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ORIGINAL ARTICLE

Raloxifene Ameliorates Liver Fibrosis of Nonalcoholic Steatohepatitis Induced by Choline-Deficient High-Fat Diet in Ovariectomized Mice

Fangqiong Luo[1] · Masatoshi Ishigami[1] · Koichi Achiwa[1] · Yoji Ishizu[1] · Teiji Kuzuya[1] · Takashi Honda[1] · Kazuhiko Hayashi[1] · Tetsuya Ishikawa[1] · Yoshiaki Katano[2] · Hidemi Goto[1]

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Abstract

Background and Aim The prevalence of nonalcoholic fatty liver disease (NAFLD) is higher in men than in women, but according to some epidemiological studies, this gender difference disappears after menopause. Estrogen therapy protects against NAFLD and nonalcoholic steatohepatitis (NASH) after menopause. We investigated the therapeutic effect of raloxifene, a second-generation selective estrogen-receptor modulator, on NASH induced by a choline-deficient high-fat (CDHF) diet in female ovariectomized (OVX) mice.

Methods Seven-week-old female C57BL/6J mice were divided into three experimental groups as follows: (1) sham operation (SHAM group), (2) ovariectomy (OVX group), and (3) ovariectomy + raloxifene (intraperitoneal injection, 3 mg/kg body weight/day; OVX + RLX group). These three groups of mice were fed a CDHF diet for 8 weeks; choline-sufficient high-fat (CSHF) diet was used as control diet. Serum biochemical indicators of hepatic function and liver histological changes were evaluated.

Results Compared with CSHF diet, ovariectomy enhances liver injury and fibrosis in CDHF diet-fed mice. Serum alanine aminotransferase (ALT) levels were significantly lower in the OVX + RLX group than in the OVX group. The OVX group developed extensive steatosis with inflammation and fibrosis. Lobular inflammatory scores and fibrosis staging in the OVX + RLX group were significantly lower than in the OVX group. Furthermore, the OVX + RLX group exhibited significantly higher expression of hepatic estrogen receptor-α, which was significantly lower in the OVX group than in the SHAM group.

Conclusions Raloxifene may ameliorate progression of liver fibrosis of NASH induced by CDHF diet in ovariectomized female mice, and up-regulation of estrogen receptor-α may play an important role in the beneficial effects of raloxifene on NASH.

Keywords Nonalcoholic steatohepatitis · Raloxifene · Ovariectomy · Estrogen

✉ Masatoshi Ishigami
masaishi@med.nagoya-u.ac.jp

Fangqiong Luo
luo_fangqiong@hotmail.com

Koichi Achiwa
kachiwa@med.nagoya-u.ac.jp

Yoji Ishizu
y-ishizu@med.nagoya-u.ac.jp

Teiji Kuzuya
tkuzuya@med.nagoya-u.ac.jp

Takashi Honda
honda@med.nagoya-u.ac.jp

Kazuhiko Hayashi
kazuh@med.nagoya-u.ac.jp

Tetsuya Ishikawa
ishikawa@met.nagoya-u.ac.jp

Yoshiaki Katano
ykatano@fujita-hu.ac.jp

Hidemi Goto
hgoto@med.nagoya-u.ac.jp

[1] Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan

[2] Department of Internal Medicine, School of Medicine, Banbuntane Hotokukai Hospital, Fujita Health University, Nagoya, Japan

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Abbreviation

SERM	Selective estrogen-receptor modulator
FFA	Free fatty acids
CDHF	Choline-deficient high-fat
CSHF	Choline-sufficient high-fat
OVX	Ovariectomized
NASH	Nonalcoholic steatohepatitis
ALT	Alanine aminotransferase
NAFLD	Nonalcoholic fatty liver disease
AST	Aspartate transaminase
SMA	Smooth muscle actin
HSC	Hepatic stellate cell
NAS	NAFLD activity score
VLDL	Very low-density lipoprotein
TGF	Transforming growth factor
ER	Estrogen receptor

Introduction

Nonalcoholic fatty liver disease (NAFLD), one of the most common causes of chronic liver disease [1], encompasses a spectrum of conditions associated with lipid deposition in hepatocytes. NAFLD ranges in severity from steatosis (simple fatty liver), to nonalcoholic steatohepatitis (NASH: fatty changes with inflammation and hepatocellular injury or fibrosis), to advanced fibrosis and cirrhosis [2].

Many studies have shown that postmenopausal women exhibit increased body weight gain, insulin resistance, hyperlipidemia, and visceral fat accumulation, all of which are known risk factors for NAFLD [3, 4]. Recent epidemiological reports have shown that NAFLD is more common in men than in women [4, 5]. In women, NASH is more prevalent in older patients, whereas in men, the prevalence of NASH increases in middle ages [6].

NAFLD occurs less frequently in women who take hormone replacement therapy (HRT) after menopause [7, 8]. Furthermore, HRT protects against hepatic fibrosis in postmenopausal women with chronic hepatitis C [9]. Estrogen also suppresses liver fibrosis in ovariectomized female rats and prevents hepatic stellate cell activation and reduced type 1 collagen production in cultured liver cells [10]. However, circulating estrogen that engages estrogen receptors promotes breast and uterine proliferation that contributes to estrogen-responsive cancers [11, 12].

Selective estrogen-receptor modulators (SERMs), which exert tissue-selective estrogen-like activity, manifest their effects by binding to intranuclear estrogen receptors and altering gene transcription [13]. In the liver, the SERMs have been proposed to function as estrogen agonists to protect hepatocytes against inflammation and control liver fibrosis [14]. Therefore, these agents might be useful for treatment of postmenopausal women with NASH or NAFLD and might avoid the increased risk of estrogen-responsive cancers induced by HRT.

Raloxifene, a second-generation SERM, exerts anti-estrogenic effects in breast and uterus and estrogenic effects in bone; hence, it is currently used for the treatment of osteoporosis in postmenopausal women. Unlike the first-generation SERM tamoxifen, raloxifene does not increase the incidence of uterine or endometrial cancer [11].

In this study, we examined the effects of raloxifene on NASH induced by a choline-deficient high-fat (CDHF) diet in mice [15]. Furthermore, to investigate the mechanism underlying the effects of raloxifene, we measured the expression levels of genes encoding estrogen receptors.

Materials and Methods

Animals and Drugs

Seven-week-old female C57BL/6J mice were obtained from SLC Japan (Hamamatsu, Japan). The animals were housed in a controlled environment (temperature 23 ± 1 °C, humidity 50 ± 10 %, 12-h light cycle/12-h dark cycle) in the institution's animal facility with free access to food and water.

Experimental Design

The mice were divided into three groups. One group underwent sham operation; the other two groups received bilateral ovariectomy (OVX) and were maintained for 1 week during recovery on a free CE2 diet (CLEA Japan, Inc., Tokyo, Japan). Thereafter, all mice were fed a CDHF diet or choline-sufficient high-diet (CSHF) diet as a control diet (Oriental Yeast CO., Tokyo, Japan) for 8 weeks. The OVX mice were treated with intraperitoneal injection of 3 mg/kg body weight of raloxifene daily [13] or vehicle control (0.9 % sodium chloride) for 10 days before killing. All studies were approved by the Animal Care and Research and Development Committees of the Division of Experimental Animals of Nagoya University.

Collection of Biological Samples

For serum analysis, blood was collected from the heart at the time of killing following a 12-h fast. Livers were resected for biochemical and histological analyses.

Biochemical Analysis

Blood chemistry analysis was entrusted to SRL, Inc. (Tokyo, Japan). Total protein, albumin, total bilirubin, free

fatty acid, total cholesterol, triglycerides, aspartate transaminase (AST), alanine aminotransferase (ALT), cholinesterase, and glucose were evaluated.

Histological Examination

Liver tissues were removed and fixed for 16 h in 4 % paraformaldehyde in phosphate-buffered saline and then dehydrated and embedded in paraffin. Liver tissue sections (3 μm) were stained with hematoxylin and eosin and Sirius red (Sigma-Aldrich, St. Louis, MO) for histological examination. The ratio of the hepatic steatosis—and Sirius red—positive areas was calculated from 10 microscopic fields for each tissue section, using the BZ-II Image Analysis Application (KEYENCE, Osaka, Japan).

mRNA Expression Analysis

Total RNA from liver tissues was isolated by the Trizol method (Life Technologies, Tokyo, Japan). Reverse transcription of total RNA for cDNA synthesis was carried out using the Revertra-Ace qPCR RT kit (TOYOBO, Osaka, Japan). Levels of mRNAs were assessed by TaqMan real-time quantitative polymerase chain reaction (qPCR) on an MX3005P sequence detection system (Agilent Technologies, Tokyo, Japan). Samples were analyzed in duplicate using commercially available TaqMan gene-specific primers: Mm03024053_m1 (transforming growth factor, beta 1 TGF-β1), Mm00433149_m1 (estrogen receptor alpha ER-α), Mm00446190 (interleukin 6, IL-6), Mm00434228 (interleukin 1 beta, IL-1β), Mm01546133 (smooth muscle α-actin α-SMA), Mm00440502 (inducible nitric oxide synthase iNOS), and Mm00446968_m1 (hypoxanthine guanine phosphoribosyl transferase, HPRT). Expression levels were normalized against the corresponding level of HPRT mRNA. PCR conditions were as follows: one cycle at 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 22 s.

Immunohistochemical Staining and Morphometric Image Analysis

Immunohistochemical staining for α-smooth muscle actin (SMA) was performed to detect activated hepatic stellate cells (HSCs). Paraffin sections of liver specimens were immunostained with anti-SMA monoclonal antibody (ab5694, 1:100, Abcam, Tokyo, Japan) or antibody against estrogen receptor alpha (MC20, 1:200, Santa Cruz Biotechnology, Tokyo, Japan) using the DAKO Envision Labeled Polymer Anti-Rabbit system (Dako Cytomation, Tokyo, Japan). Antigen retrieval was performed by heating for 5 min in antigen-reactive buffer in a microwave.

Western Blotting

Frozen tissue was homogenized in RIPA buffer (Sigma) containing complete mini protease inhibitor cocktail (Roche). Protein extracts were resolved on a SDS sample buffer. Membranes were blocked with 5 % skimmed milk (1 h, RT) and then incubated overnight at 4 °C with the anti-ERα antibody (1:100 dilution) (Santa Cruz, MC-20: sc-542) or anti-β-actin antibody (1:10,000) (Sigma-Aldrich) under gentle agitation. Subsequently, the membranes were washed (3 × 15 min) in PBS containing 1 % Tween-20 (Duchefa Biochemie) and then incubated with the anti-rabbit (1:2000 dilution) secondary antibody for 1 h at room temperature under gentle agitation. Following a repetition of the washing steps, the antibody-targeted proteins were visualized with the use of the Amersham ECL Western Blotting Analysis System (GE Healthcare) followed by autoradiograph film exposure.

Determination of Liver Lipid Levels

Total liver lipids were extracted using a modification of the method of Folch [16]. Briefly, frozen liver tissues (20 mg) were homogenized and extracted twice with the chloroform: methanol (2:1 v/v) solution. The organic layer was dried and resolubilized in chloroform. An aliquot was resuspended in isopropanol for the determination of triglyceride (TG) mass. TG levels were determined using a commercial colorimetric method (Wako Pure Chemicals Industries, Osaka, Japan).

Statistics

All analyses were performed using the Statistical Package for Social Sciences (SPSS), version 22. The data were expressed as bar graphs showing mean ± SEM. Differences between groups were determined according to Student's t test. A p value less than 0.05 was considered to reflect a statistically significant difference.

Results

CDHF Diet Enhances Liver Injury and Fibrosis in Female Mice

Both in SHAM and in OVX mice, body weight was significantly decreased in CDHF diet-fed mice compared with CSHF diet-fed mice (Table 1), liver-to-body weight ratio was significantly higher in CDHF diet-fed mice than that in CSHF diet-fed mice, while in CDHF diet-fed mice, the OVX group has lower liver-to-body weight ratio than SHAM group. Uterus weight was significantly decreased in

Table 1 CDHF diet induced liver injury in OVX mice

	CSHF diet		CDHF diet	
	SHAM ($n = 8$)	OVX ($n = 8$)	SHAM ($n = 8$)	OVX ($n = 8$)
Body weight (g)	20.21 ± 0.42	22.60 ± 0.63*	18.32 ± 0.22$^{\Delta}$	17.64 ± 0.72$^{\Delta}$
Liver/body weight (%)	4.34 ± 0.23	4.25 ± 0.20	7.016 ± 0.33$^{\Delta}$	5.66 ± 0.33*,$^{\Delta}$
Uterus weight (mg)	69.5 ± 7.26	17.3 ± 1.19*	43.10 ± 2.27	17.3 ± 1.19*
Perigonadal adipose tissue weight (mg)	0.162 ± 0.016	0.508 ± 0.055*	0.183 ± 0.013	0.190 ± 0.035$^{\Delta}$
Serum				
Total protein (g/dL)	4.60 ± 0.078	4.60 ± 0.11	4.7 ± 0.087	3.99 ± 0.42
Albumin (g/dL)	3.20 ± 0.038	3.23 ± 0.04	3.2 ± 0.04	2.63 ± 0.31
Total bilirubin (mg/dL)	0.065 ± 0.008	0.075 ± 0.012	0.170 ± 0.012$^{\Delta}$	0.34 ± 0.17
Glucose (mg/dL)	158.3 ± 21.1	143.5 ± 5.8	95.6 ± 9.8$^{\Delta}$	65.8 ± 7.5*,$^{\Delta}$
Total cholesterol (mg/dL)	79.0 ± 4.8	80.3 ± 6.3	41.1 ± 7.4$^{\Delta}$	60.1 ± 11.2
AST (IU/L)	59.38 ± 3.33	82.5 ± 7.9*	127.8 ± 7.6$^{\Delta}$	204.3 ± 41.6*,$^{\Delta}$
ALT (IU/L)	24.9 ± 3.0	51.9 ± 10.3*	143.9 ± 11.0$^{\Delta}$	167.0 ± 24.0$^{\Delta}$
Cholinesterase (IU/L)	25.5 ± 0.71	31.86 ± 1.06*	31.1 ± 0.5	29.6 ± 2.0
Triglyceride (mg/dL)	15.75 ± 2.33	16.75 ± 2.10	22.13 ± 1.29$^{\Delta}$	24.00 ± 1.39$^{\Delta}$
Free fatty acid (uEQ/L)	411.0 ± 36.8	4218 ± 25.98	544.4 ± 43.9	642.9 ± 49.2$^{\Delta}$
Histology				
Fibrosis score	0	0	0.050 ± 0.276$^{\Delta}$	2.00 ± 0.189*,$^{\Delta}$

Data are mean ± SEM

ALT alanine aminotransferase, *AST* aspartate aminotransferase

* $p < 0.05$ compared with SHAM group

$^{\Delta}$ $p < 0.05$ compared with CSHF diet

OVX mice. CDHF diet did not affect uterus weight compared with CSHF diet. In CSHF diet-fed mice, perigonadal adipose tissue weight was significantly increased in OVX group compared with SHAM group, while in CDHF diet-fed mice, perigonadal adipose tissue weight in OVX group decreased compared with CSHF diet-fed mice. Serum triglyceride, free fatty acid, and ALT and AST levels were significantly increased in CDHF diet-fed mice compared with CSHF diet-fed mice. Glucose levels were significantly lower in CDHF diet-fed mice than that in CSHF diet-fed mice, because choline deficiency improves glucose tolerance [17]. AST levels were increased in OVX-CDHF diet compared with SHAM-CDHF diet. Histological analysis showed that fibrosis staging was significantly higher in CDHF diet-fed mice than that in CSHF diet. OVX group showed significantly higher fibrosis score than SHAM group in CDHF diet-fed mice. Due to the liver damage and steaotosis, the liver-to-body weight ratio was increased in SHAM mice fed CDHF diet than that fed CSHF diet; however, it was significantly decreased in OVX mice fed CDHF diet because of the more progressive liver fibrosis in OVX group than that in SHAM group.

CDHF diet induced the liver injury and fibrosis, and ovariectomy enhanced the liver injury and fibrosis progression in CDHF diet-fed mice. Based on these findings, we investigated the effects of raloxifene on steatohepatitis and fibrosis progression in OVX mice.

Administration of Raloxifene Ameliorates Liver Injury in OVX-CDHF Mice

No mice died during the experiment. Neither OVX operation nor raloxifene supplementation influenced body weight and perigonadal adipose tissue weight in CDHF-fed mice (Table 2).

Serum AST levels were significantly higher in the OVX group than in the SHAM group and lower in the OVX + RLX group than in the OVX group, but the differences were not statistically significant. ALT levels were significantly lower in the OVX + RLX group than in the OVX group. Albumin and free fatty acid levels were significantly higher in the OVX + RLX group than in the OVX group. There were no significant changes in total protein level, total bilirubin level, triglyceride, total cholesterol, or cholinesterase levels between the three groups. Glucose level was significantly reduced in OVX mice relative to SHAM mice, and raloxifene treatment increased the glucose level in OVX mice (Table 2).

Table 2 Administration of Raloxifene ameliorates liver injury in OVX-CDHF mice

	SHAM ($n = 8$)	OVX ($n = 8$)	OVX + RLX ($n = 8$)
Body weight (g)	18.32 ± 0.22	17.64 ± 0.72	17.63 ± 0.04
Liver/body weight (%)	7.016 ± 0.33	5.66 ± 0.33*	6.09 ± 0.34
Uterus weight (mg)	43.10 ± 2.27	17.3 ± 1.19*	18.1 ± 1.26
Perigonadal adipose tissue weight (mg)	0.183 ± 0.013	0.190 ± 0.035	0.176 ± 0.014
Serum			
Total protein (g/dL)	4.7 ± 0.087	3.99 ± 0.42	4.75 ± 0.18
Albumin (g/dL)	3.2 ± 0.04	2.63 ± 0.31	3.20 ± 0.08**
Total bilirubin (mg/dL)	0.170 ± 0.012	0.34 ± 0.17	0.143 ± 0.006
Glucose (mg/dL)	95.6 ± 9.8	65.8 ± 7.5*	102.0 ± 11.0**
Total cholesterol (mg/dL)	41.1 ± 7.4	60.1 ± 11.2	52.4 ± 4.4
AST (IU/L)	127.8 ± 7.6	204.3 ± 41.6*	137.8 ± 12.2
ALT (IU/L)	143.9 ± 11.0	167.0 ± 24.0	100.0 ± 7.1**
Cholinesterase (IU/L)	31.1 ± 0.5	29.6 ± 2.0	31.0 ± 1.3
Triglyceride (mg/dL)	22.13 ± 1.29	24.00 ± 1.39	23.88 ± 1.13
Free fatty acid (uEQ/L)	544.4 ± 43.9	642.9 ± 49.2	514.0 ± 26.2**

Data are mean ± SEM

ALT alanine aminotransferase, *AST* aspartate aminotransferase

* $p < 0.05$ compared with SHAM group

** $p < 0.05$ compared with OVX diet

OVX Mice Develop Pathological NASH, but Raloxifene Treatment Ameliorates Liver Injury in OVX-CDHF Mice

CDHF-fed OVX mice exhibited pathological findings of NASH including marked steatosis, lobular inflammation, and periportal or pericellular fibrosis. Little inflammatory infiltration and fibrosis were observed in SHAM liver, whereas both inflammatory cell infiltration and periportal fibrosis were observed in OVX liver, and the severity of these symptoms was reduced in OVX + RLX mice (Fig. 1a). Assessment of liver histology using NAFLD activity score (NAS) revealed no significant difference in steatosis score in the three groups (Fig. 1b); it also showed no difference in hepatic triglyceride content in these groups (Fig. 1c). OVX livers had higher lobular inflammation scores than SHAM livers, although the difference was not significant. Inflammation score was significantly lower in the OVX + RLX group than in the OVX group (Fig. 1d). Accordingly, the NAS score was lower in the OVX + RLX group than in the OVX group (Fig. 1e). Inflammatory cytokines IL-1β increased in OVX group although there was no significant difference ($p = 0.061$), and decreased in OVX + RLX group (Fig. 2a). IL-6 mRNA expression was also significantly increased in OVX group and significantly decreased in OVX + RLX group (Fig. 2b). Meanwhile, mRNA expression of TNF-α mRNA and iNOS did not show any difference between the three groups (Fig. 2c, d).

Raloxifene Treatment Ameliorates Liver Fibrosis in OVX Mice

Sirius red staining was performed to detect fibrosis in mouse liver. Remarkable pericellular fibrosis was observed in OVX liver, but very little fibrosis was observed in SHAM and OVX + RLX groups (Fig. 3a). α-SMA, a marker for activated hepatic stellate cells (HSCs), was observed in the OVX group, whereas the SHAM and raloxifene-treated groups exhibited very slight α-SMA positivity (Fig. 3a). The RNA expression of α-SMA was also increased in OVX group than that in SHAM group and decreased in the OVX + RLX group (Fig. 3e). According to the Brunt fibrosis staging system [18], fibrosis stage was significantly higher in the OVX mice than in the SHAM mice, but the score was reduced in the OVX + RLX group (Fig. 3b). The proportion of hepatic lobule area that was Sirius red-positive, reflecting the degree of fibrosis, was significantly higher in the OVX group than in the SHAM group, but significantly lower in the OVX + RLX group than in the OVX group (Fig. 3c). TGF-β1 plays a pivotal role in hepatic fibrosis by mediating the activation of HSCs and their production of extracellular matrix proteins. In accordance with the amelioration of the hepatic fibrosis, raloxifene significantly decreased the level of TGF-β1 mRNA (Fig. 3d).

Raloxifene Treatment Increased Liver Estrogen Receptor-α Expression in OVX Mice

There are three main types of estrogen receptors: estrogen receptor (ER)-α, ER-β, and ER-γ. ER-α is the only ER

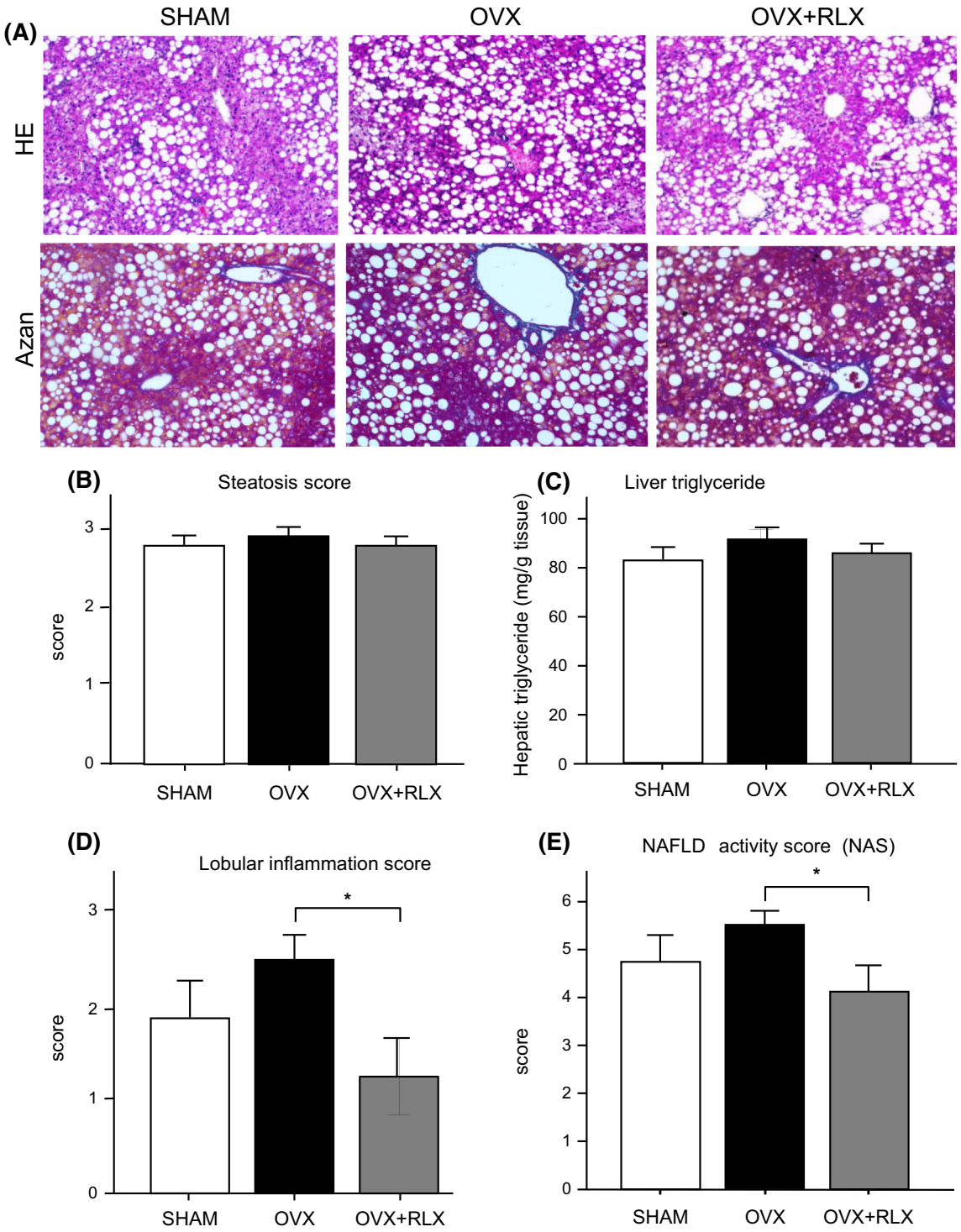

Fig. 1 OVX mice develop pathological NASH, but raloxifene treatment ameliorates liver histological injury in these mice. Sham-operated mice (SHAM), ovariectomized mice (OVX), and raloxifene-treated OVX mice (OVX + RLX) were fed a choline-deficient high-fat (CDHF) diet. Liver histology was evaluated by hematoxylin–eosin and azan staining (×200); representative slides are shown (**a**). Steatosis score (**b**), hepatic triglyceride content were assessed (**c**). Lobular inflammation score (**d**), and NAFLD activity score (NAS) (**e**) were determined. *$p < 0.05$

expressed in mouse liver [19]. Immunohistochemical staining for ER-α revealed that the protein was predominantly localized in the hepatocyte nucleus. ER-α staining in the liver was weaker in OVX mice than in SHAM mice, but more intense in raloxifene-treated OVX mice (Fig. 4a). We also confirmed by RT-PCR that the

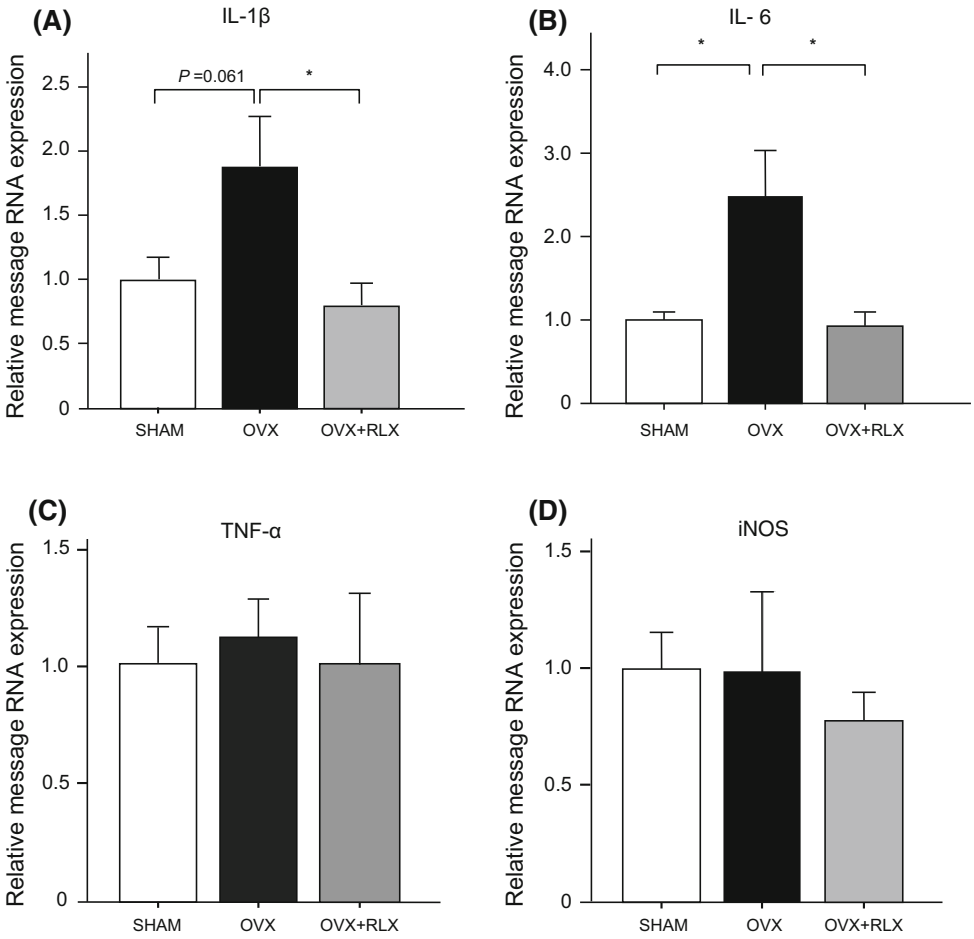

Fig. 2 Raloxifene treatment decreased liver inflammatory cytokines in OVX mice. Sham-operated mice (SHAM), ovariectomized mice (OVX), and raloxifene-treated OVX mice (OVX + RLX) were fed a choline-deficient high-fat (CDHF) diet. Relative expression of interleukin 1 beta (IL-1β), interleukin 6 (IL-6), inducible nitric oxide synthase (iNOS) and tumor necrosis factor α (TNF-α) were determined. $*p < 0.05$

relative expression of ER-α mRNA was significantly lower in the OVX group than in the SHAM group (Fig. 4b), but significantly higher in the OVX + RLX group than in the OVX group. Western blotting for ER-α protein also yielded similar results: lower liver ER-α expression in OVX group than in the SHAM group (Fig. 4c) and higher ER-α expression in raloxifene-treated OVX liver.

Discussion

NAFLD/NASH is one of the most common liver diseases in the world [1], although its pathogenesis is only partially understood. Recent reports reported accelerated progression of NAFLD/NASH in postmenopausal women [5, 20]. Moreover, postmenopausal women had an increased risk of developing more severe liver fibrosis than premenopausal women [21], indicating that estrogen deficiency can aggravate the progression of NAFLD/NASH. Choline, the major source of methyl groups in the diet, is an essential nutrient for lipid transport and production of very low-density lipoprotein (VLDL) [22] and thus could serve as a useful physiological animal model to study the progression of NASH. Fischer et al. [23] found that postmenopausal women are more susceptible to the risk of fatty liver in response to a low-choline diet and they also reported that they have a higher dietary requirement for choline than do premenopausal women [24]. Here, we demonstrated that ovariectomized mice fed a CDHF diet for 8 weeks developed NASH with increased steatosis, inflammation, and fibrosis, while SHAM mice only developed simple steatosis. We also found that raloxifene treatment mitigated liver injury and ameliorated liver histological inflammation and fibrosis in OVX mice fed by CDHF diet.

Estrogen receptor, a member of the nuclear hormone family, can be activated by estrogen to mediate gene transcription or interact with transcription factors. ER-α is the only one of the three major estrogen receptors (ER-α, ER-β, and ER-γ) to be expressed in mouse liver [19]. In rats, estrogen receptors are expressed in hepatocytes, Kupffer cells and stellate cells [25–27], but in mice the distribution of ER-α among these cell types has not been determined. Ovariectomy decreases the levels of ER-α protein and mRNA expression in hepatocytes from female

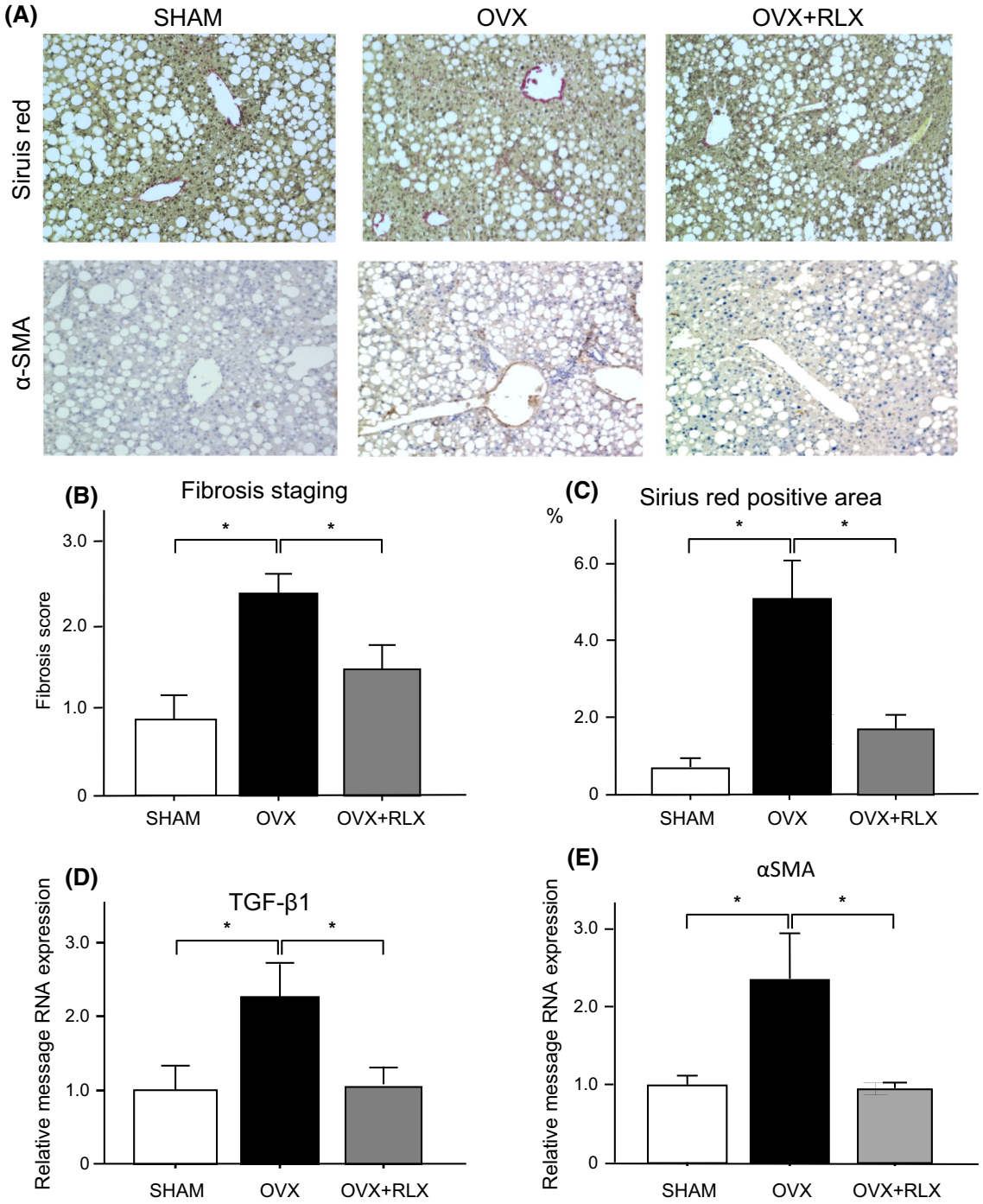

Fig. 3 Sham-operated mice (SHAM), ovariectomized mice (OVX), and raloxifene-treated OVX mice (OVX + RLX) were fed a choline-deficient high-fat (CDHF) diet. Histological hepatic fibrosis was evaluated by Sirius red staining and α-SMA immunohistochemical staining (×200); representative slides are shown (**a**). Fibrosis score (**b**), proportion of Sirius red-positive area (**c**), and relative expression of tumor growth factor beta 1 (TGF-β1) and α-SMA (**e**) were determined. *$p < 0.05$

rats, and estrogen treatment restores these levels [28, 29]. Raloxifene exerts its pharmacological action by mimicking estrogen and binding to estrogen receptor. As a selective estrogen-receptor modulator, raloxifene shows tissue-selective effects on estrogen receptor. It works as an estrogen-receptor antagonist in breast tissue, so it is used as a treatment for estrogen-sensitive breast cancer. It also acts an estrogen-receptor agonist in bone to treat for osteoporosis. Unlike tamoxifen, another type of SERMs, raloxifene showed estrogen-receptor antagonist effect on uterus,

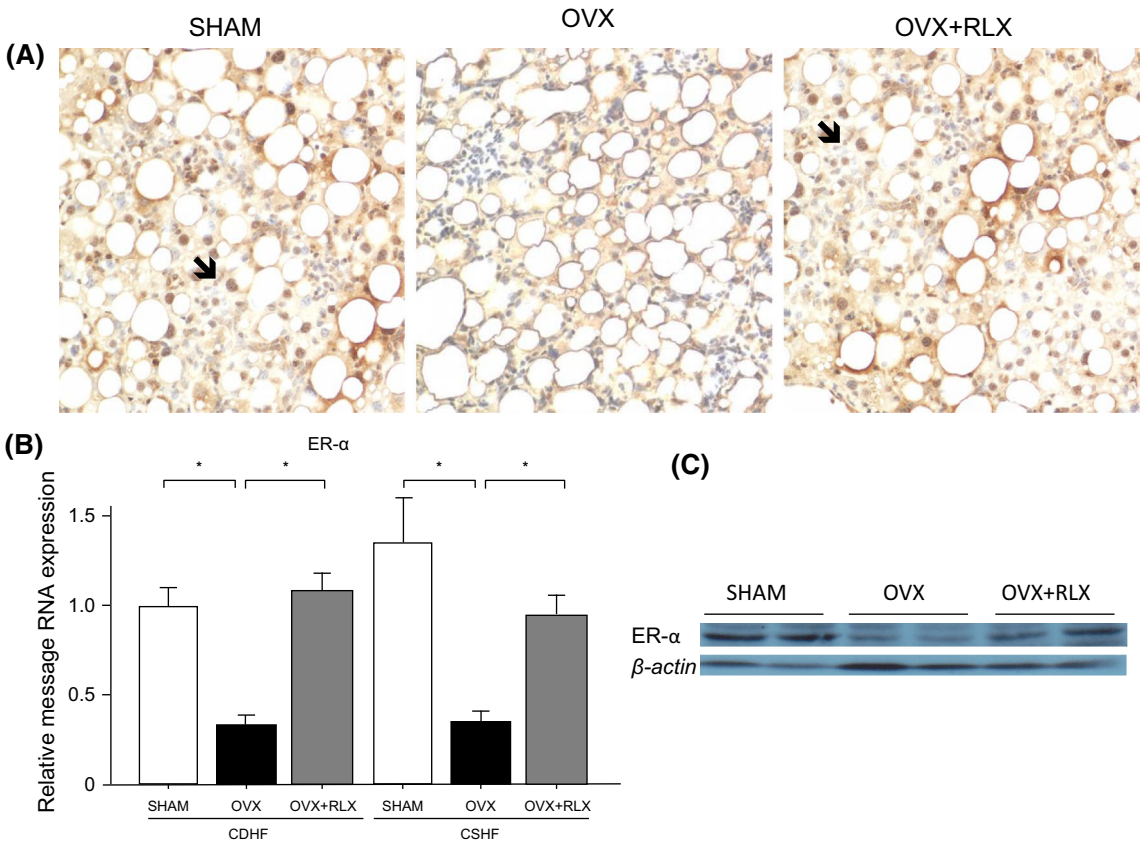

Fig. 4 Raloxifene up-regulated the estrogen receptor (ER)-α expression in the liver. **a** Sham-operated mice (SHAM), ovariectomized mice (OVX), and raloxifene-treated OVX mice (OVX + RLX) were fed a choline-deficient high-fat (CDHF) diet. Representative photomicrographs of liver subjected to immunohistological staining for ER-α are shown (**a**); *arrows* show nuclear estrogen receptor in SHAM and OVX + RLX liver. Relative expression of hepatic ER-α mRNA was assessed (**b**). Protein level of ER-α in liver was detected by Western Blotting (**c**). $*p < 0.05$

which was consistent with our finding that the uterus weight was not increased in raloxifene-treated ovariectomized mice. In liver, the effect of raloxifene on estrogen receptor remains unknown. Limited data revealed that raloxifene exerts estrogen-like effect to decrease liver fibrosis on chronic hepatitis C patients [30]. In our study, we demonstrated that raloxifene increased ER-α expression, indicating that raloxifene works as an estrogen-receptor agonist in liver.

Recent studies point to the importance of estrogen and ER-α in liver fibrosis both in vivo and in vitro. During liver fibrogenesis, HSCs play a central role [31]. Estrogen prevented reactive oxygen species and TGF-β production in cultured rat HSCs by suppressing NADH/NADPH oxidase activity [32]. In our study, fibrogenic gene, TGF-β, and α-SMA RNA expression were up-regulated in OVX mice, and raloxifene treatment improved the TGF-β and α-SMA expression. Hepatic ER was elevated after treatment with estradiol in carbon tetrachloride (CCl4)-induced fibrotic rats [29]; in that experiment, estradiol treatment led to parallel increase in the levels of ER and serum nitric oxide and the activity of hepatic nitric oxide synthase. Although no evidence supports a direct effect of raloxifene on hepatic oxidative stress, in vitro raloxifene is effective against oxidative stress-induced endothelial dysfunction [33]. In this study, iNOS mRNA expression did not show significant difference in the groups. Further research is needed to investigate the effect of raloxifene on oxidative stress in vivo.

In addition, in this study, serum glucose levels were lower in the OVX group than in the SHAM group, and these data are similar to those reported by a previous study [34]. In OVX rodent models, hepatic glycogen contents decrease [35, 36]. Twelve-hour fasting may induce a further reduction in hepatic glycogen content in OVX mice, resulting in the lower fasting serum glucose levels observed in this study.

In conclusion, raloxifene may slow or prevent the progression of liver fibrosis associated with NASH induced by CDHF diet in ovariectomized female mice, and up-regulation of ER-α may play an important role in these beneficial effects.

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Conflict of interest There is no conflict of interest with regard to this paper.

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