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Gonadectomy Influences Blood Pressure through the Kallikrein-Kinin System

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Key Words
Blood pressure · Ovariectomy · Renal cortex · Renal kallikrein

Abstract
The kallikrein-kinin system (KKS) appears to be involved in blood pressure regulation. We showed that ovariectomy (oVx) stimulates urinary kallikrein activity (UKa). So, we test whether gonadectomy (Gx) would affect blood pressure through an increase in KKS activity and which mechanism(s) were involved. We studied adult Wistar rats of either sex, with and without Gx. At baseline all groups were normotensive although the oVx mean arterial pressure (MAP) was lower than female MAP ($p < 0.05$). KKS blockade by aprotinin increased MAP ($p < 0.05$) exclusively in the oVx group. The probably mechanism(s) involved in KKS regulation (synthesis, renal content and UKa) were also studied. Previous Gx, kallikrein content (nkat/g kidney weight) and UKa (nkat/g kidney weight/day) were higher in female than in male rats: 12 ± 1.1 versus 6 ± 0.7 and 40 ± 6.8 versus 26 ± 3.4, respectively. After Gx, kallikrein content increased significantly in both orchiectomized (oRx) and oVx rats, and UKa showed a similar tendency (NS). Kallikrein synthesis did not show gender difference in non-Gx rats, but an increase after oVx was observed. KKS was found to be involved in blood pressure regulation in oVx animals. oVx may trigger the increase in kallikrein synthesis and content and UKa to act upon blood pressure.
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Introduction

Current concepts agree with the notion that hypertension results from alterations in environmental or genetic factors that might activate vasoconstrictors while depressing the activity of vasodilator systems. This imbalance appears to increase shear stress, determining an enhancement in vascular resistance that leads to an increase in arterial blood pressure. The kallikrein-kinin system (KKS) is currently considered one of the main mechanisms controlling local hemodynamics, according to growing evidence supporting this hypothesis accumulated in the past decades [1]. It is now known that kinins induce vascular smooth muscle relaxation through the action of prostacyclin and nitric oxide (NO) [2, 3]. So, exogenous kinins cause a drop in blood pressure by reducing renal vascular resistance [1]. Such decrease in vascular resistance can be blocked by the β_2-bradykinin receptor and eNOS (endothelial NO synthase) antagonists [4–6]. The regulation of regional hemodynamics by ki-

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nins, however, might be impaired in hypertension and atherosclerosis because of a NO system alteration caused by oxidative stress [1]. In this situation, vasodilation caused by bradykinin is mediated by an alternative pathway involving endothelium-dependent hyperpolarization [7]. Acute administration of kinins may also decrease renal vascular resistance and increase diuresis and natriuresis [8]. Studying rats with low-to-high sodium intake for four weeks, a positive relation between sodium excretion and urinary kallikrein was found [9]. In addition, a significant reduction in urinary sodium excretion is induced by aprotinin in hypertensive patients receiving high sodium intake [10]. Recently, Pizard et al. [11], working in tissue kallikrein-deficient mice, demonstrated that tissue kallikrein and kinin exert cardiovascular and renal protective effects. In preliminary studies [33rd Am Soc Nephrol Annual Meeting, Toronto, Canada; JASN 11; abstract 2219, 2000], we have found a significant increase in urinary kallikrein activity (UKa) in ovariectomized (oVx) compared with sham female adult Wistar rats. Thus, we hypothesized that the increase in KKS activity induced by oVx would lower blood pressure.

Besides, it would also be worth finding out where UKa changes come from (variation in either kallikrein synthesis or release) and whether the above-mentioned phenomenon also applies to orchidectomized (oRx) rats. Another unsolved problem, besides the KKS behavior according to gender, is the role of aldosterone in this experimental setting. An enhanced aldosterone level, together with an increment in UKa after gonadectomy (Gx), was observed by us in spontaneously hypertensive rats [12]. To shed some light on this question, mean arterial blood pressure (MAP), renal function and renal kallikrein synthesis, tissue content and urinary release were measured in Wistar rats of either sex, with and without gonad removal in adulthood. A better understanding of the mechanisms involved in KKS regulation would help to elucidate the role of the factors implicated in blood pressure regulation and contribute to the devise of alternative therapeutic strategies.

Methods

Thirty-two Wistar rats of either sex were studied at 150 days of age. In 50% of these animals, Gx had been performed after puberty when 60 days old. Thus, the following 8-rat groups resulted: sham males, oRx, sham females, and oVx. Animals were fed ad libitum throughout the experiment with Cargill (Alimentos Balanceados, Buenos Aires, Argentina) and had free access to tap drinking water. They were housed at 24°C with a 12:12 h dark/light cycle. Gonad removals were performed under light anesthesia at the time indicated above. The area was cleansed with an antimicrobial agent, a bilateral flank incision or a scrotum incision was made, and gonads were removed after vessel ligation. The incision was then sutured close.

At 150 days of age, a 24-hour urine collection was conducted, housing the rats in individual metabolic cages. Thereafter, they were anesthetized with sodium pentobarbital (5 mg/100 g body weight i.p.; Dr. Gray, Buenos Aires, Argentina), and the trachea, jugular vein, carotid artery, and urinary bladder were cannulated with polyethylene tubes. Glomerular filtration rate (GFR) was determined by Inutest (Laevosan Gesellschaft m.b.H., Linz, Austria). Enough inulin to provide a plasma concentration of 0.2 mg/ml was given as a primer and a sustaining infusion diluted in saline was administered at 0.036 ml/min by a Harvard syringe pump no. 944. Blank samples of blood and urine were collected, and 45 min after equilibrium was attained, 3 thirty-minute collections were made and three blood samples taken in the middle of the urine collection period. The inulin assay was carried out by the anthrone method [13]. MAP was recorded throughout the clearance studies, with a blood pressure transducer positioned in a catheter placed in the carotid artery. After completion of the clearance study a blood sample was taken for sodium, potassium and aldosterone measurements. Then the kidneys were removed, weighed and prepared for renal cortical kallikrein (RK) and RK mRNA (KLK) measurements. The studies were made in the renal cortex because kallikrein is synthesized and added to the urine in cortical segments of the nephron. For KLK and RK determinations, the kidney cortex was separated from the medulla and divided into three parts. This procedure was made using an ice-cold plate and under RNAse inhibitiors (diethylpyrocarbonate 0.1%). Immediately after, the tissues were homogenized and kept frozen until assayed. Urinary sodium and potassium concentration, osmolality and UKa were measured in the 24-hour urine collection. Plasma and urine electrolyte and urinary osmolality were measured by flame photometry and by the freezing point methods, respectively. Aldosterone plasma levels were determined by a competitive radioimmunoassay using commercial kits provided by Immunotech (Prague, Czech Republic).

Measurement of Nucleic Acids

The cortex samples were homogenized and total RNA was isolated using the SV/Total RNA Isolation System (Promega), according to the manufacturer's instructions. The total RNA samples were stored at –70°C until assayed. A ratio of optical density at 260 and 280 nm greater than 1.8 was considered a good index of the quality of the sample. Total RNA was measured by UV spectrophotometry at 260 nm, considering that one optical density unit represents 40 μg/ml. KLK expression was estimated by semiquantitative reverse transcription polymerase chain reaction (RT-PCR). To obtain cDNA by reverse transcription, 3.3 μg of total RNA of each sample were utilized. A commercial kit (Ready-To-Go You-Prime First-Strand Beads; Amersham Biosciences) and 200-pg primers (pd (N6) Random Hexamer 5′-Phosphate; Amersham Biosciences) per reaction, and 60 min incubation at 37°C, were employed. Subsequently, a semiquantitative PCR was made to amplify kallikrein and β-actin cDNAs in each sample. For this purpose, specific primers for the rat submaxillary gland PS kallikrein mRNA sequence (genbank accession M 115563), sense 5′-GGGGGTGTCCTGATAGACC-3′ and antisense 5′-GGCAGATCGATGACCTTAC-3′, designed in our laboratory, were used. These

Table 1. Renal functional data in sham and gonadectomized groups

Group	GFR, ml/min/g kidney weight	Diuresis, ml/g kidney weight/day	Na^+ Ex, mEq/g kidney weight/day	K^+ Ex, mEq/g kidney weight/day	Osmolality mosm/kg H_2O
Male	0.99 ± 0.05	5.08 ± 0.98	0.78 ± 0.11	1.41 ± 0.195	2275 ± 173
Orchidectomized	0.79 ± 0.03	4.30 ± 0.81	0.57 ± 0.13	1.25 ± 0.22	2916 ± 653
Female	0.93 ± 0.05	4.36 ± 2.17	0.34 ± 0.15	1.33 ± 0.47	2205 ± 311
Ovariectomized	0.83 ± 0.05	6.32 ± 0.42	0.64 ± 0.03	2.25 ± 0.22	2275 ± 311

GFR = Glomerular filtration rate; Na^+ Ex = urinary sodium excretion; K^+ Ex = urinary potassium excretion. Data expressed as mean ± SEM.

primers generate a 294-bp PCR product, placed inside the sequence codifying the kallikrein common loop area. As a control of constitutive mRNA, the β-actin primer sequence described by Lomez et al. [14] was used. These primers generate a 360-bp PCR product. The PCR reaction was made with 0.3 μM of each primer, 2.4 μM $MgCl_2$ reaction buffer, and one unit Taq polymerase (Promega) through 29 amplification cycles. This number of amplification cycles was selected after studying the exponential curve at different cycles (for details and additional information, see online supplementary data, www.karger.com/doi/10.1159/000245037). PCR products were detected by electrophoresis using 3% agarose gels stained with ethidium bromide, and photographed. Densitometric analyses of the obtained bands were made with the Image J Program (Wayne Rasband, National Institute of Health, USA). The KLK/β-actin ratio of each group was used to establish differences among groups. Renal kallikrein was measured in the cortex samples homogenized with buffer Tris 0.1 M (1:9, w:v), pH 8.2, that were kept at –70°C until assayed. The homogenates were frozen and thawed three times to solubilize the membrane-bound kallikrein and then centrifuged at 50,000 *g* at 4°C for 30 min; supernatant was separated. Kallikrein activity was measured in the supernatant as well as in the 24-hour urine samples by the amidolytic method [15] using the chromogenic tripeptide substrate H-D-Val-Leu-Arg-*p*-nitroanilide (S2266; Kabi Diagnostica, Sweden) at pH 8.2. The *p*-nitroanilide release was measured at 405 nm. Kallikrein activity was expressed in katal (kat) units, 1 katal being the enzyme activity that splits 1 mol of substrate per second of incubation at 37°C. For urine kallikrein determinations, the samples were previously diluted 1:250 in distilled water. In order to explore the link between blood pressure and the KKS further, an additional set of all groups of rats was studied. The response to KKS inhibitor aprotinin (Sigma, St. Louis, Mo., USA) was analyzed, and to confirm aprotinin effect, a specific β_2-receptor antagonist, HOE–140 (Sigma), was also used. Aprotinin, 30,000 kallikrein inhibitor units (KIU) i.p. [16], and HOE-140, supplied s.c. by osmotic minipumps (ALZET; Durect corp., Cupertino, Calif., USA) at a rate of 20 μg/100 g body weight, were administered [17]. Aprotinin and HOE-140 were given during the last 3 days of the experiment, enough time to block kinin effects [18].

Statistics

Results were expressed as mean ± SEM. $p < 0.05$ was considered significant. An ANOVA followed by Tukey's post hoc test was employed for comparison among groups. The least square method for correlation and the unpaired Student t test were used when indicated. All procedures were in conformance with the rules for Care and Use of Laboratory Animals approved by a Local Ethics Committee in accordance with the National Institute of Health Guide for the care and use of laboratory animals.

Results

At 150 days of age, male mean body weight (g) was 391 ± 9.5, and Gx had no effect upon it (349 ± 17.6). Female rats weighed 247 ± 7.2, and after Gx 278 ± 8.7. Kidney weight (g) in male, oRx, female, and oVx rats at the age of 150 days was 2.6 ± 0.12, 2.2 ± 0.10, 1.8 ± 0.04 and 1.8 ± 0.06, respectively. Male kidneys were heavier than oRx, female and oVx ($p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively). Male gonadectomized kidneys were also heavier than sham female or oVx kidneys ($p < 0.05$ in both cases). Plasma sodium and potassium concentrations were not different among groups. Mean values were 138.3 ± 0.86 and 3.5 ± 0.12 mEq/l for sodium and potassium, respectively. Table 1 shows the renal functional data in the different groups studied at 150 days of age. GFR, expressed in ml/min/g kidney weight, did not show any difference among groups. Daily sodium, potassium and urine osmolality were also similar in all groups. Mean blood pressure did not change significantly after oRx (sham male rats: 110 ± 5.1 vs. oRx 120 ± 4.2 mm Hg, NS). Instead, oVx rats showed significantly lower blood pressure than sham female rats (117 ± 4.8 vs. 94 ± 5.5 mm Hg, $p < 0.01$). No statistical difference was found in aldosterone levels (pg/ml) between intact (sham male: 222 ± 46.0 and sham female: 375 ± 122.0) and gonadectomized (oRx: 157 ± 46.0 and oVx: 389 ± 122.0) animals. Female renal kallikrein content (nkat/g kidney weight) doubled male RK in sham animals ($p < 0.05$). Gx

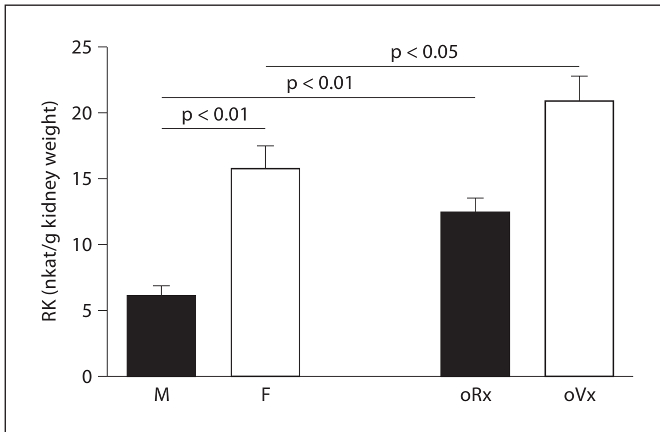

Fig. 1. RK in sham and gonadectomized rats of either sex.

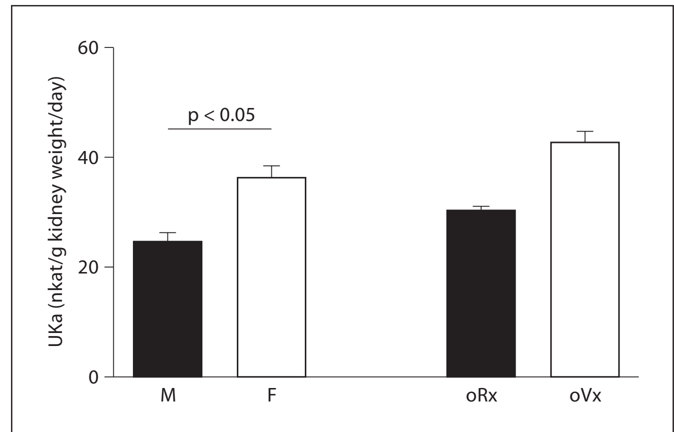

Fig. 2. UKa in sham and gonadectomized rats of either sex.

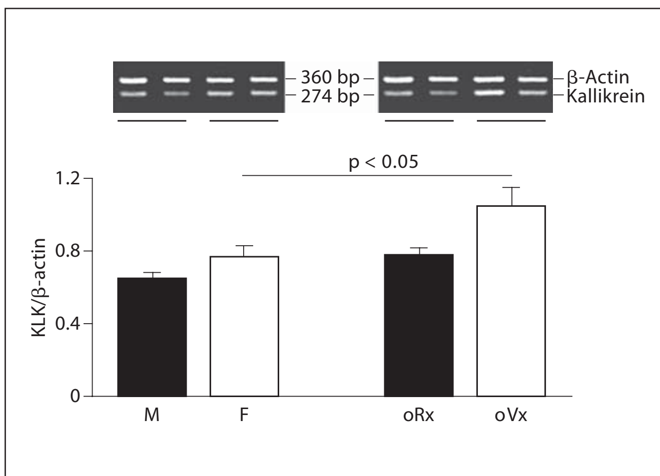

Fig. 3. KLK/β-actin in sham and gonadectomized rats of either sex. Upper panel shows kallikrein and β-actin PCR electrophoretic products and their respective size. Lower panel shows the corresponding electrophoretic densitometries in each group.

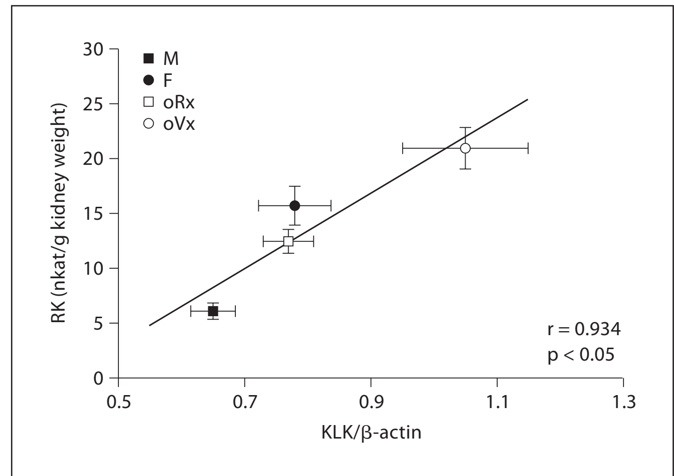

Fig. 4. Correlation between RK and KLK/β-actin. The data are mean ± SEM of each group.

increased RK in both oRx and oVx rats (male 6.1 ± 0.74 vs. oRx 12.4 ± 1.09, $p < 0.001$, and female 15.7 ± 1.74 vs. oVx 20.9 ± 1.88, fig. 1). UKa (nkat/g kidney weight/day) was also higher in female rats than in male rats ($p < 0.001$). After Gx, it showed a trend to increase (NS) in both groups. UKa was, in addition, greater in oVx rats than in oRx animals ($p < 0.05$, fig. 2). Kallikrein mRNA (expressed as KLK/β-actin) was not different between male and female rats. oRx rats did not increase kallikrein mRNA while oVx showed a higher value than female rats ($p < 0.05$, fig. 3). KLK/β-actin correlated significantly with renal kallikrein content (fig. 4; r = 0.917, $p < 0.05$), and renal kallikrein content also correlated significantly with UKa (fig. 5; r = 0.967, $p < 0.001$). After aprotinin treatment, daily UKa fell down significantly ($p < 0.001$) to 4.4 ± 1.52 nkat/g kidney weight. No effect upon baseline blood pressure was seen in sham male and female rats and oRx animals, but baseline blood pressure in oVx rats, which was the lowest of the group (94 ± 5.5 mm Hg), increased to 116 ± 6.9 mm Hg after treatment ($p < 0.05$, fig. 6). HOE-140 administration increased mean blood pressure in the same way (130 ± 2.73 mm

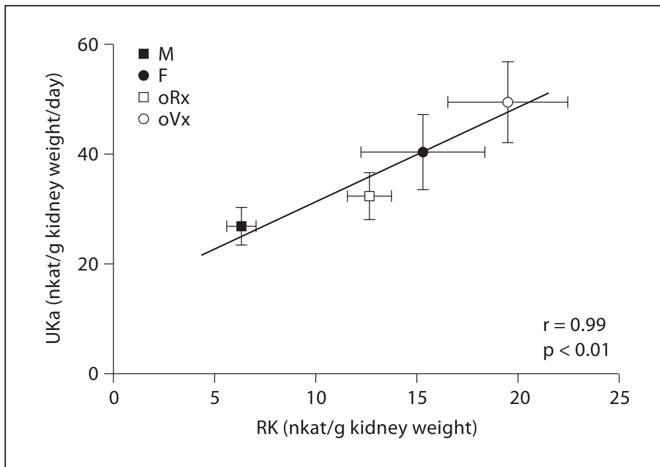

Fig. 5. Correlation between UKa and RK. Data are mean ± SEM of each group.

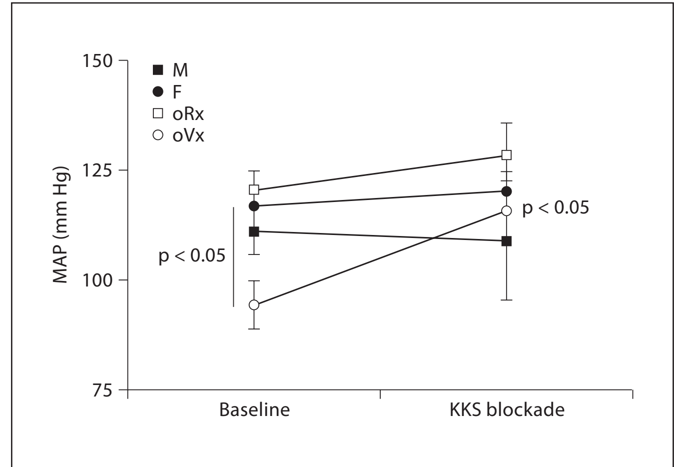

Fig. 6. MAP recorded at baseline and after KKS blockade in sham and gonadectomized rats of either sex.

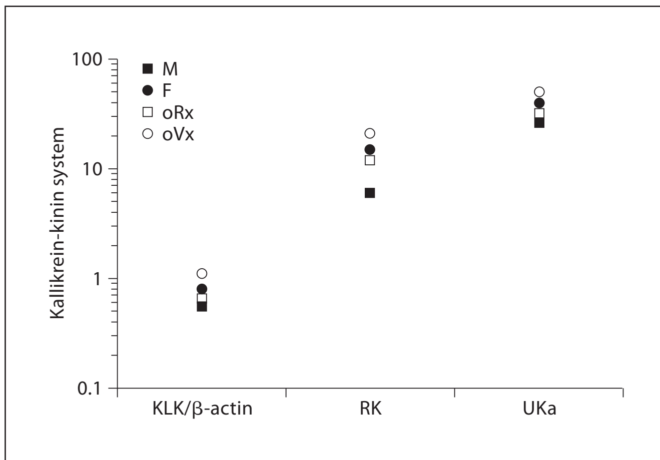

Fig. 7. Overview of KKS dynamics. KLK/β-actin, RK and UKa are expressed in arbitrary units (AU), nkat/g kidney weight and nkat/g kidney weight/day, respectively.

Hg, $p < 0.05$). Aprotinin treatment diminished GFR 35% ($p < 0.001$), from an average of 0.88 ± 0.036 to 0.61 ± 0.037 ml/min/g kidney weight. Sodium fractional excretion (%) in nontreated rats (0.34 ± 0.05) increased after aprotinin treatment in oRx and oVx groups to 1.29 ± 0.11 and 1.19 ± 0.15 ($p < 0.001$ and $p < 0.01$). Potassium fractional excretion in nontreated rats (29.4 ± 4.94) increased to 51.3 ± 3.57 and 85.5 ± 2.5 in oRx and oVx rats ($p < 0.05$ and $p < 0.001$), respectively. To understand the dynamic of KKS better, all the studied components (kallikrein synthesis, renal content and urinary excretion) were depicted together in figure 7. As can be seen, male rats have the lowest levels of the three parameters studied, while female and oVx rats exhibit the highest values.

Discussion

In the present study, the baseline KKS and the response to Gx performed in adulthood was explored in outbred Wistar rats of either sex. The results obtained showed a higher RK and UKa in female than in male rats at baseline. Gx failed to show a significant increase in UKa in male and female rats but an increase in RK was observed in both, oRx and oVx rats. RK and UKa gender difference observed at baseline were not seen in kallikrein synthesis, while Gx proved to increase KLK in female rats. These results occur without changes in renal functional data after Gx (table 1). Our results also show that oVx rats, which have the lowest baseline blood pressure, increased it after KKS blockade. Data provided for kallikrein synthesis, storage and urinary activity gave us a better insight over KKS dynamics. It appeared that at baseline and after Gx, female rats, have the KKS set at a higher level than male rats, although in some situations, this assumption comes about from a tendency (fig. 7). The renal kallikrein content at baseline and post Gx probably results from an increase in kallikrein synthesis although it proved to be significant only in the oVx group where the phenomenon is more evident. Further studies, examining discrete segments of the nephron where kal-

likrein is added to urine, would help elucidate this issue. UKa fails to increase after Gx, as it occurs in renal content, suggesting that the absence of gonad hormones does not modify the baseline pattern of kallikrein release from renal tissue into urine. The increase in tissue kallikrein, instead, may explain the lower blood pressure observed in the oVx group, probably resulting from a vasodilation and a decrease in renal vascular resistance which was reverted when KKS was blocked. The effect of KKS blockade with the β_2-receptor antagonist HOE-140 was similar to the aprotinin-induced blockade. This indicates that the bradykinin β_2-receptor is involved in the observed blood pressure regulation. These findings lend support to the results that link KKS and blood pressure regulation on the basis of clinical [19, 20] and experimental studies [21], and gene knockout animal experiments [22]. Wang et al. [23], working with two lines of transgenic mice that overexpressed human tissue kallikrein and that had developed low blood pressure, were also able to restore blood pressure after aprotinin treatment. The same group of authors [24] also showed that a substantial reduction in blood pressure in transgenic mice overexpressing human bradykinin β_2-receptor was blunted after HOE-140 administration. Other authors [25] working with normotensive nonovariectomized rats have not found any effect produced by HOE-140 administration, even in long-term treatments. In our sham normotensive rats, the HOE-140 treatment did not induce any blood pressure change either. Madeddu et al. [26] also found that ovariectomy prevented the response to vasodilator drugs at the time bradykinin β_2-receptor mRNA expression decreases and, also, that this response was restored after estrogen supplementation. The same authors [27], working with a different strain of adult oVx rats supplemented with female sexual hormones, and in a different experimental setting – a shorter period of observation (35 vs. 90 days) and older age – showed results opposite to ours, low immunoreactive kallikrein content, UKa and kallikrein mRNA after Gx. They interpreted their results in terms of the changes in sodium handling induced by ovariectomy and hormone supplementation. In our experiments, where no hormone supplementation was used, and probably because the animals' postovariectomy follow-up period was longer, no changes in water and ion excretion were seen. The diminution of GFR by KKS blockade may result from hemodynamic alterations of the vasoactive systems controlling glomerular arteriolar tone. Non-Gx rats treated with aprotinin did not significantly change the fractional sodium excretion, but the Gx animals increased the fractional sodium excretion by a factor of three. So, the increment in MAP induced by KKS blockade may not be attributed to an enhancement in sodium reabsorption.

El-Dahr et al. [28], while studying ontogenesis, found a 30% increase, as we did, in renal kallikrein activity in sham female adult rats with respect to the male animals. On the other hand, some authors have already described that estrogens regulate KLK gene expression in the anterior pituitary gland [29]. They have found that female rats have higher KLK synthesis than male rats, and that orchiectomy does not change the KLK synthesis rate. Gerald et al. [30], working on the submandibular gland, found that kallikrein mRNA was two times more abundant in male than in female rats, and that castration of male rats resulted in a decrease in KKS activity. Then, it appears that a different KKS regulation is afforded by different sexual hormones according to the organ. It has been described that high-molecular-weight plasmatic kininogen is increased by physiological amounts of natural or synthetic estrogen and diminished by high progesterone doses [31]. The synthesis of low-molecular-weight kininogen (substrate for renal KKS) has been shown to be greater in female than in male rats, and is regulated by estrogen and progesterone in ovariectomized rats whereas kallikrein gene expression has been shown to be under the influence of sexual hormones as well [32]. In this experimental setting, we found that female gonad removal stimulates KKS by increasing kallikrein synthesis and storage. On the other hand, a kallikrein-dependent low blood pressure was observed in the ovariectomized rats since KKS blockade increased blood pressure.

Acknowledgments

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