Renal effects of prolonged high protein intake and COX2 inhibition on hypertensive rats with altered renal development

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MATERIALS AND METHODS

Sprague-Dawley (SD) rats were purchased from the University of Murcia Animal Research Laboratory. The study was approved by the University Review Committee, and experimental protocols were designed according to the National Institutes of Health Guide to the Care and Use of Laboratory Animals. Rats were housed in rooms with controlled temperature (23–24°C) and a 12:12-h light-dark cycle. Food with normal sodium (0.1%) and protein (14%) content (Global diet 2014, Harlan Teklad) and water were supplied ad libitum. Female SD rats (≈230 g body wt) were placed with males, taking day 0 of pregnancy the morning that sperm was found in the vaginal smear. On postnatal day 0, litter size was fixed (8–10) to ensure similar nourishment during the suckling period. Newborn rats were treated from postnatal day 1 to postnatal day 14 with vehicle (isotonic saline) or an ARA (L-158.809, Merck Sharp&Dohme) at an oral dose of 7
mg/kg−1·day−1. Thirty-five pregnant rats gave rise to the 152 offspring used in this study.

Experimental Protocols

Renal effects of COX2 inhibition. At 3–4 and 10–11 mo of age, rats were anesthetized with pentobarbital sodium and ketamine, tracheotomized with a polyethylene catheter, and their body temperature was maintained constant throughout the experiment. Catheters were inserted into the bladder for urine collections, into the left femoral artery to measure arterial pressure (PowerLab, ADInstruments) and for blood withdrawal, and into the left femoral vein for intravenous (iv) infusions. A blood sample was collected for basal hematocrit measurement. To stabilize hematocrit levels after surgical stress, a solution of 1 ml/100 g BSA (6%; Sigma) was administered. [3H]inulin (1.5 μCi/ml; American Radiolabeled Chemicals) was given as a continuous iv infusion dissolved in isotonic saline (3 ml/h). The glomerular filtration rate (GFR) was measured by clearances of [3H]inulin. These clearances were normalized by kidney weight. A transit-time flow probe (0.7PSB282, Transonic System) was implanted in the left renal artery for measurement of renal blood flow (RBF). Renal plasma flow (RPF) changes were calculated considering RBF and hematocrit values. A 60-min stabilization period was allowed before experimental maneuvers were started.

Two twenty-minute basal clearance periods were followed by the iv administration of nimesulide at a dose (3 mg/kg) that is effective in the selective COX2 inhibition (9, 16). Thirty minutes after nimesulide infusion was initiated, two 30-min clearances were obtained. Data for the two clearances obtained during the basal period and nimesulide administration were averaged for statistical comparisons. Urine samples were collected into preweighed vials for measurements of urine flow rate (UV) and [3H]inulin. Plasma samples were collected in heparinized capillaries 5 min before the end of each clearance period to measure [3H]inulin. UV was determined gravimetrically. The number of rats in each group at 3–4 mo of age was as follows: n = 8 control males; n = 7 control females; n = 8 ARAnp-treated males; and n = 7 ARAnp-treated females. The number of rats in each group at 9–11 mo of age was as follows: n = 9 control males; n = 8 control females; n = 8 ARAnp-treated males; and n = 9 ARAnp-treated females.

Response to changes in protein intake. At 3–4 or 10–11 mo of age, normal protein intake (NPI; 14% casein; TD.00168, Harlan Teklad) was supplied for 9 days. Seventy-two hours after they started this NPI, rats were introduced in metabolic cages and after 3 days of adaptation, urine samples were collected during 3 consecutive days to evaluate changes in UV, GFR, and proteinuria during 24-h periods. Then, protein diet increased (40% casein; TD.90018, Harlan Teklad) for 28 days. At the end of this period, rats were maintained in metabolic cages for 6 days and UV, GFR, and proteinuria were determined for the last 3 days of NPI. Blood samples from the tail were obtained on the last day of NPI and the last day of HPI. Systolic blood pressure (SBP) was measured on the last day of NPI and at the end of HPI. The number of rats in each group at 3–4 mo of age was as follows: control males (n = 6); control females (n = 6); ARAnp-treated males (n = 6); ARAnp-treated females (n = 6). The number of 11–12 mo old rats in each group was as follows: control males (n = 6); control females (n = 6); ARAnp-treated males (n = 9); ARAnp-treated females (n = 10).

Blood pressure was measured in conscious rats by the tail-cuff method as described (11, 21, 22, 24) using the CODA 2 noninvasive system (Kent Scientific). To obtain an accurate reading, rats were first habituated to the measurement device. Definitive measurements began when rats remained unperturbed in the chamber throughout the inflation-deflation cycles. The SBP values in each rat are the mean value of at least 10 measurements. In previous studies (4, 24), it was found that the SBP values obtained using the tail-cuff method are highly correlated with those obtained in conscious freely moving animals using other methods (radiotelemetry and intra-arterial measurement). The filtration rate was determined by endogenous creatinine clearance. This method has been used by our group in conscious rats, and the values of GFR found were correlated with those obtained in anesthetized rats using [3H]inulin clearance (11, 12, 22, 24).

Changes in renal COX2 and nNOS expression. COX-2 and nNOS protein expression were determined by Western blotting. Briefly, after death, the renal cortex and renal medulla were dissected and homogenized, and protein (50 μg) was separated on 4–20% SDS-PAGE gels (Bio-Rad) and transferred to a nitrocellulose membrane (Hybond ECL, Amersham). The primary antibodies used were rabbit polyclonal antibodies against COX-2 (Cayman), rabbit polyclonal antibodies against nNOS (Santa Cruz Biotechnology), and a polyclonal anti-GAPDH polyclonal antibody (Sigma). Antibody binding was detected using a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology), or goat anti-mouse, IgG secondary antibody (Sigma). The bands were visualized using the enhanced chemiluminescence system ECL Plus (Amersham), and images were captured on autoradiography film and scanned and quantified with the Image J program (National Institute of Health).

Statistical Analysis

Data in text and figures are expressed as means ± SE. Significant differences between experimental periods within one group were evaluated using ANOVA for repeated measures and Fisher’s test. Significant differences between groups were examined by using ANOVA and Fisher’s test.

RESULTS

Renal Effects of COX2 Inhibition

Arterial pressure did not change in any group in response to COX2 inhibition. At 10–11 mo of age, mean arterial pressure was greater in male (133 ± 5 mmHg) and female (122 ± 4 mmHg) ARAnp-treated rats than in male (117 ± 2 mmHg) and female (111 ± 3 mmHg) control rats. Changes in GFR and RPF elicited by COX2 inhibition are shown in Fig. 1. Renal hemodynamics were similar at 3–4 and 10–11 mo of age in both groups of control rats. Renal hemodynamics were also similar in ARAnp-treated and control females at both ages. However, GFR was reduced at 3–4 mo of age in ARAnp-treated male rats compared with the GFR found in control males. At 10–11 mo of age, GFR and RPF were lower in ARAnp-treated than in control male rats.

COX2 inhibition induced a mild decrease in GFR in control males but not in control females at 3–4 mo of age, and no changes of GFR in both control groups at 10–11 mo of age. However, COX2 inhibition led to a decrease (P < 0.05) in GFR and RPF in male and female ARAnp-treated rats at both ages, renal vasconstriction being greater (P < 0.05) than that found in control rats (Fig. 1). The difference in the renal hemodynamic response to the acute COX2 inhibition between control and ARAnp-treated rats is also evident when the changes in renal vascular resistance (RVR) are examined. At 3–4 mo, RVR did not change in both control groups but increased (P < 0.05) in male (33 ± 8%) and female (28 ± 9%) ARAnp-treated rats. Similarly, at 10–11 mo of age, RVR increased (P < 0.05) in male (39 ± 7%) and female (42 ± 12%) ARAnp-treated rats.

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Figure 2 shows the SBP values in each group of rats with NPI and HPI. It can be observed that SBP was elevated ($P < 0.05$) in both sexes of ARAnp-treated rats and that the prolonged increment in protein intake did not induce changes in SBP in any group. An age-dependent increment in SBP ($P < 0.05$) was only found in ARAnp male rats. The effects of HPI on creatinine clearance are shown in Fig. 3. GFR was similar in vehicle- and ARAnp-treated rats at 3–4 mo of age and was not significantly affected by the prolonged HPI. However, at 10–11 mo of age, GFR was reduced ($P < 0.05$) during NPI in male (0.63 ± 0.07 ml/min) but not in female (0.77 ± 0.05 ml/min) ARAnp rats, with respect to the GFR values found in control groups (males: 0.90 ± 0.09 ml/min; females: 0.86 ± 0.06 ml/min). The prolonged HPI at 10–11 mo of age did not induce significant changes of GFR in male and female control rats and in female ARAnp-treated rats, but led to a decrease of GFR in male ARAnp-treated rats (0.63 ± 0.07 to 0.19 ± 0.05 ml/min, $P < 0.05$) (Fig. 3).

Changes in proteinuria in response to HPI are shown in Fig. 4. It can be observed that proteinuria was similar in the control and ARAnp-treated rats during NPI at 3–4 mo of age. Prolonged HPI led to an increment ($P < 0.05$) in proteinuria in these rats. The rise in proteinuria elicited by HPI was not significantly different in ARAnp-treated (26 ± 5 mg/day) and control (15 ± 5 mg/day) female rats. However, the increment in proteinuria was greater ($P < 0.05$) in ARAnp-treated (48 ± 6 mg/day) than in control (20 ± 6 mg/day) male rats at 3–4 mo of age. An age-dependent increment ($P < 0.05$) in proteinuria during NPI was found in male (42 ± 2 to 82 ± 8 mg/day) and female (18 ± 2 to 44 ± 7 mg/day) ARAnp-treated rats, but not in control rats of either sex. Prolonged HPI also induced an elevation of proteinuria in ARAnp-treated ($P < 0.05$) but not in control rats at 10–11 mo of age, the increment being greater ($P < 0.05$) in male (99 ± 22 mg/day) than in female (30 ± 8 mg/day) ARAnp-treated rats (Fig. 4).

Changes in Renal COX2 and nNOS Expression

No significant differences in COX2 expression in the renal cortex or renal medulla were found between sexes or between treatments at 3–4 mo of age. Differences in renal COX2 expression during NPI and changes in this expression in each group of rats at 10–11 mo of age are shown in Fig. 5. It can be observed that COX2 expression was enhanced during NPI in the renal cortex and renal medulla of male ARAnp-treated rats and only enhanced in the renal cortex of female ARAnp-treated rats compared with renal COX2 expression in their respective control groups. A sex-dependent difference in the response of renal cortical COX2 expression to HPI was found since it increased ($P < 0.05$) in both groups of male rats, but not in both groups of female rats. COX2 expression in the renal medulla increased during HPI in normotensive but not in hypertensive rats (Fig. 5).

No significant differences in renal cortical nNOS expression were found between males and females and between normotensive and hypertensive rats during NPI. nNOS expression in the renal medulla was also similar in male and female normotensive rats and was elevated ($P < 0.05$) in male but not in female hypertensive rats during NPI (Fig. 6). Prolonged HPI did not induce significant changes in nNOS in the renal cortex or renal medulla of both groups of female rats and of normotensive male rats but led to an important decrease in nNOS in the renal cortex and renal medulla of male hypertensive rats (Fig. 6).

DISCUSSION

This study presents new evidence showing that COX2-derived metabolites play an important role in the regulation of renal hemodynamics when nephrogenesis is altered during the late phase of renal development. It is shown that acute COX2 inhibition elicited a decrease in GFR and RPF that was greater in ARAnp-treated than in control rats. Contrary to what was
expected, the renal vasoconstriction induced by COX2 inhibition was similar in male and female ARAnp-treated rats and was not further enhanced when these rats aged between 3–4 and 10–11 mo. Another important new finding is that an alteration in nephrogenesis increases the renal susceptibility to prolonged HPI. This increment is sex dependent and aggravated by aging since HPI leads to a very important proteinuria and to a significant decrease in GFR in males but not in females at 10–11 mo of age.

The rise in SBP and changes in renal function in male and female ARAnp-treated rats with NPI were expected since they have already been reported by our group (11, 21, 24). These changes may be secondary to a 37% decrease in nephron number that is similar in males and females (21), but also to other renal changes elicited by the reduction of ANG II effects during the nephrogenic period (14). The inverse relationship between nephron number and SBP during adulthood is supported by many clinical and experimental studies (2, 10, 31). Based on these results, it may be proposed that it is the reduction in nephron number during nephrogenesis, rather than a decrease in nephron number after nephrogenesis is completed, what predisposes the adult to renal and cardiovascular diseases. When the renal hemodynamic results are examined together with those already reported by our group (11, 24), it may be proposed that a renal vasoconstriction occurs in male but not in female ARAnp-treated rats, and that the deterioration of renal hemodynamics is accelerated during aging only in these male rats. The difference in GFR between conscious and anesthetized ARAnp-treated male rats at 3–4 mo of age may be explained by a greater renal vasoconstriction as a consequence of anesthesia. The sex dependency in the renal adaptation to an altered nephrogenesis is confirmed by the results showing that proteinuria only increases during aging (P < 0.05) in male ARAnp-treated rats with NPI (Fig. 4). These sex-dependent differences have been reported using different models of fetal programming (8, 26), but the mechanisms involved remain to be defined.

Despite the significant number of studies evaluating the renal changes induced by an alteration in nephrogenesis, it remains unknown whether PGs are involved in the regulation of renal hemodynamics when renal development is altered. Whether this importance of PG changes during aging and whether it is sex dependent was also unknown. The role of PGs in the adaptation to a reduction in nephron number has only been examined after subtotal ablations of renal mass in male animals (7, 17, 27). The results obtained in this study present new evidence suggesting that COX2-derived metabolites are involved in the progressive and continuous renal adaptation to a decrease in nephron endowment during renal development. This notion is supported by results showing that COX2 expression is enhanced in ARAnp-treated rats (Fig. 4) and showing that COX2 inhibition induces a decrease in GFR and RPF in

Fig. 2. Systolic blood pressure in male and female control and ARAnp-treated rats at 3–4 and 10–11 mo of age, and with normal or high protein intake. #P < 0.05 vs. control rats. +P < 0.05 vs. rats at 3–4 mo of age.

Fig. 3. GFR in male and female control and ARAnp-treated rats at 3–4 and 10–11 mo of age with normal or high protein intake. *P < 0.05 vs. normal protein intake. #P < 0.05 vs. control rats.
these rats that is greater than that found in control rats (Fig. 1). The increment in COX2 seems to contribute to the compensatory adjustments to the decrease in nephron endowment that are obligatory for the preservation of normal renal function, but in the long run these adjustments seem to be central to the progressive nature of renal disease (18, 27). Since the calculated single-nephron GFR is elevated in our ARAnp-treated rats (21) and the fall in GFR after COX2 inhibition is similar to the reduction in nephron number in these rats, it may be speculated that the glomerular hyperfiltration is maintained by an elevation in PG production. Based on results reported by Pelayo and Shanley (17), the renal hemodynamic response to COX2 inhibition in our ARAnp-treated rats may be explained by vasoconstriction of the afferent arteriole.

Results obtained in preliminary studies (not shown) suggest that COX1-derived metabolites are not involved in the renal adaptation to a decrease in nephron endowment because renal COX1 expression is similar in each experimental group, and the administration of a nonselective COX inhibitor elicits changes in renal hemodynamics that are similar to those found during nimesulide administration. The notion that COX2- but not COX1-derived metabolites are involved in this renal adaptation is supported by the results obtained by Wang et al. (28) using a model of subtotal renal ablation.

This study also examines whether there are sex differences in the role of COX2-derived metabolites in the renal adaptation to an alteration in renal development during the nephrogenic period. As far as we know, no previous studies have evaluated whether PGs play a different role in males and females in the regulation of renal hemodynamics after reductions in either nephron endowment or renal mass at any age. Contrary to what was expected (8, 11, 12, 21, 24, 26, 29), the changes in renal cortical COX2 expression and the renal hemodynamic response to COX2 inhibition are similar in both sexes of ARAnp-treated rats. These results suggest that there are not sex-dependent differences in the role of COX2 in maintaining renal hemodynamics when there is a decrease in nephron endowment during renal development. Considering the results reported in previous studies (11, 13, 24), it was also unexpected that the renal hemodynamic response to COX2 inhibition is similar at both ages in ARAnp-treated rats. These results suggest that the role of COX2 in the control of renal hemodynamics does not change with age when nephron endowment has been reduced during renal development. However, COX2 expression is elevated at 10–11 but not at 3–4 mo of age in ARAnp-treated rats with respect to the expression found in control rats. Further studies are needed to elucidate why COX2 inhibition does not induce greater renal vasoconstriction in the older hypertensive rats.

Fig. 4. Proteinuria in male and female control and ARAnp-treated rats at 3–4 and 10–11 mo of age with normal or high protein intake. *P < 0.05 vs. normal protein intake. #P < 0.05 vs. control rats.

Fig. 5. COX2 expression in the renal cortical and medullary tissue in male and female control and ARAnp-treated rats with normal or high protein intake. *P < 0.05 vs. normal protein intake. #P < 0.05 vs. control rats.
Our study also examined whether renal function is more sensitive to a secondary insult such as a prolonged HPI when renal development is altered and whether this sensitivity increases with age and is sex dependent. The absence of changes in SBP after HPI was expected since the available studies have found that HP diets do not affect blood pressure (6). Prolonged HPI did not induce a significant change in GFR in both sexes and at both ages in normotensive rats and only elicited a mild but significant increment in proteinuria in normotensive rats at the younger age. The absence of changes in GFR after a prolonged HPI was unexpected because previous studies have shown that acute (12) or 2-wk (30) increments in plasma amino acid levels in male rats are accompanied by an increment in GFR. One possibility is that other compensatory mechanisms reduce GFR to normal levels when protein intake is maintained chronically elevated. The difference in the renal hemodynamic response to HPI in normotensive rats between this and other studies (6, 30) may be attributed to the time period that protein intake was maintained elevated. As expected (30), HPI is associated with an elevation in COX2 expression in the renal cortex and renal medulla of normotensive male rats and probably to an elevation in PGs. The COX2 increment in the renal cortex during HPI also occurred in male hypertensive rats and seems to be sex dependent because it was not observed in female rats (Fig. 4). As far as we know, this is the first study that has evaluated whether renal COX2 expression changes in females after a prolonged HPI. Further studies are needed to examine the mechanisms involved in the sex-dependent differences in the response of renal cortical COX2 expression to HPI.

Based on results reported previously (3, 6, 20), it was expected that the secondary insult elicited by the prolonged HPI would unmask a reduction in renal functional reserve in ARAnp-treated rats and would accelerate the aging-dependent deterioration of renal function, renal changes being greater in males than in females. Despite the fact that the decrease in nephron number during renal development is similar in both sexes (21), male rats are more sensitive to the secondary insult because the increment in proteinuria is greater in males than in females even at the younger age. The sex dependency in the renal response to a prolonged HPI is more evident in the older groups of rats since this HPI led to a decrease in GFR in male (70%) but not in female ARAnp-treated rats. The HPI-induced increment in proteinuria was also greater in male than in female hypertensive rats (Fig. 4). Our results do not allow any conclusion about the mechanisms responsible for the decrease in GFR and the greater increment in proteinuria found in male ARAnp-treated rats during HPI. However, considering the results obtained in rats after subtotal ablation of renal mass (17), it may be proposed that the increase in COX2-derived metabolites in our older hypertensive male rats could induce a dilation of the afferent arteriole. This reduction in preglomerular resistance, together with the increment in arterial pressure, would increase glomerular capillary pressure and cause a mechanical disruption of glomerular integrity and an elevation in proteinuria that finally would lead to progressive destruction of the remaining nephrons and to renal failure. Protein overload of proximal tubule cells is also harmful since it activates intracellular signals that induce an increase in vasoactive, inflammatory mediators and growth factors (1, 15). The activation of COX2 during HPI in the hypertensive males could contribute to kidney disease progression and end-stage renal failure since it may be accompanied by an elevation in reactive oxidant species production, which exacerbates proinflammatory PG action (5). Sex hormones may also be involved in the different evolution of renal function between males and females in response to the prolonged HPI. In support of this hypothesis, it has been shown that castration of male rats seems to have a beneficial effect on the development of glomerulosclerosis, whereas treatment with testosterone accelerates the disease (23). It has also been shown that estrogens reduce glomerulosclerosis susceptibility (19).

The possible involvement of nNOS in the HPI-induced increment in COX2 in the hypertensive rats with reduced renal development has also been examined. Based on results reported by Yao et al. (30) in normotensive male rats, the hypothesis was that the COX2 increment elicited by HPI is mediated by an elevation in nNOS (30). In our study, no significant elevations of nNOS are associated with the rise in COX2 induced by HPI in normotensive male rats. However, the HPI-induced increment in COX2 in hypertensive male rats is accompanied by a decrease in nNOS in cortical (45%) and
medullary (59%) renal tissue (Figs. 5 and 6). These results suggest that the increment in COX2 elicited by HPI is not mediated by an elevation of nNOS in hypertensive male rats with an alteration in renal development. The decrease in nNOS in male ARAnp-treated rats may contribute to the decrease in GFR found in these rats during HPI (25).

Considering the increased popularity of high-protein diets used in efforts to lose weight, the results reported in this study may have important clinical implications. These results strongly suggest that these diets should be clearly avoided in male patients who may have had an alteration in renal development, such as those treated with glucocorticoids to accelerate lung maturation or those with low birth weight (2, 8, 26, 31). New evidence is reported suggesting that gender in rats is an important independent contributing factor in the progression of the renal dysfunction that occurs when renal development is altered during the late nephrogenic period. The experimental model used in this study may be suitable for examining the mechanisms involved in the evolution of renal disease during aging and those that are responsible for the sex-dependent evolution of renal disease during aging.

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

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