Cigarette smoke extract induces CYP2B6 through constitutive androstane receptor in hepatocytes

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Running title: CYP2B6 induction by smoking through CAR.

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Results and Discussion: 595 words Abbreviations: CYP, cytochrome P450; CSE, cigarette smoke extract; hCAR, human constitutive androstane receptor; RT-PCR, reverse transcription polymerase chain reaction
Abstract

Smoking induces a wide range of drug-metabolizing enzymes. Among them, CYP2B6 as well as CYP1A2 is well known to be upregulated in smokers. Though the induction of CYP1A2 is mediated by arylhydrocarbon receptor, the molecular mechanisms of CYP2B6 induction by smoking remain to be fully elucidated. In this study, by preparing cigarette smoke extract (CSE), we addressed the possibility that human constitutive androstane receptor (hCAR) is involved in smoking-mediated induction of CYP2B6. In HepG2 cells, CSE induced CYP1A2 but not CYP2B6, suggesting that CYP2B6 expression is differentially regulated from CYP1A2. Compared with liver in vivo, hCAR expression is dramatically reduced in cultured hepatocytes, such as HepG2. Therefore, to reconstitute hCAR signaling pathways in vitro, we generated adenovirus vector expressing hCAR (Ad-CAR). Real time RT-PCR analyses revealed that the adenoviral transfection of hCAR resulted in the upregulation of CYP2B6 mRNA even in the absence of CSE. Interestingly, CSE stimulation augmented hCAR-mediated induction of CYP2B6. In contrast, the expression of CYP2B6 was not enhanced by adenovirus vector expressing β-galactosidase, a control vector, either in the presence or absence of CSE. In summary, hCAR mediated the CYP2B6 induction by CSE in Hep2G cells. These data suggest that smoking upregulates CYP2B6 through hCAR in vivo.
Introduction

In addition to CYP2A6, CYP2B6 is one of major nicotine-metabolizing enzymes (Yamazaki et al., 1999). Much attention has been paid to the regulation of CYP2B6 activity, because nicotine-metabolizing activity is positively associated with nicotine dependence and the understanding of nicotine dependence contributes to the promotion of smoking cessation (Ray et al., 2009). So far, several CYP2B6 genetic polymorphic mutations, which affect CYP2B6 enzyme activity, have been identified; however, the association of CYP2B6 genotypes with nicotine dependence is still controversial (Lee et al., 2007; Ring et al., 2007). Interestingly, CYP2B6 expression is upregulated in the smokers (Miksys et al., 2003), suggesting that cigarette smoke induces CYP2B6. Therefore, the understanding of molecular mechanisms for CYP2B6 induction by smoking would provide a novel insight into nicotine dependence.

Smoking induces drug-metabolizing enzymes, including CYP1A2 and 2B6, and influences pharmacokinetic profiles of wide ranges of drugs (Kroon, 2007). It has been established that cigarette smoke activates arylhydrocarbon receptor (AhR) and upregulates CYP1A2 (Shimada et al., 2002), resulting in the increased clearance of CYP1A2 substrates, such as theophylline. In contrast to CYP1A2, the involvement of AhR in CYP2B6 expression has not been proved. Previously, it has been demonstrated that CYP2B6 gene expression is regulated by human pregnane X receptor (hPXR) and human constitutive androstane receptor (hCAR) (Wang and Negishi, 2003). CYP2B6 gene is the target of hCAR and the direct binding sites for hCAR have been identified in the CYP2B6 gene promoter region (Swales et al., 2004); however, it remains to be elucidated whether hCAR is involved in CYP2B6 induction by cigarette smoke.
In this study, we have designed the experiments to address the effects of tobacco smoke on CYP2B6 expression in vitro. In analysis on the molecular mechanisms of the expression of drug metabolizing enzymes in vitro, the difficulty is derived from the phenotypic changes during the cell cultivation. In particular, it is well-known that hCAR expression is downregulated immediately after hepatocyte cultivation from liver (Pascussi et al., 2000). Therefore, hCAR signal is likely to be impaired in cultured hepatocytes. In this context, we have generated the adenovirus vectors expressing hCAR (Ad-hCAR). By transducing hCAR gene, we reconstituted hCAR signaling systems in cultured hepatocytes and analyzed the effects of cigarette smoke extract (CSE) on CYP2B6 induction in HepG2 cells. And we have revealed that hCAR expression is required for the CYP2B6 induction by CSE in HepG2 cells, but not for that of CYP1A2. These data suggest that smoking induces CYP2B6 through CAR signaling pathway.
Materials and Methods

**CSE preparation.** CSE was prepared according as described previously (Su et al., 1998), with minor modifications. Briefly, commercial cigarettes (Seven Stars, JT group, Japan) were smoked continuously through phosphate buffered saline (PBS) by the vacuum aspirator. Mainstream smoke from 10 cigarettes was drown through 20 ml of PBS, which was designated as 100 % CSE. For each experiment, this solution was diluted with PBS to the indicated concentrations.

**Generation of the adenovirus vector expressing hCAR.** The construction of the adenovirus vector was performed according to the previous report (Becker et al., 1994). Briefly, full length of human CAR cDNA was amplified by RT-PCR using human liver cDNA as the template. The sequence of the primers was as follows;

forward, GGGGAATTCATGGCCAGTAGGGAAGA TGAGCTGAGGAACTGTGTGGTA TGTGG: reverse, GGGAAGCTTTCAGCTGCAG.A TCTCCTGGAGCAGCGGCA TCATGGCAG. The PCR product was digested with EcoR1 and Hind3 and ligated into the multi-cloning site of pCCMVpLpA vector. The plasmid pACCMVpLpA-hCAR was co-transfected with pJM19 into HEK293 cells for homologous recombination.

**Cell Culture.** HepG2 cells were maintained in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FCS).

In adenoviral infection, cells were cultured with adenovirus vectors at the indicated multiplicity of infection (MOI) overnight. Culture media were changed to EMEM with 10% FCS without adenovirus
particles. Twenty-four hours later, cells were stimulated with the media containing CSE.

**Real Time RT-PCR analysis.** Real time RT-PCR was performed as described previously (Mohri et al., 2009). Total RNA (2 μg) was subjected for first strand cDNA synthesis with oligo (dT) primer. The expression of each template was quantified by real time RT-PCR with SYBR green system (Applied Biosystems). As an internal control, the expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was estimated with SYBR green system. The primer pairs used in this study were demonstrated in Supplementary Table.

**Immunoblot analysis.** Cell lysates were prepared by directly extracting proteins with SDS-PAGE sample solution. Proteins were separated with SDS-PAGE and transferred onto PVDF membrane. After blocked with 5% skim milk, the membrane was incubated with anti-hCAR (Santa Cruz Biotechnology). ECL system was used for detection.
Results and Discussion

In this study, we have introduced CSE into the analysis on the expression of drug-metabolizing enzymes. CSE has been used mainly to address the effects of cigarette smoke on tissue damage, such as endothelial dysfunction (Hoshino et al., 2005). To our knowledge, this is the first application of CSE to the analysis on the influences of cigarette smoke on drug-metabolizing enzymes. Therefore, first, we examined the effects of CSE on CYP1A2 expression in cultured hepatocytes (Fig. 1A), because CYP1A2 is well known to be induced by smoking through the activation of AhR. CYP1A2 mRNA was remarkably increased within 3 hours after CSE stimulation, indicating that CSE could be a good tool available for the study of drug metabolism. In contrast to CYP1A2, CYP2B6 mRNA was not induced by CSE in HepG2 cells (data not shown).

Since hCAR is one of transcriptional factors responsible for CYP2B6 expression, the expression of hCAR in HepG2 was examined. Real-time RT-PCR estimated the expression level of hCAR in HepG2 cells is less than 1% of that in human liver samples (Fig. 1B), consistent with the previous report that hCAR is rapidly downregulated after cultured hepatocyte preparation from liver (Pascussi et al., 2000). Therefore, to reconstitute hCAR signaling pathway in HepG2 cells, Ad-hCAR was generated (Fig. 2A). To confirm the expression of hCAR protein, HepG2 cells were infected with Ad-hCAR or Ad-βgal, a control vector. Cell lysates were prepared and immunoblotted with anti-hCAR antibody (Fig. 2B). The band with MW 40 kDa was detected in Ad-hCAR-infected HepG2 cells, as reported previously (Arnold et al., 2004), while not in Ad-βgal-infected cells.
To examine whether hCAR mediates CYP2B6 induction in response to CSE, HepG2 cells were infected with Ad-hCAR or Ad-βgal at the indicated MOI and stimulated with CSE (Fig. 3). At high MOI, adenoviral transfer of hCAR cDNA upregulated CYP2B6 mRNA even in the absence of CSE, as reported previously. Importantly, CYP2B6 expression was dramatically enhanced by CSE in Ad-hCAR- infected HepG2 cells, but not in HepG2 cells infected with Ad-βgal, a control vector. By real time RT-PCR, we have confirmed that CSE increased the expression of CYP2B6 mRNA in HepG2 cells expressing hCAR in a concentration-dependent manner (Supplementary Figure 1). Of note, the damages of the HepG2 cells expressing hCAR were observed after the stimulation with 8-10% CSE. Therefore, 7% CSE was used to stimulate the cells for this study. The most important finding of this study is that CSE enhanced CYP2B6 expression in cultured hepatocytes expressing hCAR, indicating that CSE-dependent expression of CYP2B6 is mediated by hCAR. Since the CSE increased CYP1A2 expression, either in Ad-hCAR- or Ad-βgal-infected HepG2 cells (data not shown), CSE differentially regulates the gene transcription of CYP2B6 from that of CYP1A2.

This is the first demonstration that cigarette smoke contains the activator of hCAR transcription factor and regulates the transcription of drug-metabolizing enzymes. Though smoking influences the expression for drug-metabolizing enzymes, it has been difficult to address its regulatory mechanisms of the enzyme expression without the identification of the components that activate the transcription factors for the enzymes. Here, we have successfully addressed the effects of cigarette smoke on the expression for CYP2B6 by using CSE and Ad-CAR. The in vitro system used in this study might be a promising
approach for smoking-drug interaction.

In conclusion, we have established a novel method that is available for the molecular analysis on the induction of drug-metabolizing enzymes by smoking. And, by using this system, we have revealed that cigarette smoke induces CYP2B6 through hCAR in HepG2 cells, proposing the molecular mechanisms for CYP2B6 induction by smoking \textit{in vivo}.
Authorship Contributions

Participated in research design: Y. Fujio and J. Azuma.

Conducted experiments: M. Yoshida, J. Azuma, and Y. Fujio.

Contributed new reagents or analytic tools: S. Nonen, M. Maeda and Y. Fujio.

Performed data analysis: I Washio, C. Sugiura, R. Shiga, M. Maeda and Y Fujio.

Wrote or contributed to the writing of the manuscript: I. Washio, M. Maeda and Y. Fujio.

Other: M. Maeda, S. Nonen, Y. Fujio, and J. Azuma acquired funding for the research.
References


Footnote

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Figure legends

Figure 1.  (A) CSE upregulated the expression of CYP1A2 in HepG2 cells.  (B) The expression of hCAR mRNA in HepG2 cells and human liver.

(A) HepG2 cells were culture with 7% CSE for the indicated time.  The expression of CYP1A2 was analyzed by real-time RT-PCR.  The expression of the drug metabolizing enzymes was normalized with that of GAPDH.  Data are shown as mean ± S.D. from 4 samples.  **, $p<0.01$ versus 0 hr. *, $p<0.05$ versus 0 hr.  Statistical analyses were performed by one-way ANOVA followed by post hoc multiple comparisons with Tukey-Kramer test.  (B) Total RNA was prepared from HepG2 cells, primary hepatocytes, and human liver samples.  The expression of hCAR mRNA was estimated by real time RT-PCR.  The expression level of hCAR was normalized by that in HepG2 cells.

Figure 2.  Generation of adenoviral vector expressing hCAR.

(A) Construction of adenovirus vector expressing hCAR (Ad-hCAR).  (B) HepG2 cells were infected with Ad-hCAR at the indicated MOI.  Cell lysates were prepared and immunoblotted with anti-hCAR antibody (Santa Cruz Biotechnology).

Figure 3.  CSE enhanced CYP2B6 expression in HepG2 cells infected with Ad-hCAR, but not in those with Ad-β-gal.  HepG2 cells were infected with Ad-hCAR and Ad-β-gal at the indicated MOI.  Cells were stimulated with 7% CSE for 24 hours.  The expression of CYP2B6 (A) was analyzed by real
time RT-PCR. Data are shown as mean ± S.D. from 3 independent sets of samples. *, $p < 0.05$ versus CSE(-), analyzed by paired $t$ test.
Figure 1

A

CYP1A2 / GAPDH (arbitrary units)

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B

hCAR / GAPDH (arbitrary units)

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Figure 2

**Supplementary Figure 1**

![Graph showing CYP2B6/GAPDH ratio with concentration of CSE (%) on the x-axis and ratio on the y-axis. The bars show a significant increase in CYP2B6/GAPDH with increasing concentrations of CSE.](image-url)
Drug Metabolism and Disposition (DMD #34504)


Supplementary Figure legends.

Supplementary Figure 1. CSE upregulated CYP2B6 in HepG2 cells expressing hCAR in a concentration-dependent manner. HepG2 cells were infected with Ad-hCAR at MOI 100 and cultured in the media containing 7% (vol/vol) PBS including the indicated final concentrations of CSE, for 24 hours. The expression of CYP2B6 was analyzed by real time RT-PCR. Data are shown as mean ± S.D. from three samples.

Supplementary Table 1. Primers used in real time RT-PCR analyses.

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