Hyperhomocysteinemia Activates the Aryl Hydrocarbon Receptor/CD36 Pathway to Promote Hepatic Steatosis in Mice

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Hyperhomocysteinemia (HHcy) is associated with liver diseases such as fatty liver and hepatic fibrosis; however, the underlying mechanism is still largely unknown. The current study aimed to explore the signaling pathway involved in HHcy-induced hepatic steatosis (HS). C57BL/6 mice were fed a high-methionine diet (HMD) for 4 and 8 weeks to establish the HHcy mouse model. Compared to a chow diet, the HMD induced hepatic steatosis and elevated hepatic expression of CD36, a fatty acid transport protein. The increased CD36 expression was associated with activation of the aryl hydrocarbon receptor (AHR). In primary cultured hepatocytes, high levels of homocysteine (Hcy) treatment up-regulated CD36 and increased subsequent lipid uptake; both were significantly attenuated by small interfering RNA (siRNA) knockdown of CD36 and AHR. Chromatin immunoprecipitation assay revealed that Hcy promoted binding of AHR to the CD36 promoter, and transient transfection assay demonstrated markedly increased activity of the AHR response element by Hcy, which was ligand dependent. Mass spectrometry revealed significantly increased hepatic content of lipoxin A4 (LXA4), a metabolite of arachidonic acid, in HMD-fed mice. Furthermore, overexpression of 15-oxoprostaglandin 13-reductase 1, a LXA4 inactivation enzyme, inhibited Hcy-induced AHR activation, lipid uptake, and lipid accumulation. Moreover, LXA4-induced up-regulation of CD36 and lipid uptake was inhibited by AHR siRNA in vitro in hepatocytes. Finally, treatment with an AHR antagonist reversed HHcy-induced lipid accumulation by inhibiting the AHR-CD36 pathway in mice. Conclusion: In conclusion, our results strongly suggest that HHcy activated the AHR-CD36 pathway by increasing hepatic LXA4 content, which resulted in HS. (HEPATOLOGY 2016; 00:000–000)

Nonalcoholic fatty liver disease (NAFLD) is an increasingly recognized disease status that involves a spectrum of liver damage, ranging from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis. Obesity, type 2 diabetes mellitus, and hyperlipidemia are important risk factors of NAFLD, but the pathogenesis of NAFLD is poorly understood. (1) Homocysteine (Hcy) is a toxic...
nonprotein sulfur containing amino acid, formed from methionine after removal of the methyl group on S-adenosylmethionine. Elevated plasma Hcy level (>15 μM) defines hyperhomocysteinemia (HHcy), an important and independent factor involved in several disorders. Plasma Hcy level is higher in NAFLD patients than controls. In mice, a high-methionine diet (HMD) caused HHcy and hepatic steatosis (HS). Although several mechanisms reported to be associated with HHcy disturbed lipid metabolism in different HHcy models, the underlying mechanism remains largely unknown.

HS is characterized by excess triglyceride (TG) accumulation in hepatocytes because of perturbed lipid availability and disposal. In hepatocytes, an important source of lipids is circulating free fatty acids (FFAs). FFAs are converted to TG, and excess TG is then stored in lipid droplets. CD36 is a key membrane protein, and elevated CD36 expression is involved in facilitating fatty acid (FA) uptake from the circulation by liver in pathological conditions, such as diet-induced obesity and diabetes. Recent studies have shown that increased hepatic CD36 level contributes to hepatic TG storage, and CD36 expression was also found elevated in NAFLD patients. Therefore, CD36 plays a pivotal role in development of HS.

Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that belongs to the basic helix-loop-helix/period/AHR nuclear translocator/single-minded protein family. Several metabolites of arachidonic acids (AAs) have been identified as novel endogenous ligands of AHR. Our previous studies showed that HHcy was associated with AA metabolism. AHR activation has a wide variety of toxic and biological effects. Especially, it induces spontaneous HS by up-regulating CD36 expression.

In this study, we demonstrated that activation of AHR by HHcy caused HS in vivo and in vitro. Lipoxin A₄ (LXA₄) derived from AA participates in this HHcy-induced activation of AHR, and CD36 was identified as a transcriptional target of AHR. Moreover, AHR antagonism protected mice against HHcy-induced liver lipid accumulation. Our results may have important implications for therapeutic targets of NAFLD.

**Materials and Methods**

An expanded Materials and Methods section is available in the Supporting Information.

**ANIMALS AND TREATMENTS**

Six-week-old male C57BL/6 mice were fed a 2% methionine diet (HMD) for 4 or 8 weeks, and control mice were fed a chow diet for the indicated times. For inhibiting AHR in vivo, drinking water was supplemented with the AHR antagonist, CH223191, at 10 mg/kg/day. At the end of treatment (4 or 8 weeks), mice were killed and plasma and liver were collected for analysis. Total cholesterol and TG from mouse livers and plasma were measured by use of enzymatic kits from Thermo Scientific (Waltham, MA) and Wako Life Sciences (Richmond, VA). The investigation conformed to the Guide for the Care and Use of Laboratory Animals by the U.S. National Institutes of Health (NIH Publication No. 85-23, updated 2011). Study protocols and use of animals were approved by Institutional Animal Care and Use Committee of Tianjin Medical University (Tianjin, China).

**CULTURE OF MURINE PRIMARY HEPATOCYTES**

Murine primary hepatocytes were isolated as described. Hepatocytes were cultured with RPMI 1640 medium containing 10% fetal bovine serum.
After 6-hour attachment, cells were treated for experiments. See the Supporting Information for details.

**PLASMID CONSTRUCTION AND TRANSIENT TRANSFECTION**

pCMX-Gal4-AHR plasmid was created by cloning mouse AHR-ligand-binding domain code sequences (amino acids 230-397) into the mammalian expression vector, pCMX, containing the galactose 4 (Gal4)/DNA-binding domain. For transient transfection, cells were cotransfected with different plasmids by use of Lipofectamine 2000. The $\beta$-galactosidase plasmid was cotransfected as a transfection control. See the Supporting Information for details.

**METABOLOMIC ANALYSIS**

Mouse livers were homogenized and lysed by repeated freeze-thawing cycles and preprocessed in the solvent of methanol. After centrifugation, the supernatant was extracted by ethyl acetate twice, then the upper organic phase was evaporated. The residue was dissolved in 100 $\mu$L of 30% acetonitrile. Ultra-high-performance liquid chromatography (Waters, Milford, MA) involved a 5500 QTRAP hybrid triple-quadruple linear ion-trap mass spectrometer (AB Sciex, Foster City, CA) equipped with a Turbo Ion Spray electrospray ionization source (liquid chromatography/tandem mass spectrometry) as described.

**CHROMATIN IMMUNOPRECIPITATION**

Chromatin immunoprecipitation (ChIP) assay were performed following the procedure described previously. Eluted DNA was purified for quantitative polymerase chain reaction (qPCR) using primers for mouse CD36 promoter containing AHR-binding sites (5’-AAT AACTAATCTGACCAAAGC-3’ and 5’-ATG TGCCATGTCTAAACTG-3’). See the Supporting Information for a detailed method and primer sequences.

**OIL RED O STAINING, MEASUREMENT OF HEPATIC LIPID UPTAKE, QPCR, AND WESTERN BLOTTING ANALYSIS**

See the Supporting Information for details of Oil Red O (ORO) staining, measurement of hepatic lipid uptake, qPCR, and western blotting analysis.

**STATISTICAL ANALYSIS**

Data are presented as mean $\pm$ standard error of the mean (SEM). Differences between groups were analyzed by unpaired t test or one-way analysis of variance with Bonferroni’s post-test with comparison of more than two groups. Statistical significance was set at $P < 0.05$. Statistical analysis involved use of GraphPad Prism software (version 5.01; GraphPad Software Inc., San Diego, CA).

**Results**

**HHcy INDUCED HS AND INCREASED CD36 EXPRESSION**

To determine the pathophysiological relevance of HHcy and hepatic steatosis *in vivo*, we fed 6-week-old male C57BL/6 mice an HMD for 4 or 8 weeks to establish HHcy models at different stages. Plasma Hcy level was significantly higher with both short- and long-term HMD than for controls (Table 1). Although body weight was not altered with different diets, the ratio of liver to body weight was higher with an 8-week HMD than a chow diet. Plasma levels of cholesterol and glucose were unchanged. In contrast, plasma TG level was elevated with a 4-week HMD and decreased to the control level at 8 weeks (Table 1). Hepatic lipid accumulation was increased at 4 weeks and became prominent at 8 weeks with an HMD (Fig. 1A). Hepatic TG content was increased by 25% and 75% with 4- and 8-week HMD, respectively (Fig. 1B).

Hepatic lipid accumulation results from perturbed lipid availability and disposal. To understand the mechanism by which HHcy induced HS, we examined expression of genes involved in lipogenesis, TG secretion, FA uptake, and oxidation in the liver. Messenger RNA (mRNA) and protein levels of total cellular CD36 (t-CD36) were markedly increased as early as 4 weeks with an HMD and were maintained to 8 weeks as compared to controls (Fig. 1C,D). Expression of FA transfer protein 2 and short-chain acyl-CoA (coenzyme A) dehydrogenase was elevated slightly with 8-week HMD, and that of apolipoprotein B and short-chain acyl-CoA dehydrogenase was elevated slightly with 4-week HMD. Other related genes were not affected (Fig. 1C). Because excess lipids can be utilized for bile acid synthesis, we further examined expression of genes involved in bile acid synthesis, including FXR, small heterodimer partner, Cyp7a1, Cyp7b1, Cyp8b1, and Cyp27a1, and found that mRNA levels of those
genes were merely unchanged (Supporting Fig. S1A,B). In addition, the expression of inflammatory factors, including tumor necrosis factor, interleukin-6 and CD68 were not changed by 8-week HMD (Supporting Fig. S1C). These results suggested that CD36 was an early response factor in HHcy-induced fatty liver.

**Hcy INDUCED LIPID ACCUMULATION BY UP-REGULATING CD36 IN HEPATOCYTES**

To demonstrate the cellular effects of Hcy on lipid metabolism and CD36 expression, primary cultured hepatocytes were treated with Hcy (100 \( \mu \)M) for 24 hours. Intracellular lipids were accumulated in Hcy-treated hepatocytes (Fig. 2A), which was concomitant with increased protein and mRNA levels of t-CD36 (Fig. 2B,C). As a membrane receptor, CD36 mediates lipid uptake. To determine whether Hcy induced lipid accumulation by increasing FA uptake, primary hepatocytes were incubated with BODIPY-C16, a fluorescent lipid probe that can trace cellular lipid trafficking. Lipid accumulation in hepatocytes and lipid uptake by hepatocytes were both increased with Hcy stimulation, which was reversed by CD36 small interfering RNA (siRNA; Fig. 2D,E). Thus, up-regulation of CD36 by Hcy promoted hepatocyte lipid uptake and then lipid accumulation.

**Hcy INCREASED CD36 EXPRESSION AND LIPID UPTAKE BY ACTIVATING AHR**

Given that CD36 is transcriptionally regulated by several transcriptional factors and nuclear receptors, such as liver X receptor (LXR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor gamma (PPAR\( _\gamma \)), \(^{21}\) CCAAT-enhancer-binding protein alpha or beta (C/EBP\( _\alpha \) or C/EBP\( _\beta \)), \(^{22}\) farnesoid X receptor (FXR), \(^{23}\) and AHR, \(^{17}\) we investigated the effect of these factors on Hcy-induced CD36 up-regulation. HMD feeding did not affect expression of PPAR\( _\gamma \), FXR, C/EBP\( _\alpha \), C/EBP\( _\beta \), or their target genes in mouse livers (Supporting Figs. S1A,B and S2A,B), but AHR was activated with both 4- and 8-week HMD, as evidenced by elevated levels of its target genes (Cyp1a1 and Cyp1a2; Fig. 3A). In contrast, the target genes of PXR (Cyp3a11 and Fae) were induced with 8-week HMD (Supporting Fig. S2B), which suggests a delayed regulation of PXR as compared with AHR. To further distinguish the role of AHR and PXR activation in Hcy-induced CD36 expression, we separately decreased the expression of AHR and PXR using siRNA in hepatocytes. Hcy-induced CD36 expression was abolished by AHR (Fig. 3B), but not PXR siRNA (Supporting Fig. S2C). Intriguingly, silencing PXR in hepatocytes increased expression of CD36 (Supporting Fig. S2C). Morphologically, Hcy-induced lipid deposition and FA uptake were largely abrogated by AHR knockdown (Fig. 3D). Furthermore, the AHR antagonist, CH223191, inhibited the Hcy-induced up-regulation of AHR target genes, including CD36, lipid deposition, and FA uptake in hepatocytes (Fig. 3C,E). Thus, AHR activation was involved in Hcy-induced lipid accumulation and CD36 expression.

**Hcy PROMOTED AHR BINDING TO THE CD36 PROMOTER WAS LIGAND-DEPENDENT IN HEPATOCYTES**

AHR was reported to bind directly to the dioxin response elements of target genes and regulate their expression. Mouse and human CD36 promoter regions have several putative AHR binding sites. \(^{17}\) To determine whether Hcy can facilitate AHR recruitment to the CD36 promoter, we performed a ChIP

<table>
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<th>Variables</th>
<th>Chow Diet</th>
<th>HMD</th>
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<tr>
<td>Body weight, g</td>
<td>23.95 ± 0.60</td>
<td>23.74 ± 0.45</td>
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<tr>
<td>Liver/body weight ratio</td>
<td>0.055 ± 0.001</td>
<td>0.053 ± 0.001</td>
</tr>
<tr>
<td>Plasma Hcy level, ( \mu )M</td>
<td>2.56 ± 0.76</td>
<td>29.84 ± 3.56**</td>
</tr>
<tr>
<td>Plasma TG level, mg/dL</td>
<td>57.20 ± 3.48</td>
<td>76.70 ± 8.06*</td>
</tr>
<tr>
<td>Plasma CHO level, mg/dL</td>
<td>64.65 ± 4.22</td>
<td>57.45 ± 4.81</td>
</tr>
<tr>
<td>Plasma glucose (mM)</td>
<td>8.07 ± 0.47</td>
<td>8.24 ± 0.36</td>
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Data are mean ± SEM. *\( P < 0.05; ** P < 0.01 \) versus chow diet (n = 6).
Recruitment of AHR to nucleotides $-1070 \sim -1064$ base pairs of the CD36 promoter, which contains a putative dioxin response element (TAGCCTG), was dramatically increased by Hcy in hepatocytes (Fig. 4A). The effect of Hcy on AHR activation was further verified by a marked increase in luciferase activity induced by Hcy in hepatocytes transfected with the AHR response element/luciferase reporter plasmid (Fig. 4B). 3-Methylcholanthrene (3-MC), an AHR agonist, was a positive control. Because
AHR is a ligand-activated transcription factor, we further elucidated whether Hcy-activated AHR was mediated by increasing ligands by an AHR-Gal4-luciferase system. Cotransfection of pCMX-Gal4-AHR and Gal4-luciferase reporter revealed activation of the Gal4-luciferase reporter by both Hcy and 3-MC (Fig. 4C), which suggests that Hcy activation of AHR was ligand dependent.

**LXA₄ IS AN AHR ENDOGENOUS LIGAND ACTIVATING THE AHR-CD36 PATHWAY**

Numerous correlation studies provide a potential link between AHR activation and AA metabolism, with a focus on the metabolites of AA. (24-26) In addition, Hcy participates in regulating metabolic enzyme
FIG. 3. Hcy-induced CD36 expression was mediated by AHR activation. (A) qPCR analysis of mRNA levels of hepatic AHR target genes in mice fed an HMD for 4 or 8 weeks. (B) Primary hepatocytes were transfected with siNC or siAHR, then treated with Hcy. qPCR analysis of mRNA levels of AHR target genes. (C) Primary hepatocytes were incubated with Hcy with or without the AHR antagonist, CH223191. qPCR analysis of mRNA levels of AHR target genes. (D,E) Representative ORO staining and BODIPY-C16 fluorescence images (left) and densitometry quantification (right) of lipid deposition and FA uptake in (B) and (C), respectively. Quantified data are mean ± SEM of 6-8 mice in each group or six independent experiments. *P < 0.05; **P < 0.01. Abbreviation: Ctrl, control.
FIG. 4.
activity toward AA.\(^{15,16}\) To identify metabolites of AA that are potentially involved in HHcy-induced liver lipid disturbance, we screened the change in the AA metabolite profile by a metabolomic approach in livers of 8-week HMD-fed and control mice. Levels of 15-deoxy-A12,14-prostaglandin J2 (15d-PGJ2), LXA\(_4\), and 15-oxo-eicosatetraenoic acid (15-oxo-ETE) were significantly increased in liver of HMD-fed mice (Fig. 4D). 15d-PGJ2 is derived from AA by cyclooxygenase. LXA\(_4\) and 15-oxo-ETE are metabolites of the lipoxygenase (LO) pathway of AA metabolism. Although 15d-PGJ2 is an endogenous ligand of PPAR\(\gamma\), expression of PPAR\(\gamma\) target genes did not change in liver of HMD-fed mice (Supporting Fig. S2A,B), and there is a lack of evidence suggesting the effect of 15-oxo-ETE on lipid metabolism. LXA\(_4\) is a known ligand of AHR,\(^{27}\) but its content did not change in plasma (Supporting Fig. S3A). These data suggest that LXA\(_4\) may have a local effect in liver.

To understand how HHcy increases hepatic LXA\(_4\) content, we analyzed expression of genes involved in LXA\(_4\) biosynthesis and inactivation. LXA\(_4\) is rapidly metabolized after biosynthesis and bioaction. Three major biosynthetic enzymes, 5-LO, 15-LO, and 12-LO, are involved in LXA\(_4\) formation. Then, 15-hydroxy/oxo-eicosanoid oxidoreductase catalyzes LXA\(_4\) to 15-oxo-LXA\(_4\), which is further converted to 13,14-dihydro-15-oxo-LXA\(_4\) by the action of 15-oxoprostaglandin 13-reductase 1 (Ptgr1).\(^{28}\) Analysis of genes involved in LXA\(_4\) biosynthesis and inactivation showed that HHcy directly reduced Ptgr1 expression, with no significant alteration in 5-LO, 15-LO, 12-LO, and 15-hydroxy/oxo-eicosanoid oxidoreductase expression (Fig. 4E,F). In addition, the changes of LXA\(_4\) and Ptgr1 in livers of 4-week HMD-fed mice were consistent with 8-week HMD-fed mice (Supporting Fig. S3B-D). These data indicate that HHcy may increase hepatic LXA\(_4\) content by encumbering LXA\(_4\) inactivation.

To gain further insights into whether LXA\(_4\) was essential for Hcy-induced lipid accumulation in vitro, we measured the mRNA levels of genes influencing LXA\(_4\) inactivation and found that Hcy treatment inhibited Ptgr1 expression (Supporting Fig. S4A), in accord, increased the content of LXA\(_4\) in Hcy-treated hepatocytes (Supporting Fig. S4B). Importantly, overexpression of Ptgr1 effectively inhibited up-regulation of AHR target genes, including CD36 (Fig. 5A). To further explore the potential role of LXA\(_4\) in hepatic lipid disturbance, we treated hepatocytes with 5(S),6(R)-LXA\(_4\) to mimic the function of endogenous LXA\(_4\). LXA\(_4\) at 100 nM significantly increased luciferase activity in hepatocytes transfected with AHRE or Gal4-AHR/Gal4, with 3-MC as a positive control (Fig. 5B,C). Moreover, LXA\(_4\) up-regulated expression of AHR target genes Cyp1a1, Cyp1a2, and CD36, which were abolished by AHR silencing (Fig. 5D). These results support that activation of the AHR-CD36 pathway by Hcy largely depends on LXA\(_4\). Next, we analyzed the effects of cellular LXA\(_4\) on lipid uptake and lipid deposition. Overexpression of Ptgr1 to inactivate LXA\(_4\) suppressed Hcy-induced lipid accumulation and FA uptake (Fig. 5E). Conversely, hepatocytes treated with LXA\(_4\) showed markedly increased BODIPY-C16 uptake and increased lipid deposition, which was attenuated by AHR silencing (Fig. 5F). Hence, LXA\(_4\) ligand dependently activated the AHR-CD36 pathway and played an important role in Hcy-induced lipid accumulation in liver.

**Inhibition of AHR ameliorated HS in HMD-treated mice**

To further show the crucial role of AHR on HHcy-induced HS in vivo, we treated mice with HMD with and without the AHR antagonist, CH223191 (10 mg/kg/day in drinking water, HMD+CH223191), for 4 or 8 weeks. CH223191 treatment for 4 weeks ameliorated HMD-induced hepatic lipid accumulation and TG content, which was even greater with 8-week treatment (Fig. 6A,B). Hepatic cholesterol level was decreased with 4-week CH223191 treatment, with little change at 8 weeks (Fig. 6B). Accordingly, hepatic

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**FIG. 4.** Activation of AHR by Hcy is ligand dependent. (A) ChIP-PCR analysis of AHR binding on the CD36 promoter in primary hepatocytes with or without Hcy treatment by use of AHR antibody or rabbit immunoglobulin G. (B) AHRE-luciferase reporter was transfected in primary hepatocytes with or without Hcy or 3-MC (a positive control). (C) Gal4 DBD-AHR LBD construct/Gal4-luciferase reporter system transfected in primary hepatocytes with or without Hcy or 3-MC. (D) Heatmap of AA metabolite profile in liver of mice fed an 8-week chow diet or HMD. The concentration of significantly changed metabolites is shown in the right panel. Red and green represent increased or decreased levels, respectively. (E) mRNA levels of genes related to LXA\(_4\) formation and inactivation in mice fed a chow diet or HMD for 8 weeks. (F) Western blotting analysis of protein level of Ptgr1 in mice fed a chow diet or HMD for 8 weeks. Quantified data are mean ± SEM of at least 6 mice in each group or five to six independent experiments. *\(P < 0.05\); **\(P < 0.01\). Abbreviations: Ctrl, control; IgG, immunoglobulin G.
FIG. 5.
expression of Cyp1a1, Cyp1a2, total, and membrane-bound CD36 was effectively inhibited by CH223191 treatment (Fig. 6C–E). CH223191 did not affect body weight, liver weight, and other features of mice (data not shown). Therefore, inhibiting AHR protected mice against HHcy-induced HS. Our in vitro and in vivo studies suggest that AHR activation played a prominent role in HHcy-induced HS (Fig. 6F).

Discussion

HHcy is an important and independent factor for several disorders, including atherosclerosis, diabetes, and immune activation. Health problems related to HHcy, especially cardiovascular disease, have drawn extensive attention. Previous research uncovered several mechanisms of how HHcy causes cardiovascular diseases, including increased endoplasmic reticulum stress, oxidative stress, proinflammatory effects, and DNA demethylation. HHcy also plays a vital role in liver diseases, such as fatty liver and hepatic fibrosis. However, the underlying mechanisms involved in HHcy-induced HS remain largely unknown. Our observations suggest that the AHR-CD36 pathway is a novel mechanism of HHcy-induced HS. We found that: (1) HHcy up-regulated CD36 expression in mouse liver and hepatocytes in vitro; (2) HHcy transcriptional up-regulation of CD36 is by enhancing the binding of AHR to the CD36 promoter; (3) LXA4 is involved as an endogenous ligand for AHR activation in HHcy-induced HS; and (4) activation of the AHR-CD36 pathway plays a pivotal role in HHcy-induced HS, which could be alleviated by the AHR antagonist, CH223191, in vivo.

AHR was originally characterized as a ligand-activated transcription factor that can sense environmental toxicants. Activated AHR is heterodimerized with AHR heterodimeric partner and induces expression of its target genes. Recent studies demonstrated that AHR activation in both transgenic mice and AHR agonist-treated mice led to HS. However, different from AHR transgenic mice, which were hepatocyte specific, a pharmacological AHR-activated model showed inhibited very-low-density lipoprotein/TG secretion, which might be attributed to involvement of Kupffer cells or hepatic stellate cells. Similar to AHR transgenic mice, the toxic effect of Hcy in our mice probably results from activation of AHR, its target CD36, and the consequent increase in hepatic FA uptake, which implied a possible specific effect of Hcy in liver.

Previous studies reported that CD36 mediated uptake and intracellular transport of long-chain FAs in liver, which leads to HS. Morihara et al. reported that Hcy enhanced CD36 expression in macrophages and contributed to foam cell formation. However, the level of CD36 expression accompanied by HHcy with damage to hepatic lipid homeostasis was unknown. Nuclear receptors establish a cross-talk with transcription factors controlling signaling pathways that regulate the homeostasis of lipids, glucose, inflammation, and other factors. CD36 is involved in lipid metabolism. The shared regulation of CD36 by LXR, PXR, PPARγ, C/EBPα and C/EBPβ, FXR, and AHR suggests that CD36 is a common target of these transcription factors. Many of these factors are metabolic nuclear receptors, requiring activation by endogenous metabolites. We found activation of AHR and PXR in HMD-fed mice and Hcy-treated hepatocytes, but no change in expression of LXR, FXR, PPARγ, C/EBPα, C/EBPβ, or their target genes. Notably, silencing AHR expression significantly reduced CD36 expression and markedly attenuated FA uptake and lipid accumulation in hepatocytes. In contrast, Hcy-induced CD36 expression was not inhibited by PXR silencing. This observation agreed with another report. Regarding CD36 up-regulation, results in our 4-week HMD-fed mice suggested that activation of AHR may have preceded that of other receptors. Our data support AHR playing a preferential and prominent role in the genesis and maintenance of lipid metabolism in hepatic steatosis.

An intriguing finding of the present study is that Hcy ligand dependently activated the transcriptional factor, AHR, by LXA4, thereby leading to hepatic FA uptake and lipid deposition. Paradoxically, a recent study suggested that exogenous LXA4 administration...
FIG. 6. AHR antagonist reversed HHcy-induced hepatic steatosis in mice in vivo. Male C57BL/6 mice were fed a chow diet or HMD with or without CH223191 in drinking water (10 mg/kg/d) for 4 or 8 weeks. (A) Representative ORO staining for lipid deposition in liver. (B) Hepatic TG and cholesterol (CHO) content. (C,D) Western blotting analysis of hepatic t-CD36 and membrane-bound CD36 (m-CD36) protein levels in mice with 4- or 8-week treatment. (E) qPCR analysis of mRNA levels of Cyp1a1, Cyp1a2, and CD36. Quantified data are mean ± SEM of at least 6 mice in each group. *P < 0.05; **P < 0.01. (F) Proposed mechanism of Hcy-induced activation of AHR-CD36 pathway in liver. Hcy increased hepatic content of LXA₄. As an endogenous ligand of AHR, LXA₄ promotes binding of AHR to CD36 promoter and increases expression of CD36, which accelerates FA uptake and lipid accumulation in hepatocytes.
attenuated high-fat-diet (HFD)-induced inflammation in adipose tissue by the LXA₄ receptor (ALX) and ameliorated obesity-related fatty liver. One reason for the different findings is that the biological function of LXA₄ depends on the predominant receptor in different tissues. Indeed, three major receptors showing high affinity with LXA₄ include ALX, cysteinyl-leukotriene receptor 1, and AHR, expressed mainly in white blood cells, endothelial cells, and liver, respectively. In the previous study, level of LXA₄ in adipose tissue was not affected by an HFD, and the effect of LXA₄ on fatty liver was secondary to the decreased production of Annexin AI from adipose tissue. In contrast, we found increased endogenous LXA₄ level in liver rather than plasma in HMD-fed mice, which suggests a local effect of LXA₄. In addition, elevated level of LXA₄ in liver of HMD-fed mice did not directly change expression of inflammatory factors, which further supports that ALX might not be the predominant receptor of LXA₄ in liver. Therefore, the local high concentration of LXA₄ in liver mediated Hcy-induced HS by activation of AHR, which could explain the discrepancy in findings between the previous report and our study.

NAFLD is now considered the most common liver condition in developed and developing countries. In the last decades, Hcy has been identified as a risk factor for several disorders and has become an important health issue. Our studies conducted in a murine HMD-induced HS model suggest a close association between Hcy and fatty liver. To confirm the causal relationship between HHcy and fatty liver, we not only treated primary cultured hepatocytes with Hcy in vitro, but also employed Hcy-fed mice to confirm the results. Mice were treated with or without 1.8 g/L DL-Hcy in drinking water for 8 weeks, plasma level of Hcy was increased (~4-fold), and hepatic TG content and mRNA levels of hepatic CD36 and AHR target genes were also increased (data not shown), which is consistent with the results from our HMD-fed mice. Furthermore, we found that activation of AHR to up-regulate CD36 expression by HHcy was ligand dependent and led to lipid accumulation in liver (Fig. 6F). Our study suggests a potential role of targeted AHR inhibition in liver for treating HHcy-induced fatty liver.

REFERENCES


Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28518/suppinfo.