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Aryl hydrocarbon receptor-dependent upregulation of Cyp1b1 by TCDD and diesel exhaust particles in rat brain microvessels

Aude Jacob^{1,2†}, Anika MS Hartz^{3,5†}, Sophie Potin^{1,2}, Xavier Coumoul⁶, Salah Yousif^{1,2}, Jean-Michel Scherrmann^{1,2}, Björn Bauer^{4,5} and Xavier Declèves^{1,2*}

Abstract

Background: AhR activates the transcription of several target genes including CYP1B1. Recently, we showed *CYP1B1* as the major cytochrome P450 (CYP) enzyme expressed in human brain microvessels. Here, we studied the effect of AhR activation by environmental pollutants on the expression of Cyp1b1 in rat brain microvessels.

Methods: Expression of AhR and Cyp1b1 was detected in isolated rat brain microvessels. AhR was immunovisualised in brain microvessel endothelial cells. The effect of AhR ligands on Cyp1b1 expression was studied using isolated brain microvessels after *ex vivo* and/or *in vivo* exposure to TCDD, heavy hydrocarbons containing diesel exhaust particles (DEP) or Δ^9 -tetrahydrocannabinol (Δ^9 -THC).

Results: After *ex vivo* exposure to TCDD (a highly potent AhR ligand) for 3 h, *Cyp1b1* expression was significantly increased by 2.3-fold in brain microvessels. A single i.p. dose of TCDD also increased *Cyp1b1* transcripts (22-fold) and Cyp1b1 protein (2-fold) in rat brain microvessels at 72 h after TCDD. Likewise, DEP treatment (*in vivo* and *ex vivo*) strongly induced Cyp1b1 protein in brain microvessels. DEP-mediated Cyp1b1 induction was inhibited by actinomycin D, cycloheximide, or by an AhR antagonist. In contrast, a sub-chronic *in vivo* treatment with Δ^9 -THC once daily for 7 seven days had no effect on *Cyp1b1* expression

Conclusions: Our results show that TCDD and DEP strongly induced Cyp1b1 in rat brain microvessels, likely through AhR activation.

Keywords: Aryl hydrocarbon Receptor, Cyp1b1, TCDD, diesel exhaust particles, brain microvessels

Background

The blood-brain barrier (BBB) is a dynamic neurovascular unit composed of three main cell types: brain microvessel endothelial cells sealed by tight junctions, astrocyte end-foot processes ensheathing microvessels, and pericytes sharing the basal membrane with endothelial cells. Due to tight junctions limiting paracellular diffusion and the presence of drug efflux transporters and drug-metabolizing enzymes, the BBB is both a physical and a metabolic barrier that selectively controls brain penetration of xenobiotics. Recently, we showed that

CYP1B1, a cytochrome P450 (CYP) enzyme, was the most highly expressed CYP gene in human brain microvessels [1]. Expression of genes encoding CYP enzymes is regulated in several peripheral tissues by transcription factors such as the pregnane-X-receptor (PXR), the constitutive androstane receptor (CAR), and the aryl hydrocarbon receptor (AhR) [2]. AhR is a cytosolic ligand-dependent transcription factor of the bHLH-PAS (basic helix-loop-helix/Per-Arnt-Sim) superfamily. AhR is activated by binding of several classes of structurally different aromatic compounds and environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) [3]. Briefly, upon ligand binding in the cytosol, AhR translocates into the nucleus where it heterodimerizes with its

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partner aryl hydrocarbon nuclear translocator (ARNT). The AhR/ARNT complex activates in turn xenobiotic response elements (XRE) in the promoter region of several AhR target genes, thus increasing their transcription [4,5]. AhR target genes include several genes encoding CYP enzymes such as CYP1A1, CYP1A2 and CYP1B1 [6,7], as well as ATP-binding cassette (ABC) transporters such as ABCG2 [8] and genes involved in cell cycle regulation, cell proliferation, inflammation, and apoptosis [9,10]. High levels of *AhR* mRNA were detected in human brain microvessels and in the hCMEC/D3 human cerebral microvascular endothelial cells, a promising *in vitro* model of the human BBB [1,11]. Furthermore, TCDD, the most potent AhR activator, highly induced the expression of CYP1B1 and CYP1A1 in hCMEC/D3 cells [11]. Consistent with this, AhR agonists increased Cyp1a1 and Cyp1b1 in many brain regions in rat and in blood-brain interfaces of mice and rats [12,13]. TCDD has also been shown to induce both Cyp1a1 and Cyp1b1 in cultured murine cerebral vascular endothelial cells and astrocytes [14]. Regarding Cyp1b1, this enzyme has been demonstrated to metabolise various endogenous compounds like retinol, arachidonic acid, melatonin and estrogens [7,15]. Moreover, Cyp1b1 also selectively bioactivates various PAHs (benzo[a]pyrene, DMBA, anthracenes) into intermediates that are DNA-reactive [16]. All these studies suggest that AhR control the expression of CYP1B1 at the BBB.

First, this work aimed to examine TCDD-mediated activation of AhR on Cyp1b1 expression *in vivo* in rat brain microvessels. In addition, we studied the effect of two other potential AhR ligands: Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and diesel exhaust particles (DEP). Δ^9 -THC, the psychoactive component of marijuana, has been discussed as a potential AhR ligand due to its ability to induce *Cyp1a1* expression in a murine hepatoma cell line [17]. DEP are complex environmental toxicants containing heavy hydrocarbons derived from fuel like PAHs and HAHs that people are exposed to daily and known to activate AhR [18,19].

Methods

Reagents and equipment

RNA extraction kits were purchased from Qiagen GmbH (Hilden, Germany). RT-PCR reagents were purchased from Invitrogen (Invitrogen, France). Primers were synthesized by Invitrogen Life Technologies (Invitrogen, France). The LC Fast Start DNA Master SYBR Green I kit was purchased from Roche Diagnostics (Meylan, France), and the Power SYBR Green PCR Master Mix was from Applied Biosystems (Foster City, CA, USA). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin, 50 $\mu\text{g}/\text{mL}$ in DMSO) was obtained from LGC Promochem (Molsheim, France) and Δ^9 -THC (27 mg/

mL in ethanol) was from Sigma-Aldrich (Saint Quentin Fallavier, France). Antibodies and supplies used for western blotting were: polyclonal rabbit anti-rat Cyp1a1, polyclonal rabbit anti-Cyp1b1, monoclonal mouse anti-AhR, and monoclonal mouse anti- β -actin antibodies (Abcam, Cambridge, UK and Abcam, Cambridge, MA, USA), horseradish peroxidase-conjugated monkey anti-rabbit secondary antibody and horseradish peroxidase-conjugated monkey anti-mouse secondary antibody (Amersham, Buckinghamshire, UK), and Alexa Fluor[®] 488 goat anti-mouse IgG (H+L) antibody (Invitrogen, Carlsbad, CA, USA). Proteins were detected using SuperSignal[®] West Pico Chemoluminescent Substrate (Pierce, Rockford, IL, USA). Diesel exhaust particles (DEP) were obtained from NIST [20] (Gaithersburg, MD, USA). Carbon black (CB) mock particles were a kind gift from Degussa Corporation (Akron, OH, USA). AhR antagonist (CH-223191) [21] and IgG control antibody were from Calbiochem-Novabiochem (La Jolla, CA, USA). Protein A/G Beads were from Pierce (Rockford, IL). Other chemicals and reagents were purchased from Sigma-Aldrich (France and St. Louis, MO, USA) or Invitrogen (Cergy-Pontoise, France). Equipments used were: a nucleic acid spectrophotometer (Nanodrop ND-1000, NanoDrop Technologies, USA), a programmable thermal cycler (PTC-100 programmable thermal controller, MJ research Inc., USA), a Light Cycler thermal cycler (Light-Cycler[®] instrument, Roche Diagnostics), and a 7900 HT Real-Time PCR Detection System (Applied Biosystems, Foster City, CA).

Animal treatments

All animal experiments were based on the same experimental setup. For treatment with TCDD and Δ^9 -THC, male Sprague-Dawley rats (230-250 g) were obtained from Charles River (L'arbresle, France). For treatment with DEP, male CD[®] IGS Sprague-Dawley rats (275-300 g) were obtained from Charles River (Portage, MI, USA). Rats were housed in groups of four animals per cage under standard 12:12-hour light/dark conditions (light from 8:00 a.m. to 8 p.m.) in a temperature- and humidity-controlled room. Animals had access to food and water *ad libitum*. Before using rats for experiments, they were allowed to adapt to the animal facility for 3-5 days.

The care and treatment of animals (TCDD and Δ^9 -THC treatment) were in accordance with standards and guidelines approved by the European Communities Council Directive (86/609/EEC). Animal protocols were approved by the Institutional Animal Care and Use Committees of the EPA, RTP, NC, and the University of Minnesota and were in accordance with AAALAC regulations and the Guides to Animal Use of the University of Minnesota and NIH animal guidelines.

For *in vivo* TCDD exposure, animals were dosed with a single i.p. of TCDD (25 µg/kg body weight) or control (1:9 DMSO: Corn Oil) and sacrificed 72 h after the TCDD or control injection as previously described [22,23].

For *in vivo* Δ^9 -THC exposure, Δ^9 -THC (10 mg/kg body weight) or control (1:1:18 ethanol: cremophor: saline) were administered i.p. once daily for 7 days. This *in vivo* dosing protocol was chosen for several reasons: (1) Δ^9 -THC has a half-life of about 25-36 h, which is long enough for a once daily administration, (2) Δ^9 -THC is likely a less potent AhR activator compared to TCDD, (3) the 7-day Δ^9 -THC dosing scheme is currently used in our laboratory, and we observed that it causes an addiction-like behavioral effect (unpublished data). Rats were sacrificed 12 h after the last Δ^9 -THC or control injection.

For *in vivo* DEP exposure, diesel engine exhaust was generated by operating a 30-kW (40 hp) four-cylinder indirect injection Deutz diesel engine (BF4M1008) and was collected as previously described [24]. Rats in inhalation chambers were exposed to 0.5 mg/m³ and 2 mg/m³ of this diesel engine exhaust for 5 h/day, 5 days/week for 4 consecutive weeks. Inhalation fumes contained diesel exhaust engine gas as well as diesel exhaust particulate matter. These DEP inhalation doses have been used in previous studies and are based on DEP concentrations found in ambient air during heavy traffic [25,26].

Preparation of DEP working suspension

DEP working suspension using diesel exhaust particles (SRM 2975) that were derived from diesel-powered forklifts was prepared as previously described [27]. Briefly, 2 mg of DEP was suspended in 10 mL PBS buffer, vortexed, and sonicated for 30 min at 25°C using an ultrasonic processor (Bransonic[®], Model 3510, Branson Ultrasonic Cooperation, Danbury, CT, USA). The suspension was filtered through a 0.22-µm filter (MillexGS; Millipore, Billerica, MA, USA).

Isolation of rat brain microvessels

All steps in the isolation of brain microvessels were carried out at 4°C. Rats treated with TCDD, Δ^9 -THC or DEP were anesthetized with isoflurane and sacrificed by decapitation after the last injection or euthanized by CO₂ inhalation and decapitated. Brains were immediately removed and placed in ice-cold HBSS. Cerebella, meninges, brainstems, and large superficial blood vessels were removed. Rat cortex microvessels were isolated according to a protocol that results in the lowest contamination with astrocyte and neuron mRNA and the highest yield of endothelial cells mRNA [28]. Isolated brain microvessels were also free from red blood cells,

other contaminating cells such as microglia, and cell debris [27]. Briefly, cortices were minced and homogenized with a Potter-Thomas homogenizer (Konté Glass, Vineland, NJ, USA). The resulting homogenate was centrifuged at 1000 g for 10 min, and the microvessel-enriched pellet was suspended in 17.5% dextran and centrifuged for 15 min at 4400 g at 4°C in a swinging bucket rotor. The resulting pellet was suspended in HBSS containing 1% BSA, while the supernatant containing a layer of myelin was centrifuged once more. The resulting microvessel suspension was passed through a 100 µm nylon mesh, and the filtrate was then passed through a 20 µm nylon mesh. Microvessels retained by the nylon 20 µm mesh were immediately collected and used for experiments or frozen at -80°C.

For *ex vivo* experiments, freshly isolated brain microvessels from naïve rats were incubated with different concentrations of either TCDD (25 nM) or DEP (0, 5, 50, 200 µg/mL) as previously described [27].

RNA extraction and reverse transcription

Total RNA was extracted from tissues and organs of each rat. Total RNA from isolated brain microvessels was obtained by lysing the surrounding basal lamina with proteinase K and then extracting total RNA using the RNeasy Fibrous Tissue Micro kit according to the manufacturer's instructions. RNA samples were purified from contaminating genomic DNA by treatment with DNase (RNase-Free DNase Set, Qiagen SA). RNA concentration and purity were assessed spectrophotometrically at 260 nm using a Nanodrop[®] spectrophotometer; RNA integrity was assessed by electrophoresis on a 0.8% agarose gel. One µg of total RNA was reverse transcribed into cDNA in a final volume of 20 µL. The mixture consisted of 1 µg total RNA, 500 µM of each dNTP, 10 mM DTT, 1.5 µM random hexa-nucleotide primers, 20 U RNase in ribonuclease inhibitor, and 100 U SuperScript II reverse transcriptase. Hexamers were annealed at 25°C for 10 min, products were extended at 42°C for 30 min, and the reaction was terminated by heating to 99°C for 5 min before being quick-chilled to 4°C and stored at -80°C.

Non-quantitative and quantitative RT-PCR (q-PCR)

RT-PCR for *AhR* and *Cyp1b1* was performed with *Taq* DNA Polymerase from Promega (Madison, WI, USA) using primers for rat *AhR* and *Cyp1b1* (Table 1) that were custom-synthesized by Qiagen Operon (Alameda, CA, USA). PCR products were resolved on a 2% agarose gel at 100 V for 75 min.

The effect of Δ^9 -THC and TCDD on the expression of *Cyp1a1* and *Cyp1b1* was investigated by quantitative real-time PCR (q-PCR). β -actin was used as an endogenous reference for normalizing target gene mRNA. Gene

Table 1 Sequences of primers used for RT-PCR and q-PCR

Gene	Forward primer (5'-3')	Reverse Primer (5'-3')	Length (bp)	GenBank accession*
<i>β-Actin</i>	CTGGCCCGACCTGACAGA	GCGGCAGTGGCCATCTCTC	132	NM_031144
<i>AhR^a</i>	CTCCCTCCACAGTTGGCTTTGTTTG	GATTCTGCGCAGTGAAGCATGTCAG	233	NM_013149
<i>Cyp1a1</i>	AACCCACACCTGTCACTGA	CTGGTGAACAGGGGGAT	132	NM_012540
<i>Cyp1b1</i>	GCTTGCCAGTGAGAGAGG	TTCTCAAGAATGAGCGGAA	135	NM_012940
<i>Cyp1b1^a</i>	GCAGATCAACCCTCACTCAGCAAC	GTCTGTAATAGTGGCAGGCACATCC	193	NM_012940

^a for RT-PCR

* <http://www.ncbi.nlm.nih.gov/>

expression was evaluated by q-PCR as previously described [28]. Primers were designed using OLIGO 6.42 software (Medprobe, Norway) (Table 1). PCR reactions were performed on a Light-Cycler[®] instrument using the LC-FastStart DNA Master SYBR Green I kit. cDNAs from either control or treated rats were used to generate external calibration standards for each gene. The PCR reaction mixture consisted of 1 μL LC-FastStart DNA Master SYBR Green mix, 1.2 μL of 10 mM MgCl₂, 0.5 μL of each upper and lower primer (final concentration 0.5 μM), and 1.8 μL water. The cDNAs were diluted 40-fold, and 5 μL aliquots were mixed with an equal volume of PCR mixture to yield a final volume of 10 μL. The thermal cycling conditions were 8 min at 95°C followed by 40 amplification cycles at 95°C for 5 s, 64°C for 5 s, and 72°C for 5 s. A target gene was considered to be easily quantifiable when the Ct that was obtained for the less diluted cDNA (1/20) sample was lower than 30. A Ct value of 32 was set as the detection limit. A calibration curve using serial dilutions of cDNA standard was used to determine the relative expression of *Cyp1a1* and *Cyp1b1* genes in control (vehicle) and TCDD-treated rat (n = 8 rats per group) and in control (vehicle) and Δ⁹-THC-treated rat (n = 12 rats per group). The (fold) change was expressed by the ratio (gene of interest/*β*-actin)_{treated}/(gene of interest/*β*-actin)_{control}.

Immunofluorescence

Freshly isolated brain microvessels were transferred to glass cover slips and fixed for 15 min with 3% paraformaldehyde/0.2% glutaraldehyde at room temperature. After washing with PBS, microvessels were permeabilised for 20 min with 1% (v/v) Triton X-100 in PBS and subsequently blocked with 1% BSA in PBS. Microvessels were incubated overnight at 4°C with the primary antibody to AhR (1:100). After washing with 1% BSA, microvessels were incubated for 1 h at 37°C with Alexa Fluor[®] 488-conjugated secondary IgG (1:500, 4 μg/mL; Invitrogen, Eugene, OR, USA); negative controls were incubated with secondary antibody only. Nuclei were counterstained in blue with 2.5 μg/mL DAPI for 15 min. AhR staining was visualised in green using confocal

microscopy (Nikon C1 LSC microscope unit, Nikon TE2000 inverted microscope, 40x oil immersion objective, NA 1.3, 488 nm line of an argon laser, 402 nm line of a solid state UV laser; Nikon Instruments Inc., Melville, NY, USA).

Immunoprecipitation

Isolated brain microvessels were homogenized in lysis buffer (Sigma, St. Louis, MO, USA) containing Complete[®] protease inhibitor (Roche, Mannheim, Germany). The lysate was cleared by centrifugation at 10 000 g for 15 min at 4°C. Identical amounts of pre-cleared microvessel lysates (50 μg of protein) were immunoprecipitated with 5 μg AhR antibody, 5 μg IgG antibody (IgG control), or antibody-free PBS buffer (negative control) by overnight incubation at 4°C. The immune complexes were precipitated with Protein A/G beads (Pierce, Rockford, IL, USA) and washed 3 times with RIPA buffer (Sigma, Dedham, MA, USA) and once with PBS. Immunoprecipitated proteins were eluted with LDS buffer (Invitrogen, Carlsbad, CA, USA) and analyzed by western blotting.

Western blotting analysis

For *Cyp1b1*, *Cyp1a1*, and AhR western blots, brain microvessels were homogenized in lysis buffer (Sigma, St. Louis, MO, USA) containing Complete[®] protease inhibitor (Roche, Mannheim, Germany) and centrifuged at 10 000 g for 15 min. Denucleated supernatants were used as microvessel lysates, and protein concentrations were determined. Western blots were performed using the Invitrogen NuPage[™] Bis-Tris electrophoresis and blotting system (Invitrogen, Carlsbad, CA, USA). After protein transfer, blotting membranes were blocked and incubated with primary antibody (AhR 1:100, *Cyp1b1* 1:500 (1 μg/mL), *Cyp1a1* 1:1000, *β*-actin 1:1000 (1 μg/mL)). Membranes were washed and incubated with horseradish peroxidase-conjugated ImmunoPure[®] secondary IgG (1:15,000; Pierce, Rockford, IL, USA) for 1 h. Proteins were detected using SuperSignal[®] West Pico Chemoluminescent Substrate (Pierce, Rockford, IL, USA), and bands were visualised and recorded using a BioRad Gel Doc 2000[™] gel documentation system

(BioRad, Hercules, CA, USA). Rat Cyp1a1 supersomes™ and Cyp1b1 microsomes were supplied by BD Biosciences (Woburn, MA, USA) and used as positive controls for Cyp1a1 and Cyp1b1, respectively.

Statistical analysis

Statistical analyses were done with GraphPad Prism® 4.0 software (GraphPad Software Inc., San Diego, CA, USA). Data for qPCR are expressed as means ± SD. Student's unpaired *t*-tests were performed to identify significant differences between rats treated with TCDD or

Δ⁹-THC and control-treated rats. All the tests were two-tailed and statistical significance was set at *p* < 0.05. Data received from optical density measurements from western blots are means ± SEM.

Results

AhR expression in different rat tissues and its localization in isolated rat brain microvessels

AhR transcripts (233 bp amplicon) were detected in total brain, brain microvessels, choroid plexus and peripheral tissues (Figure 1A). AhR protein was also

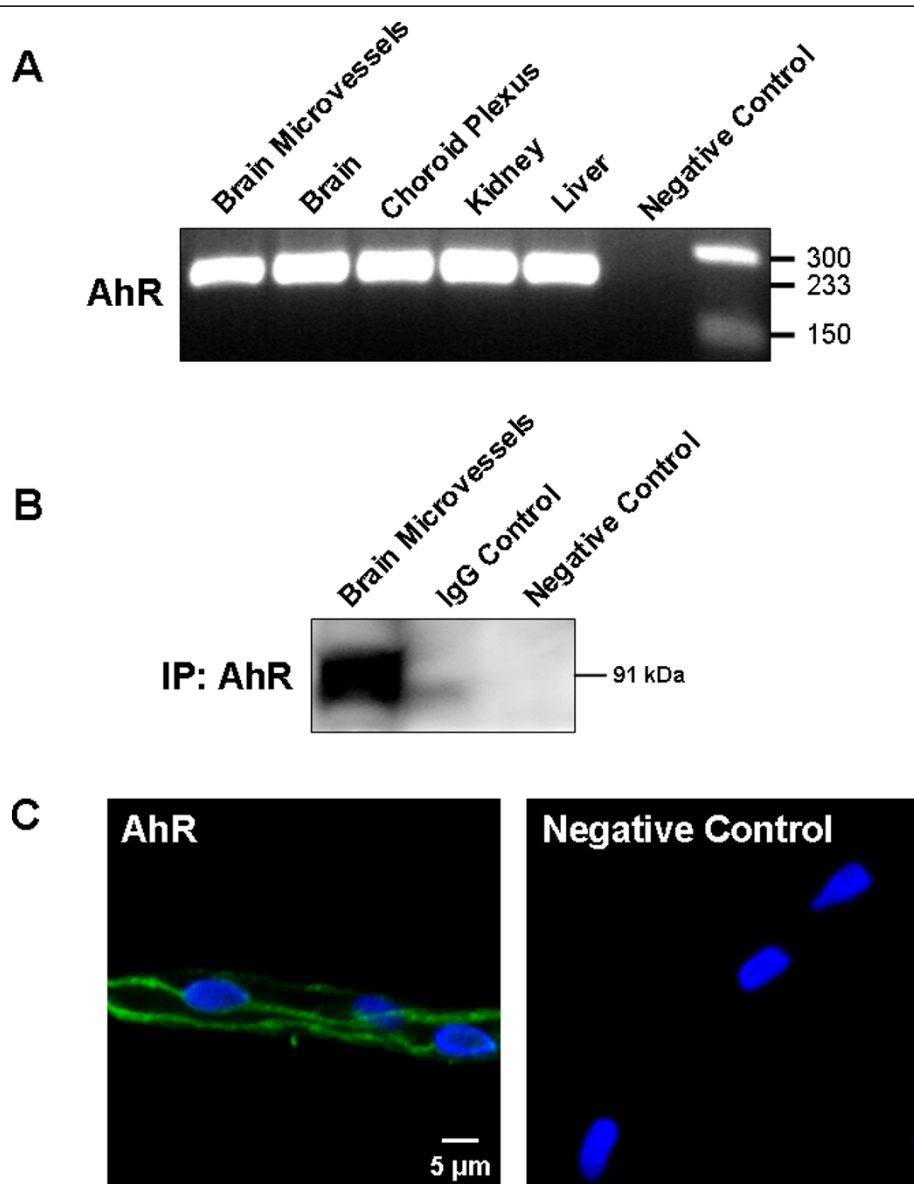


Figure 1 AhR expression in different rat tissues and AhR immunolocalization in isolated rat brain microvessels. (A) RT-PCR of liver, kidney, choroid plexus, total brain, and rat brain microvessels for rat *AhR*. The RT-PCR shows a signal for *AhR* mRNA at 233 bp. (B) Western blot of immunoprecipitated AhR from rat brain microvessels. (C) Representative image of a brain microvessel immunostained for AhR (green); nuclei were counterstained with DAPI (blue).

detected after immunoprecipitation and western blotting in brain microvessels, which showed a strong signal for AhR (Figure 1B). Importantly, immunoprecipitations without AhR antibody (negative control) or using an IgG control antibody (IgG control) instead of anti-AhR antibody showed no band demonstrating the specificity of the AhR signal. Using immunostaining we localised AhR in the cytoplasm of brain microvessel endothelial cells (Figure 1C), which is consistent with the location of this transcription factor [9,10]. Thus, AhR is expressed at both the mRNA and protein levels in rat brain microvessels.

Effect of TCDD and Δ^9 -THC on CYP expression in isolated brain microvessels

To demonstrate expression of *Cyp1b1*, we first performed both RT-PCR and western blotting. High levels of *Cyp1b1* transcript (193 bp amplicon, Figure 2A) and protein were detected in brain microvessels, total brain, liver, choroid plexus and kidney (Figure 2B). The apparent molecular weight of *Cyp1b1* protein was determined by digital analysis to be 62 kDa, a value that is consistent with the calculated molecular weight of *Cyp1b1* at 60.5 kDa.

To test if AhR induces *Cyp1a1* and *Cyp1b1* genes in isolated brain microvessels, we first incubated *ex vivo* brain microvessels from naïve rats with 25 nM TCDD, a highly potent AhR ligand, and determined the expression of *Cyp1b1* and *Cyp1a1*, two well-known AhR target genes. In control brain microvessels, *Cyp1a1* mRNA was

about 60-fold less abundant than that of *Cyp1b1*. *Cyp1b1* and *Cyp1a1* mRNA expression levels in brain microvessels were significantly increased by 2.5-fold and 11-fold as early as 3 h after *ex vivo* TCDD exposure, respectively (Figure 3A).

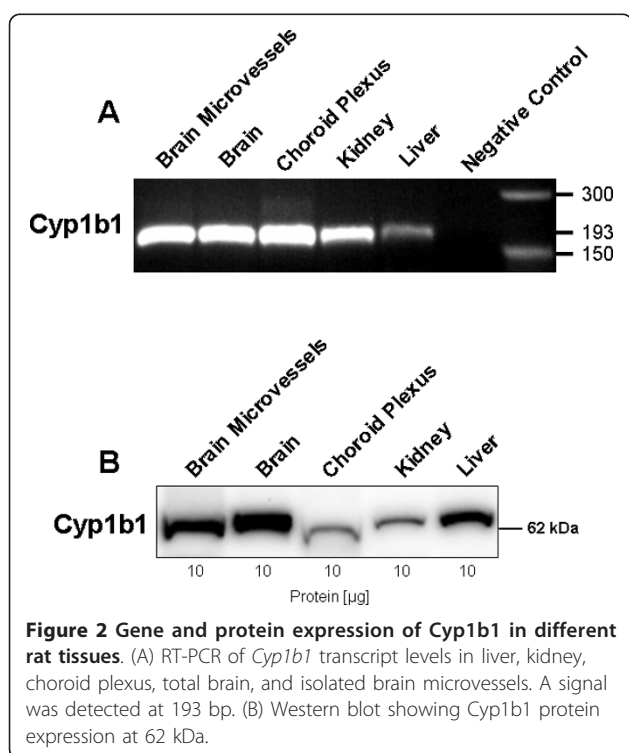
To test the effect of TCDD on *Cyp1a1* and *Cyp1b1* *in vivo*, we dosed animals with TCDD (one dose of 25 μ g/kg i.p.) and measured *Cyp1a1* and *Cyp1b1* transcript levels in isolated brain microvessels with q-PCR at 72 h after TCDD administration. TCDD substantially increased *Cyp1b1* and *Cyp1a1* mRNA levels in isolated brain microvessels by 22-fold and 260-fold, respectively (Figure 3B). In some vehicle-treated samples, *Cyp1a1* transcripts were not detectable and relative expression was therefore calculated using the lowest standard calibration curve.

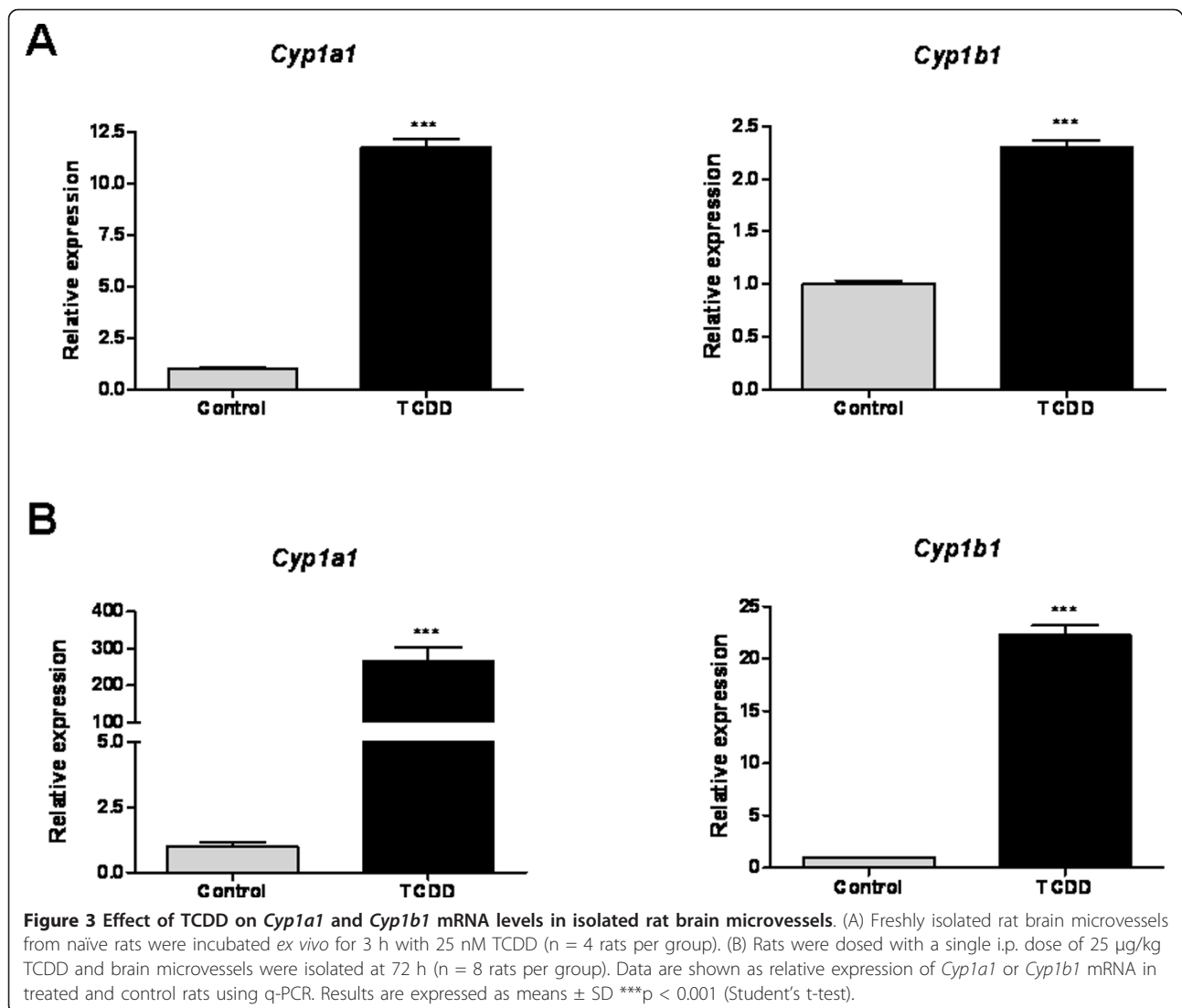
Δ^9 -THC has been discussed as a potential AhR ligand due to its ability to induce *Cyp1a1* expression in a murine hepatoma cell line [17]. Therefore, we studied whether sub-chronic treatment with Δ^9 -THC induces *Cyp1b1* and *Cyp1a1* in rat brain microvessels. Importantly, Δ^9 -THC did not change *Cyp1b1* expression (1.0 ± 0.12 in untreated rats *versus* 1.1 ± 0.18 in Δ^9 -THC-treated rats, $n = 12$ per group, data not shown). *Cyp1a1* transcripts were not quantified in brain microvessels from control- and Δ^9 -THC-treated rats.

Since TCDD strongly increased mRNA expression of *Cyp1b1* and *Cyp1a1* in isolated brain microvessels, we measured *Cyp1a1* protein levels 72 h after TCDD administration. *Cyp1a1* protein was not detected in either TCDD-treated or untreated rats. However, *Cyp1a1* was present in rat supersomesTM (positive control) at the correct molecular weight (59 kDa; Figure 4). In contrast, 12 h (Figure 5A) and 72 h (Figure 5B) after TCDD dosing, *Cyp1b1* expression was increased by $80 \pm 13\%$ and $100 \pm 8\%$ (SEM) respectively in TCDD-treated rats compared to control rats.

Effect of DEP exposure on *Cyp1b1* expression in isolated rat brain microvessels

Since *Cyp1a1* was not detected at the protein level after TCDD exposure, we focused on AhR-dependent regulation of *Cyp1b1* in the following studies. Diesel exhaust particles (DEP) are a real environmental toxicant that billions of people are exposed to on a daily basis [18]. Once inhaled, DEP can enter the circulation and translocate to tissues throughout the body. Through this route, DEP can reach the brain microvasculature and studies showed that DEP even enter the brain [29-31]. DEP consist of a carbon core with adsorbed organic chemicals such as PAHs and HAHs that are known AhR activators [18,19]. Thus, in addition to the DEP itself, the AhR-activating chemicals that are adsorbed to the carbon core also enter the blood-stream and reach the blood-brain barrier where they affect expression of

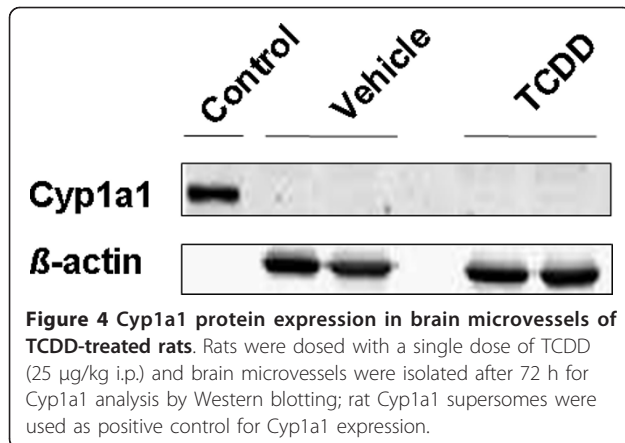




proteins [27]. Here we show that exposing rat brain microvessels to DEP for 6 h *ex vivo* increased Cyp1b1 protein in a concentration-dependent manner up to $310 \pm 16\%$ (SEM) of controls at 200 µg/ml DEP (Figure 6A; data are means from 6 densitometric measurements; brain capillaries used for western blots were pooled from 10 rats). In contrast, carbon black mock particles that are used as a negative control for particulate matter did not cause such an increase in Cyp1b1 protein in brain microvessels (Figure 6B). Note that the difference in band intensities for the controls in Figures 6A and 6B is not due to different expression levels, but to different exposure times. Figure 6C shows that Cyp1b1 protein expression was also significantly increased in brain microvessels from rats that were exposed to 0.5 and 2 mg/m³ diesel engine exhaust containing DEP for 5 h/day, 5 days/week for 4 consecutive weeks (0.5 mg/m³: $447 \pm 26\%$, 2 mg/m³: $538 \pm 40\%$ of controls; data are

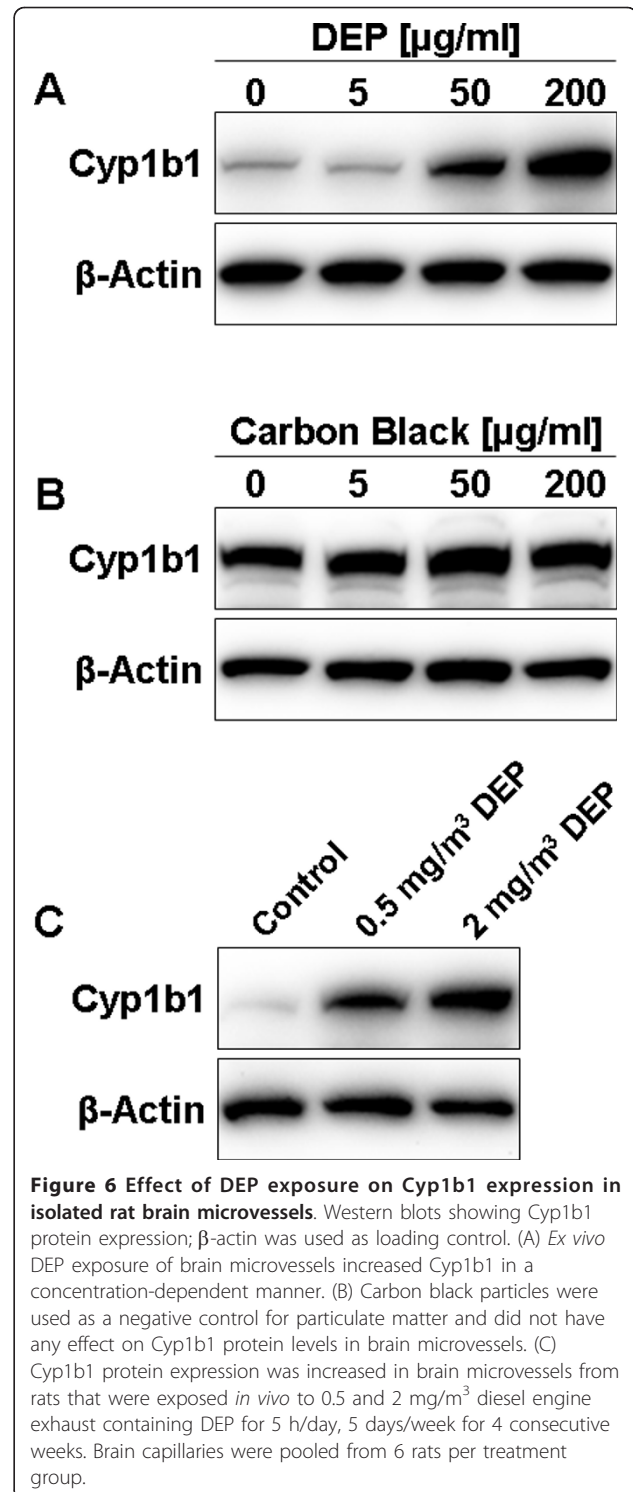
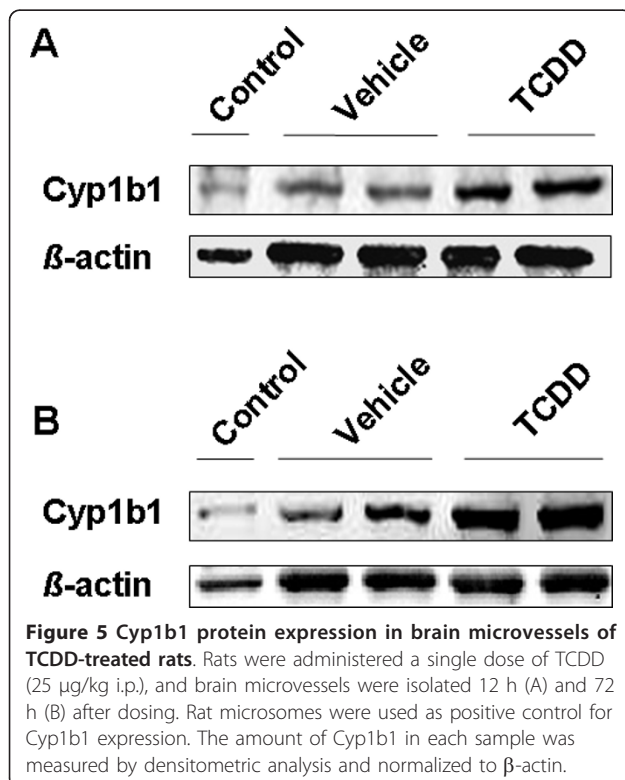
means \pm SEM from 5 densitometric measurements; brain capillaries used for western blots were pooled from 6 rats per treatment group). Note that these DEP doses are based on DEP concentrations found in ambient air during heavy traffic [25,26].

We also addressed the mechanism of DEP-mediated Cyp1b1 induction in rat brain microvessels. Inhibiting transcription with actinomycin D or inhibiting protein synthesis with cycloheximide (CHX) abolished DEP-mediated upregulation of Cyp1b1 protein expression in isolated rat brain microvessels (Figures 7A and 7B). Importantly, the use of the AhR antagonist CH-223191 blocked this DEP-mediated Cyp1b1 induction in brain microvessels (Figure 7C), indicating that this effect is AhR-dependent. Thus, these data demonstrate that DEP induces Cyp1b1 in brain microvessels *ex vivo* and *in vivo* and that this effect is likely to be AhR-dependent.

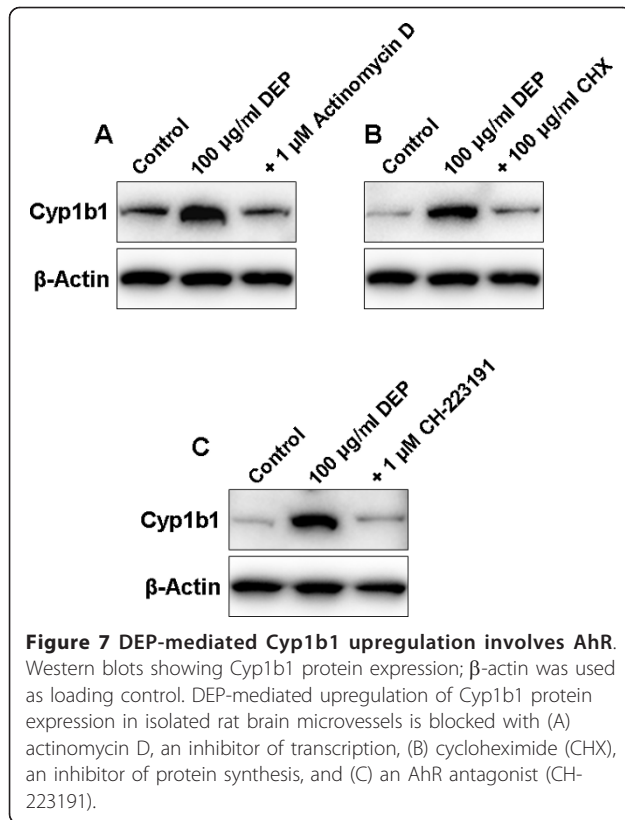


Discussion

In peripheral tissues such as liver, CYP enzymes are regulated by the same transcription factors including PXR, CAR and AhR [2]. However, few studies exist on the regulatory pathways of these enzymes at the BBB. Recently, we demonstrated that AhR transcripts are abundant in human brain microvessels [1] and that TCDD, the highly potent AhR ligand, strongly upregulated *CYP1A1* and *CYP1B1* in hMEC/D3 cells, a promising *in vitro* model of the human BBB [11]. Based on these findings, we tested if AhR pathway is involved *in vivo* in the regulation of Cyp1b1 in rat brain



microvessels. The present results show for the first time, AhR expression and immunostaining in rat brain microvessels in addition to Cyp1b1 upregulation after *in vivo* and *ex vivo* exposure to well-known AhR ligands: TCDD and heavy hydrocarbons-containing DEP. In this



way, rat brain microvessels are a critical target of AhR environmental pollutant ligands.

We first focused on AhR expression and localisation in isolated rat brain microvessels. AhR was highly expressed at both mRNA and protein level in rat brain microvessels. These findings are in accordance with the study of Filbrandt *et al.* showing that isolated murine cerebral vascular endothelial cells express abundant level of AhR protein [14]. By immunostaining, we localised AhR protein within the cytoplasm of rat brain microvessel endothelial cells corresponding to the predominant distribution of inactive (not ligand-bound) AhR [9]. Regarding Cyp1b1, expression of this enzyme at the BBB is controversial. Cyp1b1 was not detected at the mouse BBB but was immunolocalised in other BBB interfaces like the leptomeninges [12]. Furthermore, basal protein expression was undetected in primary murine vascular endothelial cells [14] whereas CYP1B1 has been localised in human brain capillaries by immunohistochemistry [32]. Here we found that Cyp1b1 is constitutively expressed in different extrahepatic rat tissues including brain and especially in brain microvessels. Our western blot analysis of Cyp1b1 expression showed that the apparent molecular weight slightly differed depending on tissue samples suggesting that transcriptional and/or post-transcriptional/translational events

might affect Cyp1b1 molecular weight. To our knowledge, alternatively Cyp1b1 spliced variants have not been described in the literature in contrast to Cyp1a1 ones reported in human brain [33].

Thus, like in human BBB, rat brain microvessels express both Cyp1b1 and AhR, being therefore a suitable model to assess AhR regulation pathway *in vivo* at this interface.

We then studied TCDD effect on AhR-target gene *Cyp1b1* expression in rat brain microvessels. Here, we report that Cyp1b1 was strongly induced by TCDD as early as 3 h after exposing *ex vivo* rat brain microvessels to 25 nM TCDD. In *in vivo* experiments, TCDD induced an early and sustained upregulation of Cyp1b1 protein expression in rat brain microvessels. In fact Cyp1b1 protein expression induction occurred at 12 h post-injection and was maintained throughout 72 h. Our results are consistent with a very recent study showing that acute TCDD treatment upregulates Cyp1b1 at the rat BBB 48 hours post-injection [34]. In our study, *in vivo* Cyp1b1 induction happened earlier (12 h after TCDD single dose) allowing less time for potential indirect mechanisms to occur.

TCDD also induced Cyp1a1 expression at the mRNA level *ex vivo* and *in vivo*. Despite high induction, we could not detect Cyp1a1 protein in isolated brain microvessels from control or TCDD-treated rats. By qPCR we found in brain microvessels from naïve rats substantially lower *Cyp1a1* mRNA levels compared to *Cyp1b1* mRNA levels (60-fold difference). Thus, *Cyp1a1* mRNA is barely detectable, which is consistent with findings in human brain microvessels [1]. Consequently, Cyp1a1 protein was also undetectable in rat brain microvessels.

However, Wang *et al.* recently detected Cyp1a1 protein expression in isolated rat brain microvessels which was increased by TCDD [34]. This suggests that discrepancies between studies may come from different technical approaches. However, using ultracentrifugation Wang *et al.* isolated brain microvessel crude membranes that include those of the endoplasmic reticulum where Cyp1a1 is localised, thus allowing Cyp1a1 enrichment and subsequent detection by Western blotting.

Δ^9 -THC has been reported to increase *Cyp1a1* mRNA in murine Hepa-1 cells through AhR pathway [17]. In the present study, we did not observe induction in *Cyp1a1* and *Cyp1b1* expression in rat brain microvessels after sub-chronic Δ^9 -THC treatment, which is consistent with our previous results showing an absence of *CYP1A1* and *CYP1B1* induction after Δ^9 -THC exposure of the human cerebral endothelial cell line hCMEC/D3 [11]. This discrepancy with experiment of Roth *et al.* [17], may be due to inter-species differences or to a difference in the dosing range. Indeed, pharmacokinetic studies showed that after sub-chronic Δ^9 -THC

treatment of rats, plasma concentrations of Δ^9 -THC were in the 2-4 ng/mL range [35], which is close to those plasma concentrations observed in humans cannabis abuser [36] and about three orders of magnitude lower than the Δ^9 -THC concentrations needed to induce Cyp1a1 *in vitro* [17]. Through marijuana consumption, users are exposed to psychoactive cannabinoids and also to toxicant-containing tar such as PAHs [37]. These AhR ligands have been reported to occur at higher levels in marijuana than in tobacco-tar [38]. Thus, even if Δ^9 -THC does not activate AhR in rats *in vivo*, PAHs-containing marijuana tar could activate AhR. Given the widespread use of marijuana around the world, further experiments are needed to assess potential AhR activation by marijuana tar at the BBB.

DEP are complex environmental toxicants consisting of a central core of elemental carbon and adsorbed organic compounds such as PAHs and HAHs [18,19]. Here we observed that a chronic inhalation exposure to DEP strongly upregulated Cyp1b1 expression *in vivo* in rat brain microvessels. *Ex vivo*, DEP also induced Cyp1b1 expression in rat brain microvessels. Furthermore, this effect was abolished by blocking transcription or translation or by using an AhR antagonist, strongly suggesting that DEP-mediated Cyp1b1 upregulation is triggered by AhR activation.

AhR involvement in toxicological responses and carcinogenesis has been widely studied in the last decades. However, increasing evidence suggests that AhR is not only a cellular sensor of environmental pollutants but also a key regulator of physiological functions. In fact, AhR is involved in reproduction, immunity, and a wide variety of basic cellular processes including proliferation, migration, adhesion, and differentiation [9,10]. In addition, AhR^{-/-} mice display abnormal vascular structures in kidney, liver, and eyes, which suggests that AhR is involved in vascular development [39]. Recently, the highly potent AhR ligand TCDD has been shown to decrease cerebral blood flow and alter brain vessel morphology in developing zebrafish, suggesting that TCDD through AhR activation may alter brain vascular physiology [40]. Regarding the physiological function of the Cyp1b1 enzyme, Cyp1b1^{-/-} mice exhibit no apparent abnormalities except in their anterior eye segment similar to what has been reported for patients with primary congenital glaucoma [15]. Interestingly, primary cultured retinal endothelial cells from Cyp1b1^{-/-} mice lose their ability to undergo capillary morphogenesis [41], suggesting that Cyp1b1 is involved in angiogenesis. Furthermore, Cyp1b1 metabolises several endogenous compounds like retinol, estrogens, and arachidonic acid [7], which is turned into epoxyeicosatrienoic acid that has vasodilatory properties [42]. Interestingly, 17- β -estradiol, a substrate of CYP1B1, increased the expression of vasoactive factors in HUVEC [43]. Taken together, these data

indicate that both AhR and Cyp1b1 could be key elements in the development and/or maintenance of a functional BBB.

Besides, Cyp1b1 is known to bioactivate procarcinogens such as PAHs into more reactive metabolites so that its induction may alter the BBB [16]. Through activating AhR, PAHs induce CYP1B1 expression and in turn promote their own activation into carcinogens. Therefore, DEP induction of CYP1B1 at the BBB may bioactivate PAHs adsorbed to DEP into genotoxic intermediates [44,45] that could increase levels of potentially harmful chemicals, which may initiate BBB dysfunction and/or brain disease.

Conclusion

The present study has shown Cyp1b1 upregulation by environmental pollutants TCDD and DEP *in vivo* in rat brain microvessels. Since BBB is likely to be a target of AhR environmental pollutant ligands, further experiments are required to assess the role AhR/Cyp1b1 play at the BBB, especially with regard to their impact in toxicological and/or pathophysiological processes. It will therefore be critical to explore whether the AhR/Cyp1b1 in the brain microvessel endothelium is beneficial or harmful to BBB function.

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Authors' contributions

AJ and AH have carried out all the experiments and written the manuscript. SY and SP have been involved in drafting the manuscript. XC, BB, and JMS have been involved in critically revising the manuscript. XD conceived the design of the study and coordinate drafting the manuscript. All authors have read and approved the final version of this manuscript.

Competing interests

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