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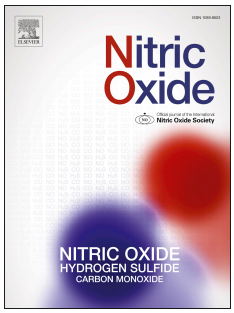

DL-propargylglycine reduces blood pressure and renal injury but increases kidney weight in angiotensin-II infused rats

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Title: DL-propargylglycine reduces blood pressure and renal injury but increases kidney weight in angiotensin-II infused rats

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Abstract

Hydrogen sulfide (H_2S), carbon monoxide (CO) and nitric oxide (NO) share signaling and vasorelaxant properties and are involved in proliferation and apoptosis. Inhibiting NO production or availability induces hypertension and proteinuria, which is prevented by concomitant blockade of the H_2S producing enzyme cystathionine γ-lyase (CSE) by D,L-propargylglycine (PAG). We hypothesized that blocking H_2S production ameliorates Angiotensin II (AngII)-induced hypertension and renal injury in a rodent model. Effects of concomitant administration of PAG or saline were therefore studied in healthy (CON) and AngII hypertensive rats.

In CON rats, PAG did not affect systolic blood pressure (SBP), but slightly increased proteinuria. In AngII rats PAG reduced SBP, proteinuria and plasma creatinine (180±12 vs. 211±19 mmHg; 66±35 vs. 346±92 mg/24h; 24±6 vs. 47±15 µmol/L, respectively; $p<0.01$). Unexpectedly, kidney to body weight ratio was increased in all groups by PAG ($p<0.05$). Renal injury induced by AngII was reduced by PAG ($p<0.001$). HO-1 gene expression was increased by PAG alone ($p<0.05$). PAG increased inner cortical tubular cell proliferation after 1 week and decreased outer cortical tubular nucleus number/field after 4 weeks. In vitro proximal tubular cell size increased after exposure to PAG.

In summary, blocking H_2S production with PAG reduced SBP and renal injury in AngII infused rats. Independent of the cardiovascular and renal effects, PAG increased HO-1 gene expression and kidney weight. PAG alone increased tubular cell size and proliferation *in-vivo* and *in-vitro*. Our results are indicative of a complex interplay of gasotransmitter signaling/action of mutually compensatory nature in the kidney.

Keywords

Hydrogen sulfide
DL-propargylglycine
Angiotensin-II
Proteinuria
Hypertension
Kidney weight

1. Introduction

Hydrogen sulfide (H_2S) is the third gasotransmitter, in addition to nitric oxide (NO) and carbon monoxide (CO), and all three are involved in similar physiological processes [1]. H_2S is endogenously produced in mammalian cells [2] and catalyzed from L-cysteine by the enzymes cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfur transferase (MST) [3; 4; 5]. CSE is mainly responsible for H_2S synthesis in the kidney, liver, and vascular endothelium and smooth muscle cells [1; 6], while CBS and MST account for the majority of H_2S generation in the nervous system [7]. CSE derived H_2S synthesis can be inhibited with DL-propargylglycine (PAG) [8; 9] or by genetic deletion of CSE [10; 11].

The role of H_2S as a vasodilator is well documented [1], but H_2S seems to act in a bell-shaped fashion; causing vasoconstriction at low concentrations, vasorelaxation at higher concentrations, and toxicity at even higher concentrations [4]. Increasing H_2S availability by two different H_2S donors decreased blood pressure and improved renal function and morphology in Angiotensin II (AngII)-infused rats [12]. Inhibiting H_2S synthesis by administration of both PAG and aminooxyacetic acid (AOA), a CBS blocker, increased mean arterial pressure (MAP) and decreased renal blood flow without affecting glomerular filtration rate indicating post-glomerular vasoconstriction while treatment with only one of these enzyme blockers did not affect MAP and renal function in healthy rats [13]. Pretreatment of rats with PAG caused an increased infarct size after myocardial ischemia induced by vasoconstriction [14]. However, hypertension and proteinuria caused by blocking NO synthesis could be prevented by concomitant PAG administration [15].

Thus the effects of modulating endogenous H_2S levels are inconsistent and might be concentration and cell type or organ dependent. As a result, the functions of endogenously produced H_2S in the healthy and diseased kidney are not well understood. In renal ischemia/reperfusion injury, inhibition of H_2S synthesis increased plasma creatinine values and mortality [10], while in toxic kidney injury, inhibiting H_2S synthesis reduced plasma creatinine and improved morphology. The latter effect was possibly caused by a reduction in inflammation [16; 17; 18]. Blocking H_2S production resulted in an increase in CO production *in-vivo* [15] and *in-vitro* [19]. Similar *in-vivo* effects on CO production were observed when H_2S inhibition was combined with NO synthase blockade [15]. Remarkably, H_2S donors are also able to increase heme-oxygenase-1 expression (HO-1) [20; 21]. Because all three gasotransmitters have vasodilatory properties [1], it is possible that CO can compensate for diminished H_2S or NO production.

Besides regulating vascular tone, H_2S is also involved in cellular processes like proliferation [22] and protein synthesis and thereby cellular hypertrophy [23]. H_2S can have inhibitory as well as stimulatory effects on proliferation. Inhibition of proliferation was observed in HEK-293 cells and smooth muscle cells overexpressing CSE and apoptosis was increased under these conditions [24; 25]. In CSE gene knockout mice, proliferation of aortic smooth muscle cells was increased compared with wild-type mice. Lowering blood pressure did not affect proliferation [26]. However, Bos et al. [10] reported that proliferation was not

affected in kidneys of CSE gene knockout mice. Furthermore, tubular proliferation was found to be decreased by PAG in adriamycin-induced kidney injury [17].

We sought to study the effects of H_2S inhibition on renal morphology and function in healthy rats and in a hypertensive renal injury model caused by AngII infusion. Based on our previous finding that PAG ameliorated hypertension and renal injury induced by NO inhibition [15], we hypothesized that PAG also decreases blood pressure and ameliorates renal injury in the AngII model. Moreover, because we found PAG to enhance renal mass and affect cell proliferation, we also studied effects of PAG on epithelial cell number and size *in-vivo* and *in-vitro*.

2. Materials and methods

2.1 Animals

Male Sprague-Dawley rats (261±20 gram, Harlan, Blackthorn, UK or Zeist, the Netherlands) were housed in a light-, temperature- and humidity controlled environment under standard conditions i.e. a 12 hour light/dark cycle and with free access to water and standard rodent chow. Protocols were approved by the Animal Ethics Committees of Utrecht University and the University of Groningen. Animal experiments were performed according to ARRIVE guidelines.

2.2 Experimental setup

PAG (dissolved in saline 30 mg/mL, 37.5 mg/kg BW) was administered daily intraperitoneally (ip) for one or four weeks in healthy rats (n=6, CON + PAG), while healthy saline treated rats served as control group (n=6, CON) as previously described [15]. Hypertension-driven renal injury was induced by AngII infusion (435 ng/kg/min, Bachem, Weil am Rhein, Germany) for three weeks via a subcutaneous osmotic minipump (model 2004, Alzet, Cupertino, CA) which were implanted under isoflurane anesthesia with buprenorphine analgesia [12]. As a control for AngII infusion, saline-filled minipumps were implanted in a group of rats (n=5, VEH). AngII-infused rats were randomly divided over intraperitoneal injections with saline (saline twice daily, n=7; AngII) and PAG (18.75 mg/kg twice daily ip, n=7; AngII + PAG). At the end of the experiment rats were sacrificed, a blood sample was taken and kidneys were excised, weighed, fixed in formaldehyde and then embedded in paraffin or snap frozen and stored at -80℃ for RNA isolation.

2.3 H_2S production in kidney

Renal H_2S production was measured exactly as previously described [15]. For all samples 5% (w/v) renal homogenates were used for measurements.

2.4 Kidney function and systolic blood pressure

Systolic blood pressure (SBP) was measured and a blood sample was collected before sacrifice. 24-hour urine samples were collected weekly (except week 3 for 4wk CON rats) by placing rats individually in metabolic cages without chow but with free access to glucose-containing water (2% w/v). Urine was collected on antibiotics to prevent formation of NO metabolites and frozen after collection. In CON groups, total protein excretion was measured using Bradford method (BioRad Laboratories, Veenendaal, Netherlands) and plasma creatinine was enzymatically measured (DiaSys PAP FS; DiaSys Diagnostic Systems, Holzheim, Germany). In VEH and AngII groups, total protein excretion was measured using pyrogallol red molybdate method [27] and plasma creatinine was measured on the Roch Modular with a standard assay from Roche (Roche Diagnostics GmbH, Mannheim, Germany). SBP was measured by tail

cuff sphygmomanometry (CON groups) or via an intra-aortic probe before sacrifice (VEH and AngII groups).

2.5 Urine malondialdehyde

Malondialdehyde (MDA) is a major breakdown product of lipid peroxides, which is generated under conditions of oxidative stress and can be measured fluorimetrically following reaction with thiobarbituric acid. A total of 20 µL urine was mixed with 90 µL of 3% SDS and 10 µL of 0.5 M butylated hydroxytoluene followed by addition of 400 µL 0.1N HCl, 50 µL 10% phosphotungstic acid and 200 µL 0.7% 2-Thiobarbituric acid. This reaction mixture was incubated for 30 minutes at 95℃. After addition of 800 µL of 1-butanol, samples were centrifuged at 960g for 10 minutes. The supernatant was fluorescently measured at 530 nm excitation and 590 nm emission.

2.6 NO metabolites

Both urine and plasma samples were added to equal volumes of methanol, vortexed, and centrifuged at 16100g on 4ºC for 20 min. The supernatant was transferred to autosampler vials and oxidation products of nitric oxide (nitrite and nitrate) were quantified by high pressure liquid ion chromatography with on-line reduction of nitrate to nitrite and post-column derivatisation with the Griess reagent (ENO-20 Analyser; Eicom, San Diego, CA) [28; 29]. For some urine samples, a further dilution with PBS (10 mM, pH 7.4) was required to stay within the linear part of the standard curve. Fractional excretions of nitrite and nitrate were calculated using the following formula: urine NOx*plasma Creatinine/plasma NOx*urine Creatinine. All concentrations in µM.

2.7 Quantitative polymerase chain reaction

Renal cDNA was isolated to determine gene expression of CSE, CBS and HO-1 using qPCR (ViiA7 Real-Time PCR system, Life technologies, Waltham, MA). The following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used: CTH (Rn00567128_m1), CBS (Rn01428016_m1) and HO-1 (Rn00561387_m1). Cycle time (Ct) values for CBS, CSE and HO-1 were normalized for mean Ct-values of ACTB (Rn00667869_m1) and CANX (Rn00596877_m1) or HPRT (Primers (Integrated DNA Technologies, Coralville, IA, USA) Forward: 5'-GCC CTT GAC TAT AAT GAG CAC TTC A-3', Reverse: 5'-TCT TTT AGG CTT TGT ACT TGG CTT TT-3' and Probe (Eurogentec, Maastricht, the Netherlands): 6-FAM 5'-ATT TGA ATC ATG TTT GTG TCA TCA GCG AAA GTG-3' TAMRA.), and expressed relative to their own control groups using the ΔΔCt method.

2.8 Renal morphology

Three µm sections were sliced of formaldehyde-fixed, paraffin-embedded kidneys. Sections were stained with primary antibodies for desmin (mouse anti-desmin NCL-DES-DER11, 1:500, Novacastra, Rijswijk, the Netherlands), Kidney Injury Molecule 1 (KIM-1, rabbit anti-Kim-1 peptide 9, 1:400, gift V. Bailly, BiogenInc,

Cambridge, MA, USA), ED-1 (mouse anti-CD68 ED-1, MCA341R AbD, 1:750, Serotec Ltd, Oxford, UK), α-Smooth Muscle Actin (α-SMA, mouse anti-SMA, clone 1A4 A2547, 1:10.000, Sigma, Zwijndrecht, the Netherlands), Collagen III (goat anti-type III Collagen, 1330-01, 1:75, Southern Biotech, Birmingham, AL, USA), Ki67 (rabbit anti-Ki67, RM-9106, 1:100, Fisher Scientific, Waltham, MA) and Podoplanin (mouse anti-Podoplanin, 11-035, Angio Bio, Del Mar, CA). Deparaffinized sections were subjected to heat-induced antigen retrieval by overnight incubation with 0.1 M Tris/HCl buffer (pH 9.0) at 80℃ (desmin, KIM-1, ED-1, αSMA) or by incubation with EDTA buffer (pH 8.0) heated by a microwave (Collagen III) or by incubation with citrate/HCl buffer (pH 6.0) at 100℃ for 20 mi nutes (Ki67, Podoplanin). Kidney sections were scanned using an Aperio Scanscope GS (Aperio Technologies, Vista, CA, USA). The extent of glomerular damage (desmin), proximal tubular ischemic damage (KIM-1) and fibrotic changes (α-SMA, Collagen III) were determined using the Aperio positive pixel analysis v9.1 algorithm. For desmin the ratio between glomerular staining intensity and total cortical glomerular area was calculated. For KIM-1, α-SMA and Collagen III the ratio between the relative cortical staining intensity and the total cortical surface area was used. Interstitial macrophages were counted manually in 30 renal cortical high powered fields (HPF). Tubular lumen area was measured in cortical tubular fields, ten fields of similar size were selected and transformed to 8-bit, a threshold was set, pictures were inverted and the percentage black area was measured using ImageJ software (Rasband, W.S., ImageJ, NIH, Bethesda, MY). In 10 and 5-8 tubular fields in the outer and inner cortex of CON rats nuclei were counted using ImageJ. Proliferating cells (Ki67) were counted in 10 and 5-8 tubular fields in the outer and inner cortex to detect epithelial proliferation. Lymph vessels were stained by Podoplanin and counted in 30 cortical fields, size of individual lymph vessels was measured as perimeter with ImageJ software. Lymph vessel perimeters were categorized by size in extra small, small, medium and large vessels [30]. Histological analysis was performed in a blinded fashion.

2.9 In-vitro assay

Human proximal tubule epithelial (HK-2) cells [31] were cultured in RPMI + GlutaMax containing 10% FCS, 1% penicillin/streptomycin and 25 mM Hepes. HK-2 cells (60% confluency) were serum starved (1% FCS) for 24 hours before adding PAG (1 and 3 mM). After 48 hours cells were trypsinized and cellular size was measured by forward scatter (FSC) using flow cytometry. Histograms of cell counts were made to discriminate between small and large cells and the percentage of large cells was calculated. Cellular surface area was measured on immunofluorescent labeled cells. Accordingly, cultured cells were fixed with 4% paraformaldehyde on coverslips. Cellular membranes were labeled with Beta-catenin (9582, 1:100, Cell Signaling, Danvers, MA). Rhodamine Phalloidin (R415, 1:40, Life Technologies, Waltham, MA) and DAPI were used to label actin filaments and nuclei respectively. Cellular size of 50 cells was measured using ImageJ software.

2.10 Statistical analysis

Data are presented as mean ± standard deviation (SD). A two-way ANOVA with Student–Newman–Keuls (SNK) as post-hoc test was performed using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA) on CON and CON + PAG groups. VEH, AngII and AngII + PAG groups were compared using a one-way ANOVA with SNK as post-hoc test, when only AngII and AngII + PAG rats were included a t-test was performed. In-vitro data was tested using a one-way ANOVA with Dunnett's as post-hoc test. $P<0.05$ was considered significant, $p>0.05$ non-significant (NS).

3. Results

3.1 PAG abolished H_2S production

H_2S production in the kidneys of PAG treated rats was abolished in both CON ($p<0.001$) and AngII ($p<0.05$) groups. AngII markedly decreased H_2S production compared to VEH rats ($p<0.01$; figure 1).

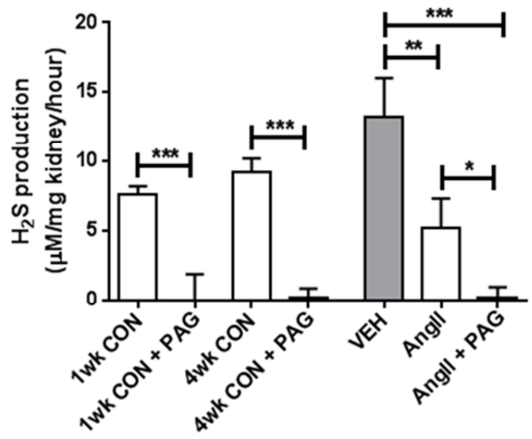

Figure 1. H_2S production in kidneys of healthy, AngII and PAG treated rats. Mean ± SD. All n=3. *p<0.05, **p<0.01, *** p<0.001

3.2 PAG improved systolic blood pressure and kidney function in AngII-infused rats

AngII infusion increased SBP and plasma creatinine measured at week 3 and proteinuria at all weeks compared to VEH rats ($p<0.01$; table 1). PAG blunted this increase in SBP and reduced plasma creatinine ($p<0.01$; table 1). At weeks 2 and 3 PAG markedly reduced AngII-induced proteinuria, with a respective median and range of 54 [20-115] vs. 329 [235-513] mg/24h at week 3. PAG administration in CON rats did not affect SBP. Plasma creatinine was slightly increased after PAG in CON rats (table 1), but was not affected by the duration of PAG (1wk vs. 4wk, NS). In CON + PAG rats proteinuria was already slightly higher at baseline compared to CON. Proteinuria increased slightly in both CON and 1wk CON + PAG rats during the course of one week. However, proteinuria was not progressive during a four week period of PAG (table 1). AngII increased urinary MDA excretion, a measure of oxidative stress, compared with VEH rats, and AngII induced oxidative stress was not affected by PAG (table 1). MDA excretion was increased by four weeks PAG compared to CON rats.

Table 1. Systolic blood pressure, plasma creatinine, proteinuria and MDA excretion in healthy, AngII and PAG treated rats. SBP and proteinuria of CON groups as published by Wesseling et al. [15].

	1wk CON	1wk CON + PAG	4wk CON	4wk CON + PAG	3wk VEH	3wk AngII	3wk AngII + PAG
n=	6	6	6	6	5	6-7	7
SBP (mmHg)	133 ± 24	135 ± 8	137 ± 7	133 ± 8	143 ± 5	211 ± 19^{bb}	180 ± 12^{aa,bb}
Creatinine (µM)	26 ± 3	53 ± 12 $^{a\ a}$	30 ± 3	41 ± 7 ^{a}	19 ± 2	47 ± 15^{bb}	24 ± 6^{aa}
Proteinuria (mg/24h)							
Baseline	11 ± 6	24 ± 4^{a}	9 ± 1	27 ± 6^{aaa}	13 ± 8	9 ± 3$^{###}$	10 ± 2
Week 1	17 ± 6*	36 ± 3^{a,}*	13 ± 3	34 ± 5^{aaa}	25 ± 14^{aa,}*	80 ± 76$^{###}$	51 ± 35***
Week 2			13 ± 1	33 ± 3^{aaa}	25 ± 17^{aaa,}**	223 ± 74$^{#}$	49 ± 37^{aaa,}***
Week 3					28 ± 25^{aaa,}*	346 ± 92$^{#}$	66 ± 35^{aaa,bb,}***
Week 4			17 ± 5*	30 ± 7^{aaa}			
MDA excretion (nmol/24h/100g)	27 ± 10	34 ± 15	24 ± 10	36 ± 5 ^{a}	20 ± 7	67 ± 15^{bb}	60 ± 22^{bb}

Mean ± SD. ^{a}p<0.05, ^{aa}p<0.01 vs. 1wk or 4wk CON or AngII group, ^{b}p<0.05, ^{bb}p<0.01 vs. VEH, *p<0.05, **p<0.01, ***p<0.001 vs. Baseline, $^{#}$p<0.05, $^{###}$p<0.001 vs. all weeks

3.3 PAG decreased plasma and urinary NO metabolites in healthy but not in AngII infused rats

PAG decreased plasma nitrite concentrations (NO_2^-, p<0.001; figure 2A) and urinary NO_2^- excretion (p<0.001; figure 2B), as well as fractional NO_2^- excretion ($FeNO_2^-$, figure 2C; Two-way ANOVA PAG effect p<0.05). AngII increased NO_2^- excretion (p<0.05) and tended to increase $FeNO_2^-$ (p=0.052), but did not affect plasma NO_2^- (figure 2A-C). No additive effects of PAG were observed concomitantly with AngII.

Plasma nitrate (NO_3^-, figure 2D), NO_3^- excretion (figure 2E) and $FeNO_3^-$ (figure 2F) all decreased after four weeks of PAG administration (all p<0.01). AngII increased plasma NO_3^- concentrations, but not NO_3^- excretion (figure 2D,E). PAG reduced AngII increased $FeNO_3^-$ (p<0.05; figure 2F).

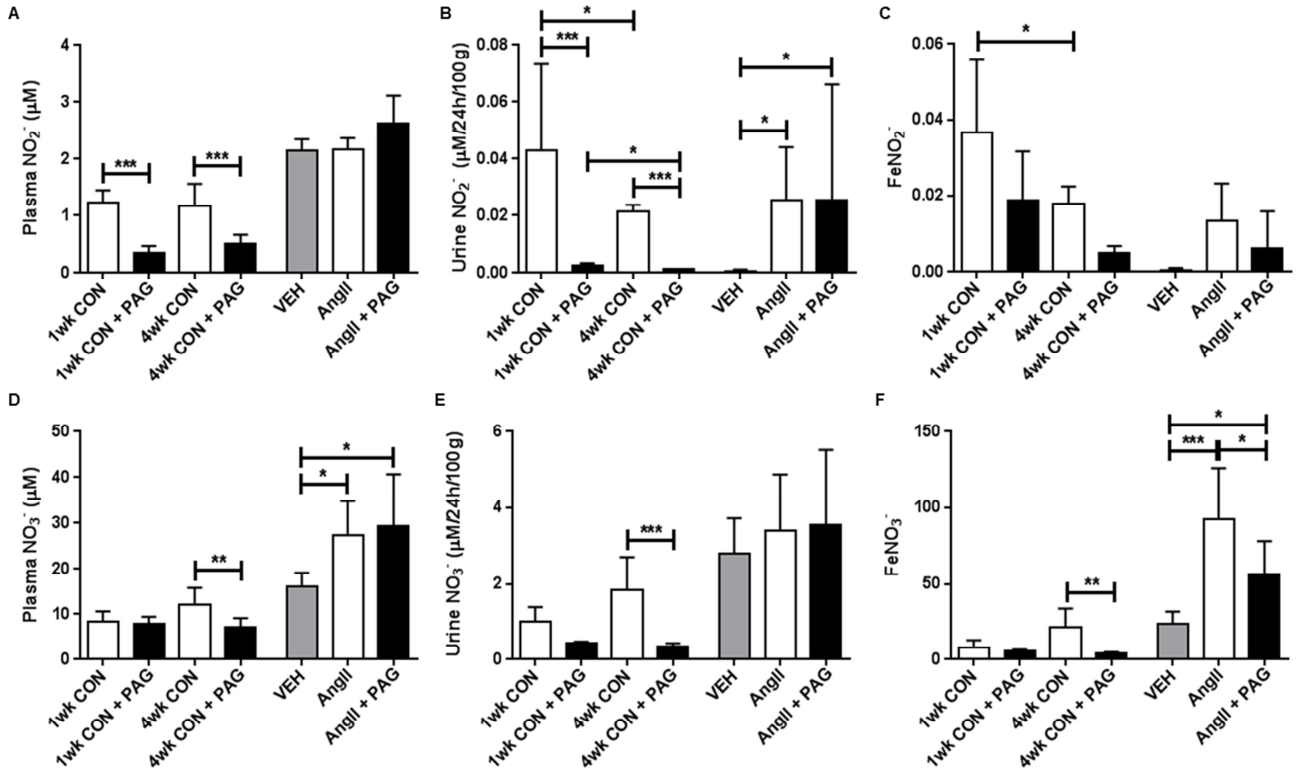

Figure 2. Plasma levels (A), renal excretion (B) and fractional excretion (C) of nitrite (NO_2^-) and plasma levels (D), renal excretion (E) and fractional excretion (F) of nitrate (NO_3^-) in healthy, AngII and PAG treated rats. Mean ± SD. 1wk CON, 4wk CON and 4wk CON + PAG n=6, 1wk CON + PAG n=3, 3 wk VEH n=5, 3 wk AngII and 3 wk AngII + PAG n=7. *p<0.05, **p<0.01, *** p<0.001

3.4 PAG increased HO-1 gene expression in healthy rats

PAG increased HO-1 gene expression in CON rats at one and four weeks (p<0.05). AngII infusion also increased HO-1 gene expression vs. VEH rats (p<0.05), however this was not significantly affected by PAG (AngII vs. AngII + PAG, NS; figure 3A). Proteinuria correlated with HO-1 gene expression in CON, VEH and AngII rats without PAG (p<0.01), but this correlation was absent in CON and AngII rats with PAG (figure 3B).

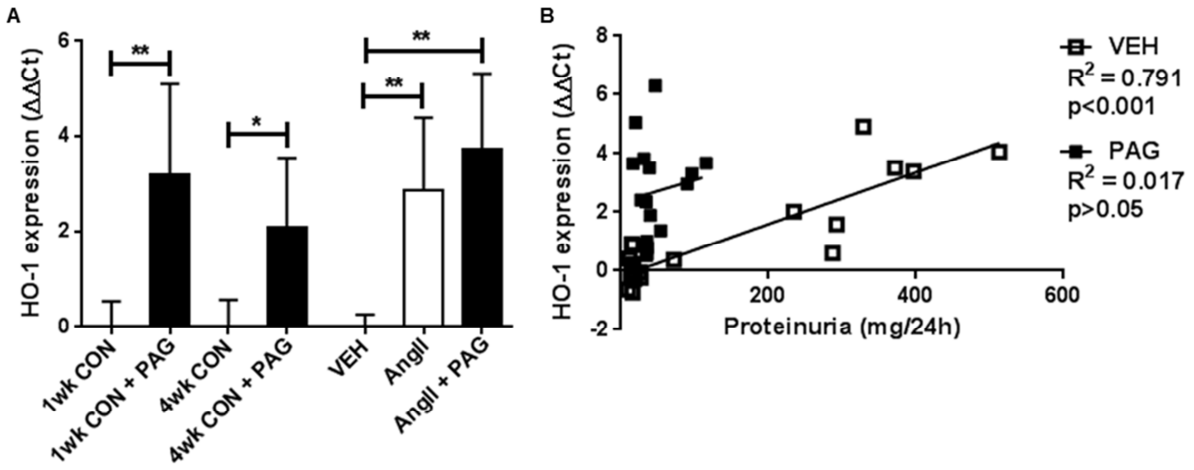

Figure 3. Relative renal HO-1 gene expression in PAG and AngII treated rats normalized to their own healthy control group (A). B: Correlation of HO-1 gene expression with proteinuria for all VEH (n=24) and all PAG (n=16) rats. Mean ± SD. CON and CON + PAG groups n=6, VEH n=5, AngII and AngII + PAG n=7. *p<0.05,**p<0.01

3.5 PAG reduced renal damage and inflammation in AngII-infused rats

No glomerular tuft damage was observed in CON or CON + PAG rats (figure 4A). AngII induced glomerular tuft damage, which was reduced by PAG (700.3 ± 111.7 vs. 421.4 ± 116.0 intensity/μm^2, p<0.01; figure 4A,B). Proximal tubular damage was also absent in CON and CON + PAG groups (figure 4C), therefore these groups were not included for other morphological damage scores. PAG decreased proximal tubular damage caused by AngII infusion, as evidenced by a decreased KIM-1 protein expression (6 ± 4 vs. 14 ± 6 intensity/μm^2, p<0.05; figure 4C,D). Furthermore, PAG reduced protein expression of α-SMA when compared to AngII-infused rats (6 ± 4 vs. 16 ± 5 intensity/μm^2, p<0.05; figure 5A,B) as well as Collagen III protein expression (16 ± 6 vs. 33 ± 9 intensity/μm^2, p<0.05; figure 5C,D). The number of interstitial macrophages per field were doubled by AngII compared to VEH (204 ± 91 vs. 86 ± 28, p<0.01), and PAG reduced macrophage numbers back to VEH level (40 ± 13 vs. 86 ± 28; figure 5E,F).

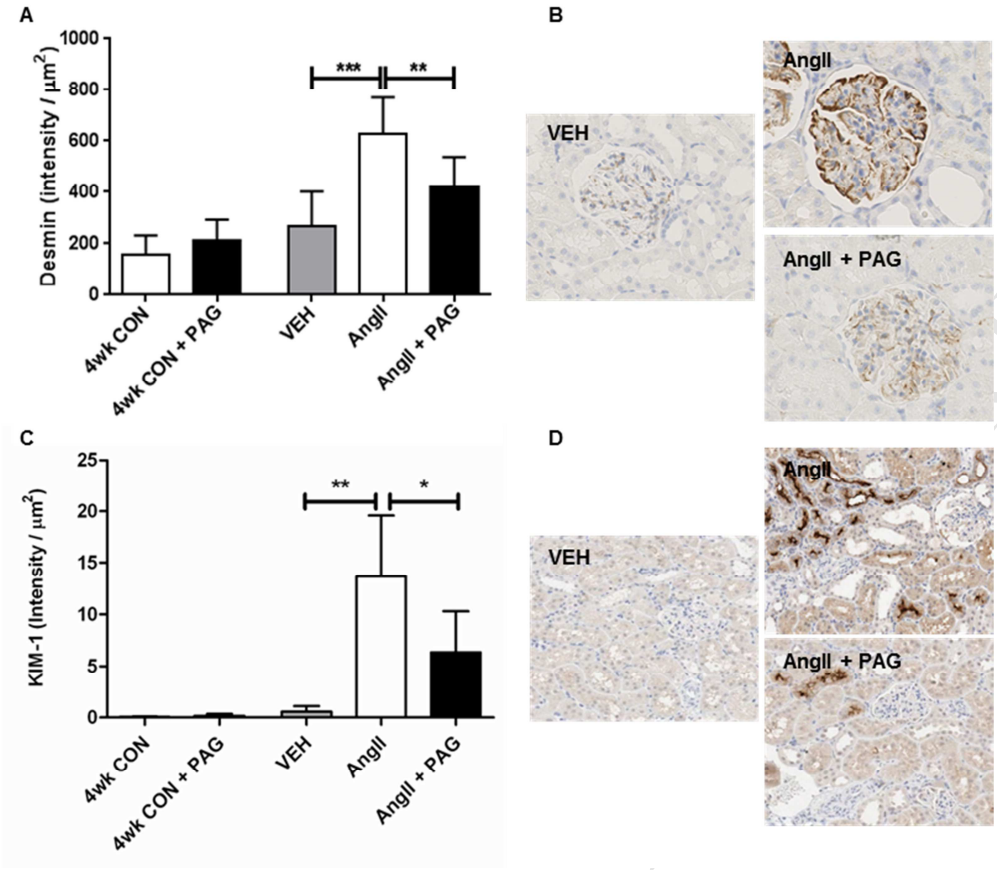

Figure 4. Glomerular tuft damage determined by desmin staining around the tuft edge (A) and proximal tubular damage determined by KIM-1 staining (C) in healthy, AngII and PAG treated rats. Representative photomicrographs of glomerular desmin (B) and KIM-1 (D) stained renal sections. Mean ± SD. VEH n=5, AngII and AngII + PAG n=7. *p<0.05, **p<0.01, ***p<0.001

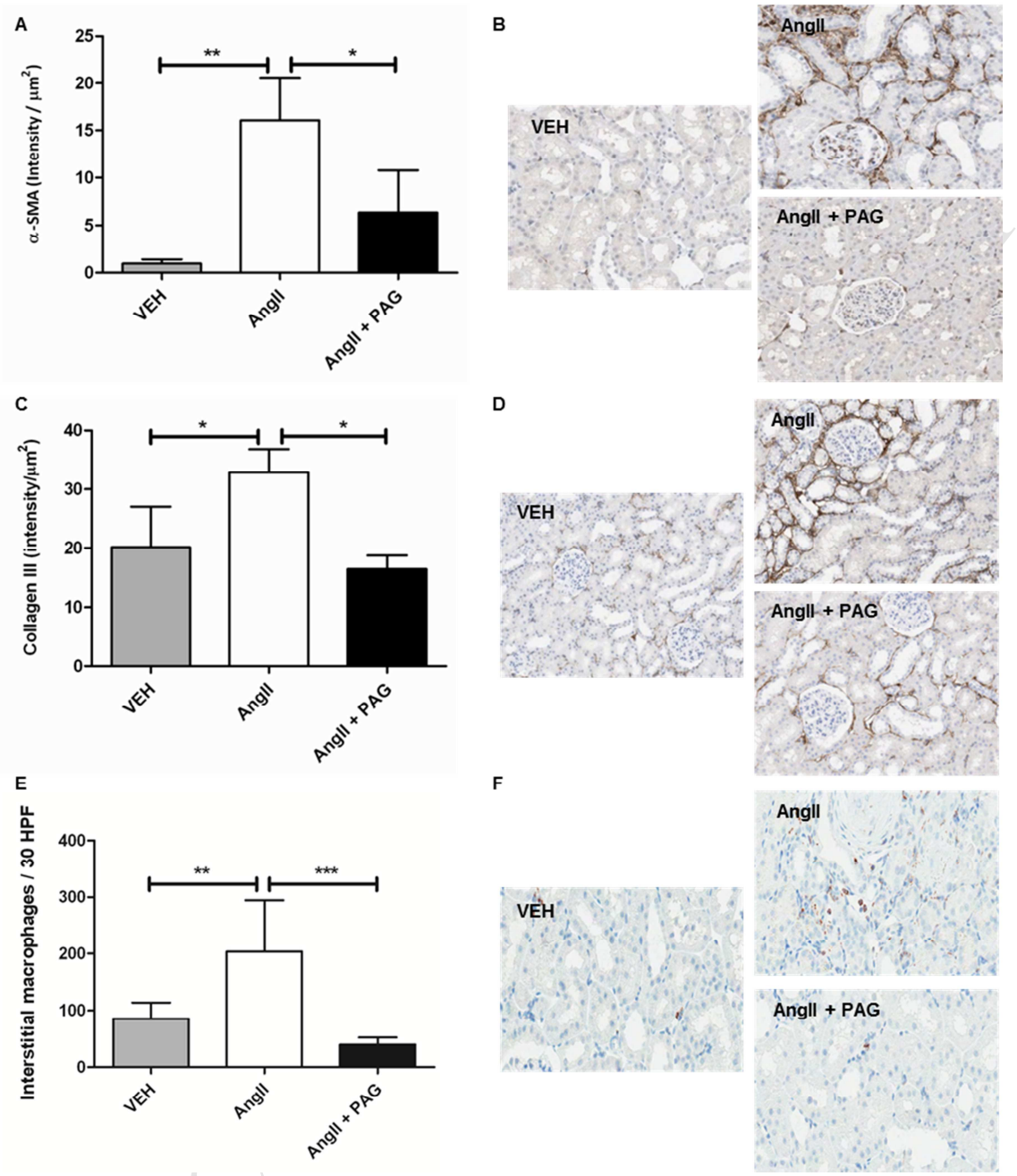

Figure 5. Pre-fibrotic interstitial damage determined by α-SMA staining (A), interstitial collagen determined by Collagen III staining (C) and interstitial macrophages determined by ED-1 staining (E) in healthy, AngII and PAG treated rats. Representative photomicrographs of α-SMA (B) Collagen III (D) and ED-1 (F) stained renal sections. Mean ± SD. VEH n=5, AngII and AngII + PAG n=7. *p<0.05, **p<0.01

3.6 PAG increased kidney weight

Surprisingly, kidney weight to body weight (BW) ratio was increased after PAG treatment in all groups (figure 6A). This was already observed after one week of PAG in CON rats (0.36 ± 0.02 vs. 0.32 ± 0.01 g/100g BW, p<0.01). The PAG-induced increase in kidney weight was not affected by the duration of PAG administration (1wk CON + PAG vs. 4wk CON + PAG, NS). AngII infusion increased kidney/BW ratio as compared to VEH rats (0.49 ± 0.02 vs. 0.38 ± 0.03 g/100g, p<0.01), and this was even further increased by PAG (0.49 ± 0.02 vs. 0.56 ± 0.07 g/100g, p<0.05).

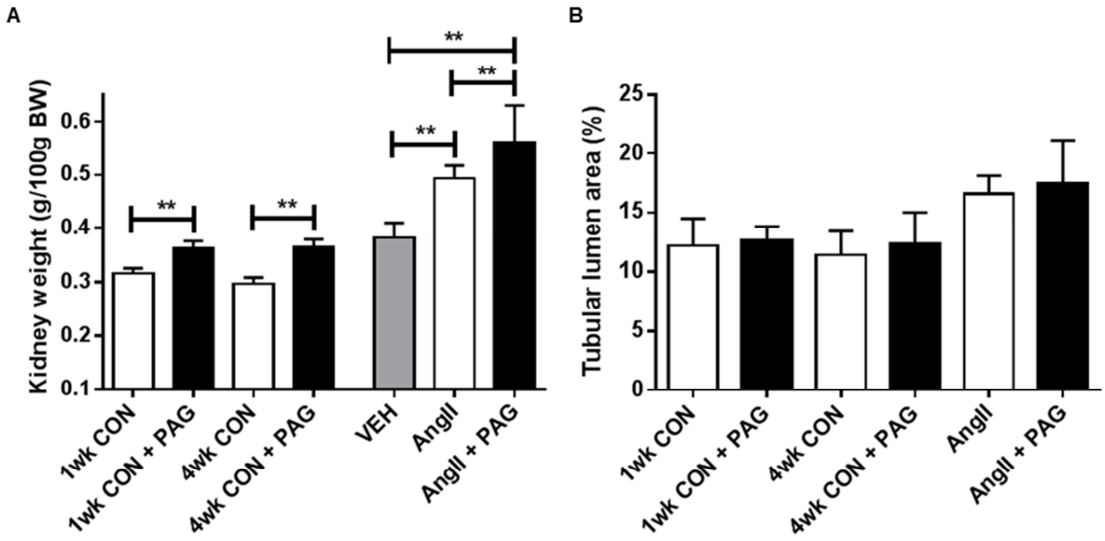

Figure 6. A: Kidney to body weight ratio in healthy, AngII and PAG treated rats. B: Tubular lumen area in healthy and AngII rats, saline or PAG treated. Mean ± SD. CON and CON + PAG groups n=5-6, VEH n=5, AngII and AngII + PAG n=6-7. **p<0.01, ***p<0.001

3.7 PAG decreased tubular cell number/field and increased tubular cell proliferation

To study the cause of the increased renal weight elicited by PAG treatment tubular lumen area, nuclei number and proliferation were investigated. Tubular lumen area was not increased in PAG-treated compared to untreated rats (figure 6B). PAG decreased tubular nucleus numbers/field in the outer cortex in CON rats after one (990 ± 70 vs. 1106 ± 144) and four weeks (1021 ± 67 vs. 1161 ± 121, p<0.05; figure 7A). In the inner cortex, PAG did not affect the number of tubular nuclei (figure 7B). As many inflammatory cells are present in kidney injury models that would result in unreliable automated counts of tubular nuclei tubular nuclei were only counted in CON rats. Tubular cell proliferation was not affected by PAG in the outer cortex for both CON and AngII groups. However, one week of PAG in CON rats considerably increased the proliferation of tubular cells in the inner cortex (84 ± 9 vs. 19 ± 2 Ki67+ cells/field, p<0.001), but not in 4wk CON + PAG and AngII rats (figure 8).

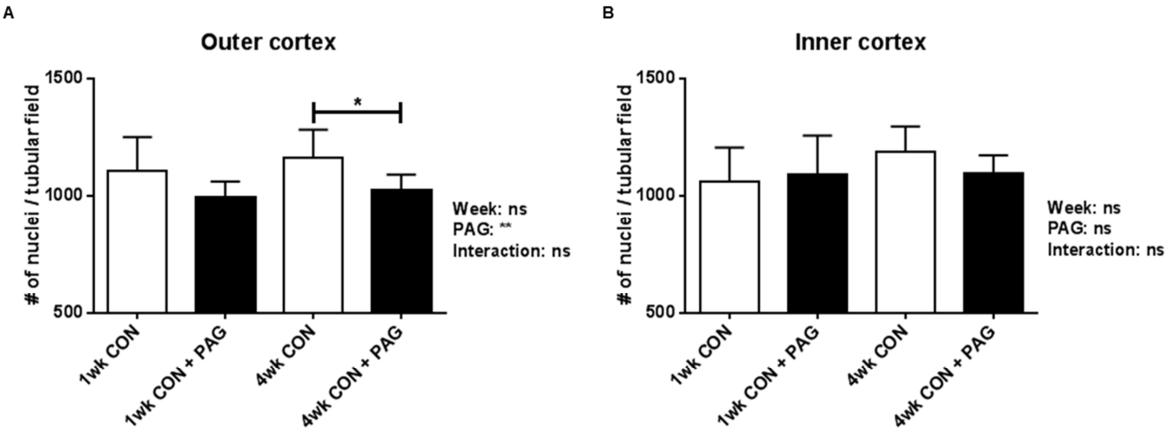

Figure 7. Tubular epithelial nucleus number per field in the outer cortex (A) and inner cortex (B) of healthy saline and PAG treated rats. Mean ± SD. CON and CON + PAG groups n=5-6. *p<0.05 **p<0.01

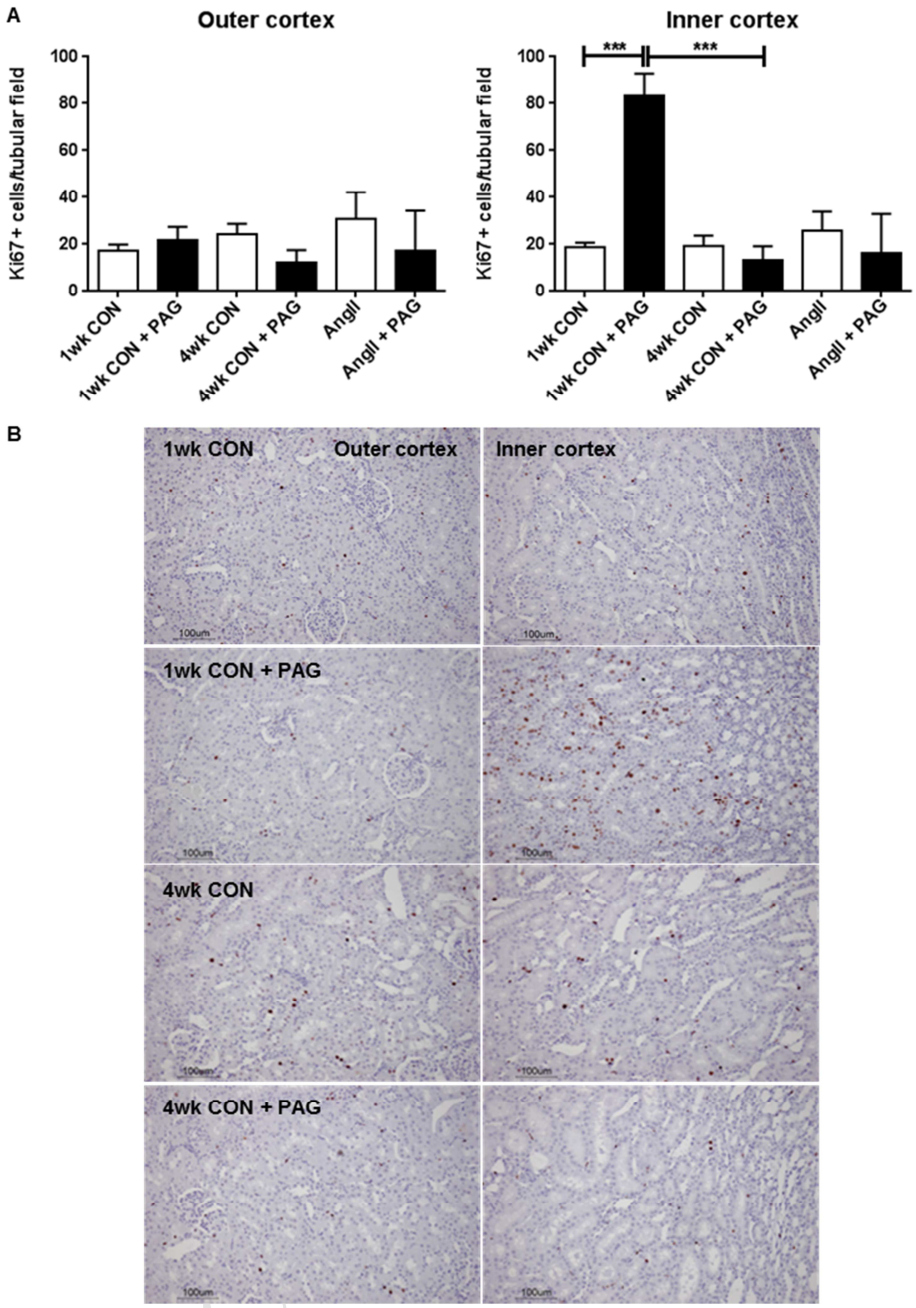

Figure 8. Tubular cell proliferation per tubular field in saline and PAG-treated rats (A) and representative photomicrographs of one or four week vehicle or PAG treated control rats in the outer cortex (left) and inner cortex (right) (B). Mean ± SD. CON and CON + PAG groups n=5-6. ***p<0.001

3.8 PAG did not directly affect lymph vessels number and size

Disturbed lymphatic vessels function and/or number (assessed by counting the number and measuring the size of lymphatic vessels) may be a cause of fluid retention in the kidney resulting in increased kidney weight. Lymph vessel number was not affected by PAG in control rats (figure 9A). After AngII infusion PAG decreased the number of lymph vessels per tubular field (2.0 ± 0.4 vs. 3.8 ± 1.1, p<0.01). Lymph vessels categorized by size were not affected by PAG (figure 9B).

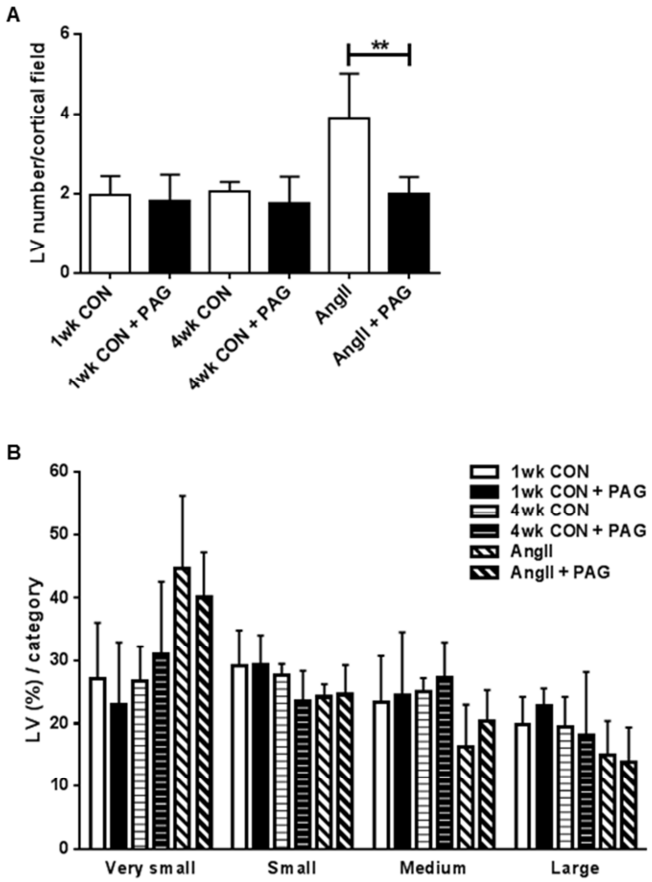

Figure 9. Renal lymph vessel number (A) and size, displayed as percentage of very small, small, medium or large sized lymph vessels (B) in saline and PAG-treated rats. Mean ± SD. CON and CON + PAG groups n=4-6, AngII and AngII + PAG n=6-7. **p<0.01

3.9 PAG increased cell size in-vitro

Proximal tubular cells were cultured with 0 (CON), 1 or 3 mM PAG for 24 and 48 hours (figure 10A). After 24 hours, no effect of PAG incubation on cellular size was observed (data not shown). However, after 48 hours, the percentage of large cells measured by flow cytometry (figure 10B) increased after culturing with

3 mM PAG compared to 0 mM (73.6 ± 9.8 vs. 54.4 ± 4.6%, p<0.05). Increased cellular size was confirmed by enlarged surface area measured on fixed cells after 48h, at 1 and 3 mM PAG (figure 10C).

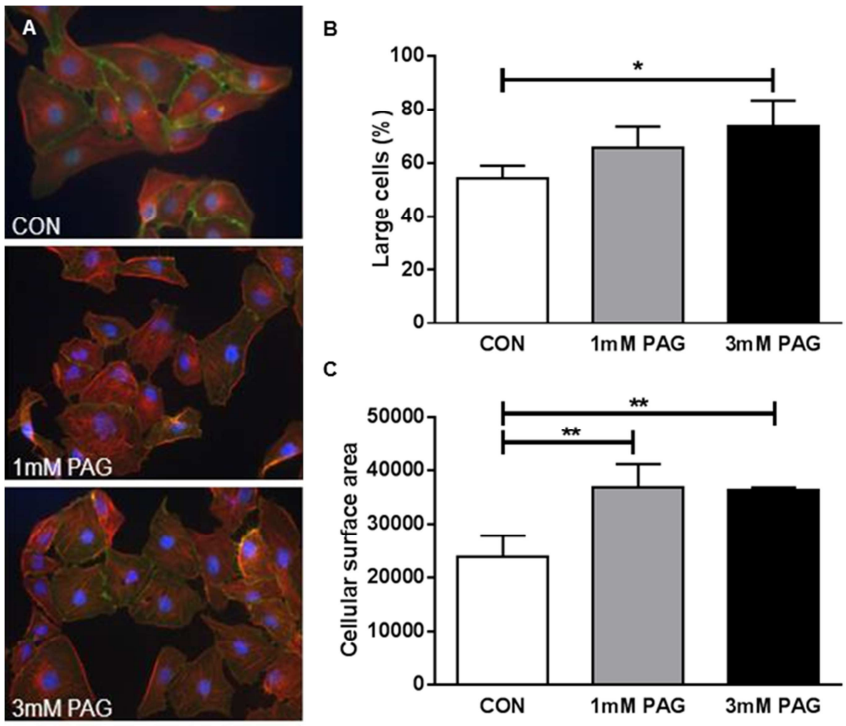

Figure 10. A: Representative pictures of HK-2 cells incubated with PAG (0, 1 and 3 mM); green-cell membrane staining (beta-catenine), red-actin staining (phalloidin), blue-nuclear stain (DAPI). PAG dose-dependently increased cell size after 48 hours in-vitro as measured by flowcytometry (B). Cellular surface area increased after PAG incubation (C). Mean ± SD. n=3. *p<0.05, **p<0.01

4. Discussion

The key findings of our study are 1) Inhibition of CSE-mediated H_2S formation by PAG reduced blood pressure and proteinuria in AngII-infused rats, which was accompanied by less kidney injury and less morphological damage. 2) In CON rats and in rats with AngII-induced kidney injury, PAG increased kidney weight, independently of effects on SBP or kidney function, and structure including lymph vessel number and size. 3) There was a marked increase in proliferation in the inner cortex after one week of PAG. Finally, 4) *in-vitro* PAG exposure increased tubular epithelial cell size.

H_2S is mainly produced from L-cysteine and L-cystathionine via the enzymes CSE, CBS or MST [32; 33]. In the kidney, H_2S is mainly produced by CSE, and renal CSE but not CBS correlates with kidney function after kidney transplantation [10]. Therefore, in the present study, we sought to inhibit CSE activity and thereby H_2S production with PAG. PAG inhibits CSE by reacting with the pyridoxal-5'-phosphate (PLP) complex [9], although PAG is known to also react with other PLP dependent enzymes. In the present study we demonstrate complete blockage of H_2S production in the kidney after PAG administration in healthy [15] and AngII-infused rats. As expected, AngII (without PAG) also decreased H_2S production because of renal injury. The finding that PAG consistently abolished H_2S production suggests that CBS and MST may also have been affected. Both the blood pressure reduction and the amelioration of renal injury by PAG treatment in AngII-infused rats are contradictory to our previous findings showing that exogenous H_2S resulted in improvement of blood pressure and less renal injury in this model [12]. Moreover, endogenous H_2S is important for vascular relaxation, by activating K_{ATP} channels on smooth muscle cells resulting in membrane hyperpolarization [34]. Although both, donating and inhibiting H_2S, reduced blood pressure after AngII infusion, only exogenous H_2S reduced oxidative stress. Reactive oxygen species (ROS) are known to be produced by AngII through NAPDH oxidase activation and ROS is probably a major player in the development of hypertension [35]. This suggests that the reduction in blood pressure by PAG is not directly ROS and AngII-mediated but is achieved via another pathway. During concomitant blockade of NO and H_2S-synthesis we previously also observed a decrease in blood pressure and less proteinuria. This appeared to be related to increased CO production [15]. Hence in the present study we focused on HO-1, an enzyme that primarily catalyzes production of CO [1].

It is known that AngII infusion in rats increases renal HO-1 expression [36; 37], which was confirmed in the present study. Increasing CO availability by the administration of an HO-inducer reduced blood pressure and renal injury caused by NO blockade or AngII infusion, whereas an HO inhibitor increased blood pressure and reduced renal function [36; 38; 39]. This corresponds to previous findings of Wesseling et al. [40] showing that inflammation and proteinuria caused by blocking NO synthesis correlates with HO-1 gene expression. The slope of the expected positive correlation between renal HO-1 expression and proteinuria [40] was greatly diminished by PAG suggesting that the strong induction of HO-1 in PAG-treated rats was directly caused by PAG. Reducing AngII- induced hypertension by losartan (an AngII receptor antagonist) also prevented an increase of HO-1 gene expression [37], suggesting that HO-1 gene

expression is also blood pressure or renal damage-dependent. This might also explain why we did not observe a further increase in HO-1 gene expression in animals simultaneously treated with AngII infusion and PAG.

Blockade of H_2S synthesis via CSE led to a decreased NO availability, judged by reductions in both circulating concentrations and urinary excretion of nitrite and nitrate. Together with the data on H_2S production, these results strongly suggest that NO production, both systemically and in the kidney, is in part dependent on H_2S production. In the presence of renal injury, a condition known to be associated with reduced whole body NO production [41; 42], in our study nitrite excretion increased while nitrate excretion did not change. This suggests intrarenal production of nitrate, perhaps by inflammatory cells; alternatively, nitrate secretion may be upregulated. Based on the marked increase in fractional nitrate excretion, the increase in plasma nitrate levels was at least partly a consequence of the fall in glomerular filtration.

Besides amelioration of renal function by H_2S donors in AngII infusion, tubular and glomerular damage and interstitial fibrosis were reduced [12]. However, in the present study inhibition of H_2S resulted also in decreased glomerular and tubular damage. Reduced renal damage and proteinuria might be partly related to the decrease in blood pressure. PAG reduced blood pressure induced by NO synthesis blockade by 15%, however proteinuria was reduced much more (81%) [15]. In AngII infused mice PAG did not affect the high blood pressure, but neither proteinuria nor renal damage were measured in this study [43]. Future work on the discrete effects of H_2S and perhaps CO on intraglomerular hemodynamics and permselectivity may help to reconcile these findings.

Although kidney function and tubular damage improved in AngII-infused rats, kidney weight increased after PAG treatment. This indicates that PAG or diminished H_2S production by blockage of CSE affects cellular processes besides having functional effects in the kidney. An increase in kidney weight can be caused by several factors, including edema formation. PAG increased pulmonary edema and decreased the expression of AQP1/5 in rats with bilateral limb ischemia [44]. One of the most important causes of edema is hampering the crucial physiological function of lymphatic vessels in controlling tissue fluid balance and hemostasis, which can result in renal edema [45], and consequently can increase kidney weight. However, in our study renal lymphatic vessel function (number and/or size) was not affected after PAG treatment. Probably the increase in lymph vessel number (lymphangiogenesis) and size in AngII-infused rats is caused by proteinuria as this has also been reported in adriamycin-induced CKD with protein excretion higher than 200mg/24h [30]. Furthermore, no tubular dilation was observed suggesting that obstruction of nephrons or the urinary tract was not involved.

Increase in cellular size may be another factor involved in the enhanced kidney weight observed. *In-vivo*, the decrease in tubular nuclei/field in the outer cortex after PAG may explain the increase in cellular size. This effect was supported by an increase in size of HK2 proximal tubular cells *in-vitro*. This effect may not be restricted to the renal epithelium, because heart weight of PAG treated mice with myocarditis was

demonstrated to increase [46], while H_2S donors reduced heart weight and cardiomyocyte size [46; 47]. Remarkably, our findings clearly dissociate kidney size from kidney injury in this model.

Although no differences were observed in electrolyte excretion between vehicle and PAG treated rats, disturbances of ion channels may result in increased cellular tonicity, resulting in larger cells. Both, osmotic swelling and hypertrophy could increase cellular size. Little is known on effects of H_2S or inhibition of H_2S on cellular size. Glucose-induced hypertrophy and protein synthesis of glomerular epithelial cells was reduced by an H_2S donor, but no effect was observed in healthy cells after incubation with an H_2S donor [23]. This might suggest that subnormal H_2S concentrations or CSE activity result in loss of control of protein synthesis, where in $CSE^{-/-}$ mice cysteine depletion led to hypermetabolism and enhanced insulin sensitivity. Weight loss could be restored by cysteine supplementation, but not by an H_2S donor suggesting a H_2S independent role of cysteine in metabolism [48].

Increased proliferation might also cause a larger kidney and thereby increase kidney weight. Tubular proliferation in the inner cortex was increased after one, but not after four weeks of PAG treatment. Incubation of glomerular mesangial cells with PAG resulted in increased proliferation [49], however in embryonic kidney cells overexpressing CSE, proliferation was also increased [24]. This indicates that effects of H_2S on proliferation, at least in the kidney, are probably time and cell type dependent, and the increase in tubular cell size and proliferation may be a fast but not progressive effect of PAG, mediated directly by diminished H_2S production.

In conclusion, the CSE inhibitor PAG reduced AngII induced hypertension and ameliorated AngII induced renal injury in rats. PAG also increased kidney weight independently of effects on SBP and renal function. More studies with different models of CKD are required to fathom the role of endogenous H_2S and CO in the kidney in order to understand how inhibition of H_2S production as well as administration of H_2S donors can show similar results in some models of renal injury. More research is also needed to define the pathways involved in PAG-induced cell enlargement and proliferation.

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Conflict of interest

None.

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Highlights NOX-15-42

- DL-propargylglycine (PAG) abolished H_2S production in the kidney
- PAG reduced systolic blood pressure and renal injury in angiotensin II-infused rats
- Independent of kidney function PAG increased kidney weight
- PAG increased tubular cell size and proliferation