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MECHANISMS OF TISSUE FACTOR INDUCTION BY THE UREMIC TOXIN INDOLE-3 ACETIC ACID THROUGH ARYL HYDROCARBON RECEPTOR/NUCLEAR FACTOR-KAPPA B SIGNALING PATHWAY IN HUMAN ENDOTHELIAL CELLS

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ABSTRACT

Chronic kidney disease (CKD) is associated with high risk of thrombosis. Indole-3 acetic acid (IAA), an indolic uremic toxin, induces the expression of tissue factor (TF) in human umbilical vein endothelial cells (HUVEC) via the transcription factor aryl hydrocarbon receptor (AhR). This study aimed to understand the signaling pathways involved in AhR-mediated TF induction by IAA.

We incubated human endothelial cells with IAA at 50 μ M, the maximal concentration found in patients with CKD. IAA induced TF expression in different types of human endothelial cells: umbilical vein (HUVEC), aortic (HAoEC), and cardiac-derived microvascular (HMVEC-C). Using AhR inhibition and chromatin immunoprecipitation experiments, we showed that TF induction by IAA in HUVEC was controlled by AhR, and that AhR did not bind to the TF promoter. The analysis of TF promoter activity using luciferase reporter plasmids showed that the NF- κ B site was essential in TF induction by IAA. In addition, TF induction by IAA was drastically decreased by an inhibitor of the NF- κ B pathway. IAA induced the nuclear translocation of NF- κ B p50 subunit, which was decreased by AhR and p38MAPK inhibition. Finally, in a cohort of 92 CKD patients on hemodialysis, circulating TF was independently related to serum IAA in multivariate analysis.

In conclusion, TF up-regulation by IAA in human endothelial cells involves a non-genomic AhR/p38 MAPK/NF- κ B pathway. The understanding of signal transduction pathways related to AhR thrombotic/inflammatory pathway is of interest to find therapeutic targets to reduce TF expression and thrombotic risk in patients with CKD.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in patients with chronic kidney disease (CKD) (Go et al. 2004; Tonelli et al. 2006). Thrombosis is a key event in CVD; and CKD is associated with an increased thrombotic risk (Daneschvar et al. 2008; Wattanakit and Cushman 2009; Carney 2016). In patients with CKD, the balance of the coagulation system is dysregulated, with higher levels of factor VII, factor VIII, thrombin and tissue factor (TF) (Jalal et al. 2010; Huang et al. 2017). Accumulation of some uremic toxins normally eliminated by the kidney is correlated with CVD and mortality in patients with CKD (Barreto et al. 2009; Moradi et al. 2013; Han et al. 2015; Storino et al. 2015). The uremic toxin indole-3 acetic acid (IAA) is an independent predictor of cardiovascular events and mortality in patients with CKD (Dou et al. 2015). IAA is a uremic indolic toxin derived from the metabolization of dietary tryptophan by the gut microbiota (Fernandez-Prado et al. 2017). Because of its protein-binding (Jourde-Chiche et al. 2009), IAA is poorly removed by dialysis (Neiryneck et al. 2013). In cultured endothelial cells, IAA promotes the expression of a proinflammatory phenotype (Gondouin et al. 2013; Dou et al. 2015). IAA also increases the endothelial expression and procoagulant activity of TF (Gondouin et al. 2013), the principal initiator of blood coagulation (Camerer et al. 1996; Ruf and Riewald 2013).

Tissue factor expression is induced by various stimuli, like pro-inflammatory cytokines, lipopolysaccharides (LPS), growth factors, or thromboxane A₂, via different transcription factors: NF- κ B, AP-1, NFAT or Egr-1 (Bode and Mackman 2014). Interestingly, we have described that indolic toxins induce TF expression by a new pathway: the aryl hydrocarbon receptor (AhR) pathway (Gondouin et al. 2013). AhR was initially described as a receptor of some environmental contaminants, especially 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Hankinson 1995; Mandal 2005); and IAA is an endogenous agonist of AhR (Heath-Pagliuso et

al. 1998). In resting cells, AhR forms a complex with HSP90, XAP2 and p23 in the cytoplasm (Heid et al. 2000; Petrusis and Perdew 2002). After ligand binding to AhR, the complex dissociates, resulting in AhR translocation into the nucleus and dimerization with the aryl hydrocarbon nuclear translocator (ARNT). The AHR/ARNT heterodimer binds to the XRE (Xenobiotic Response Element) sequences on the promoters of AhR target genes like *CYP1A1*, *CYP1A2*, or *CYP1B1* (Dolwick et al. 1993; Fujii-Kuriyama and Mimura 2005). In addition to this direct genomic pathway, a non-genomic inflammatory AhR pathway, independent of ARNT, has been described (Zhao et al. 2002; Sciallo et al. 2008; Matsumura 2009; Kim et al. 2012). AhR ligands, such as TCDD, induce the expression of proteins involved in inflammation like IL-6, RANTES, TNF, and COX-2 (Zhao et al. 2002; Sciallo et al. 2008; Matsumura 2009; Kim et al. 2012). In the non-genomic pathway, AhR acts as a signaling molecules that interacts with multiple signaling pathways (Borlak and Jenke 2008; Henklová et al. 2008; Ma et al. 2009; Puga et al. 2009a). We recently reported that a non-genomic pathway of AhR involving p38MAPK/NF- κ B signaling is crucial for the induction of endothelial COX-2 by IAA (Dou et al. 2015).

Until now, the mechanisms through which AhR controls TF expression in endothelial cells were poorly understood. The objective of the present work was therefore to determine the signaling pathways and the transcriptions factors involved in AhR-mediated TF induction by IAA.

METHODS

Endothelial cell culture

HUVEC were obtained from umbilical cord vein by collagenase digestion as described (Jaffe et al. 1973) and grown to the fourth passage in EGM-2 medium (Lonza, France) (containing 2% fetal bovine serum), under standard cell culture conditions (humidified atmosphere at 37°C, 5% CO₂). Human aortic endothelial cells (HAoEC) and cardiac-derived microvascular endothelial cells (HMVEC-C) were obtained from Lonza. HMVEC-C were grown to the fifth passages in EGM-2 MV medium (Lonza, France) (containing 5% fetal bovine serum) under standard cell culture conditions. HAoEC were grown to the fifth passage in EGM-2 medium under standard cell culture conditions.

Effect of the uremic toxin IAA

Cells were incubated in the presence of IAA (Sigma-Aldrich, France) at 50µM, the highest concentration described in uremic patients (Vanholder et al. 2003). They were treated during indicated times, with or without the AhR Inhibitor CH-229131 at 10µM (Sigma-Aldrich), the NF-κB inhibitor BAY 11-7082 at 10µM (Merck Chemicals), the PKC inhibitor Bisindolymaleimide I at 5µM (Merck Chemicals), the p38 inhibitor SB203580 at 10µM (Sigma-Aldrich), the ERK1/2 inhibitor PD98059 at 10µM (Sigma-Aldrich), and the transcription inhibitor Actinomycin D at 1µg/ml (Sigma-Aldrich). Because IAA was diluted in ethanol, ethanol 1/1000 was used as control. In some experiments, human serum albumin (LFB, France) was added in the culture medium, at the concentration found in human serum (4g/dL).

SiRNA knockdown of AhR

HUVEC were transfected with siRNA control (Negative Universal Control, Stealth™ RNAi, Life Technologies, France) or a pool containing three Silencer® Select siRNA directed against AhR

(1200, 1999 and 1998, Life Technologies, France) by magnetofection using SilenceMag beads (OZ Biosciences, France), according to the manufacturer's instructions. The knockdown of AhR was verified by western blot on cell extracts 48 hours after transfection (Supplemental Fig. 1). TF induction and p50 nuclear translocation were determined 48 hours after transfection.

RNA extraction and quantitative RT-PCR analysis of mRNA expression

Total RNA was extracted by an RNeasy mini-kit (Qiagen, France). RT was performed on 500ng of total RNA using the Takara PrimeScript™ RT reagent Kit (Ozyme, Saint Quentin en Yvelines, France) followed by qPCR on 25ng of cDNA using the Takara SYBR qPCR Premix Ex Taq (Ozyme). We quantified the following target genes: *TF*, *CYP1A1*, and *CYP1B1*. The housekeeping gene *HPRT* was used for normalization of the target gene values. The sequences of primers were as follows: TF forward: 5'TGCAGTAGCTCCAACAGTGC3', TF reverse: 5'GAGTGTATGGGCCAGGAGAA3'; CYP1A1 forward: 5'GACAGATCCCATCTGCCCTA 3', CYP1A1 reverse: 5'ATAGCACCATCAGGGGTGAG 3; CYP1B1 forward: 5' TGATGGACGCCTTTATCCTC 3', CYP1B1 reverse: 5' CCACGACCTGATCCAATTCT 3'; HPRT forward: 5'GGATTATACTGCCTGACCAAGGAAAGC 3', HPRT reverse: 5' GAGCTATTGTAATGACCAGTCAACAGG3'.

All PCR reaction efficiencies were determined with MxPro software (Agilent) and were always between 90% and 110%. The fusion curves were analyzed to assess the specificity of detected fluorescence. Fold change of mRNA expression versus control condition (ethanol) was calculated using the $2^{-\Delta\Delta Ct}$ method. The transcript for the housekeeping gene HPRT was used for data normalization.

Western blotting and densitometry analysis of Western blots

HUVEC were incubated with 50 μ M IAA, or with ethanol diluted 1/1000 (vehicle control) during 30 min, with or without the AhR Inhibitor CH-229131 at 10 μ M (Sigma-Aldrich). Some experiments were also performed on HUVECs transfected with AhR siRNA or control siRNA, and cells were stimulated with IAA 50 μ M forty-eight hours after transfection. Nuclear extracts were prepared using Cayman Chemical's Nuclear Extraction Kit (Interchim, France). Cytosolic extracts were prepared using 1ml of lysis buffer containing 20mM MOPS, 50mM β -glycerolphosphate, 50mM sodium fluoride, 1mM sodium orthovanadate, 5mM EGTA, 2mM EDTA, 1% NP40, 1mM dithiothreitol (DTT), 1mM benzamide, 1mM phenylmethanesulphonylfluoride (PMSF) and 10 μ g/mL leupeptin and aprotinin. The cells were incubated 10 min on ice and then collected with a scraper. The cytosolic extracts were obtained after centrifugation of cell lysates at 18,000 g for 15 min. Protein concentrations were measured with the Bicinchoninic Acid Kit (BCA1, Sigma-Aldrich).

Samples were mixed with LDS sample buffer and proteins were separated using 4-12% NuPAGETM protein gel (Life technologies). Proteins were transferred to nitrocellulose membrane and blocked with 5% skim milk for 1 h at room temperature. The membrane was incubated overnight at 4°C with antibodies directed against AhR (Santa Cruz, France), against NF- κ B p50 (Cell Signaling, Ozyme, France), or actin (Cell Signaling, France), and then with the secondary peroxidase-conjugated antibody (Beckman-Coulter, France). Revelations were done by chemiluminescence (ECL Western blotting substrate, Pierce). Gel images were captured using the Syngene GBox (Ozyme) and analyzed with the software *geneSys* (Ozyme).

Study of NF- κ B and AP-1 nuclear levels

After HUVEC incubation with 50 μ M IAA during 30 min, with or without the AhR Inhibitor CH-229131 at 10 μ M (Sigma-Aldrich), the NF- κ B inhibitor BAY 11-7082 at 10 μ M (Merck Chemicals), the PKC inhibitor Bisindolymaleimide I at 5 μ M, the p38 inhibitor SB203580 at

10 μ M (Sigma-Aldrich), nuclear extracts were prepared using Cayman Chemical's Nuclear Extraction Kit (Interchim, France). NF- κ B p50 was detected in nuclear extracts by Cayman Chemical's NF- κ B (human p50) combo transcription factor assay kit (Interchim). This kit is a 96-well ELISA with a specific double stranded DNA sequence containing the NF- κ B response element. NF- κ B p50 contained in nuclear extracts is detected with a specific primary antibody and a secondary antibody HRP-conjugated that provides a colorimetric readout à 450 nm.

Endothelial p65 expression in nuclear extracts was measured after 30 min incubation of HUVEC with IAA 50 μ M, and detected in nuclear extracts by the Cayman Chemical's NF- κ B (human p50/p65) combo transcription factor assay kit (Interchim).

AP-1 subunits c-Fos and c-Jun were detected in nuclear extracts by the AP1 (c-Fos/FosB/Fra1/c-Jun/JunB/JunD) transcription factor assay kit (Abcam, Paris, France).

Chromatin immunoprecipitation (ChIP) assay

HUVEC monolayers (6x10⁶ cells) were treated with IAA (50 μ M) or ethanol (vehicle) for 60 min. Cells were fixed by adding formaldehyde directly to the medium to a final concentration of 1% and incubated for 10 min at room temperature then 40 min at 4°C. ChIP assay was performed using the EZ-Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Merck Millipore, France), according to the kit instructions. Immunoprecipitations were performed overnight at 4°C with 5 μ g of rabbit IgG (control IgG) or rabbit polyclonal antibody against AhR (Santa Cruz Biotechnology, Tebu-Bio, France). Real-time PCR quantification of ChIP enrichments were run on a MX3000P instrument (Stratagene) using the Brilliant II SYBR Green QPCR Master Mix (Takara bio, France). Specific primer sequences for promoters were as follows: TF forward, 5'-GCCCTCCCTTCTGCCATAGA-3', TF reverse: 5'-CCTCCCGGTAGGAAACTCCG-3'; CYP1A1 forward: 5'-ACGCAGACCTAGACCCTTGC-3', CYP1A1

reverse: 5'-CGGGTGCGCGATTGAA-3'; CYP1B1 forward: 5'-ATATGACTGGAGCCGACTTTCC-3', CYP1B1 reverse: 5'-GGCGAACTTTATCGGGTTGA-3'. Fold enrichment was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct$ represents the difference between threshold cycles of experimental rabbit polyclonal antibodies against AhR over rabbit control IgG.

Transient transfection of HUVEC and Luciferase Promoter Assay

The human TF wild-type promoter (-227 hTF WT), the TF mutant AP-1 (-227 hTF mAP1) and the TF- κ B mutant (-227 hTF mTF- κ B) reporter plasmids in pGL2 basic were a gift from Nigel Mackman (Addgene plasmid # 15442, 15443, 15444 respectively).

HUVEC (1.5×10^5 cells) were plated into 6-well plates in EGM-2 medium 16 h before transfection. Cells were then incubated 1h before transfection with Opti-MEMTM reduced-serum medium (Gibco, Life Technologies, France). Transfection was performed using Lipofectamine 2000 (Life Technologies, France) associated with magnetofection using CombimagTM beads (OZ Bioscience, Marseille, France) according of the manufacturer's instructions. Briefly, reporter plasmids (1 μ g) were mixed with 2 μ l of Lipofectamine in 200 μ l of Opti-MEM reduced serum medium for 20 min then 1 μ l of CombimagTM beads was added to DNA-Lipofectamine complexes. The DNA-Lipofectamine-beads complexes were incubated for 20 min at room temperature, incubated with HUVEC for 1 h at 37 °C on a magnetic plate in the 5% CO₂ incubator and then replaced with growth medium. After 48h, HUVEC were treated with IAA or ethanol (vehicle control) for 6h. Cell were lysed in a reporter lysis buffer and lysates were assayed for luciferase activities using the luciferase assay system and the GloMax[®]-Multi detection system (Promega, France). The pSV- β -Galactosidase control vector (Promega) was cotransfected so that the transfection efficiencies could be normalized with the β -galactosidase activities determined using the b-Glo[®] assay system (Promega).

Patients

We performed a single center prospective study in 92 hemodialyzed patients, selected according to the following criteria: age >18 years; no cardiovascular event (myocardial infarction, stroke, peripheral vascular disease with amputation or need for angioplasty), infection, or surgical intervention (except for vascular access angioplasty) in the last 3 months; no pregnancy; no recent history of malignancy; no intake of corticosteroids or immunosuppressive agents. Patients had been dialyzed at least 3 times a week for a minimum of 6 months. Patients' blood samples were drawn before the mid-week hemodialysis session. Blood samples were drawn in BD Vacutainer® tubes containing lithium heparin for biochemical analyses, EDTA for hemoglobin measurement, citrate for TF measurement, and in BD Vacutainer® SST tubes for IS, IAA, p-cresylsulfate, and β 2-microglobulin measurement. Standard laboratory procedures were used for blood chemistry evaluations. Total IS, IAA, and p-cresylsulfate were measured by HPLC, according to Calaf et al (2011).

Informed consent was obtained from all individual participants included in the study. The study was approved by the local ethics committee and conforms to the principles outlined in the Declaration of Helsinki.

Measurement of TF by enzyme-linked immunosorbent assay

TF was quantified in citrated human plasma and in cell lysates of HUVEC with the enzyme-linked immunosorbent assay (ELISA) kit Quantikine Human Coagulation Factor III/Tissue Factor (R&D Systems, Lille, France) according to the instructions of the manufacturer.

Statistical analyses

For in vitro studies, statistical analyses were performed with the Prism (GraphPad Software Inc, CA). Significant differences were revealed by the Wilcoxon signed rank test or by the

Mann Whitney test. Data are expressed as mean \pm SEM of independent experiments performed on different cell preparations.

In CKD patients, data are expressed as mean \pm standard deviation (SD) for values with normal distribution or median (min; max) for non-normally distributed values. Numerical variables were tested for normality by the Shapiro-Wilk test. Correlations between plasma TF and continuous variables were obtained using Spearman correlation coefficients; statistical analyses were performed with the Prism (GraphPad Software Inc, CA) software. To identify factors independently associated with plasma TF, multiple linear regression analyses were performed with the R software. A p value lower than 0.05 was considered significant.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

RESULTS

Study of IAA-induced TF expression in endothelial cells

We previously demonstrated that IAA up-regulates TF expression in HUVEC (Gondouin et al. 2013). Here we studied the effect of IAA on TF mRNA and protein expression in other types of human endothelial cells that are relevant to the cardiovascular events observed in patients: arterial (HAoEC, aortic endothelial cells), and microvascular (HMVEC-C, cardiac-derived microvascular endothelial cells). In all human endothelial cells studied, IAA significantly increased the mRNA (Fig. 1A) and protein (Fig. 2B) expression of TF after 4 hours of incubation.

We then added human serum albumin at 4g/dL in the culture medium to increase the protein-bound fraction of IAA. After 4 hours of incubation, the mRNA (Fig. 1C) and protein (Fig. 1D) induction of TF by IAA was similar with or without addition of albumin.

To test whether transcription was the main way controlling TF expression after IAA stimulation in endothelial cells, we added the transcription inhibitor Actinomycin D (Fig. 1E). Actinomycin D abolished the induction of TF mRNA expression by IAA, as well as the basal expression of TF mRNA. These findings suggest that the expression of TF induced by IAA in endothelial cells is controlled at the transcriptional level.

IAA induces TF expression via AhR activation but no AhR binding on TF promoter

We confirmed the involvement of AhR in IAA-mediated TF expression using an AhR pharmacological inhibitor CH223191, and AhR siRNA. The up-regulation by IAA of TF mRNA (Fig. 2A) and protein (Fig. 2B) expression was abolished by CH223191 and by AhR siRNA. CH223191 and AhR siRNA alone had no significant effect on basal TF mRNA and protein expression (Fig. 2A and 2B).

AhR can regulate transcription directly by binding on gene promoter in a so called genomic pathway, or indirectly by activating other transcription factors in a non-genomic pathway. We first studied by CHIP experiments whether AhR binds directly to the TF promoter to induce TF expression in HUVEC. CHIP experiments demonstrated no enrichment of AhR on the promoter of TF, showing that AhR was not directly recruited to the TF promoter in endothelial cells following IAA stimulation (Fig. 2C). As expected, AhR was recruited to the *CYP1A1* and *CYP1B1* promoters, known to contain XRE sequences, after HUVEC stimulation by IAA (Fig. 2C). This confirms that IAA induces AhR recruitment to the promoters of target genes known to be up-regulated by the genomic pathway. We next analyzed the nuclear translocation of AhR by studying AhR expression in nuclear and cytoplasmic extracts after stimulation of HUVEC by IAA in presence of the AhR inhibitor CH223191. IAA increased the nuclear level of AhR, which was strongly decreased when cells were incubated with the AhR inhibitor CH223191 (Fig. 2D). In parallel, IAA caused a decrease in cytoplasmic level of AhR, which was prevented by CH223191. We next examined the effect of IAA on the up-regulation of *CYP1A1* and *CYP1B1* in HUVEC in presence of CH223191. IAA induced a marked increase in mRNA expression of *CYP1A1* and *CYP1B1*, which was abolished by the presence of CH223191 (Fig. 2E and 2F).

The NF- κ B site in TF promoter is necessary for TF induction by IAA

Because AhR did not directly bind to TF promoter after HUVEC stimulation by IAA, we studied the regulation of TF transcription using a TF promoter-luciferase reporter system, containing upstream binding sites for transcription factors known to up-regulate TF: AP-1, NF- κ B, Egr-1, and Sp1 (Oeth et al. 1997; Li et al. 2009). We focused here on transcription factors involved in TF up-regulation by inflammatory stimuli: AP-1 and NF- κ B (Bode and Mackman 2014). Luciferase plasmids containing either a wild-type TF promoter (-227 WT), or

one containing a NF- κ B non-binding mutant (-227 mNF- κ B), or one containing AP-1 non-binding sites (-227 mAP1), were transfected into HUVEC. In cells transfected with the wild-type TF construct, the luciferase activity was 70% higher in IAA-stimulated cells than in control cells (Fig. 3A). The mutation of AP-1 sites in the TF promoter construct (-227 mAP1) significantly decreased the luciferase activity in both control and IAA-stimulated cells (Fig. 3A). However, the -227-mAP-1 promoter activity was 53% higher in IAA-treated cells than in ethanol-treated control cells. This led us to study the nuclear level of the AP-1 subunits c-Fos and c-Jun in IAA-stimulated HUVEC. The nuclear level of both c-fos and c-jun was not higher in IAA-treated cells than in control cells (Supplemental Fig. 2).

We next studied the role of NF- κ B in the effect of IAA on TF transcription in using the NF- κ B non-binding mutant (-227 mNF- κ B) construct. The induction of the promoter activity by IAA was significantly lower in the mutant NF- κ B construct than in the wild-type. Interestingly, the activity of the TF promoter mutated for the NF- κ B site was similar in IAA-treated cells and in ethanol-treated control cells (Fig. 3A). In addition, in control cells, no difference in promoter activity was observed between cells transfected with the -227WT and the -227 mNF- κ B. These data indicate that the NF- κ B site is crucial for IAA-mediated TF gene transcription.

Cooperative role between AhR and NF- κ B in TF induction by IAA

Because the mutation of the NF- κ B site resulted in decrease in TF promoter activity, we studied the involvement of NF- κ B pathway in TF induction by IAA. We incubated HUVEC with IAA for 4 hours in the presence of the pharmacological inhibitor of I κ B kinase (I κ K) BAY 11-7082. BAY 11-7082 drastically decreased the induction of TF mRNA (Fig. 3B) and protein (Fig. 3C) by IAA. This suggests that NF- κ B activation is involved in TF induction by IAA. Subsequently, we studied the level of the NF- κ B p50 subunits in nuclear extracts of HUVEC

stimulated during 30 min by IAA. IAA increased the nuclear level of NF- κ B p50 (Fig. 3D) and p65 (Supplemental Fig. 3). We then analyzed the effect of pharmacological inhibitors of I κ K (BAY 11-7082) and AhR (CH223191). Nuclear translocation of p50 was inhibited by the I κ K inhibitor BAY 11-7082 (Fig. 3D). Interestingly, the increase in p50 nuclear level was also inhibited by the AhR inhibitor CH223191 (Fig. 3D) and by AhR siRNA (Supplemental Fig. 4), showing that IAA activated NF- κ B via AhR.

Involvement of p38 MAPK in IAA-mediated TF induction

We studied the involvement of Protein Kinase C (PKC) and of p38 and ERK1/2 MAP Kinases in TF induction by IAA. HUVEC were stimulated by IAA for 4 hours in presence of the PKC inhibitor Bisindolymaleimide I, the p38 inhibitor SB203580, and the ERK1/2 inhibitor PD98059. TF mRNA induction by IAA was significantly decreased by the p38 and PKC inhibitors, but not significantly modified by the ERK1/2 inhibitor (Fig. 4A). The p38 and PKC inhibitors also decreased TF protein induction by IAA (Fig. 4B). This suggests that the p38 and the PKC pathways are involved in IAA-mediated TF up-regulation.

We finally evaluated the effect of p38 and PKC inhibitors on IAA-induced NF- κ B activation. The increase in nuclear p50 level caused by IAA (Fig. 4C) was inhibited by SB203580 but not by Bisindolymaleimide I, suggesting that p38 but not PKC is involved in IAA-induced NF- κ B nuclear translocation.

Taken together, our data showed that IAA up-regulates TF expression by an inflammatory non genomic AhR/p38 MAPK/NF- κ B pathway (Fig. 5).

Circulating TF levels are independently related to IAA levels in hemodialyzed patients

We studied a cohort of 92 hemodialyzed (HD) patients (Table 1) and measured the plasma levels of circulating TF (cTF). Mean cTF values were 133 ± 66 pg/mL (median 131 pg/mL) and ranged from 36 to 410 pg/mL. At baseline, cTF levels negatively correlated with residual

renal function ($r=-0.27$, $p<0.01$), and positively correlated with serum cholesterol ($r=0.24$ $p<0.05$), HDL-cholesterol ($r=0.22$, $p<0.05$), and with the uremic toxins β 2-microglobulin ($r=0.22$ $p<0.05$) and IAA ($r=0.32$, $p<0.01$) (Table 2). In multivariate linear regression analysis in HD patients, only serum IAA (estimate =3.36, 95%CI [0.41 to 6.3], $p<0.05$) was significantly associated with cTF level (Table 3).

DISCUSSION

In this study, we demonstrated that TF induction by IAA is mediated by an AhR non genomic pathway involving p38MAPK and NF- κ B activation.

IAA is a protein-bound uremic toxin, mostly produced from tryptophan metabolism by intestinal bacteria (Fernandez-Prado et al. 2017). IAA is an endogenous AhR agonist, which has similar endothelial effects to those of exogenous AhR-activating pollutants like TCDD, benzo [a] pyrene, PCB126, and PCB77 (Sallée et al. 2014). It induces the production of reactive oxygen species, up-regulates the expression of inflammatory molecules COX-2, IL-8, ICAM-1, and MCP-1 (Dou et al. 2015), and increases the expression and the procoagulant activity of TF in HUVEC (Gondouin et al. 2013). Here, we show that IAA increases TF expression in endothelial cells from other vessel types: arterial (aortic) and microvascular (cardiac-derived). We also observed that human serum albumin did not modify IAA effect on tissue factor induction, supporting that IAA, whether free or protein-bound, induces TF up-regulation.

TF is usually not expressed by endothelial cells under normal conditions. However, it could be expressed in some pathologic conditions *in vivo* (Bode and Mackman 2014). *In vitro* induction of TF expression in endothelial cells is mediated by various intracellular signaling pathways and by the transcription factors NF- κ B, AP-1, NFAT, and Egr-1, depending on the stimulus (LPS, IL-1 β , TNF- α , thrombin and VEGF) (Bode and Mackman 2014). Using AhR siRNA, we previously described a new pathway of TF induction by IAA that is mediated by AhR activation (Gondouin et al. 2013). We confirm here that an AhR-specific antagonist, CH223191, is able to reduce IAA-mediated TF expression.

The induction of TF in response to stimuli is mainly regulated at the transcriptional level; but some stimuli may also increase the stability of TF mRNA and/or protein (Crossman et al.

1990; Brand et al. 1991; Ahern et al. 1993; Reddy et al. 2004) . The regulation of TF by IAA was well studied in vascular smooth muscle cells by the group of Chitalia. They showed that IAA prolongs the half-life of TF protein by decreasing its ubiquitination (Chitalia et al. 2013). They further demonstrated in vascular smooth muscle cells that AhR directly interacts with TF and reduces the level of TF ubiquitination (Shivanna et al. 2016), and that the ubiquitin ligase STUB1 dynamically interacts with TF (Shashar et al. 2017). In our study in endothelial cells, TF mRNA up-regulation by IAA was abolished in presence of the transcription inhibitor actinomycin D, supporting that transcription is the major mechanism of TF mRNA induction by IAA in endothelial cells. However, one cannot exclude that a posttranslational regulation of TF exists, like in vascular smooth muscle cells.

The classical genomic pathway of AhR target genes up-regulation is based on dimers of ligand-activated AhR and AhR nuclear translocator (ARNT), which directly activate gene expression. Upon ligand binding to AhR, it is translocated into the nucleus where it associates with its heterodimerization partner ARNT. The AHR/ARNT heterodimer binds to DNA sequence motif, referred to as XRE, within the promoters of target genes. In the XRE (5'-TNGCGTG-3'), a minimum core sequence of 5'-GCGTG-3' is required for AHR/ARNT binding (Swanson et al. 1995) and induction of target genes like xenobiotic-metabolizing enzymes CYP1A1 and CYP1B1 (Dolwick et al. 1993; Fujii-Kuriyama and Mimura 2005). Recently, a genomic, ARNT-independent pathway for AhR-mediated gene expression has been reported (Jackson et al. 2015), with a novel non-consensus XRE (NC-XRE) described in the promoter of PAI-1 (Huang and Elferink 2012) and in p21Cip1 promoter (Jackson et al. 2014). The AhR-dependent gene expression mediated through the NC-XRE does not involve the ARNT as a component of the NC-XRE bound AhR complex. We report here that IAA activates the classical genomic pathway of AhR in HUVEC. IAA induces AhR nuclear

translocation and AhR binding to the promoters of its target genes *CYP1A1* and *CYP1B1*, leading to upregulation of *CYP1A1* and *CYP1B1* mRNA. The AhR inhibitor CH223191 reduces AhR nuclear translocation mediated by IAA and abolishes IAA-induced expression of *CYP1A1* and *CYP1B1*. CH223191 was initially described as an inhibitor of TCDD binding to AhR, which inhibits AhR nuclear translocation induced by TCDD (Kim et al. 2006). One can suppose that CH223191 also inhibits IAA binding to AhR. Importantly, we showed that AhR does not directly bind to the TF promoter, supporting that the classical genomic pathway of AhR activation is not involved in TF up-regulation by IAA. This result agrees with the absence of description in the literature of an XRE sequence in the TF promoter, excludes the presence of a NC-XRE in the TF promoter, and suggests the activation of the non-genomic pathway of AhR by IAA.

Once activated by ligand, AhR can also elicit inflammation through the non-genomic pathway, which does not require the participation of ARNT (Matsumura 2009). We supposed that AhR activation could initiate alternative signaling pathways without direct DNA binding of AhR on TF gene promoter. It has been shown that AhR could activate others transcription factors such as AP-1 (Di Meglio et al. 2014; Ito et al. 2016) and NF- κ B (Borlak and Jenke 2008; Vogel and Matsumura 2009; Vogel et al. 2011,2013; Øvrevik et al. 2014) to drive expression of genes, by a protein kinase-dependent non-genomic route. We previously reported that IAA induces inflammatory endothelial COX-2 expression via non-genomic pathway of AhR involving NF- κ B (Dou et al. 2015). To determine the transcription factors involved in TF up-regulation by IAA, we studied IAA-induced TF transcription using a classic proximate TF promoter model, with binding sites for transcription factors AP-1, NF- κ B, Egr-1, and Sp1. We clearly demonstrated the role of NF- κ B in IAA-mediated TF expression. We showed that the NF- κ B site is essential in TF promoter activity induced by IAA. In addition,

an inhibitor of I κ B Kinase, which avoids the phosphorylation of I κ B and the subsequent NF- κ B activation, strongly inhibited TF mRNA and protein induction by IAA. Then, we showed that IAA increased the nuclear level of the NF- κ B p50 and p65 subunits, which are critical regulators of TF (Li et al. 2009). This does not exclude that other NF- κ B subunits could be activated by IAA. Interestingly, IAA-induced p50 nuclear translocation was decreased when AhR was inhibited; supporting that nuclear translocation of p50 was mediated by AhR activation. All these results demonstrated the crucial role of NF- κ B in AhR-mediated TF induction by IAA. We also observed that the mutation of the AP-1 site significantly decreased TF promoter activity in both control and IAA-stimulated cells, but IAA still increased TF promoter activity when the AP-1 site was mutated. Furthermore, IAA did not increase nuclear level of c-Jun and c-fos subunits of AP-1. These results suggest that the AP-1 site is required for basal TF expression and is only partially involved in TF induction by IAA. One cannot exclude that other AP-1 subunits may be involved, or that AP-1 cooperates with NF- κ B to induce TF expression as described in the literature for other agonists (Mackman et al. 1991; Parry and Mackman 1995; Armstead et al. 1999; Xia et al. 2013; Xia et al. 2016).

Studies show the cross-talk of AhR and multiple signal transduction pathways, notably MAP Kinases (Henklová et al. 2008 ; Puga et al. 2009a); and conventional AhR activators such as TCDD and benzo [a] pyrene activate MAP kinases (Henklová et al. 2008; Puga et al. 2009a). In our previous study, we demonstrated that IAA induces p38 MAPK and ERK phosphorylation (Dou et al. 2015). We found here that p38 MAPK, but not ERK, is involved in TF up-regulation by IAA. We previously showed that AhR inhibition decreases IAA-induced p38 phosphorylation (Dou et al. 2015), supporting that AhR is the upstream regulator of p38. Here, we observed that p38 inhibition decreased TF up-regulation by IAA. Furthermore, IAA-induced p50 nuclear translocation was decreased by inhibitors of both AhR and p38 MAPK.

Therefore, in addition to the activation of the classical genomic pathway, the binding of IAA to AhR initiates a cytoplasmic signaling pathway that activates p38 MAPK, which then activates NF- κ B to induce TF expression.

We also observed the involvement of PKC in TF up-regulation by IAA, but the absence of inhibition of p50 nuclear translocation by the PKC inhibitor suggests that PKC is not involved in the AhR/p38 cytoplasmic signaling pathway that leads to p50 nuclear translocation. PKCs are usually known for their key role in cytoplasmic signal transduction; they can also directly regulate gene expression through signal transduction within the nucleus, a process distinct from the cytoplasmic signaling pathways (Lim et al. 2015). Within the nucleus, PKCs can directly regulate gene transcription through either phosphorylating specific histone residues or phosphorylating transcription factors (Lim et al. 2015). For example, PKC α can phosphorylate NF- κ B p65 at S276, which is important for transcriptional activation of NF- κ B (Lim et al. 2015). Our results allow to hypothesize that PKC could play a role in IAA-induced TF transcription as an epigenetic regulator that could be involved in histone or NF- κ B phosphorylation, rather than as a cytoplasmic signal transducer involved in AhR/p38 signaling pathway.

TF upregulation induced by IAA has functional consequences on thrombogenicity. Indeed, the increase TF protein abundance induced by IAA, like uremic serum and the indolic uremic toxin indoxyl sulfate, is associated with increased procoagulant activity in endothelial cells and vascular smooth muscle cells (Chitalia et al 2013; Gondouin et al. 2013), and increases thrombogenicity in an ex-vivo model (Chitalia et al. 2013). An antibody against endothelial TF prevents platelet deposition on cultured endothelial cells induced by uremic serum (Serradell et al. 2001), suggesting that the presence of endothelial TF is responsible for the

enhanced platelet deposition. Patients with CKD display high levels of cTF and increased TF-dependent procoagulant activity (Gondouin et al. 2013; Shivanna et al. 2016).

cTF levels in CKD patients are related to some uremic toxins that are AhR agonists. Kynurenine levels are associated with cTF levels and hypercoagulability in HD patients (Pawlak et al. 2009); cTF levels are correlated with indoxyl sulfate in CKD patients not on dialysis (Gondouin et al. 2013). We found here that cTF level positively correlated with serum cholesterol, HDL-cholesterol, and with the uremic toxins β 2-microglobulin and IAA in simple correlation analysis. However, we did not find a positive correlation between cTF and indoxyl sulfate in HD patients, as we had previously observed in not dialyzed CKD patients (Gondouin et al. 2013). In multivariate linear regression analysis, only serum IAA was significantly and independently associated with cTF level. We previously identified IAA as an independent predictor of cardiovascular events and mortality in CKD patients (Dou et al. 2015). TF up-regulation is part of the mechanisms by which IAA may induce atherothrombosis in patients with CKD. Furthermore, cTF negatively correlated with residual renal function (in simple analysis) in HD patients in the present study, and with estimated GFR in not dialyzed CKD patients in our previous study (Gondouin et al. 2013). This suggests that the preservation of renal function in HD patients could be advantageous to prevent the increase in cTF levels.

In CKD patients, we recently showed high level of AhR agonists and an activation of the AhR pathway in cells from CKD patients (Dou et al. 2018). AhR has been proposed as a therapeutic target to reduce TF levels, for a better management of increased thrombosis in CKD (Shivanna et al. 2016). However, targeting AhR would also result in inhibition of the AhR genomic pathway that is crucial in detoxification processes. Therefore, it would be interesting to specifically target the thrombotic/inflammatory non genomic pathway of AhR

activation, while preserving the AhR genomic pathway. Thus, understanding of signal transduction pathways related to AhR thrombotic/inflammatory non genomic pathway is of interest to find therapeutic targets.

In conclusion, we demonstrated that IAA induces TF expression via an AhR/ p38 MAPK/ NF- κ B inflammatory pathway. A better understanding of the mechanisms involved in TF induction by uremia/uremic toxins could provide new therapeutic targets to reduce the thrombotic risk in patients with CKD.

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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TABLES

Table 1. Baseline characteristics of the HD population (n=92)

Age (years)	70 (23; 91)
Gender ratio (W/M)	33/59
Body Mass Index (kg/m ²)	24.0 (14.3; 47)
Kidney disease	
Glomerulonephritis	20 (22%)
ADPKD*	7 (7%)
Vascular	30 (33%)
Interstitial	11 (12%)
Other hereditary	2 (2%)
Unknown	22 (24%)
Hypertension	82 (91%)
Residual renal function	20 (21%)
Systolic Blood Pressure (mmHg)	144± 24
Diastolic Blood Pressure (mmHg)	73 ± 15
Current smokers	27 (29%)
History of cardiovascular diseases	37 (40%)
Antihypertensive drugs	61 (66 %)
Statins	30 (32%)
Antiplatelet drugs	49 (53%)
Anticoagulant drugs	23 (25%)
Erythropoietin therapy	71 (77%)
Hemoglobin (g/dL)	11.6± 1.1
Plasma TF (pg/mL)	131 (36; 410)
Serum CRP level (mg/L)	7 (0; 78)
Serum albumin level (g/L)	36.3 ± 4.4
Serum calcium level (mmol/L)	2.34 ± 0.14
Serum phosphate level (mmol/L)	1.53 (0.54; 3.17)
Serum urea level (mmol/L)	19.9 ± 5.4
Serum creatinine level (μmol/L)	759 (213; 1533)
Serum cholesterol level (mmol/L)	4.3 ± 1.0
Serum LDL-cholesterol level (mmol/L)	2.45 ± 0.87
Serum HDL-cholesterol level (mmol/L)	1.05 (0.28; 2.57)
Serum triglyceride level (mmol/L)	1.3 (0.6; 3.7)
Serum β2microglobulin level (mg/L)	32.2 (17.4; 66.9)
Serum p-cresylsulfate level (μM)	104 (4.3; 443.6)
Serum Indole-3 acetic acid level (μM)	4.3 (1.0; 19.1)
Serum indoxyl sulfate level (μM)	88.6 (0.2; 256.2)

*ADPKD: autosomal dominant polycystic kidney disease

Results are given as mean ± SD if distribution is Gaussian, or in median (min; max) if not.

Table 2. Two-tailed Spearman correlations of baseline characteristics with TF plasma levels in the HD cohort (n=92)

	TF	
	r	p
Age	0.08	0.4
Gender	-0.19	0.08
Body Mass Index	0.00	1
Kidney disease	-0.18	0.08
Residual renal function	-0.27	<0.01
Systolic Blood Pressure	-0.01	0.9
Diastolic Blood Pressure	-0.08	0.4
Current smoking	-0.05	0.6
History of cardiovascular diseases	-0.05	0.6
Antihypertensive drugs	-0.08	0.5
Statins	-0.18	0.4
Antiplatelet drugs	-0.18	0.08
Anticoagulant drugs	0.12	0.3
Erythropoietin therapy	-0.11	0.3
Hemoglobin	0.17	0.1
Serum CRP level	0.12	0.3
Serum albumin level	-0.08	0.4
Serum calcium level	-0.07	0.5
Serum phosphate level	-0.15	0.2
Serum urea level	-0.05	0.6
Serum creatinine level	0.03	0.7
Serum cholesterol level	0.24	<0.05
Serum LDL-cholesterol level	0.18	0.09
Serum HDL-cholesterol level	0.22	<0.05
Serum triglyceride level	0.04	0.7
Serum β 2microglobulin level	0.22	<0.05
Serum p-cresylsulfate level	0.07	0.5
Serum Indole-3 acetic acid level	0.32	<0.01
Serum indoxyl sulfate level	-0.1	0.3

Table 3. Multivariate linear regression analysis for evaluating the relation between independent variables and TF plasma levels in HD patients (n=92)

	TF		
	Estimate	95% CI	p-value
Age	0.18	[-0.75 to 1.12]	0.7
Gender	21.66	[-7.36 to 50.69]	0.14
Residual renal function	-23.81	[-55.45 to 7.83]	0.14
Serum Indole-3 acetic acid level	3.54	[0.66 to 6.43]	<0.05
Serum β 2microglobulin	1.1	[-0.4 to 2.61]	0.15
Serum cholesterol level	11.21	[-3.13 to 25.55]	0.12
Serum HDL-cholesterol level	3.35	[-30.86 to 37.56]	0.8

FIGURE CAPTIONS

Fig. 1 IAA induces endothelial Tissue Factor transcription

(A) The induction of TF mRNA by IAA in different types of endothelial cells: HUVEC (umbilical vein), HAoEC (aortic), and HMVEC-C (cardiac-derived microvascular) was studied by comparative RT-qPCR and expressed in mRNA fold change vs. control. Data represent the mean \pm SEM of 4 independent experiments. * $p < 0.05$, ** $p < 0.01$. (B) TF protein level in HUVEC, HAoEC, and HMVEC-C after a 4-h incubation with 50 μ M IAA was studied by ELISA. Data represent the mean \pm SEM of 4 independent experiments. * $p < 0.05$

Effect of 50 μ M IAA on Tissue Factor mRNA (C) and protein (D) induction in HUVEC, in medium containing 4% human serum albumin. Data represent the mean \pm SEM of 5 independent experiments * $p < 0.05$.

(E) Effect of the transcription inhibitor Actinomycin D (1 μ g/mL) on Tissue Factor mRNA expression after a 4-h incubation with IAA at 50 μ M. Data, expressed in mRNA fold change vs. control, represent the mean \pm SEM of 4 independent experiments. * $p < 0.05$, ** $p < 0.01$.

Fig. 2 IAA induces AHR activation but no binding of AhR on TF promoter

Effect of the AhR inhibitor CH-223191 (10 μ M) and of AhR siRNA on Tissue Factor mRNA (A) and protein (B) expression after a 4-h incubation with 50 μ M IAA. TF mRNA expression was studied by comparative RT-qPCR and expressed in mRNA fold change vs. control. TF protein expression was studied by ELISA. Data represent the mean \pm SEM of at least 5 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

C. AhR binding to the promoters of Tissue Factor, CYP1A1, and CYP1B1 was studied by chromatin immunoprecipitation (ChIP) after 1 hour of HUVEC stimulation by 50 μ M IAA. Data, expressed in fold enrichment, represent the mean \pm SEM of $n=6$ independent experiments.

D. AhR level in nuclear and cytoplasmic extracts was studied by Western blot after 30 min stimulation of HUVEC with 50 μ M IAA in presence of the AhR inhibitor CH223191 (10 μ M). Pictures are representative of 3 independent experiments.

mRNA expression of AhR-target genes CYP1A1 (E) and CYP1B1 (F) after 4-h incubation with IAA (50 μ M) was studied by comparative RT-qPCR in presence of the AhR inhibitor CH-223191 (10 μ M). Data, expressed in mRNA fold change vs control, represent the mean \pm SEM of 10 independent experiments. ** p<0.01, ***p<0.001.

Fig. 3 NF- κ B is activated by IAA and is involved in Tissue Factor up-regulation

(A) The identification of the IAA-sensitive transcription factor binding site in the TF promoter was studied using TF promoter-luciferase reporter system. Luciferase plasmids containing either a wild-type TF promoter (-227 WT), or NF- κ B non-binding mutant (-227 mNF- κ B) or AP-1 non-binding site (-227 mAP1) were transfected into HUVEC. After 6 hours of HUVEC stimulation by 50 μ M IAA, data expressed in fold induction normalized luciferase activity.

After HUVEC incubation with IAA (50 μ M) in presence of the I Kappa Kinase (I κ K) inhibitor BAY 11-7082 (10 μ M), Tissue Factor mRNA expression (B) was studied by comparative RT-qPCR and expressed in mRNA fold change vs. control; Tissue Factor protein expression (C) was studied by ELISA.

Endothelial p50 expression in nuclear extracts (D) was measured after 30 min incubation with IAA 50 μ M, in presence of the AhR inhibitor CH223191 (10 μ M) or the I κ K inhibitor BAY 11-7082 (10 μ M).

Data represent the mean \pm SEM of 6 (A) or 4 (B, C, D) independent experiments, * p<0.05, **p<0.01.

Fig. 4 Study of MAPK and PKC involvement in Tissue Factor up-regulation by IAA

mRNA expression (A) of Tissue Factor was studied by comparative RT-qPCR after 4-h incubation with IAA (50 μ M) in presence of the PKC inhibitor (Bisindolymaleimide I, 5 μ M), the p38 MAPK inhibitor (SB203580, 10 μ M), and the ERK1/2 inhibitor (PD98059, 10 μ M). Data are expressed in mRNA fold change vs. control.

Protein expression (B) of Tissue Factor was studied by ELISA after incubation with IAA (50 μ M) in presence of the PKC inhibitor (Bisindolymaleimide I, 5 μ M), and the p38 MAPK inhibitor (SB203580, 10 μ M).

Endothelial p50 level (C) was measured in nuclear extracts after 30 min incubation with IAA 50 μ M, in presence of the PKC inhibitor (Bisindolymaleimide I, 5 μ M), and the p38 MAPK inhibitor (SB203580, 10 μ M).

Data represent the mean \pm SEM of at least 4 independent experiments, * $p < 0.05$.

Fig. 5 A diagram shows the AhR-related mechanisms underlying IAA-induced TF transcription in HUVEC.

TF up-regulation by IAA in HUVEC is mediated by a non-genomic AhR/p38 MAPK/NF- κ B pathway. This up-regulation does not involved AhR binding to the TF promoter; AhR activates p38 MAPK, which mediates NF- κ B p50 nuclear translocation and binding to the TF promoter, leading to TF transcription.