High Levels of Dioxin-Like Potential in Cigarette Smoke Evidenced by *In vitro* and *In vivo* Biosensing

Ayumi Kasai,[1] Nobuhiko Hiramatsu,[1] Kunihiro Hayakawa,[1] Jian Yao,[1] Shuichiro Maeda,[2] and Masanori Kitamura[1]

Departments of [1]Molecular Signaling and [2]Biochemistry, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan

Abstract

Cigarette smoke contains low levels of agonists for the aryl hydrocarbon receptor (AhR; also called the dioxin receptor). However, little is understood about the whole potential of cigarette smoke for activating AhR. In this report, we evaluated the total "dioxin-like" activity of cigarette smoke using *in vitro* and *in vivo* reporter systems. Cigarette smoke extract (CSE) was prepared from seven cigarette brands (1-20 mg tar content) and subjected to *in vitro* bioassay based on the xenobiotic-responsive element (XRE) as the sensor and secreted alkaline phosphatase (SEAP) as the reporter. Exposure of reporter cells to CSE triggered activation of XRE in a dose-dependent manner, which was suppressed by functional inhibition of AhR. Direct, brief exposure of the cells to cigarette smoke similarly induced activation of XRE. Using 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) as the standard, the XRE-activating potential (XAP) of individual smoke was evaluated quantitatively. Positive correlation was observed between the tar content and XAP values. The XAP values estimated were extremely high with a range from 18.5 to 51.2 ng 2,3,7,8-TCDD equivalent per cigarette. To further estimate XAP of cigarette smoke *in vivo*, we generated transgenic reporter mice that secrete SEAP under the control of XRE. After exposure of the mice to smoke, serum levels of SEAP were significantly elevated within 12 hours, peaked at 24 hours, and declined thereafter. These results evidenced for the first time that cigarette smoke has unexpectedly high dioxin-like potential that triggers the AhR-XRE pathway *in vitro* and *in vivo*. (Cancer Res 2006; 66(14): 7143-50)

Introduction

Dioxins and dioxin-like compounds are causative of a wide range of pathologies, including carcinogenesis (1, 2). Most of these toxic effects are mediated by the aryl hydrocarbon receptor (AhR; also called the dioxin receptor), a ligand-activated transcription factor (3) that binds to the xenobiotic-responsive element [XRE; also called dioxin-responsive element (DRE)]. Constitutive activation of AhR via genetic manipulation causes malignant diseases and immune abnormality in mice even without exposure to xenobiotic ligands (4, 5). Because of this current knowledge, the toxicity of dioxins and dioxin-like chemicals has been evaluated by their potential for activation of AhR.

Requests for reprints: Masanori Kitamura, Department of Molecular Signaling, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Shimokato 1110, Chuo, Yamanashi 409-3898, Japan. Phone: 81-55-273-8054; Fax: 81-55-273-8054; E-mail: masanori@yamanashi.ac.jp.

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Using gas chromatography-mass spectrometric analyses, previous investigation showed that cigarette smoke contained low levels of dioxins, dioxin-like compounds, and other agonists of AhR. Those chemicals include polychlorinated dibenzo-*p*-dioxins (PCDD), polychlorinated dibenzofurans (PCDF), coplanar polychlorinated biphenyls (Co-PCB), and benzo(*a*)pyrene (6, 7). However, the whole "dioxin-like" potential of cigarette smoke for activating the AhR-XRE pathway has not been elucidated before. It is currently unclear whether or not the total level of dioxin-like activity in cigarette smoke is within permissible ranges. Quantitative analysis of individual known ligands for AhR is not useful for this purpose because cigarette smoke contains >4,800 chemicals whose actions on AhR are largely unknown (8).

As described, toxic effects of dioxins and dioxin-like compounds are mediated by AhR (9). First, those chemicals pass through the cell membrane and bind to the cytosolic AhR. The AhR-ligand complexes then translocate into the nucleus, heterodimerize with the AhR nuclear translocator, and bind to XRE, leading to transcriptional induction of dioxin-responsive genes such as *CYP1A1* (Fig. 1*A, top*). Using this molecular basis, several reporter systems have been developed by combination of XRE with reporter genes, including *luciferase, β-galactosidase*, and *enhanced green fluorescent protein* (10, 11). Recently, we reported a fast, sensitive, and economic bioassay DRESSA [DRE-based sensing via secreted alkaline phosphatase (SEAP)] that can detect and quantify dioxins and dioxin-like chemicals (12–14). In this approach, a murine hepatoma cell line was stably transfected with a SEAP gene under the control of the XRE consensus sequences or the truncated *CYP1A1* promoter that contains XRE. The established sensor clones secreted SEAP following stimulation with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) in dose-dependent and time-dependent manners (Fig. 1*A, top*). Secretion of SEAP was also induced by other activators of AhR [3-methylcholanthrene, benzo(*a*)pyrene, and β-naphthoflavone] but not by activators of other transcription factors (12). In the present investigation, we aimed at evaluating the total dioxin-like potential of cigarette smoke using the *in vitro* DRESSA assays. To further investigate *in vivo* potential of cigarette smoke for activation of the AhR-XRE pathway, we further generated transgenic mice that produce SEAP under the control of XRE. Our current results provide first evidence that cigarette smoke has extremely high levels of dioxin-like potential that triggers the AhR-XRE signaling pathway *in vitro* and *in vivo*.

Materials and Methods

Reagents. 2,3,7,8-TCDD was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and α-naphthoflavone was from Sigma-Aldrich Japan (Tokyo, Japan). These compounds were dissolved in DMSO and used for studies.

Preparation of cigarette smoke extract. Five major cigarette brands in Japan (tar content: 1, 6, 10, 14, and 20 mg) and two major brands in the

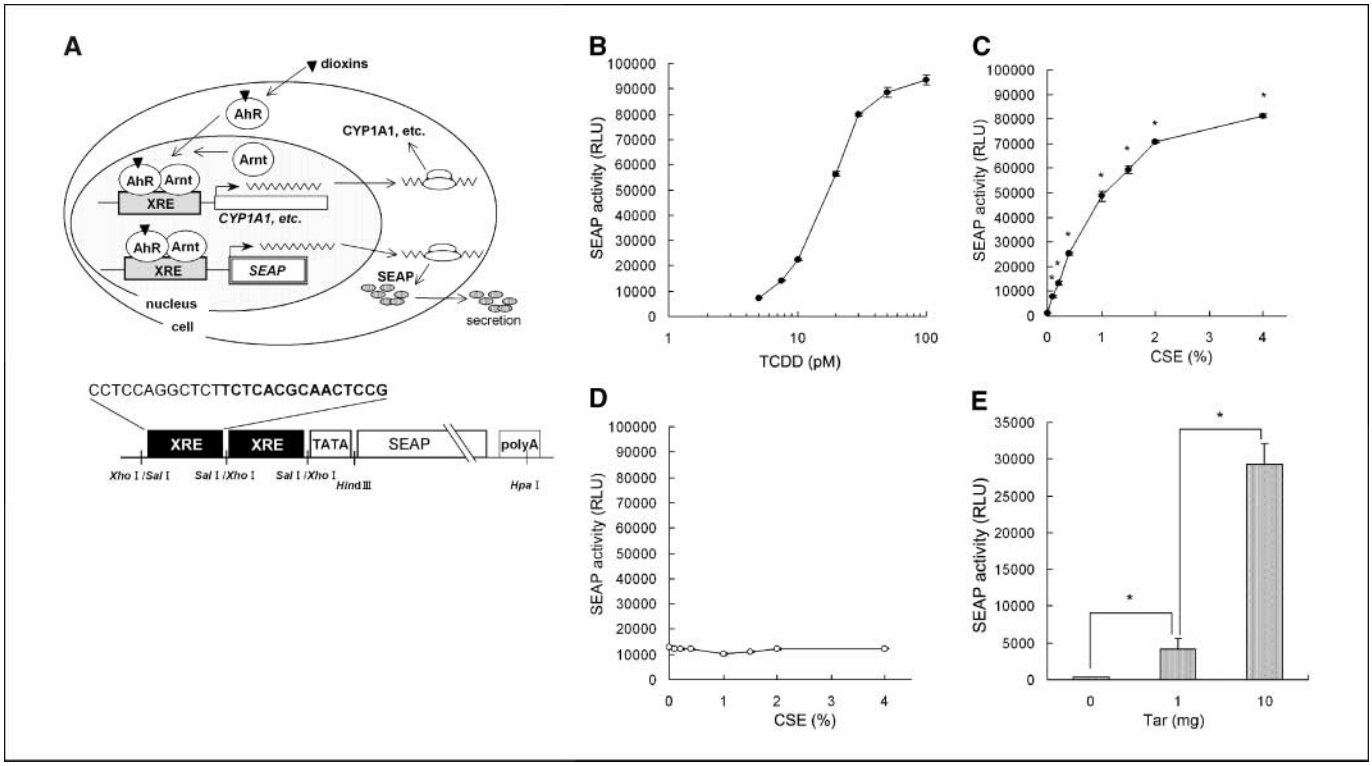

Figure 1. Activation of XRE by cigarette smoke. *A,* schematic illustrations of the AhR-XRE signaling pathway and the structure of pXRE-SEAP. Dioxins pass through the cell membrane and bind to the cytosolic AhR. The AhR-ligand complexes translocate into the nucleus, heterodimerize with the AhR nuclear translocator (Arnt), and bind to XRE, leading to transcriptional induction of dioxin-responsive genes, such as *CYP1A1*. In reporter cells stably transfected with a SEAP gene under the control of the XRE consensus sequences, secretion of SEAP is induced in response to dioxins in a dose-dependent manner. pXRE-SEAP contains two copies of the XRE consensus sequence (TCTCACGCAACTCCG) and downstream cDNA encoding SEAP (*bottom*). *B,* responses of dioxin-responsive reporter cells HeXS34 to 2,3,7,8-TCDD. HeXS34 cells stably transfected with pXRE-SEAP were treated with 2,3,7,8-TCDD at indicated concentrations for 16 hours, and activity of SEAP in culture medium was evaluated by chemiluminescent assay. *C* and *D,* responses to CSE of HeXS34 cells and HeSS8 control cells that constitutively secrete SEAP. HeXS34 cells (*C*) and HeSS8 cells (*D*) were treated with CSE prepared from a 10-mg-tar cigarette at indicated concentrations, and activity of SEAP was evaluated. *E,* responses of HeXS34 cells to cigarette smoke. HeXS34 cells without medium were directly exposed to cigarette smoke (prepared from 1-mg-tar and 10-mg-tar cigarettes) as described in Materials and Methods. After incubation for 24 hours, activity of SEAP was evaluated. All assays were done in quadruplicate. *Points/columns,* means; *bars,* SE. *, $P < 0.05$, statistically significant differences.

United States (tar content: 9 mg) were used for studies. Following the method of International Organization for Standardization, mainstream smoke was prepared from individual cigarettes using constant vacuum flow (10.5 L/min) and collected in a glass bottle (volume: 2 L) containing PBS. The smoke from one cigarette was dissolved in 100 mL PBS with vigorous shaking for 5 minutes, and cigarette smoke extract (CSE) was prepared. These original, undiluted samples were regarded as 100% CSE. Four lots of CSE were independently prepared for each cigarette brand and stored at −20°C until use. The CSE prepared from 10-mg-tar cigarette was generally used for *in vitro* experiments.

Stable transfectants. HeXS34 (Hepa-1c1c7–derived, pXRE-SEAP–transfected clone no. 34) cells were established by transfection of Hepa-1c1c7 cells with pXRE-SEAP that introduces a SEAP gene under the control of two copies of the XRE consensus sequence (14). HeDS49 (Hepa-1c1c7–derived, pDRE-SEAP–transfected clone no. 49) cells were established by transfection of Hepa-1c1c7 cells with pDRE-SEAP that introduces a SEAP gene under the control of the truncated CYP1A1 promoter containing four copies of XRE (12). HeSS8 (Hepa-1c1c7–derived, pSV40-SEAP–transfected clone no. 8) cells were also established by transfection of Hepa-1c1c7 cells with pSV40-SEAP (Clontech, Palo Alto, CA) that introduces a SEAP gene under the control of the SV40 promoter. This clone constitutively secreting SEAP was used as a control. HeXS-AhRDN9 cells were established by cotransfection of HeXS34 cells with pBS-hygro coding for the hygromycin resistance gene (15) and pEFBOS-AhR(Arg39Ile) encoding a dominant-negative mutant of AhR under the control of the elongation factor-1α promoter (a gift from Dr. Kazuhiro Sogawa; ref. 16). All cells were maintained in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS).

Transient transfection. HeXS34 cells (7,500/200 μL/well) seeded in 96-well plates were incubated overnight and transfected with small interfering RNA (siRNA) for AhR (5 pmol/well; Santa Cruz Biotechnology, Santa Cruz, CA) or control siRNA (5 pmol/well; Santa Cruz Biotechnology) using LipofectAMINE 2000 (Invitrogen). After incubation for 5 hours, the media were replaced with fresh media and incubated for 20 hours. The cells were then exposed to 1% CSE or 25 pmol/L TCDD for 24 hours. In another set of experiments, Hepa-1c1c7 cells were transiently transfected with pEFBOS or pEFBOS-AhR(Arg39Ile) together with pXRE-SEAP at 2:1 ratio and exposed to 1% CSE for 24 hours. Efficient expression of AhR(Arg39Ile) was confirmed by Northern blot analysis, as described before (17).

Exposure of reporter cells to CSE. Reporter cells (5,000/100 μL/well) were seeded in 96-well plates and exposed to 0.1% to 4% CSE for 16 hours. After the exposure, culture media and cells were subjected to chemiluminescent assay to evaluate SEAP activity and to formazan assay to assess cell viability, as described later. α-MEM containing 1% FBS was generally used for experiments. To examine the role of AhR in the activation of XRE, HeXS34 cells were pretreated with or without 1 μmol/L α-naphthoflavone (AhR antagonist) for 30 minutes and exposed to CSE.

Calculation of the XRE-activating potential. To quantify the XRE-activating potential (XAP) of cigarette smoke, 2,3,7,8-TCDD was used as the standard. Reporter cells were treated with serial concentrations of 2,3,7,8-TCDD in parallel with diluted CSE, and XAP values of individual cigarette smoke (total XRE activating potential in 100 mL CSE) were estimated as ng 2,3,7,8-TCDD equivalent (TEQ)/cigarette using the standard curve of 2,3,7,8-TCDD.

Direct exposure of reporter cells to cigarette smoke. Reporter cells (20,000/2 mL/plate) were seeded in 35-mm plates, incubated overnight, and directly exposed to mainstream smoke, as follows. Mainstream smoke prepared from 1-mg-tar and 10-mg-tar cigarettes (45 mL per 2 seconds) was collected in a plastic syringe, and the syringe was connected with a funnel (8 cm in diameter) that covers a 35-mm culture plate from which culture medium was removed. The cells were then exposed to smoke (45 mL) for 2 seconds. After the exposure, the cells were immediately fed with 2 mL α-MEM containing 1% FBS, incubated for 16 hours, and subjected to SEAP assay.

Generation of transgenic reporter mice. The 3.6-kb *Mlu*I-*Sal*I fragment of pDRE-SEAP (12) was microinjected as a transgene into the pronuclei of fertilized oocytes of C57BL/6 mice, and reporter mice were generated. To examine *in vivo* responses of the mice to AhR agonists, mice (20-25 g body weight; six male mice) were orally given with 2,3,7,8-TCDD (5 μg/kg body weight in corn oil) using feeding needles, and after 1 week, serum levels of SEAP were evaluated.

Exposure of transgenic reporter mice to cigarette smoke. Two protocols were used for exposure of reporter mice to cigarette smoke. (*a*) Mice (four male mice) were individually put in 500-mL plastic bottles laid on their sides. Mainstream smoke (45 mL) prepared from 14-mg-tar cigarettes were injected into the bottles at intervals of 15 minutes. During the exposure, mouths of the bottles (3 cm in diameter, covered with nylon mesh) were kept open for mice to breathe fresh air, which also allowed for gradual dilution of smoke in the bottles. This experimental situation mimics "passive smoking." Control transgenic mice (four male mice) were similarly kept in bottles without exposure to smoke. After the experiments, the mice were returned to conventional cages, and blood sampling (20 μL) was done from the tail vein periodically up to day 6 to evaluate the level of serum SEAP activity. (*b*) Mice (four male mice) were individually put in 50-mL conical tubes laid on their sides. Heads of mice were toward the bottoms where 12 air holes (2.5 mm in diameter) were prepared. The tube was closed with a cap where single hole was prepared for injection of cigarette smoke. In the tube, a mouse was exposed to 35 mL of mainstream smoke for 10 seconds, and then the smoke was quickly washed out thrice with 35 mL of fresh air. This exposure was repeated every 1 minute until one cigarette burned out (usually six times). This experimental setting mimics "active smoking." Using this protocol, individual mice were subjected to smoking of eight cigarettes at intervals of 15 minutes. Control transgenic mice (four male mice) were similarly kept in tubes and exposed to fresh air, instead of smoke. Blood sampling was done before and 24 hours after the exposure.

SEAP assay. Activity of SEAP in culture media and sera was evaluated by a chemiluminescent method using Great EscAPe SEAP detection kit (BD Biosciences, Palo Alto, CA), as described before (12, 13).

Formazan assay. The number of viable cells was assessed by a formazan assay using Cell Counting kit-8 (Dojindo Laboratory, Kumamoto, Japan). In brief, after collecting culture media for chemiluminescent assays, cells in 96-well plates were incubated at 37°C for 2 hours in medium containing 10% Cell Counting kit-8 assay solution. Absorbance (450 nm) of formazan generated from WST-8 was measured by Spectea Max 340 (Nihon Molecular Devices, Tokyo, Japan).

Statistical analysis. All assays were done in quadruplicate. Data were expressed as means ± SE. Statistical analysis was done using the nonparametric Mann-Whitney *U* test to compare data in different groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Activation of XRE by cigarette smoke. Reporter cells HeXS34 were established by transfection of Hepa-1c1c7 cells with pXRE-SEAP that introduces a SEAP gene under the control of two copies of the XRE consensus sequence (Fig. 1*A*). We first examined a dose-dependent response of this clone to 2,3,7,8-TCDD with a range from 5 to 100 pmol/L. In an unstimulated control condition, the level of SEAP in culture medium was 1,175 ± 37 relative light unit (RLU; mean ± SE). This level was significantly increased in response to 2,3,7,8-TCDD, and a linear, dose-dependent response was observed at concentrations between 7.5 and 30 pmol/L (Fig. 1*B*). Using HeXS34 cells, we tested effects of cigarette smoke on the activity of XRE. CSE was prepared from 10-mg-tar cigarettes, as described in Materials and Methods. The reporter cells were exposed to 0.1% to 4% CSE for 16 hours, and culture media were subjected to SEAP assay. Significant increase in the level of SEAP was observed even at the lowest concentration (0.1%, 6.7-fold, $P < 0.05$). The activation of XRE was concentration dependent up to 2% (Fig. 1*C*). At concentrations higher than 10%, CSE induced cellular damage (data not shown). To confirm specificity of the response, HeSS8 cells that constitutively secrete SEAP under the control of the SV40 promoter were exposed to the same concentrations of CSE and subjected to SEAP assay. In contrast to HeXS34 cells, the level of SEAP was constant and not significantly affected by CSE in HeSS8 cells (Fig. 1*D*).

To examine whether the activation of XRE by cigarette smoke occurs in more physiologically relevant conditions, HeXS34 cells were exposed to cigarette smoke directly. The cells without culture medium in 35-mm plates were briefly and directly exposed to 45 mL of mainstream smoke from 1-mg-tar or 10-mg-tar cigarette. After the exposure, the cells were immediately fed with medium, incubated for 16 hours, and subjected to SEAP assay. Consistent with the result using CSE, the direct exposure to smoke also induced activation of XRE (Fig. 1*E*). The activation of XRE by 10-mg-tar cigarette smoke (29,290 ± 2,862 RLU) was significantly stronger than that by 1-mg-tar cigarette smoke (4,179 ± 1,476 RLU; $P < 0.05$).

Role of AhR in the activation of XRE by cigarette smoke. Activation of XRE by a number of xenobiotic ligands, including dioxins, is mediated by AhR. We examined involvement of AhR in the activation of XRE by cigarette smoke. For this purpose, α-naphthoflavone, a partial antagonist of AhR, was used (12, 14). HeXS34 cells were pretreated with or without α-naphthoflavone (1 μmol/L) for 30 minutes and stimulated by 1% to 2% CSE prepared from 10-mg-tar cigarettes. As a positive control, 2,3,7,8-TCDD (10-50 pmol/L) that activates XRE via AhR was used. In HeXS34 cells, 2,3,7,8-TCDD induced activation of XRE in a dose-dependent manner, and this induction was abrogated by the treatment with α-naphthoflavone (Fig. 2*A, left*). CSE (1-2%) similarly induced activation of XRE comparable with that by 25 to 50 pmol/L 2,3,7,8-TCDD, and this induction was also attenuated by α-naphthoflavone (Fig. 2*A, right*). In contrast to the 2,3,7,8-TCDD-induced activation, however, CSE-triggered activation of XRE was not completely inhibited by α-naphthoflavone; that is, the inhibition was 67.8% (1% CSE) to 51.1% (2% CSE) in CSE-treated cells versus 99.4% (10 pmol/L TCDD) to 96.0% (50 pmol/L TCDD) in 2,3,7,8-TCDD-treated cells.

There are two possibilities to explain this observation. First, cigarette smoke may activate XRE, in part, independent of AhR. Second, agonistic actions of some substances in cigarette smoke may not be antagonized by α-naphthoflavone due to their differences in the interaction with AhR. Alternatively, α-naphthoflavone is a partial antagonist of AhR with weak agonistic activity (Fig. 2*A*), which could be enhanced by CSE. We therefore used different approaches to further examine roles of AhR [i.e., use of reporter cells constitutively expressing a dominant-negative mutant of AhR (AhRDN) and cells transiently transfected with AhRDN or AhR siRNA]. First, HeXS34 cells and HeXS34 cells stably expressing AhRDN (HeXS-AhRDN9) were exposed to 0.5% to 2% CSE or 10 to 25 pmol/L 2,3,7,8-TCDD, and activity of SEAP was

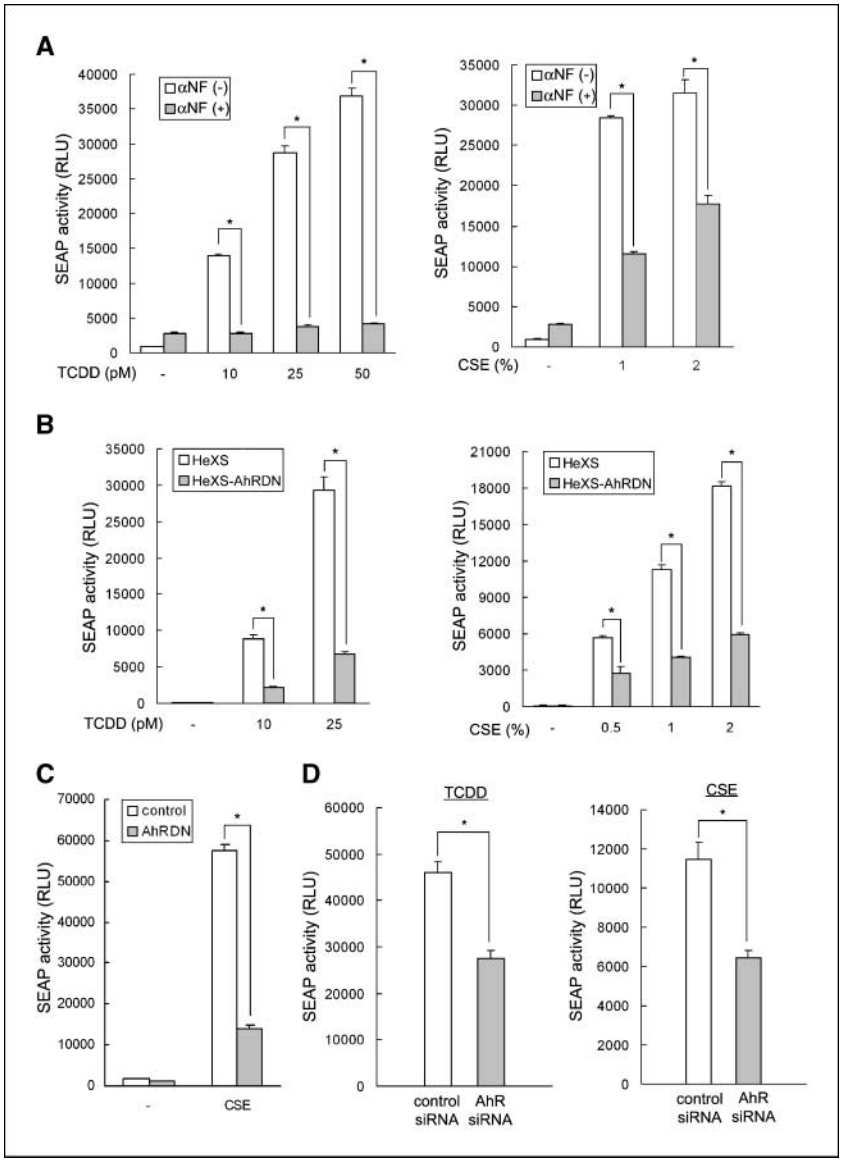

Figure 2. Role of AhR in the activation of XRE by cigarette smoke. *A,* HeXS34 cells were pretreated with or without a partial antagonist of AhR α-naphthoflavone (*αNF*; 1 μmol/L) for 30 minutes and stimulated with 10 to 50 pmol/L 2,3,7,8-TCDD (*left*) or 1% to 2% CSE (*right*) for 16 hours. Activity of SEAP in culture medium was evaluated by chemiluminescent assay. *B,* HeXS34 cells (HeXS) and HeXS-AhRDN9 cells stably expressing a dominant-negative mutant of AhR (HeXS-AhRDN) were treated with 2,3,7,8-TCDD (*left*) or 1% CSE (*right*) for 24 hours and subjected to SEAP assay. *C,* Hepa-1c1c7 cells transiently transfected with pEFBOS (control) or pEFBOS-AhRDN (AhRDN) together with pXRE-SEAP were treated with 1% CSE for 24 hours and subjected to SEAP assay. *D,* HeXS34 cells were transiently transfected with control siRNA or AhR siRNA, exposed to 25 pmol/L 2,3,7,8-TCDD (*left*) or 1% CSE (*right*) for 24 hours and subjected to SEAP assay. *Columns,* means; *bars,* SE. *, $P < 0.05$, statistically significant differences.

evaluated. Compared with control, HeXS-AhRDN9 cells exhibited blunted activation of XRE in response to both 2,3,7,8-TCDD (Fig. 2*B, left*) and CSE (Fig. 2*B, right*). The extent of inhibition was only a little higher in 2,3,7,8-TCDD-treated cells (76.1-77.2%) than that in CSE-treated cells (51.8-67.8%). Second, Hepa-1c1c7 cells were transiently transfected with an empty plasmid or the AhRDN plasmid together with pXRE-SEAP, and responses of the cells to 1% CSE were evaluated (Fig. 2*C*). Consistent with the result shown in Fig. 2*B*, AhRDN markedly suppressed the activation of XRE by CSE, and the inhibition achieved was 76.8%. Finally, the results were further confirmed by AhR siRNA. HeXS34 cells were transiently transfected with control siRNA or AhR siRNA and exposed to 25 pM 2,3,7,8-TCDD or 1% CSE. As shown in Fig. 2*D*, transfection with AhR siRNA reduced activation of XRE in response to both 2,3,7,8-TCDD (*left*) and CSE (*right*) to the same extent. These results suggested that, like 2,3,7,8-TCDD, cigarette smoke activated XRE largely through the AhR-dependent mechanism.

Quantitative assessment of XAP in cigarette smoke. To evaluate the total dioxin-like potential of cigarette smoke, five major cigarette brands in Japan were selected and used for preparation of CSE, as described in Materials and Methods. To estimate XAP values of cigarette smoke quantitatively, HeDS49 cells and HeXS34 cells were used. The sensor sequence introduced in HeDS49 cells consists of a truncated CYP1A1 promoter containing four copies of XRE and a part of the mouse mammary tumor virus long terminal repeat (12). As indicator cells, the response of HeDS49 cells to xenobiotic ligands is less selective but more sensitive than that of HeXS34 cells (12, 14). HeXS34 cells and HeDS49 cells were exposed to CSE (2% for 1-mg-tar and 5-mg-tar cigarette smoke; 1% for 10-mg-tar, 14-mg-tar, and 20 mg-tar cigarette smoke) for 16 hours, and the induction of SEAP was evaluated. Toxicity of dioxins and dioxin-like chemicals is usually assessed by their potential for activation of the AhR-XRE pathway. To quantify the XAP value of cigarette smoke 2,3,7,8-TCDD was used as the standard. In brief, reporter cells were treated with serial concentrations of 2,3,7,8-TCDD in parallel with diluted CSE, and XAP values of individual cigarette smoke (total XRE activating potential in 100 mL CSE) were estimated as ng TEQ/cigarette using

the standard curve of 2,3,7,8-TCDD. To distinguish the biologically assessed TEQ from the TEQ estimated by the physicochemical methods, we used a term "BioTEQ" (biologically assessed TEQ) to describe the results based on the DRESSA bioassays. As shown in Fig. 3*A*, a positive correlation was observed between the tar content and activation of XRE. A similar result was also obtained by the assessment using HeDS49 cells (Fig. 3*B*). The correlation coefficients were 0.835 in the HeXS-based assay and 0.840 in the HeDS-based assay. Any of CSE tested did not alter the level of SEAP in HeSS8 cells (Fig. 3*C*). Formazan assay also confirmed that the number of viable cells was not affected by the treatment with any CSE tested compared with control (data not shown).

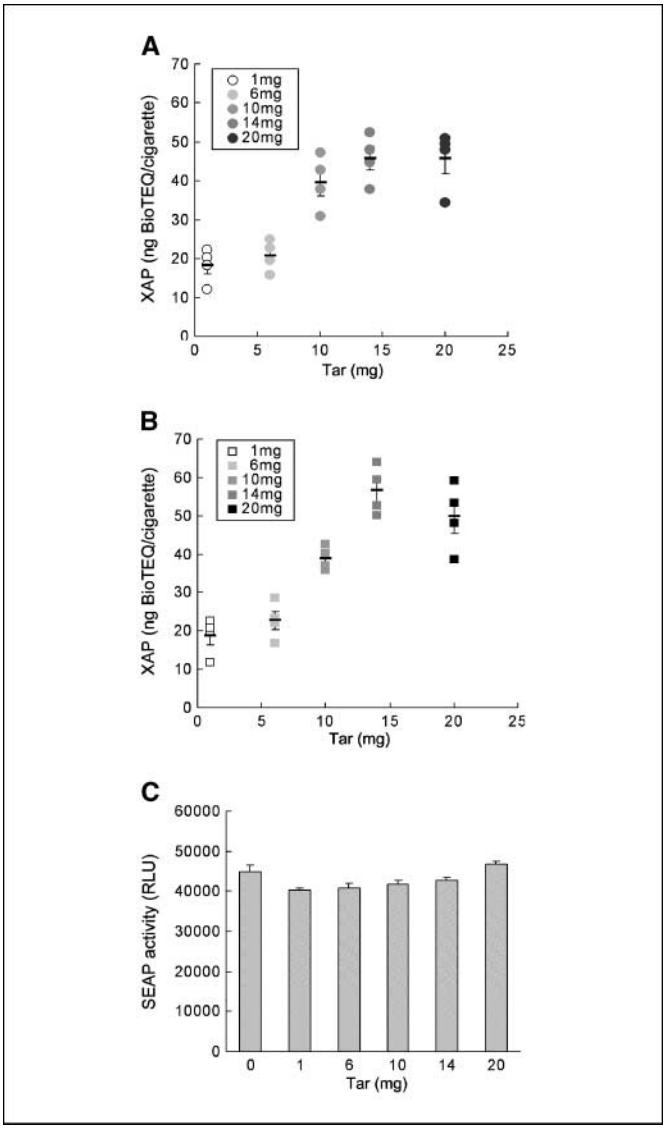

Figure 3. Quantitative assessment of XAP in cigarette smoke. Five major cigarette brands in Japan (tar content: 1, 6, 10, 14, and 20 mg) were used for preparation of CSE, as described in Materials and Methods, and used for experiments. *A* and *B,* HeXS34 cells (*A*) and HeDS49 cells (*B*) were exposed to CSE (2% for 1-mg-tar and 5-mg-tar smoke; 1% for 10-mg-tar, 14-mg-tar, and 20 mg-tar smoke) for 16 hours, and induction of SEAP was evaluated. Similarly, HeXS34 cells and HeDS49 cells were treated with serial concentrations of 2,3,7,8-TCDD (5-50 pmol/L) in parallel, and XAP for individual smoke was calculated and expressed as BioTEQ per cigarette using the standard curves of 2,3,7,8-TCDD. *C,* control HeSS8 cells were similarly treated with CSE and subjected to SEAP assay. *Points/columns,* means; *bars,* SE.

We calculated XAP values for individual cigarette smoke. The results were summarized in Table 1. The mean XAP values estimated were ranging from 18.3 ± 2.2 to 45.8 ± 3.1 ng BioTEQ/cigarette in the HeXS-based assay and from 18.8 ± 2.4 to 56.6 ± 3.2 ng BioTEQ/cigarette in the HeDS-based assay. The averages of both values ranged from 18.5 ± 1.5 to 51.2 ± 2.9 ng BioTEQ/cigarette. The tolerable daily intake (TDI) of 2,3,7,8-TCDD proposed by WHO is 1 to 4 pg/kg/d. In a person with 60 kg body weight, therefore, the XAP value generated from one 10-mg-tar cigarette was 164 to 656 times higher than the proposed TDI for dioxins, although *in vivo* half-life of AhR agonists in cigarette smoke may be shorter than that of dioxins.

Using foreign cigarette brands, we also evaluated XAP of cigarette smoke. CSE were prepared from two major cigarette brands in the United States (tar content: 9 mg) and subjected to analysis. In the HeXS-based assay, the mean XAP values estimated were 38.8 ± 0.4 and 33.8 ± 0.8 ng BioTEQ/cigarette, respectively. In the HeDS-based assay, the mean values estimated were 40.7 ± 0.8 and 34.2 ± 0.6 ng BioTEQ/cigarette, respectively. These XAP values were almost the same or only a little lower than the XAP values of Japanese 10-mg-tar cigarettes (39.3 ± 1.8 ng BioTEQ/cigarette).

Activation of XRE by cigarette smoke *in vivo*. We found that CSE has unexpectedly high levels of dioxin-like potential that triggers the AhR-XRE pathway *in vitro*. However, under *in vivo* situations, cells in the respiratory tract are exposed to cigarette smoke in a different manner, and absorption and metabolism of AhR agonists in smoke may be different from those in culture cells. The findings described, therefore, do not directly mean that inhalation of cigarette smoke activates AhR *in vivo*. To address this issue, we generated transgenic mice that produce SEAP under the control of XRE. The mice were orally given with 2,3,7,8-TCDD, and after 1 week, serum level of SEAP was evaluated. Under unstimulated conditions, reporter mice exhibited low levels of basal SEAP activity (991 ± 75 RLU, $n = 6$). One week after administration with 2,3,7,8-TCDD, the activity of serum SEAP was markedly up-regulated to 93,247 ± 20,175 RLU ($P < 0.05$; Fig. 4*A*). Using this transgenic line, direct effects of cigarette smoke were examined using two different protocols. First, reporter mice were exposed 12 times (every 15 minutes) to diluted mainstream smoke prepared from 14-mg-tar cigarettes, as described in Materials and Methods. After the exposure, blood was collected periodically up to day 6. As shown in Fig. 4*B*, serum levels of SEAP were significantly increased within 12 hours, peaked at 24 hours, and declined thereafter. Significant increase in serum SEAP activity was observed until day 4.

The experimental setting used here mimicked "passive smoking" because the mice were exposed to diluted smoke for sustained periods. In another set of experiments, reporter mice were subjected to "active smoking" consisting of brief exposure (10 seconds) to undiluted mainstream smoke with intervals for breathing fresh air (50 seconds), as described in Materials and Methods. This cycle was repeated every 1 minute until one cigarette burned out. Individual mice were subjected to smoking of total eight cigarettes at intervals of 15 minutes. Serum SEAP activity was evaluated before and 24 hours after the exposure. Consistent with the result shown in Fig. 4*B*, significant increases in the level of serum SEAP were also observed in this experimental setting (Fig. 4*C*). This result provided further evidence that exposure to cigarette smoke

Table 1. XAP of cigarette smoke

Tar content (mg/cigarette)	XAP (ng BioTEQ/cigarette ± SE)		
	HeXS34	HeDS49	Average
1	18.3 ± 2.2	18.8 ± 2.4	18.5 ± 1.5
6	20.8 ± 2.0	22.7 ± 2.4	21.8 ± 1.5
10	39.7 ± 3.5	39.0 ± 1.6	39.3 ± 1.8
14	45.8 ± 3.1	56.6 ± 3.2	51.2 ± 2.9
20	45.7 ± 3.8	49.9 ± 4.4	47.8 ± 2.8

NOTE: Data are shown as means ± SE (n = 4).

triggers activation of XRE not only *in vitro* but also in *in vivo* situations.

Discussion

Cigarette smoke contains substances that trigger activation of AhR (18, 19). Those include PCDD, PCDF, Co-PCB, and benzo(*a*)pyrene (6, 20). However, previous assessment using gas chromatography-mass spectrometric analyses showed that the levels of individual chemicals in cigarette smoke were very low and within permissible ranges. For example, Aoyama et al. evaluated the amount of dioxins (PCDD, PCDF, and Co-PCB) in mainstream and side stream smoke (20). They reported that the total amount of dioxins in cigarette smoke was ranging from 0.4 to 2.4 pg TEQ/cigarette. In a person with 60 kg body weight, therefore, the total intake of dioxins is estimated as 0.14 to 0.96 pg TEQ/kg body weight/d by smoking 20 cigarettes. It is below the TDI proposed by WHO (1-4 pg/kg/d). Consistently, Muto and Takizawa also reported that the daily intake of PCDD by smoking 20 cigarettes was ~4 pg/kg body weight/d (6). In contrast to these previous reports, we showed in this report that cigarette smoke has extremely high levels of dioxin-like potential for triggering the AhR-XRE pathway. The mean XAP values estimated were ranging from 18.5 to 51.2 ng BioTEQ/cigarette, which were 164 to 656 times higher than the TDI of dioxins proposed by WHO. Of note, the estimated values were for smoke generated from one cigarette. In a person (60 kg body weight) that smokes one box of 10-mg-tar cigarettes per day, estimated XAP values are 3,278 to 13,113 times higher than the TDI proposed by WHO. The TDI value is currently applied for the regulation of dioxins and not for other chemicals. However, it may be reasonable to apply the TDI for other toxic substances that activate AhR because (*a*) toxicity of dioxins is evaluated by their potential for binding or activating AhR, and because (*b*) constitutive activation of AhR causes malignant diseases and immune abnormality in mice even without xenobiotic ligands (4, 5). The extremely high potential of cigarette smoke for activating the AhR-XRE pathway, therefore, needs to be considered seriously, correspondingly to dioxins.

What is the reason for the discrepancy between our current results based on bioassays and the previous results by physicochemical approaches? First of all, cigarette smoke contains >4,800 chemicals whose actions on the AhR-XRE pathway are largely unknown. Our results indicated that a number of chemicals with substantial XAP may be present in cigarette smoke and act in combination as strong stimulants for XRE. In other words, dioxins analyzed by previous investigation are not major constituents responsible for the dioxin-like activity in cigarette smoke. Because of this reason, the conventional physicochemical assessment using gas chromatography-mass spectrometric analyses underestimated the risk of cigarette smoke, and bioassays may be more suitable for evaluation of its real risk. It is known that the majority of dioxins are hardly dissolved in non-organic solutions. Our results using PBS-based CSE further supported the idea that the dioxin-like potential of cigarette smoke was not due to dioxins.

The XAP estimated by the current study was unexpectedly high levels, but we may still have underestimated the XAP of cigarette smoke. First, in the present study, only mainstream smoke was focused, and side stream generated was ignored. Second, CSE was prepared using smoke passed through filters, and smoke constituents trapped by the filters were also ignored. Third, during the preparation of CSE, some smoke components could have been lost by incomplete dissolution in PBS or adsorption on the wall of plastic tubes and glass bottles, as we recently reported (21). However, it is worthwhile to note that the XAP of PBS-based CSE was not significantly different from that of DMSO-based CSE.[3] Fourth, some ligands of AhR in cigarette smoke might function as antagonists of AhR, like α-naphthoflavone. Because of these reasons, it is not unreasonable to speculate that the real XAP of cigarette smoke may be higher than that we have determined in this report.

The reliability of the DRESSA assay to detect xenobiotic ligands has been extensively tested in our previous reports (12–14). This bioassay achieves linear and dose-dependent responses exclusively to agonists of AhR (12). In the present study, 2,3,7,8-TCDD was used as the standard to evaluate XAP for individual smoke. It is because, currently, toxicity of dioxins and dioxin-like substances is evaluated based on the relative toxicity to 2,3,7,8-TCDD. To calculate the XAP values, we used a range of the standard curve that achieved linear responses. To eliminate nonspecific responses, HeXS34 cells with high ligand specificity was used in parallel with the prototypic sensor clone HeDS49. It is worthwhile to note that the XAP values independently estimated by the HeXS-based and HeDS-based systems were nearly identical in 1-mg-tar, 6-mg-tar, and 10-mg-tar cigarettes (Table 1), although the values were a little higher (not significant) in the HeDS-based method than those in the HeXS-based method in 14-mg-tar and 20-mg-tar cigarettes. In the cell-based bioassays, nonspecific damage of reporter cells may significantly affect the level of indicator protein. We excluded this possibility by confirming that cell viability was unaffected by any lots and any concentrations of CSE tested, using formazan assay. Some components in cigarette smoke could reduce secretion of active SEAP from mammalian cells without affecting cell viability. To exclude this possibility, HeSS8 cells constitutively secreting SEAP were used in parallel, and we confirmed that, at any concentrations tested, CSE did not affect secretion and activity of SEAP.

To evaluate biological effects of cigarette smoke on culture cells, cigarette smoke condensates have been generally used. However, use of smoke condensates or smoke extracts may not completely mimic *in vivo* smoking situations. We therefore evaluated the direct effect of cigarette smoke on culture cells and revealed that a single, brief exposure of smoke significantly induced activation of XRE.

[3] Our unpublished observation.

The exposure condition used (2 seconds, 45 mL) was comparable with *in vivo* exposure of the respiratory tract to smoke. Although the cells in the respiratory tract might be less sensitive to smoke than Hepa-1c1c7 cells that express AhR abundantly (22), our result is the first to show that native cigarette smoke can induce activation of the AhR-XRE pathway directly in mammalian cells. Furthermore, using transgenic reporter mice that produce SEAP under the control of XRE, we also provided evidence that inhalation of cigarette smoke triggers activation of the AhR-XRE pathway *in vivo*, and that the significant activation lasts for several days. In the experiments we did, mice were exposed to smoke only at day 0. It is probable that habitual, day-to-day smoking in humans causes sustained activation of AhR. Of note, our *in vivo* studies used exposure conditions comparable to or milder than those of previous reports (23, 24). For example, in the "passive smoking" experiments, the mice were initially exposed to >10 times diluted mainstream smoke (45 mL smoke injected into a 500-mL bottle), which was further diluted gradually because mouths of the bottles (3 cm in diameter, covered with nylon mesh) were kept open. Furthermore, during the experiments, mice were usually located in the mouths of bottles to breathe fresh air, and the actual concentration of smoke inhaled by mice may be low.

Despite the public concern about the toxic effects of cigarette smoke, little efforts have been paid for the development of assay systems for evaluating cigarette toxicity. Currently, nicotine and tar content are used as indicators, but they may not be competent for this purpose. In the present report, we examined the relationship between the tar content and the XAP value using seven cigarette brands. Although a positive correlation was observed between these variables, the content of tar was found not to be a reliable indicator for quantification of biological toxicity of cigarettes. For example, compared with 10-mg-tar cigarette, the XAP value of 1-mg-tar cigarette was not 10% but ~47%. Similarly, the XAP value of 1-mg-tar cigarette versus 20-mg-tar cigarette was not 5% but ~39%. Based on these results, we speculate that low-tar "light" cigarettes might not be so less toxic than higher-tar "mild-to-heavy" cigarettes. It is consistent with a recent report showing that the low tar/low nicotine did not mean low toxicity in cigarette smoking (25).

One approach previously used to evaluate toxicity of cigarette smoke was physicochemical quantification of individual toxicants. However, it is conceivably not competent for the realistic risk assessment because (*a*) analysis using gas chromatography-mass spectrometry is expensive and time consuming, and (*b*) as shown in this report, the real, total toxicity of numerous toxicants cannot be evaluated by this conventional approach. In contrast, the bioassay-based system is a very simple, sensitive, and economic method that is suitable for high-throughput assessment of many samples (12, 13). It enables to detect and quantify low levels of AhR agonists within 6 to 16 hours (13). Although this approach does not allow for assessment of individual chemicals, it may be advantageous to evaluate the total toxicity of cigarette smoke.

Cigarette smoke is known to cause a variety of pathologies, including cancers, obstructive pulmonary diseases, atherosclerosis, cardiovascular/cerebrovascular diseases, and developmental/reproductive abnormalities (26). Of note, many of these pathologies are also caused by dioxins and dioxin-like chemicals (2, 27). Our current finding that cigarette smoke exhibited high levels of the dioxin-like potential strongly suggests that the health problems caused in smokers are, at least in part, due to the potential of cigarette smoke to activate the AhR-XRE pathway. Previous reports showed that constitutive activation of the AhR-XRE pathway via genetic manipulation caused cancers in mice (4, 28). These results suggest that excessive, sustained activation of the AhR-XRE pathway per se is pathogenic to mammals. Based on this, the bioassay-based assessment of XAP shown in this report should provide a realistic indicator for the assessment of biological toxicity of cigarette smoke. We propose that the XAP value may be useful for (*a*) quantitative risk assessment of smoking in active and passive smokers, (*b*) comparison of toxicity among different

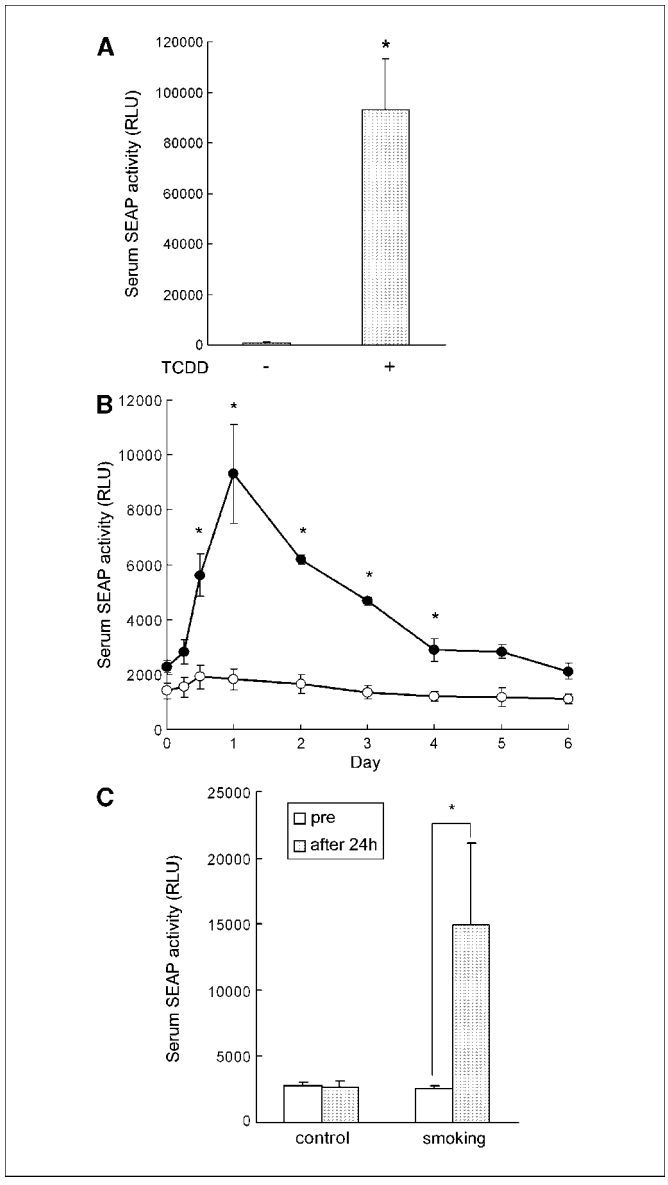

Figure 4. Activation of XRE by cigarette smoke *in vivo. A,* transgenic reporter mice (20-25 g body weight; six male mice) were orally given with 2,3,7,8-TCDD (5 μg/kg body weight), and after 1 week, serum levels of SEAP were evaluated. *B,* reporter mice kept in 500-mL bottles were exposed 12 times (every 15 minutes) to diluted mainstream smoke prepared from 14-mg-tar cigarettes ($n = 4$). As a control, mice were similarly kept in bottles without exposure to smoke ($n = 4$). After the exposure, blood was sampled periodically up to day 6 and subjected to SEAP assay. ○, without exposure to smoke; ●, exposure to smoke. *C,* reporter mice put in 50-mL tubes were exposed to undiluted mainstream smoke (14-mg tar) for 10 seconds ($n = 4$). The smoke was subsequently washed out with fresh air. This cycle was repeated every 1 minute until one cigarette burned out. Individual mice were subjected to smoking of total eight cigarettes at intervals of 15 minutes. Serum SEAP activity was evaluated before and 24 hours after the exposure to smoke. *Points/columns,* means; *bars,* SE. *, $P < 0.05$, statistically significant differences.

cigarette brands, (*c*) development of less-toxic cigarettes, and (*d*) screening of foods and chemicals that attenuate the toxicity of cigarette smoke.

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