Renin, Cyclooxygenase-2 and Neuronal Nitric Oxide Synthase in the Kidneys of Transgenic Tsukuba Hypertensive Mouse

Akira YABUKI1), Mitsuharu MATSUMOTO1), Ryozo KAMIMURA2), Kazuyuki TANIGUCHI3), and Syusaku SUZUKI1)

1)Department of Veterinary Anatomy, Faculty of Agriculture, Kagoshima University, 21–24–1 Korimoto, Kagoshima 890-0065, 2)Division of Laboratory Animal Science, Research Center for Life Science Resources, Kagoshima University, 8–35–1 Sakuragaoka, Kagoshima 890-8520, and 3)Laboratory of Paramedical Sciences, Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka 020-8550, Japan

Abstract: The transgenic Tsukuba hypertensive mouse (THM), which expresses the human renin and angiotensinogen genes, develops hypertension secondary to increased renin-angiotensin system activity. The aim of the present study was to assess expression of the renin, cyclooxygenase-2 (COX-2), and neuronal nitric oxide synthase (nNOS) proteins in THM kidneys by immunohistochemical stainings. Renin expression was decreased in the THM kidneys when compared to kidneys from heterozygotes or control mice. Although no differences were observed in nNOS expression, overexpression of the COX-2 protein was observed in the macula densa cells in THM kidneys.

Key words: cyclooxygenase-2, renin, transgenic Tsukuba hypertensive mouse

Renin is secreted from juxtaglomerular cells (JGCs) and is a key enzyme of the renin-angiotensin (RA) system that controls blood pressure. Secretion of renin from JGCs is regulated by two proteins, cyclooxygenease-2 (COX-2) and neuronal nitric oxide synthase (nNOS), which are both expressed in the macula densa cells [2, 4, 5, 10].

The Tsukuba hypertensive mouse (THM) was established by hybridization of a C57BL/6 mouse (into which the 14-kb human angiotensinogen gene, including its 1.3-kb native promoter, had been introduced) [3]. The THM develops hypertension via acceleration of RA system activity, and its blood pressure is already 30–40 mmHg higher than that of C57BL/6 as early as at 6 weeks of age [3]. Although THM is considered to be a good model for human hypertension with the renal lesions including glomerulosclerosis and fibrinoid arteritis [11], the interactions between renin, COX-2 and nNOS expression in the kidneys of THM remain unclear. Therefore, the aim of the present study...
was to assess the expression of these proteins using immunohistochemistry.

Five month old male C57BL/6CrSlc (n=3), heterozygotes carrying human renin gene (R/-; n=3), heterozygotes carrying human angiotensinogen gene (−/A; n=3), and THM (R/A; n=5) were used for the present experiments. The study was performed in accordance with the Guidelines for Animal Experimentation of Kagoshima University. Mice were maintained in a one-way airflow system room (temperature 22 ± 1°C; humidity 55 ± 10%; light period 07:00–19:00; ventilation 12 cycles/h) in the division of Laboratory Animal Science, Research Center for Life Science Resources, Kagoshima University. Animals were fed an autoclaved commercial diet (CE-2; Japan Clea, Tokyo, Japan) and supplied tap water ad libitum. Mice were sacrificed by exsanguination under anesthesia using a mixture of ketamine (Sankyo, Tokyo, Japan) and medetomidine (Meiji, Tokyo, Japan). Kidneys were quickly removed and cut perpendicular to the long axis and then fixed in Zamboni’s solution. After routine embedding in paraffin, 5-μm sections were generated for every 30 μm of tissue.

Immunohistochemistry was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, USA). Anti-recombinant renin antiserum (supplied by Dr. Murakami, University of Tsukuba) diluted 1:5,000 in 10 mM phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), anti-nNOS polyclonal antibody (Cayman Chemical, Michigan, USA) diluted 1:1,500 in 0.1% BSA/PBS, or anti-COX-2 polyclonal antibody (Cayman Chemical) diluted 1:1,500 in 0.1% BSA/PBS were used as primary antibodies. Incubation with the primary antibodies was performed overnight at 4°C. Biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1:200 in 1% BSA/PBS was used as the secondary antibody. Immunohistochemical reactivity was detected by a 3,3′-diaminobenzidine (2.5 g/l)-H₂O₂ (0.003%) solution. For antigen retrieval, all pre-treatments were performed after deparaffinization. For nNOS detection, sections were microwaved in a 10 mM citrate buffer (pH 6.0). For COX-2 detection, sections were microwaved in 50 mM Tris-HCl (pH 10.0) and then treated with 0.3% Triton X 100 in Tris-HCl. For renin detection, antigen retrieval treatment was not required [13]. In addition, livers, lungs, adrenal glands, sub-mandibular glands and testes from the mice were prepared in a similar manner for immunohistochemistry to investigate extra-renal renin expression. The number of renin-positive areas was quantified as previously described [9, 12], and analyzed using one-way analysis of variance (Bonferroni/Donn test).

Five month old male C57BL/6Cr (n=3) and THM (n=3) were used for Western blot analysis. Kidneys were homogenized in 25 mM Tris-HCl-buffered saline (pH 7.4) containing 1 mM phenylmethanesulfonyl fluoride (Sigma, Missouri, USA), 1 mM EDTA-2Na and 3 mg/ml of leupeptin (Sigma), and then centrifuged at 14,000 rpm for 10 min. Routinely prepared samples (50 μg of proteins) were separated by SDS-PAGE, then transferred to nitrocellulose membranes. After blocking, membranes were incubated with anti-recombinant renin antiserum (diluted 1:5,000 in 1% BSA/PBS) overnight at 4°C. Peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences, New Jersey, USA) diluted 1:5,000 in 1% BSA/PBS was used as the secondary antibody, and immunoreactive bands were detected by an ECL Kit (Amersham Biosciences).

Immunohistochemical findings of renin, COX-2 and nNOS are shown in Fig. 1. Positive reactions for renin were clearly observed in the JGCs of C57BL/6Cr, R/− and −/A mouse kidneys (Fig. 1a-c). However, reactions were rarely observed in kidneys of THM (Fig. 1d). As shown in Fig. 2, the number of renin-positive areas was significantly lower in the kidneys of THM when compared to those of C57BL/6Cr, R/− and −/A. In the Western blot analysis for renin expression, a single band with a molecular weight of approximately 39 kDa was detected with equal densities in homogenates from C57BL/6Cr and THM kidneys (Fig. 3). No differences in renin expression in other tissues were noted when comparing C57BL/6Cr and THM (data not shown).

Positive immunohistochemical reactions for COX-2 were observed in the macula densa cells in the kidneys of THM but not in those of C57BL/6Cr, R/− and −/A mice (Fig. 1e–h). The nNOS immunoreactivity was detected in the macula densa cells of all mouse kidneys, and no differences were observed when comparing C57BL/6Cr, R/−, −/A and THM (Fig. 1i–l).

Negative feedback of the RA system is mediated by renal COX-2 expression [2, 4, 5]. COX-2 expressed in the macula densa cells stimulates renin production in
Fig. 1. Immunohistochemical detection of renin, COX-2 and nNOS. a–d: renin. e–h: COX-2, i–l: nNOS. a, e and i: C57BL/6Cr mouse. b, f and j: heterozygote carrying human renin gene (R/–). c, g and k: heterozygote carrying human angiotensinogen gene (–/A). d, h and l: Tsukuba hypertensive mouse (THM). Juxtaglomerular cells in the C57BL/6Cr, R/– and –/A show positive reactions for renin (panels a–c). In the THM, spotted reactions for renin (arrow) are seen in a few juxtaglomerular cells (panel d). Positive reactions for COX-2 are seen in the macula densa of THM (panel h). Macula densa of C57BL/6Cr, R/– and –/A show negative reactions for COX-2 (panels e–g). Positive reactions for nNOS are seen in the macula densa, but no differences are shown between C57BL/6Cr, R/–, –/A and THM (panels i–l). Bars: 30 µm.

Fig. 2. Index of renin-positive areas. B/6: C57BL/6Cr mice. R/–: heterozygotes carrying human renin gene. –/A: heterozygotes carrying human angiotensinogen gene. THM: Tsukuba hypertensive mice. Each column represents mean ± standard error.*: Statistically significant difference vs B/6 (p<0.01).

Fig. 3. Western blot analysis of renin in the kidneys. Lanes 1–3: C57BL/6Cr mice. Lanes 4–6: Tsukuba hypertensive mice. No apparent differences are seen between C57BL/6Cr and Tsukuba hypertensive mice.
the JGCs, and COX-2 expression is inhibited by angiotensin II. A previous report demonstrated that plasma renin activity of THM was 35–50 folds higher than that of R/− or −/A mice [3]. High concentrations of angiotensin II were also demonstrated in the plasma, heart and kidney of THM [3, 6]. Based on these observations, we anticipated that the THM kidneys would show increased renin expression and decreased COX-2 expression. However, the present results contradicted our supposition. This may be because the cycle of renin synthesis-secretion in JGCs is very rapid in the THM, and thus, synthesized renin could be released rapidly without intracellular accumulation. It is likely that these alterations in cellular physiology resulted in up-regulation of COX-2 production in the macula densa cells as well. Overexpression of extra-renal renin was reported in the livers and adrenal glands of other transgenic models carrying a renin gene [1, 7, 8]. In the present study, we did not detect renin expression in livers, lungs, adrenal glands, submandibular glands or testes of THM. We suppose that synthesized renin in these organs would be rapidly released into blood, the same as that in kidneys, so no positive staining was demonstrated.

Acknowledgments

We thank Ms. S. Suruga of the Division of Laboratory Animal Science, Research Center for Life Science Resources, Kagoshima University, for animal maintenance.

References