Hypertension and Sex Differences in the Age-Related Renal Changes When Cyclooxygenase-2 Activity Is Reduced During Nephrogenesis

Fara Sáez, Virginia Reverte, Francisco Salazar, María T. Castells, María T. Llinás, F. Javier Salazar

Abstract—Several studies have proposed that cyclooxygenase-2 (COX2) is involved in the regulation of nephrogenesis and that an impaired nephrogenesis may induce the development of hypertension. This study was designed to test the hypothesis that the decrease of COX2 activity leads to a reduction in nephron number, an increase in arterial pressure, and age-dependent renal alterations that are greater in male than in female rats. Arterial pressure was measured from the first to the 16th month of life in rats treated with vehicle or a COX2 inhibitor during the nephrogenic period. Stereological and histological evaluations and renal function studies were performed at different ages. Arterial pressure increased (14%; P<0.05) and nephron number decreased (17%; P<0.05) to similar levels in male and female COX2-treated rats. However, glomerular filtration rate (31%) and renal plasma flow (25%) decreased (P<0.05) in male but not in female COX2-treated rats. A greater (P<0.05) age-dependent elevation in glomerular hypertrophy was also found in male COX2-treated rats compared with their female littermates. Glomerulosclerosis and tubulointerstitial damage in renal cortex and medulla were also significantly enhanced in male but not in female aged COX2-treated rats. Our results demonstrate that the decrease in COX2 activity during renal development leads to a reduction in nephron number and to an elevation in arterial pressure that are similar in males and females. However, the consequent age-dependent deterioration of the renal structure and renal function is only significantly enhanced in male rats.

Key Words: fetal programming © glomerulosclerosis © renal fibrosis © aging © proteinuria © cyclooxygenases © prostaglandins

The importance of cyclooxygenase-2 (COX2)–derived metabolites in the regulation of renal morphogenesis is supported by studies showing that the use of nonsteroidal anti-inflammatory drugs as tocolytics is related to renal abnormalities and renal failure in the neonate1 and that anti-inflammatory drugs as tocolytics is related to renal abnormalities and renal failure in the neonate.2 Furthermore, it is well known that COX2 appears in the developing rodent metanephros starting at embryonic day 16, and it is upregulated in the kidney during the first 2 weeks of life.6,7 The link among a suboptimal nephron endowment, hypertension, and an age-dependent development of renal damage has been shown in many studies.8–11 but nowadays it is unknown whether the reduction in COX2 activity during the nephrogenic period leads to a lower nephron number, hypertension, and a progressive deterioration of the renal structure and function.

The objective of this study was to evaluate the effects of COX2 inhibition during the nephrogenic period (COX2 Inhnp) on nephron number and on the age-dependent changes in arterial pressure, renal structure, and renal function. Because recent works of our group have reported sex differences in the long-term effects elicited by the angiotensin II type 1 receptor blockade during nephrogenesis,11–15 another objective of the present study was to find out whether there were sex differences in response to perinatal COX2 inhibition.

Materials and Methods

Sprague-Dawley rats were purchased from the animal facilities of the University of Murcia. Female Sprague-Dawley rats (250-g body weight) 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-specific inhibitor (rofecoxib, 2.4 mg/kg per day VO) was administered to dams from embryonic day 16 until delivery and then to newborn pups from postnatal day 1 to postnatal day 21. The COX2 inhibitor was administered during this period because it is known that nephrogenesis in rats takes place from midgestation until the third postnatal week.16 Solutions with or without rofecoxib were orally administered at a rate of 0.96 μL/g of body weight. On postnatal day 0, litter size was fixed between 10 and 12 pups to ensure similar nourishment during the suckling period. Litters with <10 pups were excluded. Rats had free access to normal rat chow.
Arterial Pressure Measurement

Systolic blood pressure (SBP) was measured from the first to the 16th month of life in conscious rats by the tail-cuff method, as described previously. In a previous study, it was found that the SBP values obtained using the tail-cuff method are correlated (r = 0.8) with those obtained in conscious, freely moving rats through a femoral artery catheter exteriorized at the nape of the neck.

Proteinuria and Renal Function Studies

Proteinuria was assessed at 3 to 4 and 11 to 12 months of age in the same groups of rats in which SBP was measured. Rats were kept individually in metabolic cages and had free access to powdered rat chow and drinking fluid throughout the experiments. After 2 days of adaptation, 24-hour urine samples were obtained during 3 days to examine the urinary protein excretion rate by micro-Lowry method.

Another subset of rats was used for renal hemodynamics studies. At 3 to 4, 9 to 10, and 16 to 17 months of age, rats were anesthetized with pentobarbital sodium and ketamine, tracheotomized with a razor blade and the urinary bladder were catheterized. To stabilize hematocrit levels after surgical stress, a solution of 0.5 mL/100 g BSA (6%; Sigma) was administered. [3H]inulin (2 μCi/mL; American Radio-labeled Chemicals) was given as an intravenous bolus (1 mL) and as a continuous intravenous infusion of [3H]inulin (1.5 μCi/mL) dissolved in isotonic saline (3 mL/h). Glomerular filtration rate (GFR) was measured by clearances of injected inulin. A transit-time flow probe (Transonic System) was implanted on the left renal artery for the measurement of renal blood flow. Renal plasma flow changes were calculated considering renal blood flow and hematocrit values.

After a 60-minute equilibration period, 2 consecutive 20-minute clearances were obtained, and blood samples were withdrawn to determine the inulin concentrations.

Morphological Studies

These studies were carried out in new groups of 3- to 4- and 9- to 10-month-old rats, as well as in the 16- to 17-month-old rats in which SBP was measured. Rats were anesthetized with sodium pentothal (5 mg/100 g of body weight), and the left kidney was removed. Kidneys were sliced into 2-mm-thick sections using a razor blade cutting device and then fixed in 10% formaldehyde in PBS (pH 7.4). Samples were embedded in paraffin wax, and 5-μm-thick sections were stained with hematoxylin/eosin, periodic acid-Schiff, and Masson-Trichrome. Total volumes of cortex, outer stripe of the outer medulla, inner stripe of the outer medulla, and inner medulla, and group.

Table 1. Body Weights at Birth and During the First 3 Weeks of Life in Rats Treated With Vehicle or COX2 Inhnp

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Male Birth Weight (g)</th>
<th>Female Birth Weight (g)</th>
<th>Male Third Week</th>
<th>Female Third Week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-treated, g</td>
<td>3.3 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>18.4 ± 0.8</td>
<td>17.2 ± 0.8</td>
</tr>
<tr>
<td>COX2 Inhnp–treated, g</td>
<td>3.4 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>18.5 ± 0.8</td>
<td>17.3 ± 0.8</td>
</tr>
</tbody>
</table>

Data are means ± SEs. *P < 0.05 vs vehicle-treated rats.

Table 2. Body Weight and Kidney Weight in Rats at 3 to 4, 9 to 10, and 16 to 17 Months of Age That Were Treated With Vehicle or COX2 Inhnp

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male Values</th>
<th>Female Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>339 ± 5</td>
<td>354 ± 18</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>1.02 ± 0.02</td>
<td>1.06 ± 0.04</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>487 ± 24</td>
<td>470 ± 9</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>1.37 ± 0.09</td>
<td>1.26 ± 0.07</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>530 ± 22</td>
<td>578 ± 14</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>1.60 ± 0.08</td>
<td>1.85 ± 0.10</td>
</tr>
</tbody>
</table>

*P < 0.05 vs females with the same age and treatment.

Trichrome. Total volumes of cortex, outer medulla, and inner medulla were estimated by the Cavalieri principle. Total number of glomeruli was determined by using the physical dissector/fractonator combination method. Glomerular volume was obtained by using the 2 arbitrary parallel sections technique. Approximately 20 glomeruli were measured in each rat (n = 7) to obtain the mean glomerular volume in every experimental group. To determine glomerular damage, glomerular profiles were examined in periodic acid-Schiff–stained sections and assigned to 1 of 4 groups according to the degree of damage. Approximately 500 glomerular profiles were examined per group.

Tubulointerstitial injury was scored using the MIP 4.5 image analysis software (Consulting Image Digital) in sections stained with Masson trichrome and viewed in a PC screen at a ×285 magnification. Tubulointerstitial injury was defined as tubular dilatation, tubular atrophy, thickening of the tubular basement membrane, mononuclear infiltration, and increase of collagen. The percentage of damaged cortex and medulla was estimated in square fields of 468 × 468 μm. Fifty fields were examined per each kidney zone (outer cortex, inner cortex, outer stripe of the outer medulla, inner stripe of the outer medulla, and inner medulla) and group.

Statistical Analysis

All of the values are presented as means ± SEs. Changes in SBP within a group were evaluated using ANOVA for repeated measures and Fisher’s test. Two-way ANOVA was used to evaluate the differences between groups (GB Stat, Dynamic Microsystems, Inc). P < 0.05 was considered statistically significant.

Results

Table 1 shows that the mean increment in body weight during the first 3 weeks of life was greater (P < 0.05) in litters of COX2 Inhnp–treated ones. During adult life, body and kidney weight were similar in COX2 Inhnp– and vehicle-treated rats and always greater in male than in female rats at any time of the study (Table 2).

Systolic Blood Pressure

Figure 1 shows that SBP was significantly higher in COX2 Inhnp– than in vehicle-treated rats (P < 0.05) and that there were no age- or sex-dependent changes in SBP in these rats after the third month of life.
Stereological Parameters and Histopathology

Regarding glomeruli number, it was similar in male (31.355±1063 glomeruli per kidney) and female (28.806±1885 glomeruli per kidney) vehicle-treated rats, and COX2 Inhnp led to a modest but significant (P<0.05) decrease that was not different in male (26.013±1329 glomeruli per kidney) and female (24.253±1212 glomeruli per kidney) rats (Figure 2A). When glomeruli number in both kidneys was normalized considering body weight, it was greater (31%; P<0.05) in female than in male COX2 Inhnp–treated rats (Figure 2B).

Glomerular volume was similar in male (5.0±0.3×10^5 μm^3) and female (4.3±0.3×10^5 μm^3) vehicle-treated rats at 3 months of age (Figure 3A), and with the passing of time, it increased to a greater extent in male compared with female vehicle-treated rats (P<0.05; Figure 3B and 3C). Compensatory glomerular hypertrophy in COX2 Inhnp–treated rats was already significant at 3 months in males (7.5±0.9×10^5 μm^3) and females (5.9±0.3×10^5 μm^3; Figure 3A). The age-dependent rise in glomerular volume in these rats was more important (P<0.05) in male (21.4±1.2×10^5 μm^3) than in female (12.5±0.9×10^5 μm^3) COX2 Inhnp–treated rats (Figure 3C).

Differences in glomerulosclerosis as a consequence of the COX2 treatment are presented in Table 3. At 3 to 4 months of age, there was a slight but significant increase in glomerulosclerosis in the COX2 Inhnp–treated rats that was similar in males and females, but these differences with respect to vehicle-treated rats were not significant at 9 to 10 months. However, by 16 to 17 months of age, glomerulosclerosis index raised in COX2 Inhnp–treated males but not in COX2 Inhnp–treated females when compared with the values found in vehicle rats (Table 3).

Changes in tubulointerstitial damage are shown in Table 4. No significant differences between groups were found at 3 months of age. This index of renal damage was greater in aged vehicle–treated males than in their female littermates. Treatment with the COX2 inhibitor during the nephrogenic period elicited an increase in tubulointerstitial damage in the renal cortex of 9-month–old COX2 Inhnp–treated males and in the renal cortex and outer medulla of the same group of rats at 16 to 17 months of age. No differences in tubulointerstitial damage were found in COX2 Inhnp–treated females compared with their age- and sex-matched controls (Table 4).

Renal Hemodynamics and Proteinuria

Basal renal hemodynamics and excretory function in vehicle and COX2 Inhnp–treated rats are shown in Table 5. Hematocrit was similar in vehicle and COX2 Inhnp–treated rats and did not change significantly during aging (data not shown). Renal hemodynamics were not affected as a consequence of the perinatal treatment and were similar in male and female rats at 3 months of age. Both GFR and renal plasma flow were not significantly different in vehicle and COX2 Inhnp–treated female rats at 9 to 10 and 16 to 17 months of age. However, GFR and renal plasma flow decreased (P<0.05) in 9- to 10-month-old COX2 Inhnp–treated males and remained reduced (P<0.05) at 16 to 17 months of age when compared with the values found in vehicle-treated male rats. Urinary sodium excretion and urine flow rate were similar at all of the ages in rats treated during the nephrogenic period with vehicle or the COX2 inhibitor (Table 5).

Urinary protein excretion was greater (P<0.05) in male than in female rats at both ages (Figure 4, but this sex difference is not present when proteinuria levels are normalized to body weight (data not shown). Protein excretion was similar at 3 to 4 months of age in rats treated with vehicle or the COX2 inhibitor. However, proteinuria was enhanced (P<0.05) in male and female COX2 Inhnp–treated rats at 11 to 12 months of age, when compared with the values found in male and female vehicle-treated rats (Figure 4B).

Discussion

This study introduces new data showing that the reduction of COX2 activity during the nephrogenic period leads to a modest but significant reduction in nephron number and to an increase in blood pressure that is maintained during aging.

Figure 2. Glomeruli number (A) and glomeruli number per gram of body weight (B) assessed at 3 months of age. *P<0.05 vs vehicle-treated rats of the same sex. #P<0.05 vs females with same age and treatment.
The major novel finding is that these changes are accompanied by an important age-dependent deterioration in renal function and renal structure in male but not in female rats, despite the fact that the increase in blood pressure and the number of nephrons are similar in these animals.

The role of COX2 in the regulation of nephrogenesis has been demonstrated in studies showing that COX2 is upregulated during the nephrogenic period and that the use of either COX2-specific inhibitors or nonsteroidal anti-inflammatory drugs during pregnancy is associated with renal failure, the presence of small and immature glomeruli, and the absence of brush border in proximal tubuli. In addition, several studies have reported that mice lacking COX2 develop immature glomeruli and poorly differentiated tubuli.

As reported in an interesting study performed by Yang et al., the proteinuria, renal structural damage, and development of hypertension are significantly influenced by genetic background and gender in 3 congenic lines of COX2 knockout mice. However, it was unknown whether the reduction in COX2 activity during renal development leads to a decrease in nephron number and whether this potential reduction in

Table 3. Glomerulosclerosis Index in Rats at 3 to 4, 9 to 10, and 16 to 17 Months of Age That Were Treated With Vehicle or COX2 Inh

<table>
<thead>
<tr>
<th>Variable</th>
<th>3- to 4-mo-old rats</th>
<th>9- to 10-mo-old rats</th>
<th>16- to 17-mo-old rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>COX2 Inh</td>
<td>Vehicle</td>
</tr>
<tr>
<td>% grade 0</td>
<td>93±2</td>
<td>81±1*</td>
<td>94±2</td>
</tr>
<tr>
<td>% grade 1</td>
<td>7±2</td>
<td>19±1*</td>
<td>6±2</td>
</tr>
<tr>
<td>% grade 2</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>% grade 3</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>% grade 4</td>
<td>5±0</td>
<td>4±0</td>
<td>4±0</td>
</tr>
</tbody>
</table>

Data are means±SEs.

*P<0.05 vs vehicle-treated rats of the same sex.
†P<0.05 vs females with the same age and treatment.
‡P<0.05 vs 3- to 4-month-old rats with the same sex and treatment.
§P<0.05 vs 9- to 10-month-old rats with the same sex and treatment.
The decrease in total nephron number was similar in male and female rats, but the ratio of glomeruli number per gram of body weight was greater in female than in male rats, even in the control group (Figure 2). This sexual difference could contribute to the greater degree of glomerular hypertrophy and renal damage in male compared with female rats. The decrease in nephron number in COX2 Inhnp–treated rats may be partly secondary to a reduction in the renin-angiotensin system activity. This possibility is supported by studies showing that renin release is regulated by COX2-derived metabolites\(^7\) and by studies showing that the blockade of angiotensin II type 1 receptors during the nephrogenic period elicited a 37% decrease in nephron number.\(^11\) The renal effects found in COX2 Inhnp–treated rats during the adult age could also be a consequence of the renal vasoconstriction elicited by COX2 inhibition during the postnatal period.\(^20\)

The relationship between a suboptimal nephron endowment and the development of hypertension is supported by epidemiological and experimental studies.\(^4,8–11\) Our results suggest that a 17% reduction in nephron number is enough to elicit an elevation in arterial pressure at an early age. Contrary to what was found when nephron number decreased by 37%,\(^14\) there is not a further elevation in blood pressure during aging (Figure 1) when nephron endowment is reduced by 17% (Figure 2). Most likely, a lower reduction in nephron number during renal development will not elicit an elevation in arterial pressure. In support of this possibility it has been reported that the prenatal administration of dexamethasone reduces nephron number by 13% and does not lead to an increment in arterial pressure.\(^21\) We cannot discard that the increment in arterial pressure is secondary to other effects elicited by COX2 inhibition, because it has been reported that the lack of COX2 in mice leads to abnormalities in cortical tubuli.\(^2–4\) In addition, the higher blood pressure found in COX2 Inhnp–treated rats may also be partly secondary to an enhanced response to stress\(^22\) and to the lower weight gain during the first weeks of life.\(^8,9\) As shown in Table 1, the mean increment in body weight during the first 3 weeks of life was greater in litters of vehicle-treated than in litters of COX2 Inhnp–treated rats. Thus, new studies are needed to determine the mechanisms involved in the hypertension secondary to the reduction in COX2 activity during renal development.

The glomerular hypertrophy in COX2 Inhnp–treated rats was expected because it is a compensatory response to a
reduced nephron number. This increment in glomerular volume is probably linked to a state of hyperfiltration to maintain GFR despite the lower nephron number. The mechanisms involved in this compensatory hypertrophy are unknown but seem to be sex dependent, because the increment in glomerular volume during aging was greater in males than in females.

The different degree of proteinuria found in male and female COX2 Inh np–treated rats may be secondary to a greater increase in glomerular capillary pressure in male rats that could be necessary to maintain GFR within normal levels and related to the greater glomerular hypertrophy and glomerulosclerosis found in male rats. Considering the results of this study and those reported previously, we might presume that the age-dependent deterioration of renal hemodynamics in males is directly related to the fall in nephron number during renal development. According to this hypothesis, a decrease in renal blood flow and GFR was found in COX2 Inh np–treated males starting at 9 months of age, whereas renal hemodynamics in COX2 Inh np–treated females remained unaffected. Thus, our results clearly show that a sex-dependent mechanism is acting in the progressive alteration of the renal structure and renal function that occurs during aging when there is a modest decrease in nephron number during renal development. This mechanism could be protecting the females’ glomeruli from the prolonged functional overload when there is a decrease in nephron number and/or could be involved in inducing a deterioration of glomerular integrity in males. Therefore, the decrease in GFR and renal blood flow in male rats with a modest decrease in nephron number during renal development could be secondary to a progressive reduction of functional glomeruli.

Tubulointerstitial damage increased in male but not in female rats treated with the COX2 inhibitor. This fact could be explained by the higher degree of glomerulosclerosis found in treated males. However, the contribution of other effects induced by COX2 treatment cannot be discarded, because most of the abnormalities in response to perinatal COX2 inhibition appear in the renal cortex, and tubulointerstitial damage is only significant in the renal cortex of treated males at 9 to 10 months of age.

Additional studies are required to elucidate the mechanisms involved in the development of hypertension and the sex-dependent differences in the age-related renal changes elicited by perinatal COX2 inhibition. It may be proposed that they are secondary to the effects induced by several regulatory mechanisms, such as sexual hormones, NO, and angiotensin II. In support of these possibilities, it has been shown that the sexual differences in renal damage may be explained by an decline in NO in aged male but not in aged female rats, by the damaging influence of androgens in males, and/or the protective effects of estrogens. The hypothesis that angiotensin II may be involved in the observed renal damage during aging is based on studies demonstrating the following: (1) the renin-angiotensin activity is enhanced in several experimental models with a reduced nephron endowment; (2) angiotensin II is a potent growth factor; and (3) angiotensin II effects are enhanced in the presence of androgens but reduced when estrogens are elevated. In summary, the current study emphasizes the importance of COX2-derived metabolites in the regulation of nephrogenesis. We have reported new evidence showing that a slight impairment of renal development may lead to a significant increment in arterial pressure and to a sex-dependent accelerated deterioration of the renal structure and renal function.

Perspectives
Numerous mechanisms are involved in the regulation of renal morphogenesis. The present study proposed that even a modest alteration of these mechanisms may lead to hypertension and to a progressive renal deterioration during aging and strongly supports the advice against the use of specific COX2 inhibitors as tocolytics.

Our results suggest that the secondary development of renal damage would depend not only in the extent of the insult but rather on other factors, such as body weight at birth, sex, and age. The knowledge of the importance of these factors could help in the management of hypertension and renal disease.

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Disclosures
None.

References
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