Short Communication

Cigarette Smoke Extract Induces CYP2B6 through Constitutive Androstane Receptor in Hepatocytes

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ABSTRACT:
Smoking induces a wide range of drug-metabolizing enzymes. Among them, CYP2B6 as well as CYP1A2 is well known to be up-regulated in smokers. Although the induction of CYP1A2 is mediated by the aryl hydrocarbon receptor, the 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. Real-time reverse transcription-polymerase chain reaction analyses revealed that the adrenoviral transfection of hCAR resulted in the up-regulation of CYP2B6 mRNA, even in the absence of CSE. It is interesting to note that 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 HepG2 cells. These data suggest that smoking up-regulates CYP2B6 through hCAR in vivo.

Introduction

In addition to CYP2A6, CYP2B6 is one of the 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 genetic polymorphic mutations that 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). It is interesting to note that CYP2B6 expression is up-regulated in 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 CYP2B6, and influences the pharmacokinetic profiles of a wide range of drugs (Kroon, 2007). It has been established that cigarette smoke activates aryl hydrocarbon receptor (AhR) and up-regulates 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. It has been demonstrated previously that CYP2B6 gene expression is regulated by human pregnane X receptor 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, whether hCAR is involved in CYP2B6 induction by cigarette smoke remains to be elucidated.

In this study, we have designed 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 down-regulated 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 vector expressing hCAR (Ad-hCAR). By transducing the 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. In addition, 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 the CAR signaling pathway.

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; hCAR, human constitutive androstane receptor; Ad-hCAR, adenovirus vector expressing hCAR; CSE, cigarette smoke extract; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; HEK, human embryonic kidney; MOI, multiplicity of infection; Ad-βgal, adenovirus vector expressing β-galactosidase.
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 address 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 h after CSE stimulation, indicating that CSE could be a good tool for the study of drug metabolism. In contrast to CYP1A2, CYP2B6 mRNA was not induced by CSE in HepG2 cells (data not shown).

Because hCAR is one of the transcriptional factors responsible for CYP2B6 expression, the expression of hCAR in HepG2 was examined. Real-time RT-PCR estimated that 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 down-regulated after cultured hepatocyte preparation from liver (Pascuzzi et al., 2000). Therefore, to reconstitute the 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 adenovirus vector expressing β-galactosidase (Ad-βgal), a control vector. Cell lysates were prepared and immunoblotted with anti-hCAR antibody (Fig. 2B). The band with a molecular mass of 40 kDa was detected in Ad-hCAR-infected HepG2 cells, as reported previously (Arnold et al., 2004), although not in Ad-βgal-infected cells.

Materials and Methods

CSE Preparation. CSE was prepared according to a method described previously (Su et al., 1998), with minor modifications. In brief, commercial cigarettes (Seven Stars; JT Group, Tokyo, Japan) were smoked continuously through phosphate-buffered saline (PBS) by the vacuum aspirator. Mainstream smoke from 10 cigarettes was drawn through 20 ml of PBS by application of a vacuum aspirator to the vessel containing the 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 a method described previously (Becker et al., 1994). In brief, full-length human CAR cDNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using human liver cDNA as the template. The sequences of the primers were TATGTGG (forward) and GGGAAGCTTTCAGCTGCAGATCTCCTGGGGGAATTCATGGCCAGTAGGGAAGATGAGCTGAGGAACTGTGTGG (reverse). The PCR product was digested with EcoR1 and Hind3 and ligated into the multicloning site of pCCMVpLpA vector. The plasmid pACCMVpLpA-hCAR was cotransfected with pJM19 into HEK293 cells for homologous recombination.

Cell Culture. HepG2 cells were maintained in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum.

In adenoviral infection, cells were cultured overnight with adenovirus vectors at the indicated multiplicity of infection (MOI). Cell culture media were changed to Eagle’s minimum essential medium with 10% fetal bovine serum (Applied Biosystems, Foster City, CA). As an internal control, the expression of glyceraldehydes-3-phosphate dehydrogenase mRNA was estimated with a SYBR Green system. The primer pairs used in this study were demonstrated in the supplemental table.

Immunoblot Analysis. Cell lysates were prepared by directly extracting proteins with an SDS-polyacrylamide gel electrophoresis sample solution. Proteins were separated with SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene membrane. After being blocked with 5% skim milk, the membrane was incubated with anti-hCAR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). An enhanced chemiluminescence system (ECL, Plus Western Blotting Detection Reagents, GE Healthcare Japan, Co., Ltd., Tokyo, Japan) was used for detection.

Real-Time RT-PCR Analysis. Real-time RT-PCR was performed as described previously (Mohri et al., 2009). Total RNA (2 μg) was subjected to first-strand cDNA synthesis with oligo(dT) primer. The expression of each template was quantified by real-time RT-PCR with a SYBR Green system (Applied Biosystems, Foster City, CA).

Fig. 1. A, CSE up-regulated the expression of CYP1A2 in HepG2 cells. HepG2 cells were cultured 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 glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Data are shown as means ± S.D. from 4 samples. **, p < 0.01 versus 0 h; *, p < 0.05 versus 0 h. Statistical analyses were performed by one-way analysis of variance followed by post hoc multiple comparisons with the Tukey-Kramer test. B, the expression of hCAR mRNA in HepG2 cells and human liver. 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.

Fig. 2. Generation of adenoviral vector expressing hCAR. A, construction of adenovirus vector expressing hCAR (Ad-hCAR). CMV, cytomegalovirus. 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, Inc.).
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 a high MOI, adenoviral transfer of hCAR cDNA up-regulated CYP2B6 mRNA, even in the absence of CSE, as reported previously. It is important to note that CYP2B6 expression was dramatically enhanced by CSE in Ad-hCAR-infected HepG2 cells, but not HepG2 cells infected with Ad-βgal, a control vector. We used real-time RT-PCR to confirm that CSE increased the expression of CYP2B6 mRNA in HepG2 cells expressing hCAR in a concentration-dependent manner (Supplemental Fig. 1). It is interesting to note that the damage in HepG2 cells expressing hCAR was observed after stimulation with 8 to 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. Because 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. Although 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. In this study, 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 molecular analysis of the induction of drug-metabolizing enzymes by smoking. In addition, 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 in vivo.

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