Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor regulating the expression of genes involved in xenobiotic response. Recent studies have suggested that AhR plays essential roles not only in xenobiotic detoxification but also energy metabolism. Thus, in this study, we studied the roles of AhR in lipid metabolism. Under high fat diet (HFD) challenge, liver-specific AhR knock-out (AhR LKO) mice exhibited severe steatosis, inflammation, and injury in the liver. Gene expression analysis and biochemical study revealed that de novo lipogenesis activity was significantly increased in AhR LKO mice. In contrast, induction of suppressor of cytokine signal 3 (Socs3) expression by HFD was attenuated in the livers of AhR LKO mice. Rescue of the Socs3 gene in the liver of AhR LKO mice cancelled the HFD-induced hepatic lipotoxicities. Promoter analysis established Socs3 as novel transcriptional target of AhR. These results indicated that AhR plays a protective role against HFD-induced hepatic steatosis and the subsequent lipotoxicity through the direct transcriptional regulation of Socs3 expression by AhR.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that possesses a basic helix-loop-helix/Per-Arnt-Sim domain and mediates a variety of toxic and biological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds (1–3). Upon binding to ligands, AhR forms a heterodimer with the AhR nuclear translocator (Arnt) in the nucleus. Then, this complex transactivates the target genes such as xenobiotic-metabolizing enzymes by binding to xenobiotic response element (XRE) sequences in their promoter region (4–6). In addition to direct transcriptional regulation, AhR controls gene expressions thorough interactions with other transcription factors such as nuclear factor κ-light chain enhancer, estrogen receptor, and E2F (7–9).

Recent studies using Ahr knock-out (KO) mice have suggested that AhR is required for not only xenobiotic responses but also for several endobiotic responses, including the development of tissues and reproduction (10–12). These functional roles of AhR in endobiotic responses are supported by the identification of endogenous ligands or activators (13–22).

One possible function of AhR is the regulation of energy metabolism for the following reasons. First, AhR is highly expressed in metabolically active tissues, including the liver, adipose tissue, and macrophages. Second, the expressions of AhR and its target gene are increased in obese mice (23, 24). Third, global AhR KO mice exhibit spontaneous lipid accumulation and fibrosis in the liver (11). Moreover, a comprehensive analysis of gene expression in mice treated with low-dose 2,3,7,8-tetrachlorodibenzo-p-dioxin revealed that hepatic AhR is associated with lipid, glucose, and cholesterol metabolism (25). Finally, identified endogenous ligands or activators, such as arachidonic acid metabolites, modified LDL, and glucose, are known to be involved in energy and lipid metabolism (14, 21, 22).

In this study, to better understand the role of AhR in lipid metabolism in the liver, mice with specific deletions of Ahr in the liver (AhR LKO) were subjected to a high fat diet (HFD) challenge. The results revealed that AhR plays protective roles against HFD-induced hepatic steatosis and the subsequent lipotoxicity through the direct transcriptional regulation of Socs3 expression.

Experimental Procedures

Animals, Diet, Drug Treatment, and Histology—AhR LKO mice were generated as described previously (26). Briefly, Ahrflx/flx mice were crossed to C57BL/6J mice carrying the Cre recombinase gene driven by the albumin promoter (The Jackson Laboratory, Bar Harbor, ME). Mice homozygous for the floxed allele and hemizygous for the Cre transgene (AhR LKO) were subjected to a high fat diet (HFD) challenge. The results revealed that AhR plays protective roles against HFD-induced hepatic steatosis and the subsequent lipotoxicity through the direct transcriptional regulation of Socs3 expression.

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transgenes (AhR<sup>flox/flox</sup>) were used as experimental controls. Mice were maintained at 23 ± 1 °C with 50 ± 10% relative humidity under a standard 12-h light/dark cycle with free access to water and food. Composition of a high fat diet (CLEA Japan Inc., Tokyo, Japan) is summarized in supplemental Table S1. Chow diet was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Composition of micronutrients in chow diet and high fat diet was matched (5% minerals and 1.2–1.5% vitamins). When necessary, mice received 3-methylcholanthrene (3MC) (20 mg/kg) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) by oral gavage for 3 consecutive days (27). For histology, tissues were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). For immunohistochemical analysis, liver paraffin-embedded sections were deparaffinized, rehydrated, and pretreated with 100 μg/ml proteinase K in 0.05 M Tris-HCl (pH 7.5) for 10 min at room temperature for antigen retrieval. Sections were blocked using 10% rat serum with 1% BSA in phosphate-buffered saline (PBS) for 60 min, followed by incubation with anti-F4/80 antibody (1:500; ab6640, Abcam Cambridge, MA) overnight at 4 °C. After washing, endogenous peroxidase activity was quenched using 0.3% H<sub>2</sub>O<sub>2</sub> in PBS for 10 min, and sections were incubated with anti-rat biotinylated secondary antibody, streptavidin-horseradish peroxidase ( Peroxidase Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin. To evaluate the pathological results, the stained slides (at least 15 slides per sample) were reviewed by multiple pathologists in a blinded manner. The experimental protocol was approved by the ethics review committee for animal experimentation at Nihon University.

**Metabolic Studies**—Energy expenditure (VO<sub>2</sub>, VCO<sub>2</sub>, and RQ) was measured by an indirect calorimeter (Muromachi, Tokyo, Japan). Food intake was continuously recorded on a computer and analyzed using FDMWIN software (Melquest, Toyama, Japan). Glucose tolerance test and insulin tolerance test were performed by intraperitoneal injection of glucose (1 g/kg body weight) or insulin (0.5 units/kg body weight; Lilly, respectively). Glucose levels were monitored using a TBARS assay kit (Cayman Chemical Co., Ann Arbor, MI).

**Thiobarbituric Acid-reactive Substances**—The level of thiobarbituric acid-reactive substances (TBARS) in the liver tissue was measured using a TBARS assay kit (Cayman Chemical Co., Ann Arbor, MI).

**Cell Culture**—HepG2 cells (the European Collection of Cell Culture) and RAW264.7 cells (RIKEN Cell Bank, Ibaraki, Japan) were maintained in minimum Eagle’s medium supplemented with 10% fetal bovine serum (FBS) or Dulbecco’s modified Eagle’s medium with 10% FBS, respectively.

**Knockdown and Overexpression of Socs3**—To prepare mouse Socs3 knockdown cells, RAW264.7 cells were transfected with either control stealth interfering RNA (siRNA) or stealth siRNA targeted for mouse Socs3 (Thermo Fisher Scientific) by using an X-tremeGENE siRNA transfection reagent (Roche Diagnostics, Tokyo, Japan) according to the manufacturer’s instructions. To prepare mouse Socs3-overexpressing cells, mouse Socs3 cDNA was subcloned into CMV-HA vector (Wako) were determined using a commercial assay kit according to the manufacturer’s instructions.

**Quantification of Serum and Liver FGF21 Levels**—Serum or tissue extracts prepared in PBS were processed for quantification of FGF21 levels by using the mouse/rat FGF21 ELISA kit (R&D Systems) according to the manufacturer’s instructions.

**Gene Expression Analysis**—Total RNA was extracted by use of RNAiso Plus (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer’s instructions. The cDNA was synthesized from 1.0 μg of total RNA by reverse transcriptase (Wako). Aliquots of cDNA were amplified on Stratagene Mx3000P real time PCR system (Agilent Technologies, Santa Clara, CA) using SYBR Green PCR reagents (Promega, Madison, WI). The mRNA expression levels were normalized against 36b4 and gapdh expression and are presented as relative expression levels.

**Western Blot Analysis**—Liver tissues and cells were lysed in commercial lysis buffer (Cell Signaling Technology Inc., Danvers, MA) containing 1 μM dithiothreitol and a protease inhibitor mixture. Protein concentrations were measured by the method of Bradford (30). The proteins were resolved on SDS-PAGE, transferred onto membranes, and probed with the antibodies against SOCS3, pSTAT3 (Tyr-705), STAT3, or β-actin (Cell Signaling Technology, catalog numbers 9145, 2923, 4970, and 9132, respectively). Immunoreactive proteins were visualized with electrochemiluminescence Western blotting detection reagents from Thermo Fisher Scientific Inc. (Waltham, MA).

**De Novo Lipogenesis Activity**—De novo lipogenesis activity was determined by the incorporation of [1-<sup>14</sup>C]acetate into the lipid (31, 32). In brief, mice fed a HFD for 10 weeks were anesthetized, and a liver slice was taken from each mouse. Liver slices (20–25 mg) were incubated with Dulbecco’s modified Eagle’s medium containing 0.5 mM sodium acetate and 74 KBq/ml [1-<sup>14</sup>C]sodium acetate (1.868 GBq/mmol; PerkinElmer Life Sciences) for 90 min at 37 °C. After incubation, liver slices were heated with ethanolic KOH for 1 h at 70 °C. Nonsaponified lipids were removed with petroleum ether. The aqueous solutions were acidified, and lipids were extracted with petroleum ether. Radioactivity was measured by a liquid scintillation counter (Hitachi Aloka Medical, Ltd., Tokyo, Japan).

**Biochemical Analysis of Blood**—Serum levels of non-esterified fatty acid (Wako), adiponectin (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), insulin, leptin (Morinaga Institute of Biological Science, Inc., Kanagawa, Japan), alanine aminotransferase (ALT) (Wako), and aspartate aminotransferase (AST) (Wako) were determined using a commercial assay kit according to the manufacturer’s instructions.
AhR Plays Protective Roles against Lipotoxicity

(Takara Bio). RAW264.7 cells were transfected with CMV-HA vector or CMV-HA mouse SOCS3 expression vector by using FuGENE HD (Promega).

Electrophoretic Mobility Shift Assay (EMSA)—Mouse AhR and Arnt proteins were prepared using TNT in vitro transcription and translation system (Promega). The binding reactions were performed as described previously (33). Protein-DNA complexes were resolved by electrophoresis through 5% polyacrylamide gel in 0.5× tris/borate/EDTA at 4 °C. For oligonucleotide competition experiments, unlabeled oligonucleotides were added to the reaction mixture in a 100-fold molar excess to the radiolabeled probe. EMSA probe sequences are labeled in Fig. 9C.

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was performed essentially as described elsewhere (27, 33). In brief, 12-week-old AhRflox/flox and AhR LKO male mice were treated with corn oil or 3MC (20 mg/kg) by oral gavage for 3 consecutive days. Mice were sacrificed 20 h after administration, and liver tissues were harvested. Liver tissues were homogenized and then cross-linked with formaldehyde. HepG2 cells were treated with dimethyl sulfoxide (DMSO) or 3MC (3 μM) for 16 h, cross-linked in formaldehyde, and lysed. The obtained liver and cell extracts were subjected to immunoprecipitation with anti-AhR antibody (SA210, Enzo Life Sciences, Farmingdale, NY). Parallel samples were incubated with normal IgG (sc-2027, Santa Cruz Biotechnology, Inc. Dallas, TX) as a negative control. The following PCR primers were used: mSOCS3 +137, 5′-ACCAACCCGGGAGGGGACCGAGGA-3′; hSOCS3 +137, 5′-CACCAACCCGGGAGGGGACCGAGGA-3′; hCYP1A1 +237, 5′-AAGAGGAGGTACGAGCACTCTT-3′; hSOCS3 +745, 5′-AACAGGTAAAAGAACCAGCCGCGCGATTTACGGA-3′; hCYP1A1 +745, 5′-AACAGGTAAAAGAACCAGCCGCGCGATTTACGGA-3′; mSOCS3 +745, 5′-GCGGCGCGCCCGTTTCCCTT-3′; and hSOCS3 +745, 5′-GCGGCGCGCCCGTTTCCCTT-3′. The PCR products were cloned into the pGL3-basic vector (Promega). The site-directed mutagenesis was performed by the PCR overextension method and confirmed by DNA sequencing (34). HepG2 cells were transfected with the plasmids by using a FuGENE HD (Promega) (9, 33). After 24 h of incubation, the transfection medium was replaced with fresh medium containing 3MC or DMSO. The cells lysates were extracted 16 h later and assayed with a dual-luciferase reporter assay system (Promega). The pRL-tk vector (Promega) was used as a normalization control to correct for variable transfection efficiencies. All transfections were performed in triplicate.

Statistical Analysis—When applicable, the results are presented as the means ± S.D. Statistical analysis was performed by Student’s t test or one-way analysis of variance with Tukey’s post hoc test. A value of p < 0.05 was accepted as statistically significant.

Results

Deletion of the AhR Gene in the Liver Accelerates High Fat Diet-induced Hepatic Steatosis—To understand the roles of AhR in lipid metabolism in the liver, AhRflox/flox and liver-specific AhR knock-out (AhR LKO) mice were subjected to HFD challenge for 12 weeks. As shown in Fig. 1, A–C, there were no differences in body mass, the tissue weights of the liver, the white adipose tissue and the kidney, or the state of adiposity between the two genotypes (Fig. 1, A–C). HFD feeding induced ectopic fat accumulation in the liver and the skeletal muscle (Fig. 1D). In particular, the level of hepatic triglyceride in AhR LKO mice was far greater than that in AhRflox/flox mice under the HFD condition (Fig. 1D). In contrast, this genotype-dependent increase of fat accumulation was not observed in the skeletal muscle (Fig. 1D). Analysis of the liver histology revealed that HFD feeding induced severe microvascular steatosis in AhR LKO mice (Fig. 1E). In the liver, the level of diglyceride in AhR LKO mice was significantly higher than that in AhRflox/flox mice under the HFD condition (Fig. 1F). In contrast, there was no significant difference in the level of cholesterol and phospholipid between the genotypes in the liver (Fig. 1F).

The determination of circulating biochemical parameters showed that there were no significant differences in the level of nonesterified fatty acid, insulin, glucose, FGF21, or adipocytokines such as adiponectin and leptin between the genotypes (Table 1). Also, the level of hepatic FGF21, food intake, O2 consumption, CO2 production, and RQ value in the AhR LKO mice was similar to that of AhRflox/flox mice (Fig. 1, G–I).

Hepatic steatosis is linked with altered insulin signaling. Thus, to examine whether there is a difference in insulin sensitivity between the genotypes, the mice were subjected to a glucose tolerance test and insulin tolerance test. Under chow diet conditions, AhR LKO mice exhibited improved whole-body insulin sensitivity (Fig. 2, A and B) slightly but significantly (Fig. 2, A and B). However, HFD feeding aggravated whole-body insulin sensitivity in both genotypes, and the differences of scores observed under chow condition were diminished (Fig. 2, A and B).

To gain insight into the mechanism by which the deficiency of AhR promotes HFD-induced hepatic steatosis, we examined the expression of genes related to lipid metabolism by qRT-PCR analysis. Under the HFD conditions, AhR LKO mice showed an increased expression of lipogenesis-related genes such as sterol-response element-binding protein-1c (Sreb1c) and its target genes, including sterol-coenzyme decarboxylase 1 (Scd1), acetyl-CoA carboxylase 1 (Acct1), and glycerol-3-phosphate acyltransferase 1 (Gpat1) (Fig. 3A). Also, gene expression level of adipocyte differentiation-related protein (Adrp), a lipid droplet coat protein, and hormone-sensitive
FIGURE 1. Deletion of the Ahr gene in the liver accelerates HFD-induced hepatic steatosis. AhR^flox/flox mice and AhR LKO mice were fed a chow diet or HFD for 12 weeks before being analyzed (n = 5–6). A, body weight. B, tissue weight of the liver, the epididymal white adipose tissue (WAT), and the kidney (n = 5–6). C, representative hematoxylin and eosin (H&E) staining of epididymal white adipose tissue. Magnification, × 40. Scale bar, 200 μm. D, triglyceride content in the liver and skeletal muscle. E, representative H&E staining of a liver section around the central vein. Magnification, × 100. Scale bar, 50 μm. F, content of diglyceride, phospholipid, and cholesterol in the liver (n = 5–6). G, FGF21 content in the liver (n = 5–6). H, food intake (kcal per day). I, top panel, representative daily changes of oxygen consumption (VO₂), carbon dioxide production (VCO₂), and RQ. Bottom panel, daily average of VO₂, VCO₂, and RQ (n = 5–6). #, p < 0.05 relative to AhR^flox/flox mice fed a chow diet. *, p < 0.05 relative to AhR^flox/flox mice fed a HFD.
AhR Plays Protective Roles against Lipotoxicity

lipase (Hsl) in AhR LKO mice was higher than that in AhR<sup>lox/lox</sup> mice (Fig. 3B). In contrast, no significant difference was seen in the expression level of genes involved in fatty acid uptake, β-oxidation, and gluconeogenesis (Fig. 3, C–E).

Deficiency of the Ahr Gene in the Liver Promotes HFD-induced Hepatic Inflammation—The results described above suggest that the increase of hepatic steatosis in AhR LKO mice is due to abnormal hepatic function rather than a dysfunction of other tissues or deterioration of insulin sensitivity. Hepatic steatosis is often accompanied by inflammation. Thus, in the next set of experiments, we analyzed the state of inflammation and liver damage in AhR LKO mice (Fig. 4A). Moreover, HFD feeding increased the activity of both enzymes confirmed the lower SOCS3 expression in AhR LKO mice (Fig. 5A). Also, functional loss of SOCS3 in the AhR LKO mouse liver was demonstrated by the increase in the phosphorylated form of STAT3 (Fig. 5B).

Deletion of the Ahr Gene Attenuates the HFD-dependent Induction of Socs3 Expression—In the progression of inflammation, the JAK/STAT pathway plays critical roles in the transduction of the cytokine-dependent signal. This pathway is negatively and tightly regulated by the suppressor of cytokine signal (SOCS) family (35). Among SOCS family members, SOCS3 acts as a potent negative regulator of the pro-inflammatory-induced STAT3 signaling pathway. Interestingly, HFD feeding increases the expression of Socs3 in the liver (Fig. 5A). However, the degree of HFD-induced Socs3 expression in AhR LKO mice was far lower than that in AhR<sup>lox/lox</sup> mice (Fig. 5A). Western blot analysis of liver proteins confirmed the lower SOCS3 expression in AhR LKO mice fed a HFD (Fig. 5B). Also, functional loss of SOCS3 in the AhR LKO mouse liver was demonstrated by the increase of the phosphorylated form of STAT3 (Fig. 5B).

Rescue of Socs3 Expression in AhR LKO Mice Attenuates HFD-induced Steatosis and the Subsequent Lipotoxicity—To demonstrate the development of severe HFD-induced steatosis was, at least partly, due to a decrease in SOCS3 expression in AhR LKO mice, hepatic Socs3 expression was rescued in AhR LKO mice by breeding with AhR<sup>lox/lox</sup> mice carrying the transgene of Socs3 (S3 Tg mice) (Fig. 6A). The overexpression of Socs3 in the liver of S3 Tg mice was validated by Western blotting (Fig. 6B). In addition, the reduction of the active form of STAT3 provided evidence of functional rescue of SOCS3 in S3 Tg mice and AhR LKO mice carrying transgene of Socs3 (AhR LKO/S3 Tg mice) (Fig. 6B). To evaluate the protective roles of SOCS3 for HFD-induced lipotoxicity, AhR<sup>lox/lox</sup> mice, AhR LKO mice, AhR LKO/S3 Tg mice, and S3 Tg mice were subjected to HFD challenge for 10 weeks. There was no difference in the body weight or tissue weight among the four groups...
under either a chow diet or HFD condition (Fig. 6D). Histological analysis revealed that the rescue of SOCS3 in AhR LKO mice improved the status of HFD-induced steatosis (Fig. 7A). Attenuation of HFD-induced steatosis by the rescue of SOCS3 expression was confirmed by the observation that the triglyceride level and the expression of lipogenic genes in the AhR LKO/S3 Tg mouse liver were lowered to levels similar to those in the AhRflox/flox mouse liver (Fig. 7, B and C). Physiological measurement of lipogenesis revealed that de novo lipogenesis activity in the AhR LKO mouse liver was significantly higher than that in the AhRlox/lox mouse liver (Fig. 7D). However, the increase of lipogenesis activity was attenuated by the overexpression of SOCS3 in the AhRflox/flox mouse liver (Fig. 7D). As shown in Fig. 4, the degree of the damage induced by HFD feeding in the AhR LKO mouse liver was more severe than that in the AhRlox/lox mouse liver. However, these lipotoxicities were relieved in the AhR LKO/S3 Tg mouse liver (Fig. 8, A and B). In addition, the expression of SOCS3 was induced by the activation of AhR in human hepatoma HepG2 cells in a time-dependent manner as well as a well known AhR target gene, CYP1A1 (Fig. 9B). In contrast, 3MC treatment had little effect on the expression of SOCS1 (Fig. 9B). Inspection of the 5’-flanking region of the Socs3 gene revealed the presence of an XRE in the mouse and human genome at nucleotides 166 to 171 and 49 to 54, respectively (Fig. 9C). EMSA revealed the AhR/Arnt heterodimers bound to the DNA probes containing mouse Socs3/XRE and human SOCS3/XRE (Fig. 9D). The specificity of the complex formation was confirmed by competition experiments (Fig. 9D). To determine whether AhR can mediate the transactivation via Socs3/XRE, we cloned the mouse 2.9-kb and human 1.4-kb 5’-flanking region of the Socs3 gene. As shown in Fig. 9E, 3MC treatment increased both mouse and human SOCS3 promoter activity in the presence of AhR, although these inductions were significantly suppressed by the introduction of a mutation in the XRE (Fig. 9E). Also, the results of ChIP assay confirmed the ligand-dependent recruitment of the AhR to mouse and human Socs3/XRE on the genome (Fig. 9F).
To understand the effects of Socs3 induction by AhR on cytokine signaling, HepG2 cells pre-treated with either DMSO or 3MC were exposed to IL-6. Phosphorylation of STAT3 was induced and became maximal at 15 min of the treatment with IL-6 in DMSO-treated cells. In contrast, 3MC-treated cells showed no substantial increase of phosphorylated STAT3 by IL-6 treatment (Fig. 9G).
Discussion

The results in this study revealed that deletion of the \textit{Ahr} gene in the liver leads to the development of severe hepatic steatosis and the subsequent lipotoxicity effects, including liver inflammation and injury, under the HFD condition (Figs. 1, 3, and 4). Hepatic steatosis, termed as nonalcoholic fatty liver disease, appears to be associated with abnormal lipid metabolism-related diseases such as obesity and type 2 diabetes. In patients with nonalcoholic fatty liver disease, ~60% of liver triglyceride content is derived from free fatty acid from adipose tissue, 26% from \textit{de novo} lipogenesis, and 15% from diet (36). However, the results in this study showed that there were no substantial differences in the body or tissue weight, food intake, energy expenditure, levels of circulating fatty acids or adipocytokine, and whole-body insulin sensitivity between \textit{Ahr}flox/flox mice and \textit{Ahr} LKO mice under the HFD condition. Consequently, disorder of lipid metabolism in the liver was considered to be a main cause of exacerbated ectopic fat accumulation in the tissue. Indeed, a series of gene expressions related to lipogenesis and actual \textit{de novo} lipogenesis activity were found to be up-regulated in the \textit{Ahr} LKO mouse liver (Figs. 3 and 7). In conjunction with the increased lipogenesis, one of the notable changes in the livers of \textit{Ahr} LKO mice was a lowered induction of \textit{Socs3} expression and subsequent lowered activation of STAT3 when the mice were challenged with HFD feeding (Fig. 5). SOCS3, a negative inflammatory factor, is induced by HFD in the liver, the skeletal muscle, and the adipose tissue (37–39) and inhibits inflammatory signal transduction via binding to tyrosine phosphorylation sites on cytokine receptors (40–42). Similar to
AhR LKO mice, liver-specific Socs3 KO mice fed a HFD showed the severe hepatic steatosis associated with increased de novo lipogenesis and inflammation (40). The mechanism involves HFD-dependent increase of Srebp-1c expression (40). A recent study revealed that SOCS3 acts as an inhibitor of the JAK/STAT5a pathway and disturbs lipogenesis by decreasing Srebp-1c expression (43). Also, AhR modulated the basal expression of Socs3 in spleen and brain (44). These results suggest that the lipotoxities observed following HFD feeding in AhR LKO mice were attributed to a lower level of Socs3 expression. In confirmation of this hypothesis, we demonstrated that the rescue of Socs3 in the AhR LKO mouse liver improved the degree of hepatic steatosis, inflammation, and reactive oxygen species production level similar to that in the AhR^flx/flx mouse liver (Figs. 7 and 8). We also revealed that AhR directly regulates transcription of the Socs3 gene (Fig. 9, A–F). A recent study reported by Brant et al. (44) showed that AhR is involved in a SOCS3-mediated immune response. Consequently, we are led to conclude that AhR plays protective roles against HFD-induced hepatic steatosis and the subsequent lipotoxicity via direct transcriptional regulation of Socs3 expression in the liver.

The effects of the lower level of SOCS3 in the AhR LKO mouse liver were observed only under the condition of HFD feeding but not under chow diet feeding. Also, increased liver fat and inflammation in liver-specific Socs3 KO mice were HFD-dependent phenotypes (38). Therefore, the functional significance of Socs3 induction by AhR is associated with the pathogenesis induced by HFD. Hepatic steatosis induced by HFD feeding is accompanied by chronic inflammation. Inflammation has been implicated in the progression of steatosis and liver injury through production of proinflammatory cytokines such as TNFα and IL-1β (45, 46). These cytokines promote lipogenesis and triglyceride accumulation by regulating Srebp1c expression (47). In this study, we demonstrated that
deletion of the hepatic Ahr gene increased inflammatory cell infiltration into the liver and the expression of pro-inflammatory cytokines (Fig. 4, A–C). Previous studies have similarly demonstrated that AhR inactivation induced inflammatory cell infiltration in the various tissues (48–50). These results suggest a critical role for AhR in the regulation of inflammatory responses. Similar to the phenotype of AhR LKO mice, deletion of the Socs3 gene increases infiltration of neutrophils and macrophages and heightens inflammation (40, 51–53). Given the facts that (i) expression of AhR and Socs3 is induced by HFD (Fig. 5), (ii) Socs3 is a direct transcriptional target of AhR (Fig. 9, A–F), (iii) the inflammation induced by HFD feeding is rescued by SOCS3 in the AhR LKO mouse liver (Fig. 7A and 8B), and (iv) pretreatment of HepG2 cells with AhR ligand weakened the IL-6-dependent phosphorylation of STAT3 (Fig. 9G), it would be appear that the functional significance of Socs3 induction by AhR is to suppress the progression of inflammation and the subsequent hepatic damages induced by HFD.

Hepatic steatosis is associated with insulin resistance. Previous studies have reported improved insulin sensitivity with Socs3 deletion (39–42). Under chow diet conditions, AhR LKO mice exhibited slight but significant improvement of whole-body insulin sensitivity (Fig. 2, A and B), as observed in Socs3 LKO mice (40). Therefore, the enhanced insulin actions may accelerate the early stage of lipogenesis in AhR LKO mice. However, HFD feeding markedly aggravated the insulin sensitivity in both genotypes, and as a result, the genotype-dependent differences in scores observed under chow diet conditions were diminished (Fig. 2, A and B). Thus, the HFD-dependent deterioration of hepatic steatosis in AhR LKO mice compared with AhRflox/flox mice is unlikely due to the alternation of insulin sensitivity between genotypes.

Previous studies have reported that pharmacological or transgenic activation of AhR induces hepatic steatosis (54–56). In contrast, global AhR KO mice develop spontaneous triglyceride accumulation and fibrosis in the liver (11). As shown in this study, deletion of the Ahr gene in the liver exacerbates hepatic steatosis induced by a HFD (Figs. 1, 3, and 4). These results indicate that hepatic AhR may play dual roles in the regulation of lipid metabolism. It is clear that overload of lipid in the liver is the first hit in the development of hepatic steatosis. However, the deposition of lipid in tissue is not sufficient in itself to induce hepatic damage. For example, although rescue of the Socs3 gene in AhR LKO mice improves the lipotoxicities to the level of those in AhRflox/flox mice under a HFD condition, AhR LKO mice overexpressing Socs3 still showed ectopic fat formation in the liver by HFD feeding (Fig. 7B). Thus, a second hit, such as inflammation and/or elevation of the reactive oxygen species level, is required to develop hepatic steatosis. The reports referred to above showed that pharmacologically activated or constitutively activated AhR triggers triglyceride accumulation in the liver, reflecting a role in the first hit of hepatic steatosis described above (54–56). In contrast, the results in this study demonstrated the increase of inflammation in AhR LKO mice under HFD feeding conditions. Therefore, AhR also

FIGURE 8. Rescue of Socs3 expression in AhR LKO mice attenuates HFD-induced lipotoxicity. AhRflox/flox mice, AhR LKO mice, AhR LKO/S3 Tg mice, and S3 Tg mice were fed a chow diet or HFD for 10 weeks (n = 5–7). A, activity of ALT and AST in serum. B, qRT-PCR analysis of the expression of inflammation-related genes (n = 5–7). C, TBARS level in the liver. #, p < 0.05 relative to AhRflox/flox mice fed a chow diet. *, p < 0.05 relative to AhRflox/flox mice fed a HFD. +, p < 0.05 relative to AhR LKO mice fed a HFD.
AhR Plays Protective Roles against Lipotoxicity

plays the protective roles against the second hit in hepatic steatosis development. Because the mechanism by which AhR improves HFD-induced hepatic steatosis and the subsequent lipotoxicity involves direct transcriptional regulation of Socs3 gene in vivo, activation of AhR is required. This suggests that HFD feeding or the increase of ectopic fat may produce the AhR ligands or activators. Indeed, increased adiposity elevates the level of AhR and its target genes (23, 24). Although identification of the molecules acts as the ligands or the activator produced under HFD conditions has to be elucidated in future studies, recent studies have identified various low molecular compounds as AhR endogenous ligands or activators, which include glucose, sheared LDL, cAMP derivatives, and tryptophan derivatives (13–22). These compounds and the related compounds could be contained in HFD or be derived through the metabolic pathway. It is now well recognized that AhR is, directly or indirectly, associated with several pathophysiological processes, including atherosclerosis, inflammation, immunomodulation, and cancer. Consequently, identification of an AhR ligand and/or activator in HFD may be useful for the development of treatments for metabolic liver diseases.

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AhR Plays Protective Roles against Lipotoxicity

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