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CYP1A1-mediated mechanism for atherosclerosis induced by polycyclic aromatic hydrocarbons

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) have been known to induce atherosclerosis. It has been reported that the metabolic activation of PAHs by cytochrome P450 (CYP) is an important step for PAH-induced atherosclerosis. We recently reported that PAHs down-regulated the liver X receptor (LXR) α-regulated genes via aryl hydrocarbon receptor (AHR) as one of the causes responsible for atherosclerosis induced by PAHs. Thus, the aim of this study was to clarify the role of CYP1A1 in the suppression of LXR-mediated signal transductions by 3-methlychoranthrene (MC), one of the PAHs. We found that LXR-mediated transactivation was inhibited by the PAH, but not by halogenated aromatic hydrocarbon, which is scarcely metabolized by CYP1A1. The repression of LXR-mediated signal transductions by MC was restored by co-treatment of HepG2 cells with a CYP1A1 inhibitor, α-naphthoflavone, and by the transfection of short interference RNA for CYP1A1. Based on these lines of evidence, we propose that the metabolic activation of PAHs by CYP1A1, but not the activation of AHR by PAHs, is a direct mechanism for atherosclerosis via the suppression of LXR-mediated signal transductions.

Keywords: MC; Atherosclerosis; Metabolic activation; AHR; siRNA; Quantitative RT-PCR; Luciferase assay

Polycyclic aromatic hydrocarbons (PAHs)[1] are distributed widely and persistently in our environments. PAHs are generated through the combustion of fossil fuels, wood, and other organic materials [1]. Thus, the significant amounts of PAHs are found in automobile exhaust, cigarette smoke, charcoal-broiled foods, and the by-products of industrial waste [2–4]. A variety of toxicities, including carcinogenicity and atherogenesis, are caused by PAHs [5,6]. PAHs are generally known to produce the toxic effects through the activation of AHR [7–9]. However, detailed mechanisms responsible for the toxicities have remained unknown. AHR is a ligand-activated transcription factor that controls genes, including *CYP1A1*, *CYP1A2*, and *CYP1B1* [10].

CYP1A1 is an enzyme known to bioactivate carcinogenic compounds such as B[*a*]P, one of the typical PAHs [11]. The expression of CYP1A1 is induced by PAHs including B[*a*]P and MC via AHR [10,12]. Several reports have suggested that the metabolic activation of PAHs by CYPs, including CYP1A1, is a necessary step for PAH-induced atherosclerosis [5,6].

We recently demonstrated that MC inhibited LXR-mediated signal transductions through AHR, which are known to maintain the cholesterol homeostasis [13]. In the present study, we further investigated the role of CYP1A1 in the suppression of LXR-mediated signals by MC, since MC induces CYP1A1, as a more direct mechanism for

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[1] *Abbreviations:* AH, aryl hydrocarbon; AHR, aryl hydrocarbon receptor; ANF, α-naphthoflavone; B[*a*]P, benzo[*a*]pyrene; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HAH, halogenated aromatic hydrocarbon; LXR, liver X receptor; LXRE, LXR response element; MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; PCB, 3,4,3′,4′-tetrachlorobiphenyl; Res, resveratrol; RT-PCR, reverse transcriptase-polymerase chain reaction; siRNA, short interference RNA; T1317, TO-901317; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TK, thymidine kinase.

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atherogenesis. In this paper, we show evidence indicating that CYP1A1 is a necessary factor for the MC-induced down-regulation of the LXR-target genes, suggesting that the metabolic activation of PAHs by CYP1A1 is a process critical for the repression of LXR-originated signal transductions by PAHs.

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 (BioWhittaker, Walkersville, 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. Constructions of p(LXRE)$_2$-TK-Luc and pU6-siAHR were described previously [13]. Full-length human CYP1A1 cDNA was obtained by PCR with a sense primer, hCYP1A1-*Kpn*I-S (5′-CGGGGTACCGCC ATGCTTTTC-3′), and an antisense primer, hCYP1A1-*Bam*HI-AS (5′-CG GGATCCCTAAGAGCGCAGC-3′). The resultant fragment was digested with *Kpn*I and *Bam*HI, and inserted into the *Kpn*I and *Bam*HI sites of the pcDNA 3.1 mammalian expression vector (pcDNA-hCYP1A1) (Invitrogen, Carlsbad, CA). The pU6-siCYP1A1 as an siRNA expression plasmid for silencing the *CYP1A1* gene was constructed by using the p *Silencer* 1.0-U6 siRNA Expression Vector (Ambion, Austin, TX). To construct a hairpin siRNA expression cassette, two complementary oligonucleotides were synthesized, annealed, and ligated into the blunted *Apa*I site of the p*Silencer*. The sequences of the oligonucleotides were 5′-GGACGTGCTGCAG ATCCGAAttcaagagaTTCGGATCTGCACGTCCCCTTTTT-3′ and its complement, 5′-AAAAAGGGACGTGCTGCAGATCCGAAtctcttgaaT TCGGATCTGCAGCACGTCC-3′. This sequence cassette contained the oligonucleotides encoding 23-mer hairpin sequences specific for the human CYP1A1 mRNA at the 334-356 position, a ttcaagaga loop sequence separating the two complementary domains and a TTTTT terminator at the 3′-end [14–18].

Transient transfection and luciferase assay. One 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 p(LXRE)$_2$-TK-Luc, 100 ng pcDNA-hLXRα, and 50 ng pRL-TK vector (as an internal control for transfection) by using Fugene6 (Roche Diagnostics, Indianapolis, IN). The medium was changed to fresh DMEM containing 1 μM T1317, a LXR ligand (Sigma–Aldrich, St. Louis, MO), AHR ligands including MC (5 μM) (Sigma-Aldrich), B[*a*]P (5 μM) (Sigma–Aldrich), TCDD (10 nM) (AccuStandard, New Heven, CT) or PCB (10 μM) (AccuStandard), Res (10 μM), an AHR antagonist, (Sigma–Aldrich), and ANF (1 μM), a CYP1A1 inhibitor, (Sigma–Aldrich). Cells were harvested after incubation for 36 h. Luciferase activity was measured according to the method provided by the manufacturer. When siRNA expression vector was applied, cells were transfected with 300 ng pU6-siAHR, pU6-siCYP1A1 or pU6-control, 100 ng pcDNA-hLXRα and pcDNA-hCYP1A1, 100 ng p(LXRE)$_2$-TK-Luc, and 50 ng pRL-TK vector. Twenty-four hours later, the medium was changed to DMEM containing MC (5 μM) and T1317 (1 μM). After incubation for 36 h, luciferase activity was measured.

Real-time RT-PCR analysis. HepG2 cells were transfected with 500 ng pU6-siAHR, pU6-siCYP1A1 or pU6-control. Twenty-four h after the transfection, the medium was changed to DMEM containing 10 μM MC and 1 μM T1317. After incubation for 24 h, total RNA from these cells was prepared using a GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich). Reverse transcription reaction was performed by using a First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics). Quantitative real-time PCR was performed as described previously [13].

Results and discussion

To examine whether or not the metabolic activation of PAHs by CYPs was a necessary step for the PAH-induced suppression of LXR-signal transductions, the effects of PAHs and HAHs on LXR-mediated transcriptional activity were investigated by a luciferase reporter assay (Fig. 1). When HepG2 cells were treated with MC and B[*a*]P, which are typical PAHs and readily metabolized by CYP1A1, the luciferase activity seen with p(LXRE)$_2$-TK-Luc decreased to a level of approximately 40–50% of control (Fig. 1). However, the luciferase activity was not affected by the treatment with TCDD and PCB, which are typical HAHs and scarcely metabolized by CYP1A1 (Fig. 1). These results probably support the idea that the metabolic activation of PAHs by CYPs including CYP1A1 is a key step

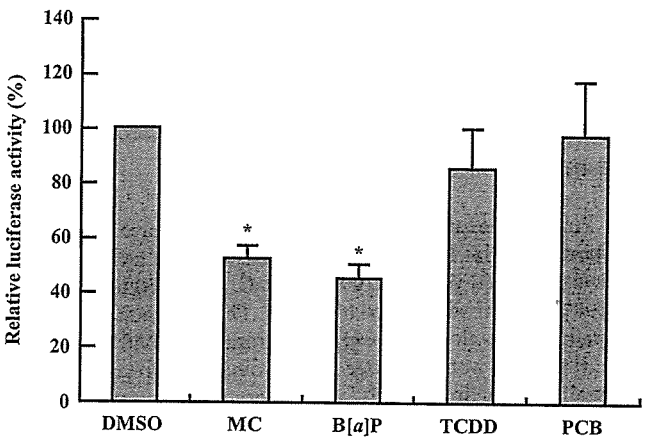

Fig. 1. Inhibition of LXR-mediated transcriptional activity by PAHs, but not by HAHs. HepG2 cells were transfected with p(LXRE)$_2$-TK-Luc and pcDNA-hLXRα in the presence of 1 μM T1317 and 5 μM MC, 5 μM B[*a*]P, 10 nM TCDD or 10 μM PCB. Luciferase activity was measured after incubation for 36 h. The values represent the average ±SD from three independent experiments. *Statistically different ($p < 0.05$) relative to the cells treated with T1317 alone.

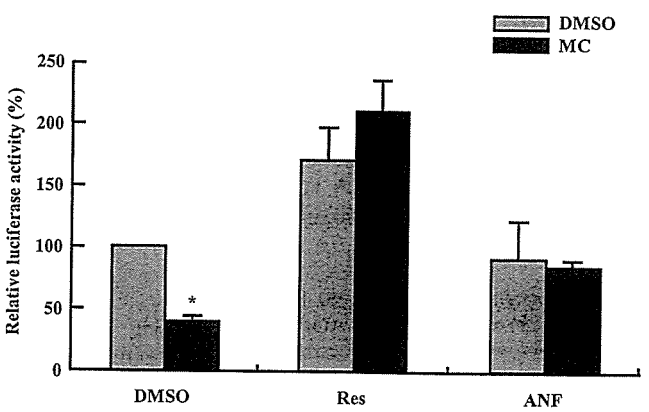

Fig. 2. Effects of Res, an AHR antagonist, and ANF, a CYP1A1 inhibitor, on the MC-induced suppression of LXR transactivation. A luciferase reporter plasmid, p(LXRE)$_2$-TK-Luc, was co-transfected into HepG2 cells with pcDNA-hLXRα. HepG2 cells were treated with Res (10 μM) or ANF (1 μM) together with or without MC (5 μM). The luciferase activity was measured after incubation for 36 h. Values in the figure represent the average ±SD from three independent experiments. *Statistically different ($p < 0.05$) relative to the cells treated with T1317 alone.

for the suppression of LXR-mediated signal transductions by PAHs.

To further support the idea that CYP1A1 was responsible for the repression of LXR-mediated signal transductions by PAHs, the effects of the inhibitor of CYP1A1 on the LXR-mediated transcriptional activity were investigated (Fig. 2). When HepG2 cells were treated with Res, an AHR antagonist [19,20], the MC-induced suppression of luciferase activity with the $p(LXRE)_2$-TK-Luc was restored (Fig. 2). This result was consistent with our previous finding that LXR-mediated signal transduction was suppressed by MC via an AHR-mediated mechanism [13]. Treatment of HepG2 cells with ANF, a CYP1A1 inhibitor, restored the inhibition by MC of LXR transactivation (Fig. 2).

To investigate if CYP1A1 played a direct role in the MC-induced suppression of LXR-mediated signal transductions, we examined the effects of CYP1A1 siRNA on the inhibition by MC of LXR transactivation (Fig. 3). When HepG2 cells were transfected with the pU6-siCYP1A1 and pU6-siAHR, the expression of mRNA for CYP1A1 decreased to a level of approximately 40% of control (Fig. 3A), indicating that both siCYP1A1 and siAHR were able to impair the metabolic activation of MC by CYP1A1. When the pU6-siAHR was transfected into HepG2 cells, the inhibition of LXR transactivation by MC was also restored (Fig. 3B). When the pU6-siCYP1A1 was transfected into HepG2 cells, the MC-induced repression of LXR-mediated transcriptional

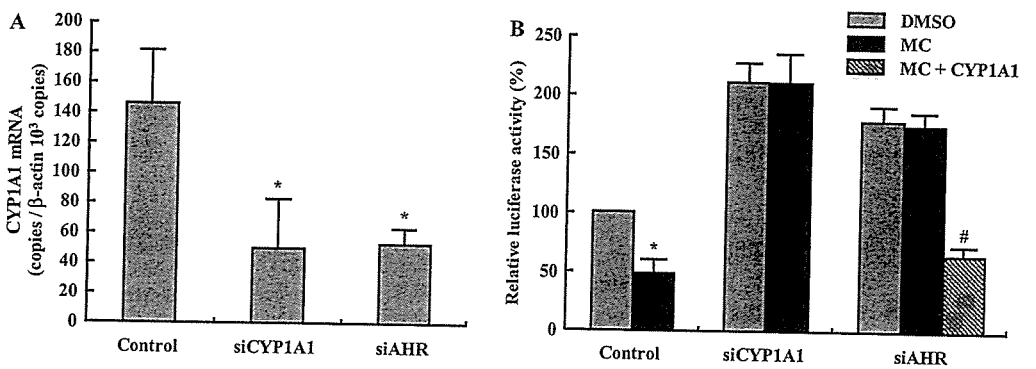

Fig. 3. Effects of siRNA for CYP1A1 on the MC-induced down-regulation of LXRα transactivation. (A) The expression of mRNA for CYP1A1 was quantified by real time RT-PCR. HepG2 cells were transfected with pU6-siAHR, pU6-siCYP1A1 or pU6-control, and then incubated with 5 μM MC. After incubation for 24 h, total RNA was prepared from these cells, and subjected to a real-time RT-PCR. (B) HepG2 cells were transfected with pcDNA-hLXRα, $p(LXRE)_2$-TK-Luc, and pU6-siCYP1A1 or pU6-siAHR. After incubation for 24 h, the cells were treated with T1317 in the presence or absence of MC. The luciferase activity was determined after incubation for 36 h. Values in the figure represent the average ±SD from three independent experiments. *Statistically different ($p < 0.05$) relative to the cells transfected with a control vector in the presence of T1317 alone. #Statistically different ($p < 0.05$) relative to the cells transfected with pU6-siAHR in the presence of T1317 alone.

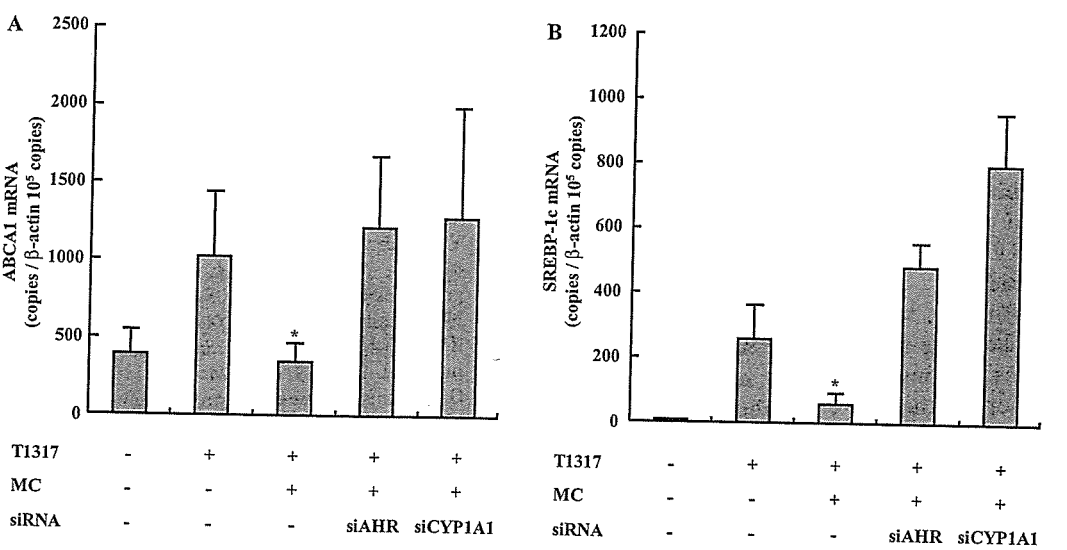

Fig. 4. Effects of siRNA for CYP1A1 on the MC-induced suppression of the expression of mRNAs for LXR-target genes. The expression of the genes regulated by LXR, ABCA1 (A) and SREBP-1c (B) was verified by real-time RT-PCR. HepG2 cells were transfected with pU6-siAHR, pU6-siCYP1A1 or pU6-control and cultured in the presence of 1 μM T1317 and 5 μM MC. After incubation for 24 h, total RNA was prepared from these cells and subjected to a real-time RT-PCR. Values in the figure represent the average ±SD from three independent experiments. *Statistically different ($p < 0.05$) relative to the cells treated with T1317 alone.

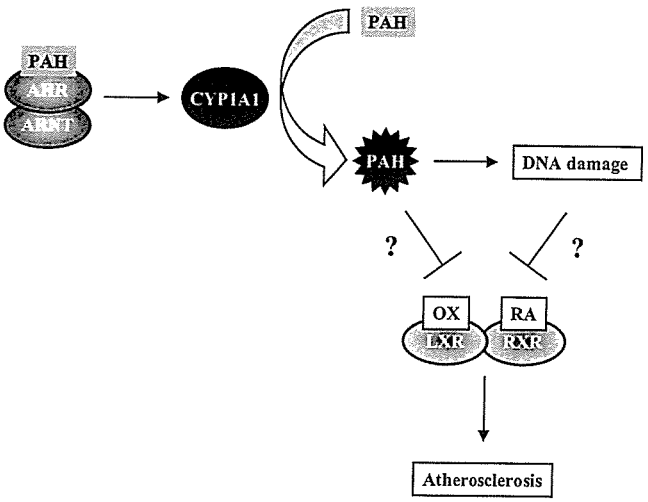

Fig. 5. Proposed mechanism(s) for the PAH-induced suppression of LXR-mediated signal transductions. PAH, polycyclic aromatic hydrocarbon; AHR, aryl hydrocarbon receptor; ARNT, AHR nuclear translocator; OX, oxysterols; RA, retinoic acid; LXR, liver X receptor; RXR, retinoid X receptor.

activity was restored (Fig. 3B). These results indicate that CYP1A1 induced by MC via an AHR-dependent mechanism plays direct roles in the MC-induced suppression of LXR-mediated transcriptional activity. In addition to the above results, we found that LXR-mediated transactivation was suppressed by MC when the pcDNA-hCYP1A1 was co-transfected into HepG2 cells with the pU6-siAHR (Fig. 3B), suggesting that CYP1A1, but not AHR, was a factor directly involved in the MC-induced suppression of LXR-mediated transcriptional activity.

To confirm that CYP1A1 is a critical factor for the MC-induced suppression of the expression of mRNAs for the LXR-target genes, we examined whether siCYP1A1 restored the MC-induced repression. The expression of mRNAs for ABCA1 and SREBP-1c, which are the LXR-targets, was induced by treatment of HepG2 cells with T1317 and was suppressed by co-treatment with MC (Figs. 4A and B). When the pU6-siAHR and pU6-siCYP1A1 were transfected into the cells, the expression levels of ABCA1 and SREBP-1c mRNAs were not suppressed by MC (Figs. 4A and B).

In the present study, we found that CYP1A1, but not AHR, played a key role in the suppression of LXR-mediated signal transductions by MC. It has been reported that the formation of the active metabolites of PAHs in White Carneau pigeons, which are susceptible to atherosclerosis, is greater than that in Show Racer pigeons, which are resistant to atherosclerosis [5]. Paigen et al. [6] reported that AKXL-38a mice, an AH-responsive strain, were more susceptible to MC-induced cancer and atherosclerosis than AKXL-38 mice, an AH-nonresponsive strain. Together with these results, it may be possible to hypothesize the mechanism of atherosclerosis induced by PAHs as follows (Fig. 5). First, PAHs bind to AHR and induce the expression of CYP1A1. Second, PAHs are metabolically activated by CYP1A1 induced by PAHs. Third, the LXR-originated signal transductions are suppressed by the active metabolites of PAHs directly or indirectly. Finally, the expression of the LXR-target genes is suppressed to cause atherosclerosis.

In conclusion, we propose in this paper that CYP1A1, but not AHR, is responsible for the MC-induced suppression of the expression of LXR-target genes, suggesting that the metabolic activation of PAHs by CYP1A1 is a process critical for atherosclerosis induced by PAHs.

Acknowledgments

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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-methlycholanthrene (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

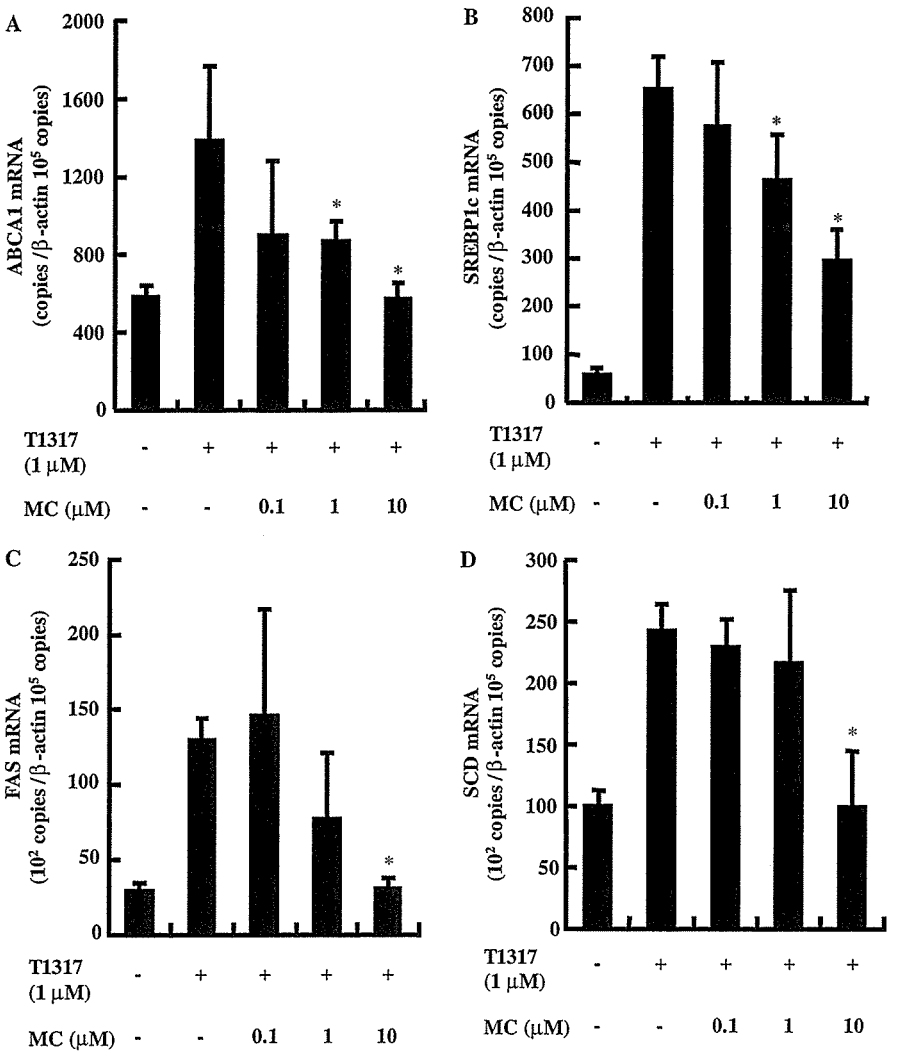

Fig. 1. Dose-dependent repression by MC of the expression of mRNAs for the genes regulated by LXRα. The expression of the genes regulated by LXRα, ABCA1 (A), SREBP-1c (B), FAS (C), and SCD (D) was verified by real-time RT-PCR. Total RNA (1 μg) prepared from HepG2 cells treated with 1 μM T1317, LXR ligand, 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

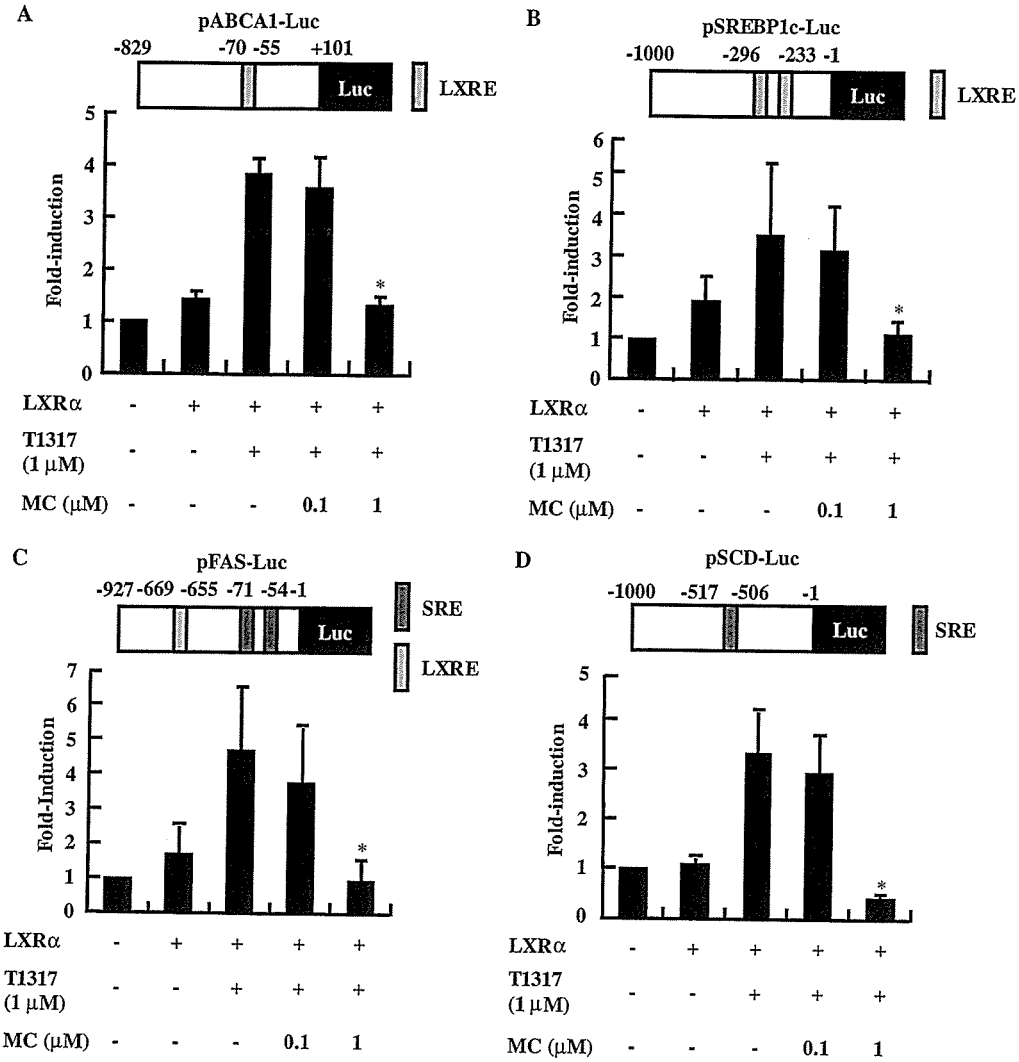

Fig. 2. Suppression by MC of the transcriptional activities of the genes regulated by LXRα. Luciferase reporter plasmids, pABCA1-Luc (A), pSREBP-1c-Luc (B), pFAS-Luc (C), and pSCD-Luc (D), were co-transfected into HepG2 cells with pcDNA-hLXRα. HepG2 cells were treated with T1317 (1 μM) and MC (0.1 and 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

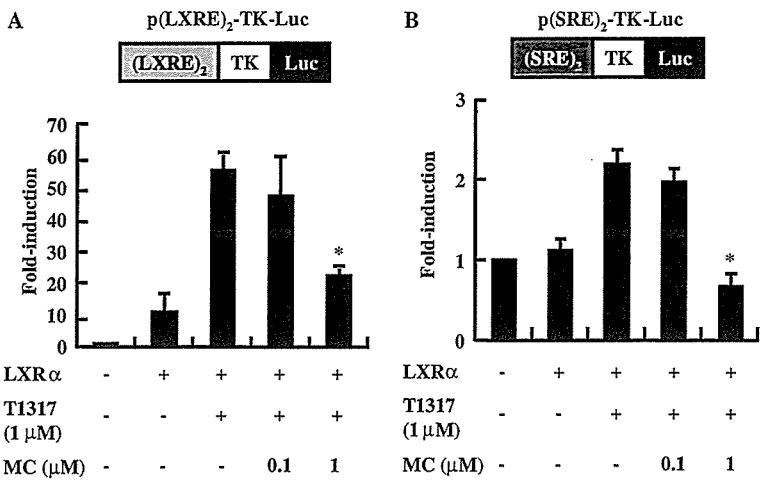

Fig. 3. Suppression by MC of transcriptional activities via LXRα and SREBP-1c. Luciferase reporter plasmids, p(LXRE)$_2$-TK-Luc (A) and p(SRE)$_2$-TK-Luc (B), were co-transfected into HepG2 cells with pcDNA-hLXRα. HepG2 cells were treated with T1317 (1 μM) and MC (0.1 and 1 μM). Luciferase activity was 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.

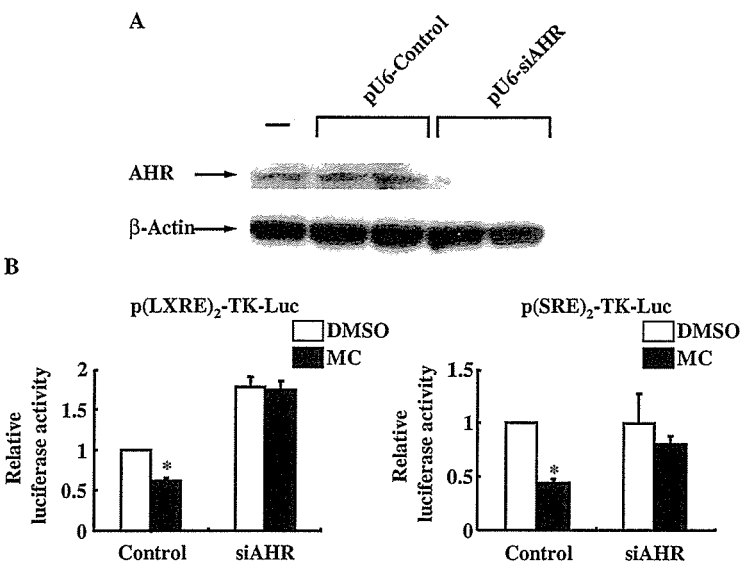

Fig. 4. Effects of siRNA for AHR on the down-regulation by MC of LXRα and SREBP-1c transactivation. (A) HepG2 cells were transfected with 300 ng pU6-control (control vector) or pU6-siAHR vector for AHR gene silencing. After 24 h, whole cell extracts (40 μg) were subjected to SDS–polyacrylamide gel electrophoresis 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.

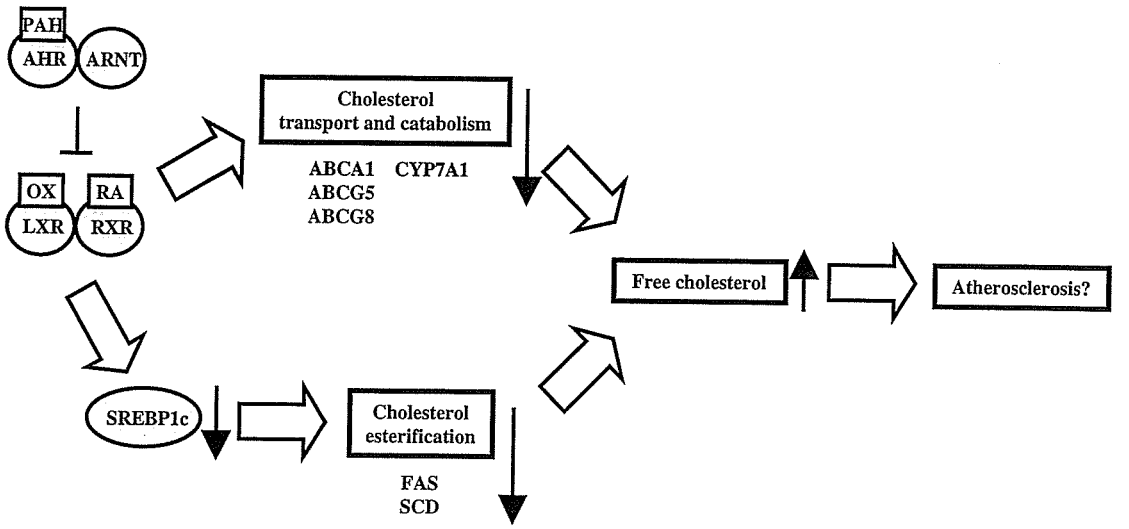

Fig. 5. Proposed molecular mechanism(s) for atherosclerosis induced by PAHs. OX, oxysterols; RA, retinoic acid.

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CYP2A6 IS A PRINCIPAL ENZYME INVOLVED IN HYDROXYLATION OF 1,7-DIMETHYLXANTHINE, A MAIN CAFFEINE METABOLITE, IN HUMANS

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ABSTRACT:

In a caffeine test previously performed with healthy Japanese volunteers, we found that the CYP1A2 index defined as urinary {5-acetylamino-6-amine-3-methyluracil (AAMU) + 1-methylxanthine (1X) + 1-methyluric acid (1U)}/1,7-dimethyluric acid (17U) was affected by the whole deleted allele of *CYP2A6* (*CYP2A6*4*). Since the high value of the CYP1A2 index could be caused by a low urinary concentration of 17U, we postulated that CYP2A6 was responsible for the 1,7-dimethylxanthine (17X) metabolism to generate 17U (17X 8-hydroxylation). Thus, the role of CYP2A6 in the 17X 8-hydroxylation was fully examined in the present study. Among 10 isoforms of human cytochrome P450 (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP3A5) expressed in *Escherichia coli* cells, CYP2A6 and CYP1A2 showed high catalytic activities for the 17X 8-hydroxylation. The 17X 8-hydroxylase activities significantly associated with coumarin 7-hydroxylase activities ($r = 0.67$, $p < 0.01$) in liver microsomes from 17 individuals, but not with ethoxyresorufin *O*-deethylase activities. Tranylcypromine, an inhibitor of CYP2A6, reduced the 17X 8-hydroxylase activities of human liver microsomes. The 17X 8-hydroxylase activities of CYP2A6.7, CYP2A6.10, and CYP2A6.11 expressed in *E. coli* cells were 12, 13, and 22% of that of CYP2A6.1, respectively. The 17X 8-hydroxylase activities were found to be low in liver microsomes from individuals possessing the deletion or mutations in the *CYP2A6* gene. Based on these data, we conclude that CYP2A6 is a main 17X 8-hydroxylase and that the catalytic activities for the 17X 8-hydroxylation are reduced by the genetic polymorphisms of the *CYP2A6* gene.

Caffeine (1,3,7-trimethylxanthine, 137X) is extensively transformed in vivo into a wide variety of metabolites in humans. Since caffeine is a commonly ingested and relatively innocuous compound, the amounts of caffeine metabolites in human urine have been determined as a useful marker to estimate the activities of drug-metabolizing enzymes involved in the caffeine metabolism, including cytochrome P450 1A2 (CYP1A2), *N*-acetyltransferase 2, and xanthine oxidase, simultaneously (Kalow and Tang, 1993).

In our in vivo caffeine test previously performed with healthy Japanese volunteers (Saruwatari et al., 2002), we found that the distribution of the CYP1A2 index defined as urinary {5-acetylamino-6-amine-3-methyluracil (AAMU) + 1-methylxanthine (1X) + 1-methyluric acid (1U)}/1,7-dimethyluric acid (17U) showed a curvilinearity. No relationship could be found between the distribution of the CYP1A2 index and the genetic polymorphisms of the *CYP1A2* gene which altered the inducibility of CYP1A2. However, we found that the whole deleted allele of the *CYP2A6* gene (*CYP2A6*4*) existed in the subjects who showed a high value of the CYP1A2 index (Saruwatari et al., 2002). Since the high value of the apparent CYP1A2 index was caused by a low urinary concentration of 17U, we assumed that CYP2A6 would be responsible for 1,7-dimethylxanthine (17X) 8-hydroxylation to yield 17U as shown in Fig. 1. Thus, we considered that the determination of urinary caffeine metabolites could be of use for the estimation of the in vivo CYP2A6 activity.

CYP2A6 is the principal enzyme involved in the metabolism of nicotine (Nakajima et al., 1996), coumarin (Miles et al., 1990), tegafur (Komatsu et al., 2000), and fadrozole (Pelkonen et al., 2000). Large interindividual variations in the activities of CYP2A6 have been noted in humans (Rautio et al., 1992). This interindividual variation can be explained, at least, in part, by the *CYP2A6* genetic polymorphisms (Inoue et al., 2000). Many variant alleles of the *CYP2A6* gene have been reported to date (http://www.imm.ki.se/CYPalleles/cyp2a6.htm). Our laboratory has found the whole gene deletion-type mutants that cause the lack of enzymatic activity (Nunoya et al., 1999a,b), as well as the single nucleotide polymorphisms (*CYP2A6*7* and *CYP2A6*11*) that reduce the in vivo and in vitro metabolic capacity (Ariyoshi et al., 2001b; Daigo et al., 2002). In addition, *CYP2A6*9*, which contains a −48T to G nucleotide substitution in the TATA box of the 5′-flanking

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ABBREVIATIONS: 137X, 1,3,7-trimethylxanthine (caffeine); P450, cytochrome P450; CPR, NADPH-P450 reductase; 17X, 1,7-dimethylxanthine; 1X, 1-methylxanthine; 17U, 1,7-dimethyluric acid; 1U, 1-methyluric acid; AAMU, 5-acetylamino-6-amine-3-methyluracil; AFMU, 5-acetylamino-6-formylamine-3-methyluracil; PM, poor metabolizer.

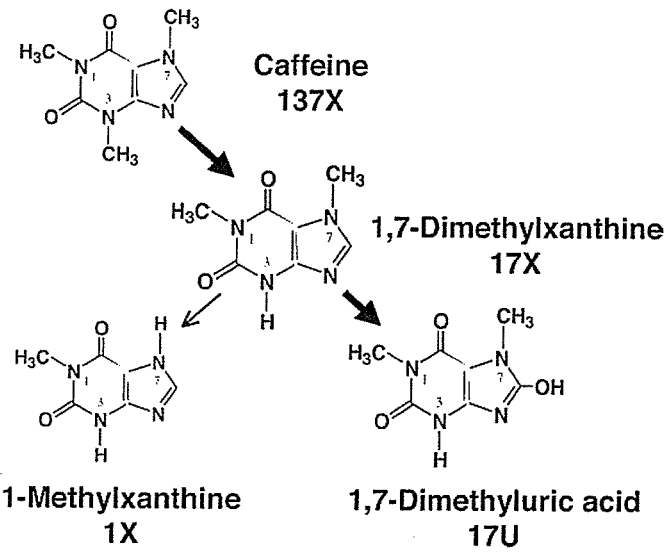

FIG. 1. Metabolic pathways of caffeine and its primary metabolite, 17X, in humans.

region of the *CYP2A6* gene (Pitarque et al., 2001), was reported to reduce the expression levels and the catalytic activities of the CYP2A6 (Kiyotani et al., 2003). Recently, the *CYP2A6*10* allele containing the amino acid substitutions of both Ile471Thr and Arg485Leu was also found (Xu et al., 2002; Yoshida et al., 2002). The subjects possessing the *CYP2A6*10* allele showed a lowered capacity to metabolize nicotine (Xu et al., 2002; Yoshida et al., 2002). Based on these genetic data, individuals can be classified into three groups, i.e., poor metabolizers (PMs), intermediate metabolizers, or extensive metabolizers, according to the genotypes. PMs for CYP2A6 have a reduced metabolic capacity, which can result in higher plasma concentration and the increased risk of adverse effects at ordinary drug dosages, whereas extensive metabolizers do not achieve therapeutic drug levels. In addition, prodrugs such as tegafur need to be metabolically activated by CYP2A6 and are therefore inactive in PMs. Actually, previous reports have shown that the in vivo metabolism of nicotine, coumarin, and tegafur was affected by the genetic polymorphisms of the *CYP2A6* gene (Daigo et al., 2002; Xu et al., 2002). Therefore, if a caffeine test was available for the phenotype analysis of CYP2A6, it would provide clinically important information for individuals. However, it has not been completely clarified as to whether CYP2A6 would be involved in the 17X 8-hydroxylation.

In this study, we investigated the role of CYP2A6 involved in the 17X 8-hydroxylation by using genetically engineered *Escherichia coli* cells expressing P450, together with NADPH-P450 reductase (CPR) and human liver microsomes. We document, herein, that CYP2A6 is a principal 17X 8-hydroxylase as well as that the genetic polymorphism of the *CYP2A6* gene affects the 17X 8-hydroxylation.

Materials and Methods

Chemicals. 4-Acetoamidophenol, 17X, 17U, and 1X (Fig. 1) were obtained from Sigma (St. Louis, MO). $NADP^+$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast Co., Ltd, (Tokyo. Japan). All other chemicals were of the highest quality commercially available.

Enzyme Preparation. Human livers were obtained from patients after pathological examination of specimens isolated after death or during surgery (Nakamura et al., 2002). The use of the human livers for this study was approved by the ethics committee of Hokkaido University. Human liver microsomes were prepared in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% (v/v) glycerol as described previously (Yamazaki et al., 1999). Commercial human liver microsomes were obtained from XenoTech (Reaction Phenotyping Kit Ver. 5; Xenotech, Lenexa, KS).

Expression plasmids carrying each P450 (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP3A5) or a mutant CYP2A6 (CYP2A6.7 or CYP2A6.11) cDNA together with CPR cDNA were constructed as described previously (Iwata et al., 1998; Ariyoshi et al., 2001a,b; Daigo et al., 2002; Yamaori et al., 2003). To introduce an amino acid substitution of CYP2A6.10, G-base was substituted by a T-base at the position of 1454 base pairs in the cDNA encoding CYP2A6.7 by the primer-directed enzymatic amplification method reported previously (Saiki et al., 1988; Ariyoshi et al., 2001b). The primers, 5′-CACtAAACTACACCATGAGCT-3′ and 5′-TAGTTTaGTGGGATCGTGGC-3′, were used to introduce the single nucleotide substitution, which codes for Arg485Leu in exon 9. The entire coding region, including the mutated site, was verified by sequencing with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Each isoform of P450 and CPR in the genetically engineered *E. coli* cells was expressed as described previously (Iwata et al., 1998; Ariyoshi et al., 2001a,b; Daigo et al., 2002; Yamaori et al., 2003). Membrane fraction was prepared from the *E. coli* cells according to the method reported by Sandhu et al. (1994). The membrane fraction was suspended in 100 mM Tris-HCl buffer (pH 7.5) containing 20% (v/v) glycerol and kept at −80°C until use. Each isoform of P450 in membranes had enough catalytic function toward typical substrates.

The contents of P450 in human liver microsomes and the membrane fraction of *E. coli* were determined spectrally by the method of Omura and Sato (1964). The protein contents were determined by using the Pierce BCA Protein Assay Kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard.

Enzyme Assays. The 17X 8-hydroxylase activity as shown in Fig. 1 was determined according to the method described below. The principal 17X concentration of 100 μM was chosen because blood concentrations of the parent compound, caffeine, were approximately 100 μM after intake of a cup of coffee (Campbell et al., 1987). A typical incubation mixture consisted of 100 mM potassium phosphate buffer (pH 7.4), 100 μM 17X, human liver microsomes (0.25 mg protein/ml), or the membrane fraction of *E. coli* expressing each P450 (80 pmol/ml) with cytochrome b_5 (80 pmol/ml), and an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 1 unit/ml glucose phosphate dehydrogenase). Inhibitory effect of tranylcypromine on 17X 8-hydroxylation catalyzed by human liver microsomes was examined at a tranylcypromine concentration of 2 μM. Incubations were carried out at 37°C for 1 h and terminated by dichloromethane/2-propanol (4:1, v/v), and then 4-acetoamidophenol was added as an internal standard. The linearity of product formation for the incubation time was confirmed with recombinant CYP2A6 (for 90 min) and human liver microsomes (for 60 min). The solvent was evaporated after removal of protein by centrifugation. The residue was dissolved in 200 μl of solvent that was used as a mobile phase, 4% (v/v) CH_3CN containing 10 mM CH_3COONa (pH 4.0), and 100 μl of the sample was injected to a high-performance liquid chromatograph (L-7100 pump, L-7200 autosampler, and L-7400 UV detector; Hitachi, Tokyo, Japan) equipped with a Mightysil RP-18 GP Aqua column (150 × 4.6 mm, 5 μm; Kanto Chemical, Tokyo, Japan). Elution was performed at a flow rate of 1.0 ml/min. The formation of 17U was monitored at a wavelength of 280 nm. The detection limit of 17X was <0.5 pmol/ml or <1 pmol of product formation/min/nmol recombinant P450 under the present conditions.

The 17X 8-hydroxylase activity of 9000*g* supernatant (S9) fraction from *E. coli* cells expressing mutant CYP2A6 was also measured by the method described above. Kinetic parameters for the 17X 8-hydroxylation were estimated with a computer program (Microcal Origin; Microcal Software, Northampton, MA), designed for a nonlinear regression analysis.

Genotyping of the *CYP2A6*. Genomic DNA was isolated from the peripheral lymphocytes obtained from 111 healthy Japanese subjects and 42 human livers according to the method of phenol-chloroform extraction followed by ethanol precipitation (Sambrook et al., 1989). Genotyping of the *CYP2A6* gene was carried out by the methods previously developed (Ariyoshi et al., 2000, 2001b; Kiyotani et al., 2003; Fujieda et al., 2004).

Caffeine Test. Apparent CYP1A2 index, defined as (AAMU + 1X + 1U)/17U, was calculated from data obtained from our in vivo caffeine test previously performed (Saruwatari et al., 2002). The sample population in this study comprised 111 CYP2A6-genotyped subjects extracted from 182 unrelated healthy Japanese in the previous caffeine test (Saruwatari et al., 2002). They were 68 males (including 16 smokers) and 43 females (including 2

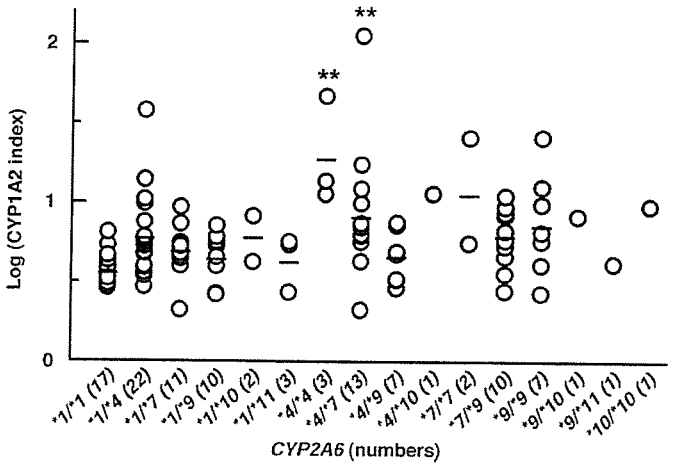

FIG. 2. Relationship between *CYP2A6* genotypes and log-transformed CYP1A2 index (AAMU + 1X + 1U)/17U obtained from 111 healthy Japanese volunteers. *CYP2A6* genotypes were determined with genomic DNA isolated from peripheral blood samples. CYP1A2 index was determined by analyzing the caffeine metabolites in urine collected 8 h after oral administration of 150 mg caffeine. The bar represents the mean value of the group. The significance of the difference for the CYP1A2 index between each genotype carrying variant alleles and *CYP2A6*1/*1* was evaluated by Dunnett's test (**, $p < 0.01$).

smokers). The age of the subjects ranged from 20 to 40 years (mean age ± S.D., 22 ± 2.3 years). The body weight of the subjects ranged from 36 to 108 kg (mean weight ± S.D., 58.2 ± 10.6 kg). The number of cigarettes smoked ranged from 3 to 20 per day per smoker (mean ± S.D., 14 ± 5.6 per day). All of the volunteers provided written informed consent. The associations between the CYP1A2 index and the *CYP2A6* genotypes were assessed by Dunnett's test. A p value <0.05 was considered to be statistically significant.

Results

Relationship between CYP2A6 Genotype and the CYP1A2 Index Obtained from Caffeine Test. Genomic DNA samples from 111 healthy Japanese subjects were analyzed for each *CYP2A6* genotype to investigate whether or not the variant alleles of the *CYP2A6* (*CYP2A6*4*, *CYP2A6*7*, *CYP2A6*9*, *CYP2A6*10* and *CYP2A6*11*) influenced the CYP1A2 index. The association between the *CYP2A6* genotype and the log-transformed CYP1A2 index is shown in Fig. 2. Analyzing the data by Dunnett's test, the apparent CYP1A2 indexes of subjects possessing *CYP2A6*4/*4* and *CYP2A6*4/*7* genotypes were significantly higher than that of subjects carrying *CYP2A6*1/*1* genotype (** $p < 0.01$). Based upon this finding, we assumed that CYP2A6 would play an important role in the 17X 8-hydroxylation.

17X 8-Hydroxylase Activities of P450s Expressed in *E. coli* Membranes. The role of 10 forms of P450 in the 17X 8-hydroxylation was examined by using *E. coli* membranes, each expressing a form of human P450 and CPR (Fig. 3). The 17X 8-hydroxylase activities of CYP1A2 and CYP2A6 were 0.24 and 0.62 nmol/min/nmol P450, respectively, at a 17X concentration of 100 μM. Thus, the 17X 8-hydroxylase activity of CYP2A6 was 2.6 times higher than that of CYP1A2, CYP2C9, CYP2C19, and CYP3A4 were also involved in the 17X 8-hydroxylation. However, these activities were extremely low (<0.012 nmol/min/nmol P450) compared with CYP1A2 or CYP2A6. Representative HPLC chromatograms for the 17X metabolism catalyzed by the CYP2A6 or CYP1A2 expressed in *E. coli* membranes are shown in Fig. 4. Although 1X, a known metabolite of 17X (Kalow and Tang, 1993; Saruwatari et al., 2002), was also seen after the incubation of 17X, 17U was the main metabolite under the present assay condition.

17X 8-Hydroxylase in Human Liver Microsomes. To further clarify the contribution of CYP2A6 and CYP1A2 to the 17X 8-hydroxylation, correlation between 17X 8-hydroxylase activities and enzyme activities for typical substrates of CYP2A6 or CYP1A2 was examined with commercial human liver microsomes. Data of activities of coumarin 7-hydroxylase (CYP2A6), ethoxyresorufin *O*-deethylase (CYP1A2), diclofenac 4′-hydroxylase (CYP2C9), *S*-mephenytoin 4′-hydroxylase (CYP2C19), and testosterone 6β-hydroxylase (CYP3A) of the human liver microsomes were obtained from manufacturer's instructions. The 17X 8-hydroxylase activities significantly correlated with coumarin 7-hydroxylase activities ($r = 0.67$, ** $p < 0.01$; $n = 17$) (Fig. 5B). In contrast, no significant correlation was observed between the 17X 8-hydroxylase activities and ethoxyresorufin *O*-deethylase activities (Fig. 5A). No significant correlation between the 17X 8-hydroxylase activities and coumarin 7-hydroxylase activities was found in the presence of antibodies to CYP2A6 (data not shown). None of the other correlations were seen among 17X 8-hydroxylase and marker activities of CYP2C9, CYP2C19, or CYP3A4 (data not shown).

We examined the inhibitory effects of tranylcypromine, an inhibitor of CYP2A6 (Draper et al., 1997), on the 17X 8-hydroxylation using 16 preparations of human liver microsomes (Fig. 6). Tranylcypromine

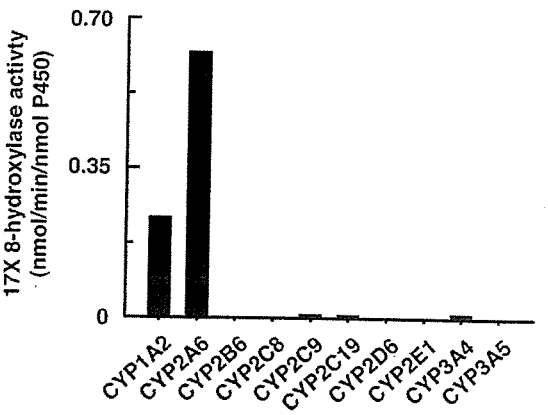

FIG. 3. Activities of 10 isoforms of P450 to metabolize 17X to yield 17U. 17X (100 μM) was incubated at 37°C for 1 h with each P450 (80 pmol/ml) expressed in *E. coli* membranes.

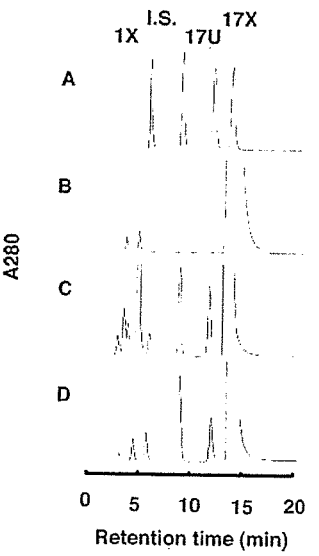

FIG. 4. Representative HPLC chromatograms of 17X metabolites catalyzed by recombinant CYP2A6. A, authentic sample peaks formed from 17X as shown in Fig. 1. B, C, and D, 17X (100 μM) was incubated at 37°C for 1 h with control membranes and CYP2A6 and CYP1A2 (80 pmol/ml), respectively.

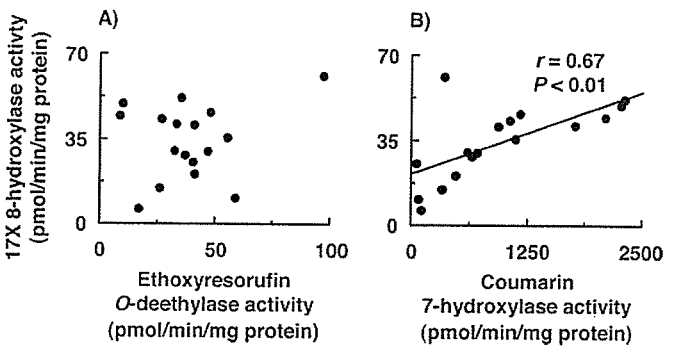

FIG. 5. Correlation between 17X 8-hydroxylase activities and enzyme activities for typical substrates of CYP1A2 or CYP2A6 in human liver microsomes. The 17X 8-hydroxylase activities of 17 human liver microsomes were plotted for ethoxyresorufin *O*-deethylase activities (CYP1A2) (A) or coumarin 7-hydroxylase activities (CYP2A6) (B).

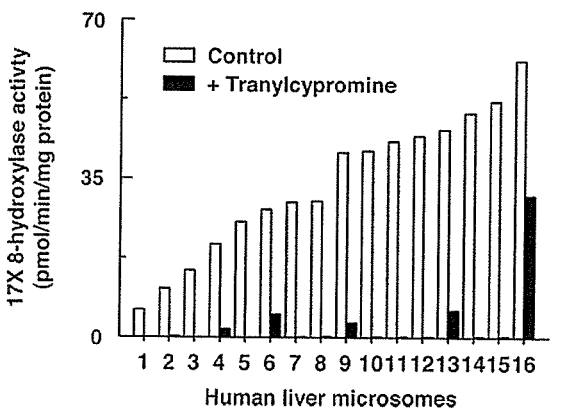

FIG. 6. Inhibitory effects of tranylcypromine on 17X 8-hydroxylation catalyzed by human liver microsomes. The 17X 8-hydroxylase activities of 16 human liver microsomes were determined in the absence (open bars) or presence (closed bars) of tranylcypromine (2 μM).

inhibited more than 80% of the 17X 8-hydroxylase activities of human liver microsomes except for one sample (sample 16).

Association between Genetic Polymorphism of CYP2A6 and 17X 8-Hydroxylase Activities of Human Liver Microsomes. To investigate the effects of the genetic polymorphism of the *CYP2A6* gene on the 17X 8-hydroxylation, we measured the 17X 8-hydroxylase activities of liver microsomes prepared from 42 Japanese subjects previously genotyped for *CYP2A6* (Fig. 7). The 17X 8-hydroxylase activities of liver microsomes from subjects carrying mutant *CYP2A6* genotype were remarkably low. The 17X 8-hydroxylase activities of liver microsomes derived from subjects genotyped as *CYP2A6*4/*4* were not detectable. Liver microsomes prepared from individuals who harbored *CYP2A6*1/*4*, *CYP2A6*1/*9*, and *CYP2A6*4/*9* showed significantly lower 17X 8-hydroxylase activities (** $p < 0.01$). Such lower activities were also seen in liver microsomes from the subjects possessing either the *CYP2A6*7*, *CYP2A6*10*, or *CYP2A6*11* alleles, with no statistical significance by limited sample numbers.

Kinetic Analysis of Drug Oxidations Catalyzed by Mutant CYP2A6 Expressed in S9 Fractions of *E. coli*. Kinetic analysis was performed for the 17X 8-hydroxylation catalyzed by the wild (CYP2A6.1) or mutant CYP2A6s (CYP2A6.7, CYP2A6.10 or CYP2A6.11) (Table 1). *The* V_{max} values of each CYP2A6 protein for coumarin 7-hydroxylation were almost the same in our preliminary study (data not shown), showing that the systems of *E. coli* cells expressing each CYP2A6 protein were catalytically active. The V_{max} and K_m values of CYP2A6.1 for the 17X 8-hydroxylation were

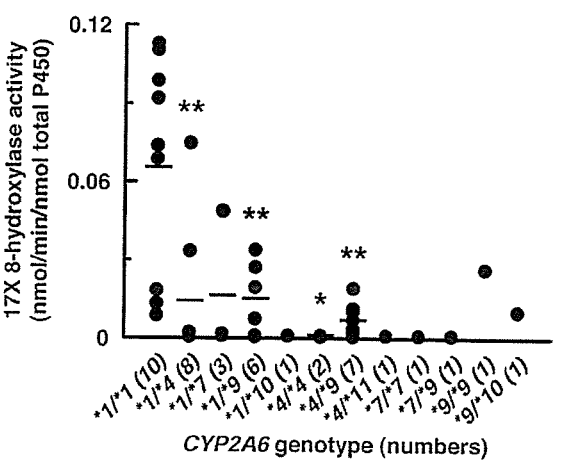

FIG. 7. Association between the genetic polymorphism of the *CYP2A6* and 17X 8-hydroxylase activities of human liver microsomes. The 17X 8-hydroxylase activities were determined in 42 human liver microsomes at 17X concentrations of 100 μM. The bar represents the mean value of the group. The significance of the difference for 17X 8-hydroxylase activities between each of the genotypes carrying *CYP2A6* variant alleles and wild-type *CYP2A6*1/*1* was evaluated by Dunnett's test (**, $p < 0.01$, *, $p < 0.05$).

TABLE 1

Kinetic parameters for 17X 8-hydroxylation catalyzed by recombinant CYP2A6 variants and CYP1A2

17X (200–2000 μM) was incubated with *E. coli* S9 fractions expressing CYP2A6 or CYP1A2. 17X 8-hydroxylase activities were determined as described under *Materials and Methods*. Kinetic parameters (mean ± S.E.) were calculated by nonlinear regression analysis.

P450	K_m	V_{max}	V_{max}/K_m
	μM	*nmol/min/nmol P450*	*μl/min/nmol P450*
CYP2A6.1	940 ± 220	0.19 ± 0.02	0.20 ± 0.05
CYP2A6.7	—	0.022[a]	—
CYP2A6.10	—	0.024[a]	—
CYP2A6.11	—	0.042[a]	—
CYP1A2	1100 ± 240	0.11 ± 0.01	0.10 ± 0.03

—, kinetic analysis could not be performed because there were no detectable activities with low concentrations of 17X.

[a] Assays were performed at a 17X concentration of 2000 μM.

0.19 ± 0.02 nmol/min/nmol CYP2A6 and 940 ± 220 μM, respectively. Kinetic parameters of CYP2A6.7, CYP2A6.10 and CYP2A6.11 for the 17X 8-hydroxylation could not be calculated because the activities of these CYP2A6 variants were not detected even at a substrate concentration of 1000 μM. Therefore, the 17X 8-hydroxylase activities at a 17X concentration of 2000 μM were shown in the table. These velocities of CYP2A6.7, CYP2A6.10, and CYP2A6.11 were 12, 13, and 22% of that of CYP2A6.1, suggesting that the 17X 8-hydroxylase activities were reduced by the genetic polymorphism of the *CYP2A6* gene.

Discussion

Caffeine is extensively converted in vivo to its 1-, 3- or 7-demethylated metabolites in humans (Kalow and Tang, 1993). Among them, the 3-demethylation of caffeine to generate 17X, as shown in Fig. 1, catalyzed by CYP1A2 is the main metabolic pathway of caffeine (Gu et al., 1992; Kalow and Tang, 1993). Since it has been reported that CYP1A2 activities account for approximately 95% of the primary systemic caffeine clearance (Kalow and Tang, 1993), several methods for determining an individual's CYP1A2 activity by using caffeine as a probe have been developed with various urinary metabolite ratios such as (17X + 17U)/137X (Butler et al., 1992), (5-acetylamino-6-formylamine-3-methyluracil (AFMU) + 1X + 1U)/17X (Grant et al.,

1983), (AFMU + 1X + 1U)/17U (Campbell., 1987), and (AAMU + 1X + 1U)/17U. Previous studies with white subjects have validated the (AAMU + 1X + 1U)/17U ratio more precisely in a variety of different conditions, revealing that this ratio was most closely correlated with the CYP1A2 activity of individuals (Kalow and Tang, 1993; Denaro et al., 1996).

When we recently performed the caffeine test and calculated the (AAMU + 1X + 1U)/17U ratio as the CYP1A2 index (Saruwatari et al., 2002), we found that the distribution of the CYP1A2 index showed a curvilinearity. No relationship could be found between the distribution of the CYP1A2 index and the genetic polymorphisms of the *CYP1A2* gene. It has been reported that CYP1A2 was induced by smoking (Pantuck et al., 1974). We also found that Japanese smokers excreted high amounts of 1X in the urine, probably by CYP1A2 induced by smoking (data not shown). The average of the CYP1A2 index of smokers in the caffeine test was significantly higher than that of nonsmokers (* $p < 0.05$, data not shown), although the smoking could not explain the curvilinearity of distribution of the CYP1A2 index completely.

We investigated the effect of CYP2A6 activity on the CYP1A2 index because urinary concentration of the 17U, which was considered to be generated from 17X by CYP2A6 (Gu et al., 1992; Nowell et al., 2002), seemed to be the most important factor to influence the CYP1A2 index. Then, we could find that the genetic polymorphism of the *CYP2A6* gene affected the apparent CYP1A2 index. In contrast to our results, the apparent CYP1A2 index was log-normally distributed in the studies with white subjects and failed to show any evidence of the genetic effects of the *CYP2A6* gene (Kalow and Tang, 1991; Carrillo and Benitez, 1994). This interethnic difference may be caused by different frequencies of the *CYP2A6* alleles between Japanese and white populations. The *CYP2A6*1*, *CYP2A6*4*, *CYP2A6*7*, *CYP2A6*9*, *CYP2A6*10*, and *CYP2A6*11* alleles are popular in a Japanese population: the frequencies of these *CYP2A6* alleles in Japanese are 42.0, 19.8, 12.6, 20.7, 4.3, and 0.7%, respectively (Fujieda et al., 2004). Based on these frequencies, 82.4% of Japanese are expected to possess any of these mutant alleles. Particularly, the frequency of Japanese subjects carrying the *CYP2A6*4/*4* genotype is estimated to be 4.3%. Xu et al. (2002) have compared the frequency of *CYP2A6* genotype between Japanese and white populations. They have reported that the frequencies of *CYP2A6*1/*1* and *CYP2A6*4/*4* were 57.1 and 7.9% in Japanese ($n = 63$), and 94.4 and 0% in whites ($n = 301$) (Xu et al., 2002), respectively, suggesting that the potential for detecting the genetic effects of *CYP2A6* was minimized when the (AAMU + 1X + 1U)/17U ratio was applied to the white population. These conclusions on the roles of polymorphic CYP2A6 in caffeine metabolism in Japanese were consistent with the previous findings, indicating a significantly decreased 17U excretion in Orientals as compared to whites (Grant et al., 1983). Since we used this ratio for the first time in a caffeine test with Japanese subjects, we were able to find the effects of the genetic polymorphisms of the *CYP2A6* gene on the apparent CYP1A2 index.

In general, coumarin and nicotine have been used for the CYP2A6 phenotyping because these substrates, at concentrations used in the phenotyping studies, were metabolized almost exclusively by CYP2A6 (Ujjin et al., 2002; Xu et al., 2002). In the present study, we propose that caffeine is useful as a probe for estimating an individual's CYP2A6 activities because the mutant alleles of *CYP2A6*, such as *CYP2A6*4* and *CYP2A6*7*, are frequently found among the subjects who showed high values of the apparent CYP1A2 index in our previous caffeine test (Fig. 2) (Saruwatari et al., 2002). In addition, caffeine is generally ingested from coffee or tea and is a relatively innocuous compound, whereas coumarin and nicotine are non-innocuous substrates toward CYP2A6. Therefore, the caffeine test is considered to be a novel and safe method to evaluate CYP2A6 phenotype in humans. In our preliminary studies, the ratio of 17U to 1X in spot urine samples under the dietary caffeine intake could be of use for phenotyping of polymorphic CYP2A6 in Japanese nonsmokers.

The roles of P450s in the 17X 8-hydroxylation (Fig. 1) have not been fully investigated except for two reports with limited information (Gu et al., 1992; Nowell et al., 2002). Gu et al. (1992) examined the isoforms of P450 responsible for the 17X 8-hydroxylation by using lysate fractions prepared from HepG2 cells expressing CYP1A2, CYP2A6, CYP2B6, CYP2E1, CYP3A4, or CYP3A5. They found that CYP1A2 and CYP2A6 catalyzed the 17X 8-hydroxylation at the 17X concentration of 1000 μM and that the activity of CYP2A6 was 1.3 times higher than that of CYP1A2 (Gu et al., 1992). In the present study, we also clarified that CYP1A2 and CYP2A6 expressed in *E. coli* membranes were involved in the 17X 8-hydroxylation at the 17X concentration of 100 μM (Fig. 4). In contrast to our result, a recent report of Nowell et al. (2002) has shown that CYP2A6 expressed in lymphoblastoid cells catalyzed the 17X 8-hydroxylation at the 17X concentration of 100 μM, whereas CYP1A2 did not. This inconsistency may be attributable to the different expression systems. When we measured the 17X 8-hydroxylase activities using *E. coli* cells, insect cells or lymphoblastoid cells expressing P450, the 17X 8-hydroxylase activities of CYP1A2 differed from the expression systems, although the activities of CYP2A6 were almost similar to each other (data not shown). CYP2A6 has been considered to be a principal enzyme responsible for the 17X 8-hydroxylation from the study of Gu et al. (1992). However, we cannot totally exclude the possibility that CYP1A2 was also the 17X 8-hydroxylase in humans, because the contents of CYP1A2 protein in human livers were approximately 3-fold higher than those of CYP2A6 (Shimada et al., 1994). Therefore, we used human liver microsomes to clarify the contribution of CYP2A6 to the 17X 8-hydroxylation in the present study.

We revealed that the 17X 8-hydroxylase activities of human liver microsomes significantly correlated with coumarin 7-hydroxylase activities (Fig. 5B), and tranylcypromine inhibited more than 80% of the 17X 8-hydroxylase activities in most of the human liver microsomes. Previous reports have demonstrated that tranylcypromine also inhibits the activity of CYP2C19 other than CYP2A6 (Inaba et al., 1985). However, the 17X 8-hydroxylase activity of CYP2C19 expressed in *E. coli* membranes was extremely low compared with CYP2A6 (Fig. 3). No significant correlation was found between the 17X 8-hydroxylase activities and *S*-mephenytoin 4′-hydroxylase (CYP2C19) activities in human liver microsomes (data not shown). Therefore, the inhibitory effects of tranylcypromine on the 17X 8-hydroxylase activities were due to the inhibition of CYP2A6 activities in human liver microsomes. Exceptionally, one liver microsomal sample (sample 16) showed a residual activity of 56% in the presence of tranylcypromine. This result might be attributable to the involvement of CYP1A2 in a part of the 17X 8-hydroxylation, because this sample showed a high activity of CYP1A2 and a low activity of CYP2A6 in the correlation analysis (Fig. 5). The 17X 8-hydroxylase activities of liver microsomes from subjects carrying mutant *CYP2A6* genotype were remarkably low (Fig. 7). Particularly, the 17X 8-hydroxylase activities of liver microsomes derived from subjects genotyped as *CYP2A6*4/*4* were not detected. Based on these data, we considered that CYP2A6 was the principal enzyme responsible for the 17X 8-hydroxylation in human livers. Nevertheless, we cannot exclude the contribution of CYP1A2 to this reaction, but it was predicted to be small.

In conclusion, we demonstrate that CYP2A6 is the principal 17X 8-hydroxylase in human livers and the genetic polymorphism of the *CYP2A6* gene has a substantial influence on the 17X 8-hydroxylation

shown in Fig. 1 in vitro and in vivo. It is proposed that caffeine test may be a useful tool for the CYP2A6 phenotyping.

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Mechanisms of chemopreventive effects of 8-methoxypsoralen against 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced mouse lung adenomas

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Recently we reported that the occurrence of lung adenoma caused by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was completely prevented by pretreatment of female A/J mice with 8-methoxypsoralen, a potent inhibitor of cytochrome P450 (P450 or CYP) 2A [Takeuchi *et al.* (2003) *Cancer Res.*, 63, 7581–7583]. Thus, the aim of this study was to confirm that 8-methoxypsoralen exhibits chemopreventive effects by inhibiting CYP2A in the mouse lung. The involvement of CYP2A in the metabolic activation of NNK in the lung was first evidenced by the fact that the mutagenic activation of NNK by mouse lung microsomes was inhibited by 8-methoxypsoralen, coumarin and antibodies to rat CYP2A1. Supporting this, the mutagenic activation of NNK was efficiently catalyzed by mouse CYP2A4 and CYP2A5 co-expressed with NADPH-P450 reductase in a genetically engineered *Salmonella typhimurium* YG7108. The expression of mRNA for CYP2A5, but not for CYP2A4 or CYP2A12, in the mouse lung was proven by reverse transcriptase–polymerase chain reaction, probably indicating that CYP2A5 present in the mouse lung was involved in the metabolic activation of NNK. In accordance with these *in vitro* data, treatment of *gpt delta* transgenic mice with 8-methoxypsoralen prior to NNK completely inhibited the mutation of the *gpt delta* gene. The *in vivo* chemopreventive effects of 8-methoxypsoralen towards NNK-induced adenoma was seen only when the agent was given to female A/J mice prior to, but not posterior to, NNK, lending support to the idea that NNK is activated by CYP2A5 in the mouse lung as an initial step to cause adenoma. The inhibition by 8-methoxypsoralen of NNK-induced adenoma was seen in a dose-dependent manner: the dose to show apparent 50% suppression was calculated to be 1.0 mg/kg. To our surprise, CYP2A protein(s) was expressed in the lesion of NNK-induced lung adenomas, probably suggesting that 8-methoxypsoralen could inhibit the possible occurrence of further mutation of the adenoma cells induced by NNK. Based on these lines of evidence, we propose that 8-methoxypsoralen inhibits the CYP2A5-mediated metabolic activation of NNK in the mouse lung, leading to the prevention of NNK-induced adenoma.

Introduction

Tobacco smoke contains >4000 components; at least 60 chemicals including nitrosamines are proven to be carcinogenic. Among them, NNK, a tobacco-specific *N*-nitrosamine, is believed to be one of the most promising candidates of lung carcinogen in humans, since NNK is known to induce lung tumors in laboratory animals such as mice, rats and hamsters (1–4). NNK is known to be metabolically activated to elicit their genotoxicity (1,5). The first activation step of NNK is thought to be the methylene or methyl hydroxylation of the carbon atom located at the α-position of the *N*-nitroso group primary mediated by P450, leading to the formation of electrophiles which can methylate or pyridyloxobutylate DNA, respectively (1). This initial event has been reported to result in the formation of O^6-methylguanine, an adduct that leads to GC→AT transitional mispairing and the subsequent activation of the K-*ras* proto-oncogene (6,7), an initiating event in tumor development.

P450 is a heme-containing enzyme responsible for the oxidation of a wide variety of exogenous compounds such as drugs, environmental pollutants and foodstuffs, and endogenous compounds including steroids, fatty acids and prostaglandins (8–10). Some of the oxidative reactions catalyzed by P450 result in the formation of reactive intermediates, which bind to endogenous macromolecules such as DNA, RNA and proteins to induce organ toxicities (11,12). Thus, the catalytic property and the content of P450 can be considered to be the determinants of the formation of reactive metabolites and the subsequent toxicity of chemicals.

CYP2A6 is one of the major members of P450 expressed in human livers (13) and to lesser amounts in the lung (14,15). This cytochrome is involved in the mutagenic activation of a wide variety of promutagens including NNK (5,16,17). The genetic polymorphism of *CYP2A6* was originally discovered as one of the causes of inter-individual differences in the metabolism of coumarin (18,19). In further study by us, we first found the novel deletion-type mutants of the *CYP2A6* gene (*CYP2A6*4A* and **4B*). Following this, we also found single nucleotide polymorphisms (CYP2A6**7* and **11*), reducing CYP2A6 enzymatic activities in a Japanese population (20–23). Combining these two concepts that CYP2A6 is capable of activating NNK and that there are genetic polymorphisms in *CYP2A6*, we hypothesized that subjects harboring these

Abbreviations: ABC, avidin-biotin complex; CPR, NADPH-cytochrome P450 reductase (EC 1.6.2.4, NADPH:ferrihemoprotein reductase); CYP, individual forms of cytochrome P450 (EC 1.14.14.1); ENU, ethylnitrosourea; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; P450, general term for cytochrome P450; PCR, polymerase chain reaction; RT, reverse transcriptase.