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Aryl hydrocarbon receptor upregulates *IL-1 β* expression in hCMEC/D3 human cerebral microvascular endothelial cells after TCDD exposure.

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Abstract:

The AhR is a cytosolic ligand-dependent transcription factor activated by both endogenous and exogenous chemicals. It can regulate expression of many target genes including some inflammatory cytokines and chemokines. To date AhR implication in the regulation of inflammatory cytokines and chemokines at human cerebral endothelium has not been addressed. In the present study, we investigated whether AhR could regulate the expression of two pro-inflammatory cytokines and one chemokine *i.e* *IL-1 β* , *IL-6* and *IL-8* in the hCMEC/D3 human cerebral microvascular endothelial cell line. Exposure to TCDD increased *IL-1 β* mRNA expression levels in hCMEC/D3. By using small interfering RNA against AhR we demonstrated that TCDD effects on *IL-1 β* expression were mediated through AhR activation. Regarding *IL-6* and *IL-8*, TCDD exposure had little or no effect on their mRNA levels in hCMEC/D3. In conclusion, our findings suggest that AhR-mediated *IL-1 β* regulation in cerebral endothelium could induce BBB breakdown and contribute to the pathogenesis of a variety of neurologic disorders.

Keywords:

Aryl hydrocarbon receptor, blood-brain barrier, hCMEC/D3, pro-inflammatory cytokines

1. Introduction

The blood-brain barrier (BBB) is the major surface of exchanges between the blood and the brain parenchyma. The primary function of the BBB is the maintenance of brain homeostasis by specifically and actively controlling exchanges of water, macronutrients, vitamins, ions and trace elements between the blood and the cerebral extracellular fluid. In addition, BBB effectively protects the brain from exposure to many xenobiotics, thanks to a strong expression of efflux transporters at both sides of brain microvessels. The BBB is mainly composed of brain microvascular endothelial cells differing from their peripheral homologs by the presence of continuous intercellular tight junctions and covered by a basal membrane wherein pericytes are embedded and on which are projected the feet of astrocytic and neuronal endings (Neuwelt *et al.*, 2008).

The integrity of BBB is essential to maintain brain physiological functions. Breakdown of the BBB observed in many brain disorders such as brain tumors, neurodegenerative diseases and infectious diseases of the central nervous system (meningitis and encephalitis) has devastating effects on brain function. Excessive release of certain neurotransmitters, pro-inflammatory cytokines, hormones and endotoxins observed during CNS pathological processes are known to increase the permeability of the BBB. For instance, pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α decreased the in vitro transendothelial electrical resistance of rat and human brain endothelial cells and increased the paracellular permeability of hydrophilic compounds by decreasing tight junction proteins expression (de Vries *et al.*, 1996; Forster *et al.*, 2008). Pro-inflammatory cytokines are synthesized during pathological processes by numerous lymphoid cells but also by non-lymphoid cells. Interestingly, brain microvascular endothelial cells, astrocytes and microglial cells can produce pro-inflammatory cytokines and therefore participate to the inflammatory response in the CNS (Ashraf *et al.*, 2014; Chakraborty *et al.*, 2010; Ronaldson and Bendayan, 2006). Release of pro-inflammatory cytokines by pig and mouse brain microvascular endothelial cells has indeed been demonstrated after exposure to diverse stimuli such as *Streptococcus suis*, metallic nanoparticles or lipopolysaccharide (LPS), respectively (Trickler *et al.*, 2014; Vanier *et al.*, 2009; Verma *et al.*, 2006).

In peripheral tissues, production of some cytokines has been linked to the aryl hydrocarbon receptor (AhR) signaling pathway (Fardel, 2013). The AhR is a cytosolic ligand-dependent transcription factor belonging to the bHLH-PAS (basic helix-loop-helix/Per-Arnt-Sim) superfamily and activated by both endogenous and exogenous chemicals. **In search for potential endogenous AhR ligands, diverse compounds have been identified in particular tryptophan derivatives (FICZ, kynurenin), bilirubin or low density lipoproteins (Opitz**

et al., 2011) Barouki et al., 2012). The best characterized exogenous ligands for the AhR include a wide variety of ubiquitous environmental contaminants such as halogenated aromatic hydrocarbons like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, Seveso dioxin) or polycyclic aromatic hydrocarbons (PAHs) like benzo(a)pyrene (Denison et al., 2011). The AhR has been historically linked to the up-regulation of xenobiotic-metabolizing enzymes expression, especially the cytochromes P450 (CYP450) of the CYP1 family, e.g. CYP1A1/1B1/1A2. However, the AhR pathway has many other physiological functions including embryonic development, cell migration or cell cycle regulation (Barouki et al., 2007; Tomkiewicz et al., 2013). More recently, the AhR has been shown to be involved in immune/inflammatory mechanisms. This notably concerns T-lymphocyte differentiation and inflammatory cytokines and chemokines secretion (Esser et al., 2009; Fardel, 2013).

High levels of *AhR* transcripts have previously been reported in both isolated human brain cortex microvessels and in the hCMEC/D3 human cerebral microvascular endothelial cell line (Dauchy et al., 2008; Dauchy et al., 2009), an *in vitro* model of the human cerebral endothelium (Weksler et al., 2005). The AhR pathway was also demonstrated actively efficient in regulating the expression of its historically well-known target genes *CYP1A1/1B1* in hCMEC/D3 cells (Jacob et al., 2015b). To date, to the best of our knowledge, the role of AhR in the regulation of pro-inflammatory cytokines and chemokines in human cerebral endothelium has not been investigated. In the present study, we investigated whether AhR could regulate the expression of two pro-inflammatory cytokines and one chemokine *i.e* *IL-1 β* , *IL-6* and *IL-8*, in the hCMEC/D3 human cerebral microvascular endothelial cell line.

2. Materials and methods

2.1 Reagents and equipments

The human cerebral microvascular endothelial cells/clone D3 (hCMEC/D3) were grown in EBM-2 medium (Lonza, Switzerland) supplemented with ascorbic acid, hydrocortisone, basic FGF (Sigma-Aldrich, France), foetal bovine serum (PAA, France) and hepes (Invitrogen, France). Cells were grown on 6-well plates (Corning, France) coated with rat tail collagen type-I (BD Biosciences, France). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) was obtained from LGC Promochem (Molsheim, France). Control-siRNA (Neg. siRNA AF 488, reference 1027284), SiRNA for AhR (Hs_AHR_5, reference SI02780148), HiPerFect transfection reagent and RNA extraction kits were purchased from Qiagen (Qiagen, Germany). RT-PCR reagents were purchased from Invitrogen (Invitrogen, France). Primers were synthesized by Invitrogen Life Technologies (Invitrogen, France). The Power SYBR Green PCR Master Mix was from Applied Biosystems (Applied Biosystems, France) and the Absolute QPCR SYBR Green ROX Mix from Abgene (Thermofisher scientific, France). Equipment used: a nucleic acid spectrophotometer (Nanodrop ND-1000, NanoDrop Technologies, USA), a programmable thermal cycler (PTC-100 programmable thermal controller, MJ research Inc., USA) and a 7900 HT Real-Time PCR Detection System (Applied Biosystems, Foster City, CA).

2.2 Cell culture

The hCMEC/D3 cells were cultured between passages 27 to 34 in 6-well plates coated with type I collagen and grown at 37°C in a humidified atmosphere of 95 % air: 5% CO₂. When cells reached confluency, they were incubated in culture medium with or without 5 or 25 nM TCDD in DMSO (0.016% v/v) for 24 h and up to 72h for cell viability assays as previously described (Jacob et al., 2015b).

2.3 Cell viability

Viability of hCMEC/D3 cells in the presence or absence of TCDD over a period of 72 h was evaluated by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT) assay as previously described (Chan et al., 2011). Following exposure to 25 nM TCDD for 72 h, cells were rinsed with warm PBS and then incubated in PBS containing 2.5 mg/mL MTT at 37 °C for 2 h. The reduced insoluble MTT was collected in DMSO and aliquoted into 96-well plates. Cell viability was assessed by comparing the absorbance (580 nm) of reduced MTT in TCDD-treated cells with that measured in control cells using SpectraMax 384 microplate

reader (Molecular Devices, Sunnyvale, CA). Cell viability in the presence of TCDD was calculated as a percent of control cells.

2.4 RNA interference

Once cells (2.5×10^5 per well) attached on 6-well plates for at least 2 hours, they were transfected with 10 nM control-siRNA or 10 nM AhR-siRNA using the HiPerFect transfection reagent according to the manufacturer's protocol. Forty eight hours after cell transfection, cells were then incubated for 24 h in culture medium with or without 25 nM TCDD.

2.5 RNA extraction and reverse transcription

Total RNA was extracted from cells using the RNeasy Plus Mini kit according to the manufacturer's instructions. 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. Total RNA (500 ng) was reverse transcribed into cDNA in a final volume of 20 μ L. The mixture consisted of 500 ng total RNA, 500 μ M of each dNTP, 10 mM DTT, 1.5 μ M 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 and quick-chilling to 4°C. cDNA were stored at -80°C.

2.6 Quantitative RT-PCR (qPCR)

PCR reactions were performed using SYBR Green fluorescence detection on a 7900 HT Real-Time PCR Detection System (Applied Biosystem, France) as previously described (Jacob et al., 2015b). The PCR reaction mixture consisted of 5 μ L Power SYBR Green PCR Master mix® (Applied Biosystem, France) and 300nM of each forward and reverse primers in a final volume of 10 μ l. Primers were designed using OLIGO 6.42 software (Medprobe, Norway) are presented in Table 1. Gene expression was evaluated using the Ct value from each sample. mRNA levels of a target gene was considered to be quantifiable when the Ct obtained for the 1/20 dilution of pure cDNA sample was lower than 31. A Ct value of 33 was taken as the detection limit. The efficacy of each PCR for all genes tested in this study was better than 95% making it possible to use the $\Delta\Delta$ Ct method for quantification of genes expression changes (Livak and Schmittgen, 2001; Pfaffl, 2001). The gene encoding TATA-box binding protein (*TBP*) was used as an endogenous reference for normalizing target gene

mRNA in each sample as follow: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$. An internal calibrator was also employed in each assay to compensate inter-PCR variations. The comparative $\Delta\Delta Ct$ parameter was calculated by the following formula:

$$\Delta\Delta C_t = (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{sample}} - (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{calibrator}}$$

For each sample, the $2^{-\Delta\Delta Ct}$ value was calculated and represents the fold change in gene expression normalized to the reference gene and relative to the internal calibrator. **For graphical representations, the $2^{-\Delta\Delta Ct}$ values of control-samples were arbitrarily defined as 1 so that $2^{-\Delta\Delta Ct}$ values of treated-samples directly indicate fold-changes in the considered gene expression relative to control-samples.**

2.7 Statistical analysis

Statistical analyses were performed applying Student's *t*-test for two group comparison or by one-way analysis of variance (ANOVA) followed by the post-hoc Tukey test for multiple comparisons. Data were analysed with GraphPad Prism® 4.0 software (GraphPad Software Inc., San Diego, CA, USA) and statistical significance was set at $p < 0.05$.

3. Results

3.1 Viability of hCMEC/D3 cells exposed to TCDD

The viability of hCMEC/D3 cells in the presence of TCDD was evaluated using a standard MTT assay. No significant reduction in cell viability following exposure to 25 nM TCDD for 72 h incubation was observed ($p > 0.05$, **Fig. 1**).

3.2 Effect of TCDD on pro-inflammatory cytokines expression in hCMEC/D3 cells

We investigated the effects of TCDD, a potent AhR ligand, on the expression of *CYP1A1*, *IL-1 β* , *IL-6* and *IL-8* using two different TCDD concentrations (5 and 25 nM) in hCMEC/D3 cells for 24 h. *CYP1A1* has been chosen as a control target gene of AhR upon AhR activation. Our qPCR analysis revealed that exposure to 5 nM or 25 nM TCDD for 24 h significantly increased by 6.7- and 8.6-fold the mRNA levels of *CYP1A1* in hCMEC/D3 cells, respectively. Regarding *IL-1 β* , its mRNA levels were increased by 1.9- and 4.9-fold in hCMEC/D3 cells exposed to 5 nM or 25 nM TCDD, respectively, with statistical significance reached at 25 nM only. TCDD had a lesser effect on *IL-6* mRNA levels: incubation for 24 h with 25 nM TCDD slightly increased *IL-6* mRNA level by 1.4-fold. TCDD exposure had no significant effect on *IL-8* mRNA levels at the concentrations and time of incubation tested (**Fig. 2**).

3.3 Effect of AhR knockdown on *IL-1 β* expression in hCMEC/D3 cells

As *IL-1 β* mRNA levels were increased with TCDD, the involvement of AhR in this TCDD-mediated increase in *IL-1 β* mRNA levels was further evaluated using small interfering RNA against AhR (AhR-siRNA). Transfected cells and untransfected cells were exposed for 24 h to control conditions or to 25 nM TCDD and the levels of *CYP1A1* (as a control gene targeted by AhR activation) and *IL-1 β* transcripts were quantitated by qPCR (**Fig. 3**). Transfection with control-siRNA or with transfection reagent alone had no effect neither on *CYP1A1* nor on *IL-1 β* expression ($p > 0.05$, data not shown). **However, as expected, transfection with the AhR-siRNA abolished the TCDD-mediated increase in *CYP1A1* mRNA levels in hCMEC/D3 (Fig. 3A).** Similarly, TCDD exposure had no effect on *IL-1 β* expression in cells previously transfected with AhR-siRNA (**Fig. 3B**). These data demonstrated that the increase in *IL-1 β* mRNA levels was mediated through AhR activation in hCMEC/D3 cells.

4. Discussion

The aryl hydrocarbon receptor (AhR) has been investigated in a wide range of human cells or tissues as a key element involved in the inflammatory response and notably through its ability to regulate pro-inflammatory cytokines and/or chemokines expression (Fardel, 2013). Such implication of AhR in the human brain has been much less studied, especially in the human cerebral endothelium, where its expression was evidenced only recently (Dauchy et al., 2008).

Since the hCMEC/D3 cell line expresses AhR (Dauchy et al., 2009; Jacob et al., 2015a) and can release pro-inflammatory cytokines under stimulation (Duperray *et al.*, 2015), we chose this *in vitro* human cerebral endothelium model to investigate the role of AhR pathway in the regulation of two pro-inflammatory cytokines and one chemokine *i.e* *IL-1 β* , *IL-6* and *IL-8* using TCDD as a potent AhR ligand.

We obtained clear evidence that TCDD increased *IL-1 β* mRNA expression levels in hCMEC/D3. These results are in agreement with previous *in vitro* studies showing that AhR agonists induced *IL-1 β* mRNA expression in various human cell types including keratinocytes (Henley et al., 2004), synoviocytes (Tamaki *et al.*, 2004), macrophages (N'Diaye et al., 2006; Sparfel et al., 2010) and umbilical vein endothelial cells (Yu *et al.*, 2015). This increase was abolished by knocking-down AhR, showing that AhR was indeed involved in the up-regulation of *IL-1 β* expression in the presence of the AhR ligand, TCDD. Our data strongly suggest that *IL-1 β* can serve a AhR target gene in the cerebral endothelium.

Regarding *IL-6* and *IL-8*, TCDD exposure had little or no effect on their mRNA levels in hCMEC/D3. These results are in contrast with a previous study reporting induction of *IL-8* and *IL-6* expression in various cell types following exposure to AhR agonists ; for a recent review we refer the reader to (Fardel, 2013). This discrepancy could be due to a cerebral endothelium specificity or to hCMEC/D3 cell line model limitations.

Taken together, our results provide the first evidence that environmental contaminants such as TCDD induce expression of the pro-inflammatory cytokine *IL-1 β* in an AhR dependent manner in the human cerebral endothelial hCMEC/D3 cell line. Even if little doubt remains regarding AhR implication in inflammatory processes, the exact mechanisms involved remain unclear. Recent data strongly support that the inflammatory effects of ligand activated-AhR are complex and multiple, involving both genomic and non genomic pathways (Fardel, 2013; Matsumura, 2009). Thus the observed modulation of *IL-1 β* expression might occur either through the classical action mode of ligand-activated AhR leading to direct activation of its target gene or through non-classical mechanisms such as AhR crosstalk with other signalling pathways (Denison et al., 2011; Puga et al., 2009). Further investigations will be necessary **to first confirm this preliminary result,**

notably at the protein level, and second to elucidate the exact mechanism underlying *IL-1 β* induction through the AhR pathway in hCMEC/D3 cell line.

Further work is also needed to identify the possible consequences of AhR-mediated IL-1 β regulation in BBB integrity and functionality. IL-1 β has been reported to induce BBB disruption by downregulating tight junction protein expression (Argaw et al., 2006) and by altering drug efflux transporter expression and activity (Poller et al., 2010). BBB breakdown is a common pathological feature in numerous neurologic diseases associated with inflammation such as multiple sclerosis, Parkinson's disease, and Alzheimer's disease. Our findings suggest that AhR-mediated IL-1 β regulation in cerebral endothelium could induce BBB breakdown and contribute to the pathogenesis of a variety of neurologic disorders.

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Disclosures

None

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Figure Legends

Fig 1: Viability of hCMEC/D3 cells exposed to TCDD.

Cells were incubated in control medium or in medium containing 25nM TCDD for 72h and cell viability was assessed by a standard MTT assay. Results are expressed as mean \pm SD of three independent experiments performed in triplicate ($p > 0.05$, unpaired Student's *t*-test).

Fig 2: Effect of TCDD on *CYP1A1*, *IL-1 β* , *IL-6* and *IL-8* expression in hCMEC/D3.

Cells were incubated in control medium or in medium containing 5 or 25 nM TCDD for 24h. Results are expressed as fold-changes in genes expression in TCDD-treated cells as compared to control cells, with control cells assigned an arbitrary value of 1. Results are means \pm SD of two independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way analysis of variance and Tukey's multiple comparison post-test).

Fig 3: Effect of AhR knockdown on *CYP1A1* (A) and *IL-1 β* (B) expression in hCMEC/D3.

Untransfected cells and cells previously transfected with 10nM AhR-siRNA were incubated in control medium or in medium containing 25 nM TCDD for 24h. Data are shown as fold-changes in *CYP1A1* (A) and *IL-1 β* (B) gene expression as compared to control cells (i.e untransfected and untreated cells), with control cells assigned an arbitrary value of 1. Results are means \pm SD of two independent experiments performed in duplicate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS not significant, one-way analysis of variance and Tukey's multiple comparison post-test).