Articles in PresS. Am J Physiol Renal Physiol (December 11, 2013). doi:10.1152/ajprenal.00535.2013

COX2 INHIBITION DURING NEPHROGENIC PERIOD INDUCES AN ANGIOTENSIN II HYPERTENSION AND SEX-DEPENDENT CHANGES IN RENAL FUNCTION DURING AGEING

Virginia Reverte
Antonio Tapia
Analia Loria
Francisco Salazar
M Teresa Llinas
F. Javier Salazar

Department of Physiology; School of Medicine, Aging Institute, Campus of International Excellence "Mare Nostrum", University of Murcia, SPAIN.

Short title: Renal effects induced by perinatal COX2 inhibition

Address for Correspondence:
F. Javier Salazar
Department of Physiology, School of Medicine
University of Murcia, 30100 Murcia, SPAIN
Tel: 34 868884881
salazar@um.es

ABSTRACT

This study was performed to test the hypothesis that angiotensin II (Ang II) contributes to the hypertension and renal functional alterations induced by a decrease of COX2 activity during nephrogenic period. It was also examined whether renal functional reserve and renal response to volume overload and high sodium intake are reduced in 3-4 and 9-11 months old male and female rats treated with vehicle or a COX2 inhibitor during nephrogenic period (COX2np). Our data show that this COX2 inhibition induces an Ang II-dependent hypertension that is similar in male and female rats. Renal functional reserve is reduced in COX2np-treated rats since their renal response to an increase in plasma aminoacids levels is abolished, and their renal ability to eliminate a sodium load is impaired ($P<0.05$). This reduction in renal excretory ability is similar in both sexes during aging but does not induce the development of a sodium-sensitive hypertension. However, the prolonged high sodium intake at 9-11 months of age leads to a greater proteinuria in male than in female (114 ± 12 μg/min vs. 72 ± 8 μg/min, $P<0.05$) COX2np-treated rats. Renal hemodynamic sensitivity to acute increments in Ang II is unaltered in both sexes and at both ages in COX2np-treated rats. In summary, these results indicate that the reduction of COX2 activity during nephrogenic period programs for the development of an Ang II-dependent hypertension, reduces renal functional reserve to a similar extent in both sexes, and increases proteinuria in males but not in females when there is a prolonged increment in sodium intake.

Key Words: COX2, sex- and ageing-dependent changes, fetal programming, hypertension, renal function, renal reserve.

INTRODUCTION

The increase in blood pressure (BP) and a deteriorated renal function as a consequence of an altered renal development are sex-dependent and probably related to the degree that renal development is affected (2,6,23,25,26,29,31,36). The involvement of cyclooxygenase-2 (COX2)-derived metabolites in the regulation of renal morphogenesis has been demonstrated in studies showing that COX2 inhibition during perinatal period reduces nephron endowment by 17% and induces the development of hypertension in both sexes but leads to a progressive deterioration of renal function in male but not in female rats (26). It is also known that COX2-deficient mice show a thin nephrogenic cortex, immature glomeruli and tubuli, and a sex-dependent increment in proteinuria (35). However, it remains unknown the mechanism involved in the hypertension secondary to a decrease in COX2 activity during nephrogenic period and whether this decrease in COX2 activity induces sex- and aging-dependent alterations in the renal response to different stimuli.

One hypothesis tested in this study is that renin-angiotensin system (RAS) plays an important role in the hypertension secondary to a reduced COX2 activity during nephrogenic period. It is also examined whether the modest reduction in nephron endowment elicited by this decrease in COX2 activity leads to a significant impairment in the renal ability to modify renal hemodynamics and to increase sodium excretion in response to appropriate stimuli, such as an increase in plasma aminoacids (AA) levels or an acute volume expansion (VE). Whether the possible attenuation in the renal excretory ability is sex-dependent during ageing and leads to the development of a sodium sensitive hypertension is also examined. Other hypothesis tested is that renal sensitivity to angiotensin II (Ang II) is enhanced in hypertensive rats with a decrease COX2 activity during renal development, being this increment in renal Ang II sensitivity sex-dependent during ageing. Studies to examine whether the previous hypotheses are correct have been performed at 3-4 and/or 9-11 months of age in rats treated with a COX2 inhibitor during the nephrogenic period (COX2np).

MATERIAL AND METHODS

Sprague Dawley (SD) rats were purchased from the University of Murcia Animal Research Laboratory. Protocols were designed according to the NIH Guide and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the University of Murcia. Food (Harlan Teklad) and water were supplied ad libitum. Female SD rats (≈230 g b.w.) were placed with a male, taking day 0 of pregnancy the morning that sperm evidence was found in the vaginal smear. Vehicle or a COX2 inhibitor (rofecoxib, 2,4 mg/kg/day) was given to dams from embryonic day 16 until delivery and to newborn pups during first three postnatal weeks since nephrogenesis in rats takes place from midgestation until the second-third postnatal week (10). Solutions with and without rofecoxib were orally administered to pups at a rate of 0,96 μL/g bw. At postnatal day 0, litter size was fixed (8-10) in order to assure similar nourishment during suckling period. Litters with <8 pups were excluded.

Arterial pressure measurement

Systolic blood pressure (SBP) was measured in conscious rats at 3-4 and 9-11 months of age by the tail-cuff method (23-26,29) using a CODA 2 non-invasive system (Kent Scientific Corporation). In order to reduce stress and to obtain an accurate reading, rats were first habituated to the measurement device and to a temperature of 30ºC for 10-15 min. Definitive measurements began when rats remained unperturbed into the chamber throughout the inflation-deflation cycles. The SBP value in each rat is the mean of at least 10 measurements taken during 2-3 days. The SBP values obtained with this method are correlated to those found in conscious freely moving rats through a femoral artery catheter (29).

Renal function studies

After overnight fasting, rats were anesthetized with 0.1 ml of ketamine (Ketolar, Parke Davis, 100 $mg.ml^{-1}$, i.m.) and 0.1 ml/100 g of penthobarbital (Pentothal, Abbott, 50 $mg.ml^{-1}$, i.p.). After tracheotomy, catheters were inserted into the bladder for collection of urine samples, and into the left femoral artery to measure mean arterial pressure (MAP) (PowerLab, ADInstruments) and for blood withdrawal. Another catheter was implanted into the left

127 femoral vein for i.v. infusions. Then, rats were placed on a temperature-regulated surgical
128 table to maintain stable their body temperature. To stabilize hematocrit level 1ml/100 g bw of
129 6% of bovine serum albumin (Sigma) was infused. [^{3}H] inulin (2 μCi/ml American
130 Radiolabeled Chemicals) was given as an i.v. bolus (1 ml) and as an infusion (1.5 μCi/ml)
131 dissolved in isotonic saline (1 ml/100 g bw/hour). A transit-time flow probe (Transonic
132 System) was implanted on the left renal artery for renal blood flow (RBF) measurement.
133 Renal plasma flow (RPF) changes were calculated considering RBF and hematocrit values.
134 Urine samples were collected for measurements of urine flow rate (UV), [^{3}H]inulin and
135 urinary sodium excretion (UNaV). Plasma samples were collected in heparinized capillaries 5
136 min before the end of each clearance period to measure [^{3}H]inulin and electrolyte
137 concentrations. A 70-min stabilization period was allowed before experiments begun.

138 **Experimental protocols**

139 **Blood pressure response to candesartan.** This angiotensin II receptor antagonist (ARA)
140 was administered by gavage (7 mg/kg/day) during three days to conscious 9-11 months old
141 rats. Systolic BP was measured during basal period (3 days) and 3 hours after each ARA
142 administration. This administration of candesartan reduced (> 80%) the SBP increment (27 ±
143 2 mmHg) induced by an i.v. Ang II infusion (30 ng/kg) (n=5).

144 **Changes in plasma renin activity (PRA).** Catheters were inserted into the femoral artery in
145 anesthetized rats (isofluorane, Abbott) to obtain blood samples to analyze PRA by using a
146 commercial RIA kit for Ang I (Diasorin).

147 **Renal response to AA infusion**. It was examined in 3-4 months old rats. Two 20-min basal
148 clearance collections were followed by an i.v. infusion of a mixed 10% AA solution (3
149 ml/hr). Five minutes after AA infusion was initiated, two 20-min clearances were obtained.

150 **Renal response to acute VE**. It was examined in 3-4 and 9-11 months old rats. Two 20-min
151 basal clearances were followed by a VE (6% b.w. during 55 min) elicited by isotonic saline
152 infusion. Two consecutive 20-min clearances were obtained during the last 40 min of VE.

Response to an increment in sodium intake. 3-4 and 9-11 months old rats were kept in individual metabolic cages to evaluate the changes in UV, glomerular filtration rate (GFR) and proteinuria during 24-h periods. After two days of adaptation, rats were maintained with a normal sodium diet (NSD) (0,4% Panlab, Spain) during 3 days. Then, sodium diet was increased (8%) (Panlab) by 7 consecutive days and decreased again to normal levels (0,4%) during 4 days (recovery period). Blood samples from the tail were obtained during basal period, last day of high sodium diet (HSD), and last day of recovery period. Systolic BP, GFR, proteinuria and UV were measured during NSD, seventh day of HSD and last day of recovery period. Systolic BP was also measured on day 3 of HSD.

Glomerular filtration rate was determined by endogenous creatinine clearance as in previous studies (24,29) and GFR values found were similar to those obtained in anesthetized rats using the [^{3}H] inulin clearance (23,25). Urine flow rate was determined gravimetrically. Proteinuria was measured by micro Lowry method (24-26,29).

Renal hemodynamic response to acute Ang II infusion. Two 20-min basal clearance periods were followed by an i.v. infusion of captopril (10 ng/Kg/min). This infusion does not modify GFR but leads to an increment of RPF (17,4 ± 1,5%, $p<0.05$) (23). An i.v. Ang II infusion (30 ng/Kg/min) was started 30 min after captopril administration begun. Fifteen minutes after the initiation of Ang II infusion, two more 20-min clearances were obtained. Renal Ang II effects were examined in captopril treated rats to discard that the possible differences found in these effects are secondary to differences in endogenous Ang II levels.

Statistical Analysis

Data in text, tables and figures are given as mean ± SE. Data from both clearances during each period in renal function studies were averaged for comparisons. Differences between experimental periods within one group were evaluated using ANOVA for repeated measures and the Fischer´s test. Differences between groups were examined with the use of ANOVA and Fischer´s test.

RESULTS

Blood pressure response to candesartan. SBP pressure was higher ($P<0.05$) in male (135 ± 2 mmHg) and female (131 ± 2 mmHg) COX2-treated rats than in male (117 ± 1 mmHg) and female (119 ± 1 mmHg) control rats at 9-11 months of age (Figure 1). Candesartan administration decreased SBP to similar levels in COX2-treated and control rats, being this fall in SBP greater ($P<0.05$) in COX2-treated (male: 36 ± 4 mmHg; female: 31 ± 4 mmHg) than in control (male; 16 ± 1 mmHg; female: 20 ± 2 mmHg) rats.

Changes in PRA. PRA (in ngAngI/ml/hr) (n = 6-8 rats/group) was similar in control (males: 7.0 ± 1.5; females: 7.7 ± 2.4) and COX2np-treated (males: 9.0 ± 1.5; females: 6.6 ± 0.8) rats at 3-4 months of age. No significant differences in PRA were also found between groups at 9-11 months of age (control males: 5.8 ± 1.1; control females: 8.4 ± 1.4; COX2np males: 8.7 ± 1.3; COX2np females: 9.5 ± 2.8).

Renal response to AA infusion. This infusion did not modify MAP in any experimental group. Figure 2 shows the renal hemodynamic responses to AA infusion in both groups of control and COX2np-treated rats. Basal renal hemodynamic and excretory functions were not different in control and COX2np-treated rats. The AA infusion led to a renal vasodilatation and hyperfiltration in control male rats since it induced an elevation in GFR (1.32 ± 0.10 to 1.93 ± 0.16 ml/min/g, $p<0.05$), and RPF (3.92 ± 0.29 to 5.01 ± 0.36 ml/min/g, $p<0.05$). The AA infusion also elicited an increment in UV (0.03 ± 0.01 to 0.05 ± 0.01 µl/min/g, $p<0.05$) and fractional excretion of sodium (FeNa) (1.16 ± 0.39 to 1.69 ± 0.52 %, $p<0.05$) in these male rats. Contrary to that found in control male rats, AA infusion to COX2np-treated male rats did not induce significant changes in GFR and RPF (Figure 2). Both, UV and FeNa were also not significantly different before (0.05 ± 0.01 µl/min/g and 1.50 ± 0.17%, respectively) and after (0.05 ± 0.01µl/min/g and 1.80 ± 0.17%, respectively) AA infusion in COX2np-treated males. In contrast to the renal hemodynamic response to AA infusion in control males, this infusion did not elicit changes in GFR and RPF in control females (Figure 2).

Nevertheless, AA infusion did induce an increment in both UV (0.03 ± 0.00 to 0.05 ± 0.00 µl/min/g, $p<0.05$) and FeNa (1.48 ± 0.13 to 2.86 ± 0.26 %, $p<0.05$) in control females. Figure 2 also shows that renal hemodynamic was unaffected by AA infusion in COX2np-treated females. As occurred in COX2np-treated males, UV and FeNa were also similar before (0.03 ± 0.01 µl/min/g and 1.72 ± 0.29%, respectively) and after (0.04 ± 0.00 µl/min/g and 1.86 ± 0.23%, respectively) AA infusion in COX2np-treated females.

Renal response to acute VE. Table 1 shows the renal response to a VE in control and COX2np-treated rats at 3-4 months of age. MAP was similar in both sexes of vehicle and COX2np-treated rats and did not change during VE. Basal GFR and RPF were similar in both sexes of control and COX2np-treated rats, and did not change in response to the acute VE. No differences in FeNa and UV were found between groups during basal period. VE led to an increment ($p<0.05$) of FeNa and UV in control and COX2np-treated rats but these increments of FeNa and UV were lower ($p<0.05$) in male (37% and 46%, respectively) and female (39% and 41%, respectively) COX2np-treated than in their respective control group. The reduction in the excretory response to VE was similar in both sexes of COX2np-treated rats.

Renal responses to VE in 9-11 months old rats are shown in table 2. As occurred at the younger age, MAP was similar before and after VE in each group of rats at 9-11 months of age. Basal GFR and RPF were similar in each group of rats and did not change during VE. Both, FeNa and UV were also similar during basal period, and increase during VE in both groups of male and female rats at the older age. However, the renal excretory ability to eliminate the sodium load was impaired ($p<0.05$) in both sexes of COX2np-treated rats with respect to that found in their age-matched control rats (Table 2).

Response to an increment in sodium intake. Figure 3 shows the SBP and proteinuria during a normal and high sodium intake in control and COX2np-treated rats at 3-4 months of age. It can be observed that basal SBP was elevated ($p<0.05$) but basal proteinuria was unchanged in both sexes of COX2np-treated rats with respect to the values in their respective control group. SBP and proteinuria did not change in response to a HSD at 3-4 months of age in both groups

of control and COX2np-trated rats (figure 2). Creatinine clearance was also similar during NSD and HSD in control males (0.71 ± 0.02 and 0.80 ± 0.02 ml/min, respectively), control females (0.81 ± 0.04 and 0.80 ± 0.07 ml/min, respectively), COX2np-treated males (0.76 ± 0.04 and 0.71 ± 0.07 ml/min, respectively) and COX2np-treated females (0.78 ± 0.03 and 0.67 ± 0.01 ml/min, respectively). SBP, proteinuria and creatinine clearance remained during the recovery period at similar levels to those found during basal period. Food intake was similar in both sexes of control and COX2np-treated rats during NSD, HSD and when sodium intake was again reduced to normal levels.

Figure 4 shows the effects of the prolonged increment in sodium intake on SBP and proteinuria at 9-11 months of age. Basal SBP was elevated in male and female COX2np-treated rats with respect to the values found in control rats, being the increment similar to that found at 3-4 months of age. As occurred at the younger age (Figure 3), the prolonged HSD did not elicit a further elevation of SBP in any group of the 9-11 months old rats (Figure 4). During NSD, proteinuria was slightly greater in COX2np-treated than in control male rats and similar in both groups of female rats. The HSD led to an elevation in proteinuria in each group at the oldest age examined in this study. Proteinuria during HSD, and the % elevation of proteinuria with respect to the basal period, were greater ($p<0.05$) in male COX2np-treated rats (114 ± 12 μg/min and 59%, respectively) than in female COX2np-treated rats (72 ± 8 μg/min and 33%, respectively) and than in male (68 ± 3 μg/min and 24%, respectively) and female (57 ± 8 μg/min and 25%, respectively) control rats. When sodium intake was reduced to normal levels, proteinuria returned to levels not significantly different to those found during the basal period. Creatinine clearance was similar during NSD and HSD in control males (1.10 ± 0.12 and 1.20 ± 0.015 ml/min, respectively), control females (0.71 ± 0.07 and 0.76 ± 0.09 ml/min, respectively), COX2np-treated males (0.94 ± 0.12 and 1.18 ± 0.12 ml/min, respectively) and COX2np-treated females (0.84 ± 0.04 and 0.78 ± 0.03 ml/min, respectively) at 9-11 months of age. Creatinine clearance remained unchanged in each group

when sodium intake decreased to normal levels. Food intake was similar in both sexes of control and COX2np-treated rats throughout the experiment.

Renal hemodynamic response to acute Ang II infusion. Ang II infusion led to an increment ($P<0.05$) in MAP that was not statistically different in both groups of male (control: 10 ± 1 mmHg; COX2np: 8 ± 2 mmHg) and female (control: 8 ± 2 mmHg; COX2np: 12 ± 4 mmHg) rats at 3-4 months of age. Similar changes in MAP were found after Ang II infusion at 9-11 months of age. Renal hemodynamic parameters during basal period were similar in both sexes of control and COX2np-treated rats at 3-4 months and did not change between the ages examined (table 3). The Ang II infusion induced a significant decrease of RPF and GFR in each experimental group. No significant differences between control and COX2np in both sexes and at both ages were found in the renal hemodynamic response to Ang II (Table 3).

DISCUSSION

This study reveals that the reduction of COX2 activity during nephrogenic period programs for to the development of an Ang II dependent hypertension and reduces renal functional reserve since the renal vasodilatory and excretory responses to increments in plasma AA concentration are abolished and the renal ability to eliminate an acute sodium load is blunted. Contrary to what has been shown in studies in which renal development is altered by affecting other mechanisms (23,29), the impairment in renal function secondary to COX2np is not associated with an increase in the renal sensitivity to Ang II or in the development of an ageing-dependent sodium-sensitive hypertension. New evidence are given supporting that renal injury is greater during aging in males than in females as a consequence of a reduction in COX2 activity during nephrogenic period since proteinuria, a hallmark of renal damage, increases more in males than in females when sodium intake is enhanced.

The BP increments in COX2np-treated rats are similar to those reported in a previous study (26) that also shows important aging-dependent deterioration in renal function and renal structure in male but not in female COX2np-treated. However, the mechanism involved in

this BP increment was unknown. Considering the well known inverse relationship between nephron endowment and the risk to develop hypertension (2,36), it can be suggested that the higher BP in COX2np-treated rats is mainly secondary to a 17% decrease in nephron number (26) but could also be a consequence of other renal changes elicited by COX2 inhibition during renal development (21). The hypothesis that Ang II is involved in maintaining this increment in BP was supported by studies showing that RAS plays an important role in the hypertension found in several models with a reduced nephron endowment (7,10,19,25,27,29). Our findings are consistent with the hypothesis that Ang II contributes to maintaining BP elevated even when there is a modest alteration in renal development. It was also examined whether this involvement was greater in males than in females because Ang II effects are modulated by sex hormones (22). A previous study in other experimental model with an altered renal development has shown that the role of Ang II in maintaining BP elevated is greater in males than in females (23). The absence of differences between both sexes of COX2np-treated rats in the decrease of BP elicited by candesartan could be explained by a small increment of Ang II effects in these rats.

This study also investigates whether the involvement of the RAS in maintaining BP elevated in COX2np-treated rats is secondary to an increase in PRA. Contradictory data have been reported with respect to changes in PRA and other components of the RAS in the hypertension secondary to different "insults" during renal development (4,10,13). These different results may be explained by the multiplicity of models employed and the sex and age at which the studies were performed. Only two studies have examined whether there are changes in PRA during ageing in animals with an altered renal development and whether these changes are sex-dependent but the results reported are also contradictory (13,23). One possibility tested was that PRA is greater in males than in females since it has been shown that PRA levels are modulated by sex hormones (22). PRA results were obtained in samples collected from anesthetized animals, as in other previous studies (4,13,23), and show that PRA is not elevated in COX2np-treated rats. Our data suggest that the regulation of PRA is

312 altered in these rats because an increased BP perse would be expected to reduce renin release
313 (16). The involvement of Ang II in maintaining BP elevated in the absence of changes in
314 PRA could be explained by changes in other components of the RAS in resistance vessels
315 and/or in renal tubules, such as AT_1 and AT_2 receptors and angiotensin converting enzyme
316 (ACE) activity (4,6,7,14,23,27,30). Further studies are needed to assess whether the
317 involvement of Ang II in the BP increment in COX2np-treated rats is related to changes in
318 oxidative stress and/or in renal sympathetic activity, as occurs in other experimental models
319 of developmental programming (15,23).

320 The absence of renal hemodynamic changes in both sexes of COX2np-treated rats at 3-
321 4 months of age was expected since the results shown are similar to those previously reported
322 (26). Total GFR and RBF are not different to those found in control rats but glomerular
323 pressures and flows are most probably elevated since these rats have a 17% reduction in
324 nephron endowment (26). This study examines whether these possible changes in glomerular
325 pressures and flows modify renal functional reserve to an extent important enough to reduce
326 the renal responses to stimuli that induce a vasodilatory response and/or an increase in renal
327 excretory ability. A previous study has shown that a 37% reduction in nephron endowment
328 elicited by a decrease of Ang II effects prevents the renal changes elicited by an increase in
329 plasma AA levels (12). The hypothesis was that a small alteration in nephron endowment
330 would not be enough to reduce renal functional reserve or that this reduction in renal reserve
331 would be evident in males but not in females. This latter possibility was supported by studies
332 showing that the effects elicited by a severe maternal protein restriction leads to similar
333 changes in adult male and female offspring (13) but the effects elicited by a modest maternal
334 protein restriction are only evident in male offspring (34).

335 The current study reveals that a modest decrease in nephron endowment reduces renal
336 functional reserve to the extent that the renal hemodynamic and excretory responses to an
337 increase in the plasma AA levels are abolished. The renal responses to the increment of
338 plasma AA in both sexes of control rats are similar to those reported (12). Further studies are

needed to evaluate why the increase in plasma AA elicits an increase of RBF and GFR in male but not in female normotensive rats. One possible explanation of why GFR and RBF do not change in response to an increase in plasma AA levels in COX2np-treated male rats is that glomerular pressures and flows are already at a level that can not increase more when submitted to a vasodilatory stimulus. Blockade of the renal hemodynamic response to an increment in plasma AA could also be secondary to the elevated glomerulosclerosis index and glomerular volumes in these rats (26) and to an alteration of the mechanisms involved in regulating this response (28,33). The mechanisms responsible for the blockade of the excretory response to the increment in AA in both sexes of COX2np-treated rats are unknown but it may be speculated that this blockade is a consequence of the tubular effects elicited by reducing COX2 activity during nephrogenic period (35). The renal excretory response to AA in COX2np-treated rats may also be secondary to an increase in Ang II since the hypertension is Ang II-dependent. It is also known that a mild elevation in Ang II reduces renal excretory ability by increasing proximal sodium reabsorption (20) and that proximal reabsorption is elevated in different models with a reduced nephron endowment (3).

The results of this study indicate that a moderate alteration in renal development also reduces the natriuretic and diuretic response to an acute VE in young adult rats of both sexes, and to a similar extent that the decrease in the renal excretory ability observed when renal development is altered by reducing Ang II effects (12,18). We predicted that this attenuation in the renal excretory ability would be enhanced in male but not in female COX2np-treated rats at 9-10 months of age. This hypothesis was supported by results showing that tubulointerstitial damage increase between both ages in male but not in female COX2np-treated rats (26) and by results showing that an alteration in renal development elicited by decreasing Ang II leads to an accelerated age-dependent impairment of renal excretory ability only in male rats (12). The results obtained did not support our hypothesis since the reduced excretory ability to eliminate the acute VE at 3-4 months of age was maintained but not enhanced at 9-11 months of age. Our results do not allow us to determine the mechanisms

involved in the reduced renal excretory but it is speculated that it may be secondary to an elevation of Ang II effects since the hypertension is Ang II-dependent and Ang II plays an important role in modulating the renal response to an acute VE (20).

It was also expected that a HSD would exaggerate the hypertension in COX2np-treated rats because an increase in BP would be needed to maintain fluid and electrolyte balance at an advanced age (8,32). In support of this hypothesis it has also been shown that an alteration in renal development elicited by a decrease of Ang II effects during nephrogenic period leads to the development of an ageing-dependent sodium sensitive hypertension (29). Conversely, we found that the prolonged HSD did not elicit a further increment of BP in COX2np-treated rats but they suggest that even a modest reduction of renal reserve renders the aged kidney more susceptible to failure when other "secondary insults" are superimposed. The increment in proteinuria during HSD was greater in male than in female COX2np-treated rats at an advanced age (figure 4) and it is well accepted that proteinuria accelerates kidney disease progression to end-stage renal failure through multiple pathways (1). The sex-dependent progression of renal damage found in this study confirms results of our group showing that tubulointerstitial damage is greater in male than in female COX2np-treated rats at 9-11 months of age (26). Our results provide new evidence that a sex-dependent mechanism is protecting females from the aging-dependent progression of renal damage that occurs as a consequence of a modest alteration in renal development. This mechanism is probably related to the greater glomerular hypertrophy and greater tubular damage found in male rats (26) and to a sex-dependent increment in the production of COX2-derived metabolites (24). Sex hormones may also be involved (22) but future studies need to examine their importance because it is unknown whether cycling is altered in animals with a developmental programming of hypertension and renal disease. Sex-dependent differences reported in this and other studies may also be secondary to mechanisms independent of sex steroids. This hypothesis is supported by studies showing that sex-dependent differences are evident long before sex hormones differ (6,9).

393 The renal sensitivity to acute increments of Ang II in COX2np-treated rats and whether
394 a possible greater renal sensitivity is sex- and/or aging-dependent in these rats has also been
395 examined. It is important since an increase in the renal sensitivity to Ang II may be involved
396 in maintaining BP elevated when systemic RAS is not activated (16). Previous studies have
397 shown that the renal hemodynamic effects elicited by Ang II are enhanced in animals with an
398 adverse renal development (19,23,27) but only one of these studies have examined whether
399 this sensitivity is different in both sexes and changes during ageing (23). That previous study
400 showed that renal AT_1 receptor sensitivity is similarly enhanced in both sexes at an adult and
401 at an advanced age (23). However, it was unknown whether the renal sensitivity to acute
402 increments in Ang II is enhanced when renal development is altered by a reduced COX2
403 activity. One possibility was that the renal Ang II effects would be sex- and/or ageing-
404 dependent since these effects are modulated by sex-hormones (17,19) and the role of Ang II
405 in the regulation of cardiovascular and renal function increases with age (5). Renal sensitivity
406 was examined in rats pretreated with captopril since ACE activity may be elevated in animals
407 with reduced nephron endowment (30). The renal hemodynamic response observed during
408 Ang II infusion to normotensive rats is similar to that reported in the only previous study that
409 has examined whether this renal response is sex-dependent during ageing (23). Contrary to
410 what was expected, the renal response to Ang II infusion was not enhanced in both sexes of
411 3-4 months old rats with an altered renal development elicited by a decreased COX2 activity.
412 It was also found that the renal hemodynamic response remained similar to that observed in
413 normotensive rats when examined at an advance age (9-11 months of age). This study present
414 novel finding showing that, contrary to what occur when renal development is altered by
415 affecting other mechanisms (19,23,27), the renal sensitivity to increments in Ang II is not
416 enhanced at an adult or advanced age when renal development is altered by decreasing COX2
417 activity. However, we can not rule out the possibility that a reduced COX2 activity early in
418 life leads to changes in some components of the intrarenal RAS, such as ACE or AT_1 and
419 AT_2 receptors, and in the postreceptor mechanisms involved in the renal response to Ang II.

Considering that human kidney development is completed before birth (3,6), the results of this study suggest that an insult inducing a reduction of COX2 activity during the third trimester of pregnancy in humans would lead to the development of an Ang II-dependent hypertension, to the reduction of renal functional reserve even at an adult young age, and to a progressive renal damage that would be greater in males than in females. The current study gives substantial support to the notion that stimuli eliciting a moderate alteration in renal development will induce a significant deterioration of renal function and an increment in arterial pressure during aging. These observations also emphasize the importance of not affecting the mechanisms involved in the regulation of renal development.

Sources of funding

This work was supported by the Dirección General de Educación y Ciencia of Ministerio de Educación y Ciencia, Spain (SAF2007-64498), and Fundación Seneca from Comunidad Autónoma of Murcia (08777/PI/08). V. Reverte was supported by Subdirección General de Redes y Centros de Investigación Cooperativa (RETICEF, RD06/0013/1020).

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

1. Abbate M, Zoja C, Remuzzi G. How does proteinuria cause progressive renal damage? *J Am Soc Nephrol* 17: 2974-2984, 2006.

2. Alexander BT. Fetal programming of hypertension. *Am J Physiol Regul Integr Comp Physiol*. 290: R1-R10, 2006.

3. Brenner BM. Nephron adaptation to renal injury or ablation. *Am J Physiol Renal Fluid Electrolyte Physiol* 249: F324-F337. *1985:*

4. Ceravolo GS, Franco MC, Carneiro-Ramos MS, Barreto-Chaves ML, Tostes RC, Nigro D, Fortes ZB, Carvalho MH. Enalapril and losartan restored blood pressure and vascular reactivity in intrauterine undernourished rats. *Life Sci* 80: 782-787, 2007.

5. Diz DI. Lewis K. Dahl Memorial Lecture. The renin-angiotensin system and aging. *Hypertension* 52: 37-43, 2008.

6. Gilbert JS, Nijland MJ. Sex-differences in the developmental origins of hypertension and cardiorenal disease. *Am J Physiol Regul Integr Comp Physiol*. 295: R1941-R1952, 2008.

7. Gwathmey TM, Shaltout HA, Rose JC, Diz DI, Chappel MC. Glucocorticoid-induced fetal programming alters the functional complement of angiotensin receptor subtypes within the kidney. *Hypertension* 57: 620-626, 2011.

8. Hall JE. Control of sodium excretion by angiotensin II: intrarenal mechanisms and blood pressure regulation. *Am J Physiol Regul Integr Comp Physiol.* 250: R960-R972, 1986.

9. Ji H, Wu X, Liu J, Ecelbarger CM, Watkins R, Arnold AP, Sandberg K. Sex chromosome effects unmasked in angiotensin II-induced hypertension. Hypertension 55: 1275-1282, 2010.

10. Langley-Evans SC, Jackson AA. Captopril normalizes systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets. *Comp Biochem Physiol* 110: 223-228, 1995.

11. Larsson L, Aperia A, Wilton P. Effect of normal development on compensatory renal growth. *Kidney Int*. 18: 29-35, 1980.

12. Loria AS, Reverte V, Salazar F, Sáez F, Llinás MT, Salazar FJ. Changes in renal hemodynamics and excretory function induced by a reduction of Ang II effects during renal devlopment. *Am J Physiol Regul Integr Comp Physiol.* 293: R695-R700, 2007.
13. Manning J, Vehaskari VM. Low birth weight-associated adult hypertension in the rat. *Pediatr Nephrol* 16: 417-422, 2001.
14. McMullen S, Gardner DS, Langley-Evans S. Prenatal programming of angiotensin type 2 receptor expression in the rat. Br J Nutr 91: 133-140, 2004.
15. Mizuno M, Lozano G, Baum M, Smith SA. Enalapril attenuates the exaggerated sympathetic response to physical stress in prenatally programmed hypertensive rats. Hypertension 62: 000-000, 2013.
16. Navar LG, Inscho EW, Majid DSA, Imig JD, Harrison-Bernard LM, Mitchell KD. Paracrine regulation of the renal microcirculation. *Physiol Rev* 76: 425-536, 1996.
17. Nickenig G, Bäumer AT, Grohè C, Kahlert S, Strehlow K, Rosenkranz S, Stäblein A, Beckers F, Smits JF, Daemen MJ, Vetter H, Böhm M. Estrogen modulates AT1 receptor gene expression in vitro and in vivo. *Circulation* 97: 2197-2201, 1998.
18. Nilsson AB, Guron GS, Adams MA, Friberg P. Renal interstitial hydrostatic pressure and urinary sodium excretion in rats with angiotensin-converting enzyme inhibitor-induced papillary atrophy. *Exp Physiol* 84: 947-955, 1999.
19. Ojeda NB, Royals TP, Black JT, Dasinger JH, Johnson JM, Alexander BT. Enhanced sensitivity to acute angiotensin II is testosterone dependent in adult male growth-restricted offspring. *Am J Physiol Regul Integr Comp Physiol* 298: R1421-1427, 2010.
20. Pinilla JM, Alberola A, González JD, Quesada T, Salazar FJ. Role of prostaglandins on the renal effects of angiotensin II and interstitial pressure during volume expansion. *Am J Physiol Regul Integr Comp Physiol.* 265: R1469-R1474, 1993.
21. Prévot A, Mosig D, Martini S, Guignard JP. Nimesulide, a cyclooxygenase-2 preferential inhibitor, impairs renal function in the newborn rabbit. *Pediatr Res* 55: 254-260, 2004.

498 22. Reckelhoff JF. Gender differences in the regulation of blood pressure. *Hypertension* 37:
499 1199-1208, 2001.
500 23. Reverte V, Tapia A, Baile G, Gambini J, Gimenez I, Llinas MT, Salazar FJ. Role of
501 angiotensin II on arterial pressure and renal hemodynamics in rats with an altered renal
502 development. Age- and sex-dependent differences. *Am J Physiol Renal Physiol* 304: F33-
503 F40, 2013.
504 24. Reverte V, Tapia A, Moreno JM, Rodriguez L, Salazar F, Llinas MT, Salazar FJ. Renal
505 effects of prolonged high protein intake and COX2 inhibition in hypertensive rats with an
506 altered renal development. *Am J Physiol Renal Physiol.* 301: F327-F333, 2011.
507 25. Saez F, Castells MT, Zuasti A, Salazar F, Reverte V, Loria A, Salazar FJ. Sex differences
508 in the renal changes elicited by Ang II blockade during the nephrogenic period.
509 *Hypertension* 49: 1429-1435, 2007.
510 26. Saez F, Reverte V, Salazar, Castells MT, Llinas MT, Salazar FJ. Hypertension and sex
511 differences in the age-related renal changes when cyclooxygenase-2 activity is reduced
512 during nephrogenesis. *Hypertension* 53: 331-337, 2009.
513 27. Sahajpal V, Ashton N. Increased glomerular angiotensin II binding in rats exposed to a
514 maternal low protein diet in utero. *J Physiol* 563: 193-201, 2005.
515 28. Salazar FJ, Alberola A, Nakamura T, Granger JP. Role of nitric oxide in the renal
516 hemodynamic response to a meta meal. *Am J Physiol Regul Integr Comp Physiol* 267:
517 R1050-R1055, 1994.
518 29. Salazar F, Reverte V, Saez F, Loria A, Llinas MT, Salazar FJ. Age- and sodium-sensitive
519 hypertension and sex-dependent renal changes in rats with a reduced nephron number.
520 *Hypertension* 51: 1184-1189, 2008.
521 30. Shaltout HA, Figueroa JP, Rose JC, Diz DI, Chappel MC. Alterations in circulatory and
522 renal angiotensin-converting enzyme and angiotensin-converting enzyme 2 in fetal
523 programmed hypertension. *Hypertension* 53: 404-408, 2009.

524 31. Tang L, Bi J, Valego N, Carey LC, Figueroa JP, Chappell MC, Rose J. Prenatal
525 betamethasone exposure alters renal function in immature sheep: sex-differences in
526 effects. *Am J Physiol Regul Integr Comp Physiol*. 299: R793-R803, 2010.
527 32. Weinberger MH, Fineberg NS. Sodium and volume sensitivity of blood pressure. Age and
528 pressure change over time. *Hypertension* 18: 67-71, 1991.
529 33. Woods LL. Mechanisms of renal hemodynamic regulation in response to protein feeding.
530 *Kidney Int* 44: 659-675, 1993.
531 34. Woods LL, Ingelfinger JR, Rasch R. Modest maternal protein restriction fails to program
532 adult hypertension in female rats. *Am J Physiol Regul Integr Comp Physiol* 289: R1131-
533 R1136, 2005.
534 35. Yang T, Huang YG, Ye W, Hansen P, Schnermann JB, Briggs JP. Influence of genetic
535 background and gender on hypertension and renal failure in COX2-deficient mice *Am J*
536 *Physiol Renal Physiol* 288: F1125-F1132, 2005.
537 36. Zandi-Nejad K, Luyckx VA, Brenner BM. Adult hypertension and kidney disease: the
538 role of fetal programming. *Hypertension* 47: 502-528, 2006.
539

FIGURE LEGENDS.

Figure 1. Systolic blood pressure (SBP) changes elicited by three consecutive days administration of an AT_1 receptor antagonist to conscious 9-11 months old rats treated with vehicle (Control) or a COX2 inhibitor during nephrogenic period (COX2np). Upper figure shows SBP changes in males (n= 6 control; n=8 COX2np) and lower figure shows SBP changes in females (n= 6 control; n=6 COX2np). * P<0.05 vs. Basal period. # P<0.05 vs. control group of the same sex.

Figure 2. Renal hemodynamic response to an aminoacids (AA) infusion in 3-4 months old rats treated with vehicle (Control) (n=8 males; n=9 females) or a COX2 inhibitor (n=9 males; n=8 females) during nephrogenic period (COX2np). * P<0.05 vs. Basal period.

Figure 3. Systolic blood pressure (SBP) and urinary protein excretion during normal sodium intake and after a 7-day increment of sodium intake in 3-4 months old rats treated with vehicle (Control) (n=6 males; n=6 females) or a COX2 inhibitor (n=5 males; n=5 females) during nephrogenic period (COX2np). # P<0.05 vs. control group of the same sex.

Figure 4. Systolic blood pressure (SBP) and urinary protein excretion during normal sodium intake and after a 7-day increment of sodium intake in 9-11 months old rats treated with vehicle (Control) (n=6 males; n=6 females) or a COX2 inhibitor (n=8 males; n=8 females) during nephrogenic period (COX2np). * P<0.05 vs. Basal period. # P<0.05 vs. control group of the same sex.

565 **Table 1.** Renal response to an acute volume expansion at 3-4 months of age in rats treated
566 with vehicle (Control) or a COX2 inhibitor during nephrogenic period (COX2np).

	Males		**Females**	
	Control n = 7	**COX2np** n = 8	**Control** n = 9	**COX2np** n = 10
GFR (ml/min/g kw)				
Basal	1.51 ± 0.11	1.57 ± 0.08	1.42 ± 0.07	1.60 ± 0.09
Vol. Expansion	1.58 ± 0.10	1.63 ± 0.07	1.47 ± 0.06	1.59 ± 0.07
ERPF (ml/min/g kw)				
Basal	4.39 ± 0.22	4.27 ± 0.22	4.05 ± 0.09	4.03 ± 0.29
Vol. Expansion	4.35 ± 0.24	4.40 ± 0.21	4.10 ± 0.15	3.98 ± 0.19
FE_{Na} (%)				
Basal	1.38 ± 0.15	1.27 ± 0.31	2.08 ± 0.43	1.98 ± 0.22
Vol. Expansion	8.02 ± 0.28*	5.47 ± 0.81*#	9.17 ± 0.61*	6.27 ± 0.47*#
UV (µl/min/g bw)				
Basal	0.18 ± 0.04	0.10 ± 0.02	0.20 ± 0.05	0.15 ± 0.03
Vol. Expansion	0.81 ± 0.03*	0.44 ± 0.06*#	0.89 ± 0.06*	0.56 ± 0.05*#

567 (GFR= Glomerular filtration rate; RPF= Renal Plasma Flow; FeNa = Fractional excretion of
568 sodium UV=; Urinary flow rate;; * $p < 0.05$ vs. Basal; # $p < 0.05$ vs. Control).
569

570

571 **Table 2.** Renal response to an acute volume expansion at 9-11 months of age in rats treated
572 with vehicle (Control) or a COX2 inhibitor during nephrogenic period (COX2np).

	Males		**Females**	
	Control n = 7	**COX2np** n = 6	**Control** n = 9	**COX2np** n = 6
GFR (ml/min/g kw)				
Basal	1.43 ± 0.08	1.28 ± 0.07	1.48 ± 0.09	1.47 ± 0.13
Vol. Expansion	1.45 ± 0.06	1.32 ± 0.07	1.49 ± 0.09	1.43 ± 0.15
RPF (ml/min/g kw)				
Basal	4.43 ± 0.21	3.95 ± 0.35	4.16 ± 0.15	4.64 ± 0.35
Vol. Expansion	4.63 ± 0.26	4.19 ± 0.52	4.29 ± 0.15	5.12 ± 0.31
FE_{Na} (%)				
Basal	0.69 ± 0.15	0.78 ± 0.10	1.22 ± 0.33	1.20 ± 0.20
Vol. Expansion	6.76 ± 0.12*	5.34 ± 0.60*#	7.10 ± 0.76*	4.74 ± 0.25*#
UV (µl/min/g bw)				
Basal	0.07 ± 0.02	0.05 ± 0.01	0.10 ± 0.02	0.08 ± 0.01
Vol. Expansion	0.65 ± 0.07*	0.42 ± 0.10*#	0.62 ± 0.07*	0.33 ± 0.05*#

573 (GFR= Glomerular filtration rate; RPF= Renal Plasma Flow; FE_{Na} = Fractional excretion of
574 sodium; UV=; Urinary flow rate; * $p < 0.05$ vs. Basal; # $p < 0.05$ vs. Control).
575

576 **Table 3.** Renal hemodynamic response to Ang II infusion in 3-4 and 9-11 months old age rats
577 treated with vehicle (Control) or a COX2 inhibitor during nephrogenic period (COX2np).
578

Males	**3-4 months old**		**9-11 months old**	
	Control n = 9	**COX2np** n = 8	**Control** n = 7	**COX2np** n = 9
Renal Plasma Flow (ml/min/gr k.w.)				
Basal	4.72 ± 0.31	4.44 ± 0.20	4.89 ± 0.60	4.93 ± 0.64
Ang II	3.39 ± 0.18*	3.02 ± 0.17*	2.51 ± 0.28*	2.73 ± 0.22*
Glomerular Filtration Rate (ml/min/gr k.w.)				
Basal	1.30 ± 0.17	1.51 ± 0.20	1.30 ± 0.16	1.16 ± 0.11
Ang II	0.99 ± 0.09*	0.98 ± 0.17*	0.99 ± 0.12*	0.92 ± 0.09*

579

Females	**3-4 months old**		**9-11 months old**	
	Control n = 7	**COX2np** n = 6	**Control** n = 7	**COX2np** n = 6
Renal Plasma Flow (ml/min/gr k.w.)				
Basal	4.47 ± 0.76	4.35 ± 0.54	4.59 ± 0.35	4.16 ± 0.49
Ang II	3.21 ± 0.52*	3.09 ± 0.34	3.10 ± 0.21*	3.17 ± 0.35*
Glomerular Filtration Rate (ml/min/gr k.w.)				
Basal	1.33 ± 0.10	1.55 ± 0.13	1.24 ± 0.12	1.30 ± 0.08
Ang II	1.04 ± 0.08*	0.99 ± 0.08*	0.79 ± 0.09*	0.77 ± 0.18*

580 # $p < 0.05$ vs. Control; * $p < 0.05$ vs. Basal period; + $p < 0.05$ vs. 3-4 months of age.
581

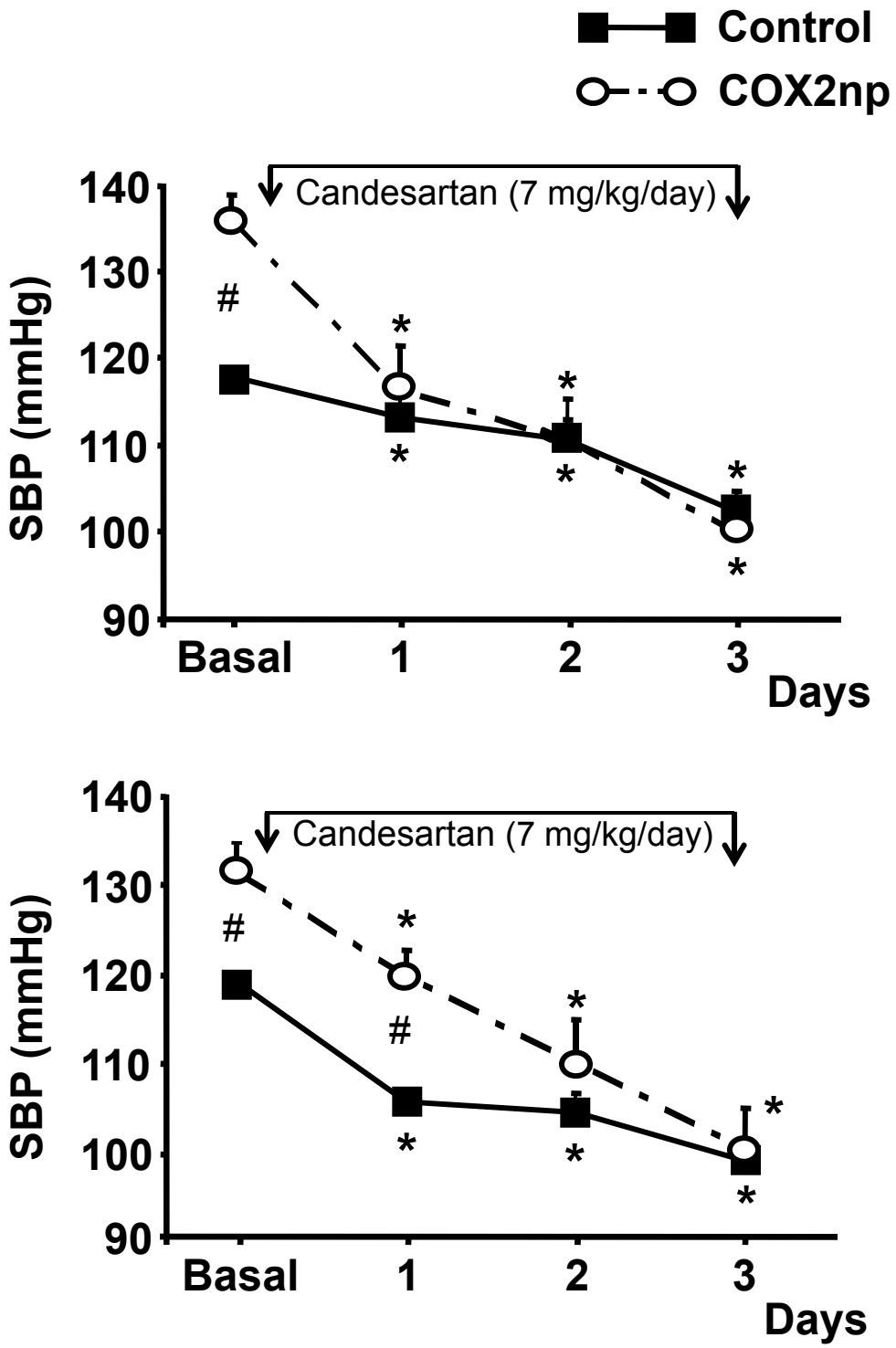
Control
COX2np
Candesartan (7 mg/kg/day)
SBP (mmHg)
140
130
120
110
100
90
Basal
1
2
3
Days
#
*

Figure 1

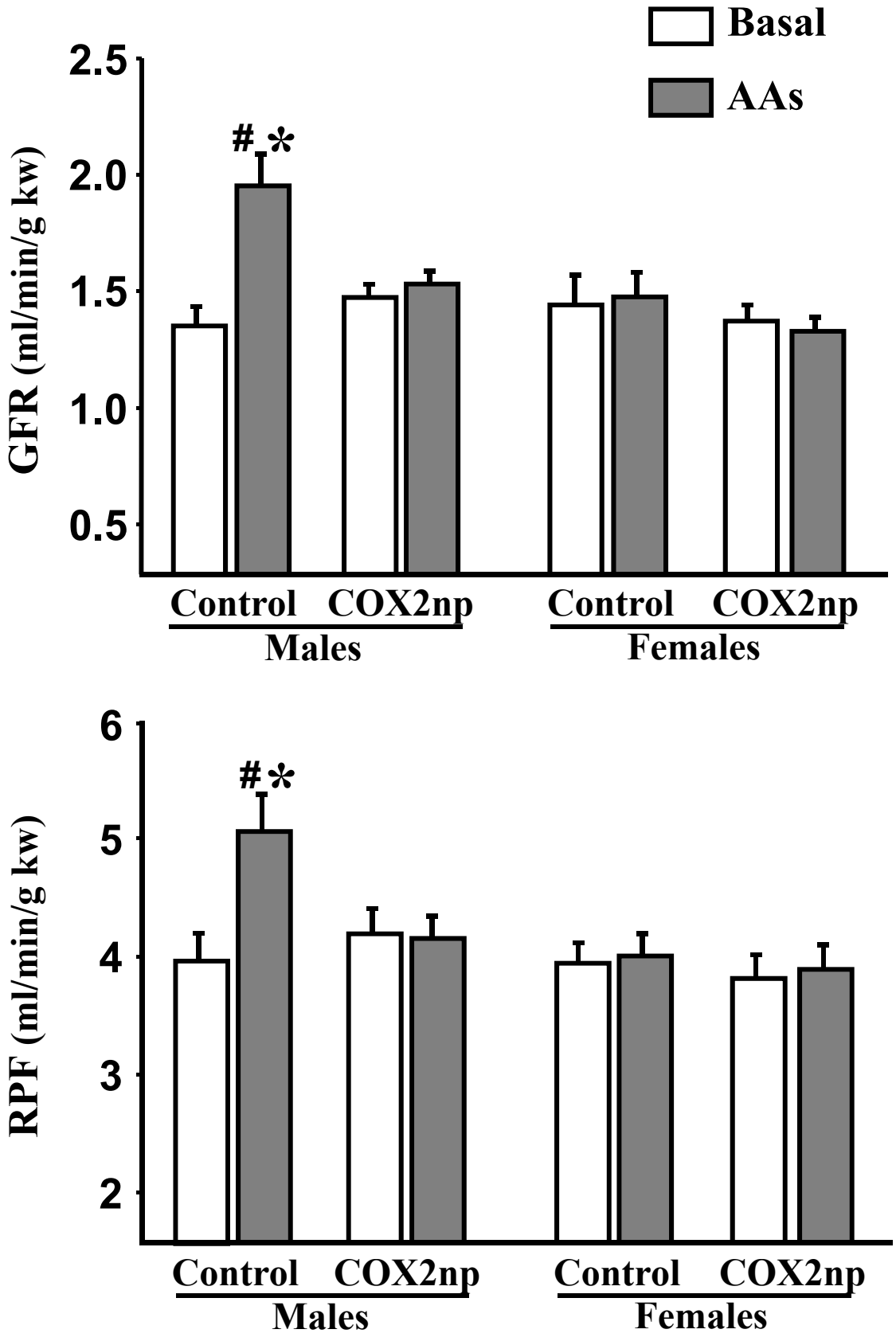
Basal
AAs
GFR (ml/min/g kw)
2.5
2.0
1.5
1.0
0.5
#*
Control
COX2np
Control
COX2np
Males
Females
RPF (ml/min/g kw)
6
5
4
3
2
#*
Control
COX2np
Control
COX2np
Males
Females

Figure 2

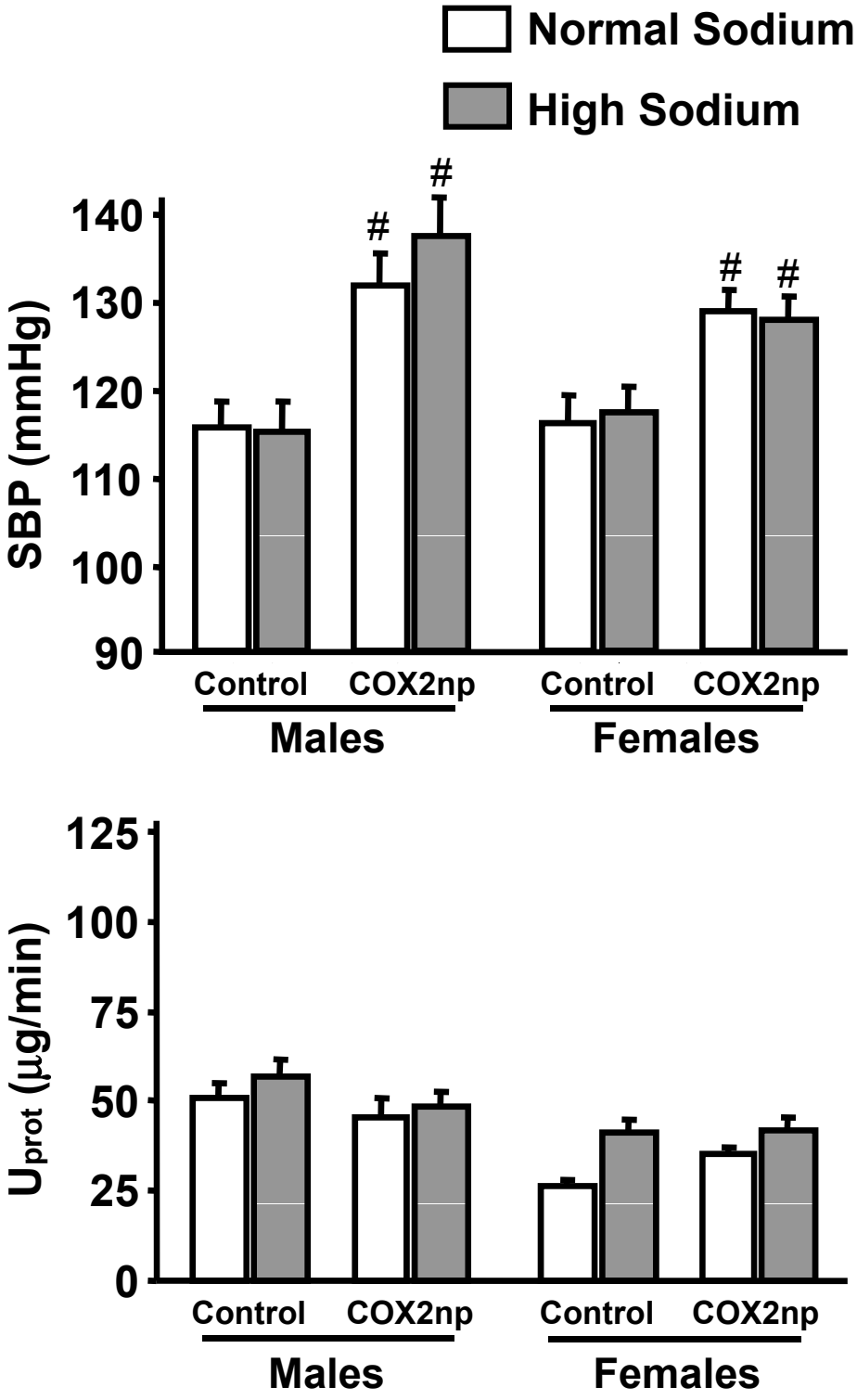
Normal Sodium
High Sodium
SBP (mmHg)
140
130
120
110
100
90
#
#
#
#
Control
COX2np
Control
COX2np
Males
Females
U_{prot} (µg/min)
125
100
75
50
25
0
Control
COX2np
Control
COX2np
Males
Females

Figure 3

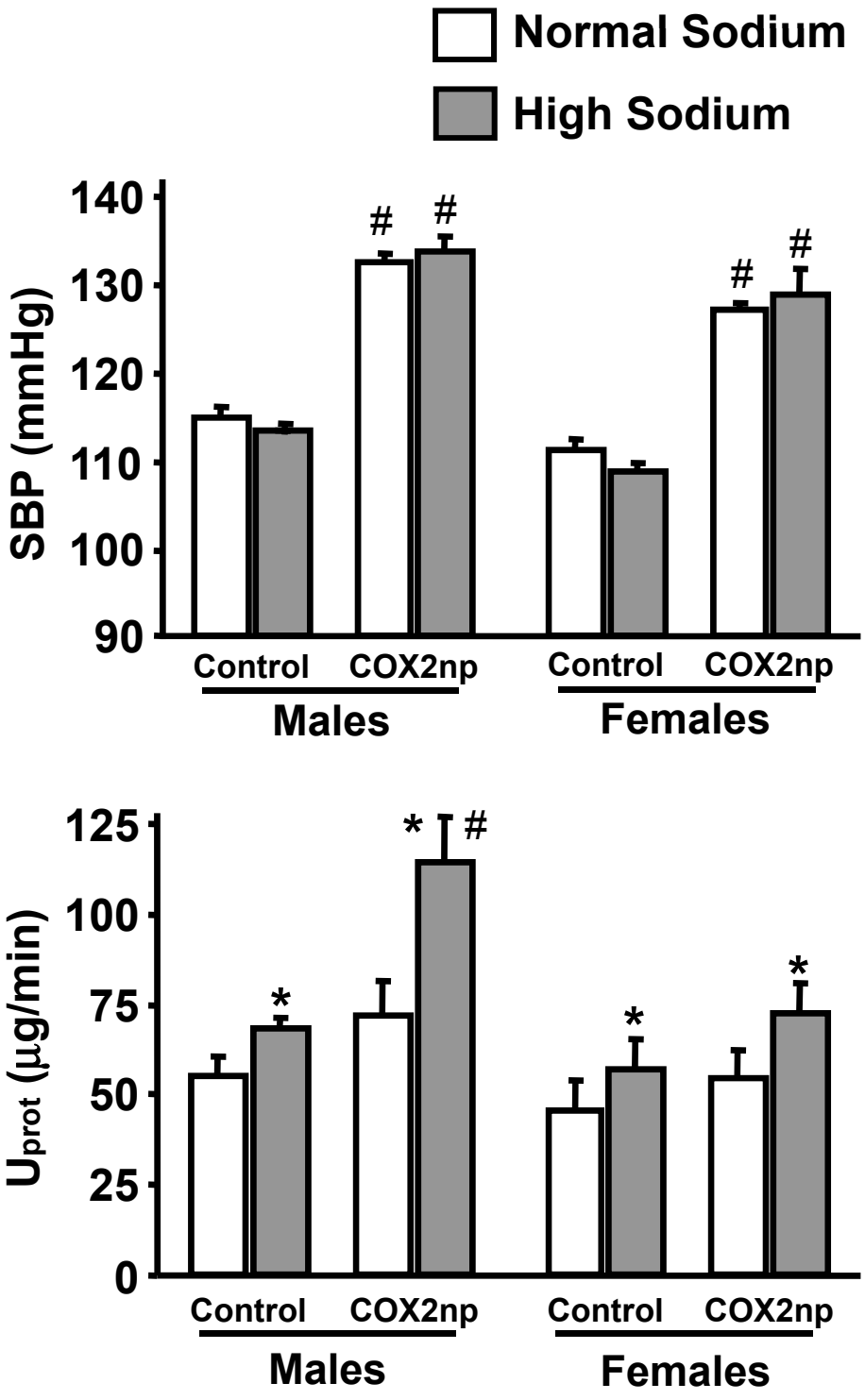
Normal Sodium
High Sodium
SBP (mmHg)
140
130
120
110
100
90
#
#
#
#
Control
COX2np
Control
COX2np
Males
Females
U_{prot} (µg/min)
125
100
75
50
25
0
*
*
#
*
*
Control
COX2np
Control
COX2np
Males
Females

Figure 4