

RESEARCH PAPER

Role of H₂O₂ in hypertension, renin-angiotensin system activation and renal medullary dysfunction caused by angiotensin II

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BACKGROUND AND PURPOSE

Activation of the intrarenal renin-angiotensin system (RAS) and increased renal medullary hydrogen peroxide (H₂O₂) contribute to hypertension. We examined whether H₂O₂ mediated hypertension and intrarenal RAS activation induced by angiotensin II (Ang II).

EXPERIMENTAL APPROACH

Ang II (200 ng·kg⁻¹·min⁻¹) or saline were infused in Sprague Dawley rats from day 0 to day 14. Polyethylene glycol (PEG)-catalase (10 000 U·kg⁻¹·day⁻¹) was given to Ang II-treated rats, from day 7 to day 14. Systolic blood pressure was measured throughout the study. H₂O₂, angiotensin AT₁ receptor and Nox4 expression and nuclear factor-κB (NF-κB) activation were evaluated in the kidney. Plasma and urinary H₂O₂ and angiotensinogen were also measured.

KEY RESULTS

Ang II increased H₂O₂, AT₁ receptor and Nox4 expression and NF-κB activation in the renal medulla, but not in the cortex. Ang II raised plasma and urinary H₂O₂ levels, increased urinary angiotensinogen but reduced plasma angiotensinogen. PEG-catalase had a short-term antihypertensive effect and transiently suppressed urinary angiotensinogen. PEG-catalase decreased renal medullary expression of AT₁ receptors and Nox4 in Ang II-infused rats. Renal medullary NF-κB activation was correlated with local H₂O₂ levels and urinary angiotensinogen excretion. Loss of antihypertensive efficacy was associated with an eightfold increase of plasma angiotensinogen.

CONCLUSIONS AND IMPLICATIONS

The renal medulla is a major target for Ang II-induced redox dysfunction. H₂O₂ appears to be the key mediator enhancing intrarenal RAS activation and decreasing systemic RAS activity. The specific control of renal medullary H₂O₂ levels may provide future grounds for the treatment of hypertension.

Abbreviations

Ang II, angiotensin II; DPI, diphenylene iodonium; EMSA, electrophoretic mobility shift assay; fEMSA, fluorescent electrophoretic mobility shift assay; GPx, glutathione peroxidase; L-NAME, nitro-L-arginine methylester; MPO, myeloperoxidase; PEG, polyethylene glycol; RAS, renin-angiotensin system; RFU, relative fluorescence units; ROS, reactive oxygen species; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase

Introduction

During the last two decades, it has been clearly established that reactive oxygen species (ROS) contribute to the pathophysiology of several diseases, not only by direct toxicity, as initially proposed in the 1950s, but also, and maybe more importantly, by altering signalling pathways that regulate cell and organ functions (Droge, 2002; Jones, 2008). Among ROS, the superoxide anion (O₂^{•-}) and hydrogen peroxide (H₂O₂) have been recognized as major signalling molecules involved in physiological and pathophysiological processes both in experimental models and humans (Ardanaz and Pagano, 2006; Paravicini and Touyz, 2006; Valko *et al.*, 2007). Arterial hypertension is a highly prevalent disease, contributing to an elevated incidence of serious cardiovascular and renal adverse events (Chobanian *et al.*, 2003). However, despite the existence of several antihypertensive drugs, the control of hypertension remains elusive in many patients and is responsible for a high mortality and morbidity worldwide. Besides the problem of non-compliance with medication, the multifactorial nature of the disease and the activation of counterregulatory mechanisms in response to antihypertensive treatment also contribute to this disappointing scenario (Chobanian *et al.*, 2003; Giles, 2006; Ferrario, 2010). Therefore, the development of new therapeutic approaches capable of controlling the multiple processes involved in the pathophysiology of hypertension remains an important and unfulfilled objective.

Emerging evidence suggests that H₂O₂ is a paracrine mediator of cardiovascular and renal dysfunction (Makino *et al.*, 2003; Ardanaz and Pagano, 2006). Pro-hypertensive effects of H₂O₂ include increased spinal sympathetic outflow (Lin *et al.*, 2003), increased vasoconstriction (Rodriguez-Martinez *et al.*, 1998; Gao and Lee, 2001; Gao *et al.*, 2003; 2004; Thakali *et al.*, 2006), vascular hypertrophy and hyperplasia (Ardanaz and Pagano, 2006; Sousa *et al.*, 2008), and decreased diuresis and natriuresis (Chen *et al.*, 2003; Asghar *et al.*, 2006). In line with this postulate, catalase, an H₂O₂-detoxifying enzyme, has been shown to reduce the pressor responses to angiotensin II (Ang II) and noradrenaline (Yang *et al.*, 2003), and to prevent the hypertension induced by the infusion of H₂O₂ in the renal medulla (Makino *et al.*, 2003). Our group has also demonstrated that vascular and urinary levels of H₂O₂ are markedly increased in a model of hypertension with activation of the renin-angiotensin system (RAS) and that the treatment with polyethylene glycol (PEG)-catalase prevents the rise in blood pressure and vascular hyperplasia (Sousa *et al.*, 2008).

Although blood pressure control is achieved through the combined action of diverse central and peripheral effector sites, overwhelming evidence suggests that renal dysfunction contributes to the pathogenesis of all forms of hypertension (Cowley *et al.*, 1995). Of note, Ang II-induced hypertension was shown to be primarily mediated by renal angiotensin AT₁ receptors (Crowley *et al.*, 2006; receptor nomenclature follows Alexander *et al.*, 2011). In addition, changes within the renal medulla were demonstrated to have an important effect on water and sodium excretion, thereby influencing the long-term control of arterial pressure (Cowley, 2008). Studies in spontaneously hypertensive rats (SHR) also suggested that renal dysfunction begins as subtle alterations in the renal medulla in the absence of changes within the renal

cortex (Cowley *et al.*, 1995). Furthermore, gene expression analysis revealed that the renal outer medulla has an increased vulnerability to non-pressor levels of Ang II (Yuan *et al.*, 2003). The RAS is a well-known regulator of sodium and water balance, as well as arterial pressure. Recently, it has been proposed that intrarenal RAS activation plays a major role in the development of hypertension and renal injury, even when there is no clear evidence of increased systemic RAS activity (Kobori *et al.*, 2007).

Ang II activates NADPH oxidases within the blood vessels, kidney and brain, leading to increased generation of ROS, such as O₂^{•-} and H₂O₂, which are involved in diverse signalling functions (Garrido and Griendling, 2009). H₂O₂ activates the transcription factor NF- κ B (Morigi *et al.*, 2002; Fardoun *et al.*, 2007; Ungvari *et al.*, 2007), which is known to regulate the expression of several genes involved in sodium homeostasis and blood pressure control, including the gene of angiotensinogen, the precursor of Ang II (Brasier *et al.*, 2000). Interestingly, Ang II-induced hypertension is associated with activation of intrarenal RAS as shown by the increased urinary excretion of angiotensinogen and intrarenal Ang II levels (Kobori *et al.*, 2004). Therefore, this study assessed the contribution of H₂O₂ to the hypertension and intrarenal RAS activation induced by Ang II. In particular, the effects of Ang II in the renal medulla were compared with those in the renal cortex. Furthermore, as H₂O₂ acts at many sites of cardiovascular regulation, we tested if PEG-catalase has an antihypertensive therapeutic effect in this model, apart from its previously demonstrated efficacy in preventing the rise in blood pressure induced by RAS activation.

Methods

Animals and experimental design

All animal care and experimental procedures complied with the European Community guidelines for the use of experimental animals (Directive 86/609/EEC and Decision 1999/575/EC). The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). Male Sprague Dawley rats (250–300 g; 64 animals in total) were used.

This study was divided in three parts.

Part I was designed to evaluate the effects of Ang II infusion (200 ng·kg⁻¹·min⁻¹, s.c. from day 0 to day 14) on systemic and renal markers of redox status and RAS activation. Saline-infused (s.c.) rats (Sham) were used as controls.

In Part II, we assessed the effects of H₂O₂ neutralization on blood pressure, redox status and RAS activation in Ang II-hypertensive rats. PEG-catalase (10 000 U·kg⁻¹·day⁻¹, i.p. bolus) was given to Ang II-infused rats from day 7 to day 14. Untreated Ang II-infused rats were used as controls. Preliminary experiments demonstrated that PEG-catalase did not affect blood pressure and H₂O₂ levels in Sham-treated rats.

Part III was designed to evaluate the effect of a 3-day treatment with PEG-catalase (10 000 U·kg⁻¹·day⁻¹, i.p. bolus, from day 7 to day 10) on RAS activation in Sham and Ang II-infused rats. In addition, we analysed the protein expression of Nox4 and also Nox1 at this earlier time point.

Ang II or saline were infused using Alzet osmotic mini-pumps (model 2002; Alza, Palo Alto, CA, USA) implanted under ketamine (60 mg·kg⁻¹) plus medetomidine (0.25 mg·kg⁻¹) (i.p.) anaesthesia. Rats were placed in metabolic cages to allow collection of urine over 24 h. Animals were killed on day 14 (Part I and II) or on day 10 (Part III) under sodium pentobarbitone (60 mg·kg⁻¹, i.p.) anaesthesia. Blood was withdrawn from the left ventricle to heparinized tubes (H₂O₂ assay) or to EDTA-containing tubes (angiotensinogen assay). Plasma was obtained by centrifugation of blood (10 min at 500× g, at 4°C; H₂O₂ assay or 20 min at 1000× g, at 4°C; angiotensinogen assay). The kidneys were perfused with saline before excision and then dissected into cortex and total medulla. Samples used for H₂O₂ measurement were promptly processed. Renal nuclear extracts were immediately prepared before storing at -80°C. The other samples were stored at -80°C until analysis.

Systolic blood pressure

Systolic blood pressure (SBP) was measured in conscious animals by non-invasive (tail-cuff) and invasive (i.a.) methods throughout the study. A photoelectric pulse detector (LE 5000, Letica, Barcelona, Spain) was used for tail-cuff measurements. Five determinations were made each time and the means used for further calculations. All the rats underwent a 7-day training period to adapt to the blood pressure measurement methodology. Direct determinations of SBP were made in some rats from each group by implantation of an indwelling catheter. The rats were anaesthetized with a mixture of ketamine (60 mg·kg⁻¹) and medetomidine (0.25 mg·kg⁻¹) (i.p.), and a polyethylene catheter (PE-10) was placed in the abdominal aorta via the left femoral artery. The distal end was joined to PE-50 tubing and tunnelled subcutaneously to the dorsum of the neck where it was exteriorized. After a recovery period, the arterial catheter was connected to a pressure transducer (TRA-021, Letica), and blood pressure signals were recorded on a polygraph (Unigraph 2000-5.6, Letica) in conscious, unrestrained animals.

Protein assay

Protein concentration was quantified by the Bio-Rad protein assay.

Evaluation of H₂O₂ levels

H₂O₂ was measured using the Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes, Alfacene, Carcavelos, Portugal). Urinary H₂O₂ excretion was evaluated in 24-h urine samples as previously described (Sousa *et al.*, 2008). Plasma H₂O₂ levels were measured in aliquots of the same sample mixed with sodium azide (52 mmol·L⁻¹) to block H₂O₂ degradation, or with catalase (1000 IU) to remove H₂O₂ in the plasma. The difference in H₂O₂ levels between the plasma azide and catalase samples reflects plasma H₂O₂ production (Lacy *et al.*, 1998; 2000).

The renal medulla or cortex were incubated in oxygenated Krebs-HEPES (composition in mmol L⁻¹: NaCl 118, KCl 4.5, CaCl₂ 2.5, MgCl₂ 1.2, KH₂PO₄ 1.2, Na-HEPES 25, NaHCO₃ 25 and glucose 5; pH 7.4), at 37°C. Sixty minutes later, H₂O₂ was measured in the incubation medium. These experiments were also performed in the presence of inhibitors of NADPH

oxidase (diphenylene iodonium, DPI, 500 µmol·L⁻¹; apocynin 500 µmol·L⁻¹), xanthine oxidase (oxypurinol, 500 µmol·L⁻¹), NOS (nitro-L-arginine methylester, L-NAME, 500 µmol·L⁻¹) and catalase (aminotriazole, 50 mmol·L⁻¹).

NADPH oxidase activity

Renal medulla or cortex were homogenized in cold HEPES buffer (25 mmol·L⁻¹) containing EDTA (1 mmol·L⁻¹), PMSF (0.5 mmol·L⁻¹) and protease inhibitors (5 µg·mL⁻¹ aprotinin, 5 µg·mL⁻¹ leupeptin and 5 µg·mL⁻¹ pepstatin). Sample homogenates (≈25 µg) were incubated with dihydroethidium (20 µmol·L⁻¹) and salmon testes DNA (0.5 mg·mL⁻¹), with NADPH (1 mmol·L⁻¹) in a microplate, at 37°C for 15 min. Ethidium fluorescence was measured at 475 nm excitation and 610 nm emission using a fluorescence microplate reader (Spectromax Gemini, Molecular Devices, Sunnyvale, CA, USA). Superoxide dismutase (SOD) (2000 IU·mL⁻¹), a O₂⁻ detoxifying enzyme, was used to confirm the specificity of the method. To determine whether NADPH oxidase was the source of O₂⁻, an inhibitor of NADPH oxidase (DPI, 500 µmol·L⁻¹) was used. The effect of L-NAME (500 µmol·L⁻¹) was also tested to exclude the possible contribution of endothelial NOS inhibition to the DPI effect. Results were expressed as the DPI-inhibitable NADPH-dependent O₂⁻ generation (fluorescence arbitrary units per min per mg of protein), after subtracting the values obtained in the presence of DPI from the corresponding sample wells in the absence of inhibitors.

Antioxidant enzyme activity

SOD, catalase and glutathione peroxidase (GPx) activities were evaluated in the renal medulla or cortex by microplate spectrophotometric assays as previously described (Sousa *et al.*, 2008).

Analysis of NF-κB activation

Nuclear extracts were prepared as previously described by Dinis-Oliveira *et al.*, (2007). Evaluation of NF-κB activation was carried by a fluorescent electrophoretic mobility shift assay (fEMSA) using Cyano dye Cy5-labelled oligonucleotide duplexes (Metabion International, Martinsried, Germany) as specific probes and an automatic DNA sequencer (ALF-Express DNA Sequencer, Amersham Pharmacia Biotech, Freiburg, Germany) for analysis. The NF-κB binding assay was performed as previously reported (Dinis-Oliveira *et al.*, 2007). Specificity of the DNA-protein complexes was confirmed by the addition of a 50-fold excess of either unlabeled specific or non-specific competitors. The subunit composition of NF-κB complexes was evaluated by preincubation of nuclear extracts with polyclonal antibodies against p50 and p65 units (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Signals were analyzed by an ALFwin 1.03 fragment analyser (Amersham Pharmacia Biotech). Binding activities were determined as arbitrary units corresponding to the area under curve and further expressed as n-fold activation vs. Sham (part I) or Ang II (part II).

Western blot analysis

Total lysate of renal medullary or cortical homogenates were used for Nox4 and Nox1 detection. The angiotensin AT₁ receptor was analysed in the membrane fraction of renal

medulla or renal cortex samples. Samples of protein (50 µg) were loaded and separated by a 12% SDS-PAGE gel electrophoresis. Proteins were transferred onto nitrocellulose membranes, blocked for 1 h and incubated overnight at 4°C with specific antibody against Nox4 (sc-21860, 1:200, Santa Cruz Biotechnology or ET3174-1, 1:200, Epitomics, Burlingame, CA, USA), Nox1 (sc-5821, 1:200, Santa Cruz Biotechnology) or AT₁ receptor (sc-1173, 1:500, Santa Cruz Biotechnology). After washing, membranes were incubated for 1 h at room temperature with fluorescent labeled secondary antibodies. All membranes were scanned with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Band intensities were normalized to β-actin (sc-47778, 1:10000, Santa Cruz Biotechnology).

Evaluation of angiotensinogen

Angiotensinogen was measured by an ELISA assay kit (Rat Total Angiotensinogen Assay Kit, IBL International GmbH, Hamburg, Germany) (Kobori *et al.*, 2008). Plasma and urine samples were collected on day 10 and on day 14. Urine samples were collected in the presence of an inhibitor cocktail (0.6 mL distilled water containing 50 µg pepstatin A, 10 mg sodium azide, 300 nmol enalaprilat and 125 µmol EDTA) to avoid further angiotensinogen formation and/or degradation (Kobori *et al.*, 2003).

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using unpaired Student's *t*-test or one-way ANOVA followed by a Tukey's multiple comparison test, where appropriate. *P* values of less than 0.05 were considered significant. Pearson's single regression analysis was used to estimate correlations between sets of parametric data.

Materials

Ketamine and medetomidine were purchased from Merial Portuguesa (Sintra, Portugal) and Laboratórios Pfizer (Oeiras, Portugal), respectively. Other drugs and chemicals were purchased from Sigma (St. Louis, MO, USA).

Results

Effects of Ang II or Ang II + PEG – catalase on SBP

The baseline SBP was similar in all experimental groups before surgery. Ang II significantly increased SBP when compared with sham-operated group ($P < 0.05$, Figure 1A). PEG-catalase had a significant ($P < 0.05$, Figure 1B) but short-term antihypertensive effect that lasted only for 3 days after the beginning of catalase administration (Figure 1B). The SBP of sham-operated rats was not affected by PEG-catalase treatment (data not shown). Under our experimental conditions, a significant correlation ($r^2 = 0.762$, $r = 0.87$, $P = 0.001$, $n = 10$) was found between tail-cuff and i.a. measurements of SBP (Figure 1C).

Effects of Ang II or Ang II + PEG – catalase on H₂O₂ levels

Ang II significantly increased H₂O₂ levels in plasma, 24 h urine and renal medulla when compared with sham-operated

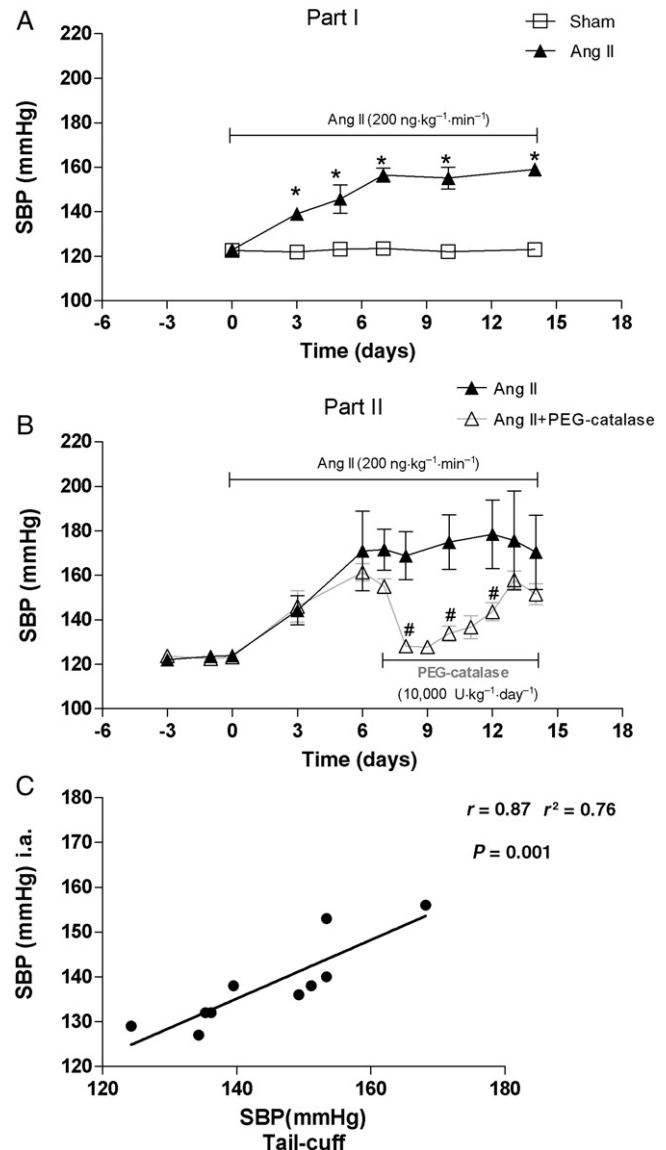


Figure 1

SBP in Sham, Ang II and Ang II + PEG-catalase-treated rats: (A, B) SBP measured by tail-cuff; (C) Correlation between SBP measured by tail-cuff and by i.a. pressures. Results are mean ± SEM (part I, $n = 15$; part II, $n = 6-9$). * $P < 0.05$ significantly different from sham, # $P < 0.05$ significantly different from Ang II.

group ($P < 0.05$, Figure 2). PEG-catalase decreased systemic, urinary and renal medullary H₂O₂ levels in Ang II-infused rats ($P < 0.05$, Figure 2). No significant changes were observed in renal cortical H₂O₂ production in Ang II- or Ang II + PEG-catalase-treated rats (Figure 2). Plasma and urinary H₂O₂ levels of sham rats were not significantly altered by PEG-catalase treatment (data not shown).

Effects of inhibitors of H₂O₂ production/metabolism on renal H₂O₂ levels

DPI and apocynin significantly reduced the renal medullary levels of H₂O₂ and abolished the difference observed

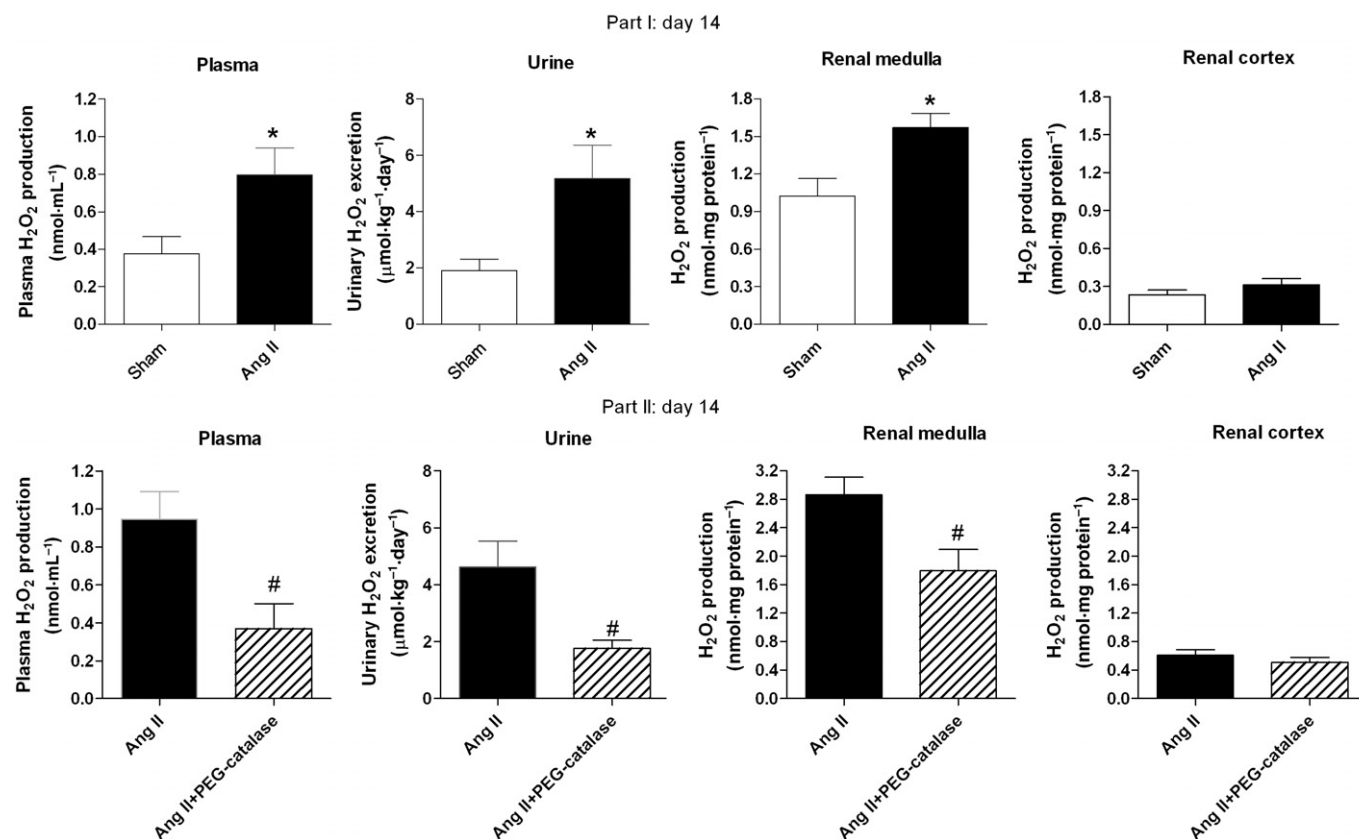


Figure 2

Plasma, urinary and renal H_2O_2 levels on day 14. Results are mean $\pm$ SEM (part I, $n = 15$; part II, $n = 6-9$). * $P < 0.05$ significantly different from sham; # $P < 0.05$ significantly different from Ang II.

Table 1

Effect of inhibitors of H_2O_2 production or metabolism on renal H_2O_2 levels

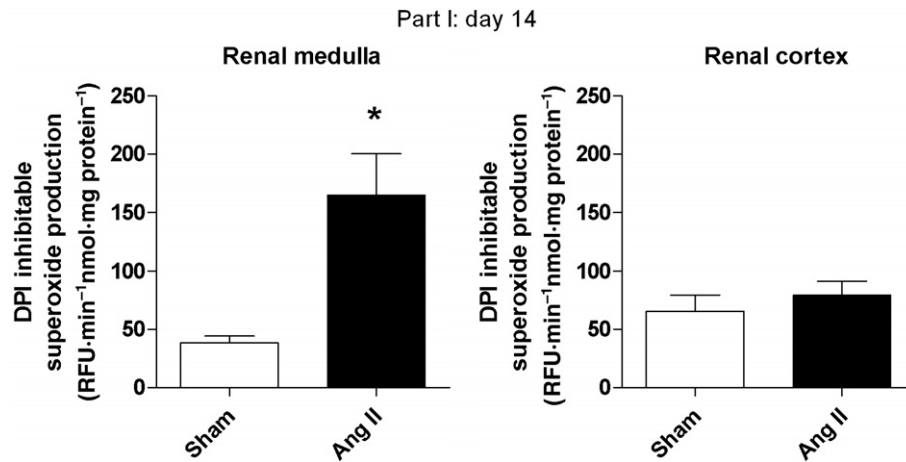
	H_2O_2 production (nmol·mg protein ⁻¹)			
	Sham	Renal medulla Ang II	Sham	Renal cortex Ang II
Krebs-HEPES (control)	1.02 $\pm$ 0.14	1.57 $\pm$ 0.11*	0.23 $\pm$ 0.04	0.31 $\pm$ 0.05
DPI, 500 μ M	0.45 $\pm$ 0.05	0.54 $\pm$ 0.05#	0.20 $\pm$ 0.07	0.26 $\pm$ 0.06
Apocynin, 500 μ M	0.29 $\pm$ 0.06#	0.31 $\pm$ 0.03#	0.04 $\pm$ 0.01	0.11 $\pm$ 0.03
Oxypurinol, 500 μ M	0.91 $\pm$ 0.14	1.07 $\pm$ 0.19	0.17 $\pm$ 0.09	0.13 $\pm$ 0.05
L-NAME, 500 μ M	1.34 $\pm$ 0.12	1.64 $\pm$ 0.19	0.40 $\pm$ 0.09	0.43 $\pm$ 0.09
Aminotriazole, 50 mM	2.58 $\pm$ 0.18#	2.63 $\pm$ 0.18#	0.79 $\pm$ 0.10#	0.71 $\pm$ 0.13#

Results are mean $\pm$ SEM. $n = 6-9$. * $P < 0.05$ vs. sham. # $P < 0.05$ vs. tissue in the absence of inhibitor.

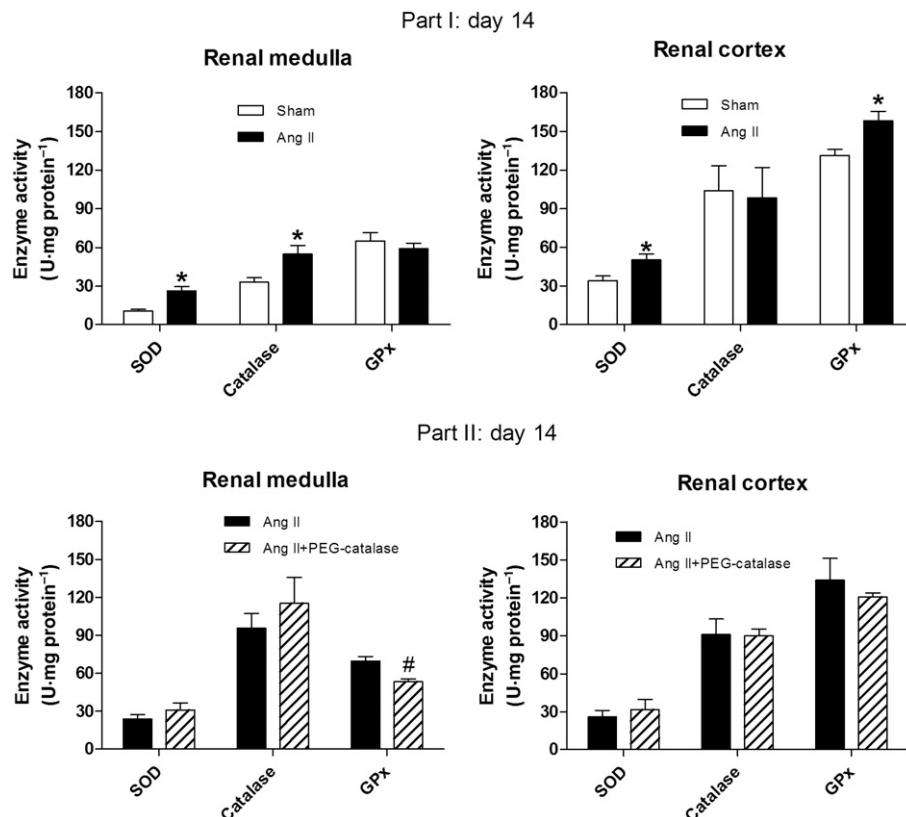
between sham-operated and Ang II-treated rats (Table 1). DPI and apocynin had no effect in H_2O_2 production in the renal cortex of Ang II-treated rats (Table 1). Aminotriazole markedly increased H_2O_2 levels both in the renal medulla and cortex (Table 1). Neither oxypurinol nor L-NAME, altered H_2O_2 production in the renal medulla and cortex (Table 1).

Effects of Ang II on renal NADPH oxidase activity

The DPI-inhibitable, NADPH-dependent, $O_2^{\cdot-}$ generation was significantly increased in the renal medulla of Ang II-hypertensive rats ($P < 0.05$, Figure 3), while no change was observed in the renal cortex (Figure 3). Furthermore,

**Figure 3**

Renal NADPH oxidase activity (as superoxide anion (O₂⁻) production) on day 14. Results are mean ± SEM (part I, *n* = 6). **P* < 0.05 significantly different from sham.

**Figure 4**

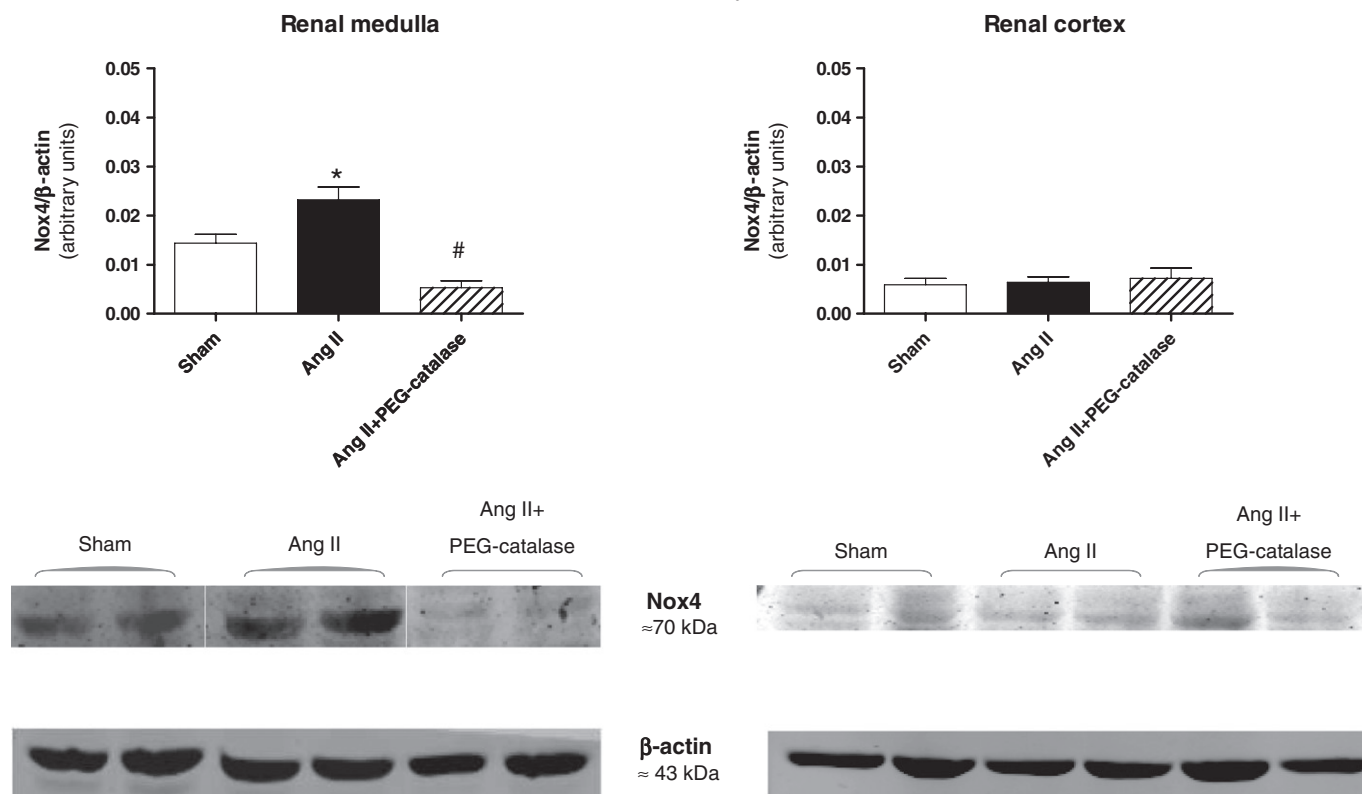
Effects of Ang II, with or without PEG-catalase, on renal antioxidant enzyme (SOD, catalase and GPx) activity on day 14. Results are mean ± SEM. **P* < 0.05 significantly different from sham; #*P* < 0.05 significantly different from Ang II (part I, *n* = 7; part II, *n* = 6–9).

L-NAME did not affect NADPH-dependent O₂⁻ generation in the renal medulla of Ang II-hypertensive rats (340.6 ± 30.2 vs. 396.5 ± 26.6 RFU·min⁻¹·mg⁻¹ protein, *P* = 0.20).

Effects of Ang II or Ang II + PEG – catalase on renal antioxidant enzyme activity

Ang II increased SOD activity both in the renal medulla and cortex (*P* < 0.05, Figure 4). Ang II raised the activity of catalase

Part I and Part II: day 14

**Figure 5**

Effects of Ang II, with or without PEG-catalase, on renal Nox4 protein expression on day 14. Data shown are representative immunoblots with summary bar graphs mean $\pm$ SEM values, part I and II, $n = 5-9$). * $P < 0.05$ significantly different from sham; # $P < 0.05$ significantly different from Ang II.

in the renal medulla and the activity of GPx in the renal cortex ($P < 0.05$, Figure 4). PEG-catalase treatment significantly decreased GPx activity in the renal medulla of Ang II-infused rats ($P < 0.05$, Figure 4), but did not change antioxidant enzyme activities in the renal cortex (Figure 4).

Effects of Ang II and PEG-catalase on renal Nox4, Nox1 and angiotensin AT₁ receptor protein expression

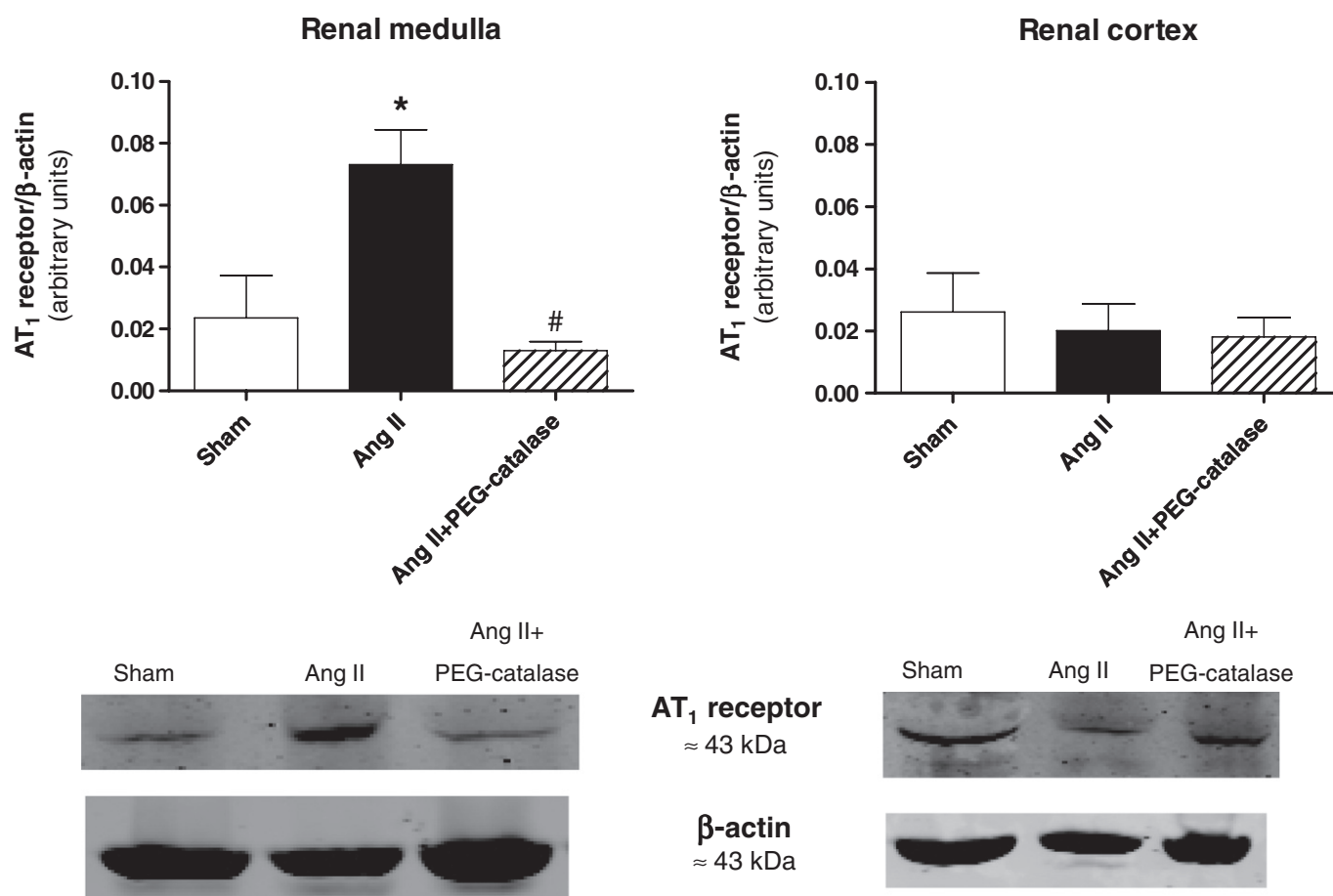
Representative immunoblots for Nox4, Nox1 and AT₁ receptors are shown in Figures 5–8. On day 14, Ang II infusion significantly increased the expression of Nox4 and AT₁ receptors in the renal medulla, but not in the renal cortex ($P < 0.05$, Figures 5 and 6). Treatment with PEG-catalase abolished these effects in the renal medulla ($P < 0.05$) and did not alter expression in the renal cortex (Figures 5 and 6). As PEG-catalase had an antihypertensive effect in the first 3 days of the treatment, we also investigated if there were changes in the expression of Nox4 and also Nox1 at this earlier time point. We observed that on day 10, Ang II-infused rats had already an increased expression of Nox4 in the renal medulla ($P < 0.05$, Figure 7), but not in the renal cortex. Again, PEG-catalase treatment markedly reduced Nox4 expression in the renal medulla ($P < 0.05$, Figure 7). No differences were

observed for renal medullary or cortical Nox1 expression between the studied groups (Figure 8).

Effects of Ang II or Ang + PEG-catalase on renal NF-κB activation

Representative fEMSA gels are shown in Figure 9 and revealed the presence of two specific DNA-NF-κB bands (Figure 9A). Both NF-κB bands (complex I and II) were reduced by the p50 antibody. In addition, the antibody against the p65 subunit abolished the signal intensity of the upper band (complex I). (Figure 9A) Although this antibody also partially interfered with the signal intensity of the lower band (complex II) (Figure 9A), this effect is due to a cross-reactivity of this polyclonal antibody with the p50 subunit (Bruggeman *et al.*, 2001). These results suggest that complex II is a p50/p50 homodimer, and complex I consists of a p50/p65 heterodimer, which is in accordance with previous results from other groups (Bourcier *et al.*, 1997; Jamaluddin *et al.*, 2000). Ang II enhanced the activation of the p50/p50 homodimer in the renal medulla, but not in the renal cortex (Table 2 and Figure 9B). Furthermore, neither the medulla nor the cortex of Ang II-hypertensive rats showed significant changes in p60/p50 heterodimer activation (Table 2 and Figure 9B). Treatment of Ang II-infused rats with PEG-catalase did not significantly reduce NF-κB activation in the renal medulla,

Part I and II: day 14

**Figure 6**

Effects of Ang II, with or without PEG-catalase, on renal angiotensin AT₁ receptor protein expression on day 14. Data shown are representative immunoblots with summary bar graphs (mean ± SEM values, part I and II, $n = 4-6$). * $P < 0.05$ significantly different from sham; # $P < 0.05$ significantly different from Ang II.

but increased both NF-κB homodimer and heterodimer binding in the renal cortex (Table 2 and Figure 9C).

Effects of Ang II and PEG-catalase on plasma and urinary angiotensinogen

On day 14, urinary angiotensinogen, a marker of intrarenal RAS activation (Kobori *et al.*, 2003; 2009), was markedly increased by Ang II infusion, when compared with sham-operated rats ($P < 0.05$, Figure 10A). In contrast, Ang II reduced plasma angiotensinogen by 50% (Figure 10A). Treatment with PEG-catalase, from day 7 to day 14, of Ang II-infused rats did not significantly change the effect of Ang II on urinary angiotensinogen (Figure 10B), but caused an eight-fold increase in plasma angiotensinogen ($P < 0.05$, Figure 10B). As PEG-catalase significantly reduced blood pressure in the first 3 days of the treatment, we also looked for changes in intrarenal or systemic angiotensinogen at this time point. PEG-catalase markedly suppressed intrarenal angiotensinogen levels ($P < 0.05$, Figure 10C) in the initial

phase of the treatment and did not alter plasma levels in Ang II-infused rats (Figure 10C). Moreover, PEG-catalase did not affect intrarenal or systemic levels of angiotensinogen in sham-operated rats (Figure 10C).

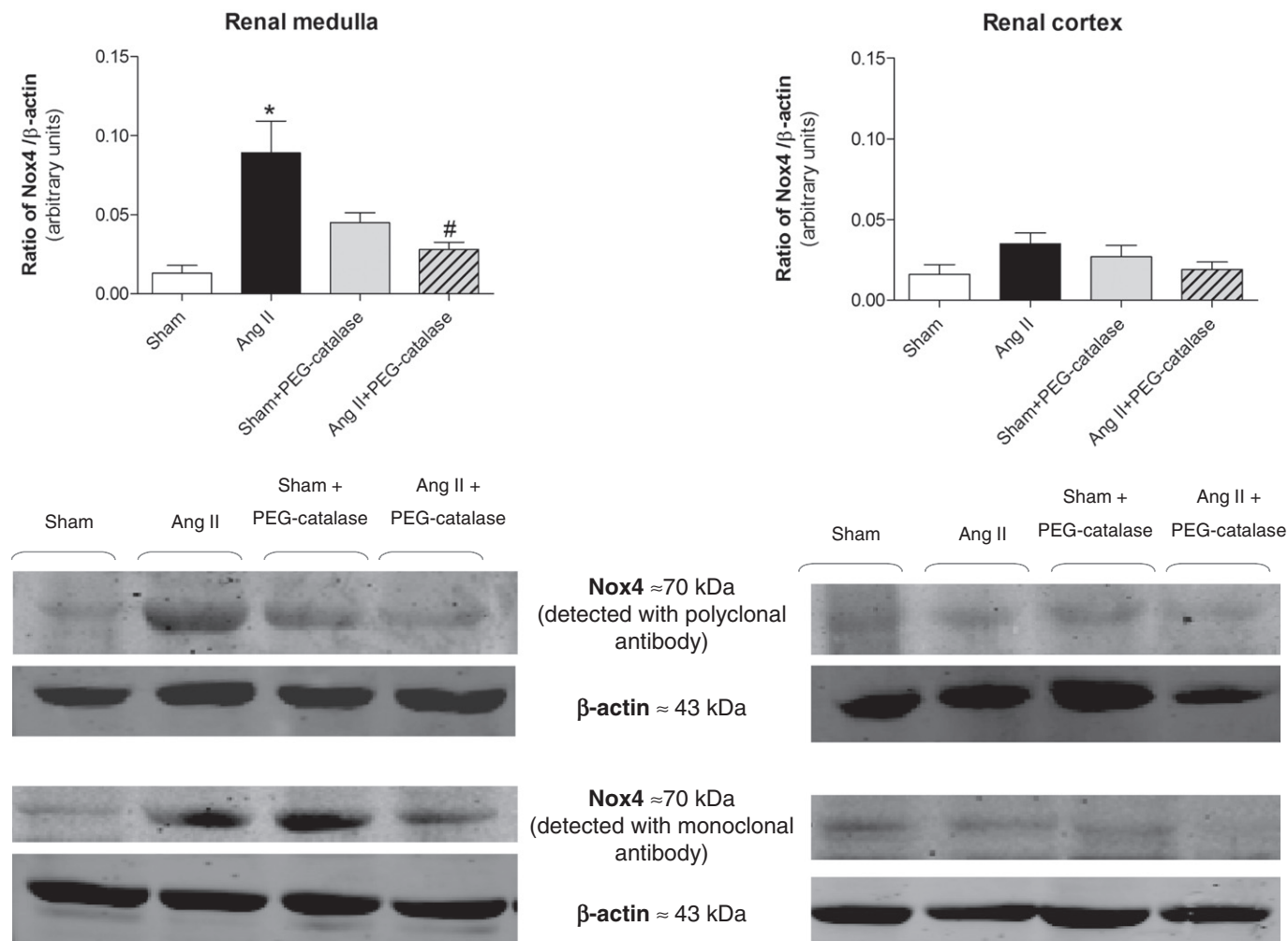
Correlation between H₂O₂ production and NF-κB homodimer activation in the renal medulla

A positive correlation ($r^2 = 0.46$, $r = 0.68$, $P = 0.0005$, $n = 22$) was observed between H₂O₂ production and NF-κB p50/p50 homodimer activation in the renal medulla (Figure 11A).

Correlation between NF-κB homodimer activation in the renal medulla and urinary angiotensinogen levels

Urinary angiotensinogen levels deviated from the normal distribution ($P < 0.001$). However, when a logarithmic transformation was applied, log (urinary angiotensinogen) values

Part III: day10

**Figure 7**

Earlier (day 10) effects of Ang II, with or without PEG-catalase, on renal Nox4 protein expression. Data shown are representative immunoblots obtained with different antibodies against Nox4. Bar graphs represent mean $\pm$ SEM values; part III, $n = 4-8$. * $P < 0.05$ significantly different from sham; # $P < 0.05$ significantly different from Ang II.

showed a normal distribution ($P = 0.50$). Hence, log (urinary angiotensinogen) values were used to evaluate the correlation between NF- κ B activation and urinary angiotensinogen. NF- κ B homodimer activation in the renal medulla was significantly and positively correlated with log (urinary angiotensinogen) ($r^2 = 0.44$, $r = 0.67$, $P = 0.025$, $n = 11$) (Figure 11B).

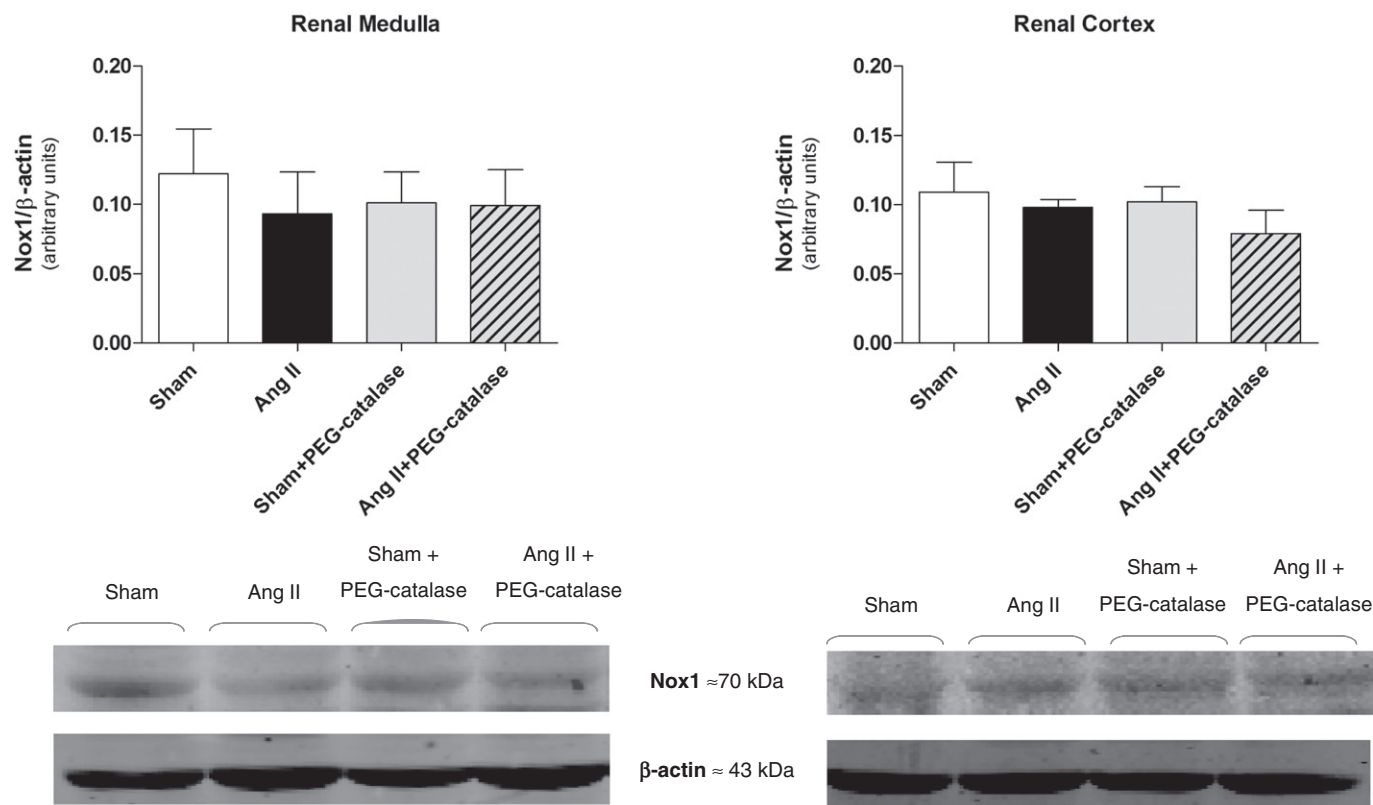
Discussion and conclusions

The present study provides strong evidence that the renal medulla is a major target for Ang II-induced dysfunction in redox signalling. We found that Ang II increases H_2O_2 production and NF- κ B activation, as well as angiotensin AT $_1$ receptor and Nox4 expression, in the renal medulla, whereas no change was observed in the renal cortex. Among the inhibitors tested, only DPI and apocynin reduced the renal

medullary H_2O_2 production. Although the use of apocynin as a specific NADPH oxidase inhibitor has been questioned (Vejrazka *et al.*, 2005; Heumuller *et al.*, 2008), the limitations described do not apply to all experimental conditions. The lack of apocynin activation due to the absence of myeloperoxidase (MPO) is not likely to occur in the kidney, as MPO expression and activity has been demonstrated in this organ (Malle *et al.*, 2003). Even though we cannot exclude a putative scavenging effect of apocynin, inhibition of H_2O_2 production was also induced by DPI which does not scavenge ROS. Furthermore, the failure of inhibitors of NOS or xanthine oxidase to reduce H_2O_2 generation excludes the contribution of these enzymes to the DPI effect. Thus, it appears that NADPH oxidases are the main sources of H_2O_2 in the renal medulla.

Renal tissues express several functional NADPH oxidase isoforms (Nox1, Nox2 and Nox4). Nevertheless, the extensive

Part III: day 10

**Figure 8**

Earlier (day 10) effects of Ang II, with or without PEG-catalase, on renal Nox1 protein expression on day 10. Data are expressed as representative immunoblots with summary bar graphs (mean $\pm$ SEM values), part III, $n = 4-7$.

abundance of Nox4 in the kidney suggests its intimate involvement in the regulation of renal-specific functions (Geiszt *et al.*, 2000; Nistala *et al.*, 2008; Garrido and Griending, 2009). Our results also suggest that in the kidney, Nox4 has a higher responsiveness to Ang II than Nox1, in contrast to what is described in vascular cells (Dikalov *et al.*, 2008). There has been controversy regarding the cortico-medullary differences in Nox4 expression (Schluter *et al.*, 2006; Orient *et al.*, 2007; Nistala *et al.*, 2008; Sedeek *et al.*, 2010). In our study, renal cortical Nox4 was barely detectable and was not affected by the treatments. On the other hand, the renal medulla had a higher Nox4 expression that was further enhanced by Ang II infusion. Although Ang II up-regulated Nox4 in renal cortical cells *in vitro* (Block *et al.*, 2008), the physiological relevance of this effect has not been sufficiently established. Furthermore, *in vivo* studies demonstrated that the renal cortex has a high resistance to Ang II-induced oxidative stress (Wesseling *et al.*, 2005), while the renal medulla exhibits a genetic vulnerability to the injurious effects of Ang II (Yuan *et al.*, 2003). Indeed, we observed that the renal cortex, besides having higher basal antioxidant activity than the renal medulla, also presented lesser changes in H₂O₂-neutralizing enzymes after Ang II infusion. While the renal medulla exhibited a marked increase in the activity of catalase, the renal cortex showed only a modest rise of GPx. These facts reflect the regional differences in renal H₂O₂ production,

since catalase and GPx are differentially required for the clearance of high or low levels of H₂O₂, respectively. Nevertheless, a similar rise in total SOD activity was observed in both kidney regions probably due to a stimulatory effect of Ang II on chaperone proteins for Cu, Zn-SOD (Gongora *et al.*, 2006).

H₂O₂ propagates its own production through the feed-forward activation of pro-oxidant enzymes, including NADPH oxidases (Ardanaz and Pagano, 2006). Recent *in vitro* studies reported that Nox2, Nox4 and Nox5 were stimulated by H₂O₂ in blood cells and cardiac fibroblasts (Colston *et al.*, 2005; El Jamali *et al.*, 2008; El Jamali *et al.*, 2010). Interestingly, we observed that a 7-day treatment with PEG-catalase almost abolished Nox4 expression in the renal medulla of Ang II-infused rats. This indicates that H₂O₂ stimulates Nox4 expression in the kidney, thus creating a vicious cycle that prolongs the redox pathological signalling.

Ang II stimulates intra-renal production of angiotensinogen through activation of AT₁ receptors, leading to increased Ang II levels in the kidney (Kobori *et al.*, 2004). This mechanism amplifies the deleterious effects of Ang II on renal function and contributes to the progression of hypertension (Kobori *et al.*, 2007). Our present data strongly suggest that renal medullary H₂O₂ mediated the Ang II-induced activation of the intra-renal RAS. We observed that Ang II-hypertensive rats had not only an increased urinary excretion of angiotensinogen but also an enhanced expression of AT₁ receptors

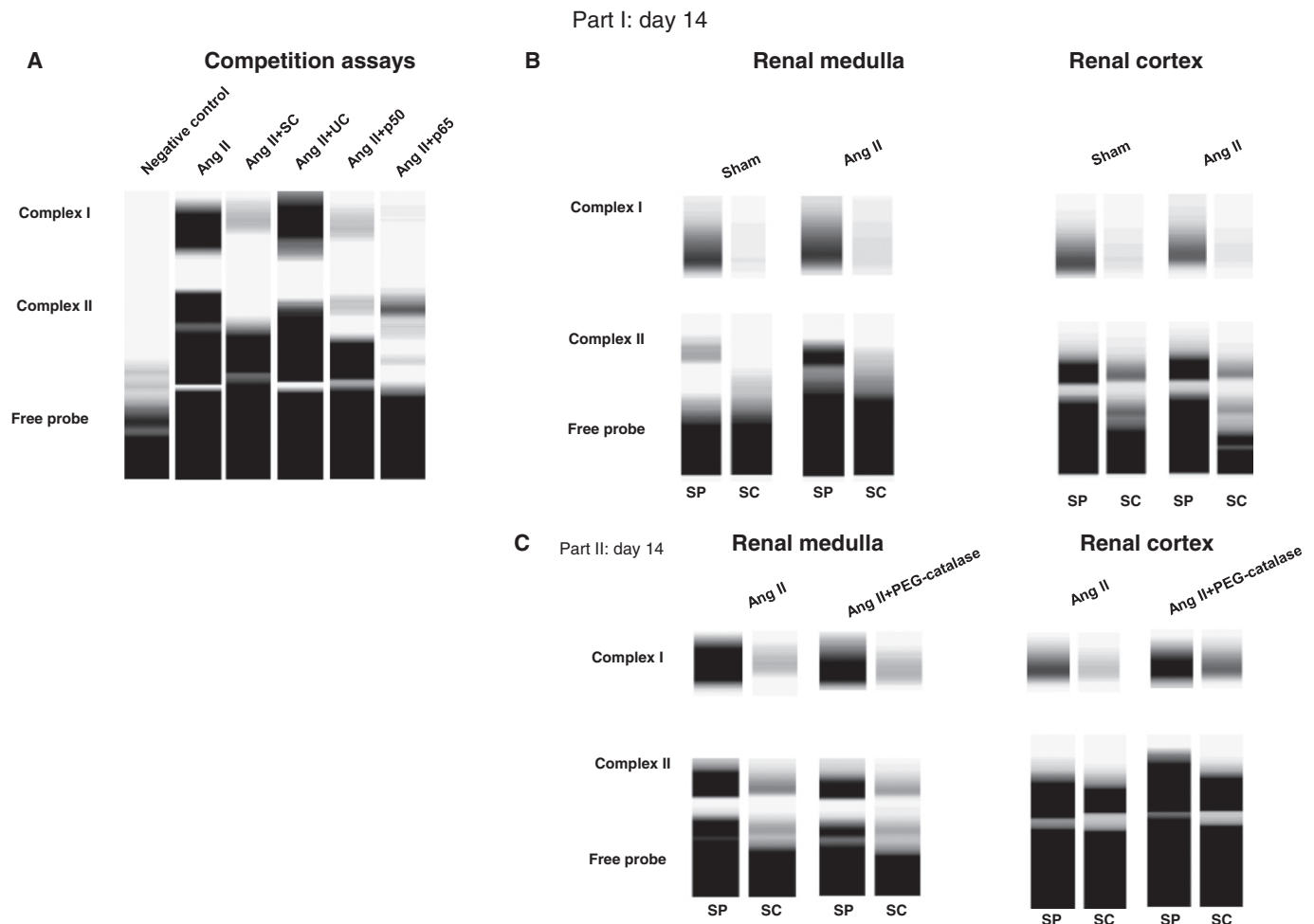


Figure 9

Representative fEMSA gels demonstrating renal NF- κ B binding: (A) competition assays showing the composition of NF- κ B complexes. SP, specific probe; SC, specific competitor; UC, non-specific competitor. (B, C) Effect of Ang II or Ang II + PEG-catalase treatment on renal NF- κ B activation on day 14.

in the renal medulla. The selective activation of renal medullary AT₁ receptors has also been described in other studies. For example, in rats with myocardial infarct, a condition associated with RAS activation in the kidney, there is an up-regulation of AT₁ receptor mRNA in the renal medulla, but not in the renal cortex (Milik *et al.*, 2006). These facts indicate that the renal medulla is an important site for the regulation of the intra-renal RAS. Of note, the reduction of renal medullary AT₁ receptor expression in rats treated simultaneously with Ang II and PEG-catalase suggests that H₂O₂ stimulates AT₁ receptor activation. In addition, H₂O₂ is known to activate NF- κ B, a transcription factor that regulates the expression of angiotensinogen and other genes involved in blood pressure homeostasis (Brasier *et al.*, 2000; Morigi *et al.*, 2002; Fardoun *et al.*, 2007). In the present study, we found that Ang II markedly activates NF- κ B homodimers in the renal medulla, but not in the renal cortex. Furthermore, no significant changes of NF- κ B p50/p65 heterodimers were observed. It is generally accepted that NF- κ B p50/p65 heterodimer complexes stimulate gene transcription, while NF- κ B p50/p50 homodimers are mostly associated with transcriptional

repression (Guan *et al.*, 2005; Pereira and Oakley, 2008). Nevertheless, Jamaluddin *et al.*, 2000 demonstrated that in hepatocytes, Ang II induces an alternative pattern of NF- κ B activation, characterized by enhanced nuclear translocation of NF- κ B homodimers and unaltered NF- κ B p50/p65 heterodimer binding. Moreover, these authors also suggested that the NF- κ B homodimer may contain latent transcriptional activity that mediates the stimulatory effect of Ang II on angiotensinogen expression. Interestingly, we observed that NF- κ B homodimer activation in the renal medulla was not only positively correlated with renal medullary production of H₂O₂, but also with the urinary excretion of angiotensinogen. This suggests that the Ang II-induced increase in intrarenal angiotensinogen production is related to the redox changes in the renal medulla, which are probably driven by H₂O₂.

It has been previously reported that hypertensive patients exhibit higher plasma levels of H₂O₂, which directly correlate with increased RAS activity and target organ dysfunction (Lacy *et al.*, 1998; 2000). Our study also shows that Ang II-hypertensive rats have increased systemic and urinary H₂O₂

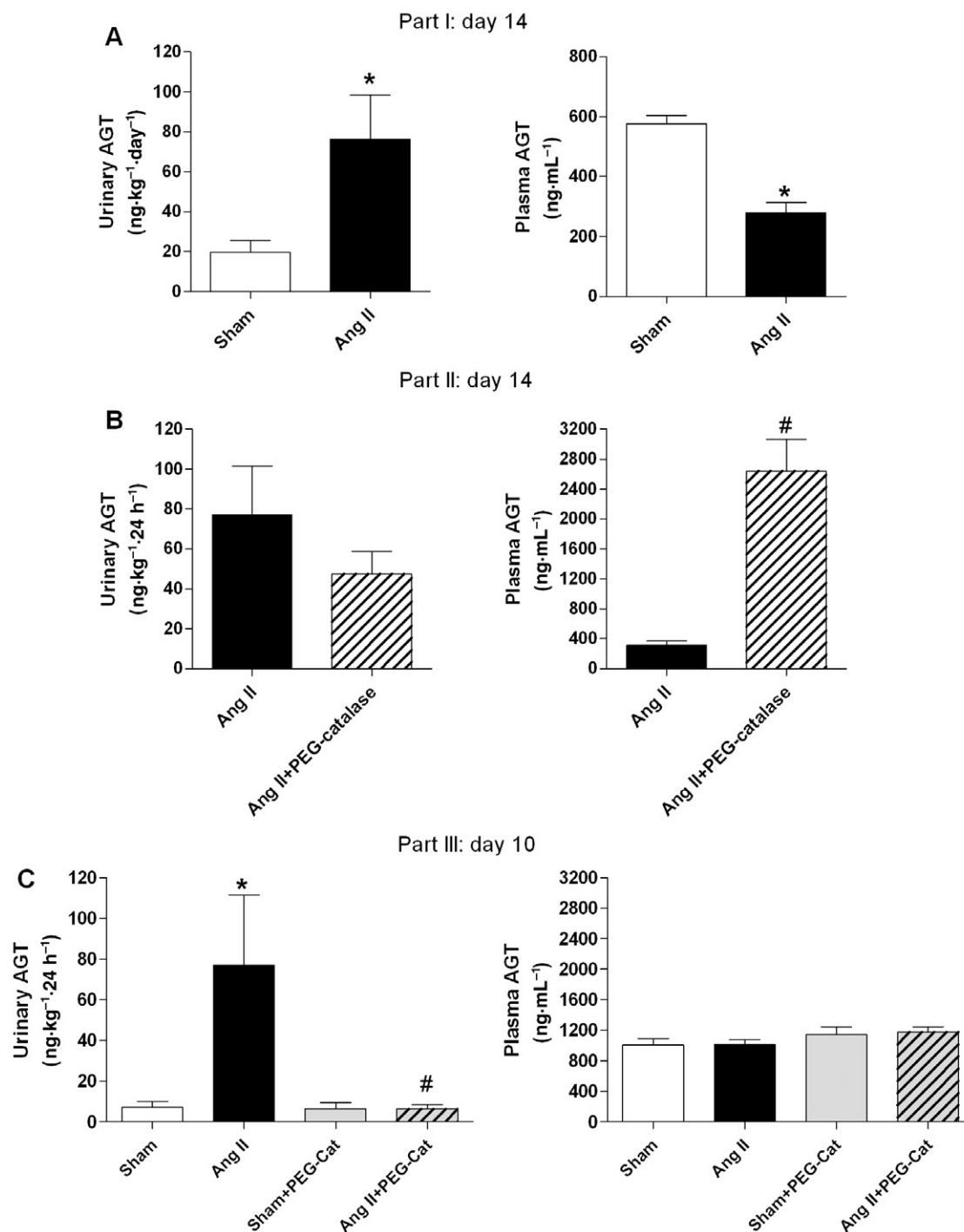


Figure 10

Effects of Ang II, with or without PEG-catalase, on urinary and plasma angiotensinogen (AGT) levels on day 14 (A and B) and on day 10 (C). Results are mean $\pm$ SEM (part I, II and III, $n = 6-9$). * $P < 0.05$ significantly different from sham; # $P < 0.05$ significantly different from Ang II.

levels. Since we previously demonstrated that PEG-catalase prevents the development of hypertension induced by RAS activation (Sousa *et al.*, 2008), we expected that it would also lower blood pressure in Ang II-infused rats. However, PEG-catalase had only a short-term effectiveness despite the sustained reduction in H₂O₂ levels. Although blood pressure was

markedly decreased during the first days of PEG-catalase administration, this effect was reversed at the end of the treatment, suggesting the existence of counter-regulatory mechanisms. Interestingly, the loss of antihypertensive efficacy of PEG-catalase was associated with a significant increase in systemic angiotensinogen levels, in opposition to the

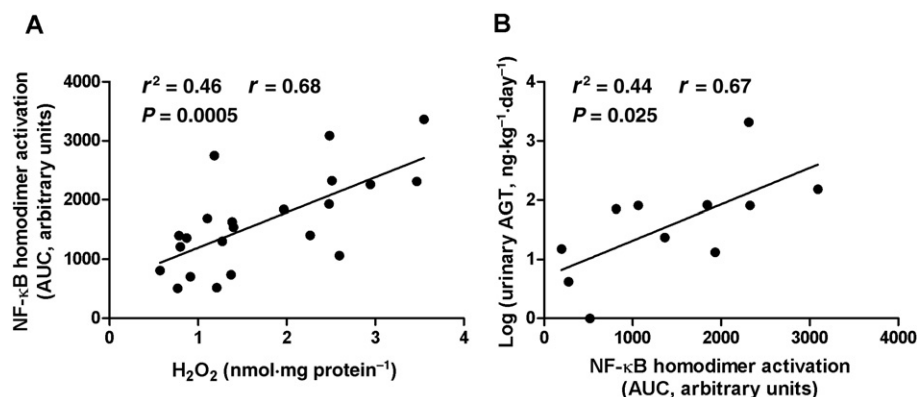


Figure 11

Correlation analysis between: renal medullary H_2O_2 and NF- κ B homodimer activation (A); renal medullary NF- κ B homodimer activation and log (urinary angiotensinogen; AGT) (B).

Table 2

Densitometric quantification of NF- κ B activation

	NF- κ B activation (n-fold vs. sham)	
	p50/p50 homodimer	p50/p65 heterodimer
Renal medulla		
Sham	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
Ang II	3.47 $\pm$ 0.34*	1.02 $\pm$ 0.27
Renal cortex		
Sham	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
Ang II	1.10 $\pm$ 0.18	1.24 $\pm$ 0.17
	NF- κ B activation (n-fold vs. Ang II)	
	p50/p50 homodimer	p50/p65 heterodimer
Renal medulla		
Ang II	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
Ang II + PEG-catalase	0.61 $\pm$ 0.11	0.86 $\pm$ 0.28
Renal cortex		
Ang II	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
Ang II + PEG-catalase	1.34 $\pm$ 0.14 [#]	1.37 $\pm$ 0.07 [#]

Results are expressed as n-fold variation vs. sham or Ang II and correspond to the mean $\pm$ SEM of 3–6 independent experiments; n = 5–9 rats per group. * P < 0.05 vs. sham. [#] P < 0.05 vs. Ang II.

down-regulation induced by Ang II alone. There is evidence that the increase of circulating angiotensinogen levels leads to a rise of blood pressure (Cholewa and Mattson, 2005). Furthermore, the angiotensinogen metabolite, angiotensin (1–12), can serve as a substrate for Ang II formation in the absence of renin (Nagata *et al.*, 2006; Ferrario, 2010). Therefore, the dysregulation of systemic angiotensinogen levels

may explain the loss of PEG-catalase antihypertensive efficacy. In contrast, the initial lowering of blood pressure is likely to be caused by the significant suppression of intrarenal angiotensinogen levels observed in the first days of PEG-catalase administration. The transient nature of this effect is probably due to the marked rise of systemic angiotensinogen, resulting in the elevation of circulating Ang II levels and subsequent increase of angiotensinogen production in the kidney. Of note, an intriguing data of our study was the enhancement of renal cortical NF- κ B activity caused by PEG-catalase. Altogether, these facts suggest that when renal medullary events are blocked, there is an alternative activation of the renal cortex by Ang II that contributes to overcome the antihypertensive effect of PEG-catalase.

Our results not only reinforce the concept that circulating and renal RAS are distinctly regulated (Kobori *et al.*, 2002; 2007), but also propose that H_2O_2 acts as a differential modulator of these systems. In line with this hypothesis is the recent finding that H_2O_2 has either stimulatory or inhibitory effects on angiotensinogen expression depending on the cell type (Okada *et al.*, 2010). The putative mechanisms whereby Ang II or H_2O_2 may increase renal or systemic RAS activation are depicted in Figure 12.

In summary, this study demonstrates that the renal medulla is a primary target of Ang II-induced redox dysfunction and highlights H_2O_2 as a key player in the amplification of Ang II effects. We conclude that increased H_2O_2 levels contribute to Ang II-induced hypertension and intrarenal RAS activation. Even so, sustained treatment with PEG-catalase had a short-term antihypertensive effectiveness probably due to the dysregulation of systemic angiotensinogen production. Thus, it appears that the regulation of systemic or intrarenal RAS activation involves fine-tuning processes dependent on H_2O_2 levels.

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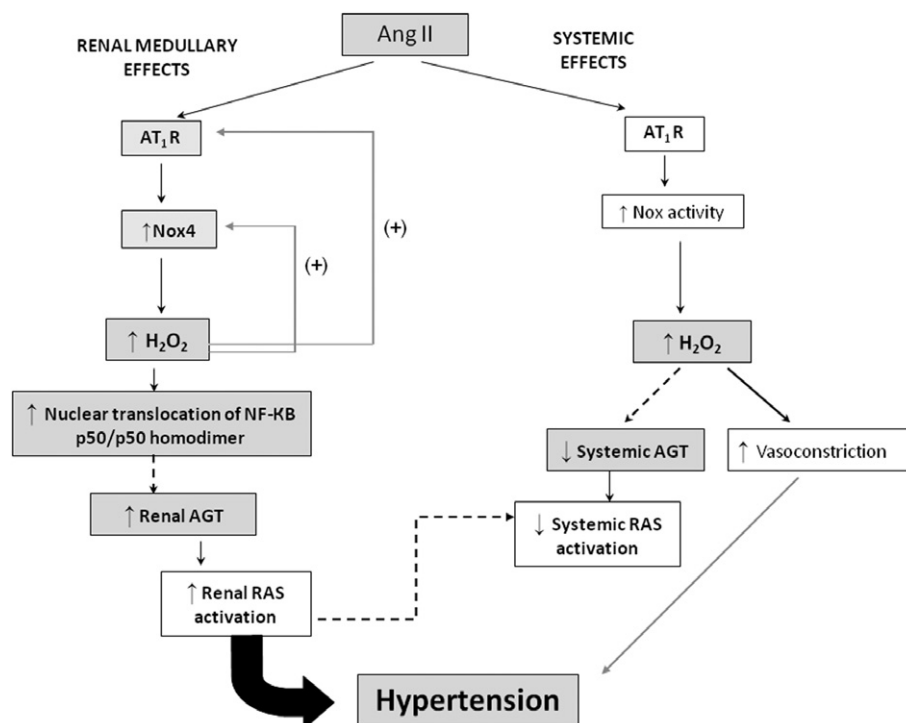


Figure 12

Putative mechanisms involved in the regulation of BP and RAS activation by Ang II and H₂O₂. Dashed arrows represent the predicted pathways for intrarenal and systemic RAS activation. Coloured (grey) rectangles indicate the effects observed in the present study. In the renal medulla, the activation of angiotensin AT₁ receptors (AT₁R) by Ang II increases NADPH oxidase-derived H₂O₂ production. H₂O₂ further enhances the sensitivity to Ang II effects by stimulating the expression of AT₁ receptors and Nox4. Increased H₂O₂ levels augment the nuclear translocation of NF-κB homodimer in the renal medulla. These events appear to trigger an increase of angiotensinogen (AGT) production in the kidney, causing a subsequent enhancement of intrarenal RAS activation that contributes to hypertension. Ang II increases systemic (non-renal) production of H₂O₂, probably through AT₁ receptor-dependent stimulation of Nox enzymes. Systemic H₂O₂ increases peripheral vasoconstriction, which also elevates blood pressure. Simultaneously, there is a reduction of systemic AGT levels by Ang II that probably attenuates the systemic RAS activity in order to prevent a sudden malignant rise of blood pressure. Down-regulation of circulating RAS may be partly mediated by systemic H₂O₂ levels and/or by renal RAS activity.

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Conflict of interest

None.

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