(4-HNE), 5 μg/ml (both Oxis International, Beverly Hills, CA, USA).

Statistical analysis

Data are given as means ± SEM. The level of statistical significance was determined by analysis of variance followed by Tukey–Kramer's test for multiple comparisons. *P* values less than 0.05 were considered significant.

Results

Effect of ESE on body weight, ALT and AST

The body weight and plasma levels of ALT and AST after 15 weeks are shown in Table 1. Body weight at week 15 was significantly lower in the diet group than in the normal group ($P < 0.01$) but was higher in the diet/ESE group than in the diet group. Plasma ALT and AST levels were significantly higher in the diet group than the normal group ($P < 0.01$ and

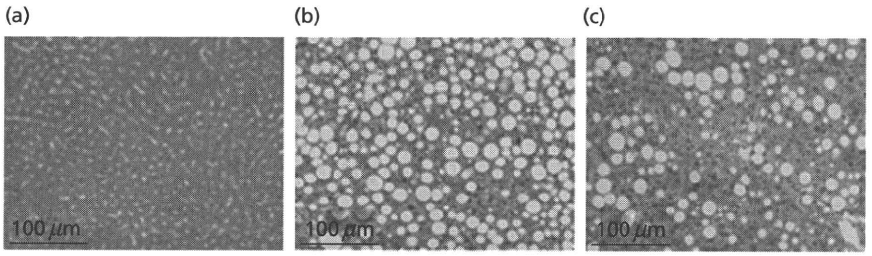

Figure 1 Effect of *E. japonica* seed extract (ESE) on diet-related fatty liver. Samples were taken from rats fed (a) a normal diet, (b) a methionine-choline-deficient (MCD) diet or (c) the MCD diet with ESE in the drinking water. Samples from rats fed the normal diet show normal hepatocytes. Samples from rats fed the MCD diet show diffuse macrosteatosis and hepatocellular ballooning. Samples from the rats fed ESE and the MCD diet show reduction in steatosis. Samples are stained with haematoxylin and eosin.

Table 2 Effect of *E. japonica* seed extract (ESE) on oxidative stress in liver tissue after 15 weeks

	Normal	MCD diet	MCD diet + ESE
SOD (U/mg protein)	332.4 ± 17.7	193.8 ± 15.1*	308.1 ± 49.0†
GPx (μmol/min per mg protein)	1315.5 ± 60.1	344.5 ± 40.1**	532.5 ± 51.5†
Catalase (μmol/min per mg protein)	2.96 ± 0.38	1.60 ± 0.14**	1.64 ± 0.06
GSH (nmol/mg protein)	186.8 ± 13.0	138.7 ± 8.4*	184.3 ± 12.0†
LPO (nmol/mg protein)	63.9 ± 4.2	126.7 ± 17.4**	73.0 ± 4.4‡

Values are means ± SEM (n = 4–6 experiments). $^{*}P < 0.05$; $^{**}P < 0.01$ vs normal group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ vs MCD diet group (Tukey–Kramer's test). GPx, glutathione peroxidase; GSH, glutathione; LPO, lipid peroxide; MCD, methionine-choline-deficient; SOD, superoxide dismutase.

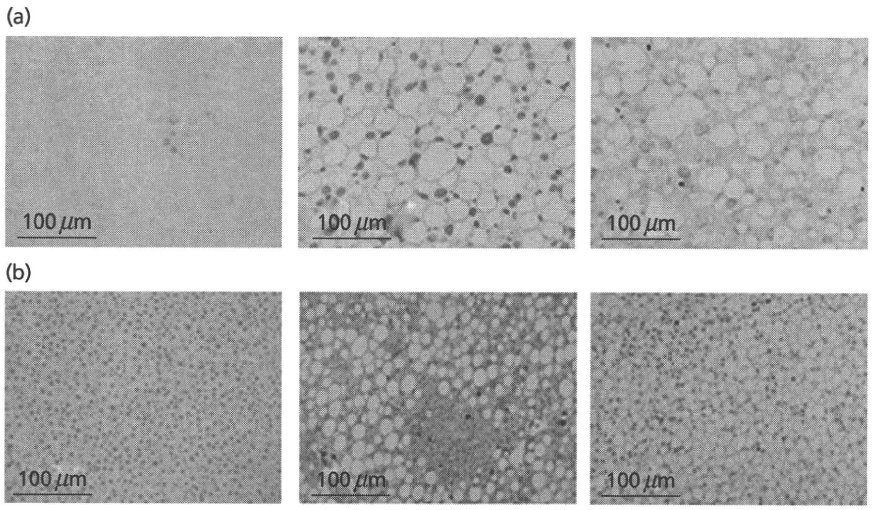

Figure 2 Effect of *E. japonica* seed extract (ESE) on expression of (a) 8-hydroxy-2′-deoxyguanosine (8-OHdG) and (b) 4-hydroxy-2-nonenal (4-HNE). Representative livers from rats fed a normal diet (left), methionine-choline-deficient (MCD) diet (centre) or MCD diet with ESE in the drinking water (right). In (a), 8-OHdG positive cells are identified by the brown nuclei in the photomicrographs. In (b) 4-HNE positive cells are identified by the brown cytoplasm in the photomicrographs.

$P < 0.05$, respectively). These increases were significantly inhibited in the diet/ESE group ($P < 0.05$ and $P < 0.05$, respectively).

Effect of ESE on diet-related fatty liver

H&E stained liver sections are shown in Figure 1. The normal group showed normal hepatic histology. The diet group showed characteristics of NASH, namely macrovesicular steatosis and hepatocellular ballooning. Slight fat deposition was seen in the diet/ESE group.

Effect of ESE on oxidative stress in liver tissue

Activities of SOD, GPx and catalase and levels of GSH and LPO in liver tissue are shown in Table 2. SOD, GPx and catalase activities were significantly lower in the diet group than in the normal group ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively). SOD and GPx activities were significantly higher in the diet/ESE group than in the diet group ($P < 0.05$ and $P < 0.05$, respectively). There was no marked difference in catalase activity between the two groups.

The level of GSH in the liver tissue was significantly lower in the diet group than in the normal group ($P < 0.05$) but was significantly higher in the diet/ESE group than in the diet group ($P < 0.05$). The level of LPO in liver tissue was significantly higher in the diet group than in the normal group ($P < 0.01$) but was significantly lower in the diet/ESE than in the diet group ($P < 0.01$).

Effect of ESE on 8-OHdG and 4-HNE expression

Immunohistochemical staining of liver tissue is shown in Figure 2. Immunohistochemical staining of 8-OHdG (Figure 2a) is used as an index of oxidative DNA damage; 4-HNE (Figure 2b) is a secondary oxidation product of high-level unsaturated fatty acids. Neither 8-OHdG nor 4-HNE was detected in the normal group. Both 8-OHdG and 4-HNE were detected in the diet group but expression of both was inhibited in the diet/ESE group.

Effect of ESE on the liver levels of TGF-β

The level of TGF-β in liver tissue is shown in Figure 3a. Levels of TGF-β were significantly higher in the diet group than in the normal group (91.5 ± 15.0 vs 32.6 ± 11.2 pg/mg protein). Levels in the diet/ESE group (34.5 ± 9.0 pg/mg protein) were significantly lower than in the diet group. There was no significant difference between the diet/ESE and normal groups.

Effect of ESE on liver fibrosis

The level of collagen in liver tissue is shown in Figure 3b. The level in the diet group was significantly higher than in the normal group (33.7 ± 4.5 vs 18.4 ± 1.7 μg collagen/mg protein). The level in the diet/ESE group (23.5 ± 1.4 μg collagen/mg protein) was significantly lower than in the diet group. There was no significant difference between the diet/ESE and normal groups.

The results of Azan staining are shown in Figure 4. The normal group showed normal hepatic histology. The diet group showed the perivenular/pericellular fibrosis that is a heterogeneous pattern of fibrosis in NASH. Slight fibrosis was seen in the diet/ESE group.

Discussion

High levels of enzymes that mop up cellular oxygen, such as SOD, GPx and catalase, are present *in vivo*. However, when the production of ROS exceeds the capacity of these enzymes, oxidative stress induces various cellular responses[20] and may also play an important role in the onset and deterioration of NASH. A study reported that, in the presence of NASH, mitochondria in hepatocytes have morphological abnormalities that are involved in the excess production of ROS.[6]

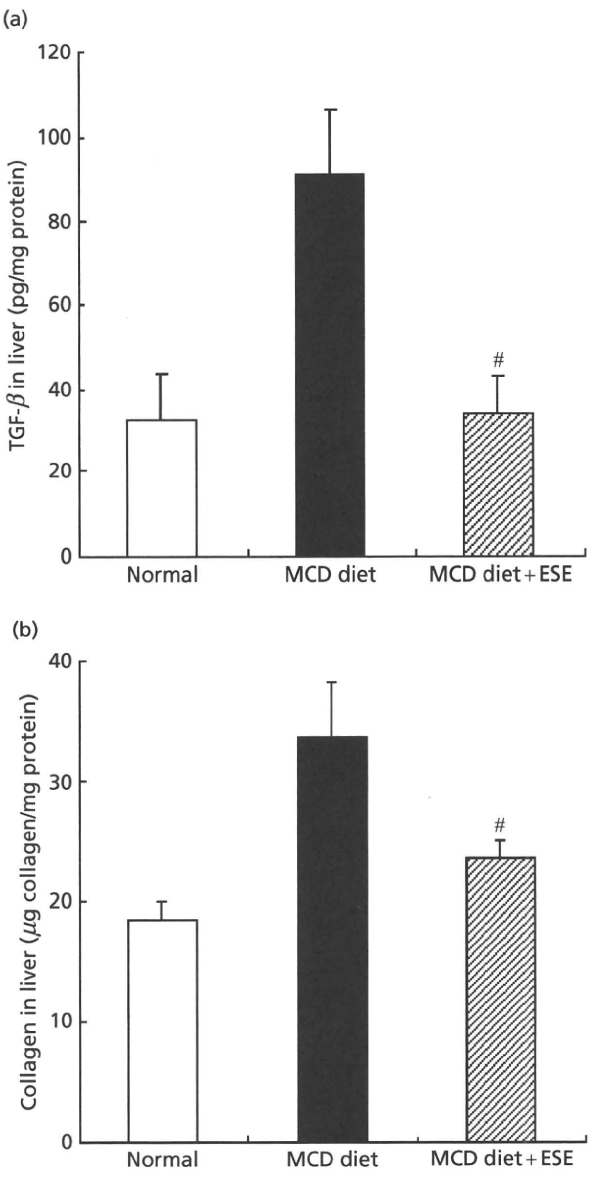

Figure 3 Effect of *E. japonica* seed extract (ESE) on liver tissue levels of (a) transforming growth factor-β (TGF-β) and (b) collagen. The significant increases in TGF-β and collagen induced by the methionine-choline-deficient (MCD) diet were reduced by concomitant treatment with ESE. Columns represent means ± SEM (n = 4–7 experiments). $^{*}P < 0.05$; $^{**}P < 0.01$ vs normal group; $^{\#}P < 0.05$ vs MCD diet group (Tukey–Kramer's test).

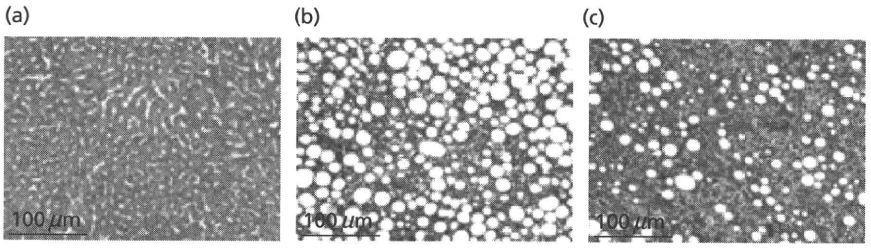

Figure 4 Effect of *E. japonica* seed extract (ESE) on liver fibrosis, demonstrated by Azan staining. Representative liver samples from rats fed (a) a normal diet, (b) a methionine-choline-deficient (MCD) diet or (c) the MCD diet with ESE in the drinking water. Collagen fibres are stained blue in the photomicrographs.

Furthermore, an excess level of fatty acids in the liver enhances mitochondrial β-oxidation, leading to oxidative stress via the excess production of CYP2E1.[21] In addition, various factors such as an excess amount of iron ions may be involved.[22,23]

In this study, we used rats fed an MCD diet as a NASH model. Decreases in the liver levels of methionine and choline promote the excess production of ROS, inducing oxidative stress and leading to hepatocellular disorder and adipose cell formation. In addition, choline deficiency affects lipid metabolism, resulting in fatty liver.[24]

We have investigated ESE because of its antioxidant actions.[14–17] In addition, the usefulness of ESE administration has also been suggested in a hyperlipidaemia model.[19]

ESE administration inhibited increases in the AST and ALT levels, preventing the onset of hepatitis. H&E staining confirmed the inhibitory effects of ESE on fat deposition in the liver. This was possibly associated with the cholesterol-reducing actions of ESE previously reported.[19] With respect to the effects of ESE on oxidative stress, ESE administration increased SOD and GPx activities and reduced oxidative stress by increasing the level of GSH (an antioxidant substance), and decreasing the LPO level. A previous study reported the hydroxyl radical and superoxide anion scavenging actions of ESE.[13]

Oxidative stress may be enhanced in the presence of a fatty liver, leading to the appearance of various types of ROS, suggesting that it can induce excessive DNA/lipid oxidation in the liver. ESE, with its antioxidant actions, inhibited the nuclear expression of 8-OHdG and intracellular expression of 4-HNE, suggesting that it reduces excessive DNA/lipid oxidation in the liver. LPO, which is generated in the presence of oxidative stress, may contribute to the progression of liver fibrosis by activating Kupffer cells, which produce collagen, and promoting the production of cytokines such as TGF-β. In our NASH model, levels of TGF-β and collagen were decreased in rats treated with ESE, suggesting that ESE has potent antioxidant actions. Azan staining confirmed the inhibitory effects of ESE administration on liver fibrosis.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Deletion of tumor necrosis factor-α receptor type 1 exacerbates insulin resistance and hepatic steatosis in aromatase knockout mice

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ABSTRACT

The relevance of estrogen functions in lipid metabolism has been suggested in patients with estrogen-signaling deficiencies. Their importance was further implied by studies in estrogen-deficient mice (ArKO mice), which progressively developed hepatic steatosis. As circulating tumor necrosis factor (TNF)-α levels are known to positively correlate with disturbances in lipid metabolism, we investigated the impact of the loss of TNF-α signaling on carbohydrate and lipid metabolism in ArKO mice. Histological examinations of the livers of mice at 5 months of age revealed that ArKO male mice lacking the TNF-α receptor type 1 (TNFR1) gene (ArKO/TNFR1KO) or both the TNFR 1 and 2 genes (ArKO/TNFR1&2KO) developed more severe hepatic steatosis than ArKO or ArKO/TNFR2KO mice. Serum analyses demonstrated a clear increase in cholesterol and insulin levels in the ArKO/TNFR1KO mice compared with the ArKO mice. Glucose- and insulin-tolerance tests further revealed exacerbation of the systemic insulin resistant phenotype in the ArKO/TNFR1KO mice. Hepatic expression of lipogenic genes including fatty-acid synthase and stearoyl-Coenzyme A desaturase 1 were more markedly upregulated in the ArKO/TNFR1KO mice than the ArKO mice. These findings indicate that under estrogen-deficient physiological conditions, hepatic lipid metabolism would benefit from TNF-α mediated signaling via TNFR1.

1. Introduction

Several lines of evidence have indicated the involvement of a variety of inflammatory processes in the development of obesity and obesity-associated pathology [1,2]. Indeed, inflammatory pathways are upregulated in obese adipose tissue, leading to increased expression of downstream cytokines. It has been proposed that tumor necrosis factor (TNF)-α is a candidate mediator of obesity-related inflammation. This is based on the following observations: 1) TNF-α is overexpressed in the adipose tissues of obese rodent models; 2) targeted deletion of the gene(s) for TNF-α or its two receptors, p55 TNF-α receptor 1 (TNFR1) and p75 TNF-α receptor 2 (TNFR2), significantly improves systemic insulin sensitivity in diet-induced obesity; 3) chronic exposure to TNF-α induces insulin resistance both in vitro and in vivo; 4) treatment with neutralizing soluble TNF receptors improves insulin sensitivity in obese rodent models [3–7]. Consistent with these animal studies, clinical studies revealed that TNF-α expression was markedly increased in adipose tissue and skeletal muscle in insulin resistant patients and that a restoration of insulin sensitivity was associated with a substantial decrease in serum TNF-α levels in obese patients [8,9].

The involvement of estrogens in the regulation of carbohydrate and lipid metabolism was robustly demonstrated by studies in genetically engineered mice including aromatase gene (*Cyp19*)-knockout (ArKO) mice [10–13]. *Cyp19* belongs to the cytochrome P450 superfamily and encodes an enzyme catalyzing the conversion of androgens to estrogens in various mammalian tissue-sites [14]. Our previous study demonstrated that disruption of *Cyp19* caused deregulation of lipid and glucose metabolism, resulting in the development of obesity, insulin resistance, and hepatic steatosis in a sexually dimorphic manner [10,15]. Furthermore, supplementation with 17β-estradiol (E2) restored the regulatory activities of the metabolism, resulting in recovery of the metabolic parameters to the levels of the wild-type (WT) mice [10,11,15]. These observations, therefore, support the fundamental role of estrogens in metabolic regulation.

We hypothesized that abolishing the signaling and function of TNF-α might improve the serum and hepatic phenotypes of ArKO mice. To test this hypothesis, ArKO mice lacking the gene(s) for TNFR1, TNFR2, or both were generated. We report here that the serum and hepatic phenotypes caused by inactivation of the aromatase gene

Abbreviations: ArKO, aromatase knockout; $ArKO_M$, aromatase knockout with minimal steatosis; $ArKO_{FL}$, aromatase knockout with hepatic steatosis; Cyp, cytochrome P450; ERα, estrogen receptor α; GTT, glucose tolerance test; ITT, insulin-tolerance test; TNF-α, tumor necrosis factor-α; TNFR1, TNF-α receptor 1; WT, wild-type

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were not ameliorated but rather exacerbated by the deletion of the TNF-α signaling pathway through TNFR1. These data demonstrate that the TNF-α mediated pathway is beneficial for glucose and fatty-acid metabolism under physiological conditions when estrogen functions are limited.

2. Materials and methods

2.1. Experimental animals

The animal experiments were carried out according to the guidelines of our Institutional Animal Regulations. All animals were maintained under a 12 h-light/dark cycle at 22–25 °C and given water and phytoestrogen-low rodent chow (NIH-07PLD, Oriental Yeast Ltd., Tokyo, Japan) ad libitum. *Cyp19* was disrupted by homologous recombination [16], and the genetic background was unified to C57BL/6j by repeated backcrossing [17]. Because of the infertility caused by inactivation of *Cyp19*, ArKO mice were generated by intercrossing with *Cyp19* heterozygous mice ($Ar^{+/-}$). TNFR double knockout (TNFR1&2KO) mice (B6;129STTnfrsf1a^{tm1Imx}Tnfrsf1b^{tm1Imx}) [18] were purchased from the Jackson Laboratory. By repeated crossing of TNFR1&2KO mice with $Ar^{+/-}$ mice, $Ar^{+/-}$/TNFR1KO, $Ar^{+/-}$/TNFR2KO, and $Ar^{+/-}$/TNFR1&2KO mice were generated. Then, ArKO/TNFR1KO, ArKO/TNFR2KO, and ArKO/TNFR1&2KO mice were obtained by intercrossing of $Ar^{+/-}$ mice with each TNFR genotype. Male mice at 2 or 5 months of age were used for this study. Their body weights were determined every 2 weeks beginning at 6 weeks of age. Their food-intake per day was measured using mice at 2 or 5 months of age.

2.2. Histological examination

Livers were removed from the mice at 2, 3, 4, or 5 months of age, fixed in a solution of 10% (v/v) buffered formalin for 24 h, dehydrated in graded ethanol, and then embedded in paraffin. The samples were cut into 3 μm-thick sections and stained with hematoxylin–eosin. The development of hepatic steatosis was evaluated qualitatively by microscopy based on the presence of liver cells filled with numerous lipid droplets.

2.3. Measurement of glucose, cholesterol, triglycerides, free fatty acids, leptin, insulin, adiponectin, and TNF-α

Tail blood was collected via vein nicks of fed mice for measurement of leptin and TNF-α, or of mice fasted for 16 h overnight with full access to water for measurement of glucose, cholesterol, triglycerides, free fatty acids, insulin and adiponectin. The concentrations of serum cholesterol, serum triglycerides, and plasma free fatty acids were measured by colorimetric methods using the Triglyceride E-Test, Cholesterol T-Test, and NEFA C-Test, respectively (Wako Pure Chemical Industries Ltd., Osaka, Japan). Serum leptin, serum insulin, plasma adiponectin, and plasma TNF-α were determined by using enzyme-linked immunoassay kits (BioVendor Laboratorni medicina a.s., Modrice Czech Republic, Mercodia AB, Uppsala, Sweden, R&D Systems, Inc., MN55413, USA, and Thermo Scientific, Rockford, IL61105, USA, respectively). Blood glucose concentration was measured with Glutest Ace and Glutest Sensor (Sanwa Kagaku Kenkyusho Co., Nagoya, Japan) before glucose challenge or at 30, 60, and 120 min after the intraperitoneal injection (ip) of glucose (2 mg glucose/g of body weight in saline) (glucose tolerance test, GTT) [15]. Insulin sensitivity was assessed using an insulin-tolerance test (ITT) as described previously [15]. The fed mice were given an ip injection of insulin from the bovine pancreas at a dose of 0.75 mU/g of body weight (Sigma-Aldrich, Inc., St. Louis, MO63103, USA, Cat. No. I1882), and blood glucose concentrations were measured at 0, 10, 30, 60, and 120 min after injection of insulin.

2.4. Secretion of TNF-α in response to lipopolysaccharide treatment

Mice at 5 months of age with mean body weights of 33.8 ± 0.7 g and 37.9 ± 0.8 g for WT and ArKO mice, respectively ($n = 5$ for each genotype) were injected ip with saline or lipopolysaccharide (LPS) (*Escherichia coli* 0127 B8, Sigma-Aldrich, Inc., St. Louis, MO) at a dose of 10 ng/g of body weight. After 60 min, 0.1 ml blood was collected from the tail vein using a tube containing 10 μl of 0.25 M EDTA, and plasma were prepared and stored at −20 °C until use. Basal plasma levels of TNF-α were also measured using untreated mice ($n = 7$) at 2, 3, 4 and 5 months of age.

2.5. Analysis of mRNA expression

Messenger RNA analysis was performed by Northern blotting or by quantitative real-time PCR (QRT-PCR) for carbohydrate responsive element-binding protein (ChREBP) and sterol regulatory element-binding protein-1 (SREBP-1). Three mice for each strain were sacrificed at 2 and 5 months of age. Liver was dissected, frozen on liquid nitrogen, and stored at −80 °C until use. Total RNA was prepared from individual liver samples by the method of Zarlenga and Gamble [19]. Equal amounts of the total RNA from the same strain were pooled. Fifteen micrograms of the pooled RNA were separated on a 0.8% agarose gel and transferred to a nylon membrane. The membranes were incubated with ^{32}P-labeled cDNA probes and analyzed on a Fuji system analyzer (Fuji Photo Film, Tokyo, Japan) for quantification of band intensity. The probes (gene symbol, accession number) used for Northern blotting were acetyl-CoA carboxylase 1 (Acaca, NM_133360); acyl-Coenzyme A dehydrogenase, medium chain (Acadm, NM_007382); acyl-Coenzyme A oxidase 1 (Acox1, NM_015729); carnitine palmitoyltransferase 1a, liver (Cpt1a, NM_013495); catalase (Cat, L25069); cytochrome P450, family 2, subfamily e, polypeptide 1 (Cyp2e1, NM_021282); cytochrome P450, family 7, subfamily a, polypeptide 1 (Cyp7a1, NM_007824); fatty acid synthetase (Fasn, NM_007988); glutathione peroxidase 1 (Gpx1, NM_008160); malic enzyme 1, NADP(+)-dependent, cytosolic (Me1, NM_008615.2); microsomal triglyceride transfer protein (Mttp, NM_008642); stearoyl-Coenzyme A desaturase 1 (Scd1, NM_009127); and glyceraldehyde-3-phosphate dehydrogenase (Gapdh, NM_008084). Quantitative changes in mRNA levels for three independent experiments were calculated by a BAS 2000 III (Fiji film Inc., Tokyo Japan) to show their ratio to the levels in the wild-type (WT) mice group.

Total RNA (1 μg) was reverse-transcribed for 1 h at 37 °C in a 25-μl final volume reaction using 200 U of M-MLV reverse transcriptase (Invitrogen Corporation, Carlsbad, CA92008, USA) according to the manufacturer's instructions. To quantify the mRNA expression levels of ChREBP and SREBP-1, QRT-PCR analysis was performed starting with 16 ng of reverse-transcribed total RNA in a final volume of 12.5-μl using SYBR® *Premix Ex* Taq™II in a light cycler instrument (Takara Bio inc., Shiga JAPAN). Primers used for ChREBP (NM_021455) were (sense) 5′-CTGGGGACCTAAACAGGAGC-3′, (antisense) 5′-GAAGCCACCCTA-TAGCTCCC-3′ (amplified size, 166 bp); for SREBP-1 (NM_011480) were (sense) 5′-ACCGTCACTTCCAGCTAGAC-3′, (antisense) 5′-CCAC-TAAGGTGCCTACAGAGC-3′ (181 bp); and for cyclophilin A (NM_008907) were (sense) 5′-ATGGCACTGGCGGCAGGTCC-3′, (anti-sense) 5′-TTGCCATTCCTGGACCCAAA-3′ (241 bp). The PCR conditions were as described [20]. The relative quantification for a given gene was corrected to the cyclophilin mRNA values.

2.6. Preparation of nuclear extracts and immunoblot analysis of ChREBP and SREBP expression

Hepatic nuclear extracts were prepared from mice at 2 and 5 months of age using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL61105, USA) according to the

Table 1
Ablation of TNFR1 accelerates fatty liver development.

Tnfr loci / Cyp19 locus	WT	TNFR1 KO	TNFR2 KO	TNFR1&2 KO
WT	0/6 (0%)	1/20 (5%)	0/7 (0%)	1/6 (17%)
ArKO	16/32 (50%)	43/48* (89%)	4/12 (33%)	11/13** (85%)

The accumulation of fat in the livers of TNFR1KO and ArKO/TNFR1KO mice was examined microscopically at various ages. The numerals over and under the bar, respectively, indicate the number of mice showing severe hepatic steatosis and the number of mice examined. The numerals in parentheses indicate the percentage of mice showing hepatic steatosis. Hepatic steatosis became evident after 4 months of age in the ArKO/TNFR1KO mice. ND indicates not determined. * $p<0.0001$, ** $p<0.02$.

manufacturer's instructions. Protein concentration was determined using BCA Protein Assay™ reagents (PIERCE, Rockford, IL61105, USA) using bovine serum albumin as a standard. Proteins (50 μg) were subjected to SDS-PAGE analysis on a 10% gel and electrotransferred onto polyvinylidene difluoride membranes (Milipore Corporation, Billerica, MA 01821, USA). Rabbit anti-SREBP-1 and anti-ChREBP polyclonal antibodies (sc-366; dilution 1:2000 and sc-33764; dilution 1:1000, respectively, Santa Cruz Biotechnology, Santa Cruz, CA95060, USA) as the primary antibody were incubated with the membranes for 12 h at 4 °C. Rabbit polyclonal anti-histone H3 antibody (H0164; dilution 1:10,000, Sigma-Aldrich, Saint Louis, MO63103, USA) was used as a loading control. The membranes were then incubated with anti-rabbit horseradish peroxidase-conjugated IgG (#7074; dilution 1:2000, Cell Signaling Technology, Danvers, MA01923, USA) as the second antibody for 1 h at 25 °C, followed by incubation with Immobilon™Western reagents (Milipore Corporation). Visualization and quantification of signals were performed using LAS-4000mini (FUJIFILM Corporation, Tokyo, Japan). The antibodies for ChREBP and SREBP-1 detected bands at 95 kDa and 70 kDa, respectively, and the relative quantification for given signals was corrected to the histone H3 values.

2.7. Statistical analysis

Data are expressed as the mean ± SEM, and analyzed using Students *t*-test, Mann–Whitney's *U* test, two-way ANOVA, and Chi-square test when they were applicable. *P* values less than 0.05 were considered significant.

3. Results

3.1. Hepatic histology

As described previously [10,15], ArKO male mice fed a diet of phytoestrogen-low chow gradually accumulated abdominal fat and developed hepatic steatosis, although there was a slight variation in the phenotype at 5 months of age; approximately 50% of ArKO mice developed hepatic steatosis (Table 1). In contrast, more than 80% of the ArKO/TNFR1KO and ArKO/TNFR1&2KO mice displayed marked microvesicular steatosis at 5 months of age (Table 1 and Fig. 1). Hepatic steatosis became evident at 4 months of age in the ArKO/TNFR1KO mice (Table 2). In contrast, deletion of TNFR2 did not exert a detrimental effect on the hepatic phenotype at 5 months of age, compared with the ArKO mice. Their wild-type (WT) siblings did not develop hepatic steatosis at this age (Table 1). Because minimum hepatic effect was observed in the ArKO/TNFR2 mice, following analyses were performed with ArKO, ArKO/TNFR1KO, TNFR1KO and WT mice.

3.2. Measurement of metabolic parameters

The mean body weights of the WT, ArKO, TNFR1KO, and ArKO/TNFR1KO mice were similar until 4 months of age. The weights of the ArKO, TNFR1KO, and ArKO/TNFR1KO mice at 5 months of age were also similar to each other, but were heavier than that of the WT mice (WT; 33.1 ± 2.0 g ($n=8$) vs. ArKO 40.4 ± 1.3 g ($n=23$) ($p<0.03$), vs. TNFR1KO; 38.5 g ± 1.5 g ($n=7$) ($p<0.06$), vs. ArKO/TNFR1KO; 40.3 ± 0.9 g ($n=6$) ($p<0.004$)) (Fig. 2). Both the TNFR1KO and ArKO/

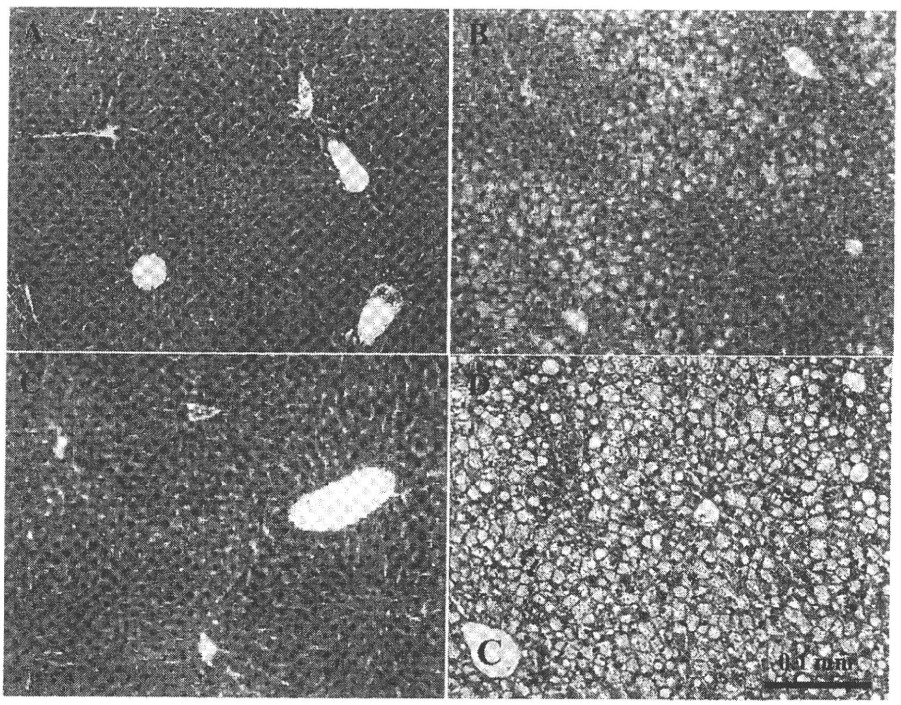


Fig. 1. Hepatic histology in ArKO, TNFR1KO, and ArKO/TNFR1KO mice. Hematoxylin and eosin staining of liver sections from fed ArKO/TNFR1KO mice at 2 months of age (A) and ArKO (B), TNFR1KO (C), and ArKO/TNFR1KO (D) mice at 5 months of age. Note that increased hepatic steatosis was evident in ArKO/TNFR1KO mice at 5 months of age, which displayed an accumulation of microvesicular lipid droplets in the regions around the central veins (c). Scale bar: 100 μm.

Table 2
Age-dependent development of fatty liver.

Age	2M	3M	4M	5M
TNFR1 KO	0/4 (0%)	ND	ND	1/20 (5%)
ArKO/TNFR1 KO	1/9 (11%)	1/9 (11%)	4/5 (80%)	43/48* (89%)

The accumulation of fat in the livers of TNFR1KO and ArKO/TNFR1KO mice was examined mic ages. The numerals over and under the bar, respectively, indicate the number of mice showing seve the number of mice examined. The numerals in parentheses indicate the percentage of mice sho Hepatic steatosis became evident after 4 months of age in the ArKO/TNFR1KO mice. ND indicates *$p<0.0001$, **$p<0.02$.

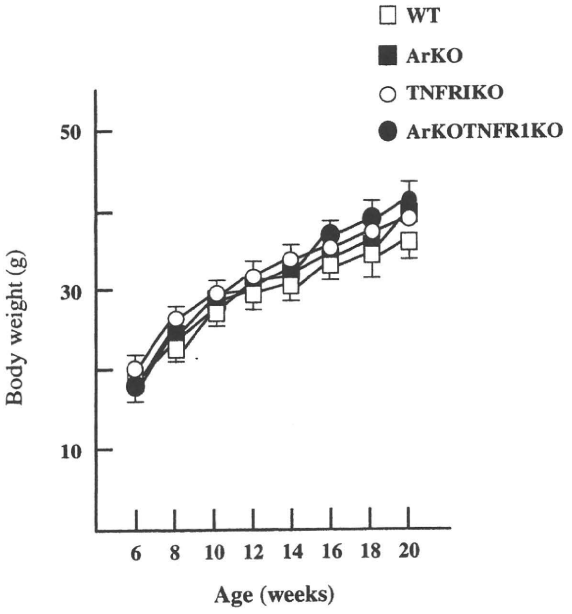


Fig. 2. Measurement of body weight. Body weights were measured every 2 weeks beginning at 6 weeks of age.

TNFR1KO mice showed increased food-intake at 2 months of age ($p<0.05$, vs. WT mice, and $p<0.001$, ArKO/TNFR1KO mice vs. ArKO mice). However, only the TNFR1KO mice exhibited a food-intake increase at 5 months of age ($p<0.05$, vs. WT mice) (Fig. 3A). Blood glucose levels were similar among the fasted animal groups at 2 months of age; whereas the fasting serum insulin levels in the TNFR1KO and ArKO/ TNFR1KO mice were significantly reduced compared to those in the WT and ArKO mice at 2 months of age ($p<0.05$ and $p<0.01$, vs. WT mice, respectively) (Fig. 3B and C). At 5 months of age, fasting blood glucose levels were significantly elevated in the ArKO and ArKO/TNFR1KO mice compared to the other two groups ($p<0.05$ and $p<0.01$, respectively) (Fig. 3B). However no significant difference was observed between ArKO and ArKO/TNFR1KO mice. There was no significant difference in fasting insulin levels between the WT and ArKO mice, although moderate pancreatic beta cell dysfunction was suggested in the ArKO mice [21]. Contrarily, the fasting serum insulin levels in the ArKO/TNFR1KO mice were four times higher than those in the WT and ArKO mice ($p<0.001$, vs. WT mice, and $p<0.001$ vs. ArKO mice, respectively) (Fig. 3C), suggesting the development of severe insulin resistance. No significant differences were observed in the circulating levels of triglycerides, free fatty acids, or cholesterol among these four groups at 2 months of age (Fig. 3D–F). The ArKO/TNFR1KO mice showed a significant elevation in serum cholesterol levels at 5 months of age compared to the other groups ($p<0.001$, vs. WT mice, and $p<0.01$, vs. ArKO mice) (Fig. 3F).

Since TNFRKO and WT mice showed significant differences in food-intake, we measured serum leptin and plasma adiponectin levels. TNFR1KO, but not ArKO/TNFR1KO mice showed significantly lower serum leptin levels as compared to the other groups at 2 months of age (Fig. 3G). Serum leptin levels significantly increased in ArKO and ArKO/TNFR1KO mice at 5 months of age ($p<0.05$, vs. WT mice, and $p<0.05$, ArKO/TNFR1KO mice vs. ArKO mice), indicating that less apparent correlation was detected between food-intake and mouse genotypes at 5 months of age. In addition, we detected no significant differences in plasma adiponectin levels among the animal groups at 2 or 5 months of age (Fig. 3H).

3.3. GTT and ITT

To investigate the effects of deletion of TNF-α signaling on glucose homeostasis in estrogen-deficient male mice, glucose tolerance tests (GTT) and insulin-tolerance tests (ITT) were performed (Fig. 4). The GTT revealed that the ArKO mice displayed reduced blood glucose elimination compared to the WT mice, as reported previously [15]. A more prominent reduction in glucose-eliminating activity was observed in the ArKO/TNFR1KO mice ($p<0.001$, ArKO/TNFR1KO vs. mice with other genotypes). Furthermore, the ArKO/TNFR1KO mice revealed severely impaired insulin sensitivity compared to the WT and ArKO mice ($p<0.05$, ArKO/TNFR1KO vs. mice with other genotypes).

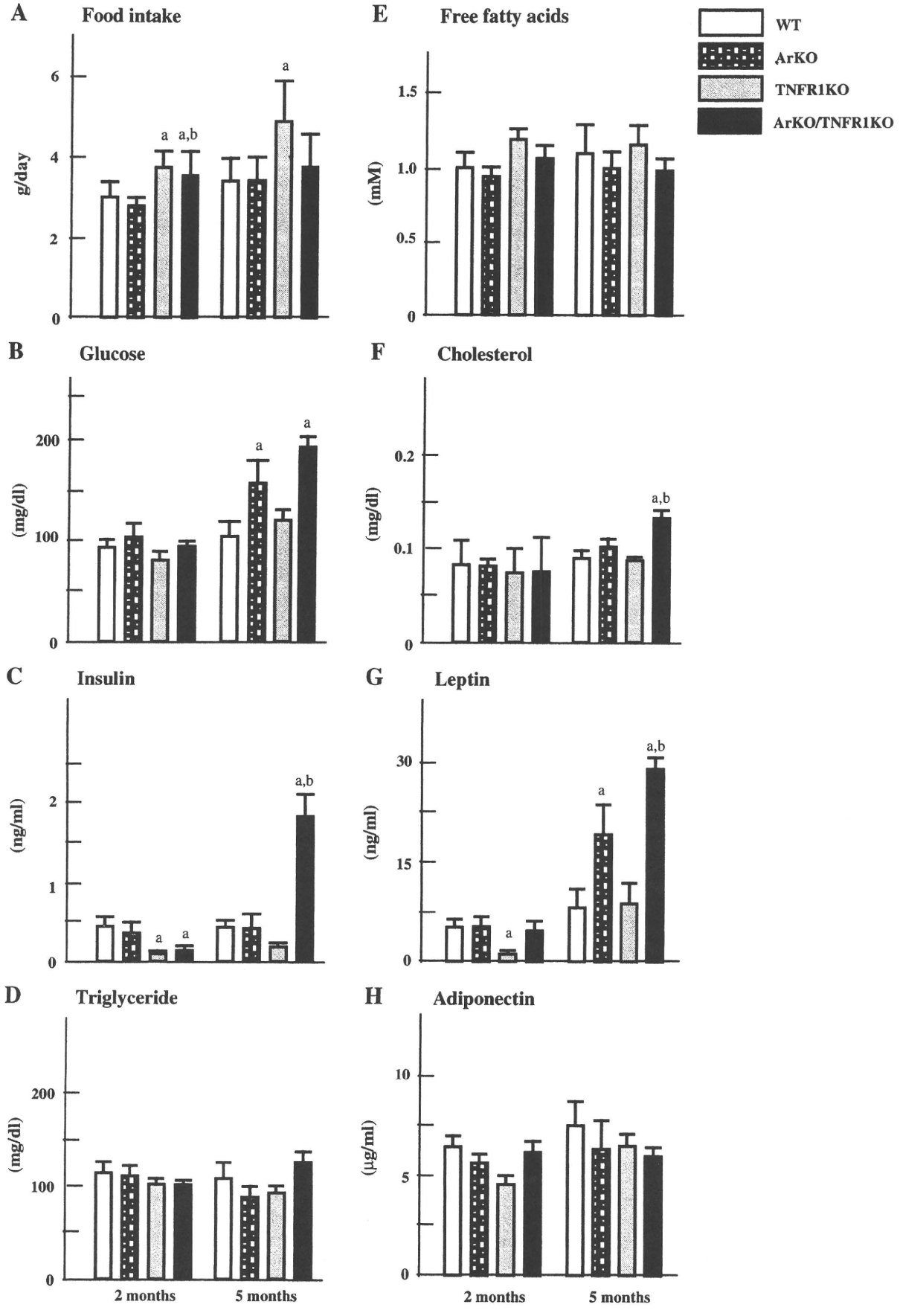
3.4. Expression of genes related to lipid metabolism in the liver

Alterations in the expression levels of a subset of genes related to lipid metabolism were examined next in the livers of the mice at 2 and 5 months of age, in order to detect the early transcriptional changes that precede the pathological manifestations of hepatic steatosis.

Enhanced mRNA expression of lipogenic and fatty-acid-oxidative genes was a characteristic feature of the estrogen-deficient mice at 2 months of age (Fig. 4A). The mRNA levels coding for the lipogenic enzymes, including Acaca, Me1, Fasn, and Scd1 were upregulated in the ArKO mice regardless of loss of the TNFR1 gene at 2 months of age compared to the levels in WT mice (1.6-fold, 1.3-fold, 5.3-fold, and 3.1-fold in the ArKO mice and 1.4-fold, 1.6-fold, 6.6-fold, and 4.6-fold in the ArKO/TNFR1KO mice, respectively) (Fig. 4C). In addition, the mRNA expression of genes encoding enzymes involving fatty-acid-oxidative reactions such as Cpt1a, Acox1, and Cat was also upregulated in the ArKO and ArKO/TNFR1KO livers (1.6-fold, 2.9-fold, and 3.1-fold in the ArKO mice and 1.4-fold, 2.2-fold, and 2.4-fold in the ArKO/TNFR1KO mice, respectively) (Fig. 4C). These results indicate that both lipogenic and fatty-acid-oxidative activities are enhanced under estrogen-deficient conditions at 2 months of age. Furthermore, the mRNA of Cyp7a1, which is a key enzyme for the clearance of cholesterol, was expressed 3.5 times more abundantly in the ArKO/TNFR1KO mice at 2 months of age.

As 50% of the ArKO mice developed severe hepatic steatosis at 5 months of age (Table 1), the ArKO mice at this age were divided into two groups for analysis of hepatic mRNA expression: one group of mice with hepatic steatosis ($ArKO_{FL}$) and the other with minimal steatosis ($ArKO_M$). The $ArKO_M$ mice revealed enhanced mRNA expression of lipogenic and fatty-acid-oxidative genes as observed in the ArKO mice at 2 months of age. In the $ArKO_{FL}$ mice, the mRNA expression of Acaca and Fasn were 0.7 and 0.5 times downregulated,

Fig. 3. Measurement of metabolic parameters. Various metabolic parameters were measured in blood samples from WT (open bar), ArKO (slashed bar), TNFR1KO (dotted bar), and ArKO/TNFR1KO (closed bar) mice at 2 and 5 months of age. (A) Food-intake (g/day), (B) fasting blood glucose ($n=11$–25), (C) fasting serum insulin ($n=5$–8), (D) fasting serum triglycerides ($n=10$), (E) fasting plasma free fatty acids ($n=10$), (F) fasting serum total cholesterol ($n=10$), (G) serum leptin ($n=7$) and (H) fasting plasma adiponectin ($n=5$–8) concentrations were measured. (a, and b, $p<0.05$ vs. WT mice, and $p<0.05$ vs. ArKO mice, respectively).


A
Food intake
g/day
6
4
2
0
a
a,b
a
B
Glucose
(mg/dl)
200
100
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a
a
C
Insulin
(ng/ml)
2
1
0
a
a
a,b
D
Triglyceride
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200
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2 months
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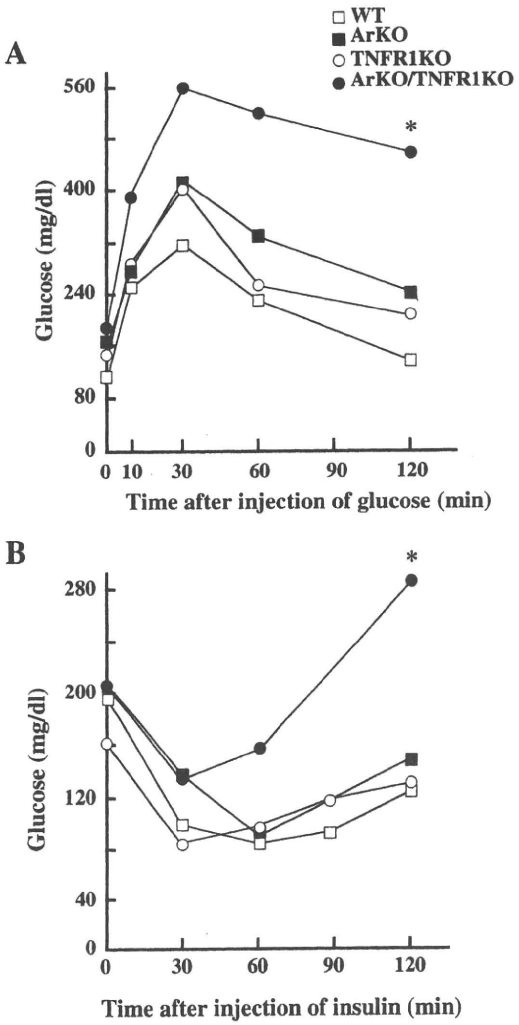

Fig. 4. ArKO/TNFR1KO mice display glucose intolerant and insulin resistant phenotypes. (A) Glucose tolerance tests were performed on WT (open squares), ArKO (closed squares), TNFR1KO (open circles), and ArKO/TNFR1KO (closed circles) mice that had been fasted for 16 h at 5 months of age. The animals were injected (ip) with 2 mg/g body weight of glucose. Blood glucose was measured before injection and 15, 30, 60, and 120 min after the injection. The results are expressed as mean blood glucose concentration ± SEM from five animals per genotype. (B) Insulin-tolerance tests were performed in fed WT, ArKO, TNFR1KO, and ArKO/TNFR1KO mice at 5 months of age. The mice were injected (ip) with 0.75 U/kg body weight of human recombinant insulin. Blood glucose was measured before injection and 30, 60, 90, and 120 min after the injection. The results are expressed as mean blood glucose concentration ± SEM. The asterisks indicate $p<0.001$, ArKO/TNFR1KO mice vs. WT mice).

respectively, and those of Me1 and Scd1 were 1.5 times upregulated compared to those in the $ArKO_M$ mice. Furthermore, the mRNA levels of genes related to fatty-acid oxidation in the $ArKO_{FL}$ mice were less abundant compared to those in the $ArKO_M$ mice except for Acadm (Fig. 4B), indicating that fatty-acid-oxidative activity was attenuated when hepatic steatosis developed in the ArKO mice, as reported previously [10]. In the ArKO/TNFR1KO mice, the expression levels of the lipogenic genes except for Acaca were sustained at high levels at 5 months of age, even after the development of severe hepatic steatosis. The expression of Gpx1 mRNA was not altered among the experimental animal groups (Fig. 5).

3.5. QRT-PCR and immunoblot analysis on expression of ChREBP and SREBP-1

Expression levels of mRNA for ChREBP and SREBP-1 and their nuclear protein contents in the livers were measured to study molecular mechanisms involved in the upregulation of lipogenic gene expression in the estrogen-deficient mice (Fig. 6). The ArKO and ArKO/TNFR1KO mice contained approximately double amounts of mRNA for the factors as compared to those of WT mice at 2 month of age ($p<0.05$, vs. WT mice, not significant between ArKO and ArKO/TNFR1KO mice). The mRNA levels for ChREBP and SREBP-1 in TNFR1KO mice were, respectively, 40% ($p<0.05$, vs. WT mice) and 70% of the WT mice levels at this age. Nuclear content of ChREBP in TNFR1KO mice, however, was 3-fold more than the other groups at 2 months of age ($p<0.05$, vs. WT mice). The nuclear contents of SREBP-1 did not show significant differences among the groups at 2 months of age.

At 5 months of age, TNFR1KO and ArKO/TNFR1KO mice, respectively, expressed twice more the amounts of mRNAs for ChREBP and SREBP-1 when compared to levels of the WT mice but without statistical significance. An increase in nuclear protein content of ChREBP was detected in ArKO mice displaying severe hepatic steatosis and TNFR1KO mice but not ArKO/TNFR1KO mice, which showed a significant decrease as compared to that of ArKO mice ($p<0.05$, vs. ArKO mice). Nuclear amounts of SREBP-1 were increased 2- to 3-fold in ArKO with severe steatosis, TNFR1KO ($p<0.005$, vs. WT mice) and ArKO/TNFR1KO mice over the WT levels. The summary of the expression analyses on ChREBP and SREBP-1 at 2 and 5 months of age was presented in Fig. 6C.

3.6. Secretion of TNF-α in ArKO mice

Estrogen-deficiency in ArKO mice might be a reason for the changes seen in their TNF-α levels, which worsened hepatic lipid metabolism. To examine this possibility, the basal plasma TNF-α levels and the levels secreted in response to LPS were determined using WT and ArKO mice showing relatively non-obese phenotypes at 5 months of age. We obtained no evidence showing significant differences in the basal plasma TNF-α levels between WT and ArKO mice at any ages examined (Fig. 7 and data not shown). Furthermore no significant differences were detected in the response to LPS-induced TNF-α levels (Fig. 7). These results indicate that estrogen-deficiency does not alter the systemic levels of TNF-α.

4. Discussion

TNF-α is a proinflammatory cytokine that plays a major role in the pathogenesis of autoimmune diseases and inflammatory disorders [22] and is also known to modulate insulin signaling in the liver either at sites downstream from the insulin receptor or through hepatic overproduction of very low density lipoproteins [23,24]. Elevation of TNF-α expression is thus thought to be associated with systemic insulin resistance [4]. However, TNF-α signaling has been demonstrated to be required for normal glucose homeostasis using db/db male mice, in which the loss of both TNFR1 and 2 genes causes severe hyperinsulinemia [25]. Thus, we wondered whether ArKO mice, which are predisposed to develop hepatic steatosis [10], would be protected from impairment of lipid metabolism by the blocking of TNF-α function.

As described previously [15], ArKO mice displayed hyperglycemia at 5 months of age. Nevertheless, GTT and ITT revealed less marked impairment in blood glucose elimination of ArKO mice when compared to the previous study [15]. Alternations in genetic background of ArKO mice, possibly owing to repeated backcrossing with C57BL/6j mice [17], might explain the phenotypic differences between the studies. The possible genetic alternations also link with the development of varying degrees of hepatic steatosis in the current ArKO mice. Nevertheless further studies are required to establish the reasons.

The hyperglycemia observed in the ArKO mice at 5 months of age might induce pancreatic β-cell dysfunction as reported recently, where E2 was demonstrated to protect insulin secretion through

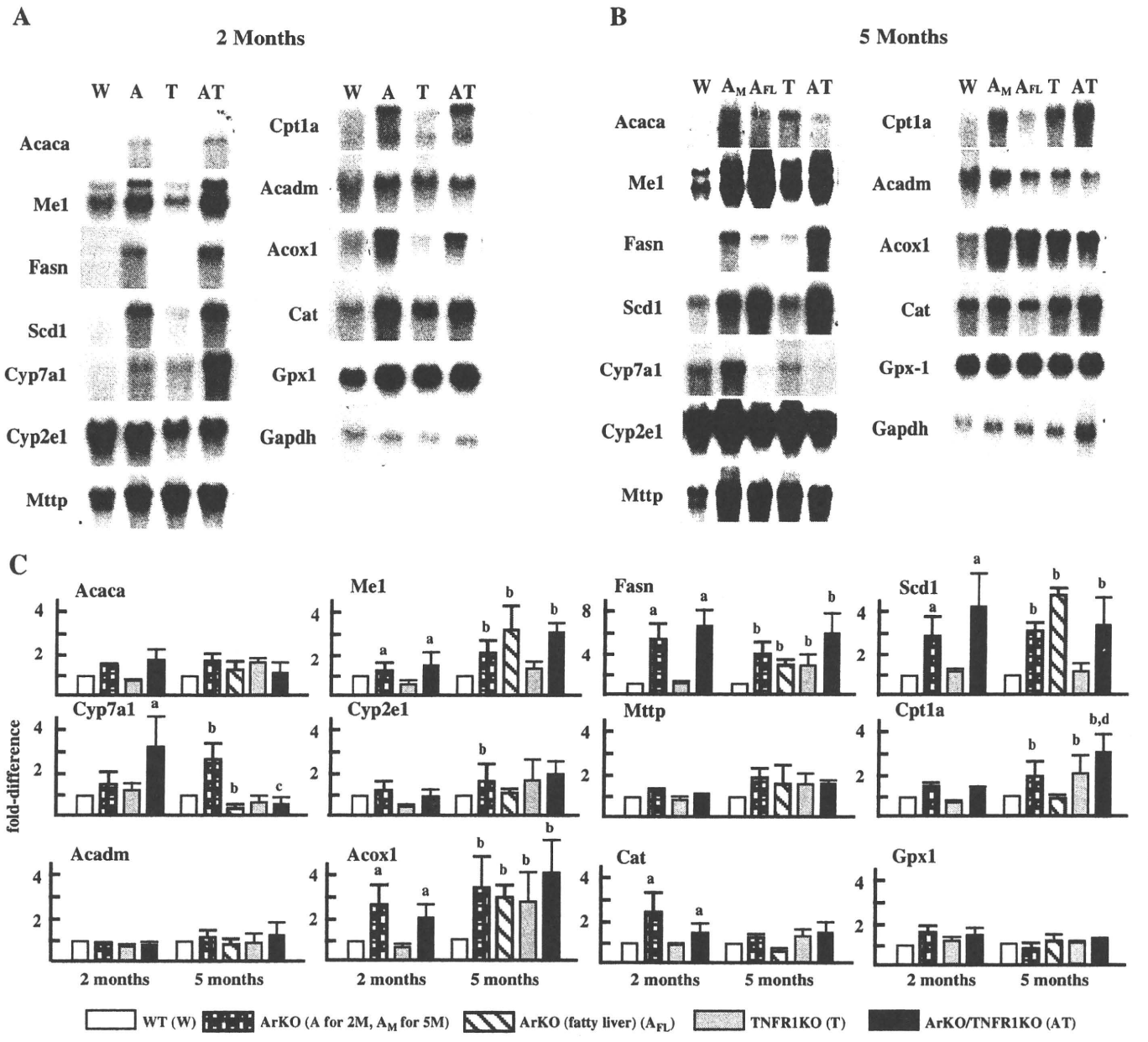

Fig. 5. Northern blot analysis. Total RNA extracted from the liver of WT (W), ArKO (A_M, A_{FL}), TNFR1KO (T), and ArKO/TNFR1KO (AT) mice at 2 (A) or 5 (B) months of age. RNA blotted onto nylon membranes were probed with ^{32}P-labeled cDNA probes as shown. GADPH was selected as a loading control. A_M and A_{FL} indicate ArKO mice that developed minimal and severe hepatic steatosis, respectively. (C) The bar-graphs illustrate fold-difference compared to the expression level in the livers of the WT mice. The bar-graphs illustrate fold-difference compared to the expression level in the livers of the WT mice. (a, b, c and d, $p<0.05$ vs. WT mice at 2 months, vs. WT mice at 5 months of age, vs. A_M mice at 5 months The expression of adiponectin is of age, and vs. A_{FL} mice at 5 months of age, respectively).

estrogen receptor (ER) α actions [21]; nevertheless, no statistically significant difference was detected in the serum insulin levels between the WT and ArKO mice. ArKO/TNFR1KO mice at this age displayed hyperinsulinemia in addition to their exacerbated glucose tolerant activity; the latter phenotype is a characteristic feature of estrogen-deficient mice and seems to be worsened by deletion of the TNFR1 gene possibly due to elevated action of gluconeogenesis. These observations indicate that the pancreatic β-cell dysregulation caused by E2 deficiency might involve a TNF-α mediated pathway that acts via TNFR1. As the ArKO/TNFR1KO mice also developed severe hepatic steatosis at this age, increased adiposity might cause hepatic metabolic abnormality and hyperinsulinemia; nevertheless, a causal relationship between hepatic lipid accumulation and systemic insulin resistance still remains debatable [26,27].

The decrease in the expression level of Fasn mRNA was marked in the ArKO mice with severe hepatic steatosis in addition to decreases in the mRNA of Cpt1a, Cat, and Cyp7a1, which is consistent with the previous observations in aged ArKO mice with hepatic steatosis [10]. By contrast, the ArKO/TNFR1KO mice, which also developed severe hepatic steatosis, maintained high levels of lipogenic gene expression including of Me1, Fasn, and Scd1. These observations suggest that TNF-α signaling through TNFR1 is required for the adaptation of hepatic cells to excessive fat accumulation via a reduction in fatty-acid synthesis. A marked elevation of Scd1 mRNA expression was notable even at the stage preceding the development of hepatic steatosis in the ArKO and ArKO/TNFR1KO mice. It has been reported that one product of Scd1, oleic acid, caused endoplasmic reticulum stress in the liver and worsened the pathology of hepatic steatosis [28]. Thus, unsaturated fatty acids, specifically monounsaturated fatty acids, synthesized in an uncontrolled fashion might be the primary event leading to metabolic abnormality in the livers of ArKO mice in the later stage of their life.

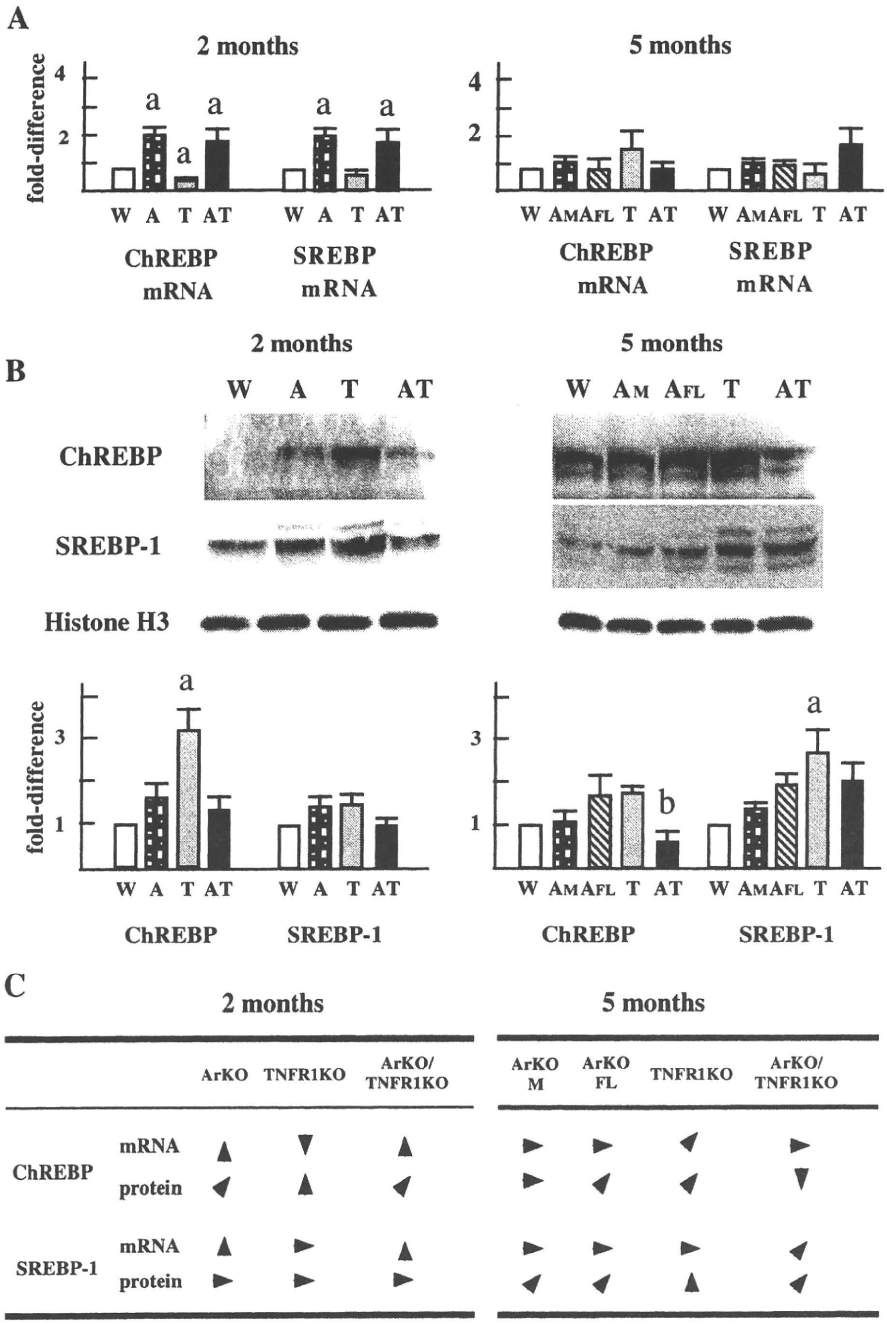

Fig. 6. Expression analysis of ChREBP and SREBP-1 in the livers. (A) Total RNA was extracted from livers of WT (W), ArKO (A, A_M, A_{FL}), TNFR1KO (T), and ArKO/TNFR1KO (AT) mice at 2 and 5 months of age. A_M and A_{FL} indicate ArKO mice that developed minimal and severe hepatic steatosis, respectively. Expression of mRNA for ChREBP and SREBP-1c were analyzed by real-time quantitative PCR, using primers described in the Materials and methods section. Results are presented as the mean ± SEM, $n = 5$/genotype. (a, $p<0.05$, vs. WT mice). Results were normalized to cyclophilin mRNA values. (B) Immunoblot analysis of ChREBP and SREBP-1 protein in hepatic nuclear extracts. Nuclear extracts were prepared from livers of mice at 2 and 5 months of age. ChREBP (95 kDa) and SREBP-1 (68 kDa) proteins were detected, respectively, with ChREBP and SREBP-1 polyclonal antibodies. Histone H3 (16 kDa) polyclonal antibody was used as loading controls to normalize the signal obtained. (a, $p<0.05$, vs. WT mice, and b, $p<0.05$, vs. ArKO mice, $n = 6$/group). Representative immunoblot images are shown. (C) Summary of the analysis. The mRNA and protein respectively indicate results from QRT-PCR analysis of total RNA and immunoblot analysis of nuclear extracts. Horizontal arrowheads, upward arrowheads, arrowheads pointing up, downward arrowheads respectively indicate no changes, increases, increases without statistical significance and decreases as compared to the expression levels of WT mice.

In the present study, we measured the expression levels of ChREBP and SREBP-1; both are transcription factors contributing to the high rate of lipogenesis in liver [29]. Expression of mRNAs for both factors was significantly elevated in ArKO and ArKO/TNFR1KO mice at 2 months of age. Nevertheless, the elevated gene expression did not clearly reflect in their nuclear contents. Inconsistencies in mRNA and nuclear protein contents of ChREBP were also observed in TNFR1KO mice at 2 months of age, where mRNA for the factor was expressed at significantly lower levels than that in the WT mice, while its nuclear protein content was elevated. These observations thus support the fact that post-transcriptional regulation on ChREBP and SREBP-1 is important for controlling their nuclear actions [30] under the estrogen-deficient conditions as well. The nuclear ChREBP and SREBP-1 protein contents in the liver of ArKO mice that developed severe hepatic steatosis were not significantly different from those in ArKO mice with minimal steatosis. Expression of mRNA for Fasn and

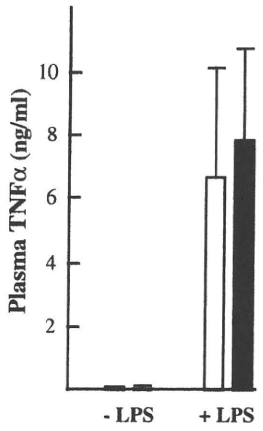

Fig. 7. TNF-α release in WT and ArKO mice after the injection of LPS. WT (open bar) and ArKO (closed bar) mice at 5 months of age were injected saline alone (−LPS) or LPS (10 ng/g of body weight) (+LPS). Plasma were prepared 1 h after the injection and used for the determination of TNF-α concentrations. Each value represents the mean ± SEM of five mice for LPS treatment and of seven mice for saline treatment.

Acaca genes, which are targets of SREBP-1 and involved in lipogenesis, was, however, induced less markedly in the former mouse models when compared to the latter. Furthermore nuclear protein contents of ChREBP in ArKO/TNFR1KO mice was significantly lower than that in the WT mice, while the former developed severe steatosis. These observations therefore suggest that nuclear protein levels of ChREBP and SREBP-1 might not necessarily associate with elevated expression of genes related to fatty-acid synthesis in the current animal models. Transcriptional activities of ChREBP and SREBP-1 are proposed to require cooperation with other nuclear factors to be functional [29]. Thus further studies including a specific technique to identify interacting factors or to inhibit hepatic expression of ChREBP and/or SREBP-1 genes are required to establish their exact roles in hepatic accumulation of lipid under estrogen-deficient conditions.

Adipose tissue-derived hormones such as adiponectin and leptin play important roles [31]; the former is known to be regulated reciprocally by TNF-α. However, in the current study, equivalent levels of plasma adiponectin were detected among the ArKO, ArKO/TNFR1KO, and WT mice, indicating that impairment in adiponectin production is not a major cause of the development of obesity in estrogen-deficient animal models in the regulation of whole-body energy homeostasis; nevertheless, it remains to be examined whether signaling systems that act through adiponectin receptors function properly under estrogen-deficient conditions. We did not detect a positive correlation between food-intake and the development of hepatic steatosis. Furthermore, serum leptin levels were elevated 2- to 3-fold in ArKO and ArKO/TNFR1KO mice compared to the levels in WT mice at 5 months of age, consistent with the previous reports [32,33]. The development of leptin-resistant phenotypes in estrogen-deficiency was also documented in mice lacking the ER-α gene [34].

The relevance of the estrogen functions in lipid metabolism in humans is suggested by patients with estrogen-signaling deficiency: a patient with an ER-α deficiency who showed glucose intolerance and hyperinsulinemia [35] and men with an aromatase gene mutation showing hyperinsulinemia [36]. Furthermore, the decline in estrogen function with the menopause is well recognized to be associated with spontaneous increases in serum proinflammatory cytokines including TNF-α, which might be the cause of the development of menopause-associated disorders such as bone loss, disturbance of vascular homeostasis, and atherosclerosis [37]. Thus, further studies on the interactions between regulatory systems mediated by estrogen and TNF-α are necessary to help the development of methods for the prevention and treatment of disorders in postmenopausal women.

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特集：炎症と動脈硬化

慢性肝疾患と動脈硬化

馬渡誠一　宇都浩文　坪内博仁

V. 各種病態と動脈硬化

慢性肝疾患と動脈硬化

馬渡誠一　宇都浩文　坪内博仁

Chronic liver disease and arteriosclerosis

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Abstract

The liver is the main metabolic organ of the body and is strongly associated with lifestyle-related diseases in which abnormal metabolism of glucose and lipid are the main manifestations. Recently, the prevalence of nonalcoholic fatty liver disease (NAFLD), including nonalcoholic steatohepatitis (NASH), has been increasing due to a higher rates of obesity. It has been reported that the presence of NAFLD/NASH and associated liver dysfunction are predictors for cardiovascular disease. In addition, attention has been paid to the link between chronic hepatitis C and lifestyle-related diseases such as obesity and insulin resistance. Atherosclerosis is an important risk factor for cardiovascular disease and is associated with lifestyle-related diseases. Thus, chronic liver disease seems to be strongly associated with atherosclerosis. Cardiovascular disease induced by atherosclerosis should be attended to along with liver cirrhosis and hepatocellular carcinoma, and medications for lifestyle-related diseases are needed in patients with chronic liver disease.

Key words: nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), chronic viral hepatitis, cardiovascular disease, lifestyle-related disease

はじめに

肝臓は生体における代謝の中心臓器であり，糖代謝異常や脂質代謝異常を呈する生活習慣病は肝臓と深いかかわりがある．最近は肥満人口の増加により，非アルコール性脂肪肝炎(nonalcoholic steatohepatitis: NASH)を含めた非アルコール性脂肪性肝疾患(nonalcoholic fatty liver disease: NAFLD)が増加している．NAFLD/NASHの存在や，それに伴う肝機能異常は心血管系疾患の予測因子である可能性が報告されている．また，ウイルス性慢性肝疾患であるC型慢性肝炎と肥満やインスリン抵抗性などの生活習慣病との関連も注目されている．動脈硬化は心血管系疾患の重要な原因の一つであり，生活習慣病とも密接に関連していることから，慢性肝疾患は動脈硬化と深くかかわっていると考えられる．

本稿では，NAFLD/NASHやC型慢性肝炎などの慢性肝疾患と動脈硬化との関連について概

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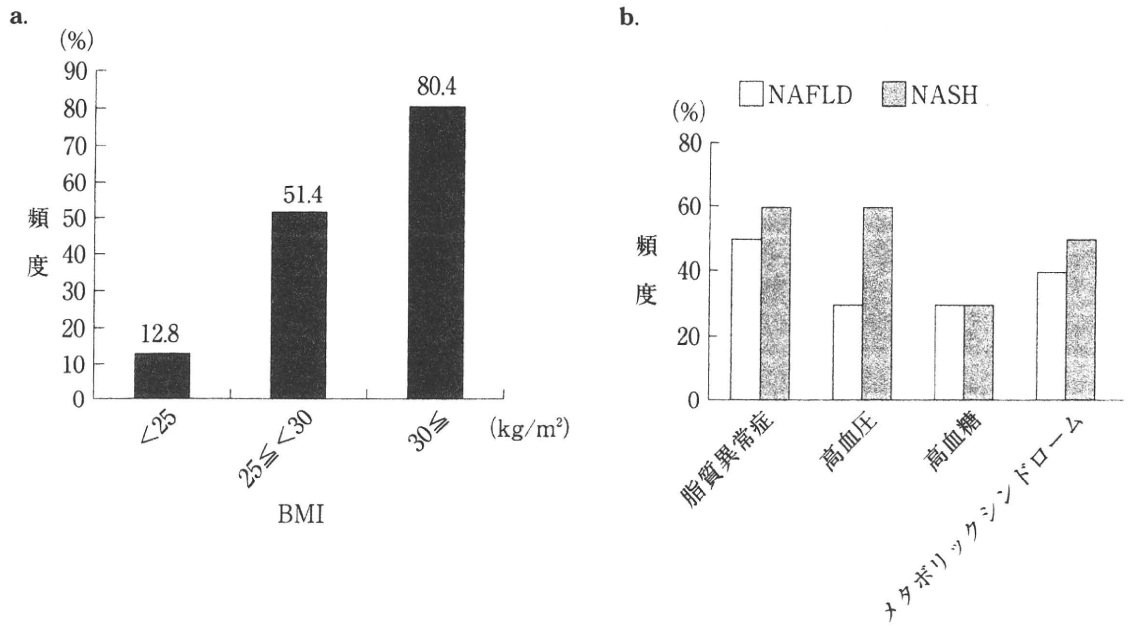

図1　肥満と脂肪肝の関連(a)およびNAFLD/NASHにおける合併症の頻度(b)
(文献[1,4]より改変)

説する.

1. NAFLD/NASHと動脈硬化

a. NAFLD/NASHの概念

ウイルス性肝疾患や自己免疫性肝疾患などがなく，明らかな飲酒歴もない(アルコール摂取量がエタノール換算で20g/日以下)にもかかわらず，アルコール性肝障害に類似した，大滴性の肝脂肪沈着を組織学的特徴とする肝障害を呈する肝疾患をNAFLDと定義する[1]．NAFLDの大部分は，肥満，糖尿病，高インスリン血症，脂質異常症を伴っており，NAFLDは肝臓におけるメタボリックシンドロームの表現型である．NASHは，NAFLDの中で，肝の脂肪化に加えて肝細胞の壊死，変性，線維化を伴っている病態であり，肝硬変に進展し，肝癌を発症する可能性がある疾患である．

NASHの発症機序として，まず，肝細胞へトリグリセリド(triglyceride: TG)が沈着し(first hit)，更に酸化ストレスなどの肝細胞を傷害する刺激が加わり(second hit)，NASHが発症するという，two-hit theory[2]が広く知られている．過食や運動不足といった過栄養状態を基盤として内臓脂肪組織にTGの過剰蓄積が起こり，それがTNF-αやアディポネクチンなどのアディポサイトカインの分泌異常やインスリン抵抗性を引き起こす．インスリン抵抗性は，NAFLD発症やNASHの進展に重要な因子である[1]．また，肝細胞障害(second hit)の機序として，酸化ストレス，腸内細菌由来のエンドトキシン，脂肪酸，アディポサイトカインなどの関与が想定されている[1]．

b. NAFLD/NASHの予後と心血管系疾患

肥満者(BMI≧25kg/m²)は，脂肪肝を高頻度に合併し，特にBMI 30kg/m²以上の人の脂肪肝の合併頻度は約80％である(**図1-a**)．また，NAFLD/NASHでは，脂質異常症，高血圧，高血糖を高率に合併している(**図1-b**)[1,3]．

NAFLD/NASHの予後については，NAFLD/NASHと診断された118人を28年間経過観察した報告では，NAFLD群，NASH群はコントロール群と比しそれぞれ1.69倍，1.86倍死亡率が高く，その死因では心血管系疾患が最も多く，肝外悪性腫瘍や肝疾患関連死がそれに次ぐ[4]．すなわち，NAFLD/NASHでは心血管系疾患がその予後に大きく関与する．

肝機能異常と心血管系疾患との関連については，約16万人を17年間経過観察した前向きコ

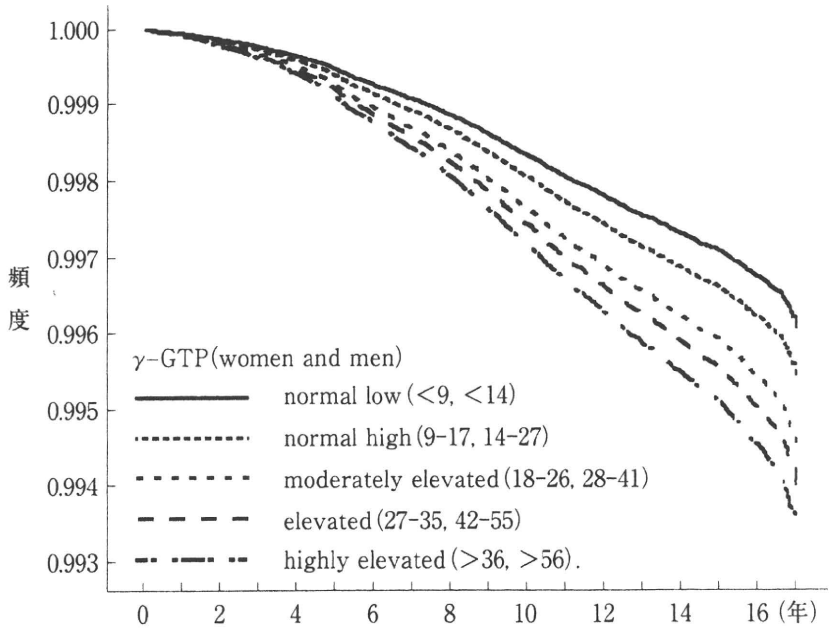

図2 γ-GTP別の心血管疾患による累積生存率
(文献[5]より改変)

ホート研究で，γ-GTPは心血管疾患死の独立した危険因子であることが報告されている(**図2**)[5]．また75gOGTT施行者を10年間経過観察したHoorn Studyの結果でも，ALT異常は，メタボリックシンドロームなどの危険因子を補正しても，冠動脈疾患の独立した危険因子である[6]．これらのことは，γ-GTPやALT異常の主な原因と考えられるNAFLD/NASHが心血管疾患死や冠動脈疾患の危険因子であることを示唆している．

NAFLDの組織学的な重症度と心血管系の危険因子である脂質異常の程度は相関し[7]，NAFLD患者はコントロール群と比較して，動脈硬化の指標である頸動脈内膜中膜肥厚度が増加し(**図3**)[8]，NAFLDの組織学的な炎症，線維化の程度が進展するほど，頸動脈内膜中膜肥厚度は増加する[9]．このように，NAFLDでは動脈硬化を合併するリスクが高く，動脈硬化の進展に伴い，予後が悪化すると考えられる．

c. 動脈硬化の原因である生活習慣病の治療とNAFLD/NASH[1]

生活習慣病は動脈硬化やNAFLD/NASHの危険因子であり，生活習慣病である糖尿病，脂質異常症，高血圧の治療は動脈硬化だけでなく，NAFLD/NASHの治療にも有効である．糖尿病治療薬であるインスリン抵抗性改善薬はNASH患者でのALT低下，脂肪肝改善(肝トリグリセリド含量)，アディポネクチン上昇などの効果が明らかにされている．また，糖尿病治療薬であるメトホルミンはNASH患者のALTを低下させる．脂質異常症治療薬のスタチン系薬剤，降圧剤のアンジオテンシン変換酵素阻害薬やアンジオテンシンII受容体阻害薬もNAFLDにおける肝障害を改善する可能性がある．これらの薬剤のNAFLD/NASHに対する効果は，生活習慣病の病態改善を介した効果と考えられるが，アンジオテンシンII受容体阻害薬は肝線維化を抑制することも報告されており，NASHの病態に直接的に作用する可能性もある．NAFLD/NASHは，動脈硬化やその危険因子と密接に関連しており，NAFLD/NASHは肝疾患としてだけでなく，動脈硬化を含めた全身疾患としてとらえることが重要である．

2. 肝硬変，ウイルス性肝炎と動脈硬化

a. 肝硬変と動脈硬化

肝硬変は原因のいかんを問わず肝障害の終末像で，肝細胞壊死と再生が繰り返された結果，肝臓全体に線維化と小葉構造の改築(偽小葉の

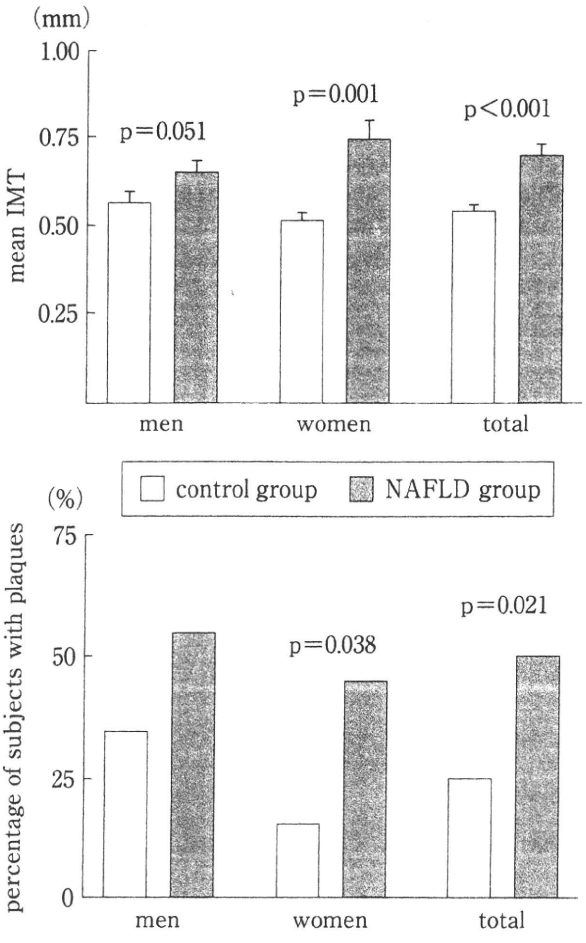

図3　NAFLDにおける頸動脈内膜中膜肥厚度とプラーク形成頻度(文献[8]より引用)

形成)が生じた状態である．肝硬変では，インスリン抵抗性を呈することが多く，糖尿病を合併する頻度も高い．インスリン抵抗性や糖尿病は動脈硬化のリスクとなるが，剖検580例での検討では，冠動脈疾患は肝硬変患者には少ないとされている[10]．その理由として，肝硬変では，血小板数，凝固因子および血清コレステロール値の低下や，血圧の低下などがみられるためと考えられる．また，肝硬変に合併した糖尿病では，一般の糖尿病患者と比べて罹病期間が短く，動脈硬化を促進するには至らないことが推測されている[11]．しかし，NASHによる肝硬変は，肝硬変に進展する以前から動脈硬化の危険因子を多数有しており，これらの因子がNASHの病態の増悪とともに冠動脈疾患の発症にも深く関与し，その頻度も増加すると考えられる[12]．また，少量のアルコール摂取は心血管系疾患を減らすといわれているが，アルコール性肝硬変は心血管系疾患の独立した危険因子であるとの報告もあり[13]，アルコールの飲酒量や飲酒頻度などアルコール摂取の詳細な状況と動脈硬化との関連についての検討が必要であると考えられる．

b. C型肝炎と動脈硬化

我が国では，肝硬変の原因としてHCV感染が最も多く約61％であり，HCV感染による肝硬変は，その他の原因による肝硬変より糖尿病の合併率が高い[14]．また肝線維化が軽度の，stageが0もしくは1のC型慢性肝炎患者では，健常人と比較しインスリン抵抗性の指標であるHOMA-IR(homeostasis model assessment-insulin resistance)は有意に高く[15]，C型肝炎ウイルス(HCV)コアタンパクはインスリンシグナル伝達経路を阻害して，インスリン抵抗性を誘導する[16]．更に，HCV感染が頸動脈プラークや頸動脈内膜中膜肥厚度に関連し，HCV感染は動脈硬化の独立した危険因子であると報告されている[17]．このように，HCVによる慢性肝炎や肝硬変ではインスリン抵抗性や糖尿病の合併頻度が健常者より高く，動脈硬化との関連が推測される．しかし，HCV感染と動脈硬化との関連性はないという報告もあり，現時点では一定の見解はない．HCV感染におけるインスリン抵抗性は，動脈硬化よりもむしろ肝脂肪化や肝線維化の進行といった，肝病変の進展に大きくかかわっていると考えられる．

3. その他の慢性肝疾患と動脈硬化

HBs抗原陽性者を17年間経過観察し，動脈硬化関連死との関連を検討した成績では，HBsAg陽性は動脈硬化関連死および心血管系疾患死の要因とならないと報告されている[18]．原発性胆汁性肝硬変(primary biliary cirrhosis: PBC)は中年女性に好発する慢性進行性の胆汁うっ滞性肝疾患で，脂質異常症を高率に有する．PBC患者における脂質異常症は，それだけでは動脈硬化の危険因子にならないと報告されているが[19]，フィブラート系製剤やスタチン系製剤といった脂質異常改善薬は，PBCそのものの病態の改善に寄与し有用である．

おわりに

慢性肝疾患の中で，特にNAFLD/NASHは動脈硬化の危険因子である生活習慣病と密接に関連することから，当然，動脈硬化性疾患とも関連している．慢性肝疾患の診療にあたっては，肝硬変や肝癌だけでなく，動脈硬化による心血管疾患の発症も念頭に置き，生活習慣病の改善を基本とした治療を行う必要がある．また，現時点では，NAFLD/NASHの標準的治療法は確立されていないのが現状であり，まず，合併する肥満，糖尿病，高血圧などの治療を適切に行うことが重要である．更にNASHを対象とした安全で有効性の高い薬剤の開発が望まれる．

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