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ORIGINAL ARTICLE

Increased type IIA secretory phospholipase A_2 expression contributes to oxidative stress in end-stage renal disease

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Abstract End-stage renal disease (ESRD) patients exhibit increased in vivo oxidative stress conceivably contributing to cardiovascular mortality. The type IIA secretory phospholipase A_2 ($sPLA_2$) has proatherogenic activity. We explored the hypothesis that $sPLA_2$ contributes to oxidative stress generation and endothelial dysfunction in ESRD patients and transgenic (tg) mice. Patients with ESRD had increased in vivo oxidative stress as assessed by plasma isoprostane levels ($p<0.001$). Active $sPLA_2$ in plasma was substantially increased compared with healthy controls (1,156±65 versus 184±5 ng/dL, $p<0.001$) and correlated with plasma isoprostanes ($r=0.61$, $p<0.001$). Correspondingly, human $sPLA_2$ tg mice display increased generation of reactive oxygen species within aortic vascular smooth muscle cells, leading to severe endothelial dysfunction (maximal vasodilation in response to 10 μmol/L acetylcholine, $sPLA_2$ 36±8%, controls 80±2% of phenylephrine-induced vasoconstriction). Increased vascular oxidative stress in $sPLA_2$ tg mice is dependent on the induction of vascular cyclooxygenase (COX)2 expression. Conversely, ESRD patients show increased formation of COX2-derived prostaglandins ($p<0.05$) correlated with plasma $sPLA_2$ ($r=0.71$, $p<0.05$). Our data indicate that increased expression of $sPLA_2$ might represent a novel causative risk factor contributing to the increased cardiovascular disease morbidity and mortality in ESRD.

Keywords $sPLA_2$ · Oxidative stress · End-stage renal disease · Endothelial dysfunction

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Introduction

Cardiovascular disease (CVD) is the single largest cause of morbidity and mortality in patients with uremia or reduced kidney function. In patients with end-stage renal disease (ESRD), age-adjusted cardiovascular mortality increases about 30-fold compared with the general population, with sudden death representing the major cardiovascular cause of death [1]. However, the underlying pathophysiological basis of these observations is not clear, and several traditional as well as nontraditional risk factors may contribute to atherosclerotic CVD in patients with ESRD [2]. Excessive oxidative stress is one of these factors that plays a central role and might represent a link between several features observed in ESRD patients that have been associated with cardiovascular morbidity and mortality, namely an increased inflammatory load and the extent of

malnutrition [3]. Increased oxidative stress also causally contributes to a reduced endothelial capacity in patients with renal insufficiency [4]. Endothelial dysfunction is thought to play a key role in the development of atherosclerotic CVD and to have independent prognostic implications [5]. Importantly, endothelial capacity is reduced already in patients with early stages of chronic kidney disease, resulting in severe endothelial dysfunction [6].

The type IIA secretory phospholipase A_2 ($sPLA_2$) is an acute-phase protein showing increased expression in acute as well as chronic inflammatory states [7]. $sPLA_2$ is expressed in response to proinflammatory stimuli in various cell types including vascular smooth muscle cells (VSMCs) [7, 8] that show constitutive expression of the enzyme that is dramatically upregulated in atherosclerotic areas [9, 10].

In experimental mouse models, $sPLA_2$ has provided a potential link between the dyslipidemia of inflammatory states and increased atherogenesis [11, 12]. Interestingly, plasma $sPLA_2$ levels are significantly increased in human populations with atherosclerotic CVD and normal kidney function [13] and predict cardiovascular events comparable or even superior to C-reactive protein [13].

The aim of this study was (1) to investigate if $sPLA_2$ expression might provide a link to oxidative stress generation in patients with ESRD, a patient population with a proinflammatory state and an excessive increase in CVD mortality, and (2) to explore potential pathophysiological consequences of $sPLA_2$ expression on vascular function in transgenic mice.

Methods

Patient recruitment

The study was approved by the local ethics committee, and informed consent was obtained from all patients. Sixty patients with ESRD who had been undergoing maintenance hemodialysis with a median of 40 (range 26–29) months were enrolled in this study. Of note, taking the average time these patients were on hemodialysis into account, this group might represent a selected group of survivors. Twenty-five subjects with normal renal function were used as a control group. The clinical and biochemical characteristics of patients and control subjects are given in Table 1. Patients were in a stable condition and free from intercurrent illness and infection for at least 3 months. As confirmed by clinical examination, patients were in a good state of health, notably without signs of malnutrition or wasting. All the patients were dialyzed on standard bicarbonate basis for 4–5 h three times weekly using biocompatible polysulfone hemodialysis membranes (F60, Fresenius Medical Care, Bad Homburg, Germany). Dialysis adequacy was estimated using kt/V values [14]. None of the patients had residual renal function (diuresis over 24 h

Table 1 Clinical and biochemical characterization of patients with ESRD and healthy control subjects

	ESRD (n=60)	Controls (n=25)	P
Age (years)	48 (25–59)	52 (34–64)	ns
Sex (male/female)	27/33	14/11	ns
Body weight (kg)	75 (58–88)	64 (59–74)	<0.05
Body mass index (kg/m^2)	23.4 (21.1–27.4)	21.3 (19.8–23.4)	ns
Duration of dialysis (months)	40 (26–83)	0	
Systolic blood pressure (mmHg)	134 (124–147)	122 (118–128)	<0.05
Diastolic blood pressure (mmHg)	75 (66–89)	73 (69–78)	ns
Hemoglobin (g/dL)	11.2 (10.2–11.5)	13.3 (12.9–5.6)	<0.05
Serum creatinine (mg/dL)	7.4 (5.6–9.8)	1.0 (0.7–1.1)	<0.05
Blood urea nitrogen (mg/dL)	29 (24–42)	15 (11–17)	<0.05
Total protein (g/L)	66 (52–82)	72 (68–75)	ns
Albumin (g/L)	31.3 (26.2–38.8)	36.2 (33.9–39.4)	ns
Cholesterol (mg/dL)	241 (199–298)	214 (178–234)	<0.05
CRP (mg/L)	5.4 (1.8–7.6)	1.2 (0.7–1.6)	<0.05
Kt/v[a]	1.4±0.2	–	
Smoking	7	0	<0.05
Diabetes	23	0	<0.05
Coronary heart disease[b]	11	0	<0.05
Family history of cardiovascular disease	23	11	<0.05
Hypertension[c,d]	2	0	<0.05
Use of acetylic salicylic acid	16	0	<0.05

Data are given as median and its range

[a] Kt/v values were estimated according to the DOQI guidelines [42]

[b] Coronary heart disease is assumed in the case of known heart attack or diagnosed by invasive procedures

[c] Hypertension for ESRD patients was defined as blood pressure above 140/90 mmHg prior to the hemodialysis session and hypertension for controls was defined according to ESH guidelines 2007

[d] Out of 60 patients, 42 were on antihypertensive medication

below 100 ml). The venous blood sample was drawn at midweek before dialysis session. Blood was immediately placed on ice and centrifuged at 4,000×*g* for 5 min at 4°C; plasma was stored at −80°C until analysis.

Quantitation of type IIA $sPLA_2$ protein mass and activity

Quantitation of $sPLA_2$ by ELISA (Cayman Chemical, Ann Arbor, MI, USA) and the $sPLA_2$ activity assay were performed essentially as previously described [15].

Cell culture studies

Human VSMCs were obtained from Promocell (Heidelberg, Germany). Cells were kept according to the manufacturer's protocol. For serum stimulation experiments, cells were washed twice with phosphate-buffered saline (GIBCO, Karlsruhe, Germany) and then incubated for 24 h in DMEM supplemented with 5% of the respective human sera as indicated.

Experimental animals

C57BL/6 J mice were obtained from Charles River (Sulzfeld, Germany). The human group IIA $sPLA_2$ transgenic mice on the C57BL/6 J genetic background used in this study have been described previously [11]. All animal experiments were approved by the responsible ethics committee of the Landesamt für Gesundheit, Ernährung und technische Sicherheit Berlin.

Arterial relaxation studies

The direct effects of acetylcholine (ACH) on arterial relaxation were evaluated in 2-mm rings of thoracic aorta from 3-month-old male $sPLA_2$ transgenic mice and wild-type controls as previously described [16]. Selected studies were performed in rings treated with indomethacin, NS398, SC-560, and tiron.

mRNA expression analyses by real-time quantitative PCR

Total RNA from cells and mouse aortas was isolated using TRIZOL. Expression analysis by real-time quantitative reverse-transcription PCR was performed as previously published [17]. mRNA expression levels presented were calculated relative to the average of the housekeeping gene cyclophilin.

Detection of intracellular O_2^- in aortic tissue

The oxidative fluorescent dye hydroethidine was used to evaluate in situ the production of superoxide [18].

Quantification of isoprostanes and 2,3-dinor-6-keto-$PGF_{1\alpha}$

Plasma and aortic levels of the isoprostane 8,12-iso-$iPF_{2\alpha}$-VI were measured by gas chromatography–mass spectrometry as previously described [19]. To assess cyclooxygenase (COX)2 activity in mice, urine was collected for 24 h from groups of animals, and 2,3-dinor-6-keto-$PGF_{1\alpha}$ levels were quantified by stable dilution isotope gas chromatography/mass spectrometry [20]. COX2 activity in humans was measured according to a protocol of Patrignani and co-workers [21].

Statistical analysis

Statistical analyses were performed using the statistical package for social sciences (SPSS Inc., Chicago, IL, USA). Data are presented as means±SEM. Statistical analysis was performed using the Mann–Whitney *U* test to compare different groups; Spearman's rank correlation coefficient was used to assess possible associations between different parameters, and the dose–response curves were compared using Friedman's test. All *P* values presented are two-tailed. Statistical significance for all comparisons was assigned at $P<0.05$.

Results

Increased oxidative stress in ESRD patients is correlated with elevated plasma $sPLA_2$ levels

First, we assessed plasma levels of type IIA $sPLA_2$ in patients with ESRD and explored a potential link to oxidative stress. Compared with controls, ESRD patients ($n=60$) undergoing hemodialysis displayed increased in vivo oxidative stress as assessed by plasma levels of the isoprostane 8,12-iso-$iPF_{2\alpha}$ (0.81±0.10 vs. 2.73±0.44 ng/mL, $p<0.001$, Fig. 1a). Patients with ESRD had on average 6.3-fold higher levels of $sPLA_2$ compared with healthy controls ($n=25$; ESRD 1,156±65 versus controls 184±5 ng/dL, $p<0.001$, Fig. 1b). Plasma $sPLA_2$ activity was 3.4-fold elevated in patients with ESRD (ESRD 141±16 vs controls 42±2U/mL, $p<0.001$, data not shown) and correlated well with plasma $sPLA_2$ concentration measurements ($r=0.50$, $p<0.001$, Fig. 1c), indicating that $sPLA_2$ in patients with ESRD is catalytically active. Plasma $sPLA_2$ levels in ESRD patients were neither correlated with the time that these patients had been undergoing maintenance hemodialysis ($r=-0.19$, ns) nor with hemoglobin ($r=-0.10$, ns) or serum albumin levels ($r=-0.12$, ns). There was a trend towards a correlation between plasma $sPLA_2$ and CRP levels ($r=0.25$, $p=0.062$). Neither treatment with statins nor ACE inhibitors or angiotensin II type 1 receptor antagonists was associated

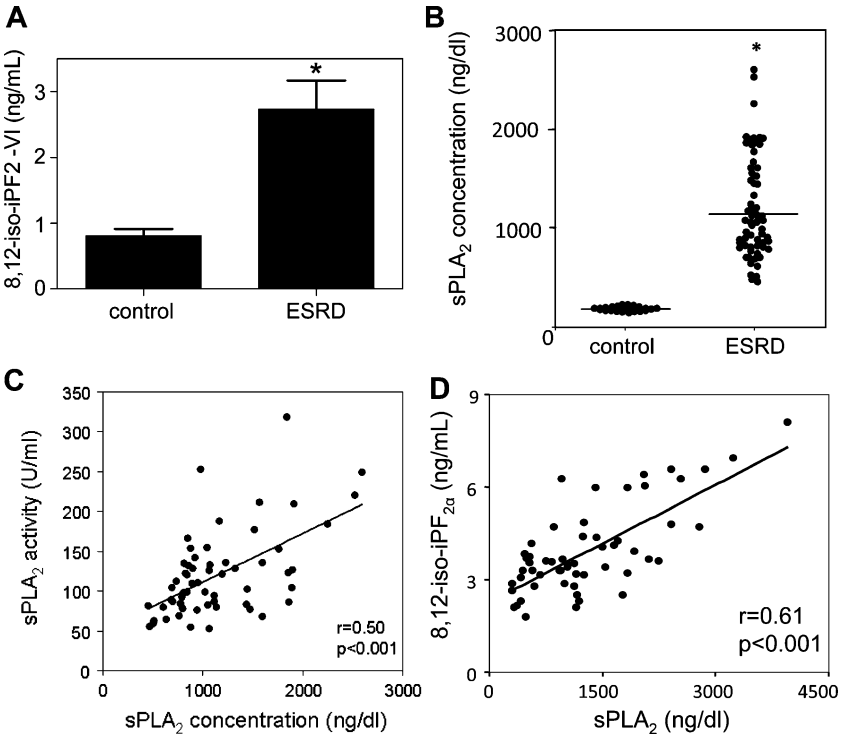

Fig. 1 ESRD patients display increased oxidative stress correlated with elevated plasma levels of $sPLA_2$. **a** Plasma levels of the isoprostane 8,12-iso-iPF_2-VI. **b** plasma levels of $sPLA_2$ in patients with ESRD (n=60) and controls (n=25). Data are given as means±SEM. **c** Correlation between plasma $sPLA_2$ levels and enzymatic activity. **d** Correlation between plasma $sPLA_2$ levels and plasma isoprostanes. *Asterisk*, significantly different from control values (at least $p<0.05$)

with a significant change in plasma $sPLA_2$ levels. Body weight in ESRD was higher than in the control group; however, no influence of body weight on $sPLA_2$ levels was observed. Higher weight in ESRD patients might indicate a status of relative good health with minor relevance of the malnutrition/inflammation complex on the observed results. Importantly, plasma isoprostanes as marker of oxidative stress were positively correlated with plasma $sPLA_2$ levels in ESRD (r=0.61, $p<0.001$, Fig. 1d).

$sPLA_2$ expression increases vascular oxidative stress in transgenic mice

Next, we investigated whether increased $sPLA_2$ expression and oxidative stress generation within the vessel wall are causatively linked. We investigated these effects in $sPLA_2$ transgenic mice which have no signs of renal failure. The plasma levels of human $sPLA_2$ in these mice are 95±7 μg/dL. Aortic levels of the isoprostane 8,12-iso-$iPF_{2\alpha}$-VI are elevated in $sPLA_2$ transgenic mice compared with wild-type C57BL/6 controls not expressing $sPLA_2$ (40.8±11.4 vs. 11.9± 1.1 pg/mg, $p<0.05$, Fig. 2a), suggesting that $sPLA_2$ expression results in increased lipid peroxidation and in vivo oxidative stress within the vascular wall.

As a further measure of vascular reactive oxygen species (ROS) generation, EtBr fluorescence was determined by confocal microscopy in aortas from $sPLA_2$ transgenic mice and controls. In contrast to controls, tissue sections of aortas from $sPLA_2$ transgenic mice showed a marked basal increase in EtBr fluorescence (Fig. 2b), indicating signifi-

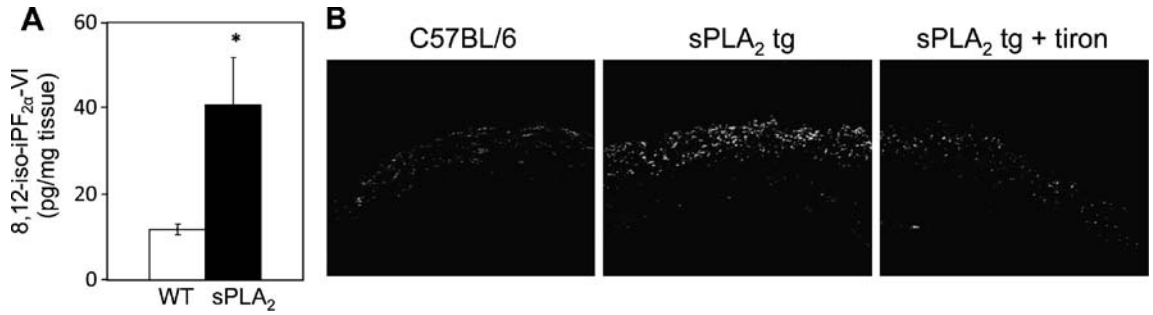

Fig. 2 $sPLA_2$ transgenic animals have increased ROS production and in vivo oxidative stress. **a** Aortic content of the isoprostane 8,12-iso-iPF_2-VI in $sPLA_2$ transgenic mice and controls (n=5). Data are given as means±SEM. *Asterisk*, significantly different from controls ($p<0.05$). **b** In situ formation of superoxide radicals in aortas from $sPLA_2$ transgenic mice ($sPLA_2$ tg) and controls (C57BL/6) using the oxidative dye hydroethidine detected by fluorescent microscopy as detailed in "Methods"

cantly increased production of ROS within the media of the vessel wall of $sPLA_2$ transgenic mice. In the presence of the free oxygen radical scavenger tiron (10 mmol/L), EtBr fluorescence in aortas from $sPLA_2$ transgenic mice was reduced to levels observed in controls.

Increased vascular oxidative stress in $sPLA_2$ transgenic mice mediates severe endothelial dysfunction

Next, we studied the potential pathophysiological effects of increased $sPLA_2$-mediated oxidative stress generation by assessing vascular function in the $sPLA_2$ transgenic mice. There is an established link between increased oxidative stress and reduced endothelial function [4]. To examine the effects of ACH (for endothelium-dependent vasorelaxation) and sodium nitroprusside (SNP; for endothelium-independent vasorelaxation) on vascular tone in $sPLA_2$ transgenic mice and controls, both substances were added to aortic rings precontracted with phenylephrine (PE 1 μmol/L; maximal vasoconstriction 8.1±0.8 mN, n=16). Maximal vasoconstriction achieved in response to 1 μmol/L PE was not significantly different in aortas from $sPLA_2$ transgenic mice and controls. ACH and SNP induced dose-dependent vasodilation in either $sPLA_2$ transgenic mice or controls (Fig. 3a, b). Maximal ACH-induced vasodilation was significantly lower in $sPLA_2$ transgenic mice compared with controls, indicating severe endothelial dysfunction in $sPLA_2$ transgenic mice (maximal vasodilation in response to 10 μmol/L ACH in $sPLA_2$ transgenic mice 36±8% and in controls 80±2% of PE-induced vasoconstriction, $p<0.05$; n=8; Fig. 3a). EC_{50} of ACH did not differ between both groups of experimental animals (EC_{50} [log mol/L]: $sPLA_2$ transgenic −6.2±0.5 and wild type −6.3±0.1, Fig. 3a). Maximal SNP-induced vasodilation in $sPLA_2$ transgenic mice and controls was not significantly different, indicating normal endothelial-independent vascular function in $sPLA_2$ transgenic mice (Fig. 3b).

We then investigated the effect of the ROS scavenger tiron (10 mmol/L) on endothelium-dependent vasodilation in $sPLA_2$ transgenic animals and controls. In the presence of tiron, the endothelium-dependent vasodilation induced by increasing doses of ACH was significantly improved (maximal vasodilation in response to 100 μmol/L ACH in $sPLA_2$ transgenic mice 36±8% of PE-induced vasoconstriction, $sPLA_2$ transgenic + tiron (10 mmol/L) 87±5% ($p<0.05$) of PE-induced vasoconstriction, Fig. 3c). In wild-type controls, the endothelium-dependent vasodilation induced by ACH was not significantly affected by the presence of tiron (10 mmol/L; Fig. 3d). The maximal vasodilation induced by SNP (100 μmol/L) either in $sPLA_2$ transgenic mice or controls was not affected by tiron (data not shown).

Increased vascular oxidative stress in $sPLA_2$ transgenic mice is dependent on COX2 expression

As a potential mechanism for the increased generation of ROS and oxidative stress within the vascular wall of $sPLA_2$ transgenic mice, we evaluated a possible functional coupling between $sPLA_2$ and cyclooxygenases. It has been previously shown in nonvascular cells that $sPLA_2$ can induce and upregulate COX2 expression [22, 23]. No significant difference was found regarding the expression of COX1 in aortas of wild-type controls and $sPLA_2$ transgenic

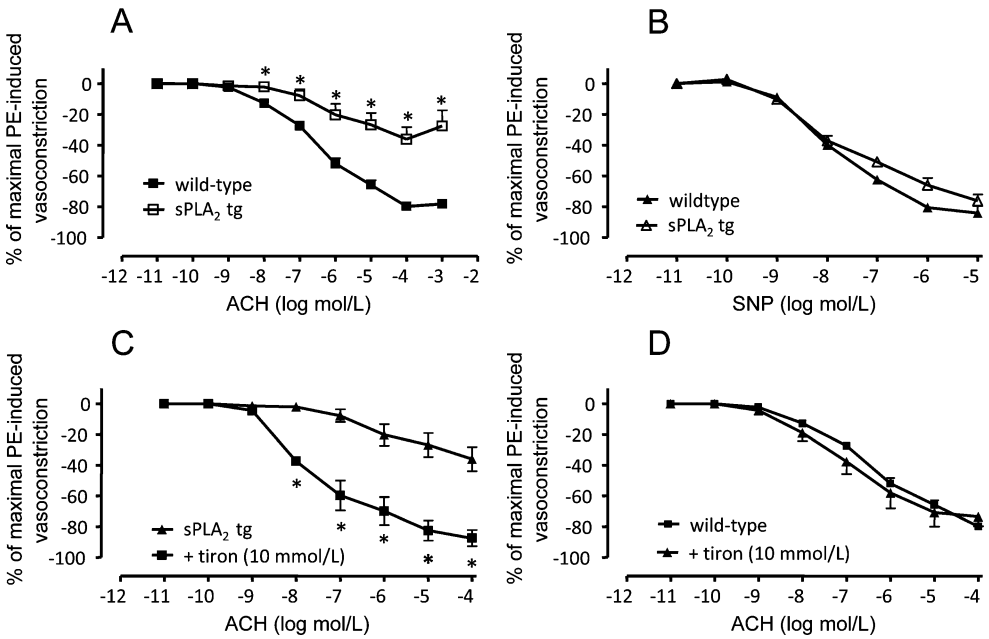

Fig. 3 $sPLA_2$ transgenic mice display severe endothelial dysfunction due to increased aortic ROS formation. Aortic rings from $sPLA_2$ transgenic mice and controls were precontracted with PE, and the direct relaxation response to ACH (**a**) and SNP (**b**) was evaluated (n=8 experiments). Data are given as means±SEM. *Asterisk*, significantly different from wild type ($p<0.05$). **c**, **d** Thoracic aortic rings from $sPLA_2$ transgenic mice (**c**) and controls (**d**) were precontracted with PE, and direct relaxation responses to ACH in the absence or presence of tiron (10 mmol/L) were evaluated (n=4 experiments each). *Asterisk*, significantly different from $sPLA_2$ values without tiron added

mice (1.00±0.09 versus 1.21±0.11, respectively, ns, data not shown). However, expression of COX2 was increased by 3.3-fold in aortas from $sPLA_2$ transgenic mice compared with controls (3.33±0.19 vs. 1.00±0.05, respectively, $p<0.001$, Fig. 4a). PGE_2 is a major cyclooxygenase product and has been shown to be involved in the progression of vascular inflammatory disease [24]. Aortic levels of the major cyclooxygenase product PGE_2 were increased by 2.3-fold in $sPLA_2$ transgenic mice compared with controls, further stressing increased cyclooxygenase activity within the vascular wall in response to $sPLA_2$ overexpression (102±21 vs. 44±9 pg/μg protein, respectively, $p<0.05$, data not shown). As further experimental support for increased vascular COX2 activity, urinary excretion of 6-keto-$PGF_{1\alpha}$ was significantly higher in $sPLA_2$ transgenic mice compared with wild-type littermates (wild type 1,258±216 vs. $sPLA_2$ 2,033±212 pg/mg creatinine, $p<0.05$, Fig. 4b).

Subsequently, we addressed the effects of altered COX activity within the vascular wall of $sPLA_2$ transgenic mice on aortic ROS production as assessed by EtBr fluorescence. Aortic ROS formation in $sPLA_2$-overexpressing vessels was dramatically reduced to the levels seen in controls by the addition of the unselective COX1/2 inhibitor indomethacin (10 μmol/L; Fig. 4c).

As a next step, the pathophysiological consequences of increased COX-dependent ROS formation within aortas of $sPLA_2$ transgenic mice were investigated. By using the unselective COX1/2 inhibitor indomethacin (10 μmol/L), the severely impaired endothelial function of $sPLA_2$ transgenic mice could be completely restored to the level observed in controls (Fig. 4d). In the presence of the selective COX1 inhibitor SC-560 (1 μmol/L), endothelium-dependent vasodilation in $sPLA_2$ transgenic mice did not change (Fig. 4d), while in the presence of the selective COX2 inhibitor NS-398 (20 μmol/L) a significant increase ($p<0.05$) in endothelium-dependent vasodilation could be observed in $sPLA_2$ transgenic mice (Fig. 4d). In wild-type controls, the endothelium-dependent vasodilation induced by ACH was not significantly affected by the presence of either indomethacin (10 μmol/L), NS-398 (20 μmol/L), or SC-560 (1 μmol/L; Fig. 4e). These data demonstrate that the severe endothelial dysfunction in $sPLA_2$ transgenic mice is dependent on increased vascular COX2 activity.

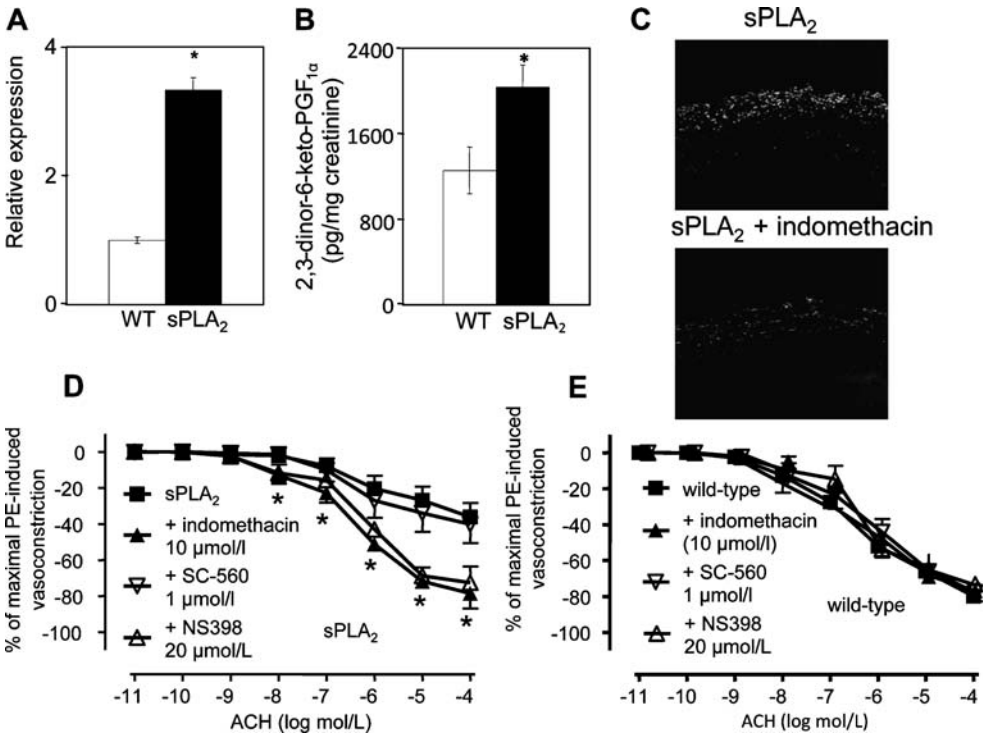

Fig. 4 $sPLA_2$ overexpression results in increased vascular COX2 expression and COX2-dependent ROS formation. **a** COX2 mRNA expression in aortas from $sPLA_2$ transgenic mice and controls determined by quantitative real-time PCR. **b** Urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ levels as marker for vascular COX2 activity measured in urine collected for 24 h from $sPLA_2$ transgenic mice and controls. Data are given as means±SEM. *Asterisk*, significantly different from controls (at least $p<0.05$). **c** In situ detection of superoxide radicals in aortas from $sPLA_2$ transgenic mice with and without addition of the COX1/2 inhibitor indomethacin (10 μmol/L for 60 min) using the oxidative dye hydroethidine. **d**, **e** Thoracic aortic rings from $sPLA_2$ transgenic mice (**d**) and wild-type controls (**e**) were precontracted with PE, and direct relaxation responses to ACH in the absence or presence of the COX1/2 inhibitor indomethacin (10 μmol/L), the specific COX1 inhibitor SC-560 (10 μmol/L), or the specific COX2 inhibitor NS-398 (20 μmol/L) were evaluated ($n=4$ experiments each). Data are given as means±SEM. *Asterisk*, significantly different from $sPLA_2$ values without added agents (at least $p<0.05$)

Increased COX2-dependent PGE2 formation in ESRD patients is correlated with plasma $sPLA_2$ levels

To investigate whether COX2 activity is also increased in ESRD patients and whether this might be linked to $sPLA_2$ levels, first, COX2-dependent production of PGE_2 within blood as a marker of COX2 activity was measured. PGE_2 generation was significantly increased in ESRD patients compared with controls (42.05±7.01 vs. 19.57±5.59 ng/mL in controls, $p<0.005$, Fig. 5a). Interestingly, COX2 activity was correlated with plasma $sPLA_2$ levels in ESRD patients ($r=0.61$, $p<0.05$, Fig. 5b). Next, we investigated the potential of serum from healthy controls with normal and ESRD patients with high levels of $sPLA_2$ (ESRD 1,462± 78 ng/dL ($n=7$) versus controls 120±16 ng/dL, $p<0.001$) to induce COX1 and COX2 expression in human aortic VSMCs: no significant difference was found regarding the expression of COX1 using serum from healthy controls compared with serum from ESRD patients (1.00±0.09 versus 1.14±0.14, respectively, ns, Fig. 5c). However, expression of COX2 was increased by 5.4-fold in hVSMC in the presence of ESRD serum compared with control serum (5.44±1.01 versus 1.00±0.05, respectively, $p<$ 0.001, Fig. 5d). These data indicate that similar pathophysiological mechanisms might be operative in ESRD patients as delineated in the human $sPLA_2$ transgenic mouse.

Discussion

In the present study, we demonstrate that circulating levels of functionally active $sPLA_2$ are dramatically increased in ESRD patients closely correlated with plasma isoprostanes, a highly sensitive marker of in vivo oxidative stress. In addition, using human $sPLA_2$ transgenic mice, we provide for the first time evidence that increased $sPLA_2$ expression negatively impacts on endothelial function via a COX2-mediated increase in oxidative stress. Furthermore, our data indicate that in ESRD patients similar pathophysiological mechanisms might be operative.

$sPLA_2$ is a cardiovascular risk factor with intrinsic proatherosclerotic biological activity. Transgenic overexpression of $sPLA_2$ in mice results in increased atherosclerosis formation already on a chow diet [12]. $sPLA_2$ decreases plasma high-density lipoprotein (HDL) cholesterol levels by increasing the HDL catabolic rate [11, 25]; $sPLA_2$ induces the formation of aggregated/fused low-density lipoprotein (LDL) particles [26], and $sPLA_2$-modified HDL loses its protective properties against LDL oxidation, which has been attributed to a loss of paraoxonase activity [27]. Macrophage expression of $sPLA_2$ significantly enhances LDL oxidation even in the absence of HDL particles [17] and is sufficient to increase atherosclerotic lesion formation [17, 28]. Our present study extends these observations by showing that $sPLA_2$ expression also contributes to vascular oxidative stress generation, resulting in severe endothelial dysfunction.

Plasma $sPLA_2$ levels are elevated in patient populations with atherosclerotic CVD, [13, 29] and are predictive for the risk of future coronary events in patients with normal kidney function [13, 30]. Notably, plasma $sPLA_2$ levels determined in these patient populations were substantially lower than the levels we found in ESRD patients. In all previous studies, there was a considerable overlap in plasma $sPLA_2$ levels between the groups of patients and controls. In the present study, however, there was a unique threshold value of circulating $sPLA_2$, clearly identifying the patients with ESRD.

Endothelial dysfunction is thought to represent an important independent clinical predictor for atherosclerosis development as well as cardiovascular events [5, 31]. This condition is already present in early stages of chronic kidney disease [6, 32] and highly pronounced in patients with ESRD [33, 34]. It is also well documented that

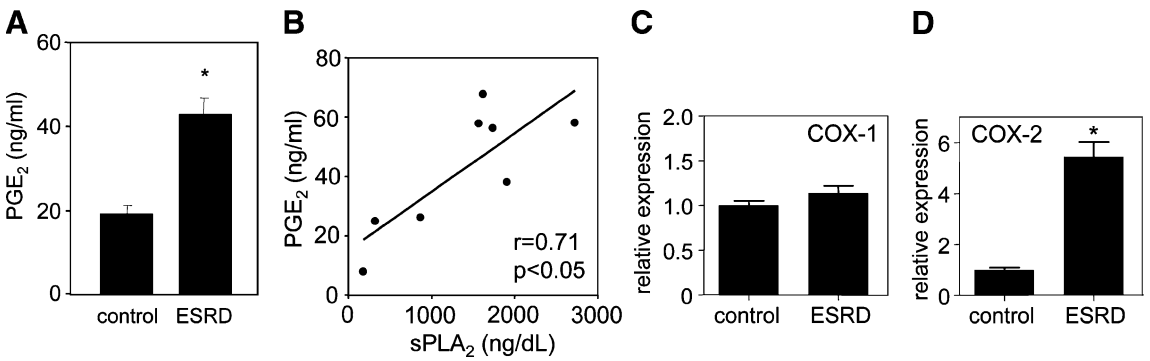

Fig. 5 ESRD patients display increased COX2 activity correlated with plasma $sPLA_2$ levels. **a** COX2-dependent PGE_2 production. **b** Correlation between plasma $sPLA_2$ levels and COX2-dependent PGE_2 formation in ESRD ($n=8$) patients. mRNA expression of COX1 (**c**) and COX2 (**d**) in human aortic VSMCs following incubation with serum from patients with ESRD and controls ($n=8$). Data are given as means±SEM. *Asterisk*, significantly different from control values (at least $p<0.05$)

patients with ESRD display increased ROS production and oxidative stress [35, 36] reflected by increased plasma isoprostane levels [37] that were in our present study correlated with plasma $sPLA_2$ levels. Increased oxidative stress substantially contributes to endothelial dysfunction since superoxide anions (O_2^-) inactivate nitric oxide (NO), a principal endothelium-derived relaxing factor [34, 38]. Consistently, overexpression of $sPLA_2$ in mice results in reduced endothelial capacity by increasing the production of ROS within the vessel wall. This increase occurs specifically within the media, the area of the vessel wall that is rich in VSMCs, one of the major cellular sources of $sPLA_2$ expression [8].

$sPLA_2$ is an extracellular phospholipase that cleaves phospholipids at the sn-2 position. The $sPLA_2$ reaction mainly yields arachidonic acid, which is then available as precursor for the production of prostaglandins [39]. Interestingly, aortas of $sPLA_2$ transgenic mice show increased expression of COX2 mRNA, an increased content of the major COX product PGE_2, and increased urinary levels of 2,3-dinor-6-keto-$PGF_{1\alpha}$, an eicosanoid indicative of increased vascular COX2 activity. On the other hand, the ESRD patients investigated also had increased COX2-dependent production of prostaglandins correlated with plasma $sPLA_2$ levels, indicating that similar molecular mechanisms might be operative in human patients. To test this hypothesis, administration of a COX2-specific inhibitor to ESRD patients would be desirable. However, for safety reasons, selective COX2 inhibitors are currently not indicated in this group of patients, as there were no studies approving these medications in ESRD. Although in ESRD patients administration of a COX2 inhibitor to establish causality is not possible due to ethical considerations, pharmacological inhibition of COX2 in $sPLA_2$ transgenic mice resulted in a significant decrease of ROS production and a normalization of the endothelial dysfunction. While the COX product PGE_2 has been demonstrated to be capable of inducing vasoconstriction [40], there is evidence that in our system the role of PGE_2 might be minor. Not only COX inhibition but also ROS scavengers improved endothelial dysfunction in $sPLA_2$ transgenic mice pointing to COX as a source of ROS rather than of PGE_2.

How the increase in COX2 expression is mediated at the transcriptional level is presently not clear. Studies performed in different contexts noted increased COX2 expression when synoviocytes [23] and mast cells [22] were incubated with purified $sPLA_2$ enzyme. In addition, positive feedback regulation of COX2 by PGE_2 has been reported [41], and it is therefore tempting to speculate that this might also contribute to linking $sPLA_2$ and COX2 expression in our experimental model. However, at the current stage, it cannot be excluded that other factors or uremic toxins contribute to the induction of COX2 in response to serum from ESRD patients.

In summary, we provide the first evidence for a dramatic increase in plasma $sPLA_2$ levels as well as enzymatic activity in patients with ESRD tightly correlated with oxidative stress. Human $sPLA_2$ expression in transgenic mice resulted in a COX2-dependent increase in ROS generation within the vascular wall and reduced endothelial capacity. Therefore, pharmacological inhibition of $sPLA_2$ in patients with ESRD might represent a novel therapeutic strategy to decrease the oxidative burden in this high-risk patient group, conceivably translating into a reduction of cardiovascular morbidity and mortality.

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