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Immunohistochemical localization of renin, NO synthase-1, and cyclooxygenase-2 in rodent kidney

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Summary. The renin-angiotensin system (RAS) and tubuloglomerular feedback (TGF) are central to the maintenance of blood pressure and body fluid composition. Renin, NO synthase-1 (NOS-1), and cyclooxygenase-2 (COX-2) are key regulators of the RAS and TGF. In the present study, to investigate species-specific differences in the RAS and TGF, we immunohistochemically and morphometrically investigated the localization of renin, NOS-1, and COX-2 in the kidneys of various laboratory rodents and comparing males with females (DBA/2Cr mice, F344/N rats, Syrian hamsters, MON/JmsGbs gerbils and Hartley guinea pigs). In all animals, renin-positive immunoreactions were observed in the vascular walls of afferent arterioles. Renin immunoreactions appeared to be more widely distributed in mice. Mice had a greater number of renin-positive arterioles than other species. NOS-1-positive reactions were detected in the macula densa (MD) of all animals. Mice had the greatest number of NOS-1-positive MD cells. In addition to NOS-1-positive reactions, COX-2-positive reactions were observed in the MD of mice, rats, hamsters and gerbils. Interestingly, guinea pigs had no COX-2-positive MD cells. Rats had the greatest number of COX-2-positive MD cells. In nephron segments excluding the MD, the immunohistochemical localization of NOS-1 and COX-2 differed markedly among not only species but also sexes within the same species. In conclusion, we determined that localization of renin, NOS-1, and COX-2 showed large species- and sex-related differences. These data suggest that the regulation mechanisms of the RAS and TGF via renin, NOS-1, and COX-2 differ among rodents.

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Introduction

In mammalian kidney, the juxtaglomerular apparatus (JGA) is a unique specialized site that consists of juxtaglomerular cells (JGCs), mesangial cells, and macula densa (MD) cells. This region effectively regulates renin secretion from JGCs and the glomerular filtration rate (GFR) via the tubuloglomerular feedback (TGF) system by sensing physiological changes including salt restriction, volume depletion, or renovascular hypertension (Celio and Inagami, 1981; Ito and Abe, 1996; Kaname and Fujita, 1996; Yabuki et al., 2004; Peti-Peterdi, 2005). It was recently determined that the renin-angiotensin system (RAS) and TGF are controlled by two key enzymes: NO synthase-1 (NOS-1) and cyclooxygenase-2 (COX-2). NOS-1, constitutive NO synthase, is primarily expressed in the MD and produces NO to continuously inhibit the vasoconstrictory response of TGF (Bachmann et al., 1995; Welch and Wilcox, 1997; Wilcox, 1998; Wang et al., 2002). It has also been suggested that NOS-1 plays an important role in the control of renin-secretion (Schricker et al., 1995). In a similar manner to NOS-1, COX-2, which is an inducible form of prostaglandin synthase, is constitutively expressed in the epithelium of distal tubules adjacent to MD cells (Harris et al., 1994). Furthermore, COX-2 is involved in the regulation of glomerular arteriolar tone and renin synthesis (Harding et al., 1997; Harris et al., 2000). It has recently been suggested that COX-2 function is under the control of NO produced by NOS-1, and is negatively regulated by angiotensin II (Cheng et al., 1999, 2000).

Mammalian kidney has an extremely complicated structure, which is closely related to its complex

function. Many studies have investigated species-specific differences in mammalian renal function. The results of these studies have suggested that the GFR (Fleck, 1999) and urine-concentrating capacity (Munkacsi and Palkovits, 1977) differ among animal species. In previous studies we have histologically and morphometrically investigated species-specific differences in kidneys, and determined that renal structures (Ichii et al., 2006a) and the length of the nephron loop (Ichii et al., 2006b) differ among rodent species (mouse, rat, hamster, gerbil, and guinea pig). Although the RAS and TGF are essential in maintaining blood pressure and body fluid composition, species-specific differences in these two central mechanisms are poorly understood. In the present study, to investigate species-specific localization of renin, NOS-1, and COX-2, which are rate-limiting enzymes of the RAS and TGF, we immunohistochemically and morphometrically studied the kidneys of mice, rats, hamsters, gerbils, and guinea pigs. Our present data provide a basis for comprehending the many functional characteristics of this organ.

Materials and methods

Animals

The present study was performed in accordance with the Guidelines for Animal Experimentation of Kagoshima University, Japan. Male and female DBA/2CrSlc mice (n=5 for each sex), F344/NSlc rats (n=4 for each sex), Slc:Syrian hamsters (n=3 for each sex), MON/JmsGbsSlc gerbils (n=3 for each sex) and Slc:Hartley guinea pigs (n=3 for each sex) were used in the present study. All animals were housed in an open system room with a one-way airflow system (temperature 22±1°C; humidity 55±10%; light period 07:00 to 19:00; ventilation 12 cycles/hr), and were given an autoclaved commercial diet (CE-2; Japan CLEA Inc., Tokyo, Japan) and tap water ad libitum. All animals were sacrificed at 3 months of age by exsanguination of the carotid arteries under anesthesia using a mixture of ketamine and medetomidine. The kidneys were quickly removed and weighed.

Tissue preparation

Central slices from the left kidneys, including the hilum, were cut perpendicular to the long axis and fixed in Zamboni's solution. After routine embedding in paraffin, 3-µm-thick sections were selected every 30 µm and used for periodic acid Schiff (PAS) stain and immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed using the Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Anti-renin polyclonal antibody (supplied by Dr. Murakami, University of Tsukuba) diluted 1:10,000, anti-NOS-1 polyclonal antibody (Cayman Chemical, Ann Arbor, MI, USA) diluted 1:1,500 and anti-COX-2 antibody (Cayman Chemical) diluted 1:800 were used as primary antibodies. Incubation with primary antibodies was performed overnight at 4°C. Biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200 was used as the secondary antibody. Immunohistochemical reactivity was detected using a 0.025% (W/V) 3,3'-diaminobenzine-0.003% (W/V) H_2O_2 solution. Immunoreactions were stopped in distilled water, and then sections were counterstained with Mayer's hematoxylin. For antigen retrieval, all pre-treatments were performed after deparaffinization. For NOS-1 detection, sections were microwaved in a 10 mM citrate buffer (pH 6.0). For COX-2 detection, sections were microwaved in the same buffer and then treated with 0.3% Triton X-100 in 10 mM phosphate-buffered saline. For renin detection, antigen retrieval was not required.

Morphometric analysis

Randomized morphometric analysis was performed by using PAS-stained sections and immunostained sections. Quantitation of the renin-positive arterioles was performed according to the previously described procedure (Oliverio et al., 1998; Yabuki et al., 2001). Briefly, the total numbers of renin-positive arterioles (A), NOS-1-positive MD cells (B) and COX-2-positive MD cells (C) in each immunostained section, and the total number of renal corpuscles (D) in PAS-stained sections (serial to immunostained sections) were determined. The indices of the number of renin-positive arterioles, NOS-1-positive MD cells and COX-2-positive MD cells were calculated as A/Dx100, B/Dx100 and C/Dx100, respectively.

Statistical analysis

Data are expressed as the mean±standard error (S.E.) and analyzed statistically by using nonparametric methods. The Mann-Whitney U test was used for comparing pairs of populations ($P<0.05$). The Kruskal-Wallis test was used for comparing three or more populations, and multiple comparisons were performed using Scheffé's method when a significant difference was observed ($P<0.05$).

Results

Renin

In all animals, renin-positive reactions were observed in the vascular walls of the afferent arterioles (AA). However, the widths of the positive reactions in the AA clearly differed among species. In rats and guinea pigs, and especially mice, these reactions were more broadly distributed in the AA and were observed from the side of the glomerulus to the distal parts of the

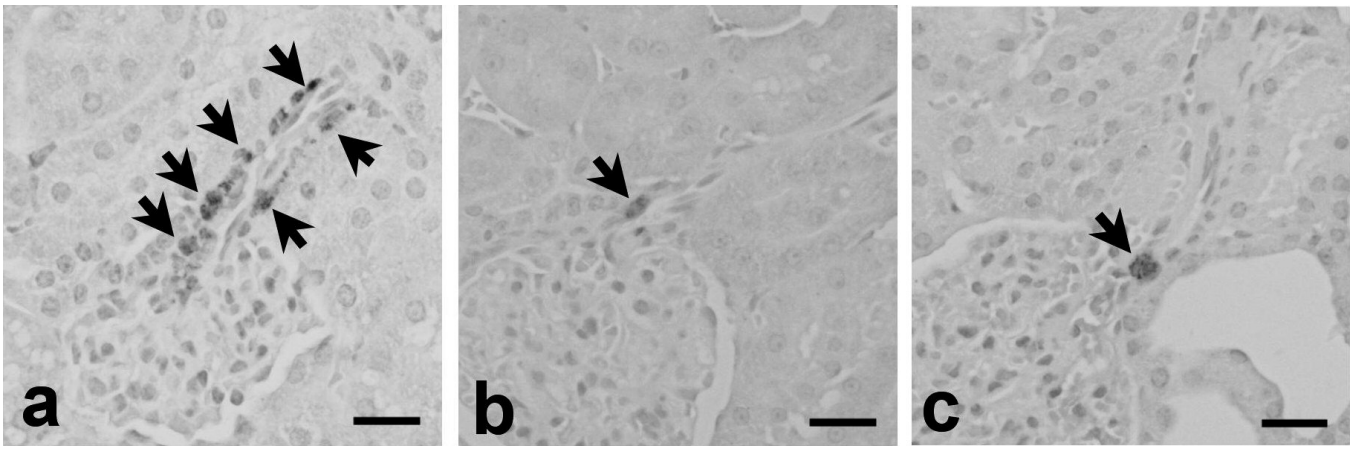

Fig. 1. Immunohistochemical detection of renin in the cortices of male rodents. Male mouse **(a)**, male hamster **(b)**, and male gerbil **(c)**. In all animals, renin-positive reactions (arrows) were observed in the vascular walls of the afferent arterioles (AA). In mice, this reaction was more widely distributed in the AA, occurring from the side of the glomerulus to the distal parts of the AA **(a)**. In hamsters and gerbils, these reactions in the AA were limited to the side of the glomerulus **(b and c)**. Bars: 20 µm.

AA (Fig. 1a). However, in hamsters and gerbils, in the AA these reactions were limited to the side of the glomerulus (Fig. 1b,c). Figure 2 shows the index for the number of renin-positive arterioles in different species, and clearly shows that species-specific differences existed. Mice of both sexes had the largest values. Significant differences were observed between mice and gerbils in both sex groups. For mice, the values of females were significantly higher than those of males.

NOS-1

In mice, rats, hamsters, and gerbils, NOS-1-positive reactions were detected in the MD of the distal tubules (Fig. 3 and Table 1). However, in guinea pigs, not only the MD, but also the distal straight tubules (DSTs) had positive reactions (Fig. 3e). Figure 4 shows the index for the number of NOS-1-positive MD cells in different

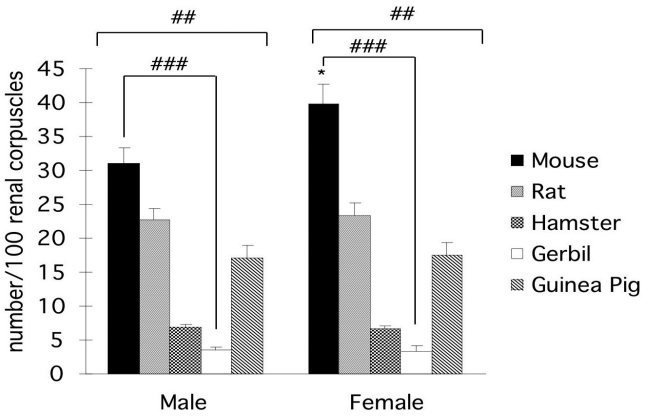

Fig. 2. Index of the number of renin-positive arterioles. Each column represents the mean ± S.E. #: Significantly different from males of the same species (Mann-Whitney U test, P<0.05). ##: Significant species difference in each sex group (Kruskal-Wallis test, P<0.05). ###: Significant differences in multiple comparisons following the Kruskal-Wallis test (Scheffé method, P<0.05).

Table 1. Summary of immunohistochemical localization of NOS-1-positive reactions in the kidneys of mice, rats, hamsters, gerbils, and guinea pigs.

	Mouse		Rat		Hamster		Gerbil		Guinea Pig	
Site	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
PCT	-	-	-	-	-	-	-	-	-	-
PST	-	+	-	-	+	+	-	-	-	-
TL	-	-	-	-	-	-	-	-	-	-
DST	-	-	-	-	-	-	-	-	+	+
MD	+	+	+	+	+	+	+	+	+	+
DCT	-	-	-	-	-	-	-	-	-	-
CCD	+	+	-	-	-	-	-	-	-	-
OMCD	-	-	-	-	-	-	-	-	-	-
IMCD	+	+	+	+	+	+	-	-	-	-
PI	-	-	-	-	-	-	-	-	+	+

PCT: proximal convoluted tubules; PST: proximal straight tubules; TL: thin limbs; DST: distal straight tubules; MD: macula densa; DCT: distal convoluted tubules; CCD: cortical collecting ducts; OMCD: outer medullary collecting ducts; IMCD: inner medullary collecting ducts; PI: papillary interstitium; +: NOS-1-positive staining; -: no NOS-1 staining.

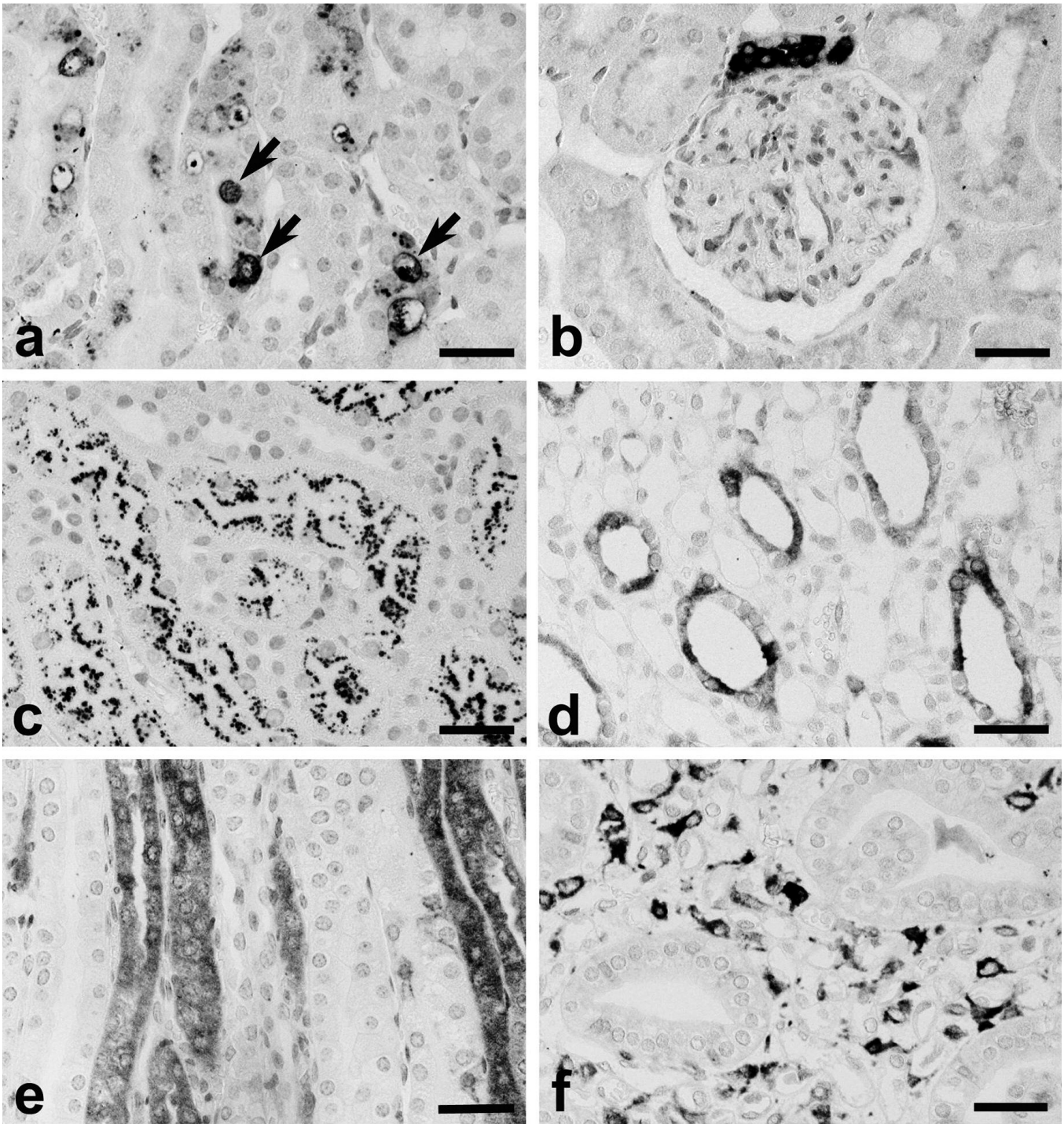

Fig. 3. Immunohistochemical detection of NOS-1 in rodent kidneys. Female mouse **(a)**, female gerbil **(b)**, male hamster **(c)**, female rat **(d)**, and male guinea pig **(e, f)**. In female mice and male hamsters, granular NOS-1 positive reactions were observed in proximal straight tubules **(a and c)**, and giant granular NOS-1 positive reactions were observed in female mice (arrows). In female gerbils, positive reactions for NOS-1 were observed in the macula densa **(b)**. In female rats, positive reactions for NOS-1 were observed in the inner medullary collecting ducts **(d)**. In male guinea pigs, positive reactions for NOS-1 were observed in the distal straight tubules and papillary interstitium **(e and f)**. Bars: 20 μm.

species, and shows that mice had the greatest values in both sex groups. For males, significant differences were observed between mice and hamsters. For guinea pigs, the values of females were significantly higher than those of males. In nephron segments excluding the distal tubules, localization of NOS-1 varied markedly with species and sex (Fig. 3 and Table 1). In the proximal tubules, granular positive reactions were detected in the proximal straight tubules (PSTs) of female mice and hamsters of both sexes (Fig. 3a,c). Positive reactions were observed in the collecting ducts (CDs) for mice, rats, and hamsters (Table 1). In rats and hamsters, positive reactions were observed in the inner medullary CDs (IMCDs) (Fig. 3d); however, in mice, positive reactions were observed in the cortical CDs and IMCDs. Positive reactions were observed in the renal papillary interstitium (PI) in guinea pigs (Fig. 3f).

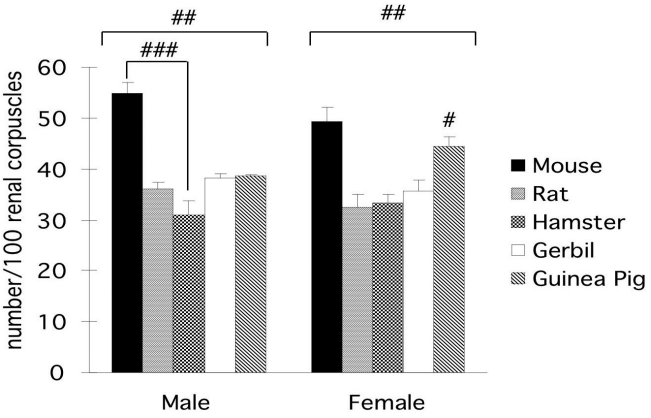

Fig. 4. Index of the number of NOS-1-positive macula densa cells. Each column represents the mean ± S.E. #: Significantly different from males of the same species (Mann-Whitney U test, $P<0.05$). ##: Significant species difference in each sex group (Kruskal-Wallis test, $P<0.05$). ###: Significant differences in multiple comparisons following the Kruskal-Wallis test (Scheffé method, $P<0.05$).

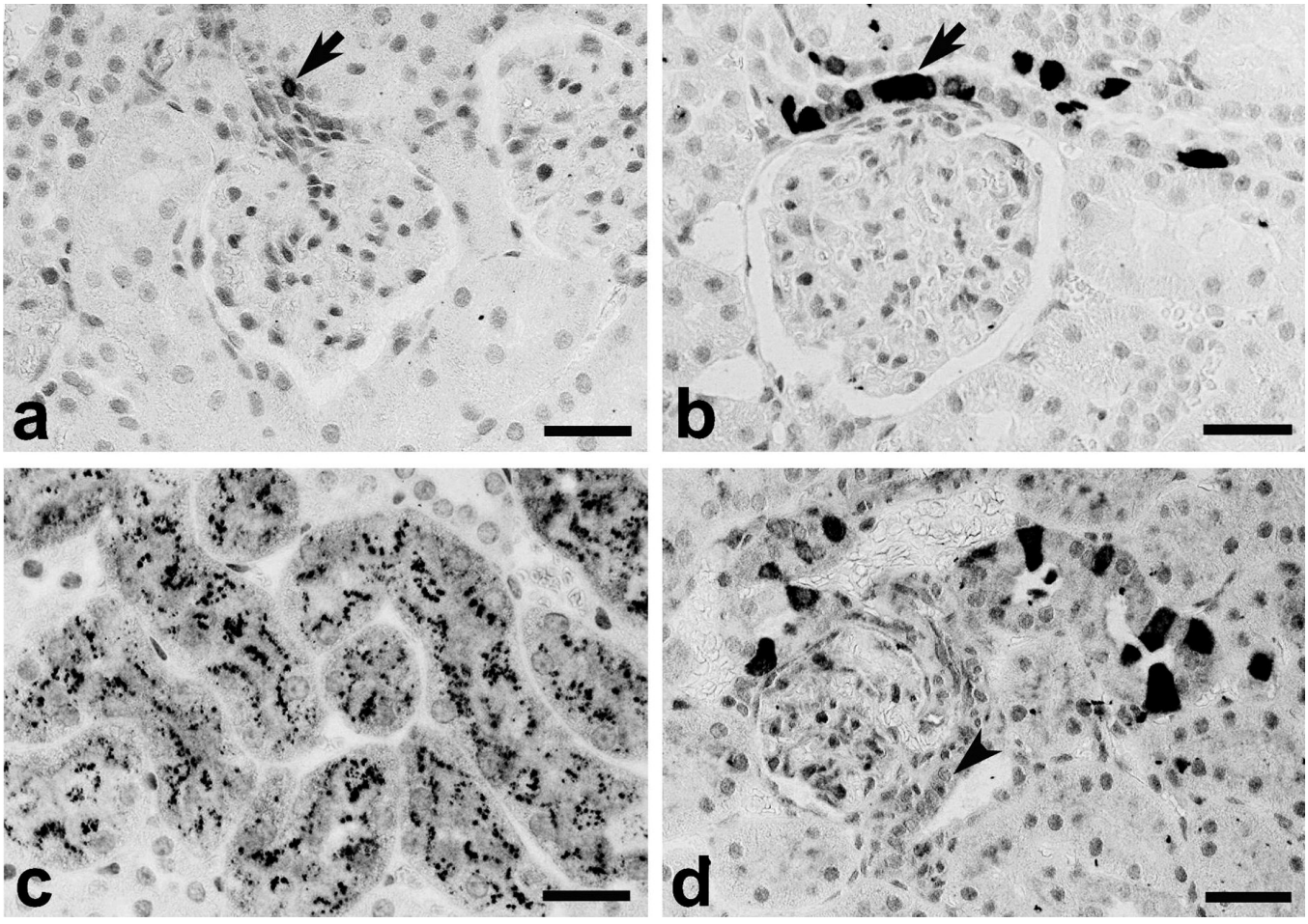

Fig. 5. Immunohistochemical detection of COX-2 in rodent kidneys. Male gerbil **(a)**, male rat **(b)**, male hamster **(c)**, and male guinea pig **(d)**. In male gerbils, positive reactions for COX-2 (arrows) were observed within a part of the macula densa (MD) **(a)**. In male rats, these reactions were widely observed in the MD **(b)**. In male hamsters, COX-2-positive granules were observed in the proximal straight tubules **(c)**. In male guinea pigs, positive reactions for COX-2 were not observed in the MD (arrowhead) but were heterogeneously seen in the cortical collecting ducts (arrows, **d**). Bars: 20 μm.

Table 2. Summary of immunohistochemical localization of COX-2-positive reactions in the kidneys of mice, rats, hamsters, gerbils and guinea pigs.

	Mouse		Rat		Hamster		Gerbil		Guinea Pig	
Site	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
PCT	-	-	-	-	-	-	-	-	-	-
PST	-	-	-	+	+	-	-	-	-	-
TL	-	-	-	-	-	-	-	-	-	-
DST	-	-	-	-	-	-	-	-	-	-
MD	+	+	+	+	+	+	+	+	-	-
DCT	-	-	-	-	-	-	-	-	-	-
CCD	-	-	-	-	-	-	-	-	+	+
OMCD	-	-	-	-	-	-	-	-	+	+
IMCD	-	-	-	-	-	-	-	-	+	+
PI	-	-	+	+	+	+	+	+	+	+

PCT: proximal convoluted tubules; PST: proximal straight tubules; TL: thin limbs; DST: distal straight tubules; MD: macula densa; DCT: distal convoluted tubules; CCD: cortical collecting ducts; OMCD: outer medullary collecting ducts; IMCD: inner medullary collecting ducts; PI: papillary interstitium; +: COX-2-positive staining; -: no COX-2 staining.

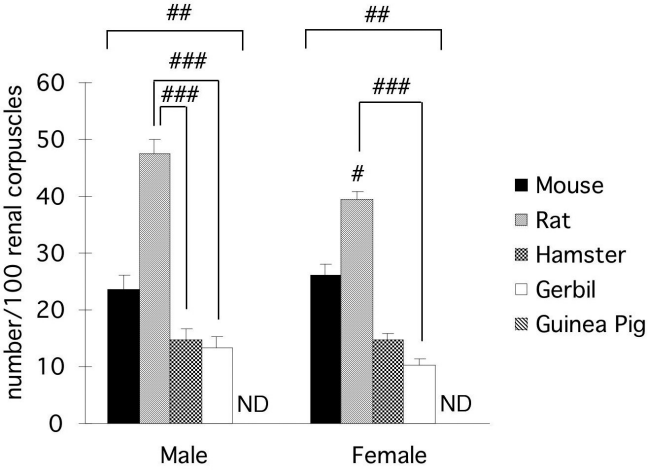

Fig. 6. Index of the number of COX-2-positive macula densa cells. Each column represents the mean ± S.E. #: Significantly different from males of the same species (Mann-Whitney U test, P<0.05). ##: Significant species difference in each sex group (Kruskal-Wallis test, P<0.05). ###: Significant differences in multiple comparisons following the Kruskal-Wallis test (Scheffé method, P<0.05). ND: not detected.

COX-2

In mice, rats, hamsters and gerbils, COX-2-positive reactions were observed in the MD (Fig. 5a,b). However, no COX-2-positive MD cells were detected in guinea pigs (Fig. 5d). Figure 6 shows the index for the number of COX-2-positive MD cells in the different species, showing that rats had the highest values in both sex groups. For males, rats had significantly higher values than hamsters and gerbils. For females, significant differences were observed between rats and gerbils. For rats, the values of females were significantly higher than those of males. In nephron segments excluding the MD, localization of COX-2 differed markedly among species and between the sexes (Fig. 5 and Table 2). In the proximal tubules, granular positive reactions were detected in the PSTs of female rats and male hamsters (Fig. 5c and Table 2). In guinea pigs, COX-2-positive cells, which were considered to be intercalated cells on the basis of their number and form, were observed over the full lengths of the CDs (Fig. 5d). Additionally, the PI had a reaction in all rodent species except mice (Table 2).

Discussion

The results of the present study morphometrically demonstrate that mice have wider distributions of renin-positive cells in the AA than do other rodents. A similarly wide distribution of renin has been previously reported in adult small ruminants and mammalian embryos (Kon et al., 1986). It has been suggested that in small ruminants, the renin-containing cells located further away from the glomerulus in the AA are sensitive to vascular innervation (beta-adrenergic) signals (Kon, 1999). Therefore, we propose that species-specific differences in the distribution of renin in the AA may be attributable to species-specific differences in the mechanisms of neuronal blood pressure regulation.

NOS-1 is also known as neuronal NOS, and is abundantly expressed in the nervous system. NOS-1 is reportedly constantly expressed in the MD of mouse, rat, guinea pig, rabbit, pig, and human kidney (Bachmann et al., 1995). Here we demonstrated for the first time that hamsters and gerbils also have NOS-1-positive MD.

Sex-related differences were observed in the number of NOS-positive MD cells, with female guinea pigs having higher values than male guinea pigs. Weiner et al. reported that renal NOS-1-activity in female guinea pigs was higher than that in males, and that this difference was caused by the effects of estrogen (Weiner et al., 1994). These findings strongly suggest that NOS-1 expression would be higher in the kidneys of female

guinea pigs than in those of males.

In distal tubular segments excluding the MD, NOS-1-positive reactions were detected in the outer medullary DSTs of guinea pigs. Furthermore, in nephron segments excluding the distal tubules, NOS-1-positive reactions were observed in the PSTs and IMCDs, and localization of these reactions differed markedly among species. Briefly, NOS-1-positive PSTs were observed in female mice and hamsters of both sexes, and NOS-1-positive IMCDs were observed in mice, rats, and guinea pigs of both sexes. Additionally, NOS-1-positive reactions were observed in the PI of guinea pigs. NOS-1 is a constitutive cytokine not a hormone, and acts locally in a paracrine/autocrine fashion. A recent study demonstrated that perfusion of 7-nitroindazole (a selective inhibitor of NOS-1) to proximal tubules increased intratubular fluid uptake in rat kidneys (Wu and Johns, 2002). Another study using arginine vasopressin-deficient rats demonstrated that NOS-1 participates in the regulation of water reabsorption in CDs (Martin et al., 2002). Furthermore, Fernandez et al. (2003) demonstrated that NOS-1 expression in IMCDs is up-regulated in spontaneous hypertensive rats compared with normotensive Wister-Kyoto rats, and suggested that NOS-1 in IMCDs participates in the regulation of blood pressure. Therefore, we propose that local NOS-1, which localizes in the PSTs, DSTs and IMCDs, may be involved in the regulation of tubular reabsorptive function or in the control of blood pressure, and our findings suggest the existence of species-specific differences in the mechanism by which tubular reabsorption and blood pressure control are maintained by NOS-1.

COX-2 is also known as inducible COX, and is expressed in inflammatory areas and tumor tissues (Shattuck-Brandt et al., 2000). In the kidney, COX-2 is reportedly constitutively expressed in distal tubules adjacent to the MD, and regulates the RAS and TGF via PGE_2 synthesis (Harris et al., 1994, 2000; Harding et al., 1997). In a previous study, clear species-specific differences in COX-2 expression were found in the MD of rats, dogs, cynomolgus monkeys, and humans (Khan et al., 1998). Briefly, the MD of rats and dogs had COX-2-positive reactions, but both primates had COX-2-negative MD. In the present study, COX-2-positive MD was observed in mice, rats, hamsters, and gerbils. The number of COX-2-positive MD cells differed among species, with rats having the highest values. Recent reports have suggested that COX-2 regulates renin synthesis, and that its function is under the control of NO produced by NOS-1 (Cheng et al., 1999, 2000; Harris et al., 2000). However, in the present study, no correlation was observed between species-specific differences in COX-2 and either renin or NOS-1 values (mice had the highest values for the latter two substances). Therefore, the higher expression level of COX-2 in rat MD may predominantly be associated with the regulation of TGF rather than the RAS via renin secretion. Furthermore, the number of COX-2-positive MD cells in male rats was higher than that in female rats. Tada et al. (2004) found that expression of COX-2 in rat renal cortex was enhanced by ovariectomy, and reduced by estrogen replacement therapy. Therefore, the sex differences in the number of COX-2-positive MD cells in rats may have resulted from COX-2 inhibition by estrogen.

COX-2-negative MD has been observed in primates such as cynomolgus monkeys and humans (Khan et al., 1998). However, in the present study, in all renal regions except for the MD, localization of COX-2 in the guinea pig was different from that in both primates. Briefly, glomerular podocytes and small blood vessels showed positive reactions in primates, while the CD intercalated cells showed positive reactions in guinea pigs. CD intercalated cells play an important role in acid-base equilibrium by secreting H^+ or HCO_3^- (Kuwahara et al., 1992). Therefore, the COX-2 that is expressed in the CDs of guinea pigs may be involved in the maintenance of acid-base balance.

COX-2-positive reactions were also observed in the PSTs and PI. The localization of immunoreactions in these segments differed markedly among species. In the PSTs, positive reactions were observed in female rats and male hamsters. COX-2 expression in the proximal tubule has been reported in reflux nephropathy (Solari et al., 2003). However, no study in the literature has clarified the function of COX-2 in the proximal tubules in normal kidney. However, there is some evidence that rat PI expresses COX-2, which participates in the maintenance of medullary electrolyte hypertonicity (Zhang et al., 2002). In the present study, COX-2-positive reactions in the PI were observed in not only rats, but also hamsters, gerbils, and guinea pigs. Interestingly, mice had no COX-2-positive PI. Therefore, our findings suggest the existence of species-specific differences in the mechanism by which electrolyte hypertonicity is maintained by COX-2. It is possible that these mechanisms may be lacking in the mouse renal medulla.

In conclusion, we identified clear species-specific differences in the localization of renin, NOS-1, and COX-2 in rodent kidneys. Our data suggest that the mechanisms by which blood pressure and body fluid composition are regulated via the RAS or TGF differ markedly among species. Our data also provide new insights into the species-specific differences that exist with respect to complex renal functions.

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