Activation of the aryl hydrocarbon receptor induces hepatic steatosis via the upregulation of fatty acid transport

Kawano, Yuki ; Nishiumi, Shin ; Tanaka, Shinwa ; Nobutani, Kentaro ; Miki, Akira ; Yano, Yoshihiko ; Seo, Yasushi ; Kutsumi, Hiromu ;…

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ACTIVATION OF THE ARYL HYDROCARBON RECEPTOR INDUCES HEPATIC STEATOSIS VIA THE UPREGULATION OF FATTY ACID TRANSPORT.

Yuki Kawano[1], Shin Nishiumi[1,2], Shinwa Tanaka[1], Kentaro Nobutani[1], Akira Miki[1], Yoshihiko Yano[1,3], Yasushi Seo[1], Hiromu Kutsumi[1], Hitoshi Ashida[4], Takeshi Azuma[1] and Masaru Yoshida[1,2,5]

[1] Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan. [2] Integrated Center for Mass Spectrometry, Kobe University Graduate School of Medicine, Kobe, Japan. [3] Division of Molecular Medicine and Medical Genetics, International Center for Medical Research and Treatment, Kobe University Graduate School of Medicine, Kobe, Japan. [4] Department of Agrobioscience, Kobe University Graduate School of Agricultural Science, Kobe, Japan. [5] Division of Metabolomics Research, Kobe University Graduate School of Medicine, Kobe, Japan.

Corresponding Author:

Masaru Yoshida

Division of Metabolomics Research,

Division of Gastroenterology,

The Integrated Center for Mass Spectrometry, Kobe University Graduate School of Medicine.

Research Build. A6-010, 7-5-1, Kusunokicho, Chuo-ku, Kobe City, Hyogo 650-0017, JAPAN

Tel: +81-78-382-6305

Fax: +81-78-382-6309

E-mail: myoshida@med.kobe-u.ac.jp

Abstract

The aryl hydrocarbon receptor (AHR) is a basic helix-loop-helix/Per-ARNT-Sim domain transcription factor, which is activated by various xenobiotic ligands. AHR is known to be abundant in liver tissue and to be associated with hepatic steatosis. However, it has not yet been elucidated how the activation of AHR promotes hepatic steatosis. The aim of this study is to clarify the role of AHR in hepatic steatosis. The intraperitoneal injection of 3-methylcholanthrene (3MC), a potent AHR ligand, into C57BL/6J mice significantly increased the levels of triglycerides and 6 long-chain monounsaturated fatty acids in the livers of mice, resulting in hepatic microvesicular steatosis. 3MC significantly enhanced the expression level of fatty acid translocase (FAT), a factor regulating the uptake of long-chain fatty acids into hepatocytes, in the liver. In an *in vitro* experiment using human hepatoma HepG2 cells, 3MC increased the expression level of FAT, and the downregulation of AHR by AHR siRNA led to the suppression of 3MC-induced FAT expression. In addition, the mRNA level of peroxisome proliferator-activated receptor (PPAR) α, an upstream factor of FAT, was increased in the livers of 3MC-treated mice. Taking together, AHR activation induces hepatic microvesicular steatosis by increasing the expression level of FAT.

Keywords:

fatty acid translocase; aryl hydrocarbon receptor; peroxisome proliferator-activated receptor α; monounsaturated fatty acid; 3-methylcholanthrene; nonalcoholic fatty liver disease.

Abbreviations:

AHR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PCB, polychlorinated biphenyl; BaP, benzo[a]pyrene; 3MC, 3-methylcholanthrene; ARNT, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin responsive element; FAS, fatty acid synthase; FAT, fatty acid translocase; L-FABP, liver fatty acid binding protein; CPT, carnitine palmitoyltransferase; MTP, microsomal triglyceride transfer protein; VLDL, very low density lipoprotein; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element

binding protein; BW, body weight; ALT, alanine aminotransferase; GC-MS, gas chromatography-mass spectrometry; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SE, standard error; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; CYP1A1, cytochrome P450 1A1; RXR, retinoid X receptor; NAFLD, nonalcoholic fatty liver disease.

1. Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-dependent basic helix-loop-helix/Per-ARNT-Sim domain transcription factor [1]. AHR is activated by various exogenous agonists, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyl (PCB), benzo[a]pyrene (BaP), and 3-methylcholanthrene (3MC) [2,3]. In addition, some endogenous ligands, for example, indigo and indirubin, are known to bind to AHR [4]. These ligands bind to cytosolic AHR, and then the activated AHR translocates into the nucleus, heterodimerizes with the AHR nuclear translocator (ARNT) and binds to specific enhancer sequences adjacent to target promoters termed "dioxin responsive elements" (DRE) [5]. Its activation leads to various toxic responses including tumor-promotion, teratogenicity, immunotoxicity, and lethality [6]. In previous studies, it was reported that AHR is abundant in liver tissue [7], which is one of the major target organs for AHR-related toxicity, such as fat accumulation, inflammation, and fibrosis, in laboratory animals [8, 9]. Boverhof *et al*. demonstrated that C57BL/6 mice gavaged with TCDD exhibited lipid accumulation in their liver tissue 7 days after exposure [9]. Korenaga *et al.* also revealed that rhesus monkeys that were subcutaneously administered TCDD for 4 years exhibited intrahepatic fatty changes [10]. However, the molecular mechanisms underlying fat accumulation induced by AHR activation have not been elucidated.

A variety of lipid metabolic pathways occur in the liver. The accumulation of lipids in the liver is also induced through several signaling pathways, and excessive lipid accumulation may lead to fatty liver. The liver is the major tissue of *de novo* lipogenesis, which is regulated by fatty acid synthase (FAS), and the upregulation of FAS causes increased triglyceride synthesis [11]. The enhanced uptake of fatty acids also leads to the accumulation of lipids in the liver. Free fatty acids enter cells via fatty acid translocase (FAT) and then bind to liver fatty acid binding protein (L-FABP), before transporting free fatty acids to mitochondria, where they will undergo metabolic conversion or be used to synthesize triglycerides [12,13]. In addition, the accumulation of lipids in the liver is induced through the downregulation of lipid catabolism, for example, via β-oxidation. Carnitine

palmitoyltransferase (CPT) 1 controls the transport of long-chain acyl-CoA into mitochondria, in which β-oxidation occurs, and reduced CPT1 activity leads to lipid accumulation accompanied by the downregulation of β-oxidation [14]. Triglycerides bind to apolipoprotein B to construct mature lipoprotein particles for secretion, and microsomal triglyceride transfer protein (MTP) plays a critical role in the assembly and secretion of very low density lipoproteins (VLDL) [15]. Therefore, decreased hepatic export of triglycerides as VLDL leads to lipid accumulation in the liver.

Nuclear factors are also important for the regulation of lipid metabolism. Fatty acid metabolism is transcriptionally regulated by two main systems; i.e., the liver X receptor (LXR) and peroxisome proliferator-activated receptor (PPAR)-related pathways. LXR activates the expression of sterol regulatory element binding proteins (SREBP)-1c, a dominant lipogenic gene regulator. On the other hand, peroxisomal, microsomal, and mitochondrial fatty acid metabolizing enzymes in the liver are transcriptionally regulated by PPARα [16]. However, the relationship between AHR activation and fatty acid metabolism has not been elucidated. Therefore, the aim of this study is to examine the role of AHR in hepatic steatosis.

2. Materials and methods

2.1. Animal treatment

All animal treatments in this study were approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations. Male C57BL/6J mice (15 weeks old, CLEA Japan, Tokyo, Japan) were housed in a temperature controlled (23-25°C) room at 60±5% humidity under a 12-h light-dark cycle and acclimatized for 7 days with a commercial chow and distilled water. The mice fed the standard chow were randomly divided into 2 groups of 5 mice each, and one group received a single intraperitoneal injection of 3MC (Sigma, Tokyo, Japan) at 100 mg/kg body weight (BW). The other group was given 0.25 mL of corn oil (Nacalai tesque, Kyoto, Japan) as a vehicle control. After 8 hours, 5 mice from each group were killed, and their livers were removed. Blood was collected via cardiac puncture, and the serum was separated by centrifugation at 1,000 × g for 10 min at 4^{o}C.

2.2. Histochemistry of the lipids in the liver

Frozen liver sections (10 μm thick) were mounted onto slides, rinsed in 60% isopropyl alcohol for 1 min, and stained with Oil Red O (Chroma-Gesellschaft Schmid GmbH & Co., Münster, Germany) dissolved in 60% isopropyl alcohol for 15 min. The sections were then rinsed in 60% isopropyl alcohol for 1 min, before the nuclei were stained with Mayer's hematoxylin for 3 min. The sections were then rinsed in water and mounted in glycerine jelly (Wako Pure Chemical Industries Ltd., Osaka, Japan).

2.3. Biochemical serum analysis

The serum concentrations of alanine aminotransferase (ALT), total bilirubin, total cholesterol, triglyceride, free fatty acids, and glucose were measured by SRL Inc. (Osaka, Japan).

2.4. Measurement of triglyceride levels in the liver

Lipids were extracted from the liver according to a modified version of the method of Bligh and

Dyer [17]. Briefly, the liver tissue was homogenized with 9 volumes of 1.15% (w/v) KCl, aliquots (0.5 ml) of homogenate were extracted with 5 mL of chloroform–methanol (2:1) solution 3 times, and the chloroform layer was then collected by centrifugation at 1,000 × g for 10 min. To remove water-soluble substances, a 1/5 volume of 0.5% (w/v) NaCl was layered onto the chloroform extract, vortexed, and centrifuged at 1,000 × g for 10 min. The resultant chloroform layer was evaporated just before use, dissolved in methanol, and its lipid levels were measured. Triglyceride levels were measured using a commercial kit (Wako Pure Chemical Industries Ltd.) according to the manufacturer's instructions.

2.5. Measurement of fatty acids by gas chromatography-mass spectrometry (GC-MS)

Commercial kits were used for the methylation and purification of fatty acids in the liver (Nacalai tesque). The resultant solution was evaporated under a stream of N_2 gas, and methyl esters of fatty acids were measured by GC-MS after being dissolved in *n*-hexane. All GC-MS analyses were performed by GCMS-2010plus (Shimadzu, Kyoto, Japan). In the gas chromatographic system, a DB-5MS column (30 m × 0.25 mm I.D., film thickness: 0.25 μm) was used. The GC column temperature was programmed to increase from 40^{o}C to 320^{o}C at a rate of 6^{o}C per minute, and the total GC run time was 50 min. The inlet temperature was kept at 280^{o}C. The helium carrier gas was used at a constant flow rate of 5.0 mL per minute. Aliquots of 1.0 μl were injected with the split ratio of the injector being 1:5. The mass conditions were as follows: ionization voltage: 70 eV; ion source temperature: 200^{o}C; full scan mode in the 35 amu -500 amu mass range with a 0.20 s/scan velocity.

2.6. Isolation of total RNA and the quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from the cultured cells or liver tissue with TRIzol Reagent (Invitrogen, Tokyo, Japan), according to the manufacturer's instructions. cDNA was prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Tokyo, Japan), and then quantitative real-time PCR was performed using the 7500 Real Time PCR System and Power SYBR Green Reagent (Applied Biosystems). The specific primers were synthesized by Hokkaido System

Science (Sapporo, Japan), and the sequences of the primers are listed in Table 1.

2.7. Western blot analysis.

To prepare cell or tissue lysate, they were homogenized with 8 volumes of lysis buffer consisting of 50 mM Tris (pH 8.0), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) sodium dodecyl sulfate (SDS) containing the protease and phosphatase inhibitors. The homogenate was stirred for 1 h on ice and centrifuged at 15,000 ×g for 20 min at 4 °C. The supernatant obtained was used as the lysate. The proteins in the lysates were separated by electrophoresis on a SDS-polyacrylamide gel and transferred onto a PVDF membrane (MILLIPORE, Massachusetts, United States) where the non-specific binding sites were then blocked. The membrane was washed with TBS containing 0.05% (v/v) Tween20 (TBST) 4 times for 5 min each and incubated with anti-FAT antibody, anti-AHR antibody, or anti-β-actin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, United States) for 1 h at room temperature. After being washed with TBST under the same conditions, the membrane was incubated with the appropriate secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. The specific immune complexes were detected with Chemi-Lumi One L (Nacalai tesque), and the density of specific bands was determined with LAS-4000 mini (FUJIFILM, Tokyo, Japan).

2.8. Cell culture and RNA interference analysis.

Human hepatoma HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Nacalai tesque) supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100μg/ml streptomycin under a humidified 95% air/5% CO_2 condition at 37^{o}C. Transfection of siRNA into HepG2 cells was performed by lipofection with Lipofectoamine RNAiMAX (Invitrogen, Tokyo, Japan), according to the manufacturer's instructions. For the collection of RNA from HepG2 cells, 250 μL of opti-MEM (Invitrogen) containing 50 pmol of AHR siRNA or control siRNA (Santa Cruz Biotechnology Inc.) as a negative control, and 250 μL of opti-MEM containing 5 μL of Lipofectoamine RNAiMAX were gently mixed and incubated for 20 min at room temperature, and

then 500 μL of the siRNA solution were prepared. After the medium from HepG2 cells (2 x 10^5 cells per 6-well plate) had been replaced with DMEM containing 10% FBS alone, AHR siRNA or control siRNA was transfected by adding 500 μL of the siRNA solution and 1500 μL of opti-MEM. After 10 hr-treatment, the medium was replaced with DMEM containing 10% FBS alone, and the cells were incubated overnight, before being treated with 10 μM 3MC for 4 hr. For the collection of proteins from HepG2 cells, 500 μL of opti-MEM containing 100 pmol of AHR siRNA or control siRNA and 500 μL of opti-MEM containing 10μL of Lipofectoamine RNAiMAX were gently mixed and incubated for 20 min at room temperature, and then 1000 μL of the siRNA solution were prepared. After the medium from HepG2 cells (4 x 10^5 cells in a 60-mm dish) had been replaced with DMEM containing 10% FBS alone, AHR siRNA or control siRNA was transfected by adding 1000 μL of the siRNA solution and 3000 μL of opti-MEM. After 10 hr-treatment, the medium was replaced with DMEM containing 10% FBS alone, and the cells were incubated overnight, before being treated with 10 μM 3MC for 24 hr. Total RNA and cell lysate were collected to perform real-time PCR and Western blotting, respectively, as described above.

2.9. Statistical analysis.

All data are expressed as the mean ± standard error (SE) of at least three independent determinations for each experiment. Statistical significance was analyzed using the Student's t-test, and a level of probability of 0.05 was used as the criterion for significance.

3. Results

3.1. Effects of 3MC on lipid levels in plasma and the liver

To investigate the involvement of AHR in hepatic steatosis, 3MC, an agonist of AHR, was used, and C57BL/6J mice were intraperitoneally given 3MC at 100 mg/kg BW. In liver histology assessed by Oil Red O staining, microvesicular fat accumulation was observed around the central vein in the 3MC-treated mice (Fig. 1A). Next, triglycerides were extracted from the liver, and the quantitative measurement of triglycerides was performed. The triglyceride level in the livers of the 3MC-treated mice was 1.5-fold higher than that in the corresponding control mice ($p<0.05$, Fig. 1B). No significant effects of 3MC on the serum levels of ALT, total bilirubin, total cholesterol, triglyceride, free fatty acid, and glucose were confirmed (Table 2).

3.2. Fatty acid constituents in the liver

To examine whether the fatty acid levels in the liver are increased by AHR activation, the concentrations of 51 fatty acids in the liver were measured by GC-MS. In our experimental conditions, 32 fatty acids were detected. In the 3MC-treated mice, the levels of 6 monounsaturated fatty acids (MUFA); i.e., C14:1, C16:1, C18:1, *trans*-C18:1, C20:1, and C22:1, and 1 saturated fatty acid (SFA); i.e., C16:0, were significantly increased in comparison to those of the corresponding control mice, whereas the levels of 1 SFA; i.e., C24:0, were significantly decreased. No significant effects of 3MC on other SFA or polyunsaturated fatty acids (PUFA) were confirmed (Table 3).

3.3. Changes in the expression levels of hepatic CYP1A1 and fatty acid-related factors

To elucidate the mechanisms by which 3MC induces hepatic steatosis, the effects of 3MC on the hepatic factors involved in fatty acid uptake, synthesis, oxidation, and secretion were examined by real-time PCR. 3MC significantly upregulated the expression of cytochrome P450 1A1 (CYP1A1) and FAT mRNA in the liver by approximately 2.0×10^4- and 2.1-fold, respectively (Fig. 2A, B), and the mRNA levels of L-FABP, CPT1, and MTP tended to be higher in the livers of the 3MC-treated mice than in those of the vehicle-treated animals (Fig. 2C, D, E). The expression level of FAS

mRNA in the livers of the mice that received 3MC tended to be decreased compared with that in the control mice (Fig. 2F). In addition, the protein level of FAT was significantly increased by approximately 1.9-fold (Fig. 3). These data demonstrate that hepatic steatosis induced by AHR activation is accompanied by upregulated FAT expression.

3.4. Changes in the expression levels of hepatic AHR, PPARα, RXRα, LXRα, and SREBP1

Next, the changes in the expression levels of hepatic AHR and the factors related to lipid metabolism were investigated by real-time PCR. In the 3MC-treated mice, significant increases in the mRNA levels of AHR and PPAR*α* were observed (Fig. 4A, B), while the levels of retinoid X receptor (RXR) *α* and LXR*α* mRNA remained unchanged (Fig. 4C, D). SREBP1 mRNA expression was downregulated without attaining statistical significance (Fig. 4E).

3.5. Suppression of FAT expression thorough the downregulation of AHR expression in HepG2 cells

It was investigated whether the 3MC-induced upregulation of FAT depends on AHR. In this study, RNA interference using AHR siRNA was performed. In AHR siRNA-transfected HepG2 cells, the downregulation of AHR mRNA and protein expression was observed, although complete knockdown of its expression was not achieved (Fig. 5A, 6). Downregulation of AHR also led to the suppression of 3MC-enhanced expression of CYP1A1 mRNA (Fig. 5B). In control siRNA-transfected HepG2 cells, 3MC increased the expression levels of FAT mRNA and protein, but no induction of FAT mRNA or protein by 3MC was observed in the AHR siRNA-transfected HepG2 cells (Fig. 5C, 6).

4. Discussion

This study was designed to examine the role of AHR in intrahepatic fat accumulation. We used 3MC as an agonist of AHR. 3MC is a synthetic compound and a highly potent member of the polycyclic aromatic hydrocarbon family of chemicals [3], which are found in cigarette smoke [18]. In a previous study, it was reported that TCDD induced hepatic steatosis as a chronic toxicity [9, 10]. However, to the best of our knowledge, this is the first study to demonstrate that an AHR agonist induces hepatic microvesicular steatosis as an acute toxicity (Fig. 1A, 1B). In the 3MC-treated mice, no liver damage was observed in examinations of their histology and serum ALT levels (Fig. 1A, Table 2). The steatosis was improved 24 hr after 3MC injection (data not shown). This may have been because 3MC is metabolized and eliminated quickly from the liver. In fact, Moorthy demonstrated that only 0.94% of the administered dose remained in the liver 1 day after the intraperitoneal injection of 3MC [19]. These results strengthen the assertion that 3MC induced hepatic microvesicular steatosis as an acute toxicity in the current study.

The most interesting finding of the current study was the upregulation of FAT expression induced by AHR activation *in vivo*. In addition, in an *in vitro* experiment, it was confirmed that the upregulation of FAT expression by 3MC is dependent on AHR (Fig. 5C, 6). Although one of the causes of hepatic steatosis is the upregulation of FAS expression, FAS mRNA expression tended to be downregulated in the 3MC-treated mice (Fig. 2F). DNA microarray analysis by Sato *et al*. also exhibited decreased FAS mRNA expression in the livers of TCDD-treated mice [20]. To the best of our knowledge, our results are the first to confirm the increased FAT expression in the livers of 3MC-injected mice (Fig. 2B, 3) and the involvement of AHR in 3MC-evoked induction of FAT expression (Fig. 5C, 6). Probably, 3MC-induced intrahepatic microvesicular steatosis results from increased levels of triglycerides, palmitic acid (C16:0), and 6 long-chain MUFA in the liver (Fig. 1B, Table 3). On the basis of a previous report [21], 3MC-induced steatosis is considered to be caused by the upregulation of FAT expression in the liver, leading to an enhanced rate of long-chain fatty acid uptake and increased triglyceride synthesis, because *de novo* lipogenesis in the livers of 3MC-treated mice was

not upregulated, and furthermore, β-oxidation and VLDL export were not downregulated (Fig. 2C, 2D, 2E, 2F). This is consistent with previous studies as outlined below. In a mouse model fed a high fat diet, increased expression of hepatic FAT protein was sufficient to exacerbate hepatic triglyceride storage and to increase the MUFA content of the liver [12, 22]. It was also reported that the expression level of FAT mRNA was upregulated in the livers of patients diagnosed with nonalcoholic fatty liver disease (NAFLD), and moreover palmitic acid (C16:0) and MUFA were the predominant fatty acids in the livers of these patients [23, 24]. Metabolic disorders, such as hyperglycemia and dyslipidemia, are observed in a mouse model of NAFLD and are well-known risk factors for NAFLD [25, 26]. However, no 3MC-induced significant alterations of the serum levels of total cholesterol, triglyceride, free fatty acid, or glucose were confirmed (Table 2). This may have been because 3MC caused acute toxicity in our experimental conditions. If mice are continuously administered 3MC, it is possible that they will exhibit metabolic disorders. Remillard *et al.* showed that TCDD causes loss of body weight and a decrease in adipose tissue weight, which is also known as wasting syndrome, and this syndrome leads to increased serum lipid levels and disordered distribution of lipids, resulting in diabetes-like symptoms [27]. TCDD is difficult to metabolize and easily accumulates in the body. Therefore, AHR activation as an acute toxicity induces hepatic microvesicular steatosis, while AHR activation as a chronic toxicity causes metabolic disorders.

Our data also demonstrated that the mRNA expression levels of PPARα and FAT were significantly increased (Fig. 2B, 4B). It was reported that FAT mRNA expression is induced by PPARα in the liver [28], suggesting that the induction of FAT expression by AHR activation is regulated through PPARα (Fig. 7). Recently, it was revealed that PPARα activation by its agonists transcriptionally induced AHR and CYP1A1 expression through PPRE sites located within its promoter [29, 30]. Although it is unknown whether the functions of PPARα are upregulated by AHR activation, the close interaction between AHR and PPARα may be mediated in the liver. Further experiments are required to elucidate the molecular mechanisms behind our findings.

In conclusion, our study highlights the importance of FAT in hepatic microvesicular steatosis caused by AHR activation. The upregulation of FAT expression results in an increased rate of long-chain MUFA uptake into hepatocytes. These findings are similar to the symptoms of patients with NAFLD. There are no established effective therapeutic modalities for NAFLD progression, and such modalities are urgently required. This study may be helpful for researchers aiming to find a novel target that can be used to prevent NAFLD progression.

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Figure legends

Fig. 1. Induction of microvesicular hepatic steatosis. C57BL/6J male mice received a single intraperitoneal injection of 3MC at 100 mg/kg BW or corn oil as a vehicle control. (A) After 8 hrs, Oil Red O and hematoxylin staining of the liver were performed (inserts: fat accumulation in the liver; magnification: 400×). C, central vein. (B) The triglyceride level in the liver was measured. Data represent the mean ± SE, n=5, and the asterisks indicate a significant difference compared with the corn oil-treated control group ($p<0.05$).

Fig. 2. The effects of 3MC on the mRNA expression of CYP1A1 and lipid catabolism-related factors in the liver. C57BL/6J male mice received a single intraperitoneal injection of 3MC at 100 mg/kg BW or corn oil as a vehicle control. After 8 hrs, the liver was removed. The mRNA expression levels of (A) CYP1A1, (B) FAT, (C) L-FABP, (D) CPT1, (E) MTP, and (F) FAS were examined by real-time RT-PCR analysis. Data represent the mean ± SE, n=5, and asterisks indicate a significant difference compared with the corn oil-treated control group ($p<0.05$).

Fig. 3. The effects of 3MC on the protein expression of FAT in the liver. C57BL/6J male mice received a single intraperitoneal injection of 3MC at 100 mg/kg BW or corn oil as a vehicle control. After 8 hrs, the liver was removed. The protein level of FAT was determined by Western blot analysis. The density of each band was quantified and normalized to the level of β-actin as an internal standard, and the fold-induction compared to the effects of corn oil treatment is indicated below each panel. Data represent the mean ± SE, n=5, and asterisks indicate a significant difference compared with the corn oil-treated control group ($p<0.05$).

Fig. 4. The effects of 3MC on the mRNA expression of AHR and nuclear receptors related to lipid metabolism in the liver. C57BL/6J male mice received a single intraperitoneal injection of 3MC at 100mg/kg BW or corn oil as a vehicle control. After 8 hrs, the liver was removed. The mRNA

expression levels of (A) AHR, (B) PPARα, (C) RXRα, (D) LXRα, and (E) SREBP1 were examined by real-time RT-PCR analysis. Data represent the mean ± SE, n=5, and asterisks indicate a significant difference compared with the corn oil-treated control group ($p<0.05$).

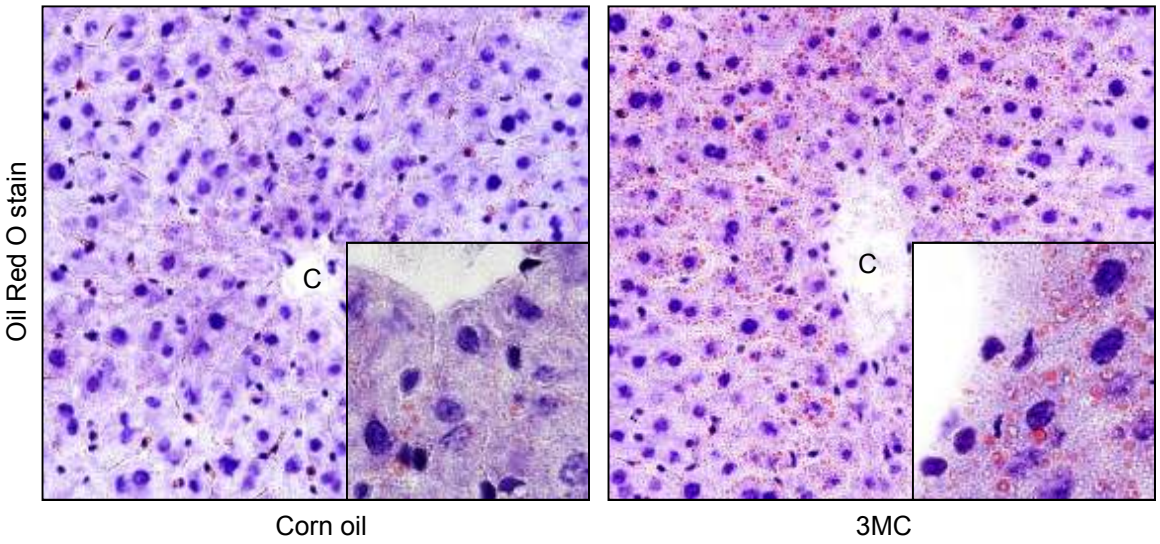
Fig. 5. The involvement of AHR in 3MC-induced expression of CYP1A1 and FAT mRNA in HepG2 cells. HepG2 cells were transfected with AHR siRNA or control siRNA, as described in Materials and Methods. The siRNA-transfected cells were treated with 10 μM 3MC or DMSO (1 μL/mL) as a vehicle control for 4 hrs. The mRNA expression levels of AHR (A), CYP1A1 (B), and FAT (C) were determined by real-time PCR and were normalized to those of β-actin as an internal standard. Data represent the mean ± SE, n=3-5, and asterisks indicate a significant difference ($p<0.05$).

Fig. 6. The effects of AHR downregulation on FAT protein expression in 3MC-treated HepG2 cells. HepG2 cells were transfected with AHR siRNA or control siRNA as described in Materials and Methods. The siRNA-transfected cells were treated with 10 μM 3MC or DMSO (1 μL/mL) as a vehicle control for 24 hrs. Proteins were prepared from the siRNA-transfected cells, and the protein levels of AHR, FAT, and β-actin were examined by Western blotting. Typical images are shown from at least triplicate determinations.

Fig. 7. Summary of the proposed mechanisms underlying hepatic lipid metabolism induced by AHR activation. AHR activation upregulates PPARα expression and subsequently FAT expression, resulting in the upregulation of fatty acid uptake and the accumulation of lipids in the liver. The increased levels of lipids in the liver lead to the downregulation of SREBP1 and subsequently FAS. However, AHR activation does not affect fatty acid transport, β-oxidation, or VLDL synthesis. The current study proposes the existence of an interaction between AHR and PPARα.

Figure 1

(A)


Oil Red O stain
C
C
Corn oil
3MC

(B)

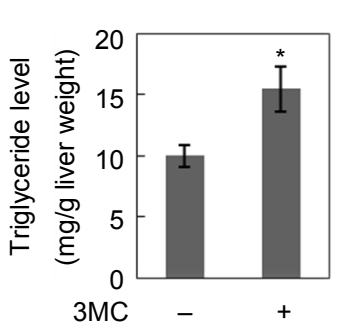

Triglyceride level
(mg/g liver weight)
20
15
10
5
0
*
3MC
–
+

Figure 2

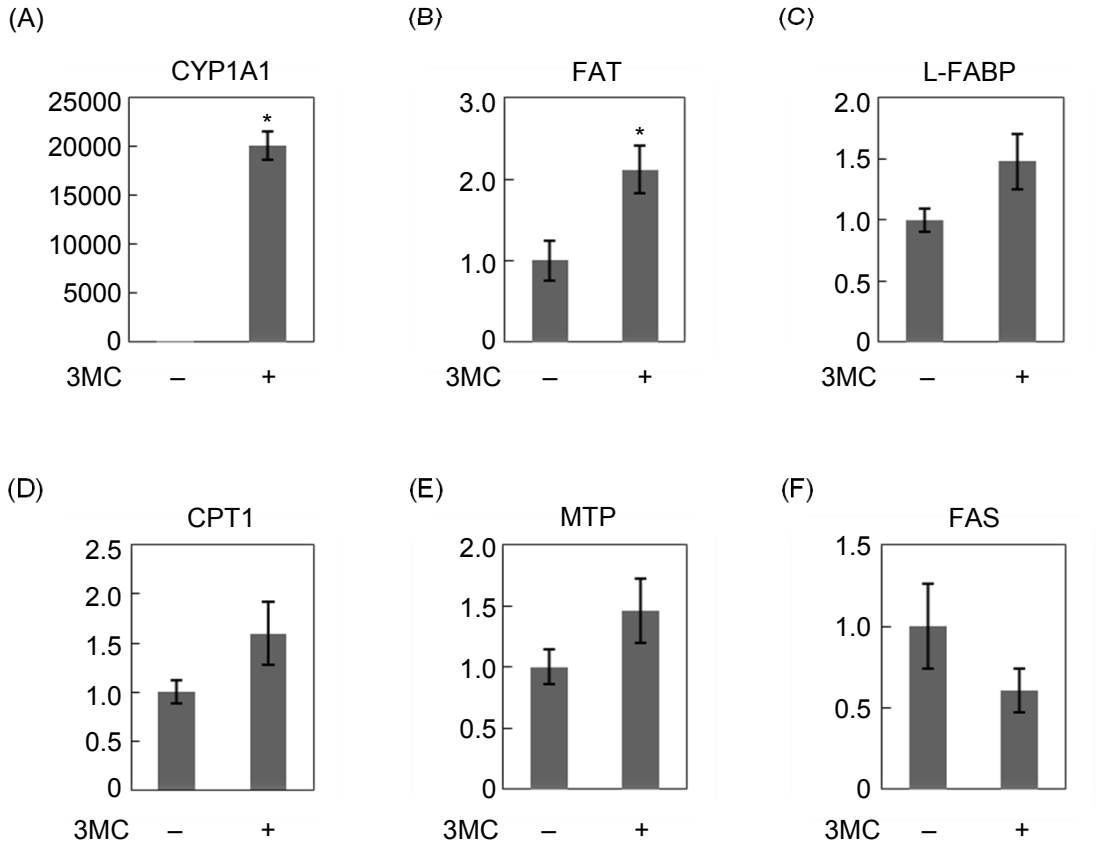

(A)
CYP1A1
25000
20000
15000
10000
5000
0
*
3MC – +
(B)
FAT
3.0
2.0
1.0
0
*
3MC – +
(C)
L-FABP
2.0
1.5
1.0
0.5
0
3MC – +
(D)
CPT1
2.5
2.0
1.5
1.0
0.5
0
3MC – +
(E)
MTP
2.0
1.5
1.0
0.5
0
3MC – +
(F)
FAS
1.5
1.0
0.5
0
3MC – +

Figure 3

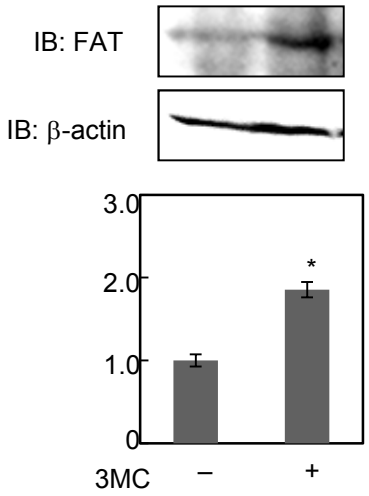


Figure 4

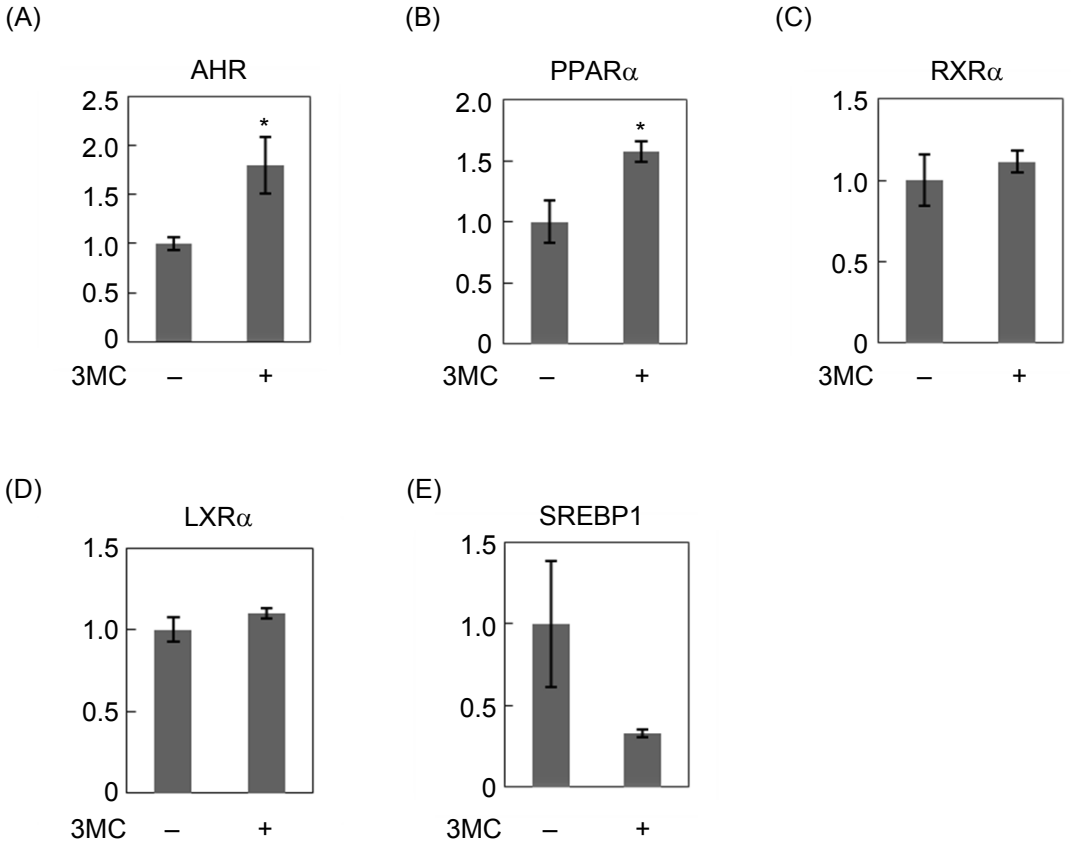

(A)
AHR
2.5
2.0
1.5
1.0
0.5
0
*
3MC – +
(B)
PPARα
2.0
1.5
1.0
0.5
0
*
3MC – +
(C)
RXRα
1.5
1.0
0.5
0
3MC – +
(D)
LXRα
1.5
1.0
0.5
0
3MC – +
(E)
SREBP1
1.5
1.0
0.5
0
3MC – +

Figure 5

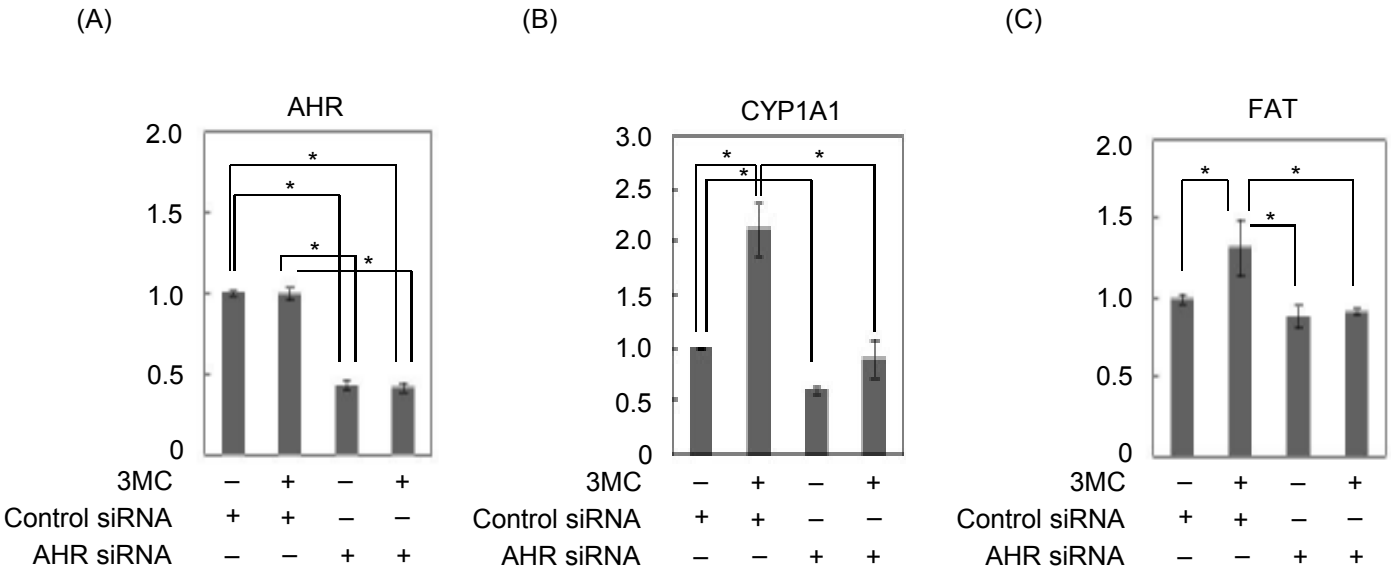

(A)
AHR
2.0
1.5
1.0
0.5
0
*
*
*
*
3MC – + – +
Control siRNA + + – –
AHR siRNA – – + +
(B)
CYP1A1
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
*
3MC – + – +
Control siRNA + + – –
AHR siRNA – – + +
(C)
FAT
2.0
1.5
1.0
0.5
0
*
*
*
3MC – + – +
Control siRNA + + – –
AHR siRNA – – + +

Figure 6

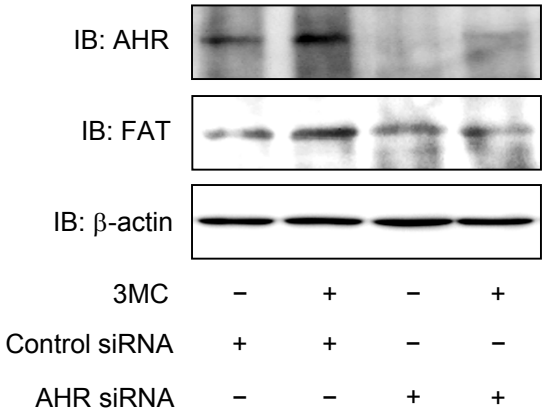


Figure 7

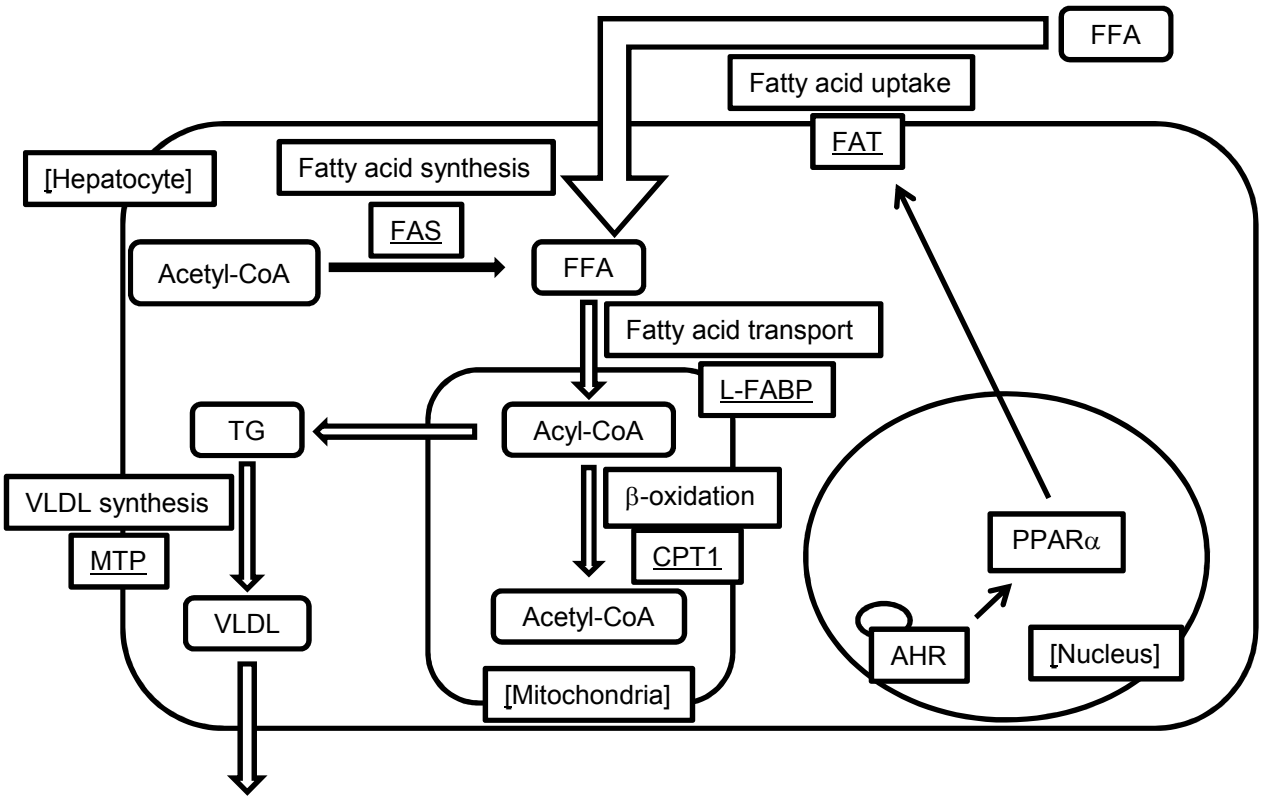

FFA
Fatty acid uptake
FAT
[Hepatocyte]
Fatty acid synthesis
FAS
Acetyl-CoA
FFA
Fatty acid transport
L-FABP
TG
Acyl-CoA
VLDL synthesis
β-oxidation
MTP
CPT1
PPARα
VLDL
Acetyl-CoA
AHR
[Nucleus]
[Mitochondria]

Table 1. Sequences of the primers used for real-time quantitative RT-PCR

	Forward primer	Reverse primer
CYP1A1	5'-ggccactttgacccttacaa-3'	5'-caggtaacggaggacaggaa-3'
FAT	5’-ttgctgccttctgaaatgtg-3’	5’-gcagaatcaagggagagcac-3’
L-FABP	5’-gtcagctgtggaaaggaagc-3’	5’-gtctccagttcgcactcctc-3’
CPT1	5’-ggaaggcaggacagagacag-3’	5’-cgggacatgatgtacagtgc-3’
MTP	5’-cactcaggcaattcgagaca-3’	5’-tatcgctttctggctgaggt-3’
FAS	5’-tgggttctagccagcagagt-3’	5’-accaccagagaccgttatgc-3’
AHR	5'-ggccaagagcttctttgatg-3'	5'-tgccagtctctgatttgtgc-3'
PPARα	5’-atgccagtactgccgttttc-3’	5’-ggccttgaccttgttcatgt-3’
RXRα	5’-ctcactgggacattgtggtg-3’	5’-ctccccaacacaggacagat-3’
LXRα	5’-agcgtccattcagagcaagt-3’	5’-cccttctcagtctgctccac-3’
SREBP1	5’-gatcaaagaggagccagtgc-3’	5’-tagatggtggctgctgagtg-3’
β-actin	5’-agccatgtacgtagccatcc-3’	5’-ctctcagctgtggtggtgaa-3’

Table 2. Hepatotoxic and metabolic parameters of the sera from mice treated with 3MC.

	Corn oil	3MC	p value
ALT (IU/L)	41.8 ± 4.76	46.8 ± 6.50	N.S.
Total bilirubin (mg/dL)	0.06 ± 0.01	0.06 ± 0.01	N.S.
Total cholesterol (mg/dL)	70.6 ± 1.80	68.4 ± 1.66	N.S.
Triglycerides (mg/dL)	16.6 ± 1.53	18.8 ± 1.07	N.S.
Free fatty acid (μEQ/L)	366.0 ± 16.58	380.5 ± 44.87	N.S.
Glucose (mg/dl)	225.2 ± 21.36	257.4 ± 8.50	N.S.

The sera were collected from the mice 8 hours after the injection of 3MC or corn oil as a vehicle control. Values are the mean ± SE (n=5/group); N.S., not significant. ALT, alanine aminotransferase.

Table 3. Fatty acid levels in the livers of mice treated with 3MC.

Fatty acids		Corn oil	3MC	p value
Saturated fatty acid	6:0	1.00 ± 1.00	1.46 ± 1.46	N.S.
	8:0	1.00 ± 0.54	1.21 ± 0.12	N.S.
	12:0	1.00 ± 1.00	2.27 ± 0.42	N.S.
	14:0	1.00 ± 0.10	1.51 ± 0.32	N.S.
	16:0	1.00 ± 0.06	2.13± 0.39	<0.05
	18:0	1.00 ± 0.03	1.13 ± 0.05	N.S.
	20:0	1.00 ± 0.07	1.02 ± 0.05	N.S.
	22:0	1.00 ± 0.01	0.95 ± 0.08	N.S.
	24:0	1.00 ± 0.08	0.74 ± 0.01	<0.05
	26:0	1.00 ± 0.06	0.88 ± 0.13	N.S.
n3	18:3	1.00 ± 0.97	4.61 ± 3.23	N.S.
	20:3	1.00 ± 0.01	1.18 ± 0.07	N.S.
	20:5	1.00 ± 0.03	1.16 ± 0.15	N.S.
	22:5	1.00 ± 0.02	1.34 ± 0.17	N.S.
	22:6	1.00 ± 0.03	0.84 ± 0.42	N.S.
n5	14:1	1.00 ± 0.53	3.29 ± 0.47	<0.05
n6	18:1	1.00 ± 0.06	1.44 ± 0.10	<0.02
	18:2	1.00 ± 0.04	0.90 ± 0.45	N.S.
	18:3	1.00 ± 0.98	2.27 ± 1.15	N.S.
	20:2	1.00 ± 0.01	1.13 ± 0.01	N.S.
	20:3	1.00 ± 0.01	1.18 ± 0.07	N.S.
	20:4	1.00 ± 0.03	1.17 ± 0.06	N.S.
	22:2	1.00 ± 0.09	0.42 ± 0.36	N.S.
n7	16:1	1.00 ± 0.05	1.70 ± 0.20	<0.05
	18:1	1.00 ± 0.04	0.90 ± 0.45	N.S.

n9	18:1	1.00 ± 0.50	0.00 ± 0.00	N.S.
	20:1	1.00 ± 0.03	1.34 ± 0.02	<0.01
	22:1	1.00 ± 0.02	1.23 ± 0.06	<0.05
	24:1	1.00 ± 0.09	0.90 ± 0.07	N.S.
n11	18:1	1.00 ± 0.01	0.90 ± 0.45	N.S.
trans	18:1 (n9)	1.00 ± 0.06	1.57 ± 0.19	<0.05
	18:2 (n6)	1.00 ± 0.04	0.90 ± 0.45	N.S.

The liver was removed from each mouse 8 hours after the injection of 3MC or corn oil as a vehicle control. The levels of fatty acids in the liver were measured by GC-MS. Lipids of various chain lengths are grouped by structural similarity. These subclasses include saturated fatty acids, terminal double bond location relative to the omega terminal (omega X = nX), and fatty acids containing *trans*-double bonds (trans). Values are the mean ± SE (n=3/group); N.S., not significant.