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ROLE OF ANGIOTENSIN II ON ARTERIAL PRESSURE AND RENAL HEMODYNAMICS IN RATS WITH AN ALTERED RENAL DEVELOPMENT. AGE- AND SEX-DEPENDENT DIFFERENCES

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Running head: Age and sex dependent effects of Ang II on fetal programming

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ABSTRACT

Numerous studies have demonstrated that angiotensin II (AngII) is involved in the hypertension and renal changes occurring as a consequence of an adverse event during renal development. However, it was unknown whether this involvement is sex- and ageing-dependent. This study examines whether the increments in arterial pressure (AP) and in the renal sensitivity to AngII are sex- and ageing-dependent in rats with an altered renal development. It also evaluates whether the AngII effects are accompanied by increments in AT_1 receptors and oxidative stress. Experiments were performed in 3-4 and 10-11 months old rats treated with vehicle or an AT_1 receptor antagonist (ARAnp) during nephrogenic period. ARAnp-treated rats were hypertensive but an age-dependent rise in AP was only found in males. Three days treatment with candesartan (7mg/kg/day) led to a fall of AP that was greater ($P<0.05$) in male than in female 10-11 months old ARAnp-treated rats. Oxidated proteins were elevated ($P<0.05$) and the decrease in AP elicited by candesartan is reduced ($P<0.05$) when these rats are also treated with tempol (18 mg/kg/day). Hypertension was not maintained by an elevation of AT_1 receptors in kidneys and mesenteric arteries. Acute renal hemodynamic response to AngII (30 ng/kg/min) was similarly enhanced ($P<0.05$) in both sexes of ARAnp-treated rats at 3-4 but not at 10-11 months of age. Our results suggest that an adverse event during nephrogenic period induces an AngII-dependent increment in AP that is aggravated only in males during ageing and that oxidative stress but not an increase in AT_1 receptor contributes to the rise in AP. This study also shows that the renal hemodynamic sensitivity to AngII is transitorily enhanced in both sexes of rats with an altered renal development.

Key Words: Angiotensin II, sex- and ageing-dependent changes, fetal programming, hypertension, renal function, oxidative stress.

INTRODUCTION

It is well accepted that an adverse event during the perinatal period predisposes an individual to the development of hypertension and renal disease later in life. The mechanisms involved in the development of hypertension and renal disease have been examined in several models of fetal programmed hypertension. Some of these models, such as those induced by the administration of glucocorticoids or a low protein intake to the pregnant mother, or by the reduction in uterine perfusion, have in common a decrease of renin-angiotensin system (RAS) activity during renal development (7,11,47). The importance of this decrease in the development of hypertension and renal disease later in life is supported by studies in which a converting enzyme inhibitor (CEI) or an AT_1 receptor antagonist (ARA) is administered during nephrogenic period (7,17-19,32,34,38). The development of hypertension and renal disease in most of these experimental models is clearly sex-dependent (7,10,22,35,44).

Although other mechanisms seem to be also involved (7,10,27,29), the RAS plays an important role in the rise of arterial pressure (AP) and in the alterations of renal function that occur at adult age in the fetal programming models currently used (2,7,14,16,20,38). This involvement has been reported in studies showing that AP decreases to normal levels when a CEI or an ARA is administered (2,7,14,38), and showing that several components of the RAS are activated (2,15,20,37). One hypothesis tested in this study was that the importance of Ang II in maintaining hypertension is sex- and ageing-dependent in rats with an adverse event during renal development. It was also expected that the possible greater involvement of Ang II on AP elevation in males is accompanied by an increase of AT_1 receptor expression in resistance vessels, and by a greater increment in oxidative stress. Our hypothesis was based on studies showing that the Ang II effects increase with age (4), are modulated by sex hormones (5,9,26-28,30,48), and are mediated by changes in oxidative stress (31,49). An increase in the renal sensitivity to Ang II effects has also been demonstrated in male animals with fetal programmed hypertension (28,37). However, it was unknown whether this increase in the renal sensitivity to Ang II is sex-dependent and changes similarly in both sexes during ageing. It was

104 also unknown whether the renal hemodynamic effects of Ang II are accompanied by changes
105 in renal AT_1 receptor expression. Thus, one aim of this study was to examine whether there are
106 sex- and ageing-dependent differences in the renal sensitivity to Ang II in rats with an altered
107 renal development. It was also evaluated whether these possible differences in the renal
108 hemodynamic response to Ang II are associated to changes in the renal AT_1 receptor
109 expression or in the oxidative stress levels.
110
111 **MATERIAL AND METHODS**

112 Sprague Dawley (SD) rats were purchased from the University of Murcia Animal
113 Research Laboratory. The study was approved by the University review committee, and
114 experimental protocols were designed according to the NIH Guide and Use of Laboratory
115 Animals. Food (Harlan Teklad) and water were supplied ad libitum. Female SD rats (≈230 g
116 b.w.) were placed with a male, taking day 0 of pregnancy the morning that sperm evidence was
117 found in the vaginal smear. At postnatal day 0, litter size was fixed (8-10) in order to assure
118 similar nourishment during suckling period. New born rats were treated from postnatal day 1 to
119 postnatal day 14 with vehicle (isotonic saline) or candesartan at an oral dose of 7 mg/kg/day.
120 Thirty-eight pregnant rats gave rise to the 168 offspring used in this study at 3-4 and 10-11
121 months of age.

122 **Experimental protocols**

123 **Arterial pressure response to Candesartan (with and without tempol).** This ARA was
124 administered by gavage (7 mg/kg/day) during three days to conscious rats. Systolic arterial
125 pressure (SAP) was measured during basal period (three days) and three hours after each ARA
126 administration. Number of rats at 3-4 months of age was: 5 control males; 5 control females; 5
127 ARAnp-treated males; 5 ARAnp-treated females. Number of rats at 10-11 months of age was:
128 7 control males; 7 control females; 6 ARAnp-treated males; 7 ARAnp-treated females. In order
129 to examine whether the fall in SAP elicited by candesartan was related to the Ang II effects on
130 oxidative stress, another set of experiments was performed in which candesartan was
131 administered by gavage to rats already treated with a scavenger of superoxide anions, tempol

(39). After a basal period of three days, tempol was administered in the drinking water (18 mg/Kg/day) during seven consecutive days to 10-11 months old conscious rats. Four days after the initiation of tempol administration, candesartan was simultaneously given (7 mg/kg/day) during three days. SAP levels were measured during basal period (three days) and each day that tempol was given. Number of rats in each group was: 8 control males; 8 control females; 8 ARAnp-treated males; 7 ARAnp-treated females.

Arterial pressure was measured in conscious rats by the tail-cuff method as described (17,18,34,35,38) using a CODA 2 non-invasive system (Kent Scientific Corporation). In order to reduce the stress and to obtain an accurate reading, rats were first habituated during several days to the measurement device and to an ambient temperature of 30ºC for 10-15 min. Definitive measurements began when rats remained unperturbed into the chamber throughout the inflation-deflation cycles. The SAP values in each rat are the mean value of at least 10 measurements taken during 2-3 days. In previous studies (3.38), it was found that the SAP values obtained using the tail-cuff method are highly correlated with those obtained in conscious freely moving animals using other methods (radiotelemetry and intraarterial).

Changes in PRA, AT_1 receptor expression, and oxidated protein levels. Catheters were inserted into the femoral artery in anesthetized rats (isofluorane, Abbott) to obtain blood samples to analyze PRA and plasma concentration of oxidated proteins. Then, kidneys and guts were removed. Mesenteric arteries were obtained as previously indicated (24). Briefly, the mesenteric arterial tree was quickly dissected in ice-cold Krebs solution under a microscope, and resistance arteries (<300 μm in internal diameter) were collected and frozen on dry ice. Plasma samples and tissues were stored at –80ºC until analyzed. Plasma renin activity (PRA) was measured using a commercial RIA kit for Ang I (Diasorin). 5 to 7 rats were included in each experimental group.

AT_1 receptor expression in the kidney and mesenteric arteries was determined by Western blotting. The method used was similar to that previously described (8). Frozen tissues were homogenized in an ice-cold homogenizing solution (in mM: NaCl 150, NaF 30, EDTA 5,

159 Na2HPO4 15, Na pyrophosphate 15, Hepes 20), containing 1% Triton X-100 and protease
160 inhibitors (P8340, Sigma), using a polytron (kidney) or a pestle-mortar homogenizer
161 (mesenteric arteries). After sample protein concentrations were quantified by the BCA method
162 (Pierce), they were adjusted and dissolved in sample buffer and subjected to SDS-PAGE.
163 Samples were analyzed several times in different combinations to allow intra- and intergroup
164 comparisons. Proteins were transferred to PVDF membranes and probed with antibodies
165 specific to AT_1 receptors (Santa Cruz). Immunoblots were revealed by chemiluminescence
166 (WestDura, Pierce) and recorded with a CCD camera (G:Box, Syngene). Densitometric
167 analysis was performed to quantify receptor expression (GeneTools, Syngene).
168 Oxidative modification of total proteins was assessed by immunoblot detection of protein
169 carbonyl groups using the 'OxyBlot' protein oxidation kit (Millipore) following the
170 manufacturer's instructions. The procedure to quantify total protein carbonyls with this kit was
171 densitometry of the oxyblot and of the Ponceau staining, followed by finding the ratio between
172 the total density in the oxyblot and the total density in the Ponceau tinction.
173 **Renal hemodynamic response to acute Ang II infusion.** After overnight fasting, rats were
174 anesthetized with 0.1 ml i.m. of ketamine (Ketolar, Parke Davis, 100 $mg.ml^{-1}$) and 0.1 ml/100
175 g i.p. of penthobarbital (Pentothal, Abbott, 50 $mg.ml^{-1}$). After tracheotomy, catheters were
176 inserted into the bladder for collection of urine samples, and into the left femoral artery to
177 measure mean arterial pressure (MAP) (PowerLab, ADInstruments) throughout the experiment
178 and for blood withdrawal. Then, one catheter was implanted into the left femoral vein for i.v.
179 infusions. Rats were placed on a temperature-regulated surgical table to maintain stable their
180 body temperature. To stabilize hematocrit level after surgical stress, 1ml/100 g bw of 6% of
181 bovine serum albumin (Sigma) was infused. [^{3}H] inulin, (2 μCi/ml American Radiolabeled
182 Chemicals) was given as an i.v. bolus (1 ml) and as a continuous infusion (1.5 μCi/ml)
183 dissolved in isotonic saline (1 ml/100 g bw/hour). A transit-time flow probe (Transonic
184 System) was implanted on the left renal artery for renal blood flow (RBF) measurement. Renal
185 plasma flow (RPF) changes were calculated considering RBF and hematocrit values. A 70-min

stabilization period was allowed before experiments begun. Two 20-min basal clearance periods were followed by an i.v. infusion of captopril (10 ng/Kg/min). In previous experiments, it was found that this infusion does not modify glomerular filtration rate (GFR) but leads to an increment ($P<0.05$) of RPF in control (17,4 ± 1,5%) and ARAnp-treated (14,7 ± 2,0%) rats. An i.v. Ang II infusion (30 ng/Kg/min) was started 30 min after captopril administration begun. Fifteen minutes after initiating Ang II infusion, two more 20-min clearances were obtained. Renal Ang II effects were examined in captopril treated rats to discard that the differences found in these effects were secondary to differences in endogenous Ang II levels. GFR was measured by clearances of [^{3}H] inulin. Urine samples were collected into preweighed vials for [^{3}H] inulin measurements. Urine flow rate (UV) was determined gravimetrically. Blood samples were collected in heparinized capillaries 5 min before the end of each clearance period to measure plasma [^{3}H]inulin. Number of rats at 3-4 months of age was: 9 control males; 8 control females; 8 ARAnp-treated males; 8 ARAnp-treated females. Number of rats at 10-11 months of age was: 7 control males; 8 control females; 9 ARAnp-treated males; 9 ARAnp-treated females.

Statistical Analysis

Data in text, tables and figures are given as mean ± SE. Data from both clearances during each period 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

Arterial pressure response to Candesartan (with and without tempol). SAP pressure was higher ($P<0.05$) in male (135 ± 2 mmHg) and female (133 ± 1 mmHg) ARAnp-treated than in male (121 ± 1 mmHg) and female (115 ± 1 mmHg) control rats at 3-4 months of age. At this age, candesartan reduced ($P<0.05$) SAP to similar levels in ARAnp-treated and control rats, being the fall in SAP greater ($P<0.05$) in ARAnp-treated than in control rats. Arterial pressure increased significantly between 3-4 and 10-11 months of age in male (135 ± 2 to 167 ± 8

mmHg) but not in female (133 ± 1 to 139 ± 1 mmHg) ARAnp-treated rats. Figure 1 shows the response of SAP to candesartan in 10-11 months old rats. It can be observed that SAP was also enhanced ($P<0.05$) in ARAnp-treated rats with respect to the values found in control rats. Candesartan administration induced a fall of SAP in each group, but the decrements were greater ($P<0.05$) in ARAnp-treated than in control rats. A sex-dependent difference in the SAP response to candesartan was found in ARAnp-treated rats at 10-11 months of age since the fall in SAP was greater ($P<0.05$) in male (47 ± 7 mmHg) than in female (28 ± 5 mmHg) ARAnp-treated rats. Figure 2 shows the percent changes in SAP elicited by candesartan with and without tempol administration at 10-11 months of age. It can be observed that tempol did not induce significant changes of SAP in male (-4 ± 2 mmHg; -3 ± 2%) and female (-1 ± 2 mmHg; -1±2%) control rats but led to a similar fall ($P<0.05$) of SAP in male (-18 ± 4 mmHg; -10 ± 2%) and female (-13 ± 3 mmHg; -9 ± 2%) ARAnp-treated rats. Candesartan administration to tempol-treated rats elicited a significant fall in SAP. Figure 2 shows that the decreases in SAP elicited by candesartan alone or with the simultaneous administration of tempol were similar in male (-28 ± 5% and -29 ± 2%, respectively) and female (-20 ± 3% and -26 ± 3%, respectively) ARAnp-treated rats. The SAP response in both sexes of control rats to candesartan and tempol was also similar to that found after the administration of candesartan alone.

Preliminary studies performed in conscious control rats (n=5) demonstrated that the administration of candesartan (7 mg/kg/day) by gavage during three days reduced (> 80%) the SAP increment (27 ± 2 mmHg) induced by an i.v. Ang II infusion (30 ng/kg). It was also found that SAP does not change between days 4 and 7 when tempol is administered alone (18 mg/kg/day) in the drinking water during seven consecutive days (n=4 in each group).

Changes in PRA, AT_1 receptor expression and oxidated protein levels. PRA (in ngAngI/ml/hr) was similar in control (males: 7.5 ± 1.6; females: 7.7 ± 2.2) and ARAnp-treated (males: 7.5 ± 2.0; females: 7.1 ± 1.6) rats at 3-4 months of age. No significant differences in PRA were also found between groups at 10-11 months of age (control males: 6.3 ± 1.1; control females: 6.9 ± 2.9; ARAnp males: 5.0 ± 1.0; ARAnp females: 6.3 ± 1.0).

241 AT_1 receptor expression in mesenteric arteries and kidneys in each group of rats are
242 shown in figure 3. It shows that AT_1 receptor expression in mesenteric arteries is independent
243 of sex, age and treatment since no significant differences were found between groups. No
244 significant differences in the renal AT_1 receptor expression were also found between males and
245 females, and between control and ARAnp-treated rats, at 3-4 months of age. Contrary to what
246 was found in mesenteric arteries, age- and sex-dependent differences were found in the renal
247 AT_1 receptor expression. This renal expression only increased ($P<0.05$) in male rats between 3-
248 4 and 10-11 months, and was greater ($P<0.05$) in control than in ARAnp-treated male rats at
249 10-11 months of age. Renal AT_1 receptor expression was also greater ($P<0.05$) in control than
250 in ARAnp-treated female rats at this age. The sex-dependent difference at 10-11 months of age
251 in this AT_1 receptor expression was found in control (males: 6.1 ± 0.6 a.u.; females: 1.0 ± 0.1
252 a.u.) and ARAnp-treated (males: 2.7 ± 0.4 a.u.; females: 0.6 ± 0.0 a.u.) rats (figure 3).
253 Oxidated protein levels in plasma and renal tissue in 10-11 months old rat are shown in
254 figure 4. Plasma oxidated proteins levels were greater ($P<0.05$) in male than in female rats.
255 These levels were also elevated ($P<0.05$) in both sexes of ARAnp-treated rats with respect to
256 those found in their respective control group. Oxidated protein levels in the renal tissue are also
257 enhanced ($P<0.05$) in male and female ARAnp-treated rats. A significant sex-dependent
258 difference in the renal oxidated protein levels was found in hypertensive but not in
259 normotensive rats (figure 4).
260 **Renal hemodynamic response to an acute Ang II infusion.** Ang II led to an increment
261 ($P<0.05$) in MAP that was not statistically different in both groups of male (control: 11 ± 1
262 mmHg; ARAnp-treated: 15 ± 2 mmHg) and female (control: 8 ± 2 mmHg; ARAnp-treated: 13
263 $\pm$ 3 mmHg) rats at 3-4 months of age. Similar changes in MAP were found after Ang II
264 infusion at 10-11 months of age. Renal hemodynamic parameters during basal period were
265 similar in each group of female rats (table 1). However, basal RPF and GFR were reduced at 3-
266 4 and 10-11 months of age in ARAnp-treated male rats when compared to the values found in

control males. An age-dependent decrease ($P<0.05$) in basal GFR was observed in both groups of ARAnp-treated rats. Ang II infusion induced a fall of RPF and GFR in each group at both ages (table 1). This acute infusion led to a renal vasoconstriction that was greater in ARAnp-treated than in control rats at 3-4 months of age. The decrease in RPF and GFR was greater in ARAnp-treated (-45 ± 3% and -43 ± 9%, respectively) than in control (-28 ± 3% and -21 ± 3%, respectively) male rats. The fall in GFR and RPF was also greater in ARAnp-treated (-42 ± 2% and -44 ± 8%, respectively) than in control (-28 ± 4% and -21 ± 5%, respectively) female rats. The difference in the renal response to Ang II at 3-4 months of age is also evident when renal vascular resistance (RVR) changes are examined (figure 5). The Ang II-induced increment in RVR was greater ($P<0.05$) in ARAnp-treated (males: 19 ± 2 mmHg/ml/min; females: 17 ± 2 mmHg/ml/min) than in control (males: 8 ± 1 mmHg/ml/min; females: 11 ± 2 mmHg/ml/min) rats. The acute Ang II infusion also elicited a fall ($P<0.05$) in RPF and GFR in each group of rats at 10-11 months of age but, contrary to what was found at the youngest age, the hemodynamic response was similar in ARAnp-treated and control rats in the oldest rats (table 1). The fact that the renal vasoconstriction elicited by Ang II was similar in these rats is also evident when the changes in RVR are examined. It can be observed in figure 5 that the Ang II-induced increment in RVR was similar in these rats (control males: 14 ± 3 mmHg/ml/min; ARAnp males: 13 ± 2 mmHg/ml/min; control females: 11 ± 1 mmHg/ml/min; ARAnp females: 9 ± 2 mmHg/ml/min) at 10-11 months of age.

DISCUSSION

The importance of Ang II in maintaining the hypertension and renal changes secondary to an adverse event during renal development has been examined in several previous studies (2,7,14-16,20,37,38). However, this is the first study examining whether there are sex and age differences in the role that Ang II plays in the hypertension secondary to an adverse event during renal development and whether this hypertension is associated to changes in AT_1 receptor expression in resistance vessels and renal tissue and changes in oxidative stress. This

is also the first study that has evaluated whether there are sex- and age-dependent differences in the renal sensitivity to an acute Ang II infusion in animals with a decrease of Ang II effects during renal development. The role of Ang II in mediating the hypertension and renal changes secondary to an alteration in renal development has been examined in adult and middle-age rats by evaluating the: A) response to the three days administration of candesartan; B) changes in PRA, and AT_1 receptor expression in mesenteric arteries and renal tissue; and C) renal hemodynamic response to an acute Ang II infusion. The importance of oxidative stress was examined by measuring the levels of one oxidative stress marker, and by comparing the AP response to candesartan with and without the simultaneous administration of tempol.

The AP and renal hemodynamic results obtained in ARAnp-treated rats before any experimental maneuver are similar to those previously reported (18,32,38) and confirm that there are important sex differences in the age-dependent increment in AP and in the renal hemodynamic changes when there is an adverse event during renal development. These changes found in ARAnp-treated rats may be secondary to a 37% decrease in nephron number that is similar in males and females (34), but also to other renal changes elicited by the reduction of Ang II effects during the nephrogenic period (19). Sex-dependent differences in the cardiovascular response to an adverse fetal environment have been reported using different models of fetal programming of hypertension (7,10,22,34,35,44). However, so far it was unknown whether the importance of Ang II in maintaining hypertension and in the regulation of renal hemodynamic is sex-dependent during ageing. One hypothesis tested in this study was that this role of Ang II would be age- and sex-dependent. This hypothesis was based on studies showing that Ang II is involved in maintaining the hypertension secondary to an adverse event during renal development (2,7,12,14,16,20,38), and on studies demonstrating that the role of Ang II in the regulation of cardiovascular and renal function increases with age (4). It was also known that the vasoconstrictor Ang II effects are modulated by sex hormones (31,52). The results obtained confirmed our hypothesis and present new evidence suggesting that the Ang II-AT_1 receptor axis is progressively activated in males ARAnp-treated rats and that it is

involved in the greater age-dependent increment of AP found in males. Only one previous study had examined the AP changes elicited by reducing the Ang II effects in both sexes of adult animals with a fetal programming model of hypertension (20). However, Manning and Vehaskari (20) did not examine whether the importance of RAS is sex-dependent at different ages because a CEI was continuously administered from the second to the tenth month of life.

This is the first study testing the hypothesis that the greater Ang II-dependence of AP in males is related to a different Ang II-dependent activation of oxidative stress in both sexes of animals with an adverse event during renal development. This hypothesis was based on studies showing that renal oxidative stress is enhanced in young offspring of mothers consuming a low protein diet during pregnancy (42), and that renal oxidative stress is enhanced in male but not in female adult rats submitted to an intrauterine growth restriction (29). It was also known that the Ang II effect on oxidative stress is enhanced in adult sheep exposed to glucocorticoids in utero (12). Our hypothesis was also supported by studies demonstrating that oxidative stress mediates the vasoconstrictor effects of Ang II (49), and that the Ang II-dependent activation of NADPH oxidase is reduced by estrogens (9) and stimulated by androgens (31). The oxidative status in our study was examined by measuring the levels of one marker of oxidative stress and the AP response to a scavenger of superoxide anions such as tempol. Taking together the results obtained (figures 2 and 4), it is proposed that an increment in oxidative stress plays an important role in maintaining hypertension secondary to an alteration of nephrogenesis during late nephrogenic period. The effect of oxidative stress on AP seems to be Ang II-dependent since the fall in AP elicited by candesartan is similar with and without the prolonged administration of tempol (figure 2). However, and contrary to what was expected, the fall in AP elicited by tempol was similar in both sexes of ARAnp-treated rats. Therefore, despite the different levels of oxidated proteins found in male and female ARAnp-treated rats (figure 4), oxidative stress does not seem to contribute to the sex-differences in the Ang II-dependent hypertension found in ARAnp-treated rats. The hypothesis that oxidative stress does not

347 account for the sex differences in the AP response to Ang II is supported by the results
348 obtained by Schneider et al (43) in mice during a 15 seconds infusion of Ang II.

349 In this study it has been examined whether the role of the RAS in maintaining
350 hypertension is secondary to an increase in PRA. No significant changes and up- or
351 downregulation of several components of the RAS have been reported in other experimental
352 models in which the hypertension is secondary to manipulations that reduce RAS activity
353 during nephrogenic period (2,14,46). Contradictory data have also been reported when the age-
354 dependent changes in PRA have been examined in male animals with fetal programmed
355 hypertension (13,20). The contradictory data may be explained by the multiplicity of models
356 employed and the sex and age at which the studies were performed. One possibility tested was
357 that PRA is greater in males than in females since it has been shown that PRA levels are
358 modulated by sex hormones (30). The results reported in this study were obtained in samples
359 collected from anesthetized animals, as in other previous studies (2,11,20,46), and show that
360 PRA is not elevated in ARAnp-treated rats when compared to the PRA values found in control
361 rats. However, our results suggest that the regulation of PRA is affected in these ARAnp-
362 treated rats because PRA levels are inappropriately elevated, since the elevation in AP should
363 be accompanied by a decrease in renin release (25).

364 To the best of our knowledge, this is the first study that has investigated whether the
365 increment in AP found in models of fetal programming of hypertension can be explained by
366 changes of AT_1 receptor expression in extrarenal resistance vessels, and whether this
367 expression is sex- and age-dependent in normotensive animals. The results obtained show that
368 AT_1 receptors expression in mesenteric arteries is similar in control and ARAnp-treated rats of
369 both sexes and at both ages examined. Therefore, these results suggest that the age- and sex-
370 dependent increment of AP that occurs as a consequence of a decrease of Ang II effects during
371 renal development is not secondary to an AT_1 receptor increment. The hypertension found in
372 ARAnp-treated rats is most probably secondary to an age- and sex-dependent increment of
373 Ang II sensitivity in resistance vessels. Sex differences in the AP response to the AT_1 receptor

antagonist at 10-11 but not at 3-4 months of age could also be explained by different levels of AT_2 receptor expression in both sexes of ARAnp-treated rats (21,22).

This study has examined whether the renal sensitivity to an acute increment in Ang II is similarly enhanced in both sexes of adult animals with an adverse renal development. It has been reported that the renal hemodynamic effects elicited by Ang II are enhanced in these adult male animals (28,37) but it was unknown whether this increase in renal Ang II sensitivity is sex-dependent. The hypothesis was that there is a sex-dependency in the greater renal sensitivity to Ang II when there is an alteration in renal development, since it has been shown that testosterone mediates the greater renal sensitivity to Ang II in young male rats submitted to a growth restriction during fetal development (28). It is also known that the Ang II-induced vasoconstriction is modulated by estrogens (26).

The results obtained suggest that renal sensitivity to Ang II is similar in both sexes of adult normotensive rats. Only one previous study has examined whether there are sex-dependent differences in the renal response to Ang II in normotensive animals (40). That study showed that the Ang II-induced increment in RVR was greater in male than in female mice. The discrepancy between the results found in both studies may be related to the fact that Ang II was infused in that previous study during only 15 seconds in mice not pretreated with a CEI. The absence in the renal vessels of a greater vasoconstrictor effect of Ang II in males has also been demonstrated in studies performed in humans (6,23). In fact, these studies showed that the renal vasoconstrictor effects of Ang II are greater in women than in men. Our study also reports novel findings suggesting that the renal hemodynamic response to an acute Ang II infusion does not change in both sexes between an adult and middle age in normotensive rats. The possible aging-dependent change in the renal response to Ang II has only been examined in male normotensive rats (1,45,53). Some of these studies also reported that the magnitude of the renal vasoconstrictor response to Ang II is not affected by age (1,53). The renal hemodynamic responses to Ang II found in our study (table 1) do not fit well with the evidence that the renal AT_1 receptor expression increases with age in male and does not change in

401 female normotensive rats (figure 3). The observed renal AT_1 receptor expression confirm the
402 results obtained in adult male and females normotensive mice (40), and in normotensive male
403 rats at different ages (41,50). However, this is the first study that has examined the ageing-
404 dependent changes of renal AT_1 receptor expression in females. A sex difference in the renal
405 activation of signaling pathways downstream of the Ang receptors (43) could contribute to the
406 observed renal response to Ang II. The different evolution of renal AT_1 receptor expression in
407 both sexes was expected, since it has been shown that estrogens and androgens modulate renal
408 AT_1 receptor expression (5,26,33). The sex-dependent difference in renal AT_1 receptors may
409 be related to the fact that female rats exhibit less age-related renal disease (1).
410 The results of this study demonstrated that the renal hemodynamic response to Ang II is
411 enhanced in both sexes of adult rats with an altered nephrogenesis (table 1, figure 5). However,
412 and contrary to what expected, the renal response was similarly enhanced in both sexes of
413 ARAnp-treated rats. This study also examined whether the greater renal sensitivity to an acute
414 Ang II infusion in ARAnp-treated rats can be explained by an increment in renal AT_1 receptor
415 expression. Contradictory findings have been reported in studies that have examined the
416 changes in renal AT_1 receptor expression at an early (≤ 1 month) (21,22,36,47) or adult (≤ 5
417 months) (10,22) age in animals with different models of fetal programming of hypertension.
418 Our results showing that the renal AT_1 receptor expression is similar in normotensive and
419 hypertensive rats and also similar in both sexes of these rats at 3-4 months of age, confirm
420 those reported in the only previous study that has examined whether there are sex-dependent
421 differences in the renal AT_1 receptor expression in animals with fetal programmed
422 hypertension (22). These results suggest that the enhanced renal hemodynamic effects are not
423 secondary to an increase in total AT_1 receptor expression. However, it can not be discarded that
424 receptors number is enhanced in each glomeruli since total renal AT_1 receptor expression is
425 normal but the number of glomeruli is reduced by 37% in ARAnp-treated rats (34). In support
426 of the hypothesis that the glomerular AT_1 receptor expression is enhanced in ARAnp-treated
427 rats, it has been shown that these rats have a glomerular hypertrophy (34) and that larger

428 glomeruli express more surface receptors (50). It is also possible that the greater renal
429 hemodynamic response to the acute Ang II infusion is secondary to an enhanced affinity and/or
430 activity of glomerular AT_1 receptors to Ang II (37). Finally, it is possible that the enhanced
431 renal vasoconstrictor response to Ang II is secondary to a fall in AT_2 receptors. In this regard,
432 it has been reported that the AT_2 receptor expression is reduced and the ratio AT_1/AT_2 is
433 enhanced, in male rats in which RAS activity decreased during nephrogenic period (21,37).

434 As previously mentioned, there are no studies evaluating whether the enhanced renal
435 sensitivity to Ang II is maintained during ageing in both sexes of animals with fetal
436 programmed hypertension, and whether this possible increment in the renal response to Ang II
437 is accompanied by similar changes in renal AT_1 receptor expression. The proposed hypothesis
438 was that the renal Ang II sensitivity would be even greater at 9-11 than at 3-4 months of age in
439 ARAnp-treated rats and that the renal AT_1 receptor expression would also be greater at the
440 oldest age. The results obtained only partly confirm our hypothesis since they suggest that the
441 renal AT_1 receptor sensitivity seems to be enhanced in these hypertensive rats at 10-11 months
442 of age. It was found that the acute Ang II infusion led to a similar renal vasoconstriction in
443 normotensive and hypertensive rats (table 1, figure 5), despite renal AT_1 receptor expression
444 was lower in hypertensive than in normotensive rats (figure 3). It is obvious that the renal
445 hemodynamic responses to Ang II can not be easily explained by the observed changes in AT_1
446 receptor expression since this expression was lower in hypertensive than in control rats, and
447 greater in male than in female ARAnp-treated rats. A postreceptor mechanism could be
448 involved in the observed sex-dependent differences in the renal Ang II effects (43). Further
449 studies are needed investigating the sex-dependent mechanisms involved in the renal response
450 to Ang II during ageing. It also remains to be investigated why renal AT_1 receptor expression is
451 lower in hypertensive rats with an adverse event during renal development than in control rats.
452 One possibility could be that the renal AT_1 receptor expression decrease in response to a high
453 local Ang II concentration (51). However, high local Ang II concentration does not seem to be
454 the explanation to the decrease of renal AT_1 receptors in ARAnp-treated rats because the renal

vasodilatation in response to a CEI in our study was similar to that found in control rats. Other possible explanation for the lower renal AT_1 receptor expression in ARAnp-treated rats is the long-term increment of renal perfusion pressure in the hypertensive rats.

In summary, this study present novel findings suggesting that a decrease of Ang II effects during nephrogenic period induces an Ang II-dependent increment in arterial pressure later in life that is significantly greater in males than in females. This hypertension is secondary to an increase in oxidative stress but not in AT_1 receptor expression in resistance vessels or renal tissue. In addition, this study reveals for the first time that the renal hemodynamic response to Ang II is similarly enhanced during adult age in males and females with an adverse event during renal development, despite the renal AT_1 receptor expression is similar to that found in the control group. Novel findings are also reported showing that the renal hemodynamic response to Ang II is only transitorily enhanced in animals with an adverse event during renal development and that renal AT_1 receptor expression is lower in hypertensive than in normotensive animals during ageing. The results reported in this study may have pathophysiological implications since RAS activity decreases during renal development as a consequence of the administration of glucocorticoids or a low protein intake to the pregnant mother, or as a consequence of placental insufficiency (7,11,47).

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Disclosures

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

Author Contributions

Most of the experiments performed in this study have been performed by Virginia Reverte with a very important collaboration of Antonio Tapia. Ignacio Gímenez and Goretti Baile contributed carrying out, analyzing and interpreting the AT_1 receptor studies. Juan Gambini contributed carrying out the studies to determine the oxidated protein levels in plasma and renal tissue. M. Teresa Llinás and F. Javier Salazar contributed in the design of the experiments performed and in the interpretation of the results obtained. Each author also contributed in the writing of this manuscript.

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638

FIGURE LEGENDS.

Figure 1. Systolic arterial pressure changes elicited by the three days administration of an AT_1 receptor antagonist to conscious 10-11 months old control and ARAnp-treated rats. * P<0.05 vs. Basal period. # P<0.05 vs. Control group.

Figure 2. Percent changes in systolic arterial pressure elicited by the prolonged administration of candesartan and/or tempol to conscious 10-11 months old control and ARAnp-treated rats. * P<0.05 vs. Basal period. # P<0.05 vs. Control group.

Figure 3. AT_1 receptor expression (arbitrary units) in mesenteric arteries and kidneys of control and ARAnp-treated rats of both sexes, and at 3-4 and 10-11 months of age. * P<0.05 vs. female rats. # P<0.05 vs. Control group. + P<0.05 vs. 3-4 months of age.

Figure 4. Oxidated proteins levels (arbitrary units) in plasma and kidneys of 10-11 months old control and ARAnp-treated rats. * P<0.05 vs. female rats. # P<0.05 vs. Control group.

Figure 5. Increments in renal vascular resistance elicited by angiotensin II in control and ARAnp-treated rats of both sexes, and at 3-4 and 10-11 months of age. # P<0.05 vs. Control group.

660

661 **Table 1.** Renal hemodynamic response to Ang II infusion in 3-4 and 10-11 months old age rats

662 treated with vehicle (control) or ARA (ARAnp) during nephrogenic period. # $p < 0.05$ vs.

663 Control group; ∗ $p < 0.05$ vs. Basal period; + $p < 0.05$ vs. 3-4 months of age.

664

Males	**3-4 months old**		**10-11 months old**	
	Control	**ARAnp**	**Control**	**ARAnp**
Renal Plasma Flow (ml/min/gr k.w.)				
Basal	4.72 ± 0.31	3.07 ± 0.23#	4.98 ± 0.72	3.15 ± 0.38#
Ang II	3.39 ± 0.18*	1.70 ± 0.16#*	2.61 ± 0.26*	2.12 ± 0.30*
Glomerular Filtration Rate (ml/min/gr k.w.)				
Basal	1.30 ± 0.17	0.89 ± 0.08#	1.36 ± 0.17	0.56 ± 0.06#+
Ang II	0.99 ± 0.09*	0.52 ± 0.10#*	1.05 ± 0.14*	0.42 ± 0.06#*

665

666

Females	**3-4 months old**		**10-11 months old**	
	Control	**ARAnp**	**Control**	**ARAnp**
Renal Plasma Flow (ml/min/gr k.w.)				
Basal	4.47 ± 0.76	4.92 ± 0.24	4.97 ± 0.47	4.53 ± 0.19
Ang II	3.21 ± 0.52*	2.86 ± 0.17*	3.15 ± 0.29*	3.16 ± 0.19*
Glomerular Filtration Rate (ml/min/gr k.w.)				
Basal	1.33 ± 0.10	1.21 ± 0.06	1.19 ± 0.10	1.02 ± 0.05+
Ang II	1.04 ± 0.08*	0.65 ± 0.07#*	0.72 ± 0.08*	0.60 ± 0.05*

667

668

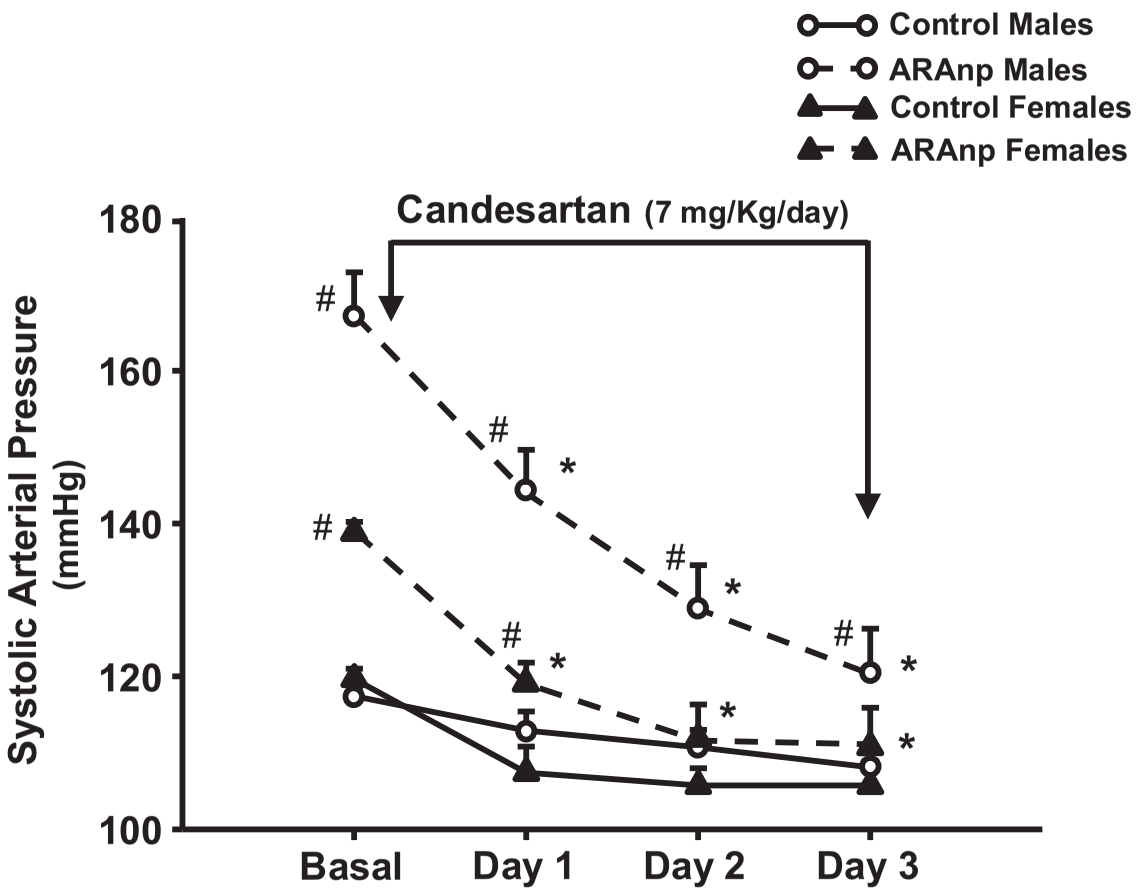
Control Males
ARAnp Males
Control Females
ARAnp Females
Candesartan (7 mg/Kg/day)
Systolic Arterial Pressure (mmHg)
180
160
140
120
100
Basal
Day 1
Day 2
Day 3
#
*

Figure 1

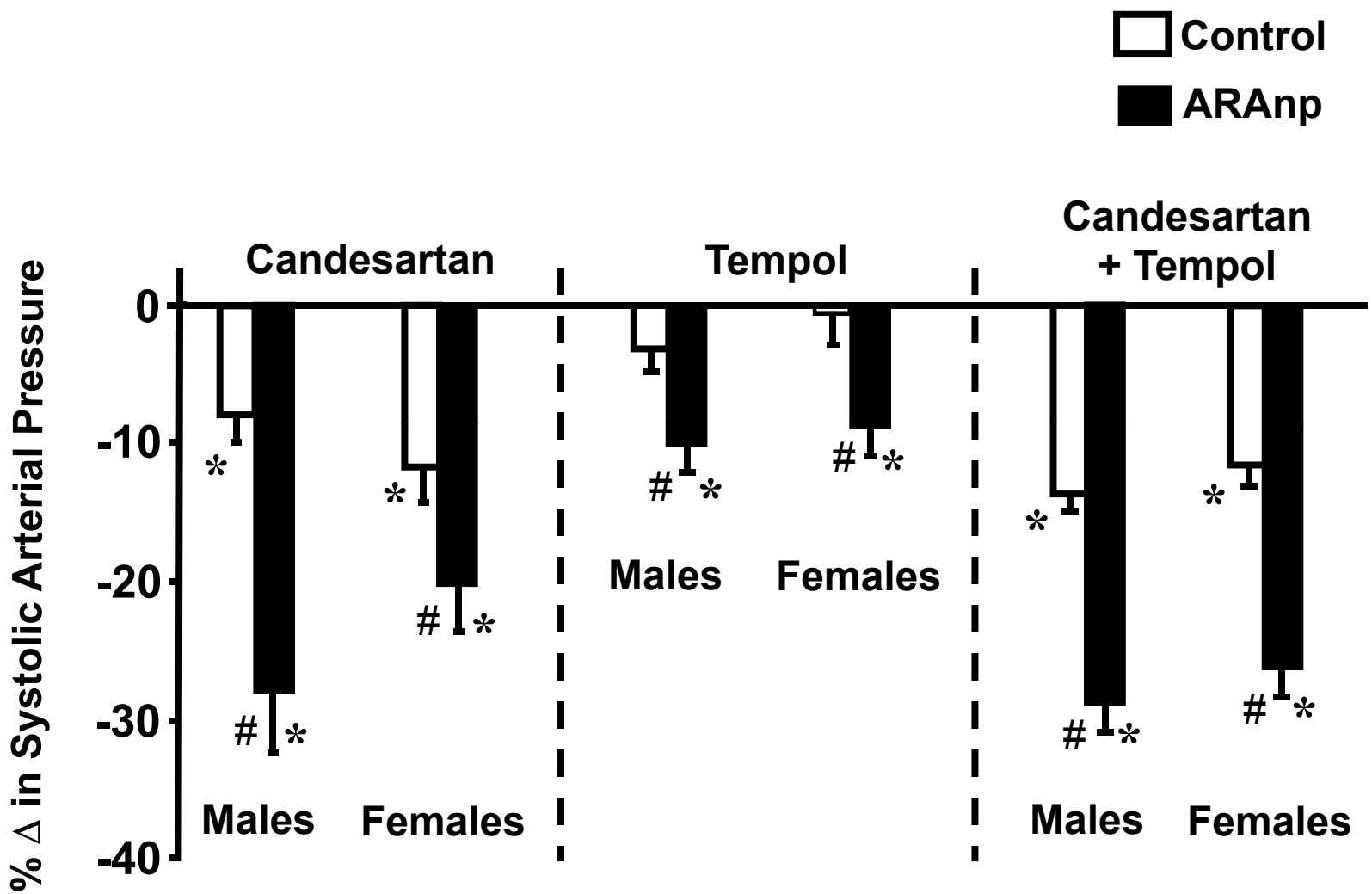
Control
ARAnp
Candesartan
Tempol
Candesartan + Tempol
% Δ in Systolic Arterial Pressure
0
-10
-20
-30
-40
Males
Females
Males
Females
Males
Females
*
#
*

Figure 2

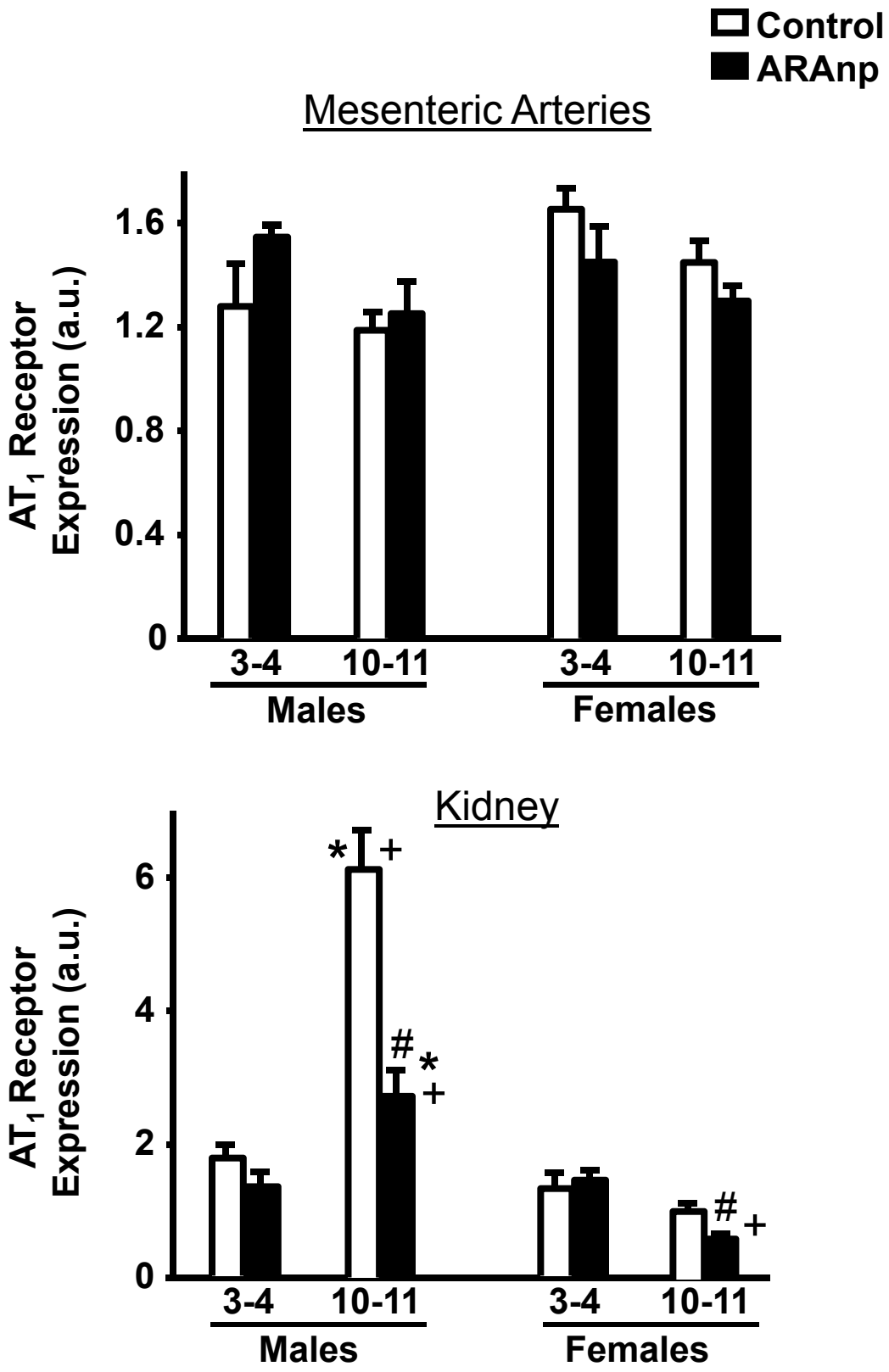
Control
ARAnp
Mesenteric Arteries
AT1 Receptor Expression (a.u.)
1.6
1.2
0.8
0.4
0
3-4
10-11
3-4
10-11
Males
Females
Kidney
AT1 Receptor Expression (a.u.)
6
4
2
0
*
+
#
*
+
#
+
3-4
10-11
3-4
10-11
Males
Females

Figure 3

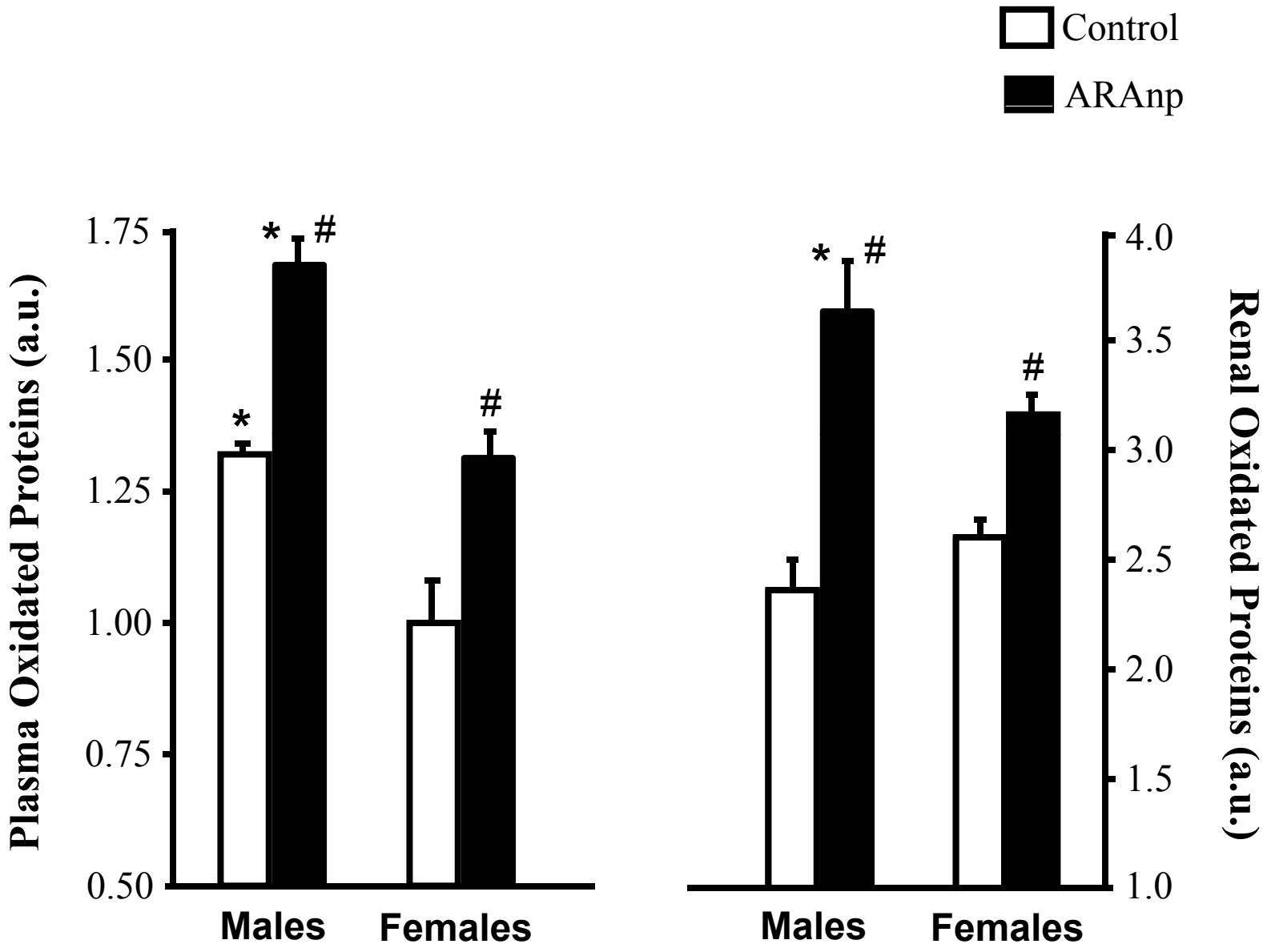
Control
ARAnp
Plasma Oxidated Proteins (a.u.)
Renal Oxidated Proteins (a.u.)
1.75
1.50
1.25
1.00
0.75
0.50
4.0
3.5
3.0
2.5
2.0
1.5
1.0
*
#
Males
Females

Figure 4

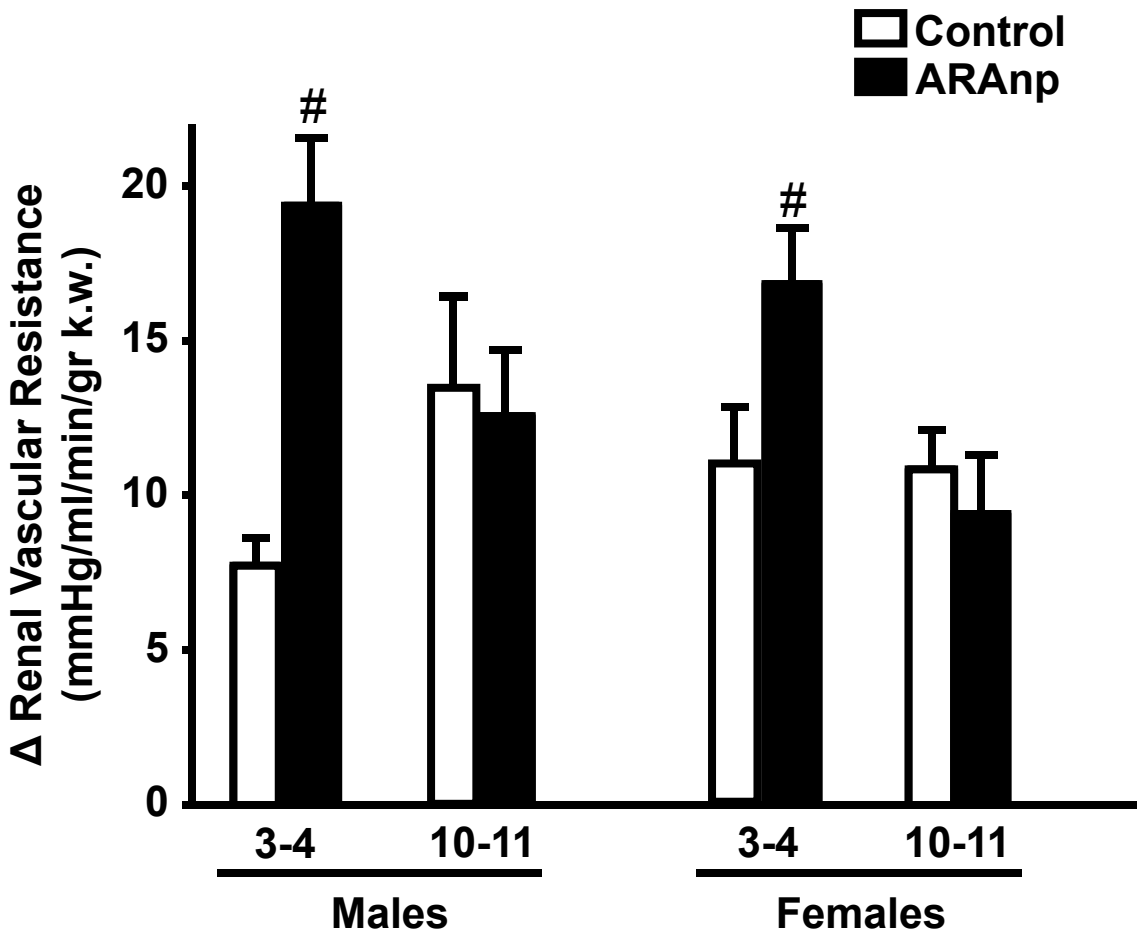
Control
ARAnp
Δ Renal Vascular Resistance
(mmHg/ml/min/gr k.w.)
20
15
10
5
0
#
#
3-4
10-11
3-4
10-11
Males
Females

Figure 5