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Species-, sex-, and age-dependent urinary excretion of cauxin, a mammalian carboxylesterase

Masao Miyazaki [a], Tetsuro Yamashita [b], Masakiyo Hosokawa [c], Hideharu Taira [b], Akemi Suzuki [a,*]

[a] *Sphingolipid Expression Laboratory, Supra-Biomolecular System Group, Frontier Research System, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan*
[b] *Department of Agro-Bioscience, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka 020-8550, Japan*
[c] *Department of Pharmaceutical Science on Life and Medicare, Chiba Institute of Science, 3 Shiomi, Choshi, Chiba 288-0025, Japan*

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Abstract

Domestic cats exhibit physiological proteinuria due to the excretion of cauxin, a carboxylesterase, into the urine. In the present report, we demonstrate that cauxin is excreted in a species-, sex-, and age-dependent manner. Although the cauxin gene is conserved in mammals, including human, mouse, and dog, urinary cauxin was found only in member of the genus *Felis* and lynx (bobcat, and lynx) and not in other Felidae (genus: *Panthera* and puma) tested. In mature cats, cauxin excretion was higher in intact males than in castrated males or in intact or spayed females. Daily cauxin excretion decreased immediately after castration. Immunohistochemistry confirmed that cauxin expression in the kidney proximal straight tubules was higher in intact males than in castrated males. Urinary cauxin was detectable by Western blotting in cats older than about 3 months, and its excretion increased with age. In a zymographic esterase assay, urine contained a major cauxin band; by contrast, kidney homogenates contained three major bands, comprising two carboxylesterases and an unidentified esterase, and one minor cauxin band. These results suggest that 1. cauxin excretion is regulated by sex hormones, such as testosterone, 2. cauxin functions as an esterase in the urine rather than in kidney cells, and 3. the decomposition products by cauxin are excreted in a species-, sex-, and age-dependent manner, as is cauxin itself.

Keywords: Domestic cat; Felidae; Urinary protein; Cauxin; Carboxylesterase; Kidney

1. Introduction

In general, mammals excrete only small amounts of protein into the urine to avoid protein leakage from the body, and proteinuria is an indicator of renal abnormality (D'Amico and Bazzi, 2003). However, we previously discovered that normal domestic cats (*Felis catus*) show physiological proteinuria (Miyazaki et al., 2003). Normal mice (*Mus musculus*) and rats (*Rattus norvegicus*) also exhibit physiological proteinuria involving the species-specific excretion of major urinary proteins (MUPs) that are members of the lipocalin superfamily and function as carriers of volatile urinary pheromones (Cavaggioni and Mucignat-Caretta, 2000; Beynon and Hurst, 2003). Since the urinary excretion of MUPs had not been reported in the cat, we investigated the cause of physiological proteinuria in the cat. We found that a novel 70 kDa protein, which we named cauxin, is excreted as a major urinary protein in normal cats (Miyazaki et al., 2003).

The deduced amino acid sequence of cauxin shares 42–47% identity with mammalian carboxylesterases (CESs, EC 3.1.1.1), that hydrolyze a wide variety of aromatic and aliphatic substrates containing ester, thioester, and amide bonds (Heymann, 1980, 1982) and are involved in the detoxification of foreign compounds and the metabolic pathways of endogenous lipids (Harrison, 1998; Satoh and Hosokawa, 1998). In addition, we found that cauxin, which is expressed in a kidney-specific manner and secreted from the renal tubular cells into the urine, has markedly different biochemical characteristics from mammalian CESs. A large number of mammalian CESs with different isoelectric points and substrate specificities have been

* Corresponding author. Tel.: +81 48 467 9615; fax: +81 48 462 4692.
E-mail address: aksuzuki@riken.jp (A. Suzuki).

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identified from liver, kidney, small intestine, brain, lung, and testis (Ronai et al., 1985; Satoh and Hosokawa, 1998; Satoh et al., 2002). CESs are localized in the lumen of the endoplasmic reticulum, except for one serum CES that is secreted from hepatic cells (Yan et al., 1995). Therefore, our findings in the cat constituted the first description of excretion of a CES-like protein into mammalian urine.

Cauxin homologues are found in the genome databases of several mammals, including human (*Homo sapiens*), crab-eating macaque (*Macaca fascicularis*), mouse, rat, dog (*Canis familiaris*), and cow (*Bos taurus*). However, these species do not excrete cauxin in the urine (Miyazaki et al., 2003). Cauxin mRNA is expressed in human liver and kidney and in mouse brain, heart, and kidney (Miyazaki et al., unpublished data). In addition, Ecroyd et al. (2005) recently found ram (*Ovis aries*) cauxin in the caudal epididymal fluid. Interestingly, ram cauxin, along with clusterin and a 17 kDa hydrophobic epididymal protein, associate with epididymal soluble prion protein in the male reproductive fluid. The biological significance of this association is unknown. These findings indicate that cauxin plays different physiological roles in different mammalian species.

Since cauxin purified from cat urine hydrolyzes the artificial substrates *p*-nitrophenylacetate and 1-naphthylacetate, we speculated that it hydrolyzes physiological substrates in the urine or renal tubular cells (Miyazaki et al., 2003). In the present report, we demonstrate that cauxin is excreted in a species-, sex-, and age-dependent manner in the cat and animals belonging to the genus *Felis* of the Felidae. Furthermore, to determine whether cauxin functions as an esterase in the urine or kidney, we performed zymographic esterase assays with urine and kidney homogenates using 1-naphthylacetate as substrate.

2. Methods

2.1. Antibodies

Rabbit polyclonal antibodies against cauxin purified from cat urine and against a C-terminal cauxin peptide were raised as described previously (Miyazaki et al., 2003). Rabbit polyclonal antibodies against megalin and CES were raised against the C-terminal region of mouse megalin and against CES purified from dog liver, respectively, as described previously (Sekine et al., 2006; Hosokawa et al., 2001). We confirmed that anti-dog CES antibody is cross-reactive with recombinant cat kidney CES protein (GenBank accession no. AB114676) expressed in COS-7 cells (data not shown). Sheep polyclonal antibody against Tamm-Horsfall glycoprotein (THP) purified from human urine was purchased from Biogenesis (United Kingdom).

2.2. Urine sample collection

Urine samples were collected from 27 normal mature cats (8 intact males, 6 castrated males, 7 intact females, and 6 spayed females; 1–6 years of age), a castrated male cat (for 1 month after castration), and three immature cats (1 male and 2 females). For the immature cats, urine samples were collected from a male and a female from 11 to 18 weeks of age, and from one female from 12 to 17 weeks of age. All urine samples were collected by direct manual pressure on the bladder. The castrated and immature cats were housed individually in metabolic cages to collect urine samples. Urine samples were also collected from eight Felidae species from the three different evolutionary lineages proposed by Johnson et al. (2006) at Gunma Safari Park (Japan). These species were bobcat (*Lynx rufus*, 3 intact males, 1 intact female; 10–14 years of age), lynx (*Lynx lynx*, 1 intact male, 3 intact females; 2–14 years of age), puma (*Puma concolor*, 2 intact males, 1 intact female; 13–23 years of age), leopard (*Panthera pardus*, 2 intact males; 9 and 10 years of age), tiger (*P. tigris*, 4 intact males, 4 intact females, 3–17 years of age), jaguar (*P. onca*, 2 intact males, 1 intact females; 3–9 years of age), snow leopard (*P. uncia*, 1 intact male, 1 intact female; 9 and 10 years of age), and lion (*P. leo*, 1 intact male, 2 intact female; 1–9 years of age). Samples were taken from urine excreted onto the ground. Each sample was centrifuged at 500 ×*g* for 5 min, and the supernatants were stored at −20 °C. The protocols for urine and kidney sample collection were approved by the Animal Research Committee of the Faculty of Agriculture of Iwate University. Experiments were conducted according to the Iwate University Guidelines for Animal Experimentation.

2.3. Analysis of urinary cauxin

Non-reducing 12%-SDS-PAGE was performed with 20 μL urine samples, except that 10 μL samples were used for cat, bobcat, lynx urine. Gels were stained with Coomassie brilliant blue R-250 (CBB). Western blotting using anti-cauxin antibody was performed with 10 μL urine samples, except that 1 μL samples were used for cat, bobcat, and lynx urine, as described previously (Miyazaki et al., 2003).

Ten-microliter urine samples from mature cats were resolved using non-reducing 12%-SDS-PAGE and stained with CBB. The 70 kDa cauxin band was analyzed using an LAS 1000 with Fujix Bioimaging software (Fuji Film, Tokyo, Japan), and the urinary cauxin concentration was calculated using a standard consisting of cauxin purified from urine, as described previously (Miyazaki et al., 2003). Urinary creatinine concentration was measured using a creatinine test (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Urinary cauxin to creatinine ratio is calculated by dividing urinary cauxin concentration (g/L) by urinary creatinine concentration (g/L). Ten-microliter urine samples from immature cats were analyzed by Western blotting with anti-cauxin peptide and anti-THP antibodies.

2.4. Immunohistochemistry

Paraffin sections were prepared from one intact and one castrated male cat, as described previously (Miyazaki et al., 2003). For fluorescence detection, deparaffinized sections were incubated first with 10% goat serum in PBS, then with anti-cauxin peptide or anti-CES antibody (each diluted 1:200), and finally with goat anti-rabbit IgG antibody coupled to Alexa Fluor-488 (Molecular Probes). Each incubation step occurred

for 1 h at room temperature. Sections were examined using an inverted fluorescence microscope (IX-50; Olympus) equipped with a digital camera (DP70; Olympus).

For double staining of cauxin and THP, deparaffinized sections were incubated first with 10% goat serum in PBS and then with anti-cauxin peptide antibody and anti-THP antibody (each diluted 1:300). Each incubation step occurred for 1 h at room temperature. The sections were then incubated with goat anti-rabbit IgG antibody coupled to Alexa Fluor-488 (Molecular Probes) and donkey anti-sheep IgG antibody coupled to Alexa Fluor-546 (Molecular Probes). Sections were examined using a Zeiss LSM 510 laser confocal microscope (Carl Zeiss, Germany).

For detection of horseradish peroxidase, endogenous peroxidase was blocked with 3% H_2O_2 in methanol. Deparaffinized sections were then incubated with 10% goat serum in PBS at room temperature for 1 h and then with anti-cauxin peptide or anti-megalin antibody (each diluted at 1:500) at room temperature for 1 h. After treatment with horseradish peroxidase-labeled polymer-conjugated anti-rabbit IgG antibodies (EnVision+, peroxidase, rabbit, Dako) for 30 min at 25 °C, the sections were treated with diaminobenzidine to visualize the antigen–antibody reaction. Finally, the sections were counterstained with hematoxylin and observed under a light microscope.

2.5. Reverse transcriptase (RT)-PCR

Total RNA was extracted from the kidneys of one 2-year-old and one 2-month-old male cat using TRIzol reagent (Gibco BRL, Grand Island, NY, USA). Messenger RNA was isolated from the total RNA using Oligotex-dT 30 Super (Takara, Osaka, Japan), and 1 μg of mRNA was used to synthesize cDNA using the SuperScript First-Strand Synthesis System (Invitrogen) in a total volume of 10 μL. Each gene was amplified from 2 μL of cDNA for 25 cycles using KOD-Plus (Toyobo). The primers for cauxin (GenBank accession no. AB045377), cat kidney CES (GenBank accession no. AB114676), and cat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession no. AB038241) were as follows (shown 5′ to 3′): Cauxin: forward, ATGTGGGTGCACCCAGGCCG; reverse, TCAGGGGA CAATGGTATTCA; cat kidney CES: forward, ATGTGG CCTCTTAGCTCTGG; reverse, TCACAGCTCAACGTG CTCTT; and GAPDH: forward, ACCACAGTCCATGCCAT CAC; reverse, TCCACCACCCTGTTGCTGTA.

2.6. Anion-exchange column chromatography separation of cauxin and cat kidney CESs

Approximately 1.5 g of frozen cat kidney were homogenized in 5 mL of 10 mM Tris-HCl (pH 7.4) containing 5 mM EDTA, and the homogenate was centrifuged at 15,000 ×*g* for 30 min at 4 °C. The supernatant fraction was removed and further clarified by centrifugation at 100,000 ×*g* for 60 min at 4 °C. The clarified supernatant fraction was applied at a flow rate of 3 mL/min to a 6-mL anion-exchange column (RESOURCE Q, Amersham Pharmacia) equilibrated with 10 mM Tris-HCl (pH 7.4). Elution with a gradient of 0–400 mM NaCl in the equilibration buffer for 12 min started 8.3 min after sample injection, and 1.5 mL fractions were collected. Aliquots (20 μL) of each fraction were resolved using native 10%-PAGE, and gels were analyzed in a zymographic assay using 1-naphthylacetate, as described previously (Miyazaki et al., 2003). Cauxin and CES in the fractions were identified by Western blotting using anti-cauxin peptide and anti-CES antibodies, respectively.

2.7. Additional methods

The phylogenetic tree was calculated using the neighbor-joining method. The amino acid sequence of the N-terminus of the 70 kDa lion protein was determined using a protein sequencer (PPSQ-21, Shimadzu). For statistical analysis, data were analyzed using the Mann-Whitney *U* test. Significance was defined as $p<0.05$.

3. Results

3.1. Phylogenetic analysis of cauxin

Genes homologous to cat cauxin were found in the genome databases of *Homo sapiens* (GenBank accession no. BC069501), *M. musculus* (AB186393), *R. norvegicus* (NM001012056), *C. familiaris* (AB186392), and *B. taurus* (XM591772), but not in those of chicken (*Gallus gallus*), South African clawed frog (*Xenopus laevis*), zebrafish (*Danio rerio*), or insects. The deduced amino acid sequences of these cauxin genes were 64–81% identical to cat cauxin and contained serine hydrolase (GESAG) and CES (EDCLY) sequence motifs.

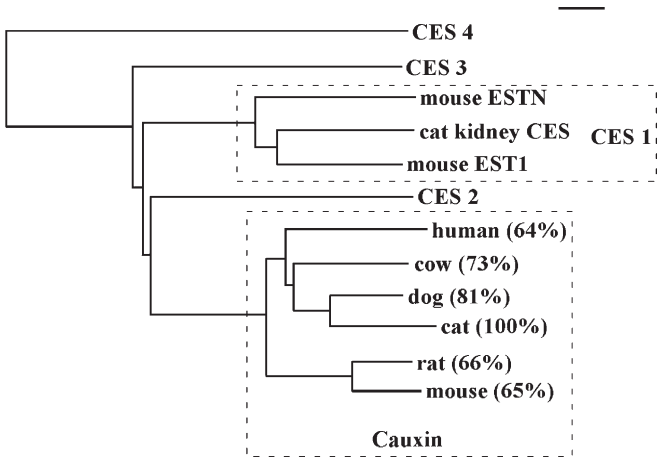

Fig. 1. Phylogenetic tree of cauxin and the CES family. The phylogenetic tree was constructed using the neighbor-joining method. The branch lengths are proportional to the relative phylogenetic distances between the proteins. The sequence identity percentages (shown in parentheses) are relative to the amino acid sequence of cat cauxin. The accession numbers of the CES sequences are as follows: CES 1: mouse ESTN (L11333), cat kidney CES (AB114676), and mouse EST1 (AK007235); CES 2: mouse similar to CES 2 (BC015290); CES 3: mouse ES-male (S64130); and CES 4: ES 46.5 kDa protein (Q99PG0). The accession numbers of the cauxin sequences are as follows: dog (AB186392), cat (AB045377), human (AAH69548), mouse (BAD35016), rat (NP001012056), and cow (XM591772).

Phylogenetic analysis indicated that cat and other mammalian cauxins are not members of the four mammalian CES subfamilies (CES 1, 2, 3, and 4) described by Satoh and Hosokawa (1998) (Fig. 1). Instead, after the CES 1 and 2 subfamilies diverged from the CES 3 family, cauxin further diverged from the CES 2 subfamily and independently evolved into the mammalian cauxins. Based on these results, we conclude that cauxin is conserved among mammals and should be categorized in a new subfamily of the mammalian CES family.

3.2. Species-dependent excretion of cauxin

In an SDS-PAGE analysis of urine obtained from eight felid species, a broad, major CBB-stained band co-migrating with cat cauxin was detected in bobcat and lynx, but not in puma, jaguar, leopard, lion, tiger, or snow leopard (Fig. 2A). Western blotting with anti-cauxin antibody confirmed that the protein detected in bobcat and lynx was cauxin (Fig. 2B). In the urine of Felidae that do not excrete cauxin, albumin was detected at 66 kDa, and, in male and female lions, a minor band was detected at 70 kDa. We analyzed the N-terminal amino acid sequence of the protein in this minor band using a protein sequencer and found that the partial amino acid sequence (APHAPGLVYRE) was similar to that of human fetuin, indicating that the lion 70 kDa protein is not cauxin. The Felidae phylogenetic tree proposed by Johnson et al. (2006) based on data for X-linked, Y-linked, and mitochondrial gene segments is shown in Fig. 2C. This tree reveals that only the close relatives of *F. catus* in the Felidae lineage excrete cauxin as a major urinary protein.

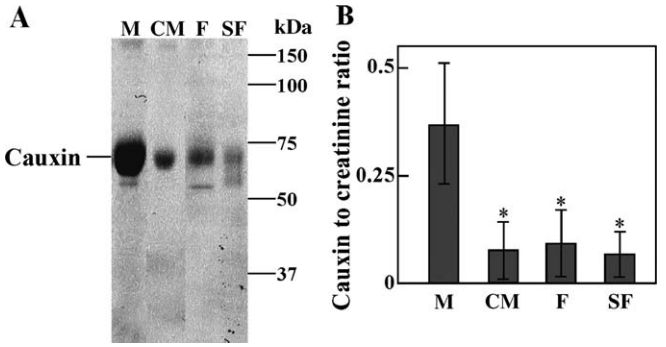

Fig. 3. Urinary concentration of cauxin in mature cats. A: Ten-μL aliquots of urine samples obtained from intact male and female (M and F), castrated male (CM), and spayed female (SF) cats were resolved by SDS-PAGE under non-reducing conditions and stained with CBB. A major cauxin band was detected. B: Each urinary cauxin to creatinine ratio (mean±standard deviation) of 8 intact males (M), 6 castrated males (CM), 7 intact females (F), and 6 spayed females (SF) was calculated by dividing urinary cauxin concentration (g/L) by urinary creatinine concentrations (g/L). *Indicates that the difference in the cauxin level between intact males and the other cats tested is statistically significant ($p<0.05$, Mann-Whitney *U* test).

3.3. Sex-dependent excretion of cauxin

SDS-PAGE analysis indicated that cauxin was a major urinary protein in all of the mature domestic cats tested (Fig. 3A). The urinary cauxin concentrations in 8 intact males, 6 castrated males, 7 intact females, and 6 spayed females were 0.87±0.19, 0.13±0.10, 0.23±0.12, and 0.04±0.03 g/L, respectively. Since urinary protein concentration varies, we calculated

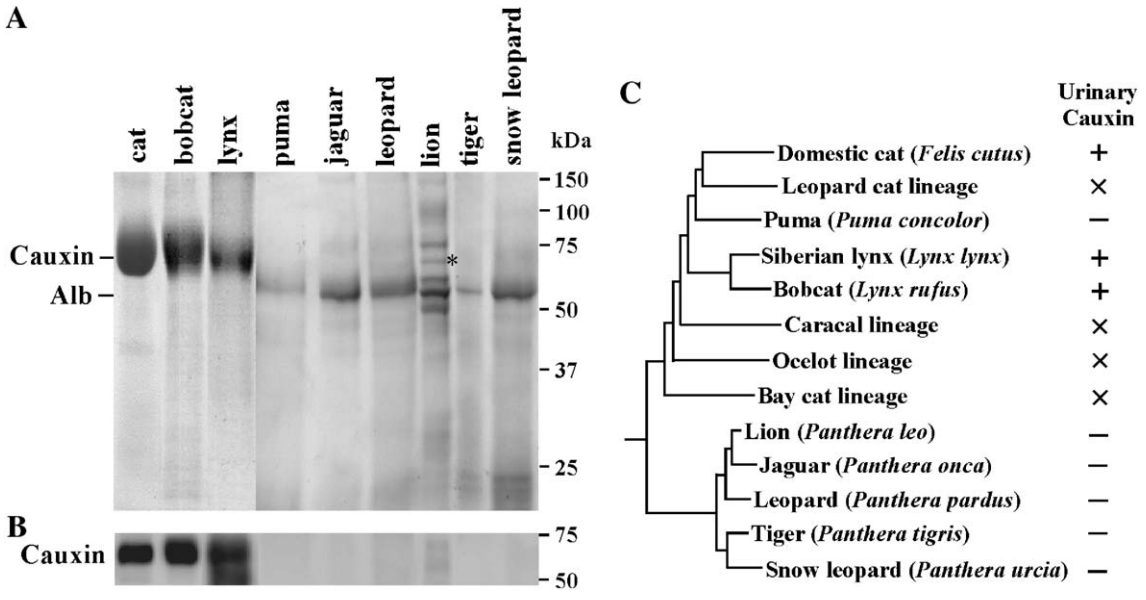

Fig. 2. Analysis of urinary proteins in the Felidae. A: Ten μL of cat, bobcat, and lynx urine or 20 μL of other members of the Felidae urine were resolved using 12%-SDS-PAGE under non-reducing conditions, and the gel was stained with CBB. All of the urine samples shown were from mature, intact males. *Indicates the lion fetuin-like protein. The molecular mass markers are shown to the right. Alb, albumin. B: One μL of cat, bobcat, and lynx urine or 10 μL of other Felidae urine were analyzed using Western blotting with anti-serum raised against cauxin purified from cat urine. The same samples are shown as in A. C: A phylogenetic tree of the Felidae modified from that proposed by Johnson et al. (2006), and detection of urinary cauxin by Western blotting with anti-cauxin antibody. The tree was constructed using data on X-linked, Y-linked, and mitochondrial gene segments. +, cauxin was detected: −, cauxin was not detected: and ×, samples were not available.

each average urinary cauxin to creatinine ratio of intact males, castrated males, intact females, and spayed females, and statistically analyzed these ratios. The cauxin level was significantly higher ($p<0.05$) in intact males than in castrated males or intact or spayed females (Fig. 3B), indicating that cauxin is excreted in a sex-dependent manner in the mature cat.

The temporal changes in the urinary cauxin concentration for a male cat (body mass 4.2 kg) for the 4 weeks following castration are shown in Fig. 4A, normalized to the urinary creatinine concentration. Cauxin excretion decreased significantly immediately after castration, and it fell to less than 10% of the pre-castration level by 4 weeks after castration. The 24-h excretion of cauxin was 116.9 mg before castration, and it decreased to 77.0, 29.1, 39.1, 25.2, and 9.1 mg at 3 days and 1, 2, 3, and 4 weeks after castration, respectively.

Immunohistochemical images of cauxin and CES in the kidney are shown in Fig. 4B–E. The samples shown in Fig. 4C and E were obtained from an intact male and a castrated male, respectively. The sample from the castrated male was obtained 33 days after castration. Each image was obtained using the same experimental conditions and exposure time with an Olympus DP70 digital camera system. The fluorescent signal of cauxin in the renal tubules was significantly stronger in the intact male (Fig. 4B) than in the castrated male (Fig. 4C), whereas the fluorescent signals of CES in the proximal tubules of the intact (Fig. 4D) and castrated (Fig. 4E) males were the same. These results indicate that the decrease in the level of cauxin excretion upon castration (Fig. 4A) is due to a decrease in the level of cauxin expression in the renal tubules.

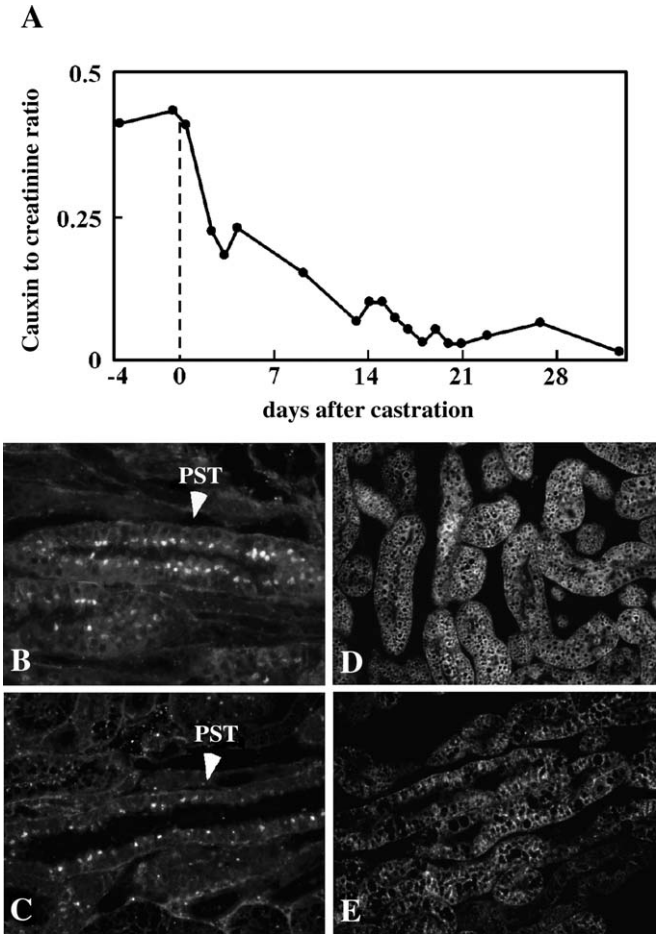

Fig. 4. The influence of castration on cauxin excretion and expression in the kidney. A: Temporal changes in cauxin in a male cat for the month following castration. The urinary cauxin to creatinine ratio was calculated by dividing urinary cauxin concentration (g/L) by urinary creatinine concentrations (g/L). B and C: Immunohistochemistry of renal sections with anti-cauxin peptide antibody. D and E: Immunohistochemistry of renal sections with anti-CES antibody. B and D are from an intact male. C and E are from a castrated male. Images in B, C, D, and E were obtained under the same conditions using a DP70 digital camera system. PST, proximal straight tubules.

3.4. Tissue distribution of cauxin

In our previous report (Miyazaki et al., 2003), we concluded that cauxin is localized in the distal tubular cells, based on our finding that cauxin-immunoreactive renal tubular cells were distributed in the inner cortex and outer medulla, and this distribution corresponds to the distribution of the distal tubules. In addition, cauxin-immunoreactive cells did not contain the lipid droplets that are characteristically observed in the proximal convoluted tubules in the cat. However, we did not further examine cauxin localization by parallel staining with distal tubule-specific markers, such as THP. Therefore, in the present study, we examined the tissue localization of cauxin in detail.

Unexpectedly, in images double-stained with anti-cauxin and anti-THP antibodies, cauxin did not colocalize with THP (Fig. 5A), indicating that cauxin does not localize at the distal tubules. Furthermore, when we examined cauxin localization by parallel staining with megalin, which localizes on the luminal side of the proximal convoluted and straight tubules (Christensen and Birn, 2001), we found that cauxin colocalized with megalin in the proximal straight tubules, where there are no lipid droplets (Fig. 5B and C). Based on these results, we conclude that cauxin localizes in the proximal straight tubular cells and is secreted from these cells into the urine.

3.5. Age-dependent excretion of cauxin

The age-dependence of cauxin expression was analyzed using RT-PCR with a GAPDH amplicon (452 bp) as a positive control. Nearly equal levels of GAPDH mRNA were observed in a 2-year-old mature male cat and a 2-month-old immature male cat (Fig. 6A). Under these conditions, cauxin mRNA was detected in the mature cat, but not in the immature cat, whereas cat kidney CES mRNA was detected in both cats.

We next examined the temporal changes of urinary cauxin in three immature cats between 11 and 18 weeks after birth. THP, which was used as a positive control, was excreted at similar levels in all urine samples (110 kDa band). In contrast, urinary cauxin was undetectable by Western blotting in the immature cats at 11 weeks of age. A weak cauxin band was detectable in the urine of the immature cats from 12–14 weeks of age, and its intensity increased with age for each cat. These results indicate that cauxin excretion is age-dependent and it increases beginning at about 3 months of age in both male and female cats.

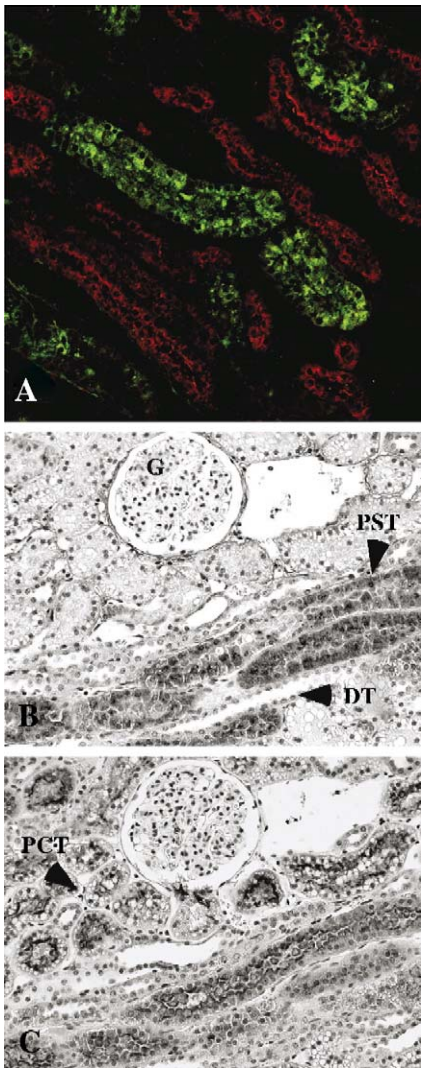

Fig. 5. Tissue distribution of cauxin. Immunohistochemistry of renal sections from a male cat was performed using anti-cauxin peptide, anti-THP, and anti-megalin antibodies. A: Merged image from immunofluorescence analysis of cauxin (green) and THP (red). Immunohistochemistry of serial paraffin sections from similar locations in the kidney was performed using anti-cauxin peptide (B) and anti-megalin (C) antibodies. G, glomerulus; PCT, proximal convoluted tubules; PST, proximal straight tubules; DT, distal tubules. All images: original magnification ×100.

3.6. Esterase activity of cauxin in the urine and kidney

In a zymographic assay of esterases, a band co-migrating with cauxin was detected in the urine (Fig. 7A, urine), but not in the 100,000 ×*g* supernatant of cat kidney homogenates, which contained three major bands: K1, K2, and K3 (Fig. 7A, kidney). When we separated these homogenates into 35 fractions using anion-exchange column chromatography and performed Western blotting on native PAGE with anti-cauxin peptide antibody, we detected a cauxin band in fractions 22–27 (Fig. 7B). This band corresponds to the minor band detected in fractions 23–25 in the zymographic assay (Fig. 7A, dashed rectangle).

Anion-exchange column chromatography separated bands K1, K2, and K3 into fractions 17–23, 25–27, and 26–32,

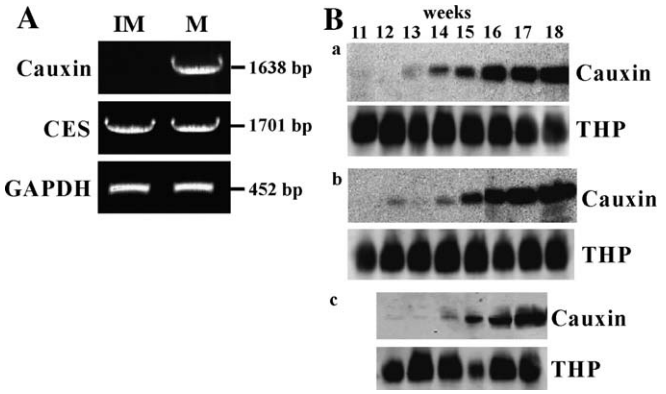

Fig. 6. Expression of cauxin and CES in cat kidney. A: RT-PCR was performed with kidney mRNA from 2-month-old (IM) and 2-year-old (M) male cats. The cDNA was synthesized from 1 μg of mRNA in a total volume of 10 μL. The genes were amplified from 2 μL of the cDNA using 25 cycles with cauxin-, CES-, and GAPDH-specific primers. B: Western blotting of a 10 μL aliquot of urine obtained from a male (a) and two female (b and c) cats was performed using anti-cauxin peptide and anti-THP antibodies.

respectively. Western blotting with anti-CES antibody on SDS-PAGE detected a major band in fractions 18–23 and a minor band in fractions 28–29, and both bands had the same apparent molecular mass (approx. 60 kDa) (Fig. 7C), suggesting that the

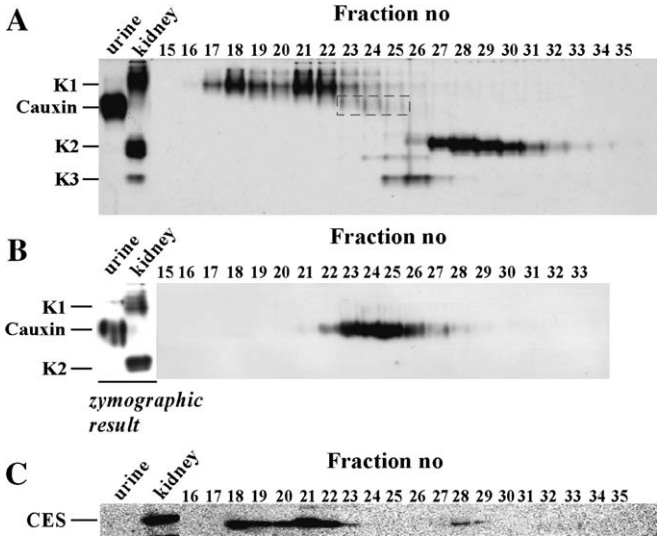

Fig. 7. Separation of cat kidney CESs and cauxin by anion-exchange column chromatography. The 100,000 ×*g* supernatant fraction of kidney homogenates was applied to an anion-exchange column, as described in ""Section 2"" Methods. Elution with a gradient of 0–400 mM NaCl in the equilibration buffer for 12 min started 8.3 min after sample injection, and 1.5 mL fractions were collected. A: A 20 μL aliquot of each fraction was subjected to native PAGE, and the gel was analyzed in a zymographic assay using 1-naphthylacetate. Urine, 20 μL of male cat urine; kidney, 20 μg of the 100,000 ×*g* supernatant fraction from male cat kidney homogenate. The locations of cauxin, K1, K2, and K3 are shown to the left of the panel. B: A native PAGE gel of chromatographic fractions 15–33 was immunoblotted with anti-cauxin peptide antibody. Urine, zymographic result for 20 μL urine; kidney, zymographic result for 20 μg of the 100,000 ×*g* supernatant fraction. C: An SDS-PAGE gel of chromatographic fractions 16–33 was immunoblotted with anti-dog CES antibody. Urine, 10 μL of male cat urine; kidney, 10 μg of the 100,000 ×*g* supernatant fraction from male cat kidney homogenate.

difference in mobilities of K1 and K2 on native PAGE was due to differences in their isoelectric points. Based on the native PAGE mobilities of K1, K2, and rat hydrolases A (57 kDa, pI 6.0) and B (59 kDa, pI 6.2) reported by Morgan et al. (1994), K1 and K2 belong to the same CES subfamilies as rat hydrolases A and B, respectively.

4. Discussion

Cauxin was initially identified in the domestic cat as a species-specific urinary protein which is the cause for physiological proteinuria (Miyazaki et al., 2003). Recently, the cauxin gene was found to be conserved in several mammals, including human, mouse, dog, and cow (Fig. 1), although only the cat excretes cauxin. Therefore, we examined whether cauxin is excreted in the urine of Felidae species other than the cat. We found that cauxin was excreted as a major urinary protein in the urine of the bobcat and lynx, but not in the urine of the other Felidae species tested (Fig. 2A and B). The bobcat and lynx are closely related to the cat in the Felidae lineage, and both belong to the same genus near to *Felis* genus (Fig. 2C). Therefore, our findings indicate that cauxin excretion is genus-dependent. However, the fact that urinary cauxin was not detected in puma is surprising, because the puma has recently been found to be more closely related to the cat than to the bobcat or lynx (Johnson et al., 2006). Future studies, including cloning of the cauxin genes for each Felidae species, should aid in determining why only the cat, bobcat, and lynx developed the machinery for cauxin excretion during evolution.

In the present study, we found that cauxin excretion is sex-dependent. Cauxin excretion levels were markedly higher in intact mature males than in castrated males or intact or spayed females (Fig. 3). Upon castration, cauxin excretion decreased immediately, and the cause was confirmed to be a decrease in the level of cauxin expression in the proximal straight tubules (Fig. 4). The plasma testosterone concentration in intact male cats has been reported to increase with age to 1.5, 6.8, and 12.6 pmol/mL at 6, 12, and 16 months after birth, respectively, and to decrease to zero after castration (Tarttelin et al., 1998). Based on this report and our findings, we postulate that the transcriptional activity of cauxin is regulated by sex hormones, such as testosterone.

Our data indicate that cauxin functions as an esterase in the urine rather than in the kidney proximal straight tubular cells. In a zymographic esterase assay, high esterase activity due to cauxin was detected in the urine (Fig. 7A, urine), whereas it was not detected in the 100,000 ×*g* supernatant fraction of kidney homogenates. This fraction contained three major bands; of these bands, two were CESs (K1 and K2) and one was an unidentified esterase (Fig. 7A, kidney). After anion-exchange column chromatography of the kidney homogenates, the zymographic assay detected very faint bands of kidney cauxin (Fig. 7A, fractions 23–25). The mobility of kidney cauxin on native PAGE corresponded to that of urinary cauxin, suggesting that the kidney cauxin detected in the zymographic assay is an active form of the esterase, as is urinary cauxin. Our previous study revealed that cauxin hydrolyzes *p*-nitrophenylacetate with a V_{max} of 7.61 μmol/mg/min, which is 10–40 times lower than the V_{max} values of CESs (Miyazaki et al., 2003). Based on these results, the esterase activity of cauxin appears to be markedly lower than that of other intracellular esterases, such as kidney CESs. Although the evolutionary factors underlying selection of cauxin, with its low esterase activity, as a urinary enzyme in the evolution of the cat are unknown, cat urine has a high esterase activity by virtue of the fact that it contains a large amount of cauxin. Therefore, we believe that the cat exploits the esterase activity of cauxin to hydrolyze physiological substrates in the urine.

In this study, we found that cauxin is excreted in a species-, sex-, and age-dependent manner in the domestic cat and its near relatives in the Felidae, such as the bobcat and lynx. In addition, we also suggest that cauxin functions as a urinary enzyme. Based on these findings, we speculate that the physiological substrates of cauxin are related to species, sex, and sexual maturation in the cat, and the decomposition products resulting from the hydrolytic reaction of cauxin are excreted in the urine and vary in a species-, sex-, and age-dependent manner, like cauxin itself.

As a candidate for decomposition products by cauxin, we are now investigating the cat-specific urinary amino acid, 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid, also known as felinine (Hendriks et al., 1995). Felinine is a putative pheromone precursor that is also excreted into the urine of the cat in a species-, sex-, and age-dependent manner (Hendriks et al., 1995). It has proposed that the precursor of felinine, 3-methylbutanol-glutathione, is hydrolyzed into felinine (Rutherfurd et al., 2002; Hendriks et al., 2004). Since cauxin is a member of the CES family which hydrolyze the amide bond of aromatic and aliphatic substrates, we hypothesize that cauxin hydrolyzes the felinine precursor to produce felinine in cat urine. Our current findings should prove useful for elucidating the physiological function of cauxin.

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