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A possible mechanism for atherosclerosis induced by polycyclic aromatic hydrocarbons

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Abstract

Polycyclic aromatic hydrocarbons (PAHs), aryl hydrocarbon receptor (AHR) ligands, induce atherogenesis. Liver X receptor (LXR) α is known to be involved in the control of cholesterol homeostasis. Thus, the purpose of this study was to investigate the effects of 3-methylcholanthrene (MC), one of the PAHs, on LXRα-mediated signal transductions. We found that expression of mRNAs for ATP binding cassette A1, sterol regulatory element binding protein 1c (SREBP-1c), fatty acid synthase, and stearoyl-CoA desaturase was suppressed by treatment of HepG2 cells with MC. A luciferase reporter assay revealed that LXRα- and SREBP-1c-mediated transactivations were inhibited by MC via AHR. Based on these lines of evidence, we propose that down-regulation of the LXRα-regulated genes by PAHs is one of the causes responsible for atherosclerosis induced by PAHs.

Keywords: PAHs; LXR; ABCA1; SREBP-1c; FAS; SCD; Quantitative RT-PCR; Luciferase assay

Polycyclic aromatic hydrocarbons (PAHs)[1] are ubiquitous environmental contaminants that originate from multiple sources, including cigarette smoke, vehicle exhaust emissions, and industrial processes [1]. PAHs induce various toxicological effects such as carcinogenesis, atherogenesis, and teratogenesis [2]. Several reports have suggested that cigarette-induced atherosclerosis is caused by PAHs [3–7]. PAHs are believed to show such toxicological effects through the activation of AHR [8–10], which is identified as a ligand-activated transcription factor that controls the genes, including the *CYP1A1*, *CYP1A2*, and *CYP1B1* genes [11].

LXRα is a member of the nuclear hormone receptor superfamily and is activated by oxysterols [12,13]. It is abundantly expressed in organs including the liver, adipose, kidney, intestine, lung, adrenals, and macrophages [14]. LXRα acts as a cholesterol sensor to transactivate the genes that govern the transport, catabolism, and elimination of cholesterol [14]. ABCA1, a typical LXR target gene [15], transports phospholipids and cholesterol, and is known as a rate-limiting step in a reverse cholesterol transport [16]. Ligand-activated LXRα also up-regulates the *SREBP-1c* gene which belongs to the bHLH-Zip family of a transcription factor [17]. SREBP-1c enhances the transcription of the genes required for fatty acid synthesis and fatty acid elongation including FAS and SCD [18,19]. FAS and SCD produce oleoyl-CoA and palmitoeyl-CoA, which

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[1] *Abbreviations:* ABC, ATP binding cassette; AHR, aryl hydrocarbon receptor; bHLH-Zip, basic helix-loop-helix-leucine zipper; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; FAS, fatty acid synthase; FBS, fetal bovine serum; LXR, liver X receptor; LXRE, LXR response element; MC, 3-methylcholanthrene; PAHs, polycyclic aromatic hydrocarbons; RT-PCR, reverse transcriptase-polymerase chain reaction; SCD, stearoyl-CoA desaturase; siRNA, short interference RNA; SRE, sterol regulatory element; SREBP-1c, sterol regulatory element binding protein 1c; T1317, TO-901317; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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are used for the esterification of cholesterol to detoxify the free cholesterol [20].

In the present study, we investigated the effects of MC, which is one of the PAHs, on the LXRα-mediated signal transductions. We show herein that the activation of AHR by MC causes the down-regulation of the expression of mRNAs for ABCA1, SREBP-1c, FAS, and SCD, which are regulated by LXRα directly or indirectly. Possible mechanism(s) by which exposure to PAHs leads to atherosclerosis will also be discussed.

Materials and methods

Cell culture. Human hepatoma-derived HepG2 cells were purchased from RIKEN (Tsukuba, Japan). The HepG2 cells were maintained in DMEM (Nissui Pharmacy, Tokyo, Japan) supplemented with 10% FBS (Bio Whittaker, Warkersville, MD), non-essential amino acids (ICN, Aurora, OH), and 1 mM sodium pyruvate (Gibco-BRL, Rockville, MD) in 5% CO_2 at 37 °C.

Plasmids. The 5′-flanking regions of the human *ABCA1* gene from −829 to +101, the human *SREEBP1c* gene from −1000 to −1, the human *FAS* gene from −927 to −1, and the human *SCD* gene from −1000 to −1 were obtained by PCR with respective sense primers, hABCA1-*Bgl*II-S (5′-GATCGATCAGATCTTAAGTTGGAGGTCTGGAGT GT-3′), hSREBP1c-*Bgl*II-S (5′-GAAGATCTGAACCCTAGAGCCT GTCACC-3′), hFAS-*Bgl*II-S (5′-GAAGATCTCGACTCCGCTCGC CACGTG-3′), and hSCD-*Bgl*II-S (5′-GAAGATCTTGACGGTTTCC ACAAAGAAG-3′), and antisense primers, hABCA1-*Hin*dIII-AS (5′-GATCGATCAAGCTTGCTCTGTTGGTGCGCGGA-3′), hSREBP 1c-*Hin*dIII-AS (5′-CCCAAGCTTGGCTCCGCGATCTGCGCC-3′), hFAS- *Hin*dIII-AS (5′-CCCAAGCTTTAGGCCGGCGCCGAC-3′), and hSCD-*Hin*dIII-AS (5′-CCCAAGCTTCGCGGTGCGTGGAG GTC-3′). The respective DNA fragments thus synthesized were digested with *Bgl*II and *Hin*dIII, and then inserted into the *Bgl*II and *Hin*dIII sites of a luciferase reporter plasmid, pGL3-basic vector (Promega, Madison, WI) to construct reporter plasmids, pABCA1-Luc, pSREBP-1c-Luc, pFAS-Luc, and pSCD-Luc. The $p(LXRE)_2$-TK-Luc was constructed by synthesizing oligonucleotides containing two copies of LXRE from mouse mammary tumor virus *LTR* gene promoter [21]. The $p(SRE)_2$-TK-Luc was constructed by synthesizing oligonucleotides containing two copies of SRE from mouse *SCD1* promoter [22]. The oligonucleotides were annealed and cloned into the *Xho*I site upstream of thymidine kinase promoter of pGL3-promoter vector (Promega). Full-length human LXRα cDNA was cloned into the *Bam*HI and *Eco*RI sites of pcDNA 3.1 mammalian expression vector (pcDNA-hLXRα) (Invitrogen, Carlsbad, CA). The pU6-siAHR as an siRNA expression plasmid for *AHR* gene silencing was constructed by using p *Silencer* 1.0-U6 siRNA Expression Vector (Ambion, Austin, TX). To construct hairpin siRNA expression cassette, two complementary oligonucleotides were synthesized, annealed, and ligated into the blunted *Apa*I site of the p *Silencer*. The sequences were 5′-GGTTTCAGCAGTCTGATGTCttca agagaGACATCAGACTGCTGAAACCCTTTTT-3′ and its complement, 5′-AGGGTTTCAGCAGTCTGATGTCtctcttgaaGACATCAG ACTGCTGAAACC-3′. This sequence cassette contained the oligonucleotides encoding 20-mer hairpin sequences specific to the human AHR mRNA at 438–458 position, a ttcaagaga loop sequence separating the two complementary domains, and a TTTTT terminator at the 3′-end [23–27].

Real-time RT-PCR analysis. Total RNA was prepared using GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich, St. Louis, MO). Reverse transcription reaction was performed by using First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Indianapolis, IN). A quantitative real-time RT-PCR was carried out with a LightCycler using FastStart Reaction Mix SYBR Green I (Roche Diagnostics). The sequences of primer pair were designed as follows:

hABCA1: sense, 5′-TTTGTTCCTGTGTATTCTCTCA-3′;
antisense, 5′-GGCAGCTTCTTGTCCTGGAG-3′,
hSREBP-1c: sense, 5′-CGGAGCCATGGATTGCACTTTC-3′;
antisense, 5′-GATGCTCAGTGGCACTGACTCTTC-3′,
hFAS: sense, 5′-AACTCCAAGGACACAGTCACCAT-3′;
antisense, 5′-CAGCTGCTCCACGAACTCAA-3′,
hSCD: sense, 5′-GGAAAGTGATCCCGGCATCGGAGA GCCAA-3′;
antisense, 5′-GACAAAATAGTAGAATACCCCCC AAAGCC-3′, and
β-actin: sense, 5′-ATTGCTGACAGGATGCAGA-3′;
antisense, 5′-AAGATCATTGCTCCTCCTGAGC-3′.

A reaction mixture contained 3 mM $MgCl_2$, 0.5 μM each primer, 1× FastStart DNA SYBR Green I mix, and 2 μL template cDNA in a final volume of 20 μL, and was collected into a LightCycler glass capillary. The details of thermal cycler program are as follows: Activation of the Taq DNA polymerase at 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 68–70 °C for 4–8 s, and 72 °C for 20 s (transition rates of 20 °C/s) and collection of the fluorescence values after each elongation step. The analysis of a melting curve was performed by annealing at 65 °C for 15 s and redenaturation by raising the temperature to 95 °C at a ramp rate of 0.1 °C/s. To correct for differences in both quality and quantity between samples, data were normalized using the ratio of the target cDNA concentration to that of β-actin.

Transient transfection and luciferase assay. The day before transfection, cells were plated at a density of 1×10^5 cells/well in a 12-well plate. Cells were transfected with 350 ng reporter plasmids (pABCA1-Luc, pSREBP-1c-Luc, pFAS-Luc, pSCD-Luc, $p(LXRE)_2$-TK-Luc or $p(SRE)_2$-TK-Luc), 100 ng pcDNA-hLXRα, and 50 ng pRL-TK vector (as an internal control for transfection) by using Fugene6 (Roche Diagnostics). The medium was changed to fresh DMEM containing MC (0.1 and 1 μM) (Sigma–Aldrich) and 1 μM T1317, a LXRα ligand (Sigma–Aldrich). Cells were harvested 36 h after starting the incubation. Luciferase activity was measured according to the method of the manufacturer. When siRNA expression vector was applied, cells were transfected with 300 ng pU6-siAHR or pU6-control, 100 ng pcDNA-hLXRα, 100 ng reporter plasmids ($p(LXRE)_2$-TK-Luc and $p(SRE)_2$-TK-Luc), and 50 ng pRL-TK vector. Twenty-four hours later, the medium was changed to DMEM containing MC (1 μM) and T1317 (1 μM). After 36 h of incubation, luciferase activity was measured.

Results and discussion

To examine whether or not the expression of genes regulated by LXRα was suppressed by PAHs, total RNA was prepared from HepG2 cells previously treated with 1 μM T1317 and 0.1, 1 or 10 μM MC. The expression of mRNAs for the genes regulated by LXRα was quantified by quantitative real-time RT-PCR method (Fig. 1). When HepG2 cells were treated with 1 μM T1317, the expression of mRNAs for the LXRα-target genes including ABCA1 and SREBP-1c was induced (Figs. 1A and B). The expression levels of ABCA1 and SREBP-1c mRNAs were increased by treatment with 1 μM T1317 and decreased by the co-treatment with

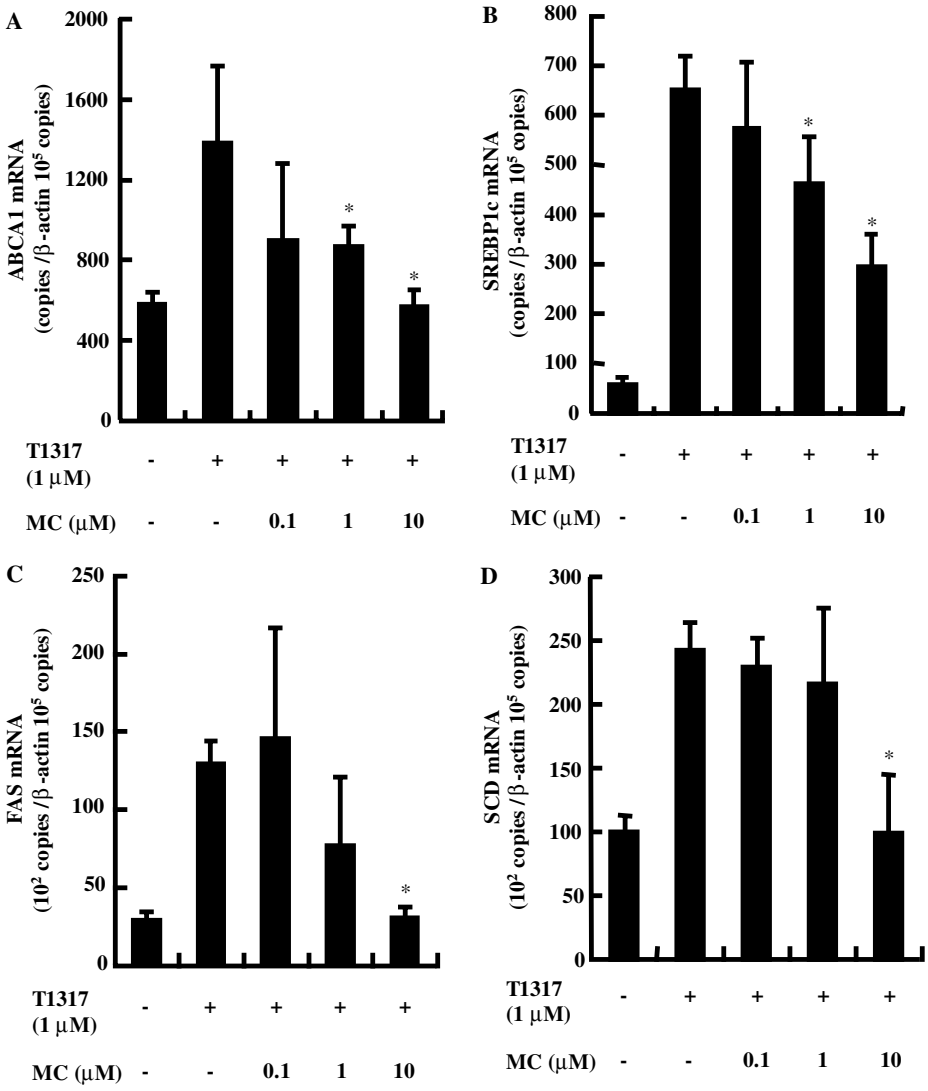

Fig. 1. Dose-dependent repression by MC of the expression of mRNAs for the genes regulated by LXRα. The expression of the genes regulated by LXRα, ABCA1 (A), SREBP-1c (B), FAS (C), and SCD (D) was verified by real-time RT-PCR. Total RNA (1 μg) prepared from HepG2 cells treated with 1 μM T1317, LXR ligand, and MC at various concentrations (0.1, 1, and 10 μM) was subjected to a real-time RT-PCR. The values represent the average ± SD from three independent experiments. *A statistically significant difference ($p < 0.05$) relative to the cells treated with T1317 alone.

MC in a dose-dependent manner (Figs. 1A and B). The expression of mRNA for FAS, which is both the LXRα- and SREBP-1c-target genes [18], was also induced by T1317 in HepG2 cells and decreased by MC (Fig. 1C). In the case of SCD, which is the SREBP-1c-target gene [19], the mRNA expression was induced by T1317, and depressed by MC, dose-dependently (Fig. 1D). These results indicate that the expression of mRNAs for the LXRα-regulated genes induced by the activation of LXRα was suppressed by MC, suggesting that the LXRα-originated signals were repressed by PAHs.

To further support the possibility that the transcription of the genes regulated by LXRα was suppressed by PAHs, the effects of MC on the transcriptional activity of LXRα- and SREBP-1c-target genes were examined by a luciferase reporter assay using a reporter plasmid including pABCA1-Luc, pSREBP-1c-Luc, pFAS-Luc, and pSCD-Luc (Fig. 2). When HepG2 cells were transfected with pcDNA-hLXRα in the presence of 1 μM T1317, the luciferase activity seen with pABCA1-Luc, pSREBP-1c-Luc, pFAS-Luc or pSCD-Luc was 3.8-, 3.6-, 4.7- or 3.4-fold higher than that seen in the absence of T1317, respectively (Figs. 2A–D). The luciferase activity seen with pABCA1-Luc, pSREBP-1c-Luc, pFAS-Luc or pSCD-Luc in the presence of 1 μM T1317 was decreased to a level of approximately 20–30% by 1 μM MC (Figs. 2A–D). Thus, it indicated that the transcription of essentially all of the LXRα and SREBP-1c target genes was suppressed by MC.

To further investigate the effects of MC on the transcriptional activation through LXRα and SREBP-1c, a luciferase reporter assay was performed by using

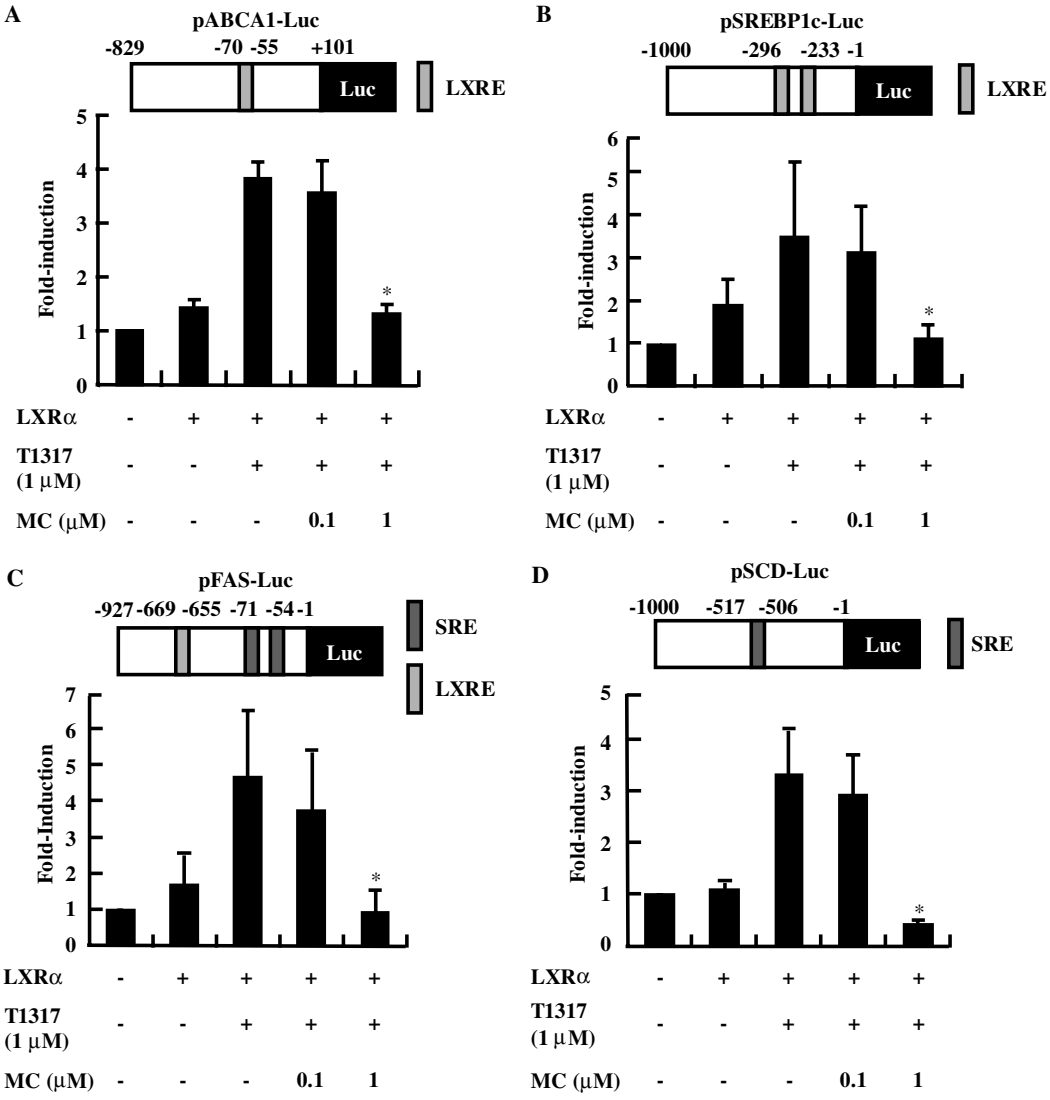

Fig. 2. Suppression by MC of the transcriptional activities of the genes regulated by LXRα. Luciferase reporter plasmids, pABCA1-Luc (A), pSREBP-1c-Luc (B), pFAS-Luc (C), and pSCD-Luc (D), were co-transfected into HepG2 cells with pcDNA-hLXRα. HepG2 cells were treated with T1317 (1 μM) and MC (0.1 and 1 μM). Luciferase activity was measured 36 h after incubation. The values represent the average ± SD from three independent experiments. *A statistically significant difference ($p < 0.05$) relative to the cells treated with T1317 alone.

p(LXRE)$_2$-TK-Luc and p(SRE)$_2$-TK-Luc (Figs. 3A and B). When HepG2 cells were transfected with the p(LXRE)$_2$-TK-Luc or the p(SRE)$_2$-TK-Luc, and pcDNA-hLXR, the luciferase activity seen with the p(LXRE)$_2$-TK-Luc or the p(SRE)$_2$-TK-Luc was increased 55- or 2.2-fold by treatment with T1317, respectively (Figs. 3A and B). When HepG2 cells were treated with MC, the luciferase activities decreased to a level of 30–50% compared to that of control (Figs. 3A and B).

It has been reported that PAHs produce toxic effects through the activation of AHR [8–10]. To confirm if AHR plays key roles in the repression of the transcriptional activation through LXRα and SREBP-1c by PAHs, we examined the effects of siRNA expression plasmid to impair the expression of the *AHR* gene (Fig. 4). When HepG2 cells were transfected with pU6-siAHR, the protein level of AHR decreased (Fig. 4A), indicating that the siAHR could impair the effects of MC though AHR. Subsequently, we investigated whether the suppression of LXRα and SREBP-1c transactivation by MC was blocked by using the pU6-siAHR in HepG2 cells. The pU6-siAHR reversed the inhibition of LXRα and SREBP-1c transactivation by MC (Fig. 4B).

In the present study, we found that the transcriptional activity of the genes regulated by LXRα such as ABCA1, SREBP-1c, FAS, and SCD was down-regulated by MC depending on AHR. It has been reported that LXR regulates the ABC transporter genes including *ABCA1*, *ABCG5*, and *ABCG8*, which are responsible for cellular cholesterol efflux and dietary cholesterol absorption [14,28,29], and *CYP7A1* gene, which is

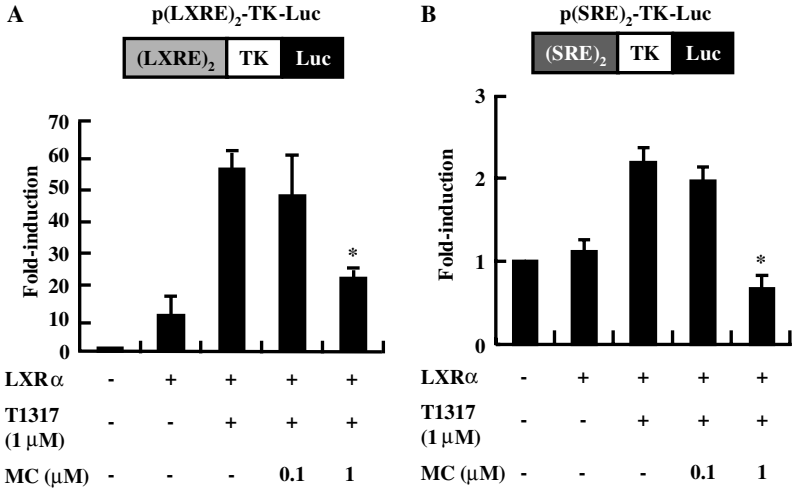

Fig. 3. Suppression by MC of transcriptional activities via LXRα and SREBP-1c. Luciferase reporter plasmids, $p(LXRE)_2$-TK-Luc (A) and $p(SRE)_2$-TK-Luc (B), were co-transfected into HepG2 cells with pcDNA-hLXRα. HepG2 cells were treated with T1317 (1 μM) and MC (0.1 and 1 μM). Luciferase activity was measured 36 h after incubation. The values represent the average ± SD from three independent experiments. *A statistically significant difference ($p < 0.05$) relative to the cells treated with T1317 alone.

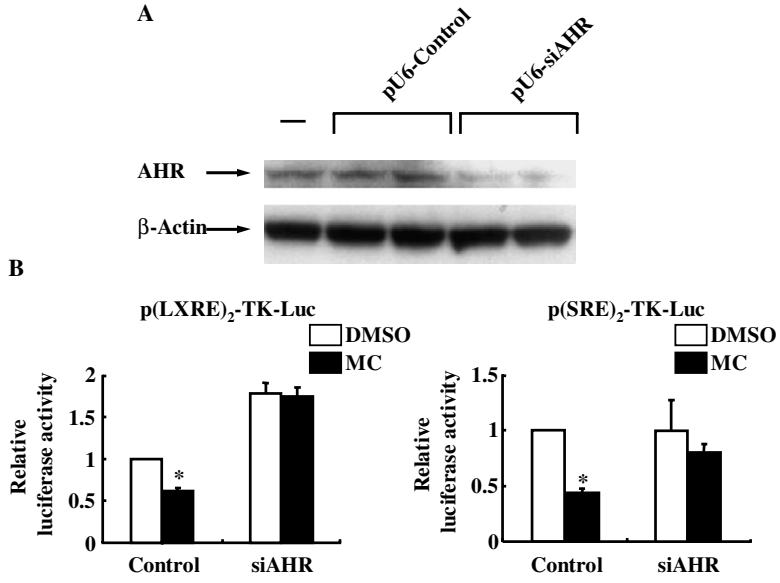

Fig. 4. Effects of siRNA for AHR on the down-regulation by MC of LXRα and SREBP-1c transactivation. (A) HepG2 cells were transfected with 300 ng pU6-control (control vector) or pU6-siAHR vector for AHR gene silencing. After 24 h, whole cell extracts (40 μg) were subjected to SDS–polyacrylamide gel electrophoresis and immunoblotting with AHR and β-actin antibodies. (B) HepG2 cells were co-transfected with pcDNA-hLXRα, pU6-siAHR, and $p(LXRE)_2$-TK-Luc or $p(SRE)_2$-TK-Luc. After 24 h incubation, the cells were treated with T1317 (1 μM) in the presence or absence of MC (1 μM). The luciferase activity was determined after 36 h of incubation. All transfection data represent means of at least three independent experiments. *A statistically significant difference ($p < 0.05$) relative to the cells treated with T1317 alone.

involved in cholesterol catabolism [13]. Miyazaki et al. [20] reported that FAS and SCD were responsible for the esterification of cholesterol to produce oleoyl-CoA and palmitoyl-CoA, which is the detoxification pathway of free cholesterol. Thus, it is possible to assume that the atherosclerosis is induced by PAHs through the following mechanisms: (1) The exposure to PAHs causes the increase of free cholesterol level in plasma because of the suppression of LXR-target genes by PAHs via AHR (Fig. 5). (2) The detoxification of cholesterol is inhibited by PAHs via the down-regulation of the *FAS* and *SCD* genes, which are SREBP-1c-target genes, by PAHs through AHR (Fig. 5).

In conclusion, we demonstrated in this paper that the expression of the genes regulated by LXRα was suppressed by treatment with MC due to the disruption of LXRα-mediated transactivation via ligand-activated AHR. These molecular mechanisms probably account for the cause responsible for the atherosclerosis induced by PAHs.

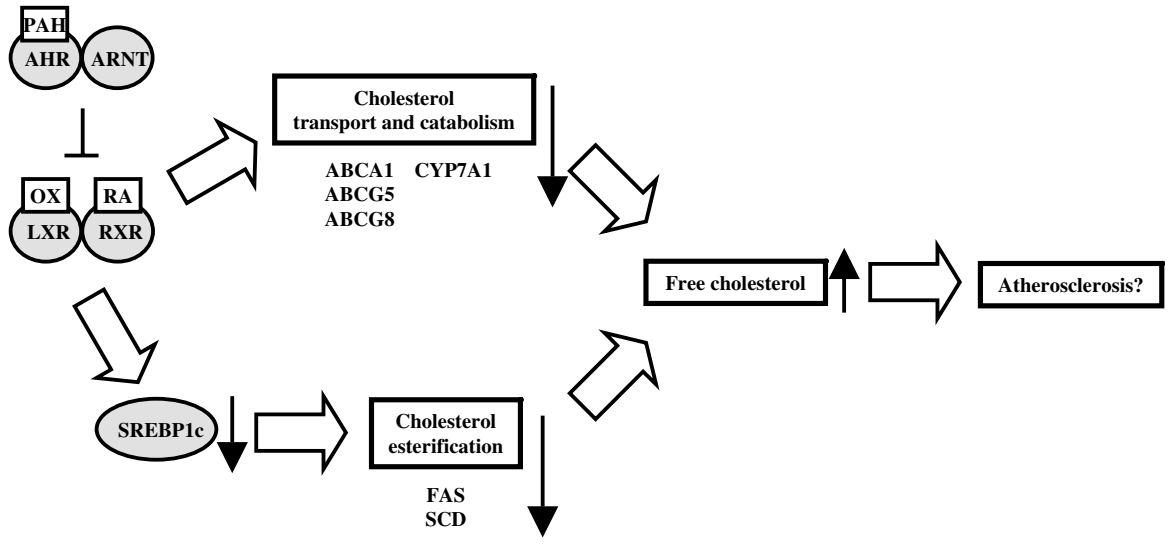

Fig. 5. Proposed molecular mechanism(s) for atherosclerosis induced by PAHs. OX, oxysterols; RA, retinoic acid.

Acknowledgments

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