

# Increased Klk9 Urinary Excretion Is Associated to Hypertension-Induced Cardiovascular Damage and Renal Alterations

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**Abstract:** Early detection of hypertensive end-organ damage and secondary diseases are key determinants of cardiovascular prognosis in patients suffering from arterial hypertension. Presently, there are no biomarkers for the detection of hypertensive target organ damage, most outstandingly including blood vessels, the heart, and the kidneys.

We aimed to validate the usefulness of the urinary excretion of the serine protease kallikrein-related peptidase 9 (KLK9) as a biomarker of hypertension-induced target organ damage.

Urinary, plasma, and renal tissue levels of KLK9 were measured by the Western blot in different rat models of hypertension, including angiotensin-II infusion, DOCA-salt, L-NAME administration, and spontaneous hypertension. Urinary levels were associated to cardiovascular and renal injury, assessed by histopathology. The origin of urinary KLK9 was investigated through in situ renal perfusion experiments.

The urinary excretion of KLK9 is increased in different experimental models of hypertension in rats. The ACE inhibitor trandolapril significantly reduced arterial pressure and the urinary level of KLK9. Hypertension did not increase kidney, heart, liver, lung, or plasma KLK9 levels. Hypertension-induced increased urinary excretion of KLK9 results from specific alterations in its tubular reabsorption, even in the absence of overt nephropathy. KLK9 urinary excretion strongly correlates with cardiac hypertrophy and aortic wall thickening.

KLK9 appears in the urine in the presence of hypertension as a result of subtle renal handling alterations. Urinary KLK9 might be potentially used as an indicator of hypertensive cardiac and vascular damage.

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**Abbreviations:** ACE = angiotensin converting enzyme, DOCA = deoxycorticosterone acetate, KLK = human tissue kallikrein-related peptidase family, L-NAME = L-nitro-arginine methyl ester, NAG = N-acetyl-beta-D-glucosaminidase, SHR = spontaneously hypertensive rats.

## INTRODUCTION

Hypertension is the leading risk factor for morbidity and mortality throughout the world,<sup>1</sup> and it is likely the single most important modifiable risk factor for cardiovascular disease.<sup>2,3</sup> Hypertension produces functional and structural alterations in the heart and blood vessels. Local vascular damage is responsible for secondary alterations in specific target organs, such as the brain, the kidneys, and the retina.<sup>4-6</sup> Early detection of hypertensive end-organ damage is a key determinant of cardiovascular prognosis in hypertensive patients.<sup>7</sup> Early detection facilitates damage prevention, slowing and even regression with adequate therapy where organ damage is still at a reversible stage. At present, there are no single and inexpensive biomarkers for the detection of hypertension-induced vascular and organ damage (heart, kidney, retina). Thus, biomarkers of end-organ damage measurable in easily obtaining samples such as urine will be of great usefulness in cardiovascular risk assessment of hypertensive patients.

The human tissue kallikrein-related peptidase family (KLK) consists of 15 highly conserved serine proteases, which are encoded by the largest uninterrupted cluster of protease genes in the human genome.<sup>8</sup> Several members of the KLK family have been related with hypertension. Recently, Jiang et al<sup>9</sup> showed that a polymorphism in the KLK1 gene may be involved in the development of essential hypertension. Plasma KLK3 gene polymorphisms have been associated to end-stage renal failure.<sup>10</sup> But to date there are no studies linking urinary excretion of other KLK family members with the presence of hypertension and associated target organ damage.

Recently, our research group has focused attention on understanding whether changes in the urinary excretion of these most unknown KLKs are related to hypertension, diabetes, and chronic renal disease. Our pilot experiments showed that the urinary excretion of the serine protease kallikrein-related peptidase 9 (KLK9) is increased in an experimental model of hypertension in rats. Therefore, we further investigated KLK9 in different models of genetic and induced hypertension,

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the mechanism of its increased urinary excretion, and its potential relation with target organ damage.

## METHODS

All reagents were purchased from Sigma (Madrid, Spain), except where indicated otherwise.

### Animals and Experimental Protocol

Male Wistar and spontaneously hypertensive (SHR) rats (225–250 g body weight, 2 months old) were kept in an aseptic atmosphere with controlled temperature and light cycles in the facilities of the Animal Experimentation Service of the University of Salamanca, with free access to food and water. Wistar rats were provided by the Animal Experimentation Service of the University of Salamanca (Salamanca, Spain). SHR rats were obtained from Charles River (Barcelona, Spain). Rats were assigned to the following experimental groups:

- (1) Wistar rats followed during 4 months,  $n = 10$
- (2) SHR rats analyzed during 4 months,  $n = 20$
- (3) Wistar rats treated with angiotensin II (288 mg/kg body weight/day in micro osmotic pumps) during 14 days,  $n = 7$
- (4) Wistar rats treated with deoxycorticosterone acetate (DOCA; 53 mg/kg body weight/week intramuscularly) during 7 weeks with free access to water containing 0.01 NaCl,  $n = 9$
- (5) SHR rats treated with hydralazine (20 mg/kg body weight/day in drinking water) during 6 weeks,  $n = 11$
- (6) SHR rats treated with trandolapril (0.7 mg/kg body weight/day in drinking water; Knoll AG, Ludwigshafen, Germany), during 6 weeks,  $n = 11$
- (7) Wistar rats treated with *L*-nitro-arginine methyl ester (*L*-NAME; 40 mg/kg/day in drinking water) during 3, 6, and 9 weeks,  $n = 16$

Systolic blood pressure was monitored in conscious animals by the tail-cuff method (Cibertec, Madrid, Spain). Rats were allocated in individual metabolic cages for 24-h urine sample collection. Urine was cleared by centrifugation, and it was stored at  $-80^{\circ}\text{C}$  until use.

Blood samples were obtained in heparinized capillaries at different time points by a small incision in the tail tip. Blood was centrifuged and plasma was kept at  $-80^{\circ}\text{C}$  until use. At the end of the experimental treatments, rats were anesthetized and perfused through the aorta with saline solution (0.009 NaCl) with 0.001 heparin. Immediately, the kidneys, heart, liver, and lungs were dissected. Hearts and kidneys were weighted. One half from each organ was frozen in liquid nitrogen and subsequently kept at  $-80^{\circ}\text{C}$  for protein and mRNA extraction. The other half was fixed in buffered 0.037 *p*-formaldehyde for histological studies.

These studies were approved by the Bioethics Committee of the University of Salamanca, and animals were treated following the Recommendations of the Helsinki Declaration on the Advice on Care and Use of Animals referred to in: law 14/2007 (3 July) on Biomedical Research, Conseil de l'Europe (published in Official Daily N. L358/1-358/6, 12.18.1986), Spanish Government (Royal Decree 223/1988, (14 March) and Order of 10.13.1989, and Official Bulletin of the State b. 256, pp. 31349–31362, 10.28.1990).

### Characterization of Renal Function

Plasma and urinary creatinine were measured with the automatic analyzer Reflotron (Roche Diagnostics, Barcelona,

Spain; lower detection limit of 0.028 mmol/L). Urine protein concentration was measured by the Bradford method.<sup>11</sup> Urine *N*-acetyl-beta-D-glucosaminidase (NAG) content was determined by a colorimetric method with a commercial kit (Roche Diagnostics, Basel, Switzerland) based on the conversion of 3-cresolsulfonylphthalate-*N*-acetyl- $\beta$ -D-glucosaminide into the purple 3-cresol-cresolsulfonylphthalate. Albuminuria was measured with a commercial ELISA following the manufacturer's instructions (Bethyl Laboratories, Montgomery, TX).

### Osmotic Pump Implantation and Angiotensin II Administration

Wistar normotensive rats were anesthetized with inhalational isoflurane (Isoba, Schering-Plough S.A., Madrid, Spain) to facilitate the subcutaneous implantation—on the left side of the back—of osmotic pumps (Alzet model 1002, Durect Corporation, Cupertino CA) containing angiotensin II in 0.01 N acetic acid at a concentration sufficient to yield an infusion rate of 288 mg/kg/day during 14 days, according to the manufacturer's instructions.

### Urinary Excretion Studies With *in Situ* Perfused Kidneys

SHR rats were anesthetized and an extracorporeal circuit for kidney perfusion was set up, as described elsewhere,<sup>12</sup> with some modifications. Briefly, the renal artery, vein, and ureter of the right kidney were ligated. The renal artery and vein of the left kidney and the urinary bladder were cauterized. Oxygenated and warm ( $37^{\circ}\text{C}$ ) Krebs–dextran solution [40 g/L of dextran (molecular weight 64–76 k) in the Krebs solution (118.3 mM NaCl, 4.7 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ , 0.026 mM EDTA, 11.1 glucose,  $\text{pH} = 7.4$ )] was perfused through the renal artery at 3 mL/min and was discarded through the renal vein. Urine fractions were collected from a catheter placed in the urinary bladder, starting before the perfusion with Krebs (when blood was still passing through the kidney), and during 2 h after perfusion with Krebs-dextran started. All urine samples were kept at  $-80^{\circ}\text{C}$  until assayed by Western blot for the presence of KLK9.

### Urinary Excretion Studies After Megalin Inhibition

In another set of experiments, Wistar rats were anesthetized and after a single injection of sodium maleate (400 mg/kg) through the jugular vein, urine fractions were collected from the bladder every 10 min, for up to 1 h.<sup>12</sup>

### Gene Expression Analysis

Total RNA was isolated as described previously.<sup>13</sup> Reverse transcriptase-PCR-amplification of rat KLK9 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed with the following primers: for KLK9 5'-GACCACTGGTGTG-CAAAGG-3' and 5'-CGGTGTTTTCAATCCAGTCC-3' (conditions:  $1 \times (95^{\circ}\text{C} \times 5')$ ,  $30 \times (95^{\circ}\text{C} \times 1' + 55.9^{\circ}\text{C} \times 1' + 72^{\circ}\text{C} \times 1')$ ,  $1 \times (72^{\circ}\text{C} \times 5')$ ; for GAPDH, 5'-AACTCCCTCAA-GATTGTCAGCAA-3' and 5'-GTGGTCATGAGCCCTTCCA-3' (conditions:  $1 \times (95^{\circ}\text{C} \times 5')$ ,  $30 \times (95^{\circ}\text{C} \times 1' + 61^{\circ}\text{C} \times 1' + 72^{\circ}\text{C} \times 1')$ ,  $1 \times (72^{\circ}\text{C} \times 5')$ ). After the amplification, 1 aliquot from each PCR sample was separated by agarose electrophoresis, and visualized and photographed with a transilluminator (Bio-Rad, Berkeley, CA) under ultraviolet illumination.

## Western Blot Analysis

Urinary, plasma, and tissue KLK9 expression was determined by Western blot. Tissue proteins were extracted by homogenization in the lysis buffer. [25 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mmol/l NaCl, 0.01 Igepal CA-630, 10 mmol/l MgCl<sub>2</sub>, 1 mmol/l EDTA, 0.02 v/v glycerol, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 5 mmol/l phenylmethylsulfonyl fluoride, 5 mM NaF, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, pH = 7.5]. Protein content of the extracts was determined by the Lowry assay (Dc protein assay, BioRad, Hercules CA). Plasma samples were dealbuminized with a column-based, commercial kit based on the immunological retention of rat albumin (Qproteome Murine Albumin Depletion Kit, Quiagen, Venlo, The Netherlands). 100 µg protein from each kidney extract, 40 µl from each albumin-free plasma sample, or a fraction of urine representing the same daily urine output from each sample were used for Western blot assays. Samples were separated by electrophoresis in 0.1–0.15 acrylamide gels (Mini Protean II system, BioRad, Berkeley, CA). Western blot assays were performed as previously described.<sup>12</sup> Membranes were probed with anti-KLK 9 antibody (dilution 1/1000, Santa Cruz Biotechnologies, Santa Cruz, CA), anti-KLK 14 antibody (dilution 1/1000, Santa Cruz Biotechnologies), and anti-KLK 3 (dilution 1/1000, Millipore, Billerica, MA). Secondary antibodies were donkey anti-goat IgG-horseradish peroxidase (HRP) conjugated antibody (dilution 1/10,000, Santa Cruz Biotechnologies) and goat-anti-rabbit-HRP conjugated from BioRad.

## Histological Studies

5-µm tissue sections from buffered formalin fixed, paraffin-embedded tissues were stained with hematoxylin and eosin. Photographs were taken under an Olympus BX51 microscope connected to an Olympus DP70 color, digital camera (Olympus, Hicksville, NY).

## Cardiac Hypertrophy and Wall-to-Lumen Ratio Quantification

Cardiac hypertrophy was evaluated after by measuring the heart weight/body weight ratio. The enlargement of the aortic wall was analyzed by measuring the wall-to-lumen ratio with the Axiovision software (Carl Zeiss, Jena, Germany). Radium was obtained from the inner perimeter (radius = perimeter/2π). The wall-to-lumen ratio was calculated by wall thickness divided by internal (lumen) diameter, as previously described.<sup>14</sup>

## Immunofluorescence

Immunohistofluorescence was performed on buffered formalin fixed, paraffin-embedded tissues, which were cut into 5-mm-thick slices. Before immunofluorescence staining, samples were deparaffinized in xylene and rehydrated in graded ethanol and distilled water. After these previous steps, samples were permeabilized at 100°C and 1.5 bars for 3 min. Blocking (non-fatted milk 0.02, Triton X-100 0.001 in PBS) was carried out for 1 h. Samples were incubated with anti-KLK9 antibody (dilution 1/100) for 1 h and, after a washing step, they were incubated with a secondary antibody Alexa Fluor 488 anti-goat (Molecular Probes, Eugene, OR) (dilution 1/1000). Nuclei were stained with 2 mM Hoechst 33258 (Molecular Probes) for 5 min. Cover slips were mounted on slides using Prolong gold antifade (Molecular Probes). Confocal images were taken using a Zeiss Axiovert 200M microscope and a Zeiss LSM 510 confocal

module (Zeiss, Oberkochen, Germany), with a HeNe laser with 543-excitation for rhodamine and Hg laser with 365-excitation for DAPI. All images were obtained with identical parameters for intensity, pinhole aperture, and so on.

## Statistical Analysis

Data are represented as the mean ± standard error of *n* experiments performed, as indicated in each case. The chi-square test was used to analyze associations between qualitative variables. The Student *t* test for independent samples was used to compare quantitative variables for 2 groups, and 1-way analysis of variance (ANOVA) was used for >2 groups, once the normality of the data was assessed (by kurtosis normality of residuals test), followed by the post hoc Tukey's test for multi-group comparisons. The Pearson correlation test was used to analyze associations between quantitative variables. A *P* ≤ 0.05 was considered statistically significant.

## RESULTS

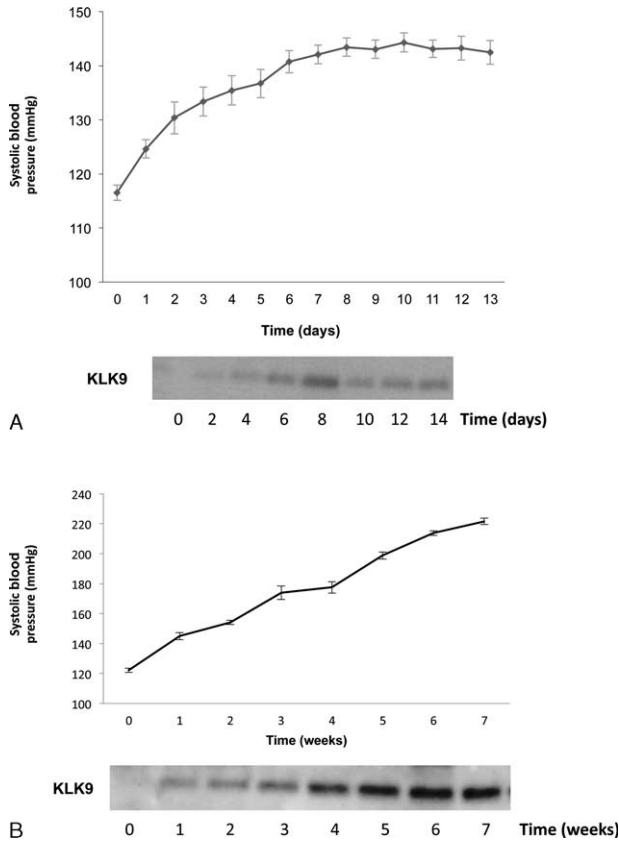
### KLK9 Urinary Excretion Is Increased in Angiotensin II Infusion-Induced Hypertension and in DOCA-Salt Hypertensive Rats

We induced hypertension in rats by the infusion of angiotensin II (288 mg/kg/day) through subcutaneously implanted osmotic pumps. This experimental maneuver promoted an increase in blood pressure levels of ~20 mm Hg, which is stabilized approximately after 5 to 6 days of angiotensin II treatment (Fig. 1A). After analyzing by Western blot the expression of KLK9 in urine, we observed that this protein is also excreted in urine 5 to 6 days after the start of angiotensin II infusion.

In order to study whether the increase in urinary KLK9 was due to hypertension or to exposure to angiotensin, we analyzed KLK9 urinary excretion in an angiotensin II-independent model of hypertension, the DOCA salt model.<sup>15</sup> The DOCA-salt treatment (53 mg/kg body weight/week) promoted a significant increase in blood pressure levels from the first week (Fig. 1B), which grew progressively during the 7-week experimental period. We observed that after 1 week of hypertension, KLK9 appeared in urine, and that its urinary excretion remained high until the end of the experimental period (Fig. 1B).

### KLK9 Urinary Excretion Is Increased in SHR

In order to discard that the increased urinary excretion of KLK9 was due to experimental artifacts, we analyzed its presence in urine in SHR. Western blot analysis of urine samples from SHR (obtained 4 months after the onset of hypertension) and Wistar normotensive controls showed a large urinary excretion of KLK9 in hypertensive animals that was almost undetectable in Wistar rats (Fig. 2A). We also analyzed the urinary excretion of other KLKs. Figure 2B shows that, unlike KLK9, the urinary excretion of KLK3 and KLK14 is greater in Wistar than in SHR rats. Systolic blood pressure was high in SHR throughout the 3 months of the study (Fig. 2C), and this increase was reduced by antihypertensive drugs, especially by the angiotensin converting enzyme (ACE) inhibitor trandolapril (Fig. 2D). Moreover, urinary excretion of KLK9 was significantly reduced after 3 weeks of trandolapril treatment (Fig. 2E). Serum creatinine, urinary albumin, and total protein excretion were similar in SHR and Wistar rats in the period studied (data not shown).



**FIGURE 1.** Effect of angiotensin II infusion during 13 days (A) and effect of DOCA-salt treatment during 7 weeks (B) on the time course of systolic blood pressure and KLK9 urinary excretion. Figure shows representative images of 3 independent Western blots. Data in graphs are expressed as mean  $\pm$  SEM of 7 rats (angiotensin II) and 9 rats (DOCA-salt). DOC-A = deoxycorticosterone acetate, KLK9 = kallikrein-related peptidase 9, SEM = standard error of the mean.

### Plasma is the Source of Urinary KLK9

We analyzed the potential sources of KLK9 by studying its level in plasma and several tissues by PCR and Western blot. The KLK9 mRNA expression was similar in kidney and heart from SHR and Wistar rats (Fig. 3A). Similarly, KLK9 protein abundance was similar in kidney, heart, and liver from SHR and Wistar rats (Fig. 3B). KLK9 immunofluorescence staining was similar in kidneys of SHR and Wistar rats (Fig. 3C). There are no differences in KLK9 plasma levels between SHR and Wistar rats (Fig. 3B). In order to demonstrate whether the blood or the renal parenchyma was the source of urinary KLK9, we performed in SHR an “in situ” renal perfusion substituting the blood with Krebs buffered solution (free of proteins but with a high-molecular-weight dextran to compensate for the oncotic pressure of the blood) at 37°C for several hours. Figure 3D shows that the urinary excretion of KLK9 disappears when blood is substituted with Krebs, suggesting that urinary KLK9 comes from the blood and not from the renal tissue. To assess whether KLK9 is reabsorbed by the megalin transport system in the proximal tubule, we injected maleate—which sheds megalin from the renal brush-border membrane<sup>16</sup>—in the blood through the jugular vein. After 10 min of maleate administration, KLK9

(as well as KLK3 and KLK14) appears in the urine of Wistar rats, but after another 10 min all these KLKs disappear from the urine (Fig. 3E), suggesting that these KLKs are reabsorbed in the renal tubule via the megalin receptor, but in the absence of this receptor, their reabsorption is performed in a few minutes by an alternative transport system.

### Urinary KLK9 Excretion Correlates With Cardiac Hypertrophy and Aorta Wall Thickening

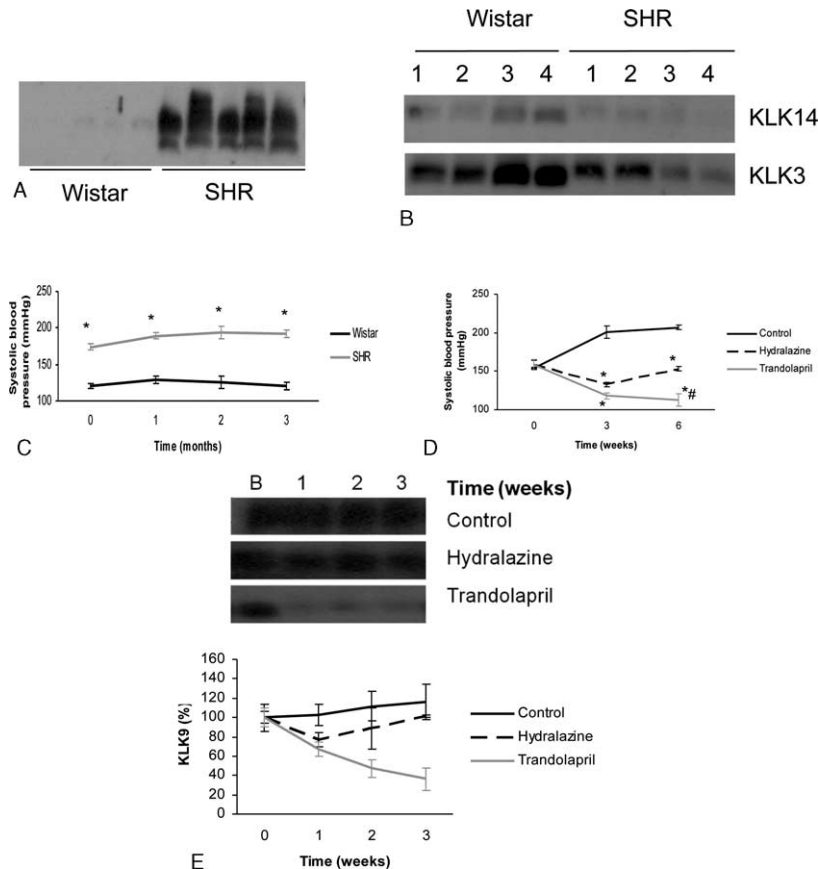
Cardiac hypertrophy was observed in SHR (Fig. 4A), which was prevented after 3 weeks of treatment with trandolapril (Fig. 4A). Urinary excretion of KLK9 strongly correlated with cardiac hypertrophy in SHR and with its reduction after antihypertensive treatment (Table 1). Treatment with the vasodilator hydralazine, and especially with trandolapril during 3 and 6 weeks, significantly prevented the thickening of the aorta wall (wall-to-lumen ratio) observed in untreated SHR rats (Fig. 4B), which was also strongly correlated with the urinary excretion of KLK9 (Table 1).

### KLK9 Urinary Excretion is Increased in L-NAME-Induced Hypertension

In order to widen the scope of hypertension models in which to test the association of hypertensive events and KLK9 excretion (to further generalize the associations or to limit them to specific scenarios), we also analyzed the urinary excretion of KLK9 in another, etiologically different experimental model of hypertension, namely Wistar rats treated with the nitric oxide synthesis inhibitor L-NAME during 9 weeks. L-NAME administration increased both arterial pressure (Fig. 5A) and KLK9 urinary excretion (Fig. 5B) with respect to untreated rats, without inducing any significant difference between the groups in urine flow, proteinuria, or NAG urinary excretion (data not shown). KLK9 appeared in the urine after 4 weeks of induced hypertension (Fig. 5B). The KLK9 protein expression was similar in cardiac and renal tissue from either L-NAME treated or normotensive rats (Fig. 5C). Cardiac hypertrophy and thickening of the aorta wall were also observed in L-NAME treated rats with respect to controls (Fig. 5D and E), and these effects also strongly correlated with urinary KLK9 level (Table 1).

### DISCUSSION

KLK9 is a member of the kallikrein-related peptidase family, which have diverse physiologic functions in many tissues.<sup>17,18</sup> KLK9 is primarily expressed in thymus, testis, spinal cord, cerebellum, trachea, mammary gland, prostate, brain, salivary gland, ovary, and skin.<sup>19</sup> Human KLK9 mRNA is differentially expressed in ovarian and breast cancer, having a prognostic value.<sup>20</sup> However, there are no studies linking KLK9 with hypertension. We show that KLK9 urinary excretion is increased in 4 different experimental models of hypertension induced in rats by AII, DOCA-salt, and L-NAME, and in SHR. In addition, urinary KLK9 excretion is reduced after treatment with the ACE inhibitor trandolapril. All these data link the urinary excretion of KLK9 with hypertension. This behavior is not characteristic of other KLKs, as it is not observed for KLK3 or KLK14. KLK9 appears in urine in the absence of renal function deterioration (without changes in serum creatinine, urinary albumin, NAG excretion, or total protein excretion). As such, KLK9 tubular handling alteration seems to be a very early event in the course of renal deterioration.



**FIGURE 2.** KLK9 level detected by Western blot in the urine from SHR and Wistar rats (A), KLK3 and KLK14 levels in the urine from SHR 4 months after the development of hypertension (B), time course of systolic blood pressure in SHR and Wistar rats (C), effect of hydralazine and trandolapril treatment on systolic blood pressure in SHR (D), and effect of hydralazine and trandolapril treatment on KLK9 urinary excretion in SHR (E). Images show representative Western blots performed with samples obtained from 4 to 5 different experimental animals (A, B), or representative blots of 4 experiments performed in similar conditions (E) (see Methods for details). Data in graphics are expressed as mean + SEM of 11 to 20 SHR rats and 6 to 10 Wistar rats. \**P* < 0.001 versus control (Wistar rats); †*P* < 0.001 versus hydralazine treatment. KLK = kallikrein-related peptidase, SEM = standard error of the mean, SHR = spontaneously hypertensive rats.

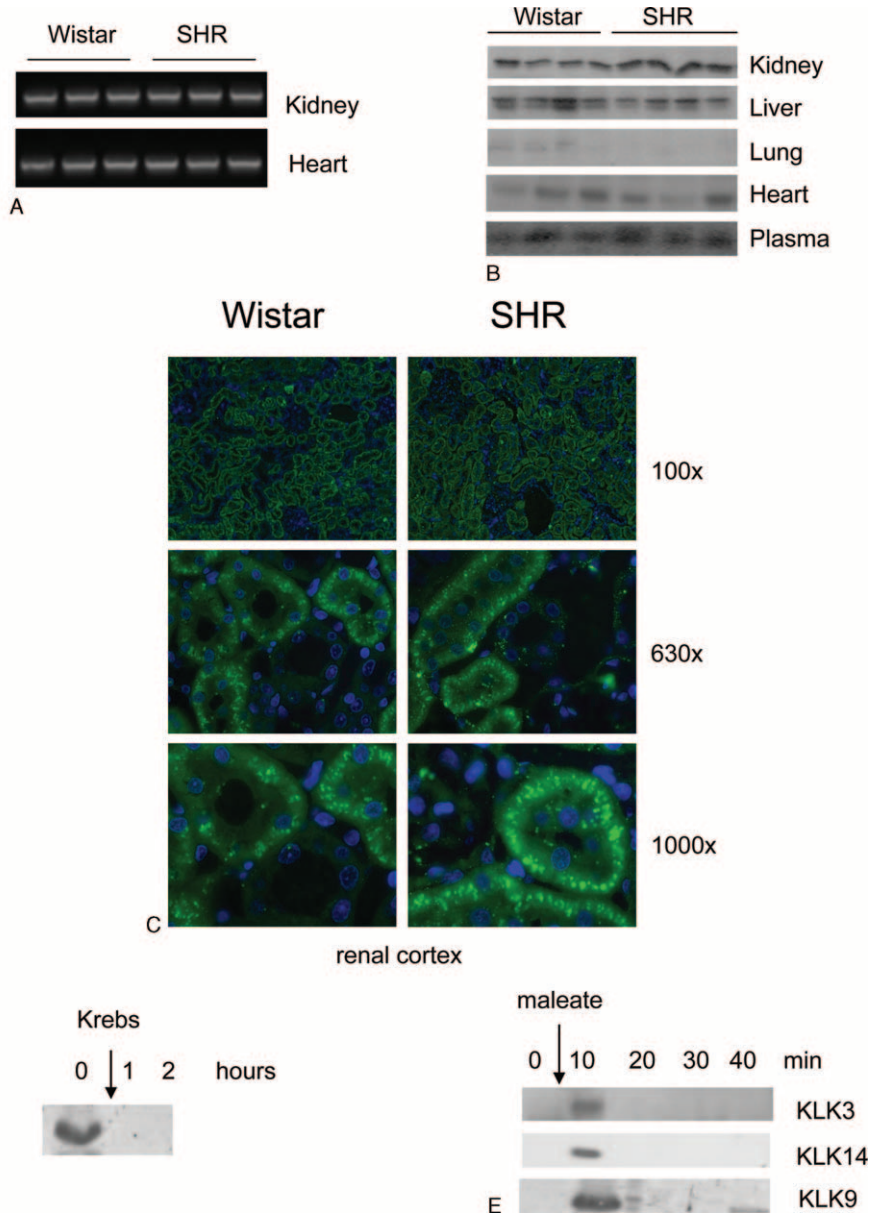
We do not have a clear explanation for the fact that the time course of increased KLK9 urinary excretion is desynchronized from that of arterial pressure increase in the L-NAME model. However, our results suggest that a subchronic influence of hypertension is needed to produce the tubular alterations leading to the specific increase in KLK9 excretion. In the L-NAME model, blood pressure rise and maintenance follow a biphasic pattern, where the first phase is largely angiotensin independent, and the second one is angiotensin dependent.<sup>21</sup> Accordingly, KLK9 urinary excretion might be regulated or modulated by undetermined humoral, hypertension-induced (or hypertension-associated) events.

Our study shows no differences in KLK9 plasma levels and in the expression of KLK9 in the kidney, liver, heart or lung, regardless of whether rats are hypertensive or normotensive. This suggests that the hypertension-induced increase in urine KLK9 may be due to changes in renal handling of the substance, whose reabsorption would be lower in the presence of hypertension. This is supported by our experiments performed on *in situ* perfused kidneys, which show that urinary KLK9 comes from the blood by filtration. In a similar context, hypertension alters the renal handling of different substances: a genetically determined defect in urinary kallikrein excretion is associated

with greater blood pressure sensitivity to salt, possibly due to altered renal sodium handling.<sup>22</sup> Renal calcium transport in the renal tubule is also modified in the presence of hypertension.<sup>23</sup> The detection of KLK9 in urine probably due to hypertension-induced changes in tubular reabsorption mechanisms, even before the development of hypertensive nephropathy, suggests that this serine protease might be an early biomarker of hypertensive nephropathy.

KLK9 is reabsorbed in the tubule by the megalin-associated transport system, a multiligand binding receptor, and transporter found in the plasma membrane of epithelial cells.<sup>24</sup> However, KLK9 also uses an alternative or redundant recapture system in the tubule. Therefore, megalin does not appear to be essential for KLK9 reabsorption in the kidney. We did not observe any other tubular alterations by the time urinary excretion of KLK9 was already increased, as even the urinary excretion of a very sensitive marker of tubular damage, NAG, is not increased. Consequently, we hypothesize that the tubular alteration induced by hypertension causing KLK9 urinary excretion might be an earliest event in hypertensive tubular injury and hypertensive nephropathy.

We also identified a strong statistical correlation between both cardiac hypertrophy and the increase in the thickness of the



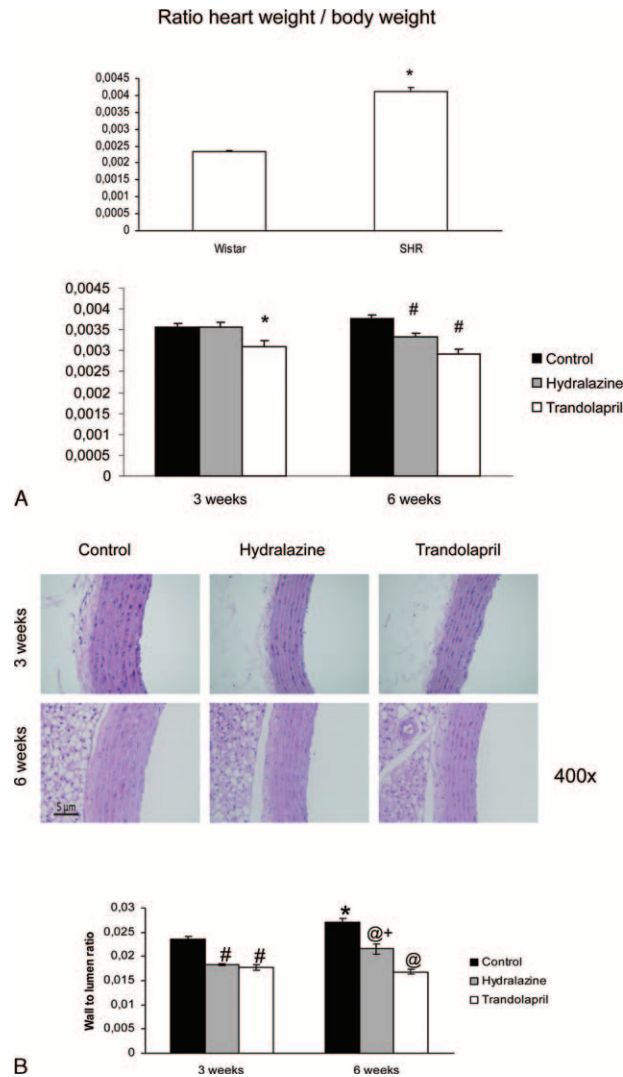
**FIGURE 3.** KLK9 mRNA expression in the kidney and heart of SHR and Wistar rats evaluated by PCR (A), Western blot analysis of KLK9 protein expression in the kidney, heart, liver, lung, and plasma from SHR and Wistar rats (B), KLK9 immunofluorescence in the renal cortex from SHR after 3 months of follow-up (C), the KLK9 level in the urine from in situ perfused kidneys in SHR (D), and after megalin inhibition in Wistar rats (E). Pictures in A–B show blots performed with samples obtained from 3 to 4 different experimental animals, and D–E show representative blots of 3 experiments performed in similar conditions (see Methods for details). KLK9 = kallikrein-related peptidase 9, PCR = polymerase chain reaction, SHR = spontaneously hypertensive rats.

aorta wall with KLK9 urinary excretion values in SHR and after L-NAME treatment in Wistar rats, a correlation that was also observed after antihypertensive treatment in SHR. Further experimental confirmation of this statistical correlation would suggest a possible use of KLK9 as an indicator of hypertension-induced cardiovascular damage. Interestingly, this correlation is not dependent on the level of blood pressure, but poses an independent maker of cardiovascular damage.

Adaptive and pathological hypertension-associated cardiovascular remodeling has long been known to have a strong blood pressure-independent, humoral component.<sup>25,26</sup>

Accordingly, we propose that undetermined hypertension-induced (or hypertension-associated) humoral alterations underlie and link both the renal effects leading to increased KLK9 urinary excretion and cardiovascular remodeling, so that urinary KLK9 could be a surrogate marker of cardiovascular organ damage.

In conclusion, our study shows that KLK9 appears in the urine in the presence of hypertension in stages before the onset of overt renal damage, due to renal handling alterations. Urinary KLK9 should be a putative indicator of target organ damage (cardiac hypertrophy, arterial wall thickening) associated with

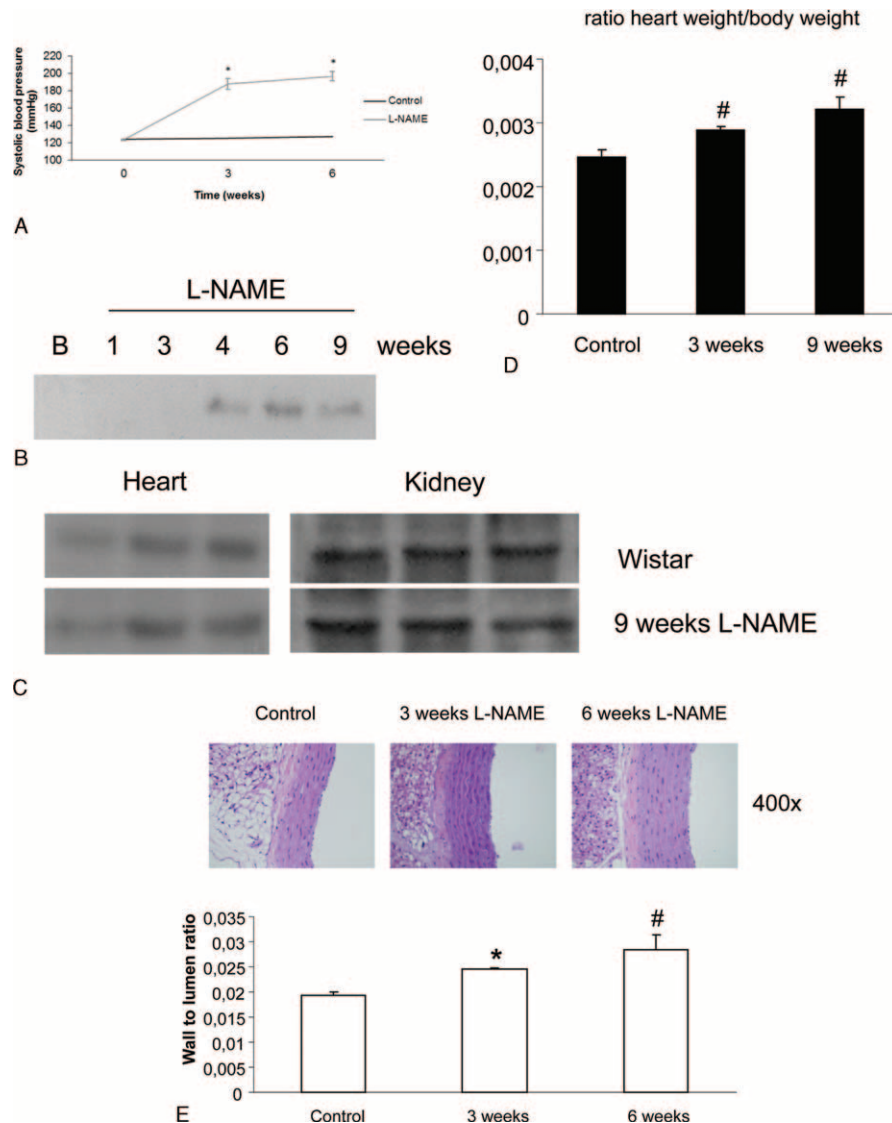


**FIGURE 4.** Changes in heart weight in SHR after 4 months and followed by antihypertensive treatment (A) and effect of hydralazine and trandolapril treatment on the thickening of the aorta wall in SHR (B). B shows representative microphotographs of hematoxylin-eosin staining (magnification: 400×). Data in graphs are expressed as mean + SEM of 4 experiments. \**P* < 0.001 versus control (Wistar rats) and control 3 weeks (A) and *P* < 0.05 versus control 3 weeks (B); †*P* < 0.001 versus control 6 weeks (A) and *P* < 0.01 versus control 3 weeks (B); ‡*P* < 0.01 versus control 6 weeks; §*P* < 0.05 versus hydralazine 3 weeks. SEM = standard error of the mean, SHR = spontaneously hypertensive rats.

**TABLE 1.** Pearson Correlations Between Urinary KLK9 and Cardiac Hypertrophy (Heart/Body Weight Ratio) and Arterial Wall Thickening (Wall to Lumen Ratio)

	SHR vs. Wistar Rats		Antihypertensive Treatment vs. SHR Control		L-NAME Treatment vs. Wistar Control	
	<i>R</i>	<i>P</i> Value	<i>R</i>	<i>P</i> Value	<i>R</i>	<i>P</i> Value
Heart/body weight	0.991	0.000	0.500	0.015	0.991	0.000
wall to lumen ratio	0.886	0.008	0.495	0.022	0.886	0.008

KLK9 = kallikrein-related peptidase 9, L-NAME = L-nitro-arginine methyl ester, *P* value < 0.05, statistically significant differences; *R* = correlation coefficient, SHR = spontaneously hypertensive rats.



**FIGURE 5.** Effect of L-NAME treatment on time course of systolic blood pressure (A), effect of L-NAME treatment on KLK9 expression in urine (B), kidney and heart (C) in Wistar rats, detected by Western blot, changes in heart weight in L-NAME-treated rats (D), and effect of L-NAME treatment on the thickening of the aorta wall (E). B–C show representative blots of 3 experiments performed in similar conditions (see Methods for details). E shows representative microphotographs of hematoxylin-eosin staining (magnification: 400 $\times$ ). Data in graphs are expressed as mean + SEM of 4 to 16 experiments. \* $P < 0.001$  versus control (Wistar rats, A) and  $P < 0.01$  versus control (E); † $P < 0.005$  (D) and  $P < 0.05$  (E) versus control. KLK = kallikrein-related peptidase 9, L-NAME = L-nitro-arginine methyl ester, SEM = standard error of the mean.

hypertension, and a very early indicator of progression to hypertensive nephropathy. Further experiments are needed to elucidate the mechanisms that modify KLK9 renal handling and the causative relation with underlying hypertension-associated factors and mechanisms.

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