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Cytochrome P450 1A1 (CYP1A1) protects against nonalcoholic fatty liver disease caused by Western diet containing benzo[a]pyrene in mice

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Abstract

The Western diet contributes to nonalcoholic fatty liver disease (NAFLD) pathogenesis. Benzo[a]pyrene (BaP), a prototypical environmental pollutant produced by combustion processes, is present in charcoal-grilled meat. Cytochrome P450 1A1 (CYP1A1) metabolizes BaP, resulting in either detoxication or metabolic activation in a context-dependent manner. To elucidate a role of CYP1A1-BaP in NAFLD pathogenesis, we compared the effects of a Western diet, with or without oral BaP treatment, on the development of NAFLD in *Cyp1a1*($-/-$) mice versus wild-type mice. A Western diet plus BaP induced lipid-droplet accumulation in liver of *Cyp1a1*($-/-$) mice, but not wild-type mice. The hepatic steatosis observed in *Cyp1a1*($-/-$) mice was associated with increased cholesterol, triglyceride and bile acid levels. *Cyp1a1*($-/-$) mice fed Western diet plus BaP had changes in expression of genes involved in bile acid and lipid metabolism, and showed no increase in *Cyp1a2* expression but did exhibit enhanced *Cyp1b1* mRNA expression, as well as hepatic inflammation. Enhanced BaP metabolic activation, oxidative stress and inflammation may exacerbate metabolic dysfunction in liver of *Cyp1a1*($-/-$) mice. Thus, Western diet plus BaP induces NAFLD and hepatic inflammation in *Cyp1a1*($-/-$) mice in comparison to wild-type mice, indicating a protective role of CYP1A1 against NAFLD pathogenesis.

Keywords

Benzo[a]pyrene; Western diet; Nonalcoholic fatty liver disease; Bile acids; Lipogenesis; Inflammation

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Disclosure

The authors declare no conflicts of interest.

1. Introduction

The polycyclic aromatic hydrocarbon benzo[*a*]pyrene (BaP) is a prototypical environmental pollutant produced by combustion processes such as cigarette smoke, charcoal-grilled food and coke ovens. BaP is implicated as a causative agent in malignant, inflammatory and metabolic diseases, including lung cancer and atherosclerosis (Miller and Ramos, 2001; Uno and Makishima, 2009). BaP activates the aryl hydrocarbon receptor (AHR) and induces expression of genes involved in xenobiotic metabolism, such as the three cytochrome P450 1 (CYP1) monooxygenases (CYP1A1, CYP1A2 and CYP1B1) (Nebert, 2017). CYP1A1 is involved in both BaP detoxication and metabolic activation, the latter resulting in DNA adduct formation and reactive oxygen species (ROS) production (Miller and Ramos, 2001; Shimada and Fujii-Kuriyama, 2004). In addition to CYP1A1, AHR activation induces expression of other phase I enzymes, such as CYP1A2, CYP1B1 and NAD(P)H:quinone oxidoreductase-1 (NQO1), along with phase II enzymes, including glutathione *S*-transferase-A1 (GSTA1) (Nebert et al., 2000). Sequential reactions by phase I and phase II enzymes induce effective detoxication of BaP (Shimada, 2006; Uno and Makishima, 2009).

Oral BaP administration induces a higher level of BaP-DNA adduct formation in *Cyp1a1*(–/–) mice (Uno et al., 2004), and CYP1A1 overexpression suppresses BaP-DNA adduct formation in hepatocytes (Endo et al., 2008). Thus, oral BaP-induced CYP1A1 activity mediates phase I metabolism in BaP detoxication. In contrast, CYP1A1-null mutant hepatoma cells are resistant to BaP toxicity (Hankinson et al., 1991). Conversion of CYP1-mediated metabolites of BaP by epoxide hydrolase leads to further metabolic activation to potent carcinogens such as BaP-7,8-diol-9,10-epoxide (Conney, 2003; Shimada, 2006; Shimada and Fujii-Kuriyama, 2004). Compensatory induction of CYP1B1 in *Cyp1a1*(–/–) mice enhances BaP metabolic activation (Nebert et al., 2013; Uno et al., 2006). Therefore, the coordinated expression of phase I and phase II enzymes is required for efficient BaP detoxication.

Nonalcoholic fatty liver disease (NAFLD) is a major health problem in Western countries, and is a hepatic metabolic disease caused by various factors, including dietary factors such as excess intake of energy, carbohydrates and lipids (Yasutake et al., 2014; Zelber-Sagi et al., 2011). Higher meat intake, specifically grilled meat, is also associated with an increased risk of NAFLD (Miele et al., 2014; Zelber-Sagi et al., 2011). Western-style food items, such as barbecued and fried meats and smoked dried beef, contain significant concentrations of BaP (Anastasio et al., 2004; Aygun and Kabadayi, 2005; Lee and Shim, 2007). The total daily intake level of BaP in a typical Western diet is 125 ng/person/day, corresponding to the levels derived from cigarette smoking (20–40 ng/cigarette) (Uno and Makishima, 2009). In addition to dietary factors, cigarette smoking is a risk factor for NAFLD (Hamabe et al., 2011), and large amounts of smoking residue are deposited in the gastrointestinal tract (Nebert et al., 2013). These findings suggest that dietary BaP might be a factor linking NAFLD pathogenesis with the Western diet. Further, AHR activation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induces NAFLD in mouse models (Angrish et al., 2012; Lee et al., 2010), indicating that BaP increases the incidence of NAFLD by AHR activation. However, it remains unknown how the oral BaP-induced enzyme CYP1A1 is involved in the

pathogenesis of NAFLD. In the present study using *Cyp11a1*($-/-$) mice, we investigated the role of CYP11A1 in NAFLD induced by the Western diet combined with oral BaP.

2. Materials and methods

2.1. Animals and treatment

C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan), and *Cyp11a1*($-/-$) mice on >99.8% C57BL/6J genetic background were generated, as described previously (Dalton et al., 2000; Uno et al., 2014). All experiments were conducted using 6- to 7-week-old male mice. Mice were maintained under controlled temperature (23 ± 1 °C) and humidity (45–65%) with free access to water and a control diet (CE-2; CLEA Japan) or a Western diet (CE-2 supplemented with 1.25 g/100g cholesterol, 0.5 g/100g sodium cholate, 12.5 g/100g cocoa butter) (Miura et al., 2001; Uno et al., 2014). BaP (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in corn oil, and the pellets were soaked in control corn oil or BaP-containing corn oil (1.0 mg/ml) for at least 24 hours. The daily oral BaP doses were estimated to be ~12.5 mg/kg/day (Uno et al., 2014). We fed wild-type mice and *Cyp11a1*($-/-$) mice a control diet or a Western diet containing control corn oil or BaP-containing corn oil for 3 or 15 weeks ($n = 3-4$ for each group). Blood and tissue samples were collected after euthanization with CO₂ inhalation. The experimental protocol adhered to the Guidelines for Animal Experiments of the Nihon University School of Medicine and was approved by the Ethics Review Committee for Animal Experimentation at the Nihon University School of Medicine.

2.2. Biochemical tests

Plasma total cholesterol, triglyceride and bile acid levels were quantified with Cholesterol E-Testwako (Wako Pure Chemical Industries, Osaka Japan), Triglyceride E-Testwako (Wako Pure Chemical Industries) and Total Bile Acid Testwako (Wako Pure Chemical Industries), respectively. Liver samples (~0.1 g) were homogenized in phosphate-buffered saline, and lipids were extracted with a mixture of chloroform, methanol and water (2:1:0.8) and subjected to biochemical analysis.

2.3. Histology

Liver samples were fixed with 10% paraformaldehyde and embedded with paraffin. Sections were stained with hematoxylin and eosin, immunostained with anti-F4/80 antibody (Nikken Seil Co., Ltd., Fukuroi, Japan), a Histofine mouse stain kit (Nichirei Corporation, Tokyo, Japan) and an AEC substrate kit for peroxidase (Vector Laboratories, Burlingame, CA), and then visualized by counterstaining with hematoxylin.

2.4. Reverse transcription and real-time quantitative polymerase chain reaction

Total RNA extraction was performed using the acid guanidium thiocyanate/phenol/chloroform method, and cDNAs were synthesized using the ImProm-II Reverse Transcription system (Promega Corporation, Madison, WI) (Uno et al., 2014). Real-time polymerase chain reactions were performed on the ABI PRISM 7000 Sequence Detection System (Thermo Fisher Scientific, Waltham, MA) using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). Primer sequences for mouse genes included *Abcb11* (bile-

salt-exporter pump; BSEP), *Abcc3* (multidrug resistance-associated protein-3; MRP3), *Abcc4* (MRP4), *Slc51a* (organic solute transporter- α ; OST α), *Slc51b* (OST β), *Slc10a1* (Na⁺/taurocholate-cotransporting polypeptide; NTCP), *Slco1a1* (organic anion-transporting polypeptide-1A1; OATP1A1), *Slco1b2* (OATP1B2), *Cyp7a1*, *Cyp8b1*, *Cyp27a1*, *Cyp11a1*, *Cyp11a2*, *Cyp11b1*, *Tnf* (tumor necrosis factor; TNF), and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), as reported previously (Nishida et al., 2009; Ogura et al., 2009; Uno et al., 2006). Primers for other genes (positive and negative strands, respectively) were as follows: *Abcg5* (ATP-binding cassette (ABC) transporter-G5; ABCG5), 5'-GTT GTG AGA TTC TCG TGG TC-3' and 5'-TGG GCA GGT TTT CTC GAT GA-3'; *Abcg8*, 5'-ATC TCC AAG CTG TCG TTC CT-3' and 5'-GGT AGA TCG CAT AGA GTG GA-3'; *Srebf1* (sterol regulatory element-binding protein-1c; SREBP-1c), 5'-ATC GGC GCG GAA GCT GTC GGG GTA GCG TC-3' and 5'-ACT GTC TTG GTT GTT GAT GAG CTG GAG CAT-3'; *Scd1*, *Scd2*, *Scd3* and *Scd4* (common primers for all four stearyl-CoA desaturase (SCD) genes), 5'-TAC CGC TGG CAC ATC AAC TT-3' and 5'-CCA GTT CTC TTA ATC CTG GC-3'; *Acc* (acetyl-CoA carboxylase; ACC), 5'-GGT GGT TCT TGG GTT GTG AT-3' and 5'-CAG CCA AGC GGA TGT AAA CT-3'; *Lpl* (lipoprotein lipase; LPL), 5'-CTT GAT TTA CAC GGA GGT GG-3' and 5'-CCT AGC ACA GAA GAT GAC CT-3'; *Ephx1* (epoxide hydrolase-1; EPHX1), 5'-CCT GGA AGA TCT GCT GAC TA-3' and 5'-TGG GCA CAA AGA CCT TCA TC-3'; *Gsta1*, 5'-CAT CCA CCT ACT GGA AGT TC-3' and 5'-CCA TCA ATG CAG CTT CAC TG-3'; *Nqo1*, 5'-ATG CTG CCA TGT ACG ACA AC-3' and 5'-TGA ATC GGC CAG AGA ATG AC-3'; *Cybb* [cytochrome b-245, beta polypeptide; CYBB), 5'-CAG AAG ACT CTG TAT GGA CG-3' and 5'-TGG TGA AAG AGC GGA GTT AG-3'; *Nox4* (NADPH oxidase-4; NOX4), 5'-TCG AGA CTT TTC ATT GGG CG-3' 5'-TCC CAT ATG AGT TGT TCC GG-3'; and *Duox2* (dual oxidase-2; DUOX2), 5'-TCC ATC GCT GGA TTG CTA TG-3' and 5'-AAC TTG GAC CCG TCA TTC AC-3'. The mRNA values were normalized to the *Gapdh* mRNA levels.

2.5 Western blotting

Plasma membrane fractions from pooled liver samples (n = 3–4 for each group) were prepared with the Plasma Membrane Extraction Kit (Abcam, Cambridge, United Kingdom), subjected to SDS-polyacrylamide gel electrophoresis (20 g/lane), and transferred to a nitrocellulose membrane. Western blot analysis was performed using a polyclonal anti-MRP4 (Santa Cruz Biotechnology, Santa Cruz, CA), a polyclonal anti-OATP1A1 antibody and a monoclonal anti-LPL antibody (Abcam), visualized with the ECL Prime Western Blotting Detection Reagents (GE Healthcare, Chalfont St. Giles, United Kingdom) as reported previously (Uno et al., 2006).

2.6. Measurement of TNF- α protein levels

TNF- α protein levels in liver homogenates were determined with the Quantikine ELISA Mouse TNF- α Immunoassay (Bio-Techne, Minneapolis, MN), and were adjusted with total protein levels measures with a BCA protein assay kit (Thermo Fisher Scientific).

2.7. Statistical analysis

Data are presented as mean $\pm$ S.E.M. We performed one-way ANOVA followed by Tukey's multiple comparisons, or unpaired two-group Student's *t* test, to assess significant differences.

3. Results

3.1. Combined treatment with Western diet and oral BaP induces hepatic steatosis in *Cyp1a1*($-/-$) mice

To investigate the role of BaP metabolism by CYP1A1 in the pathogenesis of NAFLD, we first fed wild-type mice and *Cyp1a1*($-/-$) mice a Western diet containing BaP for 15 weeks. The Western diet, oral BaP treatment, or both combined did not induce histological changes in wild-type mice, which have the intact *Cyp1a1* gene. While the Western diet alone, or oral BaP alone, had no effect, combined treatment induced accumulation of lipid droplets in the livers of *Cyp1a1*($-/-$) mice (Fig. 1A). Oral BaP increased hepatic cholesterol, triglyceride and bile acid levels in *Cyp1a1*($-/-$), but not wild-type mice, and the combined treatment (Western diet plus BaP) further increased cholesterol and bile acid levels in livers of *Cyp1a1*($-/-$) mice (Fig. 1B). Plasma cholesterol and triglyceride levels were not increased by the Western diet, with or without BaP, and were similar in wild-type mice and *Cyp1a1*($-/-$) mice (Fig. 1C). However, oral BaP increased plasma bile acid levels in *Cyp1a1*($-/-$), but not in wild-type mice. Combined treatment with Western diet and oral BaP elevated bile acid levels further in these mice. Although we observed hepatic lipid accumulation in *Cyp1a1*($-/-$) mice fed a Western diet with BaP for 15 weeks, these mice did not have increased plasma alanine aminotransferase levels (Fig. 1D). The combined Western diet and BaP treatment did increase plasma aspartate aminotransferase levels, a similar finding observed in *Cyp1a1*($-/-$) mice receiving a high dose of BaP (Uno et al., 2004), suggesting that these mice have damage in extrahepatic tissues. We next examined the effect of treatment with a Western diet and BaP for 3 weeks, and found that the combined treatment increased plasma aspartate aminotransferase and alanine aminotransferase levels and hepatic and plasma bile acid levels in *Cyp1a1*($-/-$) mice to levels much higher than in wild-type mice (Fig. 1D and supplementary Fig. 1). As reported previously (Uno et al., 2004), BaP treatment for 3 weeks decreased weight gain in *Cyp1a1*($-/-$) mice (Fig. 1E). The combined treatment with a Western diet and BaP induced weight loss in *Cyp1a1*($-/-$) mice but not in wild-type mice. The combined treatment did not decrease food intake both in wild-type mice and *Cyp1a1*($-/-$) mice. Interestingly, *Cyp1a1*($-/-$) mice fed a Western diet decreased food intake compared to wild-type mice, and addition of BaP to the Western diet increased food intake. Thus, the Western diet containing BaP induces hepatic steatosis, which is associated with dysregulated bile acid and lipid metabolism, and liver cell damage, in *Cyp1a1*($-/-$) mice.

3.2. Effect of combination of the Western diet and oral BaP on expression of bile acid and cholesterol transporter genes

Cyp1a1($-/-$) mice fed a Western diet with BaP showed hepatic steatosis associated with lipid accumulation, specifically cholesterol and bile acids (Fig. 1). Since we observed liver dysfunction in *Cyp1a1*($-/-$) mice fed a Western diet with BaP for 3 weeks, we next examined the effect of 3-week treatment with a Western diet and BaP on expression of genes

involved in bile acid and cholesterol transport. Bile acids are secreted into the bile via the canalicular transporter BSEP (encoded by *Abcb11*) and, particularly in the context of cholestasis, to the systemic circulation via the basolateral transporters MRP3 (encoded by *Abcc3*), MRP4 (encoded by *Abcc4*), and the heterodimer of OST α (encoded by *Slc51a*) and OST β (encoded by *Slc51b*) (Zollner et al., 2006). Among these genes, *Abcc4* mRNA levels were markedly enhanced in *Cyp11a1*($-/-$) mice fed a Western diet with BaP (Fig. 2A). Expression of *Slc51b* was also induced by the combined treatment, but its levels were similar between wild-type mice and *Cyp11a1*($-/-$) mice. Expression of *Abcc3* and *Slc51a* mRNA levels were decreased in *Cyp11a1*($-/-$) mice fed a Western diet with BaP, compared to *Cyp11a1*($-/-$) mice treated with BaP alone, or fed a Western diet alone. *Abcb11* mRNA levels did not change in wild-type or *Cyp11a1*($-/-$) mice under any dietary condition.

Circulating bile acids are taken up by hepatocytes via NTCP (encoded by *Slc10a1*), OATP1A1 (encoded by *Slco1a1*) and OATP1B1 (encoded by *Slco1b1*) (Zollner et al., 2006). Expression of these import transporters was significantly decreased in *Cyp11a1*($-/-$) mice fed a Western diet with BaP (Fig. 2B). The combined treatment with a Western diet and BaP also increased MRP4 protein levels and decreased OATP1A1 protein levels in the liver of *Cyp11a1*($-/-$) mice (Fig. 2D and supplementary Fig. 2). These findings suggest that decreased expression of *Abcc3* and *Slc51a* mRNA might lead to elevated bile acid levels in hepatocytes and that alterations in *Abcc4* expression and import transporters may protect hepatocytes from bile acid accumulation, leading to increased bile acid levels in the blood circulation.

ABCG5 and ABCG8 mediate biliary excretion of cholesterol in hepatocytes (Yu et al., 2003). A Western diet alone increased *Abcg5* and *Abcg8* mRNA levels in both wild-type and *Cyp11a1*($-/-$) mice (Fig. 2C). Addition of BaP to the Western diet decreased the levels of these transcripts in liver of *Cyp11a1*($-/-$) mice (Fig. 2C). Diminished expression of *Abcg5* and *Abcg8* mRNA may account for the cholesterol accumulation in liver of *Cyp11a1*($-/-$) mice.

3.3. Effect of a Western diet and oral BaP on expression of genes involved in bile acid and lipid synthesis

We next examined the expression of bile-acid-synthesizing enzyme genes. Interestingly, BaP treatment increased *Cyp7a1* expression, and the Western diet plus BaP combined repressed its expression, but there was no difference between wild-type and *Cyp11a1*($-/-$) mice (Fig. 3A). The Western diet, with or without BaP, decreased *Cyp8b1* expression in both wild-type and *Cyp11a1*($-/-$) mice. These changes may reflect cellular responses to protect against accumulated bile acids. We saw no significant change in *Cyp27a1* expression following in all of the dietary conditions (Fig. 3A).

The Western diet increased expression of the lipogenic genes *Srebf1* (encoding SREBP-1c), *Scd* (which includes *Scd1*, *Scd2*, *Scd3* and *Scd4*), and *Acc* in both wild-type and *Cyp11a1*($-/-$) mice (Fig. 3B). Although addition of oral BaP did not further increase these expressions, mRNA levels of *Scd* and *Acc* in *Cyp11a1*($-/-$) mice fed the Western diet with BaP were higher than those in wild-type mice. Treatment with a Western diet alone or oral BaP alone induced slightly higher *Lpl* mRNA levels in *Cyp11a1*($-/-$) mice, compared to wild-type mice,

and the combination of Western diet plus BaP caused a striking increase of *Lpl* mRNA expression in *Cyp1a1*($-/-$), but not wild-type mice (Fig. 3B). These findings are consistent with the occurrence of hepatic steatosis in *Cyp1a1*($-/-$) mice that had received the Western diet with BaP. We observed increased LPL protein levels in *Cyp1a1*($-/-$) mice fed a control diet compared to wild-type mice (Fig. 3C). BaP treatment decreased LPL protein expression in *Cyp1a1*($-/-$) mice.

3.4. Effect of combined Western diet plus oral BaP treatment on expression of genes involved in BaP metabolism

We had previously reported that the levels of oxidative stress markers are elevated in the serum of *Cyp1a1*($-/-$) mice fed a Western diet with BaP, compared with that in wild-type mice (Uno et al., 2014). BaP metabolic activation is associated with ROS production (Miller and Ramos, 2001), and increased CYP1B1 activity enhances metabolic activation in *Cyp1a1*($-/-$) mice (Uno et al., 2006). Hence, we examined the expression of genes involved in BaP metabolism. Treatment with the Western diet and/or oral BaP did not change mRNA levels of *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Ephx1*, *Gsta1* or *Nqo1* in wild-type mice (Fig. 4). As reported previously (Uno et al., 2006), oral BaP treatment did increase mRNA levels of *Cyp1a2* and *Cyp1b1* in liver of *Cyp1a1*($-/-$) mice. While the Western diet did not change hepatic expression of *Cyp1a2* or *Cyp1b1*, expression of *Cyp1a2*, but not *Cyp1b1*, was decreased in *Cyp1a1*($-/-$) mice receiving the Western diet plus oral BaP (Fig. 4). *Cyp1a1*($-/-$) mice fed the Western diet had increased *Ephx1* mRNA levels, but addition of oral BaP slightly decreased *Ephx1* expression, although this change was not statistically significant. *Gsta1* mRNA levels demonstrated a statistically significant elevation in *Cyp1a1*($-/-$) mice fed Western diet plus BaP, and *Nqo1* expression showed the same pattern but was not statistically significant (Fig. 4). These results show that combined treatment with Western diet and BaP changes the hepatic expression of BaP-metabolizing enzymes in *Cyp1a1*($-/-$) mice.

3.5. Effect of combined Western diet plus oral BaP treatment on liver inflammation

The combination of a Western diet and oral BaP increased mRNA levels of *Cybb* (a subunit of the NOX2 complex), *Duox2* and *Tnf* and protein levels of TNF- α in liver of *Cyp1a1*($-/-$) but not wild-type mice (Fig. 5, A and B). By contrast, *Nox4* expression was decreased in *Cyp1a1*($-/-$) mice receiving the combined treatment, compared with those fed the Western diet without BaP (Fig. 5A). BaP treatment induced appearance of F4/80-positive cells in liver of *Cyp1a1*($-/-$) mice, and the combined Western diet plus BaP further increased F4/80-positive cells in these mice (Fig. 5C). These findings were not observed in wild-type mice. Thus, the combination of a Western diet and BaP induces an inflammatory response, in addition to lipid accumulation, in liver of *Cyp1a1*($-/-$) mice.

4. Discussion

In this study, we show that oral BaP induces liver dysfunction in *Cyp1a1*($-/-$) mice fed a Western diet, which exacerbates hepatic steatosis and inflammation while these effects are not seen in wild-type mice. We provide experimental evidence supporting involvement of

the BaP-containing Western diet in the pathogenesis of NAFLD. Our results therefore demonstrate a protective role of CYP1A1 against NAFLD development.

More than a decade of oral BaP studies have made it clear that BaP-induced toxicity or cancer is dependent on the dose of BaP, acute versus chronic dosage as a function of time, route-of-administration, organs targeted, and genetic background of the animal (Nebert et al., 2013). Oral BaP detoxication has been shown to be mediated by CYP1A1 located in the proximal small intestine rather than hepatic CYP1A1 (Shi et al., 2010). The current results in *Cyp1a1*($-/-$) mice demonstrate the protective role of CYP1A1 against NAFLD induced by a Western diet with BaP. Therefore, lack of hepatic and gastrointestinal CYP1A1 in *Cyp1a1*($-/-$) mice fed the Western diet plus oral BaP is suggested to result in NAFLD.

The findings in this study present a number of lines of supporting evidence. *Cyp1a1*($-/-$) but not wild-type mice receiving the Western diet plus BaP show increased hepatic cholesterol, triglyceride and bile acid levels. Disturbance of bile acid homeostasis is associated with NAFLD (Chow et al., 2017). Increased hepatic expression of the bile acid export transporters *Abcc4* and *Slc51b*, combined with decreased mRNA levels of bile acid import transporters *Slc10a1*, *Slco1a1* and *Slco1b2*, as well as bile-acid-synthesizing enzymes *Cyp7a1* and *Cyp8b1* (Figs. 2 and 3) were all observed in *Cyp1a1*($-/-$) but not wild-type mice receiving the Western diet plus BaP. The combined treatment also increased MRP4 protein levels and decreased OATP1A1 protein levels in the liver of *Cyp1a1*($-/-$) mice (Fig. 2). These changes can protect hepatocytes from bile acid toxicity and may lead to increased bile acid levels in the circulation. In contrast, hepatic *Abcc3* and *Slc51a* mRNA levels were diminished in *Cyp1a1*($-/-$) mice fed a Western diet with BaP, compared to these mice treated with Western diet alone, or BaP alone (Fig. 2). In hepatocytes, the farnesoid-X receptor (FXR) and pregnane-X receptor (PXR) are able to sense excessive amounts of bile acids and regulate bile acid transport and synthesis (Chiang, 2009; Makishima et al., 1999; Staudinger et al., 2001; Xie et al., 2001). FXR suppresses bile acid import and synthesis by inhibiting expression of *Slc10a1*, *Cyp7a1* and *Cyp8b1*, and enhances bile acid export by inducing expression of *Abcb11*, *Slc51a* and *Slc51b*, whereas PXR induces bile acid export through *Abcc3* and *Abcc4* expression (Lefebvre et al., 2009). In mice receiving oral BaP, induction of *Abcb11*, *Abcc3* and *Slc51a* expression is ineffective and fails to protect against bile acid toxicity. Dysregulated expression of these genes may cause increased bile acid levels in the liver. Furthermore, inflammation and oxidative stress in the liver of *Cyp1a1*($-/-$) mice (Fig. 5) is likely to influence bile acid transport and metabolism (Aitken et al., 2006).

The Western diet also increased expression of cholesterol export transporter genes *Abcg5* and *Abcg8*, as well as lipogenic genes *Srebf1*, *Scd* and *Acc* (Figs. 2 and 3). The Western diet used in our experiments contained high cholesterol levels, which activate the oxysterol receptor liver-X receptor (LXR), which in turn up-regulates expression of these genes (Peet et al., 1998; Tontonoz and Mangelsdorf, 2003). While increased ABCG5 and ABCG8 transporters can protect hepatocytes from cholesterol accumulation, induction of these lipogenic genes may enhance hepatic steatosis, which has been reported in mice treated with synthetic LXR ligand (Chisholm et al., 2003; Schultz et al., 2000). However, *Srebf1*, *Scd* and *Acc* mRNA levels in *Cyp1a1*($-/-$) mice were similar to, or only slightly higher than,

those in wild-type mice (Fig. 3), and the marked steatosis in *Cyp1a1*($-/-$) mice fed a Western diet plus BaP cannot be explained by these gene expression changes. Interestingly, *Lpl* mRNA levels were drastically elevated in *Cyp1a1*($-/-$), compared to wild-type mice, fed the combined treatment (Fig. 3). Up-regulation of the *Lpl* gene is known to be elicited by LXR activation and AHR activation in the liver of mice (Angrish et al., 2012; Minami et al., 2008; Zhang et al., 2001), and hepatic LPL over-expression causes fatty liver (Kim et al., 2001). These findings suggest that the combined treatment with a Western diet plus BaP induces hepatic steatosis by activating AHR and LXR signaling, resulting in *Lpl* induction in *Cyp1a1*($-/-$) mice. Unexpectedly, the combined treatment decreased LPL protein levels in the liver of *Cyp1a1*($-/-$) mice (Fig. 3). A potent AHR activator, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, suppresses LPL activity in 3T1-L1 murine preadipocytes (Olsen et al., 1998). The discrepancy between mRNA and protein expression changes of LPL may due to cellular dysfunction induced by BaP toxicity, such as enhanced oxidative stress, or differential effects of AHR activation. Further studies are needed to elucidate mechanisms of dysregulation of genes involved in transport and metabolism of bile acids and lipids in *Cyp1a1*($-/-$) mice fed the Western diet with BaP.

Treatment with BaP alone increased and combined treatment with a Western diet and BaP decreased plasma triglycerides levels in wild-type mice, while these treatments did not change hepatic triglyceride levels (Fig. 1). Treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induces hypertriglyceridemia in rats in a strain-dependent manner (Walden and Schiller, 1985), and also in rabbits through suppression of adipose tissue LPL (Brewster et al., 1988). BaP may increase plasma triglyceride levels by affecting extrahepatic tissues. The combined treatment with a Western diet and BaP also decreased plasma triglyceride levels, but increased hepatic triglyceride levels. Activation of the bile acid receptor FXR suppresses very low density lipoprotein secretion (Hirokane et al., 2004; Liu et al., 2016). Bile acid accumulation may contribute to decreased plasma triglyceride levels in mice treated with a Western diet plus BaP. Hepatic inflammation and oxidative stress induced by BaP metabolic activation may also influence dysregulation of lipid metabolism.

In the liver of *Cyp1a1*($-/-$) mice, BaP treatment induced the expression of *Cyp1a2* and *Cyp1b1* (Fig. 4), as reported previously (Uno et al., 2006). The combined treatment with a Western diet and BaP decreased expression of *Cyp1a2*, but not *Cyp1b1* (Fig. 4). Because CYP1A1 opposes and CYP1B1 promotes BaP toxicity (Endo et al., 2008; Uno et al., 2006), the combined treatment with a Western diet and BaP is likely to decrease BaP detoxification and enhance metabolic activation. Although expression of other genes involved in BaP metabolism (*Gsta1* and *Nqo1*) was increased, hepatic mRNA levels of the inflammation-associated genes *Cybb*, *Duox2* and *Tnf* and hepatic TNF- α protein levels were elevated in *Cyp1a1*($-/-$) mice fed the Western diet with BaP (Figs. 4 and 5). Furthermore, F4/80-positive cells accumulated in the liver of these mice (Fig. 5), indicating inflammation that is associated with Kupffer cell activation. These findings suggest that enhanced BaP metabolic activation, due to the absence of CYP1A1 and the presence of other enzymes such as CYP1B1, induces ROS production and inflammatory responses in *Cyp1a1*($-/-$) mice receiving the combined treatment. The combined treatment with a Western diet and BaP for 3 weeks induced liver injury in *Cyp1a1*($-/-$) mice, but these mice had ameliorated liver damage with showing NAFLD and dysregulated lipid metabolism after the 15-week

treatment (Fig. 1). Further studies are needed to elucidate time-dependent changes in NAFLD pathogenesis.

Induction of CYP1A1 by AHR activation had been considered to be required for BaP-induced toxicity, because the original studies using mutant Hepa-1 cells demonstrated that BaP-elicited toxicity is diminished in cells lacking a functional CYP1A1 or AHR (Hankinson et al., 1991). Moreover, *Ahr*-knockout mice are resistant to BaP-induced carcinogenicity in the skin, and these mice showed no enhanced *Cyp1a1* gene expression in skin or liver after subcutaneous BaP injection (Shimizu et al., 2000). Induction of exogenous CYP1A1 expression in CYP1A1-deficient cells restores the formation of BaP-DNA adducts (Maier et al., 2002). On the other hand, overexpression of CYP1A1, as well as CYP1A2, decreases BaP-DNA adduct formation (Endo et al., 2008). The role of CYP1A1 in the intestine-liver axis and the effect on NAFLD pathogenesis warrant further investigation. In conclusion, a Western diet containing BaP, found in grilled meat, increases the risk of NAFLD and nonalcoholic steatohepatitis, and CYP1A1 plays a protective role against NAFLD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BaP	benzo[<i>a</i>]pyrene
AHR	aryl hydrocarbon receptor
CYP	cytochrome P450
ROS	reactive oxygen species
NQO1, NAD(P)H	quinone oxidoreductase-1
GSTA1	glutathione <i>S</i> -transferase-A1
NAFLD	nonalcoholic fatty liver disease
BSEP	bile salt export pump
MRP	multidrug resistance associated protein
OST	organic solute transporter

NTCP	Na ⁺ /taurocholate-cotransporting polypeptide
OATP	organic anion-transporting polypeptide
TNF	tumor necrosis factor
ABC	ATP-binding cassette
SREBP-1c	sterol regulatory element-binding protein 1c
SCD	stearoyl-CoA desaturase
ACC	acetyl-CoA carboxylase
LPL	lipoprotein lipase
CYBB	cytochrome b-245, beta polypeptide
NOX	NADPH oxidase
DUOX2	dual oxidase 2
FXR	farnesoid X receptor
PXR	pregnane X receptor
LXR	liver X receptor

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Highlight

A Western diet with benzo[*a*]pyrene induces hepatic steatosis in *Cyp1a1*(*-/-*) mice. Hepatic steatosis in these mice is associated with lipid and bile acid accumulation. Bile acid export and lipid metabolism gene expression is dysregulated in these mice. These mice also have hepatic inflammation, likely due to enhanced benzo[*a*]pyrene toxicity.

CYP1A1 protects against nonalcoholic fatty liver disease induced by benzo[*a*]pyrene.

Fig. 1. Combined treatment with the Western diet and BaP induces hepatic steatosis in *Cyp1a1*(-/-) mice. (A) Hematoxylin-and-eosin staining of liver samples. (B) Hepatic cholesterol, triglyceride and bile acid levels. (C) Plasma cholesterol, triglyceride and bile acid levels. (D) Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. (E) Body weight changes and food intake. Wild-type (WT) and *Cyp1a1*(-/-) mice were fed a control diet versus Western diet, with or without dietary BaP for 3 weeks (D and E) and 15 weeks (A, B, C and D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA followed by

Tukey's multiple comparisons); $\dagger\dagger P<0.01$, $\dagger\dagger\dagger P<0.001$ compared to wild-type mice fed the same diet (Student's *t*-test).

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Fig. 2.

Hepatic expression of genes involved in bile acid and cholesterol transport. (A) mRNA levels of bile-acid-export transporters BSEP (*Abcb11*), *Mrp3*, *Mrp4*, OST α (*Slc51a*) and OST β (*Slc51b*). (B) mRNA levels of bile-acid-import transporters NTCP (*Slc10a1*), OATP1A1 (*Slco1a1*) and OATP1B2 (*Slco1b2*). (C) mRNA levels of cholesterol-export transporters *Abcg5* and *Abcg8*. (D) Protein levels of MRP4 and OATP1A1. Wild-type (WT) and *Cyp1a1*($-/-$) mice were fed the control diet versus Western diet, with or without dietary BaP, for 3 weeks. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA followed by

Tukey's multiple comparisons); $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ compared to wild-type mice fed the same diet (Student's t test).

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Fig. 3. Hepatic expression of genes involved in bile acid and lipid synthesis. (A) mRNA levels of bile-acid-synthesis enzymes *Cyp7a1*, *Cyp8b1* and *Cyp27a1*. (B) mRNA levels of lipogenesis genes SREBP-1c (*Srebf1*), SCD (*Scd1*, *Scd2*, *Scd3* and *Scd4*), *Acc* and *Lpl*. (C) Protein levels of LPL. Wild-type (WT) and *Cyp1a1(-/-)* mice were fed the control diet versus Western diet, with or without BaP, for 3 weeks. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparisons); † $P < 0.05$, †† $P < 0.01$ compared to wild-type mice fed the same diet (Student's *t* test).

Fig. 4.

Hepatic expression of genes involved in BaP metabolism. mRNA levels of *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, epoxide hydrolase 1 (*Ephx1*), *Gsta1* and *Nqo1* in wild-type (WT) and *Cyp1a1*(-/-) mice receiving the control diet versus Western diet, with or without BaP, for 3 weeks. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA followed by Tukey's multiple comparisons); † $P < 0.05$, ††† $P < 0.001$ compared to wild-type mice fed the same diet (Student's *t* test). n.d., not detected.

Fig. 5. Combined treatment with the Western diet plus BaP induces hepatic inflammation in *Cyp1a1*(-/-) mice. (A) mRNA levels of NOX2 (*Cybb*), *Nox4*, *Duox2* and *Tnf*. (B) Hepatic levels of TNF-α protein. * $P < 0.05$, ** $P < 0.01$ (one-way ANOVA followed by Tukey's multiple comparisons); † $P < 0.05$, †† $P < 0.01$ compared to wild-type mice fed the same diet (Student's *t* test). (C) Immunostaining of liver with anti-F4/80 antibody. Wild-type (WT) and *Cyp1a1*(-/-) mice fed the control diet versus Western diet, with or without BaP, for 3 weeks.