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Alternative pathways for angiotensin II production as an important determinant of kidney damage in endotoxemia

Rodolfo Mattar Rosa[1], Juliana Almada Colucci[1], Rodrigo Yokota[1], Roseli Peres Moreira[1], Danielle Sanches Aragão[1], Amanda Aparecida Ribeiro[1], Danielle Yuri Arita[1], Ingrid K. M. Watanabe[1], Zaira Palomino[1], Tatiana Sousa Cunha[2], Dulce Elena Casarini[1]

1- Medicine Department, Nephrology Division, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brasil.
2- Science and Technology Department, Instituto de Ciência e Tecnologia, Universidade Federal de São Paulo, São José dos Campos, Brasil.

Running title: **Kidney angiotensin II production in endotoxemia**

Corresponding Author:
Dulce Elena Casarini
E-mail: casarini.elena@unifesp.br
Phone: +55 11 5904-1685
Address: Rua Botucatu, 740, Vila Clementino, São Paulo, SP, Brazil
Nephrology Division, Medicine Department - 2nd floor
Zip code 04023-900

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Abstract

Sepsis is an uncontrolled systemic inflammatory response against an infection and a major public health issue worldwide. This condition affects several organs, and, when caused by Gram-negative bacteria, kidneys are particularly damaged. Due to the importance of renin-angiotensin system (RAS) in regulating renal function, in the present study, we aimed to investigate the effects of endotoxemia over the renal RAS. Wistar rats were injected with E. *coli* lipopolysaccharide (LPS) (4 mg/kg), mimicking the endotoxemia induced by Gram-negative bacteria. Three days after treatment, body mass, blood pressure and plasma nitric oxide (NO) were reduced, indicating that endotoxemia triggered cardiovascular and metabolic consequences and that hypotension was maintained by NO-independent mechanisms. Regarding the effects in renal tissue, inducible NO synthase (iNOS) was diminished, but no changes in the renal level of NO was detected. RAS was also highly affected by endotoxemia, since renin, Angiotensin-Converting Enzyme (ACE) and ACE2 activities were altered in renal tissue. Although these enzymes were modulated, only Angiotensin (Ang) II was augmented in kidneys, Ang I and Ang 1-7 levels were not influenced by LPS. Cathepsin G and chymase activities were increased in the endotoxemia group, suggesting alternative pathways for Ang II formation. Taken together, our data suggests the activation of non-canonical pathways for Ang II production and the presence of renal vasoconstriction and tissue damage in our animal model. In summary, the systemic administration of LPS affects renal RAS, what may contribute for several deleterious effects of endotoxemia over kidneys.

Introduction

Sepsis is among the biggest challenges in medicine, since it is difficult to diagnose and treat. In developed countries, 6 to 30% of the patients from intensive care units (ICUs) may develop sepsis (21). Epidemiological studies from the USA show that 215 thousand deaths per year are due to sepsis and was described that the incidence of sepsis is growing by approximately 8.7 % per year (3, 21, 26, 36–38). This condition begins with an infection that leads to systemic inflammatory response syndrome (SIRS). Gram-negative bacteria are mainly responsible for sepsis cases in ICUs, however, the infection *per se* is not the cause of death; in fact, organ dysfunction arising from SIRS is the real responsible (21, 27, 31, 34, 36, 40).

The host's immune system recognizes endotoxins from Gram-negative bacteria, which are chemically classified as lipopolysaccharides (LPS). In the cell membrane, LPS binds to the acceptor CD14 and the toll-like receptor 4 (TLR-4) triggers an intracellular signaling that leads to pro-inflammatory cytokines release, like interleukins 1-beta (IL-1β), 6 (IL-6) and Tumor Necrosis Factor alpha (TNF-α) (17, 24, 26, 31). Systemic releasing of endotoxins and increased expression of TLR-4 in non-immune cells may initiate secondary responses that contribute to septic shock by increasing nitric oxide (NO) production and vascular permeability, leading to systemic hypotension. This condition affects tissue perfusion, coagulation cascade and causes organ dysfunction and failure (1, 10, 17, 20).

Mesangial cells from glomerulus express CD-14 and TLR-4, allowing LPS binding to the cell surface and the triggering of molecular and physiological effects of septic shock, like the local release of cytokines, systemic decrease of blood pressure and renal vasoconstriction. Taken together, these complications may lead to acute kidney injury (AKI) and renal failure (1, 3, 4, 20, 38). In order to study this complication, exogenous administration of LPS in animals is one of the models for mimicking AKI secondary to septic shock (13, 26, 34, 40). In several animal models of sepsis, as well as in human patients, renal blood flow (RBF) is quite variable. Although hypotension and RBF are important factors for renal injury

progression, LPS injection is capable of inducing this condition even in the absence of significant hemodynamic changes (4, 39).

Previous studies from our group suggested the participation of the renin angiotensin system (RAS) in AKI secondary to endotoxemia. Treatment with captopril, an inhibitor of the angiotensin-converting enzyme (ACE), prevented the deleterious effects of endotoxemia in LPS-injected animals. Moreover, some RAS components were modulated in human mesangial cells exposed to LPS (1, 18, 20). Despite all of these data, little is known about the real importance of the RAS in endotoxemia.

In recent times, the understanding of RAS has increased, giving a new level of complexity to this system. Classically, renin cleaves angiotensinogen (AGT) producing Angiotensin (Ang) I, which is converted by ACE to Ang II, a peptide with proliferative and vasoconstrictor actions. Alternative pathways for non-renin-dependent Ang II formation were described, being tonin and cathepsin G the enzymes responsible for this release from AGT and chymase is an enzyme able to cleave Ang I generating Ang II. Nevertheless, the angiotensin-converting enzyme 2 (ACE2) was identified as being homologous to ACE but responsible for cleaving Ang II into Ang (1-7), that presents anti-proliferative and vasodilator effects. Thus, the balance between Ang II and Ang (1-7) levels is important for controlling RAS activity (2, 9, 14, 15, 23, 28, 33).

Besides its action in controlling vascular tonus, today it is well established that RAS influences other physiological functions, because Ang II activates signaling pathways involved in tissue injury, inflammation, fibrosis, free-radicals production, immune cells activation, adhesion molecules expression and cytokines production. It was also proved that Ang (1-7) counter-regulates these effects too by reducing leukocytes migration and activating fibrinogenic pathways, for instance (32, 33, 39).

Therefore, in view of the pathophysiological effects triggered by endotoxemia and the importance of RAS in controlling renal homeostasis and inflammation, the aim of the present study was to evaluate renal RAS under LPS systemic actions in Wistar rats.

Methods

Experimental model

Male Wistar rats from Experimental Models Development Center [CEDEME, *Escola Paulista de Medicina, Universidade Federal de São Paulo* (UNIFESP)] weighting 300-400g and aging 12 weeks were used. Animals were housed in boxes under controlled conditions (22±2ºC; light/dark 12h/12h; 60% humidity), with food and water *ad libitum*. All procedures were performed in accordance with Ethics in Research Committee (#0368/11). Endotoxemic group was injected with E. *coli* LPS (0111:B4, catalogue #L4391 *Sigma-Aldrich*, USA) (4mg/kg, i.p.) and control group received saline (0.9% NaCl, i.p.). Three days after treatment, animals were decapitated; blood and kidneys were collected.

Tail cuff blood pressure and body

Animals were weighted in a B6000 scale (*Micronal S.A.*, São Paulo, Brazil) and tail cuff blood pressure was assessed by the *NIBP Controller* apparatus, linked to the *PowerLab System* and to the signal transducer *MLT125R* (*AD Instruments*, Dunedin, New Zealand). These measurements were performed in the afternoon, three days before (day -3), in the day of (day 0) and three days after (day 3) treatment.

Urine collection

Two days after treatment, animals were housed in individual metabolic cages with food and water *ad libitum*. 24-hours urine was collected and samples were centrifuged (1000 rpm, 10 minutes, 4ºC), volume of urine was measured and they were stored at -20ºC.

Serum, plasma and tissue collection

Blood and kidneys were collected immediately after euthanasia. Blood was collected into dry or EDTA tubes (*BD Vacutainer*, New Jersey, USA), centrifuged (3000 rpm, 15 minutes, 4ºC) and supernatant was stored at -80ºC. Kidneys were quickly removed, washed in phosphate buffered saline (PBS) and stored in 10% buffered formalin or frozen at -20°C or -80ºC.

Creatinine clearance

Creatinine was measured in urine and serum by Enzymatic Method (*Creatinina K kit*, *Labtest Diagnóstico*, *Lagoa Santa*, Brazil), which is based on Jaffe's reaction principle associated to the application of a correction index (8). This methodology minimizes the interference on creatinine determination. The experiment was performed according to manufacturer's instruction and absorbance (510 nm) was read in two time points (30 and 90 seconds) at the *Bio-200* apparatus (*Bioplus, Barueri*, Brazil). Creatinine clearance was based on the following formula:

Clearance (mL/min) = $\left(\frac{U}{S}\right) x\ VM$

U: Creatinine concentration in urine (mL/min)

S: Creatinine concentration in serum (mL/min)

VM: 24h-urine volume (mL) in 1440 min

Protein quantification

Protein concentration was estimated for homogenates by Bradford method (5), using Comassie Blue reagent (1:5, *Bio-Rad*, USA). Absorbance (595 nm) was read at the *Infinite 200* apparatus (*Tecan*, Switzerland). Calculation was based on a bovine serum albumin standard curve; values were expressed in mg/mL.

Plasma cytokines quantification

Pro-inflammatory cytokines (IL-1β, IL-6 e TNF-α) were quantified in EDTA plasma by *Luminex xMAP* system (*Millipore*, USA). The immunoassay was performed according to the manufacturer's instructions, using specific antibodies and fluorophores immobilized over microsphere's surface. Briefly, three different sets of microspheres were created into a color-based code, and detection was done by adding phycoeritrin to the assay. Finally, cytokines were detected at the *Luminex 200* apparatus (*Luminex Corp*, USA), which is able to recognize this color code. Data was analyzed in software *xPotent/Analist 4.2*; values were expressed in pg/mL.

Plasma and renal NO quantification

NO was quantified in plasma and kidneys by colorimetric assay using improved Griess method (*BioAssay*, USA). Plasma samples were collected in EDTA tubes and renal tissues were homogenized into PBS (1g tissue: 10mL buffer). Homogenates were centrifuged twice (15000 rpm, 10 minutes, 4ºC). Supernatants were collected and stored at -80ºC. Before the assay, samples were deproteinized. The assay was performed according to manufacturer's instructions. Absorbance (540 nm) was read at the *Stat Fax 2010* apparatus (*Awareness Technology*, USA) and the calculation was based on a standard curve using *MultiCalc* Software; values expressed in μM. For kidney samples, values were normalized to protein concentration (μmol/mg).

Reactive Oxygen Species (ROS) dosage

ROS were quantified in renal tissues by solid phase ELISA (*Blue Gene Biotech*, China). Samples were homogenized and stored as previously de4scribed for renal NO quantification. The competitive enzymatic immunoassay technique uses monoclonal anti-ROS antibodies and ROS-HRP conjugates. The assay procedures followed manufacturer's instructions, using coated plates and specific reagents. Absorbance (450 nm) was read at the Stat Fax 2010 apparatus and the calculation was done based on a

standard curve using MultiCalc Software. ROS values were normalized by protein concentration (µmol/mg).

Angiotensin peptides quantification

Angiotensin peptides were quantified by High Performance Liquid Chromatography (HPLC), according to the method previously described by our group (1, 29). Renal Tissues were homogenized using a protease inhibitor cocktail *Complete Mini* (Roche, USA) and pepstatin. Angiotensin peptides were extracted from renal tissue homogenates. Calculation of angiotensin levels was based on a standard curve; values were expressed in pmol/mL and later normalized by tissues weight (pmol/g).

Immunohistochemistry (IHC)

After fixation in 10% buffered formalin, renal tissues were immunostained by the peroxidase-antiperoxidase method with antibodies against Ang II and Ang (1-7) (1:50), a generous gift from Dr. Preenie Senanayake (*Eye Cole Institute - Cleveland Clinic*, Cleveland, Ohio, USA). The method was performed as previously described (29).

Enzymatic activities

Renin

Renin activity was determined by HPLC, according to the method previously described (35). Kidney samples were homogenized in 50 mmol/L Tris buffer pH 7.5 (1 g tissue: 10 mL buffer) containing protease inhibitors (10 mmol/L EDTA, 1.0 mmol/L PMSF, 3 µmol/L E64, 1.5 mmol/L, o-phenanthroline). Homogenates were centrifuged twice (15000 rpm, 15 minutes, 4ºC). Samples (20 µL) were incubated at 37ºC with substrate (tetradecapeptide, 1 nmol/L) in buffering conditions containing the same inhibitors described above at pH 6, reaction was stopped, samples were collected at 0 and 30 minutes and injected in the HPLC system. Chromatographic profile was compared to standard and values were

normalized by protein concentration and expressed in nmol/min/mg of protein. We used as assay control the incubation of homogenate in presence of protease inhibitors containing also aliskiren and pepstatin (10 µmol/L).

ACE

Kidney samples were homogenized in borate buffer pH 7.2 (sodium borohidride 0.4 mol/L, sucrose 0.34 mol/L, NaCl 0.9 mol/L, and protease inhibitor PMSF 1mmol/L, 1 g tissue:10 mL buffer). Homogenates were centrifuged twice (15000 rpm, 15 minutes, 4ºC) and supernatants were stored at -20ºC. ACE activity was determined in spectrofluorimeter (*Hitachi F-2000*, Japan), using the fluorescent substrate Abz-YRK-EDDnp (10 µmol/L; excitation 320 nm; emission 420 nm). The substrate was added to the buffer (Tris 100 mmol/L, NaCl 50 mmol/L, $ZnCl_2$ 10 µmol/L, pH 7.1) and fluorescence was read (10 minutes, 37ºC). Then, 10 µL of sample was added and read again. Finally, the inhibitor captopril (10 µmol/L) was added for checking ACE's activity. Arbitrary units were registered and calculations were done. Values were normalized by protein concentration (nmol/min/mg of protein).

ACE2

Kidney samples were homogenized in 50 mmol/L Tris buffer pH 7.5 (1 g tissue: 10 mL buffer) containing proteases inhibitors (*Complete mini EDTA free, Roche,* USA). Homogenates were centrifuged twice (15000 rpm, 15 minutes, 4ºC) and supernatants were stored at -20ºC. ACE2 activity was determined in spectrofluorimeter (*Tecan*, Switzerland), using the substrate Mca-APK-Dnp (30 µmol/L, excitation 320 nm, emission 420 nm). Buffer (Tris-HCl 50 mmol/L, NaCl 1 mol/L, $ZnCl_2$ 10 µmol/L, captopril 10 µmol/L, pH 6.5) and samples (2 µL) were pre-incubated for 30 minutes in the presence or the absence of ACE2 inhibitor (DX600, 20 µmol/L). Substrate was added and samples were read at 0 and 60 minutes. Arbitrary units were registered, calculations were done based on a fluorescence standard curve (OmniMMP) and the time point 0 was used as internal blank. Values were normalized by the protein concentration (nmol/min/mg of protein).

Neprilysin, cathepsin G and chymase

Kidney homogenate was prepared as described above in the respective buffers used to quantify neprilysin, cathepsin and chymase activities. The fluorogenic substrates were produced by AminoTech Pesquisa e Desenvolvimento (Brasil).

The neprilysin activity was measured after pre-incubation of 10 µL of kidney homogenate from control and endotoxemia groups with captopril (10 µmol/L), aliskiren (10 µmol/L) at 37°C for 30 minutes in 50 mmol/L Tris/HCl buffer, pH 7.4, in plates from the *Infinite 200* apparatus (*Tecan*, Switzerland), followed by Abz(d)R-G-L-EDDnp (10 µmol/L) (Abz=ortho-amino benzoic acid; EDDnp= 2,4-dinitrophenyl ethylenediamine) substrate addition. Fluorescence was measured continuously at λ_{ex}=320 nm and λ_{em}= 420nm. The same procedure was carried out using 50 nmol/L of the specific inhibitor Thiorphan. The proteolytic activity was expressed as µM/min/mg of protein.

Cathepsin G activity was measured using the substrate Abz-DRVYIHPFHLLVYSQ-EDDnp (10 µmol/L). An aliquot of samples from kidney homogenate from control and endotoxemia groups was pre-incubated in 50 mmol/L sodium phosphate buffer, pH 6.0, at 37^{o}C for 30 min containing a pool of inhibitors *Complete mini (Roche,* USA), aliskiren and captopril (10 µmol/L) directly in the plates of *Infinite 200* apparatus (*Tecan*, Switzerland), followed by the addition of 10 µmol/L of the substrate. The hydrolysis was monitored for 10 min. Fluorescence was measured continuously at λ_{ex}=320 nm and λ_{em}= 420nm. The activity was expressed as µM/min/mg of protein.

Chymase activity was quantified using the substrate Abz-AIKFFSAQ-EDDnp (10 µmol/L). The homogenates from kidney of control and endotoxemia groups were incubated directly in the plates of the *Infinite 200* apparatus (*Tecan*, Switzerland). An aliquot of samples (10 µL) were incubated in 100 mmol/L Tris/HCL buffer, pH 7.4 at 37^{o}C. The amount of Abz released after the hydrolysis of the substrate was measured at λ_{ex}=320 nm and λ_{em}= 420nm. The same procedure was performed in the presence of 100 µmol/L of the specific inhibitor chymostatin. The enzyme activity was defined as the amount of substrate sensitized by chymostatin and corrected by protein

concentration of each sample. The activity was expressed as μM/min/mg of protein.

Western blotting analysis (WB)

Kidney samples were homogenized, centrifuged and stored as described previously for ACE activity assay. Samples (50 μg) went through electrophoresis in polyacrylamide gel (acrylamide 7.5% or 10%) with sodium dodecyl sulfate (SDS-PAGE) (16). Proteins were electro-transferred to nitrocellulose membranes (*Hybond*, *GE Healthcare*, USA) and incubated overnight at 22ºC with primary antibodies anti-AGT (1:1000, *rabbit*, *Millipore*, *catalog #MABC123*, USA), anti-Renin (1:500, *mouse*, *Santa Cruz*, *catalog #sc-365484*, USA), anti-ACE (1:250, biotinylated, *R&D Systems*, *catalog #BAF1513,* USA), anti-ACE2 (1:250, biotinylated, *R&D Systems*, *catalog #BAF933*, USA), anti-iNOS (1:200, rabbit, *Santa Cruz*, *catalog #sc-650*, USA) or anti-β-actin (1:5000, *mouse*, AbCam, *catalog #mAbcam8226*, USA). Secondary antibody anti-mouse or anti-rabbit IgG (1:2000, *GE Helthcare*, Switzerland) was used when necessary. Subsequent steps were performed with streptavidin-alkaline phosphatase system (*Amersham Pharmacia Biotech*, Sweden). Bands were reveled with NBT/BCIP substrates (*BioRad*, USA). Optical densitometry was analyzed and quantified by the *GS-800 Calibrated Densitometer* and *Quantity One Software* (*BioRad*, USA). Protein expression was measured in pixels/mm^2 and normalized by β-actin expression.

Statistical analysis

Values were represented as mean (X) ± standard error of the mean (SEM). Data from endotoxemic group were compared to control group by two-tails non-dependent t-Test. The significance level (p-value) of 5% was considered statistically significant.

Results

LPS dose was determined by dose-response tests in small groups of animals (n=3). LPS was injected in Wistar rats intraperitoneally in the following doses (mg/kg): 1, 2, 4 and 8. Twenty-four hours after treatment, the group LPS 8 mg/kg reached 100% of mortality, but animals treated with lower LPS doses survived through the three-day treatment, as expected in our protocol. Blood pressure and body weight were measured twice before LPS injection and once 3 days after treatment. All tested doses reduced body weight, but only the doses 2 and 4 mg/kg affected blood pressure. Thus, as long as rodents are resistant to LPS effects, we chose LPS 4 mg/kg for the following steps of our work (data not shown).

A larger group of animals was injected with LPS 4 mg/kg and both blood pressure (Figure 1.A) and body mass (Figure 1.B) were reduced three days post-treatment, as expected. Creatinine clearance was similar between groups, suggesting that glomerular filtration rate (GFR) was not affected by endotoxemia (Figure 1.C). Plasma cytokines (Figure 2.A) presented great internal variation; thus, no statistical significant alteration induced by LPS was observed. Although, plasma NO (Figure 2.B) was highly reduced in endotoxemic animals ($p<0.01$).

Angiotensin peptides were quantified in renal tissue, Ang II level was increased by LPS but Ang I and Ang (1-7) levels remained unchanged between groups (Figure 3A). Endotoxemia did not affect ROS and NO levels in renal tissue (Figures 3.B and 3.C); still Inducible Nitric Oxide Synthase (iNOS) protein expression was decreased by LPS treatment (Figures 3.D and 5). Immunohistochemistry for Ang II and Ang (1-7) showed the same profile of peptides production found by HPLC, with higher levels of Ang II in endotoxemia group and unaltered Ang (1-7) level between groups. Both peptides presented intracellular staining, especially in cytosol from renal tubules cells (Figure 4).

Concerning protein expression of RAS components, single bands were detected in AGT, renin and ACE2 WB, but none of them had their expression affected by LPS treatment (Figures 5.A 5.B, 5.D). Regarding ACE2, the 68 kDa-band is in accordance with the molecular mass previously described by

our team (2). Two bands of ACE were detected with different molecular masses (120 kDa and 65 kDa), indicating the presence of two isoforms, as previously found by our team (29). In the present work, only the 120 kDa isoform was down regulated (Figures 5.C).

RAS enzymes activities in renal tissue were also affected by LPS injection, while renin and ACE presented reduced activities in endotoxemic animals, ACE2 activity was increased in LPS-treated animals (Figure 6). The renin activity tested was inhibited by aliskiren 86% and 97% in control and endotoxemia groups, respectively. Additionally, the activity was inhibited by pepstatin 71.5% and 76% in control and endotoxemia groups, respectively.

Based on the increased levels of Ang II and reduced renin activity, we tested the alternative RAS enzyme pathways for peptide generation quantifying cathepsin G and chymase activities. The cathepsin G activity was significantly higher in the endotoxemia group when compared to control (1.80 vs 2.37 μM/min/mg of protein) ($n=3$, $p<0.05$), and also for chymase activity (0.50 μM/min/mg of protein vs 0.60 μM/min/mg of protein, $n=3$, $p<0.0001$) (Figure 6), suggesting that these enzymes contributed to Ang II formation.

Discussion

The effects of endotoxemia over the renal Renin-Angiotensin System (RAS) were studied in the present work, since kidneys are greatly affected during that condition. Endotoxemia was mimicked in rats by lipopolysaccharide (LPS) injection, causing the alteration of physiological parameters related to the cardiovascular function and modulation of some RAS components in the kidneys. Endotoxemic renal tissue presented reduction of renin and Angiotensin-Converting Enzyme (ACE) activities, ACE2 increased activity and augmentation of Angiotensin II renal levels. Cathepsin G and chymase activities were increased suggesting the activation of alternative pathways for angiotensin II production in kidneys during endotoxemia, what may lead to renal dysfunction.

Sepsis is a highly complex disease, which might trigger several types of host responses, depending on a number of factors. Although these

characteristics restrain the possibility of fully mimicking that condition, animal models are still essential tools for studying biological processes. In sepsis studies, LPS injection was chosen as one of the main methods for mimicking endotoxemic shock and its renal outcomes (26, 27, 40).

Since high doses of LPS provoke cardiovascular collapse and low doses induce hyperdynamic response (26, 27), we tested several doses of LPS (data not shown). We standardized 4 mg/kg for the subsequent treatments, since that dose reduced blood pressure and body mass three days after treatment, indicating cardiovascular and metabolic alterations. These complications are typical from endotoxemia, since they are triggered by disturbances of hemodynamic, substrate turnover, hormonal pattern and protein catabolism (6).

Our data is in accordance to Tsai *et al,* that observed reduction of blood pressure after intravenous LPS injection, reaching low levels in the first hours of treatment (33). Despite this hypotension, creatinine clearance remained unchanged, indicating that GFR was not affected. Previous studies also showed that creatinine clearance was preserved 24h after LPS injection in Wistar rats (24). It is important to highlight that the triggering of AKI does not require renal hemodynamics alterations (1).

Concerning systemic inflammation, endotoxemia did not affect plasma pro-inflammatory cytokines levels, what can be explained by the specie-specific sensitivity to LPS. Rodents are more resistant to LPS effects than primates, presenting brief augmentation of cytokines levels. Therefore, endotoxemia induction in rodents requires higher LPS doses, what greatly activates innate immune system but causes early and transient release of pro-inflammatory cytokines (27, 40). Thus, our prolonged treatment might have allowed the cytokines levels returning back to normal. Besides, the unchanged cytokines levels could also be related to the maintenance of GFR after LPS injection, since they contribute to systemic hypotension and renal injury (24).

Plasma NO greatly reduced in endotoxemic group, indicating that NO-independent mechanisms maintained the hypotension, like prostacyclin pathway. In this regards, the kallikrein-kinin system becomes important, because the activation of the type 1 bradykinin receptor (B_1R) induces

prostacyclin release. Furthermore, LPS and vascular dysfunction, an early event from endotoxemia, activate this system, and it was demonstrated that the B_1R blockage partially reverts the hypotension in sepsis animal models (22). Besides, it was already reported that there are iNOS-dependent and iNOS-independent pathways to trigger LPS-induced hypotension and death (19).

Other authors presented results similar to ours (19, 39). Their data suggest that NO-independent pathways might be important controllers of vascular tonus during endotoxemia. In these previous studies, typical cardiovascular effects of LPS were observed even in the absence of changes in NO levels and/or iNOS modulation. These works support our findings, in which LPS-treated animals presented decreased blood pressure despite the reduced levels of plasma NO and renal iNOS expression.

After the physiological characterization of our model, we analyzed the influence of endotoxemia specifically over renal RAS, since it is independently regulated from the systemic RAS and an important controller of renal injury progression (15, 39). In fact, endotoxemic kidneys presented favorable environment for tissue damage, since renal Ang II was augmented and NO production was compromised, with decreased iNOS expression and maintenance of NO level. According to Boffa *et al*, animals injected with LPS and treated with Ang II, NOS inhibitors and norepinephrine present similar vascular reaction to control animals (3), suggesting that increased levels of Ang II highly contribute to renal vasoconstriction and damage, due to the imbalance between vasoconstrictors and vasodilators.

Previous findings from other groups reported the augmentation of Ang II levels and alteration of NO production in kidneys after LPS treatment (13). Indeed, Ang II mediates tissue injury, since it may act through two types of receptors, AT_1R and AT_2R, which are differentially expressed in endotoxemia. LPS increases AT_1R expression (39) and, according to our data, Ang II is mainly located in renal tubules. Taken together, it suggests that Ang II triggers deleterious effects in endotoxemic kidneys, like vasoconstriction, endothelial damage, cellular growth and fibrosis. Besides, it was demonstrated that RAS blockage attenuates renal damage induced by LPS (18, 25), supporting this idea.

Vascular effects of Ang II are also related to NAD(P)H oxidase production and ROS generation. It is not completely clear how Ang II acts, but it is known that there are two phases of ROS production: one is early and transient and the other is late and sustained. The first phase is due to NAD(P)H acute activation by Ang II and the second phase depends on this early event, being a consequence from the augmented expression of different NAD(P)H subunits (12, 23). Unaltered ROS levels may be related to our three-day treatment, which could not have been enough to reach the late phase of ROS production in kidneys. This might be a compensatory mechanism as an attempt for reducing the deleterious effects of endotoxemia.

After quantifying angiotensin peptides, we evaluated other components of the system. Endotoxemia induced reduction of renin activity without effects over protein expression. Previous works showed results similar to ours, when human mesangial cells are exposed to LPS; renin activity reduces without alteration of its protein expression or AGT's (1). The mechanism whereby LPS modulates renin activity remains unknown; however LPS activates several biochemical pathways and mesangial cells express the required molecular machinery for LPS binding. Thus, the activation of signaling pathways or the production of some factors by LPS stimulus might influence renin activity. Another possibility is that LPS could affect renin directly, by inhibiting the enzyme itself or by modulating its co-factors.

Regarding ACE, reduction of protein expression and enzyme activity suggest a physiological attempt of regulating renal function, since ACE controls renal vascular tonus by producing the vasoconstrictor Ang II and degrading the vasodilator bradykinin (11). The modulation of ACE could have resulted from the action of factors and peptides released during endoxemia, since changes in microenvironment influence several biological processes. The shedding mechanism is also capable of regulating ACE biological activity and LPS induces the enzyme shedding in human umbilical vein endothelial cells, according to English *et al* (11). Previous works from our group identified ACE shedding in several cells types, like mesangial cells, proximal tubules cells and collecting duct cells (7, 28). Thus, in our model, endotoxemia could have induced ACE shedding, modulating its expression and activity.

Another possible regulator of Ang II level is ACE2 and, since LPS increased its activity, higher amount of Ang II could have been degraded. It is expected that augmented degradation of Ang II by ACE2 cleavage would cause growth of Ang (1-7) production, however, our data shows no statistic difference on Ang (1-7) levels between control and endotoxemia groups. This situation suggests that ACE2 is mainly acting on other peptides, for instance, Ang I. Besides the maintenance of ACE2 protein expression levels, factors released locally may influence enzyme activities directly. No changes in protein expression between groups with increase in activity of one of them were detected. It is important to note that this can occur considering that proteins are flexible and rapidly fluctuating molecules whose structural mobility have functional significance and can stimulate the activity. Also we can emphasize that an enzyme's substrate-binding affinity may vary with the binding of small molecule effectors thereby changing the enzyme's catalytic activity activating or inhibiting it physiologically. Increased ACE2 activity may represent an attempt of controlling tissue damage induced by augmented Ang II, playing a protective role in kidneys (9, 14).

We found that cathepsin G and chymase activities increased significantly in the endotoxemia group suggesting a parallel pathway for non-renin-dependent Ang II formation.

Taken together, our data suggest that alternative pathways for Ang II production are activated in LPS-treated animals, due to increased levels of Ang II not accompanied by changes of Ang I levels or ACE activity. For instance, cathepsin G generate Ang II directly from Ang I or AGT. Chymase is another example, since it converts Ang I to Ang II and has been described in several tissues. Still, during endotoxemia, cathepsin G stands out, because it is highly expressed by monocytes and neutrophils, becoming important for inflammatory response regulation. Thus, that enzyme may also contribute to local Ang II production, modulating blood flow and cellular aggregation. Furthermore, cathepsin G generates Ang II in the same magnitude as ACE does and its emergence in kidneys is strongly related to inflammatory processes (30).

In the present study the effects of systemic LPS administration over renal RAS were studied. It is important to highlight that a long-term LPS

565 treatment was used, in comparison to short-term, used by most works. We
566 found some unique results, like diminished NO levels in plasma despite
567 reduced blood pressure. Since our animal model reproduces mainly the renal
568 effects of sepsis and RAS regulates renal function, we tried to elucidate the
569 effects of endotoxemia specifically over renal RAS.
570 In conclusion, our treatment was capable of influencing renal RAS and
571 activating some compensatory mechanisms, as an attempt of minimizing
572 LPS-induced damage. Reduced renin and ACE renal activities indicate that
573 alternative pathways, through the cathepsin G and chymase activities are
574 important for Ang II production and the increased ACE2 renal activity
575 suggests that its degradation is compromised in our endotoxemia model.
576 Thus, the high Ang II levels found in renal tissue may be related to local
577 vasoconstriction and tissue damage, both complications triggered by LPS.
578

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Conflict of interests' statement

None declared.

Figure 1. Analysis of Blood Pressure, Body Mass and Creatinine Clearance. Endotoxemic and control groups' physiological parameters were analyzed and compared as described in methods. *Tail cuff blood pressure (**A**)* and *body mass* (***B***) remained unchanged between groups three days before and on the day of LPS treatment (day -3 and 0, respectively); both parameters were reduced and three days after LPS injection (day 3) LPS. *Creatinine clearance (**C***) was calculated according to the dosages performed in urine collected from the second to the third day after LPS injection and in serum collected during euthanasia, no statistical difference was found between groups. (*n=6)* X±SEM, Endotoxemia vs. Control *p≤0.05.

Figure 2. Quantification of NO and Cytokines in plasma. *Pro-inflammatory Cytokines* (IL-1β, IL-6 and TNF-α) (***A***) and *NO* (***B***) levels were analyzed and compared between endotoxemia and control groups, as described in methods. Three days after LPS treatment plasmatic NO levels were importantly reduced in endotoxemic animals, but no effects were observed over plasma cytokines levels. (*n=6*) X±SEM, Endotoxemia vs. Control **p≤0.01.

Figure 3. Quantification of Angiotensin Peptides, ROS, NO and iNOS in renal tissue. *Angiotensin peptides* [Endotoxemia (*n=10)* vs Control (*n=6)* X±SEM *p<0.05] *(**A***), *ROS (**B***) and *NO (**C***) levels were quantified in renal tissue and compared between endotoxemic and control animals as described in methods. *iNOS (**D***) *WB* presented a single band with 130 kDa for both groups. Three days after LPS treatment, Ang II level was increased and iNOS protein expression was reduced in renal tissue of endotoxemic group (*n=6,* X±SEM, Endotoxemia vs. Control *p≤0.05).

Figure 4. Immunohistochemistry for Angiotensins II and 1-7 in renal tissue. Ang II (***A***) and Ang (1-7) (***B***) are mainly observed in the cytosol of tubular cells in both renal cortex and medulla, but only Ang II was augmented in LPS-treated animals.

Figure 5. Protein expression by WB in renal tissue. *AGT (**A**)* presented a single band with 74 kDa. *Renin (**B**)* presented a single band with 39 kDa. ACE (***C***) presented two bands with 120 kDa and 65 kDa. ACE2 (***D***) presented a single band with 68 kDa. Among the RAS components represented in this figure, only the 120 kDa-ACE was down regulated in renal tissue three days after LPS injection, with no effects over protein expression from the other RAS components. (*n=6*) X±SEM, Endotoxemia vs. Control *p≤0.05.

Figure 6. Enzymatic activities in renal tissue. *Renin (**A**)*, *ACE (**B**), ACE2 (**C**), Cathepsin G (D), Chymase (E)* activities were analyzed and compared as described in methods. Both renin and ACE presented reduced activities and ACE2, cathepsin and chymase showed increased activities in renal tissue of endotoxemic group three days after LPS treatment. (*n=3*) X±SEM, Endotoxemia vs. Control *p≤0.05, **p≤0.01 and ***p≤0.0001.

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Figure 1. Analysis of blood Pressure, Body Mass and Creatinine Clearance

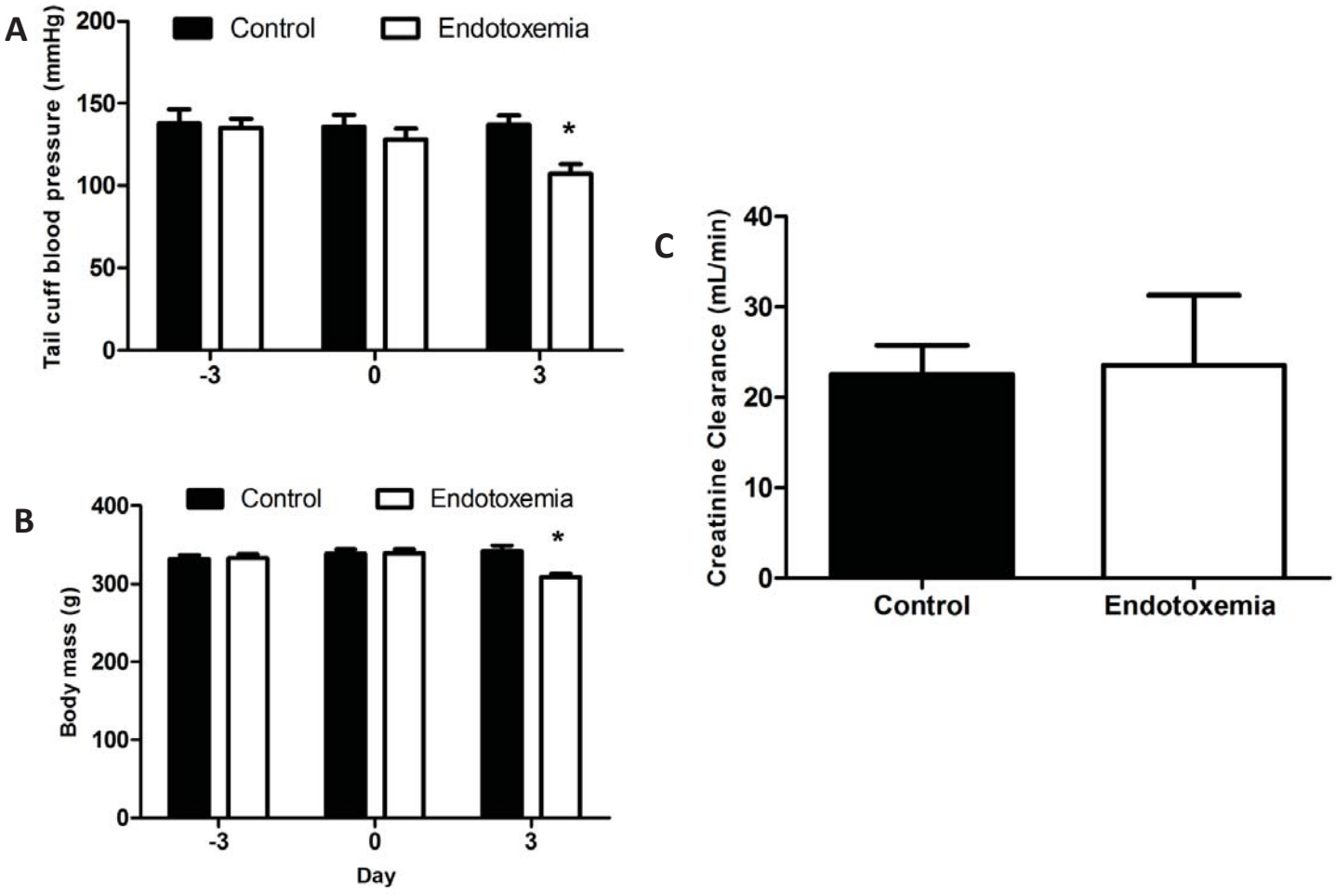


Figure 2. Quantification of NO and Cytokines in plasma

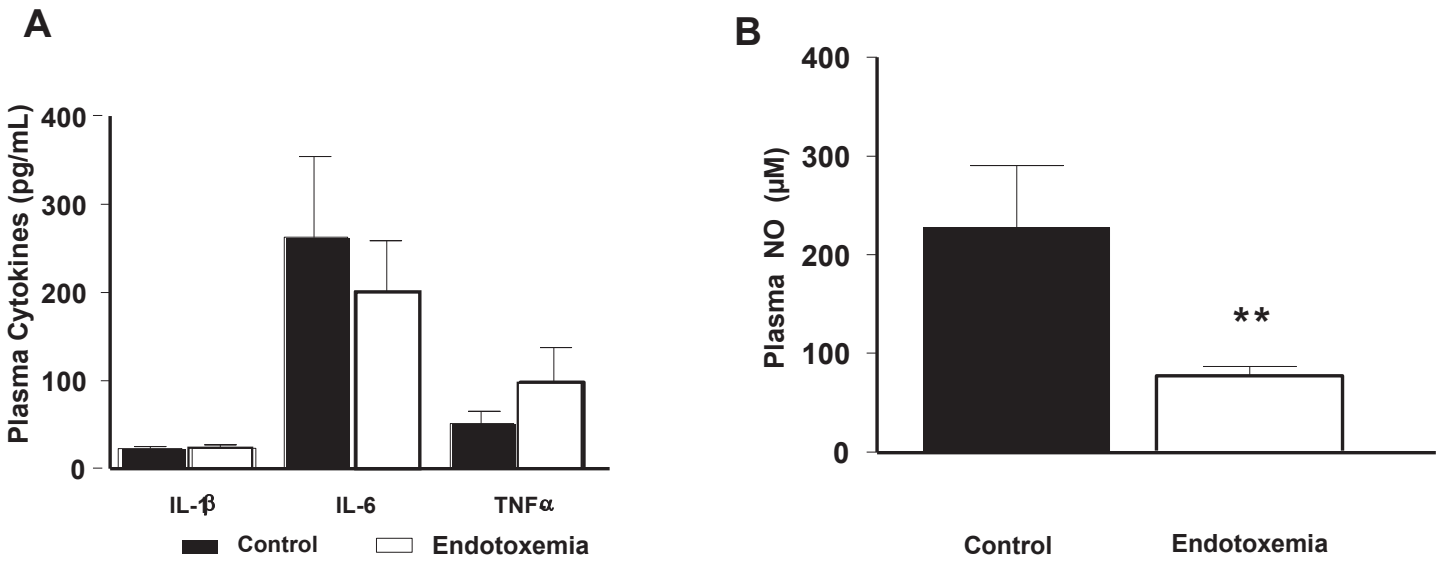
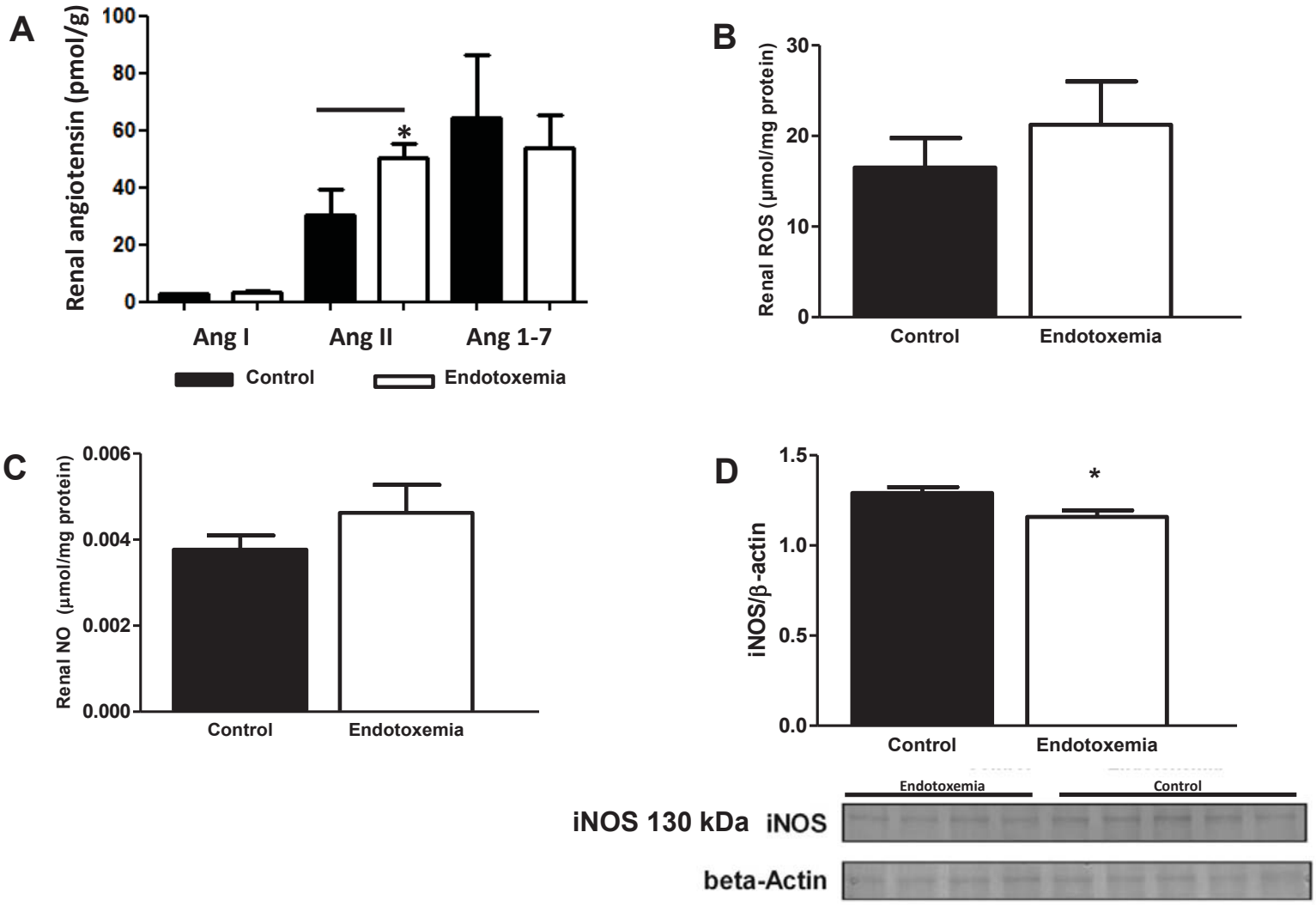


Figure 3. Quantification of Angiotensin peptides, ROS, NO and iNOS in renal tissue



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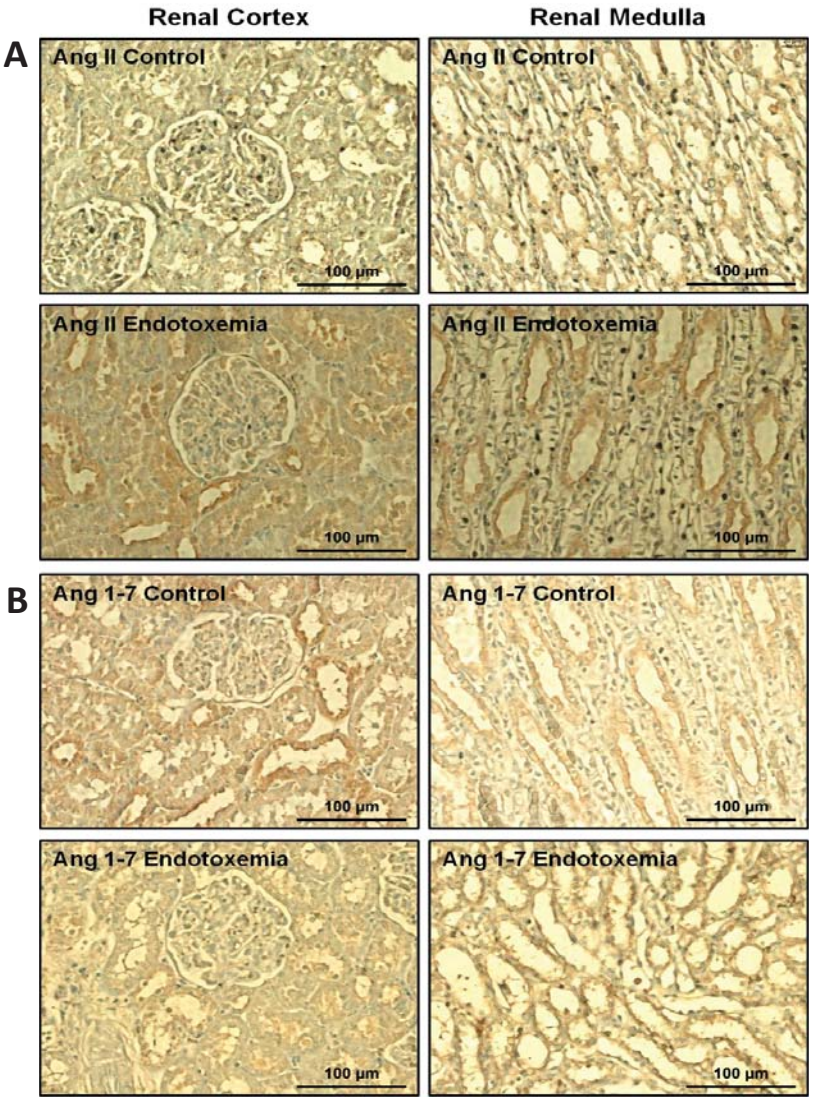

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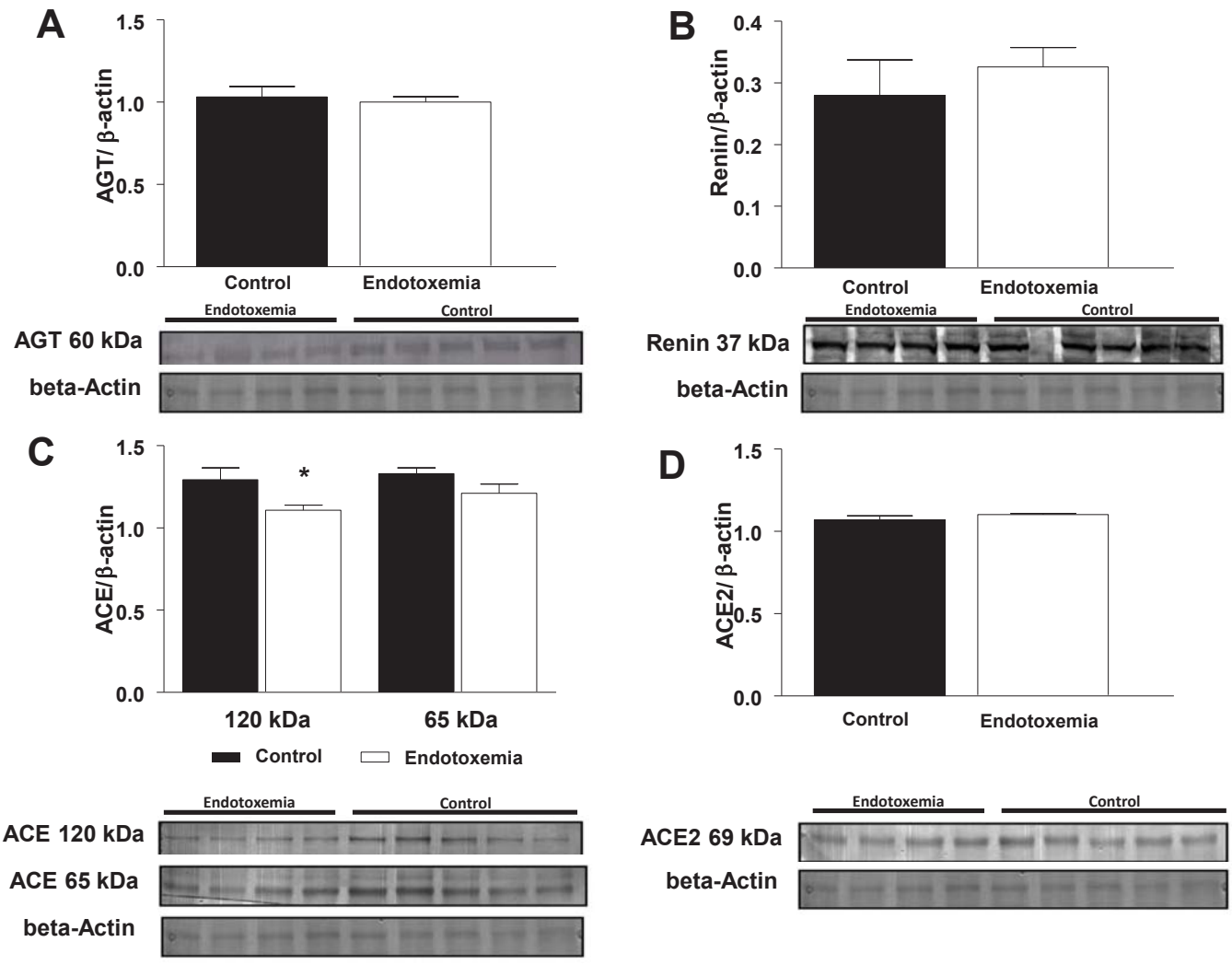

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