Constitutive active/androstane receptor promotes hepatocarcinogenesis in a mouse model of non-alcoholic steatohepatitis

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The nuclear receptor constitutive active/androstane receptor (CAR) acts as a sensor of toxic byproducts derived from the endogenous metabolism and exogenous chemicals. We previously reported that CAR is responsible for exacerbating hepatic injury and fibrosis in a dietary model of non-alcoholic steatohepatitis (NASH) via upregulation of lipid peroxidation. In this study, we investigated the pathological roles of the CAR in the development of hepatocellular carcinoma in NASH. CAR+/+ and CAR−/− mice were fed methionine- and choline-deficient (MCD) diet after tumor initiation with a single dose of the genotoxic carcinogen diethylnitrosamine (DEN) at 2 weeks of age. Interestingly, the MCD diet dramatically promoted DEN-induced hepatocarcinogenesis in CAR+/+ mice. However, the deletion of CAR leads to a significantly lower tumor incidence and smaller tumor diameter. Hepatocytes of MCD-treated-CAR+/+ mice showed a significantly higher staining frequency of Ki-67, a marker of cell proliferation, and exhibited a higher expression of c-Myc and FoxM1 transcripts compared with MCD-treated CAR−/− mice. Immunohistochemistry revealed the nuclear translocation of CAR thus suggesting that the activation of CAR signaling increased in the hepatocytes of CAR+/+ mice fed MCD diet. In addition, in vitro experiments using the CAR stably expressed cell line with TCPOBOP have suggested that CAR activation directly leads to cell proliferation. Survival was significantly lower in the CAR+/+ mice fed the MCD diet in comparison with the CAR−/− mice. Taken together, these results suggest that CAR may therefore play a critical role in the hepatocarcinogenesis of the murine NASH model via the upregulation of cell proliferation.

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide (1). In 70–90% of patients, HCC develops with a background of chronic liver disease or inflammation (2–4). The most well-known risk factor for HCC is hepatitis C virus infection, although many other risk factors for HCC have been defined, including hepatitis B virus infection, alcoholic liver injury and non-alcoholic steatohepatitis (NASH) (2–4). The risk factors and etiologies vary among geographical regions, although the majority of cases are related to hepatitis B virus and hepatitis C virus infections. In developed countries, high consumption of alcohol and non-alcoholic fatty liver disease (often in the context of metabolic syndromes) are the most prevalent risk factors (4). With a better control of hepatitis, the number of patients with NASH or metabolic syndrome-associated HCC is increasing. Although simple steatosis generally follows a benign course, ~10% of patients with non-alcoholic fatty liver disease progress to NASH, and 8–26% of these individuals develop liver cirrhosis (5). Retrospective data suggest that, after cirrhosis develops, 4–27% of NASH cases progress to HCC (6).

The development of HCC is a multistage process, involving initiation, promotion and progression (7). In rodents, HCC can be initiated by genotoxic carcinogens (e.g. nitrosamines) and subsequent promotion by non-genotoxic agents such as phenobarbital (PB), 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane and 17α-ethylɣ-estradiol (8–10). Such rodent models have been used to provide insight into HCC development and, in particular, the promotion of liver tumors in the absence of genetic mutations.

The nuclear receptor, constitutive active/androstane receptor (CAR), was first identified as a PB-activated transcription factor that regulates genes encoding hepatic xenobiotic-metabolizing enzymes such as cytochrome P450 (CYP) (11–15). Although CAR was initially characterized as a xenosensor, it is now evident that CAR also triggers pleiotropic effects on physiological or pathological functions, including lipid metabolism, glucose homeostasis, apoptosis and inflammation (15–18). Yamamoto et al. (19) used CAR−/− mice to investigate the role of CAR in the development of HCC and revealed that the nuclear receptor CAR mediates liver tumor promotion by PB. CAR appears to be a molecular target for PB promotion, and the activation of the receptor CAR is an essential requirement for liver tumor development (19,20).

Nevertheless, the pathophysiological mechanisms leading to the development of NASH and HCC remain unclear (21–23). A two-hit theory of NASH has been proposed, with hepatic steatosis as the first hit, and the triggering host or environmental factor(s) as the second hit, precipitating a cascade of events leading to cell necrosis, inflammation and fibrosis (24,25). We previously reported that CAR activation causes a worsening of hepatic injury and fibrosis in a dietary model of NASH via the induction of lipid peroxidation (26). In the rodent methionine- and choline-deficient (MCD) dietary model of NASH (27–29), the CAR translocated to the nucleus, leading to increased target gene transcription (26). Nuclear translocation of the receptor is the first step in CAR activation by PB (11,12). Therefore, the MCD diet produces a similar effect to PB stimulation and may result in HCC through a similar mechanism. However, the precise relationship between CAR and the development of HCC from NASH is still not fully understood. In the present study, we used CAR-null mice to investigate the role of the receptor in the development of HCC from NASH in mice fed an MCD diet.

Materials and methods

Materials

Diethylnitrosamine (DEN), 1,4 bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) were purchased from Sigma Chemicals Co. (St Louis, MO). The MCD diet and control diets were both purchased from Funabashi Farm (Chiba, Japan). All other chemicals were obtained from commercial sources at the highest purity grade, which are as available.

Animals and treatment

The CAR+/+ and CAR−/− mice used in this study were generated as described previously (19). All mouse procedures were performed in accordance with the guidelines for animal care and use established by Gunma University Graduate School of Medicine. Germ line transmission of the disrupted allele was detected by polymerase chain reaction (30). Male mice from each genotype were given a single intraperitoneal injection of DEN (5 mg/kg) at 2 weeks of age and were then randomly assigned into experimental groups (Figure 1). Beginning at 8 weeks of age, the mice were fed either a plain MCD diet or an MCD diet supplemented with choline bitartrate (2 g/kg) and ω-methionine (3 g/kg); the latter was designated as the control diet. A schematic presentation of the treatment protocol is shown in Figure 1. During the experimental period, the
individual body weights and food intake were recorded three times per week. Mice were killed, and sera and livers were collected and saved for analysis. Samples were collected at 12, 24 or 40 weeks after beginning the MCD diet (n = 8 in each group). To ascertain the effects on overall survival, additional mice were observed until 60 weeks while on the MCD diet (n = 12 in each group).

**Blood chemistry**

The serum alanine aminotransferase, alkaline phosphatase, triglyceride, total cholesterol and glucose levels were all measured with an auto-analyzer (Hitachi 7170; Hitachi Ltd, Tokyo, Japan). The serum levels of insulin were measured using the Merckodia Mouse Insulin ELISA kit (Merckodia AB, Uppsala, Sweden) following manufacturer’s instructions.

**Histology and immunohistochemistry**

Macroscopic liver lesions were visually counted. For the histological examinations, liver tissue specimens were fixed in 10% formalin, embedded in paraffin and then were stained with hematoxylin–eosin and were evaluated for hepatocellular proliferative lesions according to the established criteria by an investigator blinded to the identity of the samples (31–33). The immunohistochemical analyses for Ki-67 and CAR were performed using the avidin-biotin-peroxidase complex method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) using a Ki-67 (DakoCytomation, Glostrup, Denmark) or CAR (Perseus Proteomics, Tokyo, Japan) antibody. The sites of peroxidase binding were determined using the diaminobenzidine method. For each group, 10 areas were examined for Ki-67-positive hepatocytes at a magnification of ×200. Blinded investigators (S.K. and H.N.) evaluated the slides and counted the Ki-67-positive hepatocytes/hpf. Similarly, nuclear staining for CAR was assessed in 10 areas for each group in a blinded fashion (investigators S.K. and H.N.) at ×200 magnification. Sirius red staining was performed according to the usual method and the area of the Sirius red-positive area was measured using the ImageJ software program (National Institutes of Health, Bethesda, MD) in nine microscopic fields at ×200 magnification and the mean ± SD are thus shown. The area of the tumor per section was measured using the ImageJ software program in 10 macroscopic fields.

**In situ analysis of liver apoptosis by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end-labeling assay**

In order to examine the apoptotic cells, a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end-labeling assay (TUNEL) was performed for each group. TUNEL-positive hepatocytes/hpf were evaluated using a Ki-67 (DakoCytomation, Glostrup, Denmark) or CAR (Perseus Proteomics, Tokyo, Japan) antibody. The sites of peroxidase binding were determined using the diaminobenzidine method. For each group, 10 areas were examined for Ki-67-positive hepatocytes at a magnification of ×200. Blinded investigators (S.K. and H.N.) evaluated the slides and counted the Ki-67-positive hepatocytes/hpf. Similarly, nuclear staining for CAR was assessed in 10 areas for each group in a blinded fashion (investigators S.K. and H.N.) at ×200 magnification. Sirius red staining was performed according to the usual method and the area of the Sirius red-positive area was measured using the ImageJ software program (National Institutes of Health, Bethesda, MD) in nine microscopic fields at ×200 magnification and the mean ± SD are thus shown. The area of the tumor per section was measured using the ImageJ software program in 10 macroscopic fields.

**Reverse transcription–polymerase chain reaction**

Total RNA was extracted from the liver or culture cells and the subsequent syntheses of first strand complementary DNA were performed using the TRIzol reagent (Invitrogen, Carlsbad, CA) and the SuperScript™ premultiplication system (Invitrogen), respectively. Complementary DNAs were amplified using the following sets of primers: mouse c-myc messenger RNA (mRNA), 5′-CCACACAGGACCTCTGA-3′ and 5′-GGGGTTCCTGTTCAC-3′; human c-myc mRNA, 5′-TCCTCCATCCAGGTGGT-3′ and 5′-TCGAGGAGAGCATGGA-3′; mouse forkhead box m1 (FoxM1) mRNA, 5′-GCACTTGGTATCACGAG-3′ and 5′-AACACGAGGACTGTTC-3′; human FoxM1 mRNA, 5′-TCCCTTGGAAAGGTGGG-3′ and 5′-AGAAAGATTCGACGAC-3′ and CYP2B6 mRNA, 5′-AAGCCGATTGTCTGGTGA-3′ and 5′-TGAGGATGGTGTGGAGAAG-3′. One-twentieth of each complementary DNA synthesized from 5 μg of RNA was subjected to real-time PCR using SYBR green dye (PE Applied Biosystems, Foster City, CA).

**Western blotting analysis**

Mouse livers were homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 50 mM Tris–HCl) containing complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Transblot membranes were incubated with an anti-c-Myc antibody (Millipore Corporation, Temecula, CA). After incubation with secondary antibodies, the immunoreactive bands were visualized using an enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

**Cell culture, cell number and cell proliferation assay**

Y1m7 cells, a stable cell line expresses CAR, were established from HepG2 cells that were transfected with a mouse pcDNA3.1-CAR-V5-His expression vector and were selected for neomycin resistance (34). The cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). To determine the effect of CAR stimulation on the cell number, the cell viability of Y1m7 cells and the parental HepG2 cells were determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide viability assay. Briefly, 5 × 10⁴ Y1m7 or HepG2 cells per well were cultured in 96-well plates in 100 μl of culture medium, and after 24 h, 250 nM of TCPOBOP was added to the wells. At 48 h, 10 μl of 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide in phosphate-buffered saline was added to each well, and the cells were cultured for 4 h at 37°C. The supernatant was removed, and 100 μl/well of dimethylsulfoxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added. The number of viable cells was converted through the measurements of absorbance using a microplate reader (Fosech, Tokyo, Japan) at 550 nm with a reference wavelength of 650 nm. The cell viability was calculated as a percentage in comparison with the titer of the control.

A cell proliferation analysis was performed using Cell proliferation ELISA, 5-bromo-2-deoxyuridine (BrdU) (Colorimetric) (Roche Applied Science), according to the manufacturer’s protocol. Cell proliferation was investigated by measuring BrdU incorporation during DNA synthesis. Briefly, 5 × 10⁴ Y1m7 or HepG2 cells per well were cultured in 96-well plates in 100 μl of culture medium, and after 24 h, 250 nM of TCPOBOP was added to the wells. At 24 h prior to the end of the stimulation period, BrdU labeling solution was added to each well at a final concentration of 10 μM. After 48 h of TCPOBOP treatment, the cells were fixed (30 min) and then incubated for 90 min at room temperature with substrate solution and the luminescence was measured using a microplate reader at 450 nm with a reference wavelength of 690 nm. The BrdU labeling index was calculated as a percentage in comparison with the titer of the control.

**Data analysis**

All experimental data are shown as the means ± SDs. The significance of the differences was determined by a one-way factorial analysis of variance for each group. Survival curves were calculated by Kaplan–Meier method, and the significance of the differences was determined by Log-rank test. The level of significance for all statistical analyses was set at P < 0.05.

**Results**

**Effects of the MCD diet on CAR+/+ and CAR−/− mice body weight**

Despite a higher food intake relative to their body weight, both CAR+/+ and CAR−/− mice fed the MCD diet lost body weight in comparison with the animals fed the control diet (supplementary Table S1 is available at Carcinogenesis Online), consistent with the findings of a previous study (24,26). Food and water consumptions were measured BrdU incorporation during DNA synthesis. Briefly, 5 × 10⁴ Y1m7 or HepG2 cells per well were cultured in 96-well plates in 100 μl of culture medium, and after 24 h, 250 nM of TCPOBOP was added to the wells. At 24 h prior to the end of the stimulation period, BrdU labeling solution was added to each well at a final concentration of 10 μM. After 48 h of TCPOBOP treatment, the cells were fixed (30 min) and then incubated for 90 min at room temperature with substrate solution and the luminescence was measured using a microplate reader at 450 nm with a reference wavelength of 690 nm. The BrdU labeling index was calculated as a percentage in comparison with the titer of the control.

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Online. However, there were no differences in those levels between the CAR+/- and CAR+/+ mice fed MCD diet for 12, 24 or 40 weeks (supplementary Table S2 is available at Carcinogenesis Online).

The incidence of liver tumors and the tumor area per section
Eight mice from each group were necropsied at 12, 24 and 40 weeks after beginning the MCD diet to assess tumor development, and liver tissue samples were taken to examine gene induction. Proliferative liver lesions had already developed in the MCD-treated CAR+/- mice at 12 weeks. However, no proliferative liver lesions were observed in mice from any of the other groups (Table I). Representative macroscopic findings in the liver at 24 weeks are shown in Figure 2. Thorough visual analysis of the livers revealed large lesions and high tumor multiplicity in all MCD-treated CAR+/- mice at 24 weeks (Figure 2A). The liver tumor incidence was significantly increased in mice fed the MCD diet compared with those fed the control diet. Furthermore, the CAR+/- mice on the MCD diet developed more advanced liver tumors in comparison with the other groups (Figure 2A).

The largest diameter of tumors in CAR+/- mice fed the MCD diet was significantly (2-fold) larger than the largest tumors observed in the CAR-/- mice fed the same diet at 24 weeks (P < 0.05, Figure 2B and Table I). Representative features of liver sections at 24 weeks are shown in Figure 2C. The area of the tumor per section was measured using the ImageJ software program and the area of the tumor per section in the CAR+/- mice fed the MCD diet was 2.6-fold higher than that of the CAR-/- mice fed the same diet (P < 0.05, Figure 2D).

Concuring the hepatic fibrosis, Sirius red staining revealed increased hepatic fibrosis in the both the CAR+/- and the CAR-/- mice fed the MCD diet compared with mice fed the control diet at 24 weeks (P < 0.05; supplementary Figure S1 is available at Carcinogenesis Online). Increased hepatic fibrosis was observed in CAR+/- mice in comparison with CAR-/- mice (P < 0.05). Perivenular and pericellular fibrosis were more clearly observed in CAR+/- mice than in CAR-/- mice. Representative histological features of liver tumors developed in this DEN–MCD model are shown in Figure 2E. Various sizes of the nuclei large cells that are also varied in size and shape, extensive fatty regeneration, increased cell intensity and mitosis were all observed. We diagnosed these tumors to be moderately well differentiated hepatocellular carcinomas.

Ki-67 and CAR immunostaining and mRNA expression of cell cycle-related genes

Immunohistochemical analyses of the Ki-67 labeling index in tissue samples indicated that the number of proliferative cells was increased in CAR+/- mice fed the MCD diet at 24 weeks (Figure 3A and B). Because Ki-67 immunostaining is indicative of highly proliferative and malignant tissues, these results suggest that there was an increase in liver tumor promotion by MCD in the CAR+/- mice. The initial step of CAR-mediated gene expression is the nuclear translocation of the receptor, and therefore nuclear staining for CAR was evaluated by immunohistochemistry for each group at 24 weeks. Confirming that the MCD diet affects CAR signaling, we observed that nuclear accumulation of CAR increased in the livers of CAR+/- mice fed the MCD diet (Figure 3C and D). TUNEL staining demonstrated the TUNEL-positive cells to significantly increase in the both CAR+/- and CAR-/- mice fed the MCD diet in comparison with the control diet (Figure 3E and F, P < 0.01). Although it did not reach statistical significance, the mean number of the TUNEL-positive cells increased in the CAR+/- mice in comparison with the CAR-/- mice fed the MCD diet.

To further investigate the increase in proliferation indicated by Ki-67 staining, we examined the mRNA expression of cell cycle-related genes using real-time polymerase chain reaction. Because c-Myc and FoxM1 have previously been reported to mediate liver hyperplasia induced by CAR (35), the mRNA expression of these two genes was evaluated in non-malignant liver tissues at 24 weeks. The mRNA expression of both c-Myc and FoxM1 were significantly elevated in the CAR+/- mice fed the MCD diet (Figure 4). Furthermore, western blotting analyses of c-Myc in malignant and non-malignant liver lesions at 40 weeks of MCD diet administration revealed that c-Myc was significantly elevated in CAR+/- mice fed the MCD diet in comparison with CAR-/- mice fed the same diet (P < 0.05).

Survival curves of CAR+/- and CAR-/- mice

The mice were maintained on the MCD or control diet until moribund (as indicated by difficulty breathing, rapid growth of internal mass (es) and/or severe dehydration), when they were humanely killed. The CAR+/- mice on the MCD diet began to succumb at 38 weeks, and all of the mice had died or were killed by 57 weeks from the start of MCD diet administration (Figure 5). All mice bore multiple large liver tumors. Although the CAR-/- mice on the MCD diet began to succumb at 42 weeks of MCD diet administration, their overall survival time was significantly better than that of the CAR+/- mice (P < 0.05).

Effect of CAR stimulation by TCPOBOP on cell proliferation and the mRNA expressions of c-Myc and FoxM1 in vitro

To determine the effect of CAR stimulation on the cell numbers, the cell viability of Ym17 cells stably expressing CAR and the parental HepG2 cells was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide viability assay (Figure 6A). No differences were observed in HepG2 cell proliferation by treatment with the CAR agonistic ligand, TCPOBOP. Conversely, the cell number of the Ym17 cell line significantly increased in comparison with the parental HepG2 cells (P < 0.05). Furthermore, treatment with TCPOBOP significantly increased cell proliferation in Ym17 cells (P < 0.05). Furthermore, to determine the effect of CAR stimulation on cell proliferation, the DNA synthesis of Ym17 cells and HepG2 cells was determined by a BrdU assay (Figure 6B). No differences were observed in the HepG2 cell proliferation by treatment with the CAR agonistic ligand, TCPOBOP. However, treatment with TCPOBOP significantly increased the cell proliferation in Ym17 cells (P < 0.05). CYP2B6 mRNA expression, one of the typical CAR target genes, was examined as a positive control (Figure 6C). The basal CYP2B6

Table I. Area of tumor per liver section and the mean largest diameter of tumors in CAR+/- and CAR-/- mice fed the MCD or control diet for 12, 24 and 40 weeks

<table>
<thead>
<tr>
<th>Mice</th>
<th>Diet</th>
<th>Area of tumor per liver section (%)</th>
<th>Mean of largest diameter of tumors (mm)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>12 Weeks</td>
<td>24 Weeks</td>
</tr>
<tr>
<td>CAR+/-</td>
<td>Control</td>
<td>—</td>
<td>10.3 ± 4.8a,b</td>
</tr>
<tr>
<td></td>
<td>MCD</td>
<td>2.1 ± 2.4a,b</td>
<td>7.1 ± 4.8b</td>
</tr>
<tr>
<td>CAR-/-</td>
<td>Control</td>
<td>—</td>
<td>2.5 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>MCD</td>
<td>8.6 ± 5.6b</td>
<td>4.6 ± 3.2</td>
</tr>
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Data represent the mean ± SD for eight mice in each group.

*P < 0.05 compared with the control diet.

*P < 0.05 in CAR+/- mice in comparison with the CAR-/- mice.
mRNA expression in Ym17 cells was significantly increased in comparison with HepG2 cells \( (P < 0.05) \), whereas those of Ym17 cells significantly increased following TCPOBOP stimulation \( (P < 0.05) \). The mRNA expressions of c-Myc also significantly increased due to TCPOBOP stimulation for 24 h in Ym17 cells (Figure 6D). However, this increase in expression was not observed in HepG2 cells. Furthermore, the mRNA expressions FoxM1 also significantly increased following 48 h of TCPOBOP stimulation in Ym17 cells (Figure 6E). As a result, c-Myc and FoxM1 appear to be related to the cell proliferation in Ym17 cells in the same manner as that observed in animal models.

**Discussion**

In this study, we demonstrated that the MCD diet promotes DEN-induced hepatocarcinogenesis compared with a control diet and that the nuclear receptor CAR is an important factor in promoting hepatocarcinogenesis in a dietary model of NASH with MCD diet.
Moreover, we have also shown that the promoting effects of CAR may be mediated by the activation of the c-Myc-FoxM1 pathway, which regulates cell proliferation. We previously reported that the nuclear receptor CAR accelerates liver injury and fibrosis (26). However, the MCD diet itself was not sufficient to induce liver tumors. Therefore, mice at 2 weeks of age were injected with DEN and were fed thereafter

![Image](https://academic.oup.com/carcin/article-abstract/32/4/576/2463525)

**Fig. 3.** (A) Immunohistochemical staining for Ki-67 in the liver of mice fed the MCD or the control diet for 24 weeks. (B) The number of Ki-67-positive cells per view. At ×200 magnification, 10 areas of Ki-67-positive cells were measured in a blinded fashion for each group. Eight animals were included in each group. The Ki-67-positive cells per high-powered field were counted, and the data are presented as the means ± SDs. (C) Immunohistochemical staining for CAR at 24 weeks. Nuclear staining for CAR was positive, whereas cytoplasmic staining was negative; ×1000 magnification. (D) The number of cells positive for nuclear staining for CAR per field with ×200 magnification. Ten fields were evaluated for CAR staining in a blinded fashion for each group. Eight animals were included in each group. The CAR-positive cells per high-powered field were counted, and the data presented are the means ± SDs. *P < 0.05 in comparison with the control diet. **P < 0.05 in comparison with the control diet. (E) The TUNEL staining for the livers of the Wt and Tg mice after 24 weeks on either the control or MCD diet. (F) The number of TUNEL-positive cells was counted in 10 randomly selected fields at 200-fold magnification in each group. The TUNEL-positive cells significantly increased in the both mice fed the MCD diet in comparison with the control diet. *P < 0.05 compared with the control diet. **P < 0.05 in CAR+/+ mice compared with CAR−/− mice.
with MCD diet. To our knowledge, this is the first report to confirm that the MCD diet potentiates DEN-induced hepatocarcinogenesis.

The MCD diet caused steatosis, hepatocyte injury, inflammation, liver fibrosis and cell proliferation in the liver in comparison with the control diet. Six animals were included in each group.

Fig. 4. (A) C-Myc mRNA expression in non-malignant liver lesions of mice fed the MCD or the control diet for 24 weeks. The data represent the mean ± SD. Six animals were included in each group. *P < 0.05 in comparison with the control diet. #P < 0.05 in CAR\(^{+/+}\) mice compared with CAR\(^{-/-}\) mice. (B) The FoxM1 mRNA expression in non-malignant liver lesions at 24 weeks. Six animals were included in each group. (C) A western blot analysis of c-Myc in tumor and non-tumor liver tissues in mice fed the MCD diet for 40 weeks. Six animals were included in each group. C-Myc expression levels in malignant and non-malignant liver tissues were significantly elevated in CAR\(^{+/+}\) mice fed the MCD diet in comparison with CAR\(^{-/-}\) mice fed the same diet (P < 0.05).

Fig. 5. Survival curves of the CAR\(^{+/+}\) and CAR\(^{-/-}\) mice. The MCD diet was continued until the mice were humanely killed. The CAR\(^{+/+}\) mice on the MCD diet started to succumb at 38 weeks, and all the mice had died or were killed at 57 weeks from the beginning of MCD diet administration. All the deceased mice bore multiple large liver tumors. Although the CAR\(^{-/-}\) mice on the MCD diet started to die at 42 weeks from MCD diet administration, their overall survival was significantly better than the CAR\(^{+/+}\) mice (n = 12 animals in each group, P < 0.05).

Fig. 6. The effect of CAR stimulation by TCPOBOP treatment on cell proliferation and c-Myc expression. (A) The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay of Ym17 cells stably expressing CAR and parental HepG2 cells following stimulation with TCPOBOP. No differences were observed in cell proliferation by treatment with the CAR agonistic ligand TCPOBOP in HepG2 cells. The cell proliferation of Ym17 cells significantly increased in comparison with that of HepG2 cells (n = 16 in each group, #P < 0.05). Furthermore, TCPOBOP significantly increased the cell proliferation of Ym17 cells (^P < 0.05). (B) The BrdU labeling assay of Ym17 cells and HepG2 cells following stimulation with TCPOBOP. No differences were observed in the cell proliferation after treatment with the CAR agonistic ligand TCPOBOP in HepG2 cells. TCPOBOP significantly increased the cell proliferation of Ym17 cells (n = 16 in each group, *P < 0.05). (C) CYP2B6 expression in Ym17 and HepG2 cells after 24 h of TCPOBOP treatment. The basal expression of CYP2B6 mRNA in Ym17 cells significantly increased in comparison with HepG2 cells (n = 6 in each group, #P < 0.05). Furthermore, CYP2B6 expression was significantly increased in Ym17 cells after TCPOBOP stimulation (n = 6 in each group, *P < 0.05). (D) The mRNA expression of c-Myc in Ym17 and HepG2 cells after 24 h of TCPOBOP treatment. The c-Myc mRNA expression significantly increased following TCPOBOP stimulation in Ym17 cells (n = 6 in each group, *P < 0.05). However, no such increases were observed in HepG2 cells. (E) FoxM1 expression in Ym17 and HepG2 cells after 48 h of TCPOBOP treatment. The FoxM1 expression significantly increased after TCPOBOP stimulation in Ym17 cells (n = 6 in each group, #P < 0.05). However, no such increased expression was observed in HepG2 cells.
mice fed the control diet. Furthermore, reactive oxygen species production, generation of oxidative DNA damage via 8-hydroxydeoxyguanosine and tumor necrosis factor-alpha production were all elevated in MCD-fed mice in comparison with control diet-fed mice (26). We hypothesize that these results might partly account for the promoting effect of the MCD diet compared with the control diet. Furthermore, methionine and choline deficiency following a single initiating carcinogen dose were reported to enhance enzyme-altered pre-neoplastic foci formation in rats (36,37). Fullerton et al. (38) reported that dietary supplementation with methionine and choline protected against the formation of liver carcinomas in DEN-treated PB-fed mice. Aberrant methylation, consisting of DNA hypomethylation and/or promoter gene CpG hypermethylation, have been implicated in the development of a variety of solid tumors, including HCCs (37–40). Because methionine and choline deficiency lead to aberrant methylation (37–40), a similar mechanism might be involved in the results observed in the current study. However, further studies will confirm this mechanism.

Non-genotoxic carcinogens such as PB stimulate cell proliferation and suppress apoptosis, leading to tumor development. Yamamoto et al. (19) revealed that CAR is the molecular target of PB and that activation of this receptor is essential for liver tumor promotion by PB. In this study, we showed the deletion of CAR also leads to a significantly lower tumor incidence and smaller tumor diameter. The drastic acceleration of hepatocarcinogenesis in DEN-MCD-treated CAR+/− mice suggests that CAR acts as a promoter of DEN-induced hepatocytes. One mechanism by which CAR accelerates hepatocarcinogenesis in DEN-MCD-treated CAR+/− mice is through the dysregulation of cell growth. Blanco-Bose et al. (35) reported that the CAR agonist TCPOBOP induced cell proliferation which was mediated by c-Myc and FoxM1 and induced liver hyperplasia. In addition, they showed that the c-Myc-FoxM1 pathway may be downstream of CAR receptor signaling, leading to heightened hepatocyte proliferation. In the current study, we demonstrated that MCD-fed CAR+/− mice exhibited increased hepatocyte proliferation compared with MCD-treated CAR−/− mice, as measured by Ki67 staining. Moreover, we have demonstrated that c-Myc and FoxM1 are elevated in MCD-treated CAR+/− mice, lending further support to the hypothesis that there is a CAR-c-Myc-FoxM1 pathway, which may contribute to the promoting effect of CAR. Another possible mechanism may be that the anti-apoptotic effect of CAR is due to Bcl-2 (15) and Mcl-1 (15) upregulation, thereby increasing the survival of the initiated cells that would otherwise succumb to mutation-induced death. However, the number of apoptotic cells increased in the CAR+/− mice in comparison with the CAR−/− mice fed the MCD diet although it did not reach the statistical significance in this study. As a result, the anti-apoptotic effect of CAR was not observed in this DEN-MCD model. The increased apoptotic cells may be due to the immune response of the host as a response to the increased hepatocarcinogenesis in the MCD-fed CAR+/− mice.

A recent study by Dong et al. (41) revealed that activation of CAR by the agonistic ligand TCPOBOP significantly reduces serum glucose levels and improves glucose tolerance and insulin sensitivity. In addition, CAR activation dramatically improves steatosis by inhibiting hepatic lipogenesis and inducing beta-oxidation (41,42). Gao et al. (43) also reported that CAR can prevent or reverse obesity and that treatment with TCPOBOP improves insulin sensitivity. Because a loss of insulin sensitivity has been suggested to underlie the development of NASH, improvement in fatty liver and insulin sensitivity by CAR activation may be related to the observed improvement of NASH. Conversely, we have previously reported that CAR lead to increased hepatic injury and fibrosis as a result of lipid peroxidation upregulation (26). These two seemingly conflicting results may be the result of differences in experimental conditions, including the dietary model of NASH with or without TCPOBOP activation, the strains of mice and other factors. The authors used ob/ob mice and a high-fat diet and studied insulin resistance and inflammation. However, in the current study, the MCD diet resulted in NASH via a blockade of triglycerides export from the liver or reactive oxygen species production and was not caused by insulin sensitivity (44). Indeed, insulin levels did not change between CAR+/− mice and CAR−/− mice in the current study. Otherwise, reactive oxygen species production induced by CAR in the MCD diet may overcome the improvement in insulin sensitivity by CAR. In addition, we previously reported that there are differences in the susceptibility to NASH between different species and sexes. We choose C3H/He mice for this study because of their increased susceptibility to liver carcinogenesis compared with C57BL/6N mice (45). However, this may have affected the outcome of the study because the C57BL/6N strain has a higher susceptibility to NASH than the C3H/HeN mice (46). Gao et al. (43) used a C57BL/6J-SvJ129 mixed background mice and a high-fat diet. These strain differences in susceptibility to NASH may have resulted from differing insulin sensitivity.

On the other hand, Breuker et al. (47) recently reported that CAR and/or pregnane X receptor activating drugs and xenobiotics may promote aberrant hepatic de novo lipogenesis, potentially leading to fatty liver diseases and insulin resistance via the regulation of lipogenic gene thyroid hormone-responsive spot 14 protein. This result is also conflicting in lipogenesis and insulin sensitivity and resemble to our result. As a result, further investigation will evaluate the role of CAR on insulin sensitivity or lipid homeostasis.

In conclusion, the present study has revealed that the MCD diet-induced model of NASH promotes liver tumors in CAR+/− mice, indicating that the nuclear receptor CAR mediates the liver tumor promotion in NASH. Once CAR-mediated pathways of tumor promotion are defined at the molecular level, the receptor may be useful as a drug target for the prevention of HCC in patients with NASH.

Supplementary material

Supplementary Figure S1 and Tables S1 and S2 can be found at http://carcin.oxfordjournals.org/

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References

CAR promotes NASH-induced hepatocarcinogenesis


