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THE CARDIOVASCULAR EFFECT OF THE UREMIC SOLUTE INDOLE-3 ACETIC ACID

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ABSTRACT

In chronic kidney disease (CKD), uremic solutes may induce endothelial dysfunction, inflammation, and oxidative stress, leading to increased cardiovascular risk. We investigated whether the uremic solute indole-3 acetic acid (IAA) predicts clinical outcomes in CKD patients and has prooxidant and proinflammatory effects.

We studied 120 CKD patients. During the **median study period of 966 days**, 29 patients died and 35 experienced a major cardiovascular event. Kaplan-Meier analysis revealed that mortality and cardiovascular events were significantly higher in the higher IAA group ($> 3.73 \mu\text{M}$) than in the lower IAA group ($\text{IAA} < 3.73 \mu\text{M}$). Multivariate Cox analysis demonstrated that serum IAA was a significant predictor of mortality and cardiovascular events, after various adjustments including age and male gender; cholesterol, systolic blood pressure, and smoking; CRP, phosphate, body mass index, and albumin; diastolic blood pressure and history of cardiovascular disease; uremic toxins p-cresyl sulfate and indoxyl sulfate. Of importance, IAA level remained predictive of mortality when adjusted for CKD stage.

IAA levels were positively correlated with markers of inflammation and oxidative stress, respectively C-reactive protein and malondialdehyde. Induction of inflammation and oxidative stress by IAA was corroborated in cultured human endothelial cells. IAA activated an inflammatory non genomic AhR/p38MAPK/NF- κ B pathway that led to the induction of the proinflammatory enzyme cyclooxygenase-2. In addition, IAA increased production of endothelial reactive oxygen species.

In conclusion, serum IAA is an independent predictor of mortality and cardiovascular events in CKD patients. In vitro, IAA induces endothelial inflammation and oxidative stress and activates an inflammatory AhR/p38MAPK/NF- κ B pathway.

INTRODUCTION

Chronic inflammation and oxidative stress are key mechanisms in endothelial dysfunction and atherosclerosis that lead to cardiovascular disease in patients with chronic kidney disease (CKD) (1). The uremic syndrome of CKD is attributed to the progressive retention of uremic solutes, which are normally eliminated by the kidneys (2, 3). Serum levels of some uremic solutes are correlated with cardiovascular events and mortality in CKD patients (4). Uremic solutes have been identified as inducers of oxidative stress, inflammation, and endothelial dysfunction (4-6). Indole-3 acetic acid (IAA) is a protein-bound uremic solute from tryptophan metabolism (7). Its serum level is increased in patients with CKD (3), with a mean uremic concentration of about 5 μ M (8), and the highest concentration of 50 μ M (8). IAA belongs to the family of indolic uremic solutes (3), such as indoxyl sulfate (3, 5). IAA is an agonist of the transcription factor aryl hydrocarbon receptor (AhR) (9, 10), which regulates the cell response to environmental xenobiotics such as 2,3,7,8 tetrachlorodibenzo(p)dioxin (TCDD) (11). AhR activation by exogenous ligands promotes vascular inflammation, oxidative stress (5, 12), and atherosclerosis (13) and plays a role in cardiovascular diseases (14). However, the vascular toxicity of IAA has not been thoroughly studied. We recently reported that IAA induces endothelial tissue factor expression, leading to a procoagulant effect (9), and increases the mRNA expression of the enzyme cyclooxygenase-2 (COX-2), which is primarily responsible for the synthesis of inflammatory prostanoids (15).

In the present work, we test the hypothesis that IAA is associated with mortality and cardiovascular events in CKD patients. In addition, we demonstrate in vitro that IAA induces endothelial oxidative stress and inflammation by activating an AhR/p38MAPK/NF- κ B pathway, which leads to COX-2 up-regulation.

RESULTS

We studied 120 patients with CKD. The baseline characteristics, according to the median IAA level (IAA < 3.73 μ M vs. IAA > 3.73 μ M), are presented in Table 1. The group of patients with higher IAA serum levels had lower estimated glomerular filtration rate (eGFR) and more dialyzed patients (Table 1). The patients with higher IAA serum levels were older, had higher serum C-reactive protein (CRP) and malondialdehyde (MDA), and lower triglycerides and diastolic blood pressure (Table 1).

IAA levels were significantly higher in patients with stage 3-5 CKD ($p < 0.05$) and stage 5D CKD ($p < 0.0001$) than in controls (Fig. 1). IAA mean values were $1.9 \pm 0.8 \mu\text{M}$ [range 0.7-3.9 μM], $3.3 \pm 3.2 \mu\text{M}$ [range 0.6-15.1 μM], and $6.3 \pm 4.9 \mu\text{M}$ [range 0.9-18.5 μM] respectively in controls, in patients with stage 3-5 CKD, and in patients with stage 5D CKD (Fig. 1).

At baseline, IAA levels positively correlated with age ($r=0.25$, $p < 0.01$), CRP ($r=0.28$, $p < 0.01$), MDA ($r=0.32$, $p < 0.01$), and history of cardiovascular diseases ($r=0.25$, $p < 0.01$); IAA levels negatively correlated with diastolic blood pressure ($r=-0.16$, $p < 0.01$) and triglycerides ($r=-0.22$, $p < 0.05$) (Table 2). In the 47 patients with stage 3 to 5 CKD, IAA levels negatively correlated with eGFR estimated by the MDRD simplified formula ($r=-0.31$, $p < 0.05$) (Table 2).

IAA predicted the risk of death and cardiovascular events in patients with CKD

After a mean follow up of 804 ± 346 days, median (range): 966 (9-1100), 29 patients died (18 from cardiovascular causes, 4 from infectious causes, and 7 from other causes). Among the patients who died, 27 were dialyzed. Thirty-five patients displayed a cardiovascular event, which included a new non fatal cardiovascular event (myocardial infarction $n=11$, peripheral vascular event with amputation or need for angioplasty $n=2$, and stroke $n=4$) or death from cardiovascular cause ($n=18$). Among the patients who displayed a cardiovascular event, 30 were

dialyzed. During the follow-up, 33 patients dropped out the study, 22 because of kidney transplantation and 11 because of loss to follow-up.

The Kaplan-Meier analysis revealed that all-cause mortality (Fig. 2A) and cardiovascular events (Fig. 2B) were significantly higher in patients with high IAA levels (IAA > 3.73 μM) than in those with low IAA levels (log-rank comparison of the curves: $p = 0.024$ and $p = 0.013$, respectively). Note that there was no difference in mortality between patients with high and low IAA levels in the group of dialysis patients. In unadjusted Cox regression analysis, higher age, dialysis status, lower diastolic blood pressure, and a history of cardiovascular disease were also significantly associated with an increased risk of death and cardiovascular events (data not shown). In addition, CRP was significantly associated with the risk of death (data not shown).

We performed univariate and multivariate Cox analyses with the Box-Cox-transformed IAA entered as a continuous variable. In the unadjusted model, higher serum levels of IAA were associated with an increased risk of death (Table 3) and cardiovascular events (Table 4). After adjusting the different models for age and male gender (model 1); cholesterol, systolic blood pressure, and smoking (model 2); CRP, phosphate, body mass index (BMI), and albumin (model 3); diastolic blood pressure and history of cardiovascular disease (model 4), serum level of IAA remained a significant predictor of an increased risk of death (Table 3) and of cardiovascular events (Table 4). Even when adjusted for CKD stage (model 5), level of IAA remained a predictor of death (Table 3) but not of cardiovascular events (Table 4).

We finally compared the predictive power of IAA with that of uremic toxins p-cresyl sulfate and indoxyl sulfate. In univariate Cox analyses, p-cresyl sulfate predicted overall mortality and cardiovascular events, and indoxyl sulfate predicted mortality (not shown). In multivariate analyses that entered IAA, p-cresyl sulfate, and indoxyl sulfate as continuous variables, IAA

remained a significant predictor of mortality and cardiovascular events whereas p-cresyl sulfate and indoxyl sulfate did not (Tables 5 and 6).

IAA increased COX-2 expression and activity

The correlation between IAA level and the inflammatory marker CRP led us to study the proinflammatory effect of IAA in cultured vascular endothelial cells. We first studied in vitro the effect of IAA on the up-regulation of the inflammatory molecule COX-2 in different types of human endothelial cells. In cardiac-derived microvascular (HMVEC-C), coronary artery (HCAEC), aortic (HAoEC), and human umbilical vein (HUVEC) endothelial cells, IAA increased COX-2 mRNA expression (Fig. 3A). In addition, the mRNA expression of inflammatory molecules IL-8, ICAM-1, MCP-1, and to a lesser extent IL-6, was significantly increased by IAA in HUVEC (Supplemental Fig. 1).

We verified that incubation with IAA was not associated with cellular cytotoxicity. IAA did not decrease HUVEC viability at the concentrations studied (5, 10, 25, and 50 μ M) (Supplemental Fig. 2) and did not increase LDH release at 50 μ M (Supplemental Fig. 3).

We then studied how IAA affected COX-2 expression. Dose-response experiments showed that IAA at concentrations of 10, 25, and 50 μ M significantly increased COX-2 mRNA expression after 4-h incubation (Fig. 3B). A similar increase in COX-2 mRNA was observed when human serum albumin (HSA) at the concentration of 4g/dL was added in the culture medium (Fig. 3C). Kinetic study showed that COX-2 mRNA expression increased significantly after 1 hour of incubation with IAA, reached a plateau between 4 and 8 hours, and returned to basal levels after 24 hours (Fig. 3D). By Western blot, we showed that IAA at 50 μ M increased endothelial COX-2 protein level (Fig. 3E). Kinetic study showed that COX-2 protein level increased after 4 hours of incubation with IAA, was maximal at 8 hours, and decreased at 24 hours (Fig. 3E).

To study COX-2 activity, we measured PGE2 production after IAA stimulation. An increased PGE2 concentration could be detected in the supernatants after 8 hours of incubation, and the increase reached significance after 24 hours of incubation (Fig. 3F).

We finally studied whether IAA affected the expression of the cyclooxygenase COX-1. COX-1 mRNA was not modified after 4-h incubation with IAA at 50 μ M (Supplemental Fig. 4).

IAA activated an AhR/p38 MAPK/NF- κ B signaling pathway involved in COX-2 up-regulation

Because IAA is an AhR agonist, we verified that IAA induced the up-regulation of AhR target genes in HUVEC. IAA markedly increased the mRNA expression of *CYP1A1* (Fig. 4A), *CYP1B1* (Fig. 4B), and *AHRR* (Fig. 4C). This induction was completely abolished by the AhR inhibitor CH-223191 (Fig. 4A, 4B, 4C). The p38 MAPK inhibitor, SB203580, did not decrease IAA-induced *CYP1A1*, *CYP1B1*, and *AHRR* mRNA expression (Fig. 4A, 4B, 4C).

We studied the involvement of AhR activation in IAA-induced COX-2 up-regulation. COX-2 mRNA up-regulation by IAA was abolished by the AhR inhibitor CH-223191 (Fig. 4D). We then depleted AhR levels in HUVEC by transfection of siRNA targeting AhR mRNA, which dramatically decreased AhR levels after 72 hours (Fig. 4E). *CYP1A1*, *CYP1B1*, and *AHRR* induction by IAA was also significantly reduced (Supplemental Fig. 5E, 5F, 5G). Of importance, lowering the amount of AhR strongly inhibited IAA-induced COX-2 mRNA expression (Fig. 4F).

To study the signaling pathways activated by IAA, we first determined whether IAA could trigger MAPK activation. IAA induced p38 MAPK (Fig. 5A) and ERK1/2 (Fig. 5B) phosphorylations in HUVEC that were transient and robust, peaking at 15 min. IAA-induced p38 phosphorylation was abolished by the AhR inhibitor CH-223191 (Fig. 5C), suggesting IAA induced p38 MAPK phosphorylation through AhR activation.

We analyzed the involvement of MAPK in IAA-induced *COX-2* mRNA up-regulation. *COX-2* mRNA up-regulation was decreased by the p38 inhibitor SB203580 but not by the ERK1/2 inhibitor PD98059 (Fig. 5D), suggesting that the p38, but not the ERK pathway, is involved in *COX-2* up-regulation.

We finally evaluated NF- κ B activation. IAA caused an increase in nuclear p50 (Fig. 5E) and p65 level (not shown). The p38 inhibitor blocked IAA-induced p50 nuclear translocation (Fig. 5E), suggesting that IAA activated NF- κ B via p38 phosphorylation. In addition, IAA-induced *COX-2* mRNA expression was completely abolished by the NF- κ B inhibitor BAY 11-7082 (Fig. 5F).

To confirm the involvement of the AhR/p38 MAPK/NF- κ B pathway in *COX-2* up-regulation, we studied the increase in *COX-2* protein level induced by IAA in presence of the AhR inhibitor CH-223191, the p38 inhibitor SB203580, and the NF- κ B inhibitor BAY 11-7082. All inhibitors decreased *COX-2* protein induction by IAA (Fig. 6).

IAA increased endothelial ROS production

To determine whether IAA had a prooxidant effect, we studied the ability of IAA to increase endothelial ROS production. Treatment of HUVEC with IAA at uremic concentrations of 10, 25, and 50 μ M significantly enhanced ROS production. No increase was observed with the IAA concentration of 5 μ M, which is the highest concentration found in control subjects (Fig. 7A). The increase in ROS production induced by IAA started to be significant after 4 hours and up to 24 hours (Fig. 7B). ROS production induced by IAA was moderately inhibited by the AhR inhibitor CH-223191 (Fig. 7C) and markedly decreased by the ROS scavenger N-acetylcysteine (Fig. 7C).

DISCUSSION

In this study, we show that the uremic solute IAA is a predictor of mortality and major cardiovascular events in CKD. In addition, we found that serum IAA positively correlates with CRP and malondialdehyde, used to evaluate inflammation and oxidative status. Finally, we provide experimental data demonstrating the proinflammatory and the prooxidant effects of IAA on cultured endothelial cells.

The extremely high prevalence of cardiovascular disease in patients with CKD suggests the involvement of uremia-specific factors, such as uremic solutes (16-19). Here, we showed that patients with higher IAA display higher rates of mortality and cardiovascular events. More important, we demonstrated that serum IAA is a significant predictor of cardiovascular events and mortality. IAA is therefore a new member within the group of uremic solutes associated with mortality and cardiovascular events in CKD patients.

Little is known about the factors influencing IAA serum levels in CKD patients. IAA is produced by intestinal bacteria from tryptophan, but it is also produced by cells. One can suppose that diet plays a role in IAA levels. However, in HD patients, IAA levels are not related to Protein equivalent of total Nitrogen Appearance (PNA), a reliable estimate of dietary protein intake (20). IAA is bound to albumin with a high affinity (21), and the protein binding of IAA is about 80% (22). IAA is partly removed during hemodialysis, with a reduction rate of about 45% during session (22). Dialysis therapy, efficiency, and method may also influence IAA levels. However, predialysis concentrations of IAA seem to depend on residual renal function rather than on dialysis adequacy, assessed by Kt/V_{urea} (20).

This observational clinical study has some limits. First, the relation to outcomes is only observational. Second, clinical endpoints were correlated with a single serum IAA measurement

at the inclusion, without information about IAA intra-individual variability. In the literature, no study is specifically focused on intra-individual variability of IAA. Only data retrieved from an interventional study on sevelamer revealed no significant change in IAA level in CKD patients over 16 weeks (23). Third, our data obtained in a small group of patients have to be confirmed in larger CKD populations. Notably, the absence of difference in mortality between dialysis patients with high and low IAA levels has to be confirmed in larger cohorts of dialysis patients. Fourth, it was difficult to dissociate the predictive value of IAA from that of low eGFR and CKD severity, because eGFR and percentage of dialysis patients were different between the high and the low IAA groups. Note that serum IAA level increases with CKD stage but is poorly associated with eGFR in CKD patients not on dialysis (24). In our study, when adjusted with eGFR, IAA was not predictive of mortality and cardiovascular events. This was probably because analysis was limited to the 47 non-dialyzed CKD patients, **which were the only patients with laboratory values available for eGFR calculations.** To perform the statistical analysis on the whole population of 120 patients, we used CKD stage rather than eGFR to correct for renal function. In the model adjusted for CKD stage, serum level of IAA was still a significant and independent predictor of increased risk of death. In addition, IAA level remained predictive of mortality and cardiovascular events when adjusted for other protein-bound uremic toxins, p-cresyl sulfate and indoxyl sulfate. This suggests that IAA was a predictor on its own rather than just a reflection of the group of protein-bound toxins.

To demonstrate that IAA is a real mediator of vasculotoxicity and not merely a marker of impaired renal function, we studied the IAA effects on cultured endothelial cells, focusing on its prooxidant and pro-inflammatory effects. In vitro, IAA mediated endothelial oxidative stress by increasing ROS production. IAA also increased the expression of endothelial inflammatory

genes: IL-6, IL-8, ICAM-1, and MCP-1. Finally, IAA increased endothelial COX-2 expression and function.

A mechanism to explain IAA cellular effects could be AhR activation. Indeed, IAA is a ligand of the transcription factor AhR, whose activation is involved in atherogenesis (13), vascular inflammation (13), and oxidative stress (25). Upon ligand binding, AhR translocates into the nucleus, and binds to xenobiotic-responsive elements (XRE) within the promoters of target genes (11) such as *CYP1A1*, *CYP1B1*, *AHRR*, and *COX-2* (26). AhR activation also initiates alternative signaling pathways called “inflammatory non genomic pathways” without direct DNA binding of AhR. We previously reported that IAA induces AhR nuclear translocation in HUVEC (9). Here, we showed IAA up-regulates *CYP1A1*, *CYP1B1*, and *AHRR*. IAA induced AhR activation, which subsequently activated an inflammatory signaling pathway involving p38 MAPK and NF-κB activation.

IAA induced inflammation notably through COX-2 up-regulation, without modification of COX-1 expression. Although COX-1 and COX-2 share a high level of homology, their expressions and activities are differentially regulated, and they can function independently within the same cell type (27). Because COX-2 leads to the synthesis of prostanoids involved in inflammatory states (15), the IAA-induced overexpression of COX-2 may cause an imbalance in prostanoid synthesis. Studies support the notion that the COX-2/PGE2 axis has an important role in atherosclerosis (28-30). In animal models of CKD, COX-2 is up-regulated (31, 32) and associated with a proinflammatory and prooxidant profile (31, 32). Serum from CKD patients increases COX-2 expression in smooth muscle cells (33) and induces endothelial p38 MAPK and NF-κB activation (34). In our cultured endothelial cells, IAA increased COX-2 level and PGE2 production, induced

NF- κ B activation, and increased oxidative stress, mimicking the inflammatory and pro-oxidative profile found in CKD.

Endothelial COX-2 is regulated by several signaling pathways, including ERK1/2 and p38 MAPK (35), with subsequent activation of transcription factors such as AP-1 (36) and NF- κ B (35). COX-2 production is also regulated by AhR via a genomic and a non genomic pathway (37, 38). Here, we demonstrated the crucial involvement of AhR and subsequent p38 MAPK/NF- κ B activation in IAA-induced COX-2 up-regulation. Further studies on COX-2 promoter activation would be of interest to better understand COX-2 regulation by IAA.

AhR activation by exogenous ligands results in induction of endothelial oxidative stress, notably through CYP1 (12, 14). Here, we demonstrated that IAA increases ROS production, which was moderately decreased by the AhR inhibitor, suggesting the involvement of other pathways. In addition to CYP1, several sources of ROS exist, notably the mitochondrial electron transport chain, NADPH oxidase, and cyclooxygenases (39-41). Note that IAA also has a prooxidant effect in renal tubular cells (42). Via prooxidant, proinflammatory, and prothrombotic (9) mechanisms, IAA could act as a mediator of endotheliotoxicity of uremia.

In conclusion, IAA has proinflammatory and prooxidant effects in endothelial cells and activates a signaling pathway related to AhR that leads to COX-2 up-regulation. These biological effects are clues to explain the association between IAA concentration and the increased risk of mortality and cardiovascular events in patients with CKD.

METHODS

Patients

We performed a single center prospective study in 120 patients with CKD, enrolled between April 2007 and July 2010, selected according to the following criteria: age >18 years; no diabetes mellitus; no cardiovascular event, 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. CKD patients on dialysis had been dialyzed at least 3 times a week for a minimum of 6 months. Standard laboratory procedures were used for blood chemistry evaluations.

CKD stages 3, 4, and 5 were defined according to eGFR. CKD stage 3 (n=16) was defined as $30\text{mL}/\text{min}/1.73\text{m}^2 < \text{eGFR} < 60\text{mL}/\text{min}/1.73\text{m}^2$, stage 4 (n=17) as $15\text{mL}/\text{min}/1.73\text{m}^2 < \text{eGFR} < 30\text{mL}/\text{min}/1.73\text{m}^2$, and stage 5 (n=14) as $\text{eGFR} < 15\text{mL}/\text{min}/1.73\text{m}^2$. Stage 5D (n=73) was defined as patients on dialysis.

During the study period, clinical events, including overall mortality and cardiovascular events, were recorded. The causes of death were categorized as cardiovascular, infectious, or other. Cardiovascular events included death from cardiac cause, non fatal myocardial infarction, non fatal stroke, and non fatal peripheral vascular disease with amputation or need for angioplasty. Note that patients without clinical event might have subclinical cardiovascular disease, because this disease was not systematically explored by imaging.

Patients were compared with 51 control subjects with normal renal function, no diabetes, no cardio-vascular event, and no chronic medication. They were recruited by the Centre d'Investigation Clinique of the Assistance Publique Hôpitaux de Marseille. Their median age was 64 years (range 49-85 years) and their gender ratio was 25 W/26 M.

All participants gave their written informed consent. The study was approved by the local ethics committee and conforms to the principles outlined in the Declaration of Helsinki.

Statistical analyses

Data are expressed as mean \pm standard deviation (SD) for values with normal distribution or median (min; max) for non-normally distributed values. All numerical variables were tested for normality by the Shapiro-Wilk test. Statistical analyses were performed with the Prism (GraphPad Software Inc, CA) and the MedCalc (Medcalc, Belgium) softwares. Differences were considered significant when p was less than 0.05. Baseline variables were compared by chi-square tests for categorical variables, t -tests for continuous variables with Gaussian distributions, and Mann Whitney tests for continuous variables with non Gaussian distributions. Correlations between IAA levels and continuous variables were obtained using Spearman correlation coefficients. The Kaplan-Meier method was used to estimate the cumulative event-free rate for the time to overall mortality and the first cardiovascular event in patients with IAA level above and below the median (3.73 μ M). The log-rank test was used to compare the Kaplan-Meier curves. Univariate and multivariate analyses of overall mortality and cardiovascular events were performed using a Cox proportional hazard model as a function of serum IAA as continuous variable. Variables with non Gaussian distribution were normalized by Box-Cox transformation when used in tests that assume normally distributed variables.

In multivariate analyses, models were adjusted for demographics (age and male gender; model 1), Framingham cardiovascular risk factors (cholesterol, systolic blood pressure, and smoking; model 2), factors associated with mortality in CKD patients (CRP, phosphate, BMI, and albumin; model 3), confounding factors associated with mortality in univariate analysis (diastolic blood pressure and history of cardiovascular disease; model 4), and CKD stage (model 5). Univariate

and multivariate analyses were also performed to compare IAA, p-cresyl sulfate, and indoxyl sulfate.

For in vitro experiments, significant differences were revealed by the Wilcoxon signed rank test or by the Mann Whitney test. Data are expressed as mean \pm SEM or mean to max box and whiskers plot of independent experiments performed on different cell preparations. Statistical analyses were performed with the Prism software. A p value lower than 0.05 was considered significant.

Measurement of IAA, p-cresylsulfate, and indoxyl sulfate

IAA, p-cresylsulfate, and indoxyl sulfate were measured in serum by HPLC using a reversed phase column, an ion-pairing mobile phase, and isocratic flow, as described (43).

Malondialdehyde assay

Malondialdehyde was analyzed in serum by HPLC using the Chromsystems reagent kit (Chromsystems, Germany) according to the manufacturer's instructions.

Endothelial cell culture

HUVEC were obtained from umbilical cord vein by collagenase digestion as described (44) 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). The endothelial nature of HUVEC was confirmed by flow cytometry using endothelial specific markers CD31, CD144, Von Willebrand Factor, and CD146, and by negative staining of the leukocyte marker CD45.

HAoEC, HCAEC, and HMVEC-C were obtained from Lonza. Routine endothelial characterization and analysis of purity of HAoEC, HCAEC, and HMVEC-C were performed by immunofluorescent staining by the supplier. HCAEC and HMVEC-C were grown respectively to the fourth and 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.

Treatment with the uremic solute IAA

Cells were treated with IAA (Sigma-Aldrich, France) at concentrations of 5, 10, 25, and 50 μ M, with and without different inhibitors (supplemental Table 1). Five μ M IAA corresponds to the highest concentration of IAA found in control subjects; 10 and 25 μ M are in the range of IAA concentrations found in uremic patients; 50 μ M is the highest IAA concentration described in uremic patients (8). Because IAA was diluted in ethanol, ethanol 1/1000 was used as a vehicle 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 (Life Technologies, France) by magnetofection using SilenceMag beads (OZ Biosciences, France), according to the manufacturer's instructions. The knockdown of AhR was confirmed by reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) 72 hours after transfection. Dose-responses and transfection efficiency of AhR siRNA are described in supplemental methods and displayed in supplemental Fig. 5. The induction of COX-2, Cyp1A1, Cyp1B1, and AHRR mRNA by IAA was determined 72 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 1 μ g of total RNA using the AffinityScript™ multiple temperature cDNA synthesis (Agilent, France)

followed by qPCR on 10ng of cDNA using the Brilliant II SYBR Green QPCR Master Mix (Agilent). We quantified the following target genes: *COX-2*, *Cyp1A1*, *Cyp1B1*, *AHRR*, *AHR*, *IL-6*, *IL-8*, *ICAM-1*, *VCAM-1*, and *MCP-1*. The housekeeping gene *HPRT* was used for normalization of the target gene values. The sequences of primers are displayed in Supplemental Table 2. 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.

Western blotting and densitometry analysis of Western blots

HUVEC were lysed in lysis buffer containing protease inhibitors. Protein concentrations were measured with the Bicinchoninic Acid Kit (BCA1, Sigma-Aldrich). After saturation, the membrane was incubated with antibodies directed against COX-2, phospho-p38, p38, phospho-ERK1/2, ERK1, or actin (all from Cell Signaling, Ozyme, 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). Results were expressed as a ratio between values obtained with IAA and control normalized with values obtained with actin. The effect of AhR inhibition on p38 phosphorylation was studied with the RayBio® Cell-based p38 MAPK ELISA kit (Tebu-bio, France).

PGE2 measurement

PGE2 was measured in the supernatants of HUVEC using the Prostaglandin E2 assay kit (R&D Systems, France).

Measurement of ROS production

Intracellular ROS production was detected at t0 and after 5 hours of incubation by measuring the fluorescence of 6-carboxy-H2DCF-DA-di-AM (Invitrogen, France) by the Cytofluor Series

4000 (Applied Biosystems), with the excitation filter at 485nm and the emission filter at 530nm.

Fluorescence intensity was expressed in arbitrary units.

Study of NF- κ B activation

Nuclear extracts were prepared using Cayman Chemical's Nuclear Extraction Kit (Interchim, France). NF- κ B p50 and p65 were detected in nuclear extracts by Cayman Chemical's NF- κ B (human p50/p65) combo transcription factor assay kit (Interchim).

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FIGURE LEGENDS

Figure 1. Serum levels of IAA are increased in patients with CKD.

Plasma IAA levels are higher in stage 3-5 and stage 5D CKD patients than in control subjects.

Figure 2. Kaplan-Meier estimates of cumulative survival (A) and major cardiovascular events (B) of all patients according to serum IAA level above and below the median of 3.73 μ M.

(A) $p = 0.024$ in a log-rank comparison of the curves

(B) $p = 0.013$ in a log-rank comparison of the curves

The number of patients indicated in the tables is the number of patients still followed at the indicated time.

Figure 3. IAA induces endothelial COX-2

(A) The induction of COX-2 mRNA by IAA in different types of endothelial cells: HMVEC-C (cardiac-derived microvascular), HCAEC (coronary artery), HAoEC (aortic), and HUVEC (umbilical vein) was studied by comparative RT-qPCR and expressed in mRNA fold change vs. control. Data represent the mean \pm SEM of 4 independent experiments. * $p < 0.05$. (B) Dose-effect of COX-2 mRNA induction by IAA in HUVEC after 4-h incubation was studied by comparative RT-qPCR and expressed in mRNA fold change vs. control. Data represent the mean \pm SEM of 5 independent experiments. * $p < 0.05$, *** $p < 0.001$.

(C) Dose-effect of COX-2 mRNA induction by IAA in HUVEC in medium containing 4% human serum albumin. Data represent the mean \pm SEM of 5 independent experiments * $p < 0.05$. (D)

The kinetics of COX-2 mRNA expression induced by 50 μ M IAA in HUVEC were studied by comparative RT-qPCR and expressed in mRNA fold change vs. control. Data represent the mean \pm SEM of 5 independent experiments. * $p < 0.05$. (E) COX-2 protein induction by IAA in HUVEC was studied by Western blot. Data, expressed as normalized COX-2/ β actin ratio vs control,

represent the mean \pm SEM of 3 independent experiments. * $p < 0.05$. (F) PGE₂ was measured by ELISA in the supernatants of HUVEC stimulated with 50 μ M IAA. Data are expressed as mean to max box and whiskers plot. * $p < 0.05$.

Figure 4. The AhR pathway is involved in COX-2 mRNA up-regulation by IAA

mRNA expression of AhR-regulated genes CYP1A1 (A), CYP1B1 (B), and AHRR (C) after 4-h incubation with IAA was studied by comparative RT-qPCR in presence of an AhR inhibitor (CH-223191) or a p38 inhibitor (SB203580). (D) mRNA expression of COX-2 after 4-h incubation with IAA was studied by comparative RT-qPCR in presence of CH-223191. (E) mRNA expression of AhR studied by comparative RT-qPCR in HUVEC transfected with control siRNA or AhR siRNA. (F) mRNA expression of COX-2 induced by IAA in HUVEC transfected with control siRNA or AhR siRNA. Data, expressed in mRNA fold change vs control, represent the mean \pm SEM of 3 (A, B, C), 4 (D), or 5 (E, F) independent experiments. * $p < 0.05$, *** $p < 0.001$.

Figure 5. Study of MAPK and NF- κ B involvement in COX-2 up-regulation by IAA

The phosphorylations of p38 (A) and ERK1/2 (B) were measured in HUVEC by Western blot after incubation with IAA during 15, 30, and 45 min. (C) IAA-induced p38 phosphorylation was measured by ELISA after 15 min in presence of the AhR inhibitor CH-223191. (D) mRNA expression of COX-2 after 4-h incubation with IAA in presence of a p38 inhibitor (SB203580) and an ERK inhibitor (PD98059) was studied by comparative RT-qPCR. Data are expressed in mRNA fold change vs. control. (E) Endothelial p50 expression in nuclear extracts was measured after cell incubation with IAA 50 μ M, with and without the p38 inhibitor SB203580. (F) mRNA expression of COX-2 after 4-h incubation with IAA in presence of the NF- κ B inhibitor BAY 11-7082 was studied by comparative RT-qPCR and expressed in mRNA fold change vs. control. Data represent the mean \pm SEM of 3 (A, B), 4 (C, D, F), and 6 (E) independent experiments, * $p < 0.05$.

Figure 6. The AhR/p38MAPK/NF- κ B pathway is involved in COX-2 protein induction

COX-2 protein level was studied by Western blot after 8-h incubation with 50 μ M IAA, in presence of the AhR inhibitor CH-223191, the p38 inhibitor SB203580, and the NF- κ B inhibitor BAY 11-7082. Data are expressed as normalized COX-2/ β actin ratio vs. control and represent the mean \pm SEM of 3 independent experiments. * $p < 0.05$.

Figure 7. IAA induces ROS production in HUVEC

(A) ROS production induced by IAA was measured by cytofluorimetry after 5-h incubation with 5, 10, 25, and 50 μ M IAA. (B). Kinetic study of endothelial ROS production induced by IAA. (C) IAA-induced endothelial ROS production was measured after 5-h incubation in presence of N-acetylcysteine (NAC 10mM) and of the AhR inhibitor CH-223191 (10 μ M). Data represent the mean \pm SEM of 5 (A), 4 (B), and 6 (C) independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

TABLES

Table 1. Baseline characteristics of the study population.

	All patients (n=120)	IAA< 3.73 μM	IAA>3.73 μM	p	
Age (years)	67 (23; 91)	64 (23; 87)	71 (32; 91)	<0.05	
Gender ratio (W/M)	46/74	25/35	21/39	0.5	
Body Mass Index (kg/m ²)	24.1 (14.3; 47)	24.4 (16.9; 47)	23.7 (14.3; 39.8)	0.053	
Dialyzed patients (%)	73 (61%)	43%	78%	<0.05	
eGFR ^a (mL/min/1.73m ²)	26 ± 12	28 ± 11	19 ± 13	<0.05	
Kidney disease	Glomerulonephritis	23 (19%)	11 (18%)	12 (20%)	0.8
	ADPKD*	9 (8%)	5 (8%)	4 (7%)	0.7
	Vascular nephropathy	34 (28%)	13 (22%)	21 (35%)	0.2
	Interstitial nephropathy	24 (20%)	14 (23%)	10 (17%)	0.4
	Other hereditary	5 (4%)	4 (7%)	1 (1%)	0.2
	Unknown	25 (21%)	13 (22%)	12 (20%)	0.8
Hypertension	110 (92 %)	55 (92%)	55 (92%)	1	
Systolic Blood Pressure (mmHg)	142± 23	145 ± 24	140 ± 23	0.3	
Diastolic Blood Pressure (mmHg)	76 ± 14	78 ± 14	73 ± 14	<0.05	
Current smokers	48 (40%)	27 (45%)	21 (35%)	0.4	
History of cardiovascular diseases	42 (35%)	16 (27%)	26 (43%)	0.1	
Antihypertensive drugs	87 (73 %)	43 (72%)	44 (73%)	0.9	
Statins	38 (32%)	16 (27%)	22 (37%)	0.3	
Antiplatelet drugs	47 (39%)	17 (28%)	30 (50%)	0.057	
Anticoagulant drugs	27 (23%)	12 (20%)	15 (25%)	0.5	
Erythropoietin therapy	67 (56%)	26 (43%)	41 (68%)	0.06	
Serum CRP level (mg/L)	5 (0.1; 78)	4 (0.1; 30)	7 (0.1; 78)	<0.01	
Hemoglobin (g/dL)	12 ± 1.4	12 ± 1.6	11.9 ± 1.2	0.7	
Serum albumin level (g/L)	36 ± 4	35.9 ± 3.4	36 ± 4.6	0.8	
Serum calcium level (mmol/L)	2.33 (1.84; 2.66)	2.34 (2.06; 2.55)	2.32 (1.84; 2.66)	0.8	
Serum phosphate level (mmol/L)	1.28 (0.54; 3.17)	1.22 (0.65; 2.91)	1.44 (0.54; 3.17)	0.06	
Serum cholesterol level (mmol/L)	4.79 ± 1.26	4.94 ± 1.33	4.63 ± 1.17	0.2	
Serum LDL-cholesterol level (mmol/L)	3.09 ± 1.08	3.02 ± 1.11	3.18 ± 1.06	0.4	
Serum triglyceride level (mmol/L)	1.44 (0.37; 5.96)	1.65 (0.37; 5.96)	1.22 (0.37; 3.67)	<0.05	
Serum malondialdehyde level ^b (nmol/L)	200 (100; 500)	150 (100; 500)	215 (120; 430)	<0.01	
Serum IAA level (μmol/L)	3.73 (0.6; 18.58)	1.86 (0.6; 3.71)	6.67 (3.75; 18.58)	<0.0001	

^a estimated glomerular filtration rate calculated by MDRD formula for non-dialyzed CKD patients (n=47)

^b measured only in 68 patients (40 patients in the IAA<3.73 μg/mL group, and 28 patients in the IAA>3.73 μg/mL group) randomly chosen in the different CKD stage populations

*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. Spearman correlations of baseline characteristics with IAA serum concentrations

	Serum IAA	
	r	p
Age	0.25	<0.01
Gender	0.07	0.4
Body Mass Index	-0.14	0.10
eGFR ^a	-0.31	<0.05
Systolic Blood Pressure	-0.01	0.8
Diastolic Blood Pressure	-0.16	<0.01
Current smoking	-0.09	0.3
History of cardiovascular diseases	0.25	<0.01
Serum CRP level	0.28	<0.01
Hemoglobin	-0.03	0.7
Serum albumin level	0.14	0.12
Serum calcium level	-0.03	0.7
Serum phosphate level	0.14	0.12
Serum cholesterol level	-0.11	0.20
Serum LDL-cholesterol level	0.05	0.5
Serum triglyceride level	-0.22	<0.05
Serum malondialdehyde ^b level	0.32	<0.01

^a calculated for non-dialyzed CKD patients (n=47)

^b measured only in 68 patients randomly chosen in the different CKD stage populations

Table 3. Multivariate Cox regression analysis of risk factors for overall mortality – Box-Cox normalized serum IAA entered as continuous variable

Models of patient survival	RR	95% CI	p
Unadjusted	2.44	1.34 to 4.46	0.004
Model 1 ^a	1.95	1.05 to 3.65	0.036
Model 2 ^b	2.19	1.15 to 4.14	0.017
Model 3 ^c	2.01	1.01 to 4.00	0.047
Model 4 ^d	2.09	1.11 to 3.93	0.022
Model 5 ^e	2.46	1.35 to 4.50	0.003

^a Model 1 was adjusted for age and male gender

^b Model 2 was adjusted for cholesterol, systolic blood pressure, and smoking

^c Model 3 was adjusted for CRP, phosphate, BMI, and albumin

^d Model 4 was adjusted for diastolic blood pressure and history of cardiovascular disease

^e Model 5 was adjusted for CKD stage

Table 4. Multivariate Cox regression analysis of risk factors for cardiovascular events – Box-Cox normalized serum IAA entered as continuous variable

Major cardiovascular event	RR	95% CI	P
Unadjusted	2.16	1.26 to 3.71	0.005
Model 1 ^a	1.81	1.04 to 3.14	0.037
Model 2 ^b	1.91	1.10 to 3.32	0.023
Model 3 ^c	1.89	1.08 to 3.33	0.027
Model 4 ^d	1.77	1.02 to 3.09	0.043
Model 5 ^e	1.39	0.77 to 2.48	0.270

^a Model 1 was adjusted for age and male gender

^b Model 2 was adjusted for cholesterol, systolic blood pressure, and smoking

^c Model 3 was adjusted for CRP, phosphate, BMI, and albumin

^d Model 4 was adjusted for diastolic blood pressure and history of cardiovascular disease

^e Model 5 was adjusted for CKD stage

Table 5 Multivariate Cox regression analysis of risk factors for overall mortality in a model with Box-Cox normalized serum IAA, indoxyl sulfate, and p-cresylsulfate entered as continuous variables

Models of patient survival (event 29/120)	RR	95% CI	p
IAA	2.04	1.05 to 3.95	0.035
Indoxyl sulfate	1.05	0.95 to 1.16	0.368
p-cresylsulfate	1.05	0.97 to 1.12	0.211

Table 6 Multivariate Cox regression analysis of risk factors for cardiovascular events in a model with Box-Cox normalized serum IAA, indoxyl sulfate, and p-cresylsulfate entered as continuous variables

Major cardiovascular event (event 35/120)	RR	95% CI	p
IAA	1.95	1.09 to 3.50	0.026
Indoxyl sulfate	0.99	0.90 to 1.08	0.784
p-cresylsulfate	1.03	0.96 to 1.10	0.369

Figure 1

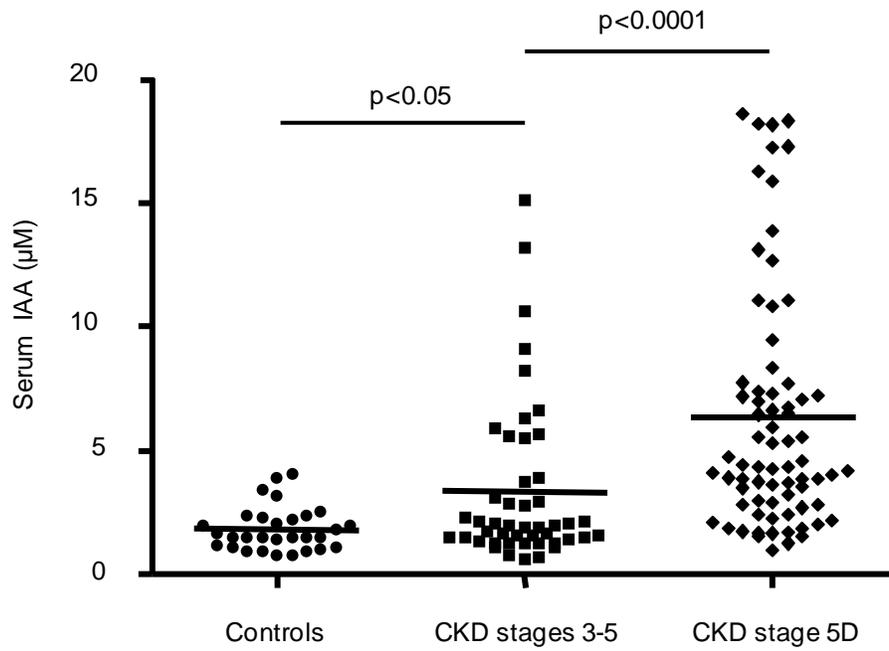
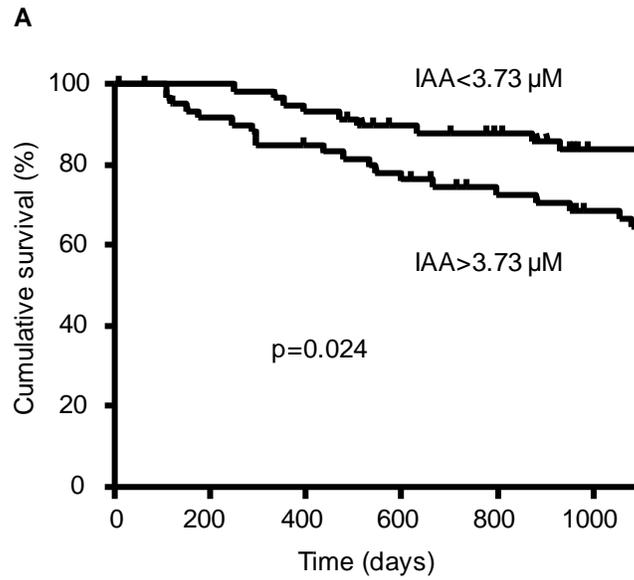
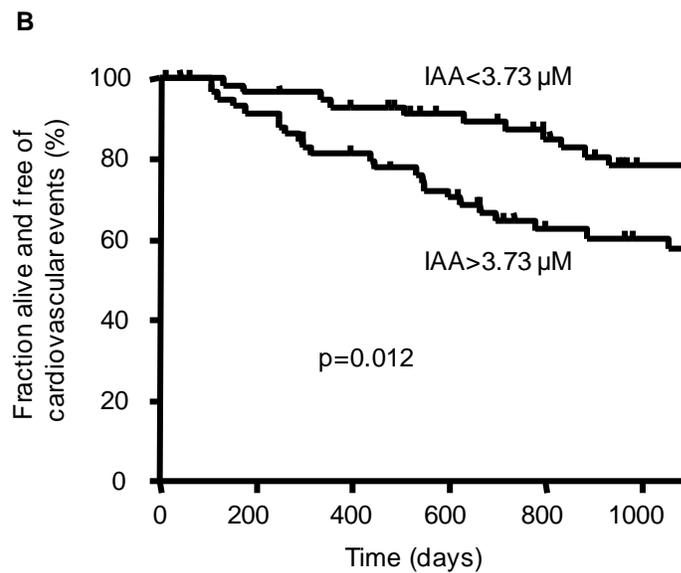


Figure 2



IAA < 3.73 μ M	60	58	54	48	44	35
IAA > 3.73 μ M	60	54	49	44	38	34



IAA < 3.73 μ M	60	56	52	46	40	31
IAA > 3.73 μ M	60	54	46	39	29	26

Figure 3

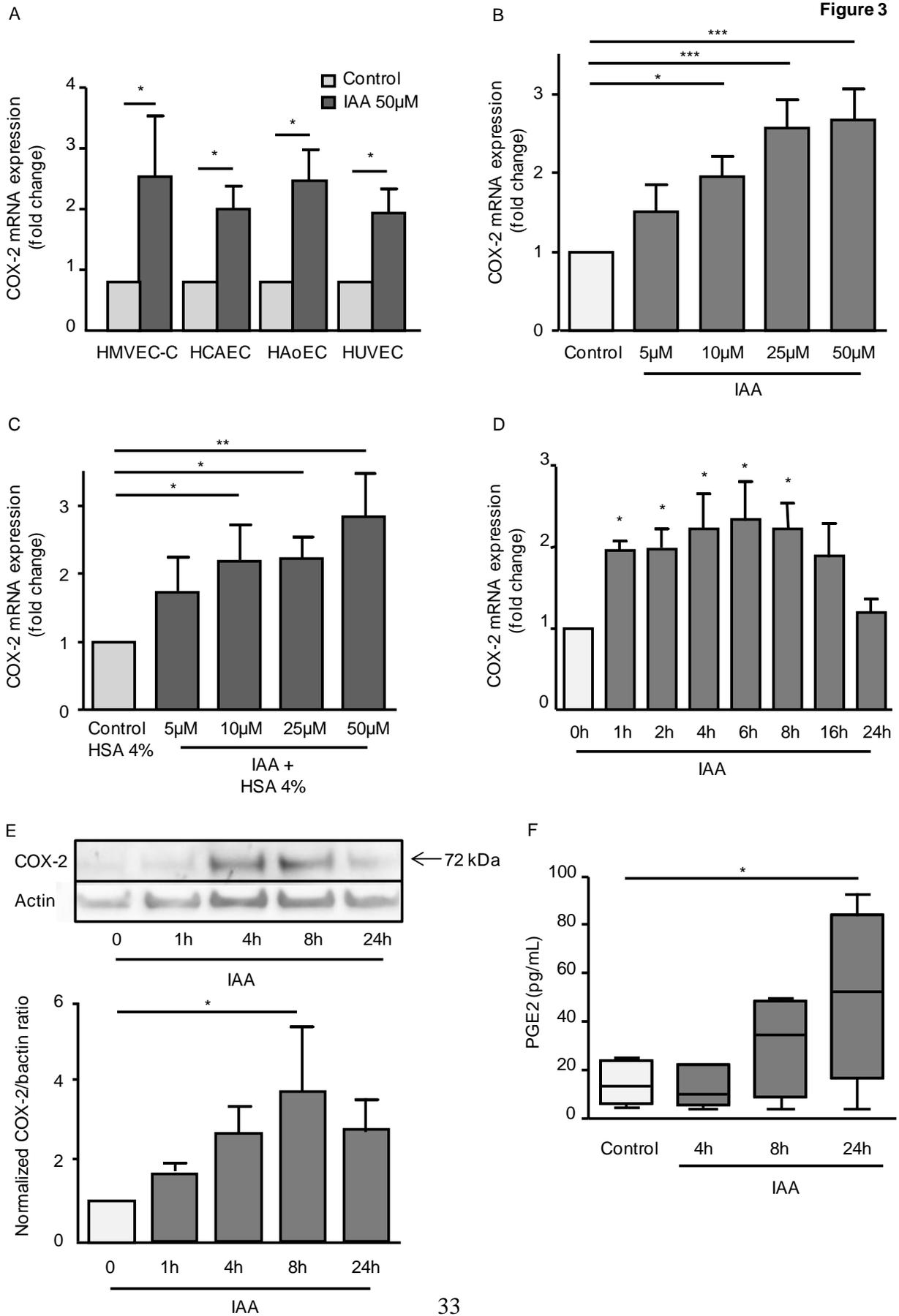


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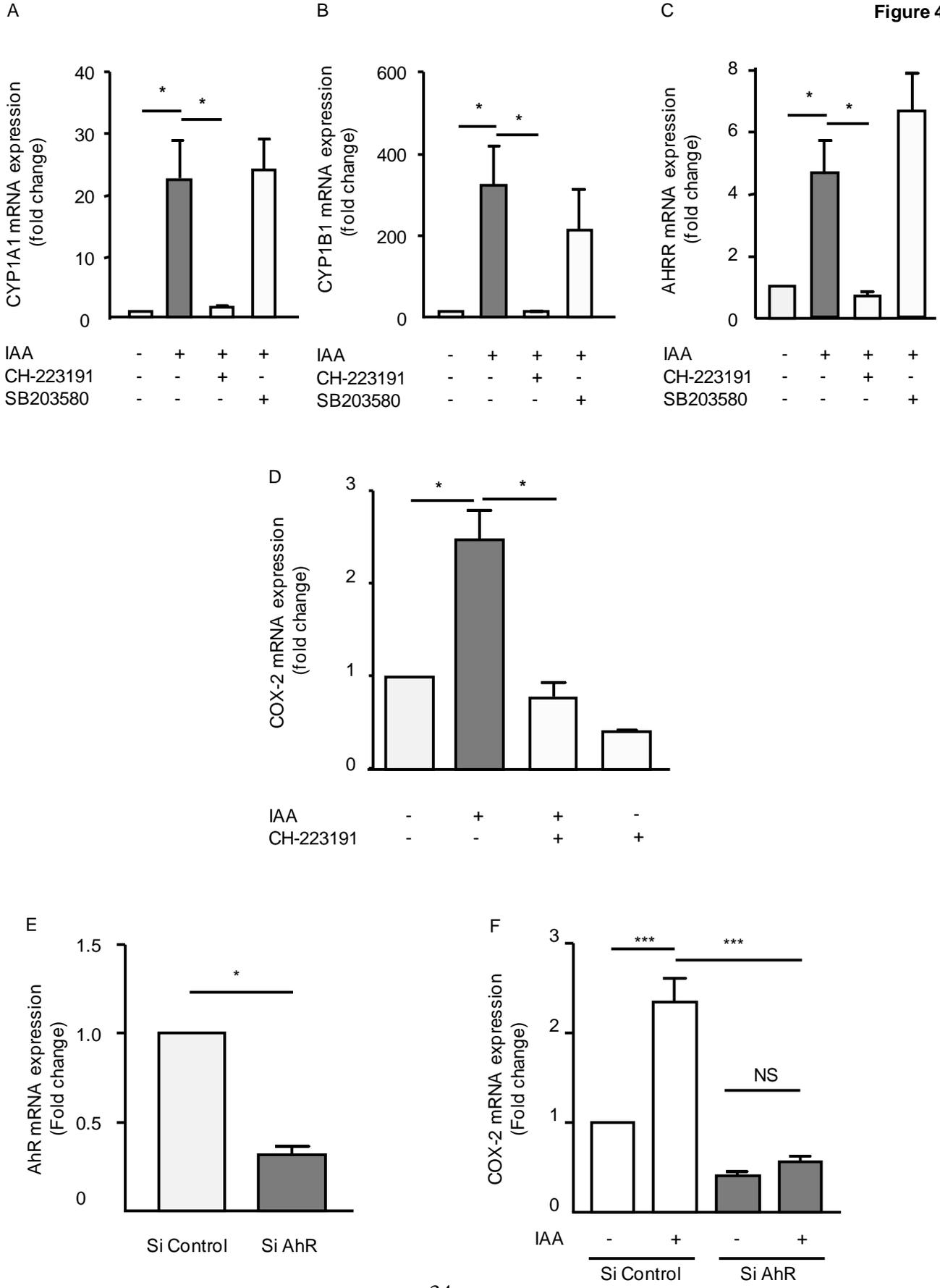


Figure 5

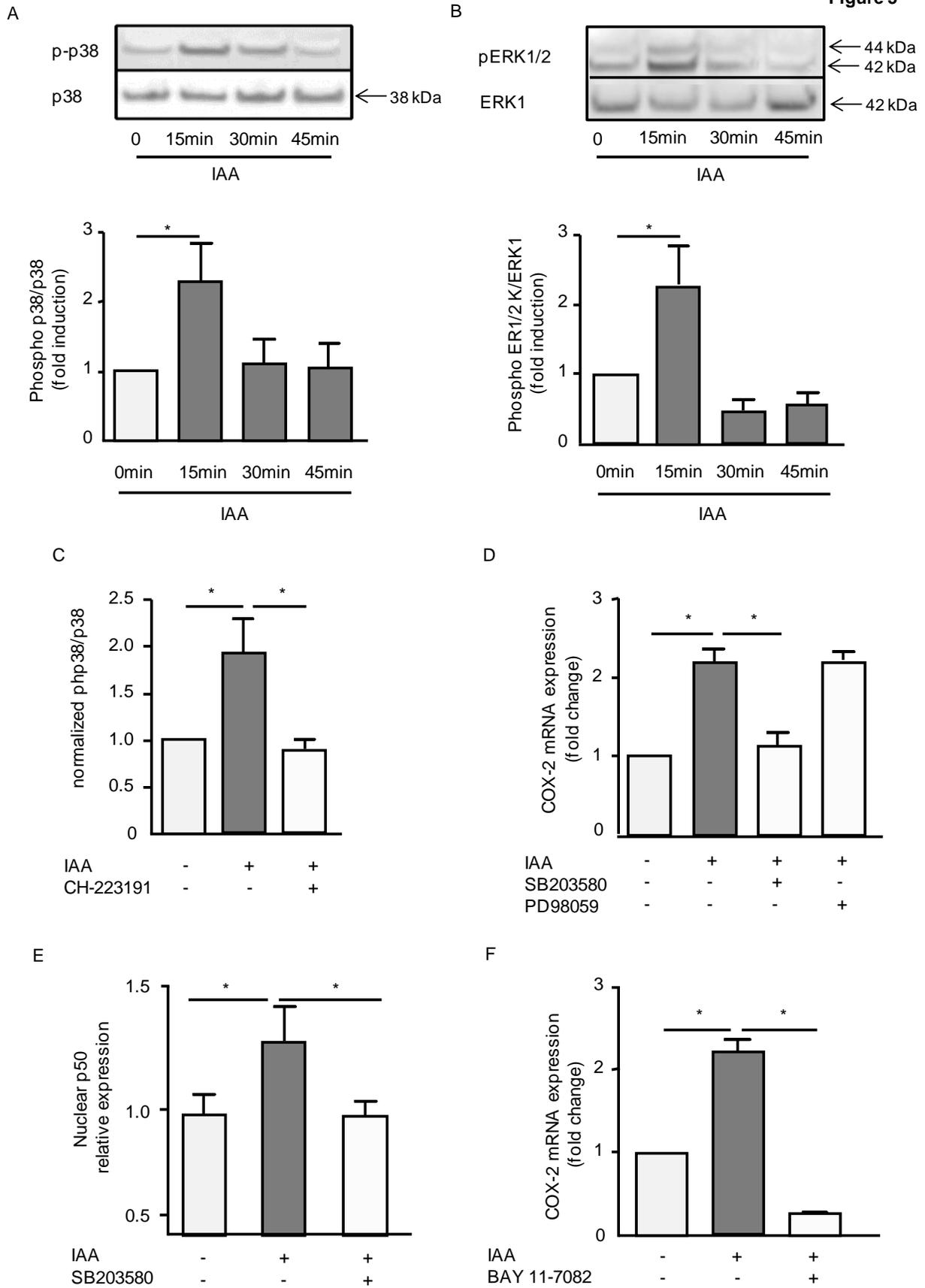
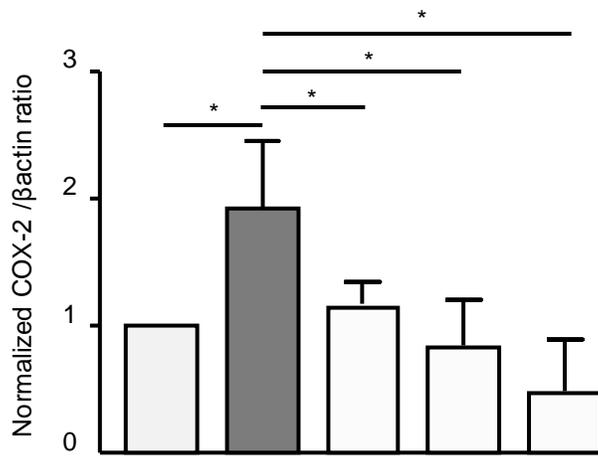


Figure 6



IAA 50 μ M	-	+	+	+	+
CH-223191	-	-	+	-	-
SB203580	-	-	-	+	-
BAY 11-7082	-	-	-	-	+

Figure 7

