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Nuclear Factor Erythroid 2–Related Factor 2 Deficiency Results in Amplification of the Liver Fat-Lowering Effect of Estrogen[S]

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ABSTRACT

Transcription factor nuclear factor erythroid 2–related factor 2 (Nrf2) regulates multiple biologic processes, including hepatic lipid metabolism. Estrogen exerts actions affecting energy homeostasis, including a liver fat-lowering effect. Increasing evidence indicates the crosstalk between these two molecules. The aim of this study was to evaluate whether Nrf2 modulates estrogen signaling in hepatic lipid metabolism. Nonalcoholic fatty liver disease (NAFLD) was induced in wild-type and *Nrf2*-null mice fed a high-fat diet and the liver fat-lowering effect of exogenous estrogen was subsequently assessed. We found that exogenous estrogen eliminated 49% and 90% of hepatic triglycerides in wild-type and *Nrf2*-null mice with NAFLD, respectively. This observation demonstrates that Nrf2 signaling is antagonistic to estrogen signaling in hepatic fat metabolism; thus, *Nrf2* absence results in striking amplification of the liver fat-lowering effect of estrogen. In addition, we found the association of trefoil factor 3 and fatty acid binding protein 5 with the liver fat-lowering effect of estrogen. In summary, we identified Nrf2 as a novel and potent inhibitor of estrogen signaling in hepatic lipid metabolism. Our finding may provide a potential strategy to treat NAFLD by dually targeting Nrf2 and estrogen signaling.

Introduction

Estrogen, via its nuclear receptors estrogen receptor (ER) α and ERβ and membrane-bound ER, regulates energy homeostasis in addition to its well established essential roles in reproduction (Barros and Gustafsson, 2011; Xu et al., 2011). Many studies have demonstrated that, irrespective of gender, estrogen (predominantly relying on ERα) exerts body weight-lowering, adiposity-lowering, and antidiabetic effects through systematic and tissue-specific mechanisms in both rodents and humans. The pivotal roles of estrogen signaling in the metabolic network were thoroughly reviewed by several groups recently (Roepke, 2009; Barros and Gustafsson, 2011; Mauvais-Jarvis et al., 2013; Frank et al., 2014; Kim et al., 2014; Palmer and Clegg, 2015). The liver is one of the key organs coordinately controlling energy homeostasis, including lipid metabolism. Hepatic estrogen signaling is critical for maintaining lipid homeostasis in the liver (Han et al., 2014; Shen and Shi, 2015). Nonalcoholic fatty liver disease (NAFLD), primarily manifested by excessive fat accumulation in hepatocytes, has become the most common chronic liver disease worldwide (Demir et al., 2015). A number of studies consistently showed that estrogen displays a fat-lowering effect by presumably inhibiting lipogenesis and promoting lipolysis in the liver in a variety of animal models, including genetically manipulated mice, ovariectomized rodents, spontaneous obese mice, and rodents with diet-induced metabolic syndrome (Jones et al., 2000; Hewitt et al., 2004; Gao et al., 2006; Bryzgalova et al., 2008; Lundholm et al., 2008; Pedram et al., 2013; Zhang et al., 2013a; Han et al., 2014). However, the mechanisms behind those observations, especially how the fat-lowering action of estrogen is modulated in the liver, remain elusive.

As a leucine zipper motif-containing transcription factor, nuclear factor erythroid 2–related factor 2 (Nrf2) essentially mediates the cellular response against redox stress. Its target genes include those encoding cytoprotective proteins, such as detoxifying enzymes, glutathione synthesis enzymes, and antioxidant proteins, thereby positively or negatively contributing to carcinogenesis, drug resistance, and many degenerative diseases (Dodson et al., 2015; Huang et al., 2015; Suzuki and Yamamoto, 2015; Tebay et al., 2015). Depending on a disease condition, activation or suppression of Nrf2 is believed to be a promising therapeutic strategy. In addition, Nrf2 regulates carbohydrate and lipid metabolism, participating in the development and progression of NAFLD despite contradictory findings in the literature (Chambel et al., 2015; Tebay et al., 2015).

Several lines of evidence suggest a unidirectional positive or negative effect of estrogen signaling on Nrf2 activity in a cell

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ABBREVIATIONS: DIO, diet-induced obesity; E2, estradiol-17β; ER, estrogen receptor; HFD, high-fat diet; I/R, liver ischemia and reperfusion; LXR, liver X receptor; NAFLD, nonalcoholic fatty liver disease; NQO-1, NAD(P)H:quinone oxidoreductase 1; PCR, polymerase chain reaction.

type– or tissue–dependent manner. Estrogen stimulates Nrf2 activation in epithelial cells of the mammary gland, cerebral nerve tissue, primary myocardial cells, umbilical vein endothelial cells, ovarian epithelial carcinoma, and breast cancer cells (Liao et al., 2012; Yu et al., 2012; Zhang et al., 2013b; Gorrini et al., 2014; Meng et al., 2014; Wu et al., 2014). In contrast, estrogen potently inhibits the activities of Nrf2 target proteins (glutathione-*S*-transferase and quinone reductase), specifically in the uterus, possibly via its ligand-dependent interaction with Nrf2 (Ansell et al., 2004, 2005). The liver highly expresses both ERα and Nrf2, both of which are involved in hepatic lipid metabolism (Chan et al., 1996; Kuiper et al., 1997). We were interested in whether there is a connection between Nrf2 and estrogen signaling in NAFLD.

In our studies, NAFLD was induced in wild-type and Nrf2-null mice fed a high-fat diet (HFD), which was followed by an estradiol-17β (E2) treatment regimen. We found that the lack of Nrf2 results in a marked increase in hepatic ERα basal expression and striking amplification of the liver fat-lowering effect of E2. These findings demonstrate Nrf2 as a new and potent negative modulator of estrogen signaling in hepatic lipid metabolism.

Materials and Methods

Animal Care and Use. *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ male mice of a C57BL6/129SV mixed background were used for the study (Chan et al., 1996). Standard rodent chow and drinking water were provided ad libitum throughout the experiment. To induce NAFLD, mice were given a HFD (TD.88137; Harlan Laboratories, Madison, WI) for 16 weeks. Afterward, mice received vehicle (sesame oil), E2 (25 μg/mouse; Sigma-Aldrich, St. Louis, MO) (Bourassa et al., 1996), progesterone (1 mg/mouse; Sigma-Aldrich) (Walter et al., 2005), or a combination of these two steroid hormones (25 μg E2 and 1 mg progesterone/mouse) subcutaneously once daily for 3 weeks, while they were continuously fed the HFD. Animals were euthanized 4 hours after the last dosing. All of the animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Protocols for the care and use of animals were approved by the Indiana University–Purdue University Indianapolis Animal Care and Use Committee.

Histology and Immunohistochemistry. Liver specimens embedded in paraffin were sliced at 5-μm thickness and stained with hematoxylin and eosin to examine liver histology. Liver sections were subjected to immunostaining with a fatty acid binding protein 5 (FABP5) antibody (GTX12109; GeneTex, Irvine, CA) or a trefoil factor 3 (TFF3) primary antibody (TFF331-A; Alpha Diagnostic, San Antonio, TX) according to the manufacturer's instructions. Optimal cryo embedding medium-embedded liver specimens were cut into 10-μm thickness and stained with Oil Red O to visualize intracellular lipid droplets.

Measurement of Liver Lipid Content. Hepatic triglycerides, total cholesterol, and free fatty acids were quantified using commercially available kits (ab65336, ab65359, and ab65341; Abcam, Cambridge, MA) according to the manufacturer's instructions.

Mouse Fatty Liver RT^2 Profiler Polymerase Chain Reaction Array. Total RNA was extracted from the livers using TRIzol regent (Invitrogen, Carlsbad, CA). cDNAs were synthesized from 500 ng total RNA for each sample using the RT^2 First-Strand Kit (330401; Qiagen, Valencia, CA). The Mouse Fatty Liver RT^2 Profiler PCR Array, which profiles the expression of 84 key genes involved in the mechanisms of NAFLD and hepatic insulin resistance, and RT^2 SYBR Green ROX qPCR Master Mix were purchased form Qiagen (PAMM-157Z, 330520). The amplification reactions were carried out with the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). Fold changes in gene expression between different groups were calculated using the comparative threshold cycle method in the PCR Array Data Analysis template from Qiagen.

Quantitative Real-Time Polymerase Chain Reaction. Total RNA was isolated from each frozen liver using TRIzol reagent according to the manufacturer's protocol (Invitrogen). cDNAs were synthesized from 1 μg total RNA of each sample using a Verso cDNA Kit (Thermo Scientific, Rockford, IL), diluted four times with water, and subjected to quantitative real-time polymerase chain reaction (PCR). TaqMan Universal PCR Master Mix and the primers and TaqMan MGB probes of mouse Nrf2 (Mm00477786_m1), NAD(P)H: quinone oxidoreductase (NQO)-1 (NQO-1) (Mm01253561_m1), ERα (Mm00433149_m1), ERβ (Mm00599821_m1), TFF3 (Mm00495590_m1), FABP5 (Mm00783731_s1), and albumin (Mm00802090_m1) were purchased from Applied Biosystems. The amplification reactions were carried out with the ABI Prism 7900 sequence detection system (Applied Biosystems) with initial hold steps (50°C for 2 minutes followed by 95°C for 10 minutes) and 40 cycles of a two-step PCR (92°C for 15 seconds and 60°C for 1 minute). The comparative threshold cycle method was used for the relative quantification of the amount of mRNA in each sample normalized to the albumin transcript levels.

Western Blot Analysis. Liver homogenates (10 μg) were separated by PAGE under reducing conditions. Proteins from the gels were electrophoretically transferred to polyvinylidene difluoride membranes. Antibodies against NQO-1 (ab80588; Abcam), ERα (SC-514910; Santa Cruz Biotechnology, Santa Cruz, CA), FABP5 (GTX12109; GeneTex), and glyceraldehyde-3-phosphate dehydrogenase (SC-130656; Santa Cruz Biotechnology) were used as probes. Immune complexes were detected using the enhanced chemiluminescence system (Pierce, Rockford, IL).

Statistical Analysis. Data are shown as means ± S.D. Statistical analysis was performed using a one-way analysis of variance or *t* test. Significant differences were defined when $P < 0.05$.

Results

***Nrf2* Absence Results in a Strikingly Enhanced Liver Fat-Lowering Effect of Estrogen in Mice with NAFLD.** To determine whether Nrf2 modulates estrogen signaling in hepatic fat metabolism, we fed adult male wild-type and *Nrf2*-null mice a HFD for 4 months to induce NAFLD. Afterward, we treated mice with both genotypes with vehicle, E2, progesterone, or a combination of E2 and progesterone once per day for 3 weeks. During the steroid hormone treatment period, mice were continuously fed the HFD. Progesterone often exerts opposite effects to estrogen in many tissues (Diep et al., 2015). Hence, progesterone served as a potential negative control, whereas the combination of the two steroid hormones was used as a potential antagonistic control.

We first examined the liver size and hepatic histology. We found that Nrf2 absence caused a slight but significant reduction in liver-to-body weight ratio in mice fed a normal chow, as reported (Huang et al., 2010; Zou et al., 2014) (Fig. 1). The HFD induced liver enlargement and massive hepatic fat accumulation in both wild-type and Nrf2-null mice in the vehicle control group (Figs. 1 and 2). Compared with the vehicle control, exogenous estrogen resulted in deceases in liver size and hepatic fat deposition in both genotype groups of mice, exerting a liver fat-lowering effect, as demonstrated by others (Gao et al., 2006; Bryzgalova et al., 2008; Han et al., 2014) (Figs. 1 and 2). Notably, histologic assessment revealed that this effect was evidently enhanced when *Nrf2* was lacking, which was manifested by a further reduction in liver fat accumulation in *Nrf2*-null mice (Fig. 2). This finding indicates that Nrf2 counteracts with estrogen signaling in hepatic lipid metabolism. Relative to the vehicle control,

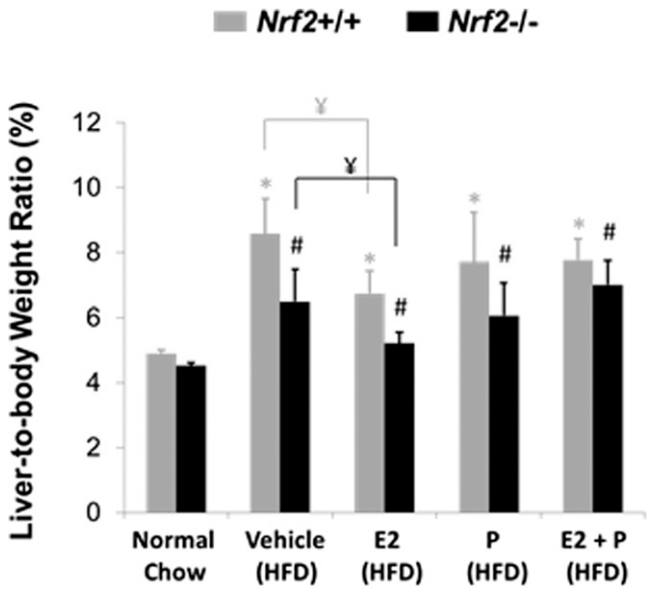

Fig. 1. Liver-to-body weight ratios. Adult male wild-type ($Nrf2^{+/+}$) and *Nrf2*-null ($Nrf2^{-/-}$) mice were fed a HFD for 16 weeks. Afterward, vehicle (sesame oil), E2 (, 625 μg/kg), progesterone (P, 25 mg/kg), or a combination of these two steroids (625 μg/kg E2 and 25 mg/kg progesterone) was injected subcutaneously once daily for 3 weeks. At 4 hours after the last dosing, mice were weighed and then euthanized for sample collection. $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice fed a normal chow served as homeostasis controls. The liver-to-body weight ratios were calculated and are shown. $^{*}P < 0.05$, compared with normal chow-fed $Nrf2^{+/+}$ mice; $^{\#}P < 0.05$, compared with normal chow-fed $Nrf2^{-/-}$ mice; $^{¥}P < 0.05$, compared as indicated. $n = 5$.

irrespective of genotype, exogenous progesterone did not overtly affect liver size and fat deposition (Figs. 1 and 2). However, regardless of genotype, the liver fat-lowering effect of estrogen was largely prevented in the estrogen and progesterone combination treatment group (Fig. 2), indicating that progesterone antagonizes the action of estrogen in eliminating hepatic fat content. Together, our findings demonstrate that Nrf2 negatively modulates estrogen signaling in NAFLD.

To further demonstrate the above findings, we performed Oil Red O staining on liver sections to visualize fat deposition. As a result, compared with the vehicle control, estrogen administration reduced liver fat staining in wild-type mice and, strikingly, almost diminished that in Nrf2-null mice (Fig. 3A). We subsequently quantified hepatic lipid contents. Relative to normal chow, the HFD induced excessive accumulation of triglycerides, total cholesterol, and free fatty acids in the liver, which were equivalent quantitatively between wild-type and Nrf2-null mice (Fig. 3B). In wild-type mice, relative to the vehicle control, exogenous estrogen decreased hepatic triglycerides by 49% and free fatty acids by 25% but did not significantly affect total cholesterol (Fig. 3B). When *Nrf2* was absent, exogenous estrogen reduced hepatic triglycerides by 90%, free fatty acids by 54%, and total cholesterol by 37% (Fig. 3B). The data together demonstrate that the liver fat-lowering effect of estrogen is drastically heightened because of the lack of *Nrf2*, indicating that Nrf2 is antagonistic to estrogen signaling in hepatic fat metabolism.

***Nrf2* Absence Causes a Marked Increase in Exogenous Estrogen-Induced Hepatic Estrogen Signaling in Mice with NAFLD.** We evaluated hepatic Nrf2 signaling by assessing the mRNA expression of *Nrf2* and its typical target gene *NQO-1* in the liver (Chan et al., 2001) (Fig. 4A). As expected, in normal chow-fed mice, *Nrf2* deficiency led to a reduction in the basal level of hepatic NQO-1 mRNA. HFD feeding highly activated Nrf2 in wild-type mice, relative to normal chow feeding, which was manifested by 8-fold and 6-fold upregulation in *Nrf2* and *NQO-1* gene expression, respectively. As a result of *Nrf2* genetic deletion, NQO-1 mRNA did not respond to the HFD. The data demonstrate that *NQO-1* expression is solely controlled by Nrf2 in this disease condition, reliably reflecting Nrf2 activity. In wild-type mice with NAFLD, compared with the vehicle control, estrogen administration did not significantly alter Nrf2, while marginally increased NQO-1, transcript level. The data indicate that exogenous estrogen slightly increased HFD-induced activation of hepatic Nrf2. In addition, excessive estrogen did not significantly influence Nrf2-dependent regulation of hepatic *NQO-1* gene in this disease. This observation indicates that estrogen signaling does not independently control NQO-1 mRNA expression. Furthermore, we showed that NQO-1 mRNA expression was consistent with its protein expression in the liver in various conditions (Fig. 4B). Taken together, we demonstrate that hepatic Nrf2 is highly activated in NAFLD and exogenous estrogen can somewhat further increase hepatic Nrf2 activity in this condition.

We next assessed hepatic ER signaling by evaluating the expression of *ERα*, *ERβ*, and *TFF3* genes (Fig. 4A) and ERα protein (Fig. 4B) in the liver. *TFF3* is an estrogen-responsive gene; thus, its mRNA expression was used to estimate ER activity (May and Westley, 1997; Gruvberger et al., 2001). In normal chow-fed mice, *Nrf2* deficiency caused a 6.5-fold increase in hepatic ERα mRNA expression. Both wild-type and *Nrf2*-null mice fed the normal chow displayed variable expression of hepatic ERα protein. Notably, in normal chow-fed mice, *Nrf2* absence resulted in a 4.5-fold elevation in the hepatic TFF3 mRNA level, indicating higher ERα activity. This finding suggests that Nrf2 may be a suppressor of ERα signaling in the homeostasis condition. Compared with normal chow feeding, HFD feeding increased and reduced hepatic ERα mRNA expression in wild-type and *Nrf2*-null mice, respectively, resulting in equivalent ERα mRNA levels between the two genotypes. Relative to normal chow feeding, HFD feeding induced overt increases in hepatic ERα protein expression in both wild-type and Nrf2-null mice. Taken together, these data indicate that mRNA and protein of hepatic ERα differentially respond to the disease state in their expression. Compared with normal chow feeding, HFD feeding did not affect Nrf2-dependent basal TFF3 transcript expression, showing that HFD does not alter Nrf2-dependent basal activity of hepatic ERα. Remarkably, relative to the vehicle control in wild-type mice, E2 administration elevated hepatic TFF3 mRNA expression by 122-fold in the presence of *Nrf2* and by 480-fold in the absence of *Nrf2*. Immunohistochemical analysis showed that hepatic TFF3 protein was mainly induced in hepatocytes (Fig. 4C). The data demonstrate that the lack of *Nrf2* leads to an astonishing enhancement of exogenous estrogen-induced hepatic ER signaling in NAFLD. Collectively, these findings demonstrate that Nrf2 antagonizes hepatic estrogen signaling and Nrf2 deficiency thereby results in amplification of the clearing effect of exogenous estrogen on liver fat. In addition, the hepatic TFF3 level positively correlates with the liver fat-lowering effect of estrogen. ERβ mRNA was barely detected across the

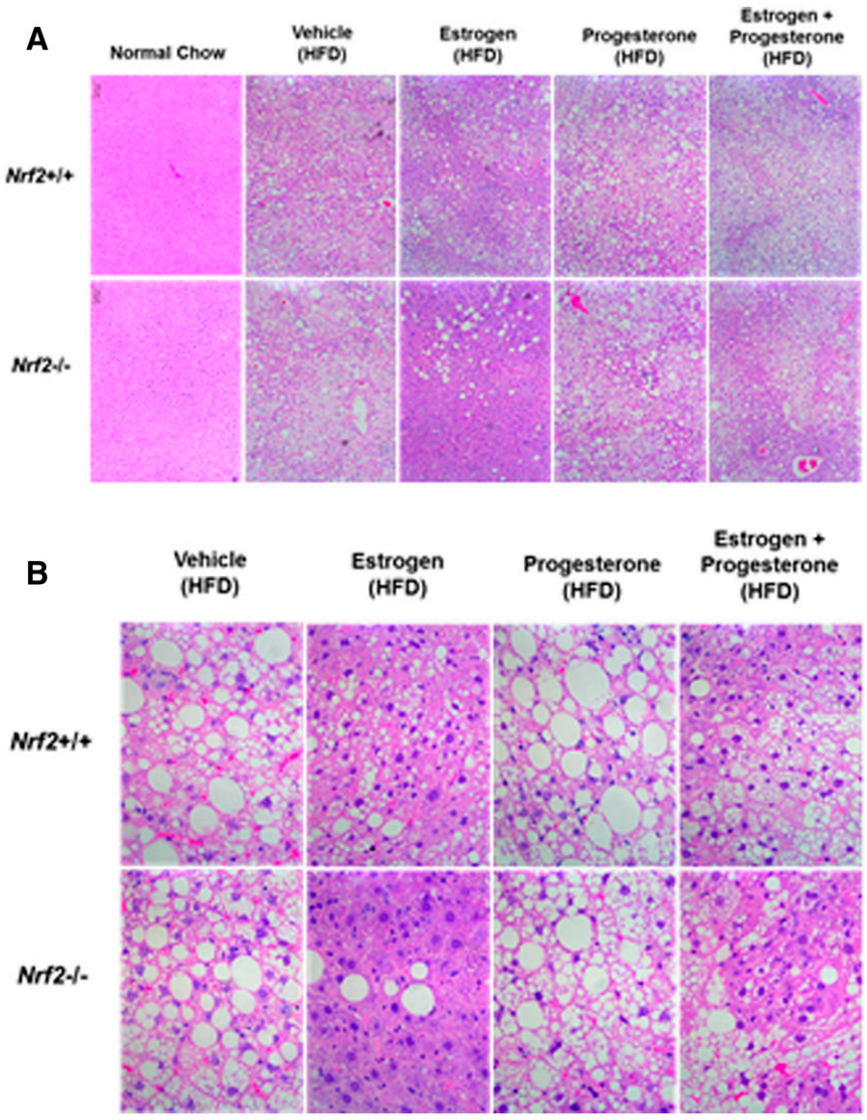

Fig. 2. Histologic assessments of liver sections. Livers were collected from the experiment described in Fig. 1. Formalin-fixed and paraffin-embedded liver sections were stained with hematoxylin and eosin and were visualized at low magnification (A) and high magnification (B). Original magnification, ×100 in A; ×400 in B.

samples (data not shown), indicating that ERα dominantly mediates the action of estrogen in the liver.

FABP5 Is Induced in an Nrf2- and Exogenous Estrogen-Dependent Manner in Mice with NAFLD. To gain further insight into the above findings, we analyzed hepatic expression of 84 key genes involved in the mechanisms of NAFLD and hepatic insulin resistance by a Mouse Fatty Liver RT^2 Profiler PCR Array in both genotype groups of mice with NAFLD (Supplemental Table 1). Surprisingly, we found that only FABP5 exhibited both Nrf2- and exogenous estrogen-dependent changes. The data were validated by quantitative real-time PCR (Fig. 5A). In normal chow-fed mice, *Nrf2* deficiency resulted in a 6.5-fold increase in hepatic FABP5 mRNA expression. The data indicate that Nrf2 suppresses FABP5 basal expression in the liver in the homeostasis condition. Compared with normal chow feeding, HFD feeding suppressed hepatic *FABP5* gene expression in both genotype groups of mice. Compared with vehicle controls, E2 treatment led to increases in hepatic FABP5 mRNA irrespective of genotype in mice with NAFLD. Notably, the magnitude of exogenous estrogen-induced elevation of hepatic FABP5 transcription in Nrf2-null mice was 2.4-fold higher than that in wild-type mice. Western blotting and immunohistochemical analyses of hepatic FABP5 protein further demonstrated the finding at the protein level and revealed its expression in hepatocytes (Figs. 4B and 5B). The data indicate that 1) FABP5 is a novel estrogen-responsive gene in the liver, 2) Nrf2 limits exogenous estrogen-induced elevation of hepatic FABP5, and 3) FABP5 positively correlates with the liver fat-lowering effect of estrogen.

Discussion

These studies identified Nrf2 as a potent suppressor of estrogen signaling in the liver. We provided two pieces of evidence to support our notion. First, in a homeostasis condition, *Nrf2* deficiency resulted in enhanced hepatic estrogen signaling, manifested by elevated expression of its target gene *TFF3*. Second, in NAFLD, the lack of *Nrf2* led to drastically increased activation of hepatic estrogen signaling by E2, reflected by a markedly elevated *TFF3* induction and highly enhanced liver fat-lowering effect of E2. The exact mechanism underlying the crosstalk between Nrf2 and estrogen signaling must be further investigated. It has been reported that Nrf2 directly interacts with ERα at the protein

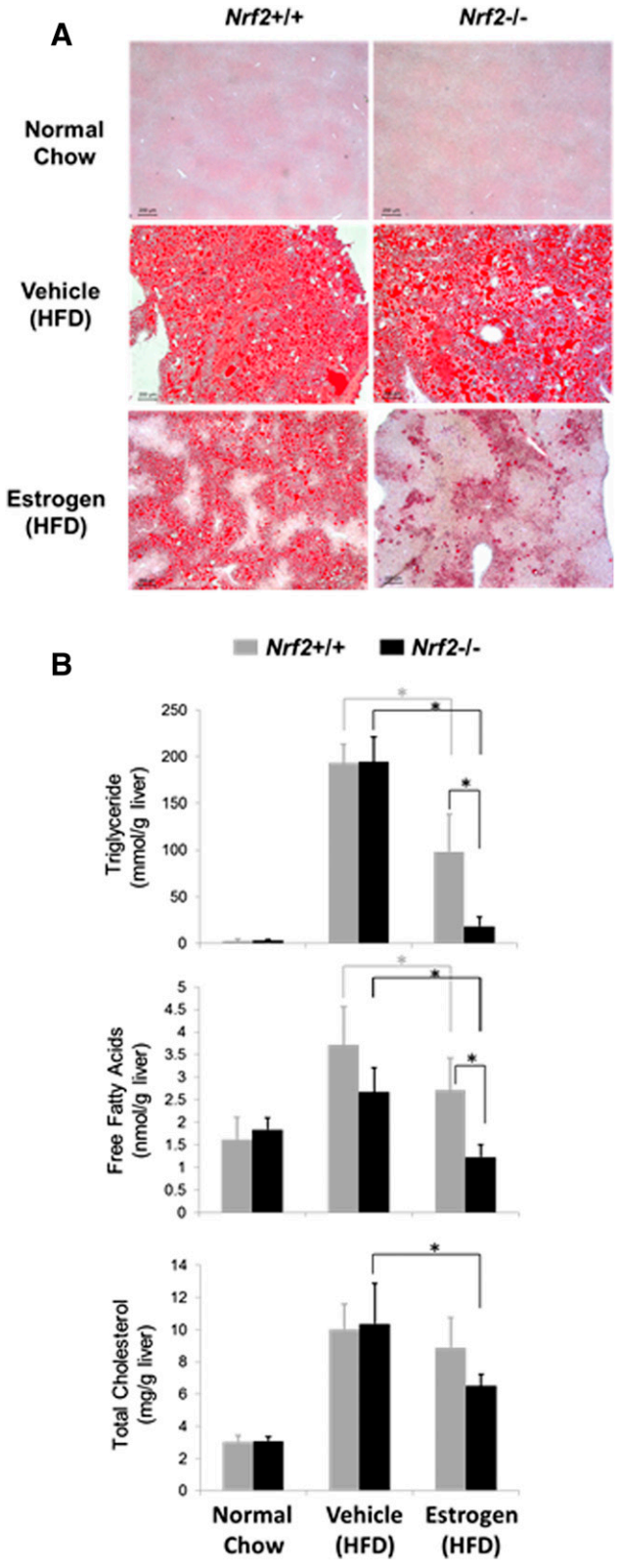

Fig. 3. Liver fat assessments. Livers were harvested from the experiment indicated in Fig. 1. (A) Oil Red O staining of liver fat. Frozen liver sections were stained with Oil Red O. Liver fat was stained red. (B) Quantification of liver fat contents. Frozen livers were used for measuring the concentrations of hepatic triglycerides, free fatty acids, and total cholesterol with various commercially available kits. $^*P < 0.05$. $n = 5$.

level in the uterus (Ansell et al., 2004, 2005). Xie et al. revealed a feedback mechanism between Nrf2 and estrogen in liver ischemia and reperfusion (I/R) Guo et al., 2015. I/R-induced Nrf2 activation directly upregulates estrogen sulfotransferase and in turn increases estrogen breakdown, limiting Nrf2 activity and gender-dependently affecting I/R injury (Guo et al., 2015). In addition, the crosstalk of Nrf2 signaling with the PTEN-GSK-3-β-TrCP pathway and the p62-mediated autophagy pathway has been demonstrated (Komatsu et al., 2010; Ni et al., 2014; Rojo et al., 2014; Taguchi et al., 2014). Han et al. (2014) uncovered an ERα/liver X receptor (LXR)/sterol regulatory element-binding protein (SREBP) 1 pathway modulating hepatic lipid metabolism (Han et al., 2014). They demonstrate that ERα interacts with LXR and inhibits LXR-dependent transcription of *SREBP1*, a critical fatty acid biosynthesis regulator, contributing to estrogen's liver fat-lowering effect. In our studies, we found that estrogen treatment did not significantly alter hepatic *SREBP1* expression compared with $Nrf2^{+/+}$ and $Nrf2^{-/-}$ HFD-fed mice (Supplemental Table 1). This finding suggests that Nrf2-dependent enhancement of the liver fat-lowering effect of estrogen may not be via this ERα/LXR/SREBP1 pathway. Here, we created a novel connection between the Nrf2- and ERα-mediated signaling pathway in lipid metabolism. We believe that our finding will provide a critical mechanistic aspect in understanding liver health and disease, because these two molecules are all essential for maintaining liver homeostasis. Moreover, we demonstrate a potential therapeutic strategy of dual targets (inhibiting Nrf2 and activating ERα) for NAFLD, which has emerged as the most common chronic liver disorder without a clinical cure in developed countries (Sanyal, 2015).

Our studies revealed the positive correlation of TFF3 with the liver fat-lowering effect of estrogen. We observed that the amplification of the liver fat-lowering effect of E2 in *Nrf2*-null mice with NAFLD was accompanied by further increased hepatic TFF3 induction. TFF family members including TFF1, TFF2, and TFF3 are secretory peptides without a known receptor (Busch and Dünker, 2015). Information regarding their expression, regulation, and function is expanding. TFF3 is expressed along the gastrointestinal tract, promotes wound healing of the gastrointestinal epithelium, and is associated with gastroadenocarcinoma progression (Xiao et al., 2015). It is also expressed in pancreatic β cells and oxytocin-secreting neurons in the hypothalamus, related to cell growth and learning and memory (Jagla et al., 2000; Fueger et al., 2008; Shi et al., 2012). This peptide is induced in biliary duct epithelial cells in biliary diseases (Srivatsa et al., 2002; Nozaki et al., 2004; Sasaki et al., 2007; Jiang et al., 2010). The *TFF3* gene maps to the Obq4 obesity quantitative trait locus, which is highly associated with total calorie intake; indeed, the expression of Obq4 in the intestine is regulated by food intake (Kumar and Smith Richards, 2008; Ge et al., 2015). Hepatic *TFF3* expression is increased in hepatic steatosis and decreased in db/db mice and mice with diet-induced obesity (DIO), suggesting its involvement in lipid metabolism (Guillén et al., 2009; Xue et al., 2013). Adenovirus-mediated 5.5-fold overexpression of mouse TFF3 in the liver reduces blood glucose and improves glucose tolerance in db/db mice and ameliorates insulin resistance in DIO mice (Xue et al., 2013). Adeno-associated virus-mediated overexpression of human TFF3, reaching 900 ng/ml in 2 weeks after virus infection, improves glucose tolerance in

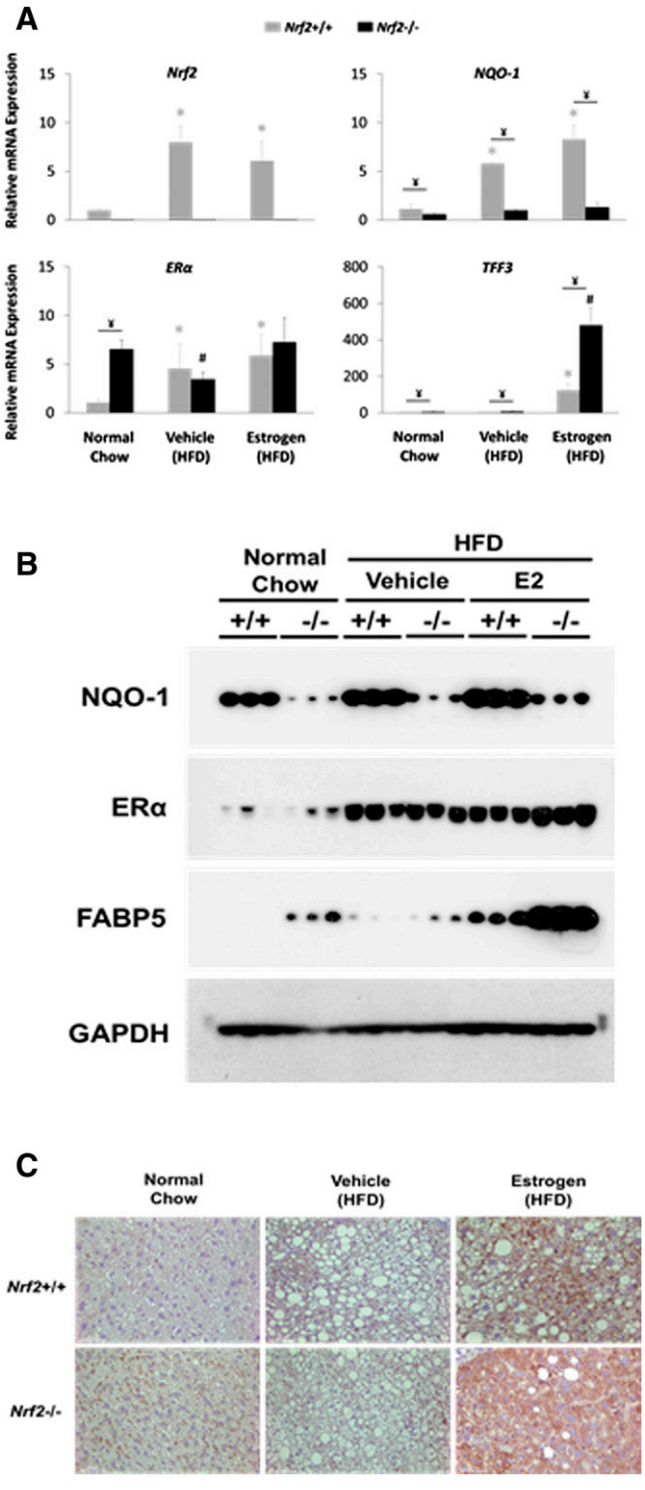

Fig. 4. (A) mRNA expression of hepatic Nrf2, NQO-1, ERα, and TFF3. Total RNA was extracted from each liver collected from the experiment illustrated in Fig. 1. Hepatic mRNA expression of the genes indicated was quantified with quantitative real-time PCR. The hepatic mRNA level of each gene in normal chow-fed $Nrf2^{+/+}$ mice was set to 1. $^{*}P < 0.05$, compared with normal chow-fed $Nrf2^{+/+}$ mice; $^{\#}P < 0.05$, compared with normal chow-fed $Nrf2^{-/-}$ mice; $^{¥}P < 0.05$, compared between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. $n = 5$. (B) Protein expression of hepatic NQO-1, ERα, and FABP5. Total liver lysates were prepared from the livers collected from the experiment described in Fig. 1. Western blotting was performed with the antibodies against the proteins indicated. The lanes represent samples prepared from individual mice. (C) Hepatic expression and distribution of TFF3 protein. Formalin-fixed and paraffin-embedded liver sections prepared from the experiment indicated in Fig. 1 were subjected to immunostaining with a TFF3 antibody. TFF3 protein was stained dark brown.

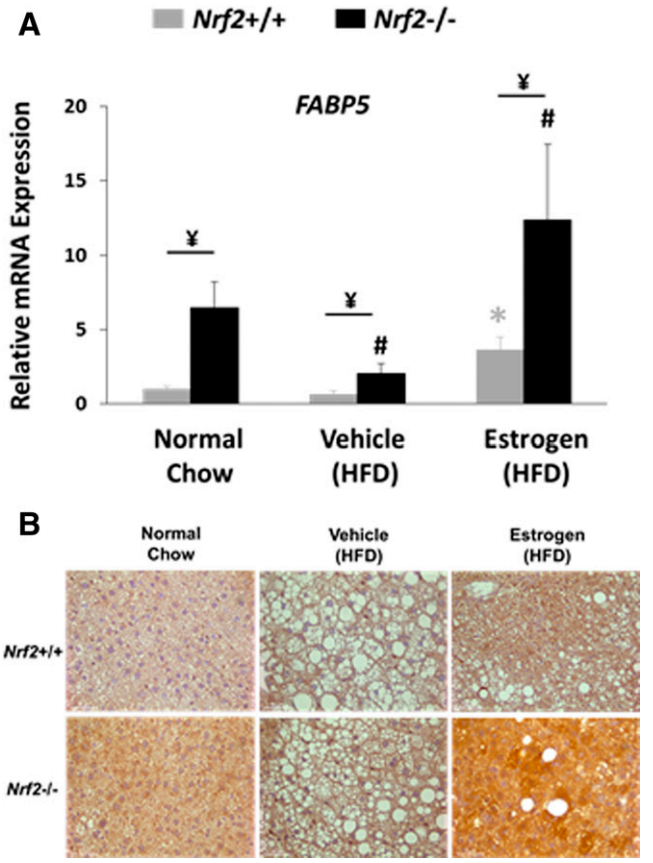

Fig. 5. Nrf2- and estrogen-dependent induction of FABP5 in the liver. (A) Hepatic expression of FABP5 mRNA. Total RNA samples prepared from the experiment described in Fig. 4 were used for evaluating the transcript levels of hepatic FABP5 with quantitative real-time PCR. Hepatic mRNA expression of the gene in normal chow-fed $Nrf2^{+/+}$ mice was set to 1. $^{*}P < 0.05$, compared with normal chow-fed $Nrf2^{+/+}$ mice; $^{\#}P < 0.05$, compared with normal chow-fed $Nrf2^{-/-}$ mice; $^{¥}P < 0.05$, compared between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. $n = 5$. (B) Hepatic expression and distribution of FABP5 protein. Formalin-fixed and paraffin-embedded liver sections prepared from the experiment indicated in Fig. 1 were subjected to immunostaining with a FABP5 antibody. FABP5-expressing cells were stained dark brown.

DIO mice without affecting body weight, fasting insulin, and levels of triglycerides, cholesterol, and leptin in the blood (Ge et al., 2015). These two studies highlighted the critical role of TFF3 in maintaining glucose homeostasis. However, it is not clear whether TFF3 overexpression affects hepatic lipid contents and whether human TFF3 is equipotent to mouse TFF3 in mice. Thus, whether TFF3 regulates hepatic lipid metabolism and mediates the liver fat-lowering effect of estrogen warrants further investigation.

Our studies also showed the association of FABP5 with the liver fat-lowering effect of estrogen. In mice with NAFLD, compared with the vehicle control, exogenous E2 increased hepatic FABP5 expression in wild-type mice and further increased its expression in *Nrf2*-null mice. It is evident that the level of FABP5 expression is positively correlated with the liver fat-lowering effect of estrogen. FABP5 belongs to a family of intracellular fatty acid binding proteins with tissue-specific distribution and functions. They bind unesterified long-chain fatty acids and regulate lipid metabolism (fatty acid transport, metabolism, and storage), inflammation, and energy homeostasis (Thumser et al., 2014; Hotamisligil and Bernlohr, 2015). FABP5 has been shown to interact with nuclear receptor

peroxisome proliferator-activated receptor, exhibiting properties of anti-inflammation and antioxidative stress (Berger et al., 2012; Gally et al., 2013). Overexpression of FABP5 in adipose tissue results in increased lipolysis, decreased fat mass, and potentiated insulin resistance (Hertzel et al., 2006). In the liver, FABP5 displays an anti-inflammatory action by regulating macrophage phenotypes (Moore et al., 2015). Consistent with a report showing that FABP5 is negatively regulated by Nrf2 in normal and fatty livers (Chartoumpekis et al., 2013), we additionally revealed that hepatic FABP5 positively responds to estrogen signaling in NAFLD. Notably, we found that FABP5 is the only one exhibiting both Nrf2- and E2-dependant alterations among the examined 84 genes that are associated with fat metabolism and insulin signaling. Taken together, these findings suggest an Nrf2/ERα/FABP5 regulatory pathway that controls hepatic lipid homeostasis. Hence, the function of FABP5 in liver lipid metabolism needs further investigation.

Authorship Contributions

Participated in research design: Rui, Zou, Zhang, Yang, Dai.
Conducted experiments: Rui, Zou, Lee, Nambiar, Lin.
Performed data analysis: Rui, Zou, Lee, Lin, Dai.
Wrote or contributed to the writing of the manuscript: Rui, Zou, Dai.

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