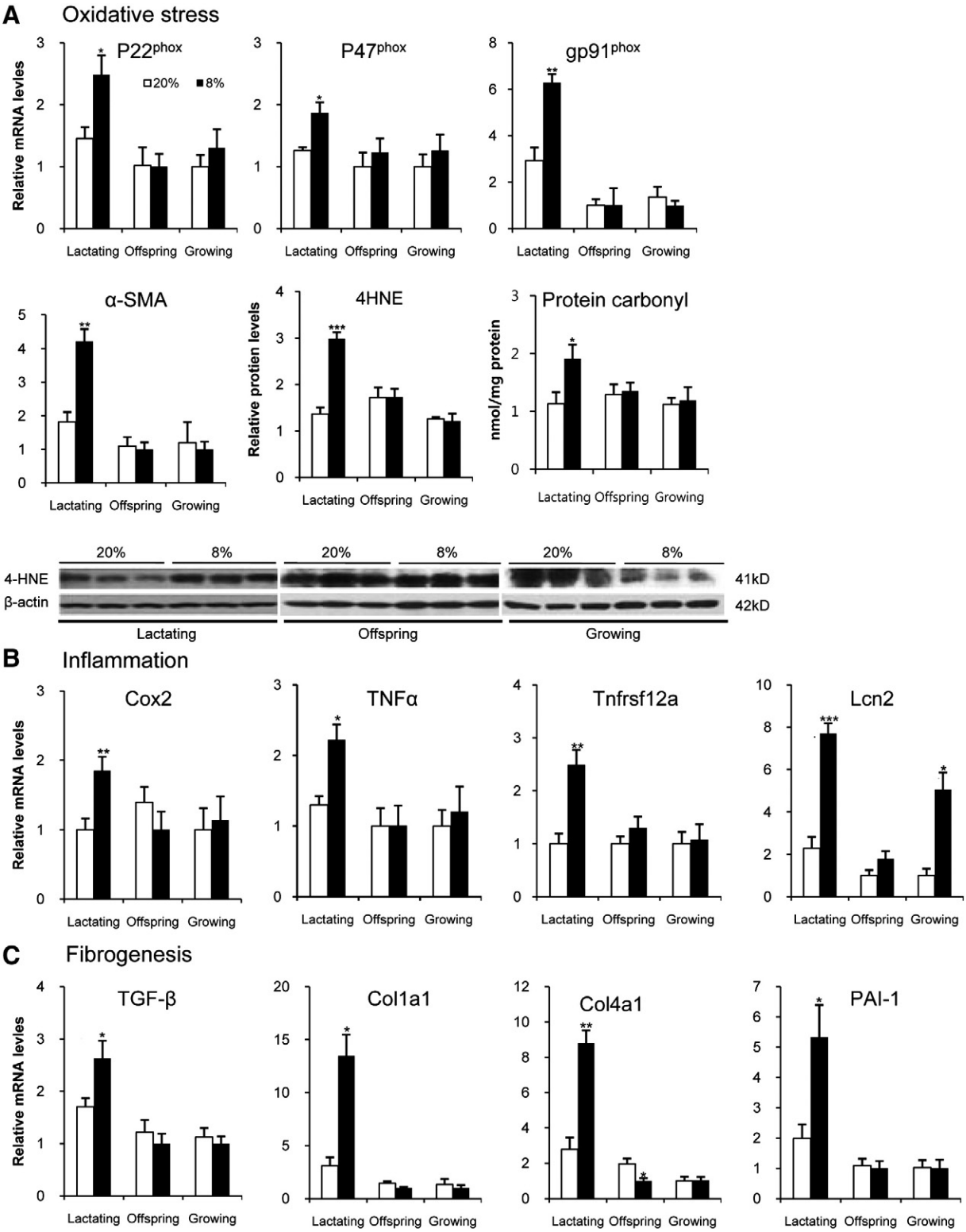

Fig. 4. Expression of genes or markers for oxidative stress (A), inflammation (B) and fibrosis (C) in the liver of lactating (23 days), offspring (23 days) and growing rats (22 days) fed or nourished with a 20P diet (20%) or an 8P diet (8%). Hepatic mRNA levels were determined by real-time PCR and normalized with β-actin. 4-HNE was determined using Western blotting and normalized with β-actin. Hepatic protein carbonyls were determined as described in "Materials and methods." Values are the mean±S.E.M. (n=4–6). *P<.05, **P<.01, ***P<.001. p22^{phox}, cytochrome b-245, alpha polypeptide; p47^{phox}, neutrophil cytosolic factor 1; gp91^{phox}, cytochrome b-245, beta polypeptide; α-SMA, α-smooth muscle actin; Cox2, cyclooxygenase 2; Tnfrsf12a, tumor necrosis factor receptor superfamily, member 12a; Lcn2, lipocalin 2; Col1a1, collagen, type I, alpha 1; Col4a1, collagen, type IV, alpha 1; PAI-1, plasminogen activator inhibitor-1.

energy in protein-restricted lactating rats was 34.4%, 106% and 87% of control rats, respectively. Thus, major cause of hepatic steatosis and other changes in lactating rats fed low-protein diet is likely due to 65.5% of PR compared with control, although there may be minor contribution from slightly low consumption of energy. In growing rats fed low-protein diet, actual consumption of protein, carbohydrate and

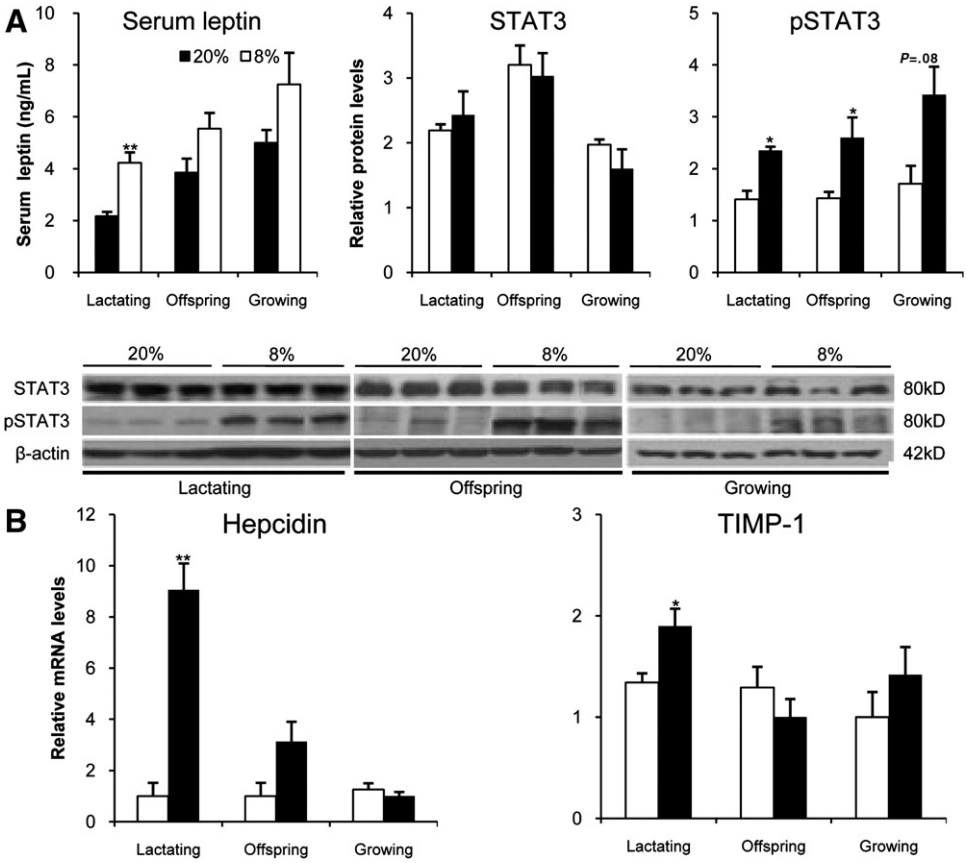

Fig. 5. Leptin-STAT3 signaling in the liver of lactating (23 days), offspring (23 days) and growing rats (22 days) fed or nourished with a 20P diet (20%) or an 8P diet (8%). Hepatic mRNA levels were determined by real-time PCR and normalized with β-actin. Hepatic protein levels were determined by Western blotting and normalized with β-actin. Values are the mean ±S.E.M. (n=4–6); *P<.05, **P<.01. pSTAT3, phosphorylated STAT3.

energy was 41%, 133% and 109% of control rats, respectively. Thus, both PR and excess carbohydrate intake may be responsible for the development of mild hepatic steatosis in growing rats fed low-protein diet. There was minor difference in sucrose contents between control and low-protein diet. We compared occurrence of NAFLD between rats fed 10% sucrose/low-protein diet and 23% sucrose/low-protein diet. Both low-protein diets with 10% and 23% sucrose induced same degree of a severe steatosis with mild lobular inflammation, ballooning degeneration and fibrosis (Table 3), indicating that minor difference in sucrose contents did not affect degree of NASH in low-protein diets.

The quantity and types of carbohydrate in the diet have been suggested to influence on hepatic steatosis and body fat accumulation [24,25]. Our low-protein diet contains 32% of sugar of the total carbohydrate content. Previously, it has been also shown that *de novo* lipogenesis is minimal when the carbohydrate in the diet has a starch: sugar of >50:50 [26].

Another important finding is that the offspring of dams fed the low-protein diet also displayed NAFLD with simple steatosis. By restricting the dietary protein of lactating dams, milk yield and milk protein contents were decreased by 80% and 85%, respectively. Thus, a major cause of the hepatic steatosis observed in the offspring seemed to be protein malnutrition, because of a decrease in milk yield and milk protein contents. Our results demonstrate that protein malnutrition effect on lactating dam was transmitted to their offspring.

A fatty liver by protein malnutrition is also seen in human. Kwashiorkor is a form of severe protein–calorie malnutrition in infants, and fatty liver is one of most striking characteristics of kwashiorkor [12]. The increased incidence of protein–calorie malnutrition has been noted among breast-fed infants [28]. A study has reported an association between prolonged breast-feeding (>12 months of age) and an increased risk of malnutrition [29]. Our dietary PR model suggests that protein malnutrition of lactating woman may affect a development of fatty liver for breast-feeding baby as well as the mother herself.

Our results demonstrate that impairment of lipid metabolism, including increased lipogenesis, decreased FAO and reduced VLDL secretion, is involved in the development of marked hepatic steatosis in protein-restricted lactating rats. In contrast, offspring and growing rats showed minor impairment of gene expression for FAO and VLDL secretion. The mild hepatic steatosis observed in low-protein groups of offspring and growing rats seemed to be due to decreased FAO and impaired VLDL secretion.

Our results show that PR increased serum leptin levels in lactating rats. A previous study also reported an increase in serum leptin levels in protein-restricted lactating rats [5]. Adipose tissues are a major source of leptin synthesis. Thus, increased body fat accumulation with PR may contribute to elevated leptin levels in protein-restricted lactating rats. Our observation that food intake was decreased in protein-restricted rats demonstrates that elevated leptin was responsible for suppression of food intake, via neuronal control of the hypothalamus. Serum leptin levels were not significantly changed by PR in growing rats. This may explain no change in food intake by PR in growing rats.

An interesting finding of our study was that the same dietary PR method could lead to either simple hepatic steatosis or further development to NASH, depending on the physiological stage of the rats. We attempted to elucidate the mechanisms involved in the development of NASH by comparing molecular markers of NAFLD among lactating rats, offspring and growing rats that were fed a low-

protein diet. One of the common mediators of the inflammatory and fibrogenic processes in NASH is oxidative stress, due to generation of ROS [30]. In the liver, ROS can be generated thorough several mechanisms, including mitochondria, Cyp2E1, lipoxygenase and NADPH oxidase [31]. Among them, we found no change in the expression of lipoxygenase or Cyp2E1 genes in protein-restricted lactating rats. Furthermore, we observed decreased expression of genes for mitochondrial FAO. Taken together, our data demonstrate that PR does not alter ROS-generating systems in hepatocytes alone. Instead, we observed up-regulation of p22phox, p47phox and gp91phox genes, key components of NADPH oxidase, in protein-restricted lactating rats, whereas expression of these genes was not altered in offspring or growing rats. The NADPH oxidase complex is an important source for ROS generation in both Kupffer cells and HSCs [22]. We found up-regulated gene expression of α-smooth muscle actin, an activated HSC marker, and increased levels of both 4 HNE protein and protein carbonyls, oxidative stress markers, in the livers of protein-restricted lactating rats. Thus, our findings demonstrate that oxidative stress induced by ROS generated by increased NADPH oxidase due to activation of HSCs or Kupffer cells or both cell types is responsible for the development of NASH in lactating rats.

In the current study, PR during lactation activated the leptin/STAT3 signaling pathway, resulting in up-regulation of expression of hepcidin and TIMP-1, target genes of STAT3. Leptin activates NADPH oxidase and increases the production of ROS in hepatic HSCs [32], and NADPH oxidase mediates the fibrotic and inflammatory effects of leptin on HSCs [33]. Kupffer cells are also implicated in the generation of NADPH oxidase by several stimuli (including TNFα) to produce ROS, which exert proinflammatory and profibrogenic effects [22]. A recent study demonstrated that leptin mediated HSC activation and liver fibrosis through indirect effects on Kupffer cells and that these effects were mediated by TGFβ [8]. Leptin up-regulates inflammatory hepcidin expression in hepatocytes through the JAK2/STAT3 signaling pathway [6]. Thus, our results showing up-regulation of NADPH oxidase, TNFα, TGFβ and hepcidin genes by PR suggest that elevated leptin levels and activation of STAT3 pathway are responsible for oxidative stress, inflammatory cell infiltration and fibrosis in the liver.

In conclusion, we provide evidence that dietary PR during lactation induces steatohepatitis in rats. We also provide evidence that PR during lactation activates the leptin/STAT3 signaling pathway, resulting in up-regulation of downstream target gene expression responsible for induction of oxidative stress, inflammation and fibrosis. Our study highlights the importance of clinical monitoring of protein malnutrition in lactating mothers to reduce the incidence of liver damage in the mother and development of kwashiorkor in infants in developing countries.

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2011.04.002.

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