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Ubiquitous localization of leukotriene A_4 hydrolase in the rat nephron

Akihide Nakao, Tsuyoshi Watanabe, Nobuya Ohishi, Akiko Toda, Kenichiro Asano, Shigeo Taniguchi, Kazuo Nosaka, Eisei Noiri, Takako Suzuki, Tatsuo Sakai, Kiyoshi Kurokawa, Takao Shimizu, and Satoshi Kimura

First and Third Department of Internal Medicine, Department of Molecular Biology and Biochemistry, Faculty of Medicine, University of Tokyo, and Department of Anatomy, School of Medicine, Juntendo University, Tokyo; and Department of Internal Medicine, Tokai University School of Medicine, Isehara, Kanagawa, Japan

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Background. Leukotriene (LT) B_4 is a well-known inflammatory mediator and is implied to play some roles in glomerulonephritis. Although LTA_4 hydrolase, a final-step key enzyme to produce LTB_4, is located in glomerular mesangial cells, as well as in leukocytes, platelets, and endothelial cells, its precise distribution in the kidney other than in mesangial cells remains unknown. Therefore, we have investigated the localization of mRNA, protein, and enzyme activity of LTA_4 hydrolase in the rat kidney.

Methods. Microdissection reverse transcriptase–polymerase chain reaction was used for the determination of LTA_4 hydrolase mRNA. The enzyme protein was detected by Western blot, and immunohistochemistry was performed. Finally, LTA_4 hydrolase activity and LTB_4 were assayed in kidney tissues.

Results. LTA_4 hydrolase mRNA was detectable in all microdissected nephron segments of the cortex and outer medulla. The corresponding size of ~ 70 kDa protein was shown in descending order in the inner medullary > outer medullary ≥ cortical homogenates. The immunohistochemical study demonstrated the ubiquitous presence of the enzyme in all nephron segments of cortex, outer medulla, and inner collecting tubules. LTA_4 hydrolase activity was detected in the inner medullary ≥ outer medullary ≥ cortical tissue homogenates. LTB_4 was demonstrated in the inner medullary > outer medullary ≥ cortical tissues during the basal condition, and was time-dependently increased by stimulation with arachidonic acid and ionomycin in the cytosolic fraction from outer medulla and in the glomerular suspension.

Conclusions. These results strongly suggest that renal tubular cells as well as glomerular cells have an LTB_4-forming potency, which may participate in physiological and pathophysiological roles in the kidney.

Leukotriene (LT) B_4 is a bioactive lipid mediator with chemotactic and chemoattracting activities on leukocytes, and thereby participates in various inflammatory diseases [1, 2]. It also has been reported to play an important role in some animal models of glomerulonephritis [3, 4]. The initial step of LTB_4 biosynthesis is a liberation of arachidonic acid from membrane phospholipids by the action of phospholipase A_2, and the resultant free arachidonic acid is converted to LTB_4 by the sequential action of 5-lipoxygenase (LO) with 5-LO–activating protein and LTA_4 hydrolase [5, 6]. The key enzyme for LT's formation, 5-LO, biosynthesizes LTA_4 and is thought to be exclusively present in leukocytes. Red blood cells and platelets are known to form LTB_4 by the transcellular metabolism of LTA_4 hydrolase generated in leukocytes [7, 8]. It has been reported that isolated rat renal glomeruli possess synthetic LTB_4 potency [9, 10] such that immune-injured isolated glomeruli release a high amount of LTB_4 [3], and that the depletion of resident macrophages in the glomeruli reduces LTB_4 generation [11]. These results suggest that LTB_4 can be synthesized by resident glomerular macrophages/monocytes. However, LTB_4 biosynthetic pathways in other cellular components in the kidney, such as tubular cells and interstitial cells, have not been reported.

In this study, we demonstrate a ubiquitous distribution of LTA_4 hydrolase mRNA, the enzyme protein, and the enzyme activity in tubular as well as in glomerular cells of the rat kidney. We also detected a comparative amount of LTB_4 in rat kidney tissues during both the basal and stimulated conditions.

METHODS

Material

Collagenase (type 1) was the product of Sigma (St. Louis, MO, USA). Avian myeloblastosis virus (AMV) reverse transcriptase, random hexamer, ribonuclease (RNase) inhibitor, "DNA tailing kit," digoxigenin (DIG)-dUTP, positively charged nylon membrane, chemiluminescent DIG detection kit, and DIG-labeled DNA weight marker (pBR328 *Bgl* I+ pBR328 *Hif* I) were purchased from Boehringer Mannheim (Mannheim, Ger-

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many). *Taq* polymerase was purchased from Takara Shuzo (Kyoto, Japan). NuSieve 3:1 agarose was the product of FMC BioProducts (Rockland, ME, USA). Oligonucleotides used for polymerase chain reaction (PCR) and Southern hybridization were synthesized by Funakoshi Co. Inc. (Tokyo, Japan).

Microdissection

Male Sprague-Dawley rats (5 to 7 weeks old) were used in this study. Microdissection of nephron segments was performed as previously reported [12]. In brief, the left kidney was perfused from the abdominal aorta with a microdissection solution (containing 137 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 5 mM glucose, 5 mM lactate, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, as well as 0.1% bovine serum albumin, and pH adjusted to 7.4 by NaOH) containing 0.1% collagenase and was cut with a razor blade, and a tissue slice was incubated with collagenase for 30 minutes at 37°C. In a microdissection solution (4°C), the nephron was dissected into eight segments: glomerulus, proximal convoluted tubule, proximal straight tubule, medullary thick ascending limb, cortical thick ascending limb, distal convoluted tubule, cortical collecting duct, and outer medullary collecting duct. These nephron segments (20 glomeruli or about 2–3 mm of each tubular segment) were rinsed carefully in another dish and were transferred into a 500 μl tube. Then, 100 μl of a denaturing solution [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol] were added, and the tube was well vortexed. Five micrograms of tRNA were added as carrier, and nucleic acids were precipitated with ethanol (200 μl) in the presence of 10 μl of 3 M sodium acetate (pH 5.3). After precipitation at −20°C, the tubes were centrifuged, and the pellets were rinsed once with 70% ethanol and air dried. Both RNA and DNA in the sample were totally recovered by this procedure.

Reverse transcription–polymerase chain reaction and sequence analysis of the RT–PCR product

Reverse transcription–polymerase chain reaction (RT-PCR) was performed using precipitated nucleic acid from microdissected segments as previously reported, with minor modification [13]. The dried pellet was dissolved in the RT solution, and it was divided into two aliquots: one for LTA_4 hydrolase and the other for β-actin (positive) or RT (negative) control. After the addition of 0.5 μl of AMV reverse transcriptase (or 0.01% gelatin as a negative control) to each tube, RT was performed at 42°C for 60 minutes. The final volume of RT buffer was 10 μl, and its composition was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM dNTP, 4 μM random hexamer, 2 unit/μl RNase inhibitor, 1.25 units/μl AMV. The sequence of sense PCR primer (SE) was 5′-CAGTCACAGGAGGATAAT-3′, which corresponds to bases 131 to 148, and that of antisense primer (AS) was 5′-GGAGTGAGCCACTGAAGG-3′, which corresponds to bases 346 to 363 of rat LTA_4 hydrolase cDNA [14]. The sense PCR primer of β-actin was 5′-TCCTAGCACCATGAAGATC-3′, corresponding to bases 2845 to 2863 (exon 5 of rat β-actin gene), and the antisense PCR primer of β-actin was 5′-AAACGCAGCTCAGTAACAG-3′, corresponding to bases 3140 to 3158 (exon 6 of rat β-actin gene) [15]. These combinations of primers were designed to span introns so that the PCR products of the expected size were exclusively amplified from the cDNA, which had been reversely transcribed from the specific mRNA. The predicted size of RT–PCR product from cDNA of rat β-actin and LTA_4 hydrolase is 190 bp [13] and 233 bp, respectively. The final volume of PCR mixture was 50 μl, and the composition was as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 nM each sense and antisense primers, 0.2 mM dNTPs, and 0.025 unit/μl *Taq* polymerase. The PCR conditions were as follows: 40 cycle of 94°C (30 seconds), 59°C (20 seconds), 72°C (10 seconds) for LTA_4 hydrolase, and 32 cycle of 94°C (30 seconds), 59°C (30 seconds), and 72°C (30 seconds) for β-actin. The RT–PCR product corresponding to the LTA_4 hydrolase mRNA from rat kidney was directly sequenced from both directions using a 373 Sequencer (Applied Biosystems, Foster City, CA, USA) with the same primers as those used for PCR.

Southern hybridization

Ten microliters of each PCR product was detected by Southern hybridization after electrophoresis [13]. For Southern hybridization, PCR products were electrophoresed on NuSieve 3:1 agarose gel and blotted to a nylon membrane using 20 × 3 M NaCl, 0.3 M sodium citrate, pH 7.0 (SSC) after alkalization. The oligonucleotide probe, in which the sequence was 5′-TCAACGGACAAGAAGTCAAATACACTCTTG-3′ corresponding to bases 200 to 230 of rat LTA_4 hydrolase mRNA, was labeled with DIG-dUTP using a DNA tailing kit. Hybridization of DIG-labeled oligonucleotide was performed at 65°C for 12 hours. The hybridized nylon membrane was washed in 2 × SSC at room temperature for 10 minutes and then in 0.1 × SSC at 65°C for 30 minutes. The hybridized bands were detected using a DIG luminescent detection kit following the manufacturer's instructions.

Western blotting analysis

Rats were killed under anesthesia with intraperitoneal injection of pentobarbital, and lungs and kidneys were removed after perfusion with Hank's buffered solution (138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM $Na_2HPO_4 \cdot 7H_2O$, 5.0 mM KCl, 0.3 mM KH_2PO_4, 1.3 mM $CaCl_2$, 0.5 mM

$MgCl_2 \cdot 6H_20$, 0.4 mM $MgSO_4 \cdot 7H_2O$, pH 7.4) from the abdominal aorta. Kidney tissue was separated into three parts: cortex, outer medulla, inner medulla. Glomeruli were collected by sieving methods from the renal cortex. Each tissue was homogenized with three volumes of phosphate-buffered saline containing 10 mM ethylenediaminetetraacetic acid and using a microhomogenizer; this was then centrifuged 10,000 × g at 4°C for 15 minutes. The supernatants (100 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide gel) [16] and were transferred to a nitrocellulose membrane. The membrane was blocked with Block Ace (Yukijirushi, Hokkaido, Japan) and then was incubated with the affinity-purified antihuman LTA_4 hydrolase antibody (2 μg/ml as IgG), and finally, the membrane was incubated with affinity-isolated antirabbit IgG conjugated with horseradish peroxidase (1:5000 dilution). The immunoreactive bands were visualized with 3,3′-diaminobenzidine and hydrogen peroxide as substrates [17].

Immunohistochemical staining

Immunohistochemical staining was performed by the methods described previously [17], with slight modifications. The kidney removed by the method described earlier here was fixed with 20% (wt/vol) neutral-buffered formalin and was embedded in paraffin. Tissue blocks were sliced into 6 μm coronal sections and mounted on Polypep glass slides (Sigma). After deparaffinization with xylene and the consecutive removal of picric acid with ethanol (70%, 90%, 100%), sections were boiled with 10 mM citrate buffer, pH 6.0, for 5 minutes and were then incubated with 0.1% trypsin (type I; Sigma), and 0.1% $CaCl_2$ in 0.05 M Tris-HCl buffer pH 7.6 for 25 minutes at 37°C. Tissue sections were immersed in 1% normal goat serum for 30 minutes at room temperature. Tissue samples on glass slides were then incubated for 30 minutes at room temperature with 50 μl of the affinity-purified antibody (2 μg/ml as IgG) or normal rabbit IgG (2 μg/ml). After washing three times with phosphate-buffered saline pH 7.4, sections were processed successively using SLAB (R) 2 kit (Dako, Carpinteria, CA, USA), according to the manufacturer's manual. Finally, immunoreactive LTA_4 hydrolase was visualized with *NBT/BCIP* (Boehringer Mannheim, Mannheim, Germany) as a substrate for alkaline phosphatase. Control samples were stained with 1% methyl green for clear visualization.

Assay of LTA_4 hydrolase activity

Leukotriene A_4 hydrolase activity was measured as described previously with minor modification [18]. Forty microliters of 10,000 × g supernatant obtained by the method described earlier in this article were mixed with 10 μl of 0.1 M Tris-HCl buffer (pH 7.6) and were prewarmed for several minutes at 37°C. Then 1 μg of LTA_4 in 1 μl in ethanol was added. After one minute of incubation, 117 μl of stopping solution (0.1% acetic acid in methanol containing 0.3 nmol of prostaglandin B_2 as an internal standard) were added. After being kept at −20°C for at least 20 minutes, the mixtures were centrifuged at 10,000 × g for 10 minutes. A 50 μl aliquot of the supernatant was directly injected onto high pressure liquid chromatography (HPLC). The conditions were as follows: column, TSK-ODS 80TM, 0.46 × 15 cm (Tosoh, Tokyo, Japan); solvent, methanol/water/acetic acid (70/30/0.05, vol/vol/vol); flow rate, 1 ml/min; column temperature, 35°C; UV monitor, 270 nm. PGB_2 and LTB_4 were eluted at approximately 7 and 11 minutes, respectively, and the amount of LTB_4 formed was calculated from the peak ratio of LTB_4/PGB_2.

Assay of LTB_4 content

The LTB_4 assay was performed by the combination method of column extraction and enzyme immunoassay (EIA). After being cut into three parts, each tissue was frozen in dry ice/acetone immediately after weighing and was stored at −20°C until assay. On the day of assay, each tissue was homogenized with nine volumes of ice-cold ethanol using a microhomogenizer, and 1 ml of aliquot was centrifuged by 10,000 × g for five minutes at 4°C. The supernatant was diluted with nine volumes of acetic acid (0.1 N) and was applied to a Bond Elute C2 column pre-equilibrated with 2 ml of ethyl acetate, 4 ml of methanol, and 8 ml of water successively. Then the column was washed with 6 ml of water, 4 ml of ethanol, and 4 ml of hexane, in that order. LTB_4 was eluted with 1.5 ml of ethyl acetate twice and was evaporated under N_2. After resuspension with EIA buffer, LTB_4 was assayed using the EIA kit according to the manufacturer's manual (Cayman Chemical, Ann Arbor, MI, USA).

Glomerulus obtained by the sieving method [19] was resuspended in Hank's buffered saline and was aliquoted in tubes. After centrifugation at 300 × g for five minutes, the supernatants were aspirated, and 300 μl of Hank's buffered saline containing 30 μM of arachidonic acid and 2.5 μM of ionomycin were added to start the reaction. The tubes were incubated at 37°C for the indicated time, during which they were swirled to keep the glomeruli in suspension. The reaction was stopped by the immersion of the tubes in boiled water for five minutes, and they were centrifuged at 10,000 × g for 10 minutes. The supernatants were stored at −20°C until assay. Precipitated glomeruli were assayed for protein after being dissolved with 0.1 N NaOH. In the experiments in which 5-LO inhibitors, nordihydroguaiaretic acid (NDGA), and AA-861 (a gift from Takeda Pharmaceutical Company, Tokyo, Japan) were used, glomeruli were preincubated for 10 minutes with these 5-LO inhibitors (NDGA, 4 μM; AA-

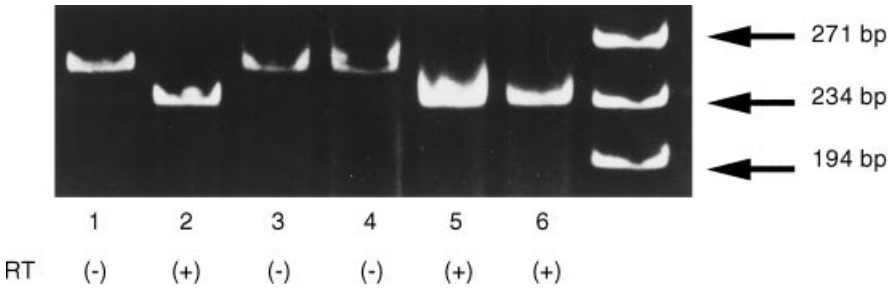


Fig. 1. Polymerase chain reaction (PCR) products from rat kidney or lung. Nucleic acids were extracted from rat kidney or lung, and amplified by PCR with or without RT as described in the Methods section. RT (+) indicates PCR with RT, and RT (−) indicates PCR without RT. Lanes 1 and 2, lung homogenate tissues; Lanes 3 to 6, kidney homogenate tissues. Arrows indicate the size of the markers.

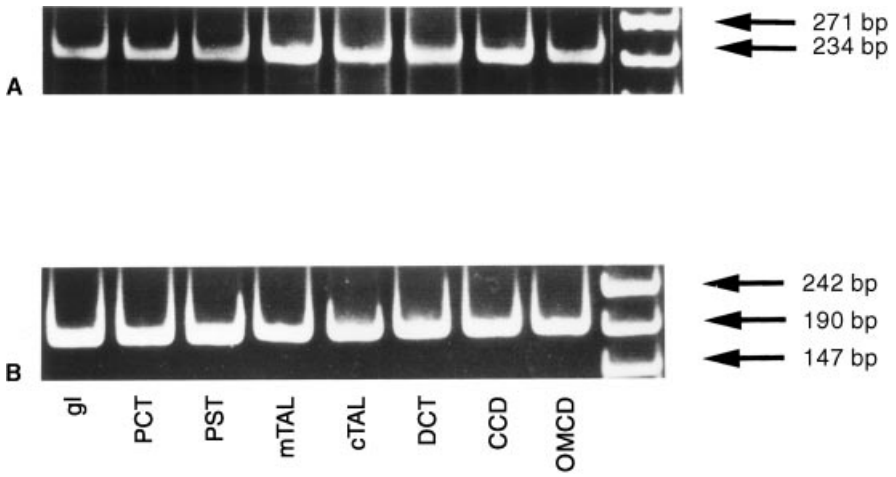


Fig. 2. Distribution of LTA_4 hydrolase mRNA along the microdissected nephron segments. (*A*) RT-PCR products from microdissected samples using the primers specific for LTA_4 hydrolase as described in the **Methods** section. (*B*) RT-PCR products using the primers for rat β-actin. Abbreviations are: gl, glomerulus; PCT, proximal convoluted tubule; PST, proximal straight tubule; mTAL, medullary thick ascending limb; cTAL, cortical ascending limb; DCT, distal convoluted tubule; CCD, cortical collecting tubule; OMCD, outer medullary collecting tubule. Arrows indicate the size of markers.

861, 0.2 μM) at 37°C, and were then incubated for 30 minutes further after the addition of arachidonic acid (30 μM) and ionomycin (2.5 μM). In the experiment with tubules and interstitial cells, the outer medulla was cut out as described earlier, and the solution was homogenized with three volumes of Hank's buffered saline. After centrifugation at 10,000 × g for 10 minutes the supernatants were aliquoted in tubes, and arachidonic acid and ionomycin were added in a final concentration of 30 μM and 2.5 μM, respectively. The reaction was stopped exactly as in the glomeruli experiment. Protein concentration of the supernatants was assayed by the method described later in this article. These supernatants were adjusted to approximately pH 3 to 4 by the addition of six volumes of 0.3 N acetic acid, and were then extracted and used for the LTB_4 assay as described earlier. The recovery rate of exogenously added ^{3}H-labeled LTB_4 in tissue and supernatants was 96.4 ± 0.4% and 82.9 ± 0.7%, respectively.

Miscellaneous

The amount of protein was determined with the Protein Assay Kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Statistical analysis was performed by analysis of variance, and *P* values of less than 0.05 were denoted to be significant.

RESULTS

RT–PCR determination and Southern hybridization of LTA_4 hydrolase mRNA and direct sequencing of the RT–PCR product

We designed the set of PCR primers according to the cloned sequences of LTA_4 hydrolase cDNA from rat mesangial cells reported by Makita et al [14]. The size of RT–PCR products in our system is predicted to be 233 bp. Although rat genomic DNA of LTA_4 hydrolase has not been cloned, cloned human genomic DNA of the enzyme was revealed to have two introns with 54 bp and 74 bp inside the sequences we attempted to amplify [20]. Thus, the size of PCR products from rat LTA_4 hydrolase genomic DNA using our set of primers should be longer than that of the RT–PCR product from the mRNA of the rat LTA_4 hydrolase, if the rat genomic DNA has an intron(s) as the human counterpart. A single band with the size of 233 bp was detected in each RT–PCR product from the sample of rat lung and kidney (Fig. 1, lanes 2, 5, and 6). The size of each PCR product without RT was larger than that from cDNA, suggesting that it was derived from LTA_4 hydrolase genomic DNA (Fig. 1, lanes 1, 3, and 4). The RT–PCR product with 233 bp corresponding to LTA_4 hydrolase mRNA was detected in all the nephron segments of the cortex and

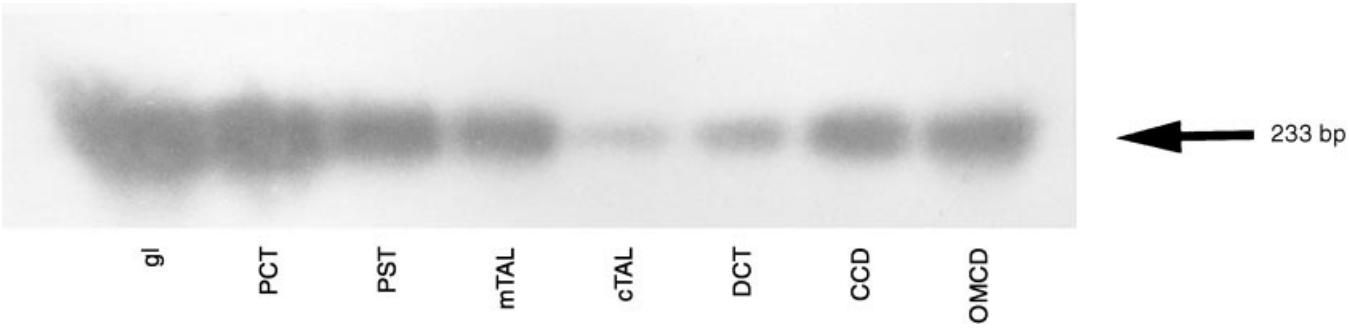


Fig. 3. Southern hybridization of RT-PCR products of nephron segments. Abbreviations are in Fig. 2.

outer medulla (Fig. 2A). The relative amount of RT–PCR product for LTA_4 hydrolase in comparison to that for β-actin (Fig. 2B) could not be determined, because our RT–PCR condition was not strictly quantitative. The specificity of RT-PCR products of 233 bp was also confirmed by Southern hybridization, as shown in Figure 3. Furthermore, the result of sequencing of the RT–PCR product showed a 100% match to the nucleotide sequences from 131 bases to 363 bases of the cloned rat LTA_4 hydrolase (data not shown) [14].

Western blotting and immunohistochemical analysis of LTA_4 hydrolase protein

Western blotting analysis using affinity-purified antibody against human LTA_4 hydrolase [17] revealed a band of approximately 70 kDa protein as the most prominent protein in the cytosolic fraction of lung, followed in order by the glomeruli obtained by the sieving method, inner medulla, outer medulla, and cortex . Because the molecular mass of rat LTA_4 hydrolase protein was reported to be in the range of 68 to 70 kDa [21] and rich in the lung [22], this approximate 70 kDa protein seems to be LTA_4 hydrolase protein (Fig. 4). Immunoreactive LTA_4 hydrolase was detected in all of the nephron segments in the cortex and outer medulla in the rat kidney slice by immunohistochemistry, using the same antibody (Fig. 5 A–C). In glomeruli, immunologic reactivity of LTA_4 hydrolase was localized in mesangial cells and glomerular visceral epithelial cells (arrow and arrowhead, respectively; Fig. 5A). Proximal tubules were also stained by the specific antibody against LTA_4 hydrolase (Fig. 5A). Photomicrographs of the outer medulla showed immunostaining in proximal straight tubules (Fig. 5B). Figure 5C showed an immunoreactivity in the inner medullary collecting tubules (IMCTs). The staining of thin descending limb cells (TDLs) seemed very weak or negative, compared with IMCTs, although faint signals of TDLs might be because of their thin cytosolic area.

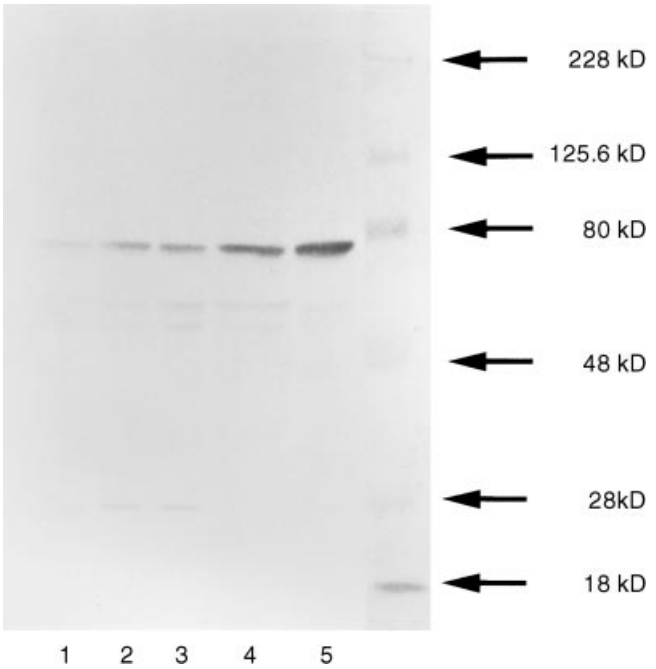


Fig. 4. Western blotting analysis using the affinity-purified antibody against LTA_4 hydrolase. Homogenate of each part of kidney, glomeruli, and lung were separated on SDS-PAGE and electrophoresed, then transferred to a nitrocellulose membrane. Proteins were immunostained by using affinity purified anti-LTA_4 hydrolase antibody. Lane 1, cortex; lane 2, outer medulla; lane 3, inner medulla; lane 4, glomeruli; lane 5, lung.

LTA_4 hydrolase activity and LTB_4 content in the kidney tissue

The specific activity of LTA_4 hydrolase was highest in the inner medulla, followed by the outer medulla and cortex (Fig. 6), which corresponded to the amount of LTA_4 hydrolase protein in these three parts of the kidney (Fig. 4). LTB_4 was detectable in the inner medullary tissue > outer medullary tissue > cortical tissue (Fig. 7). This LT was also present in glomeruli as well as in the mixture of tubular and interstitial cells even under the basal condition (Fig. 8). When stimulated with arachi-

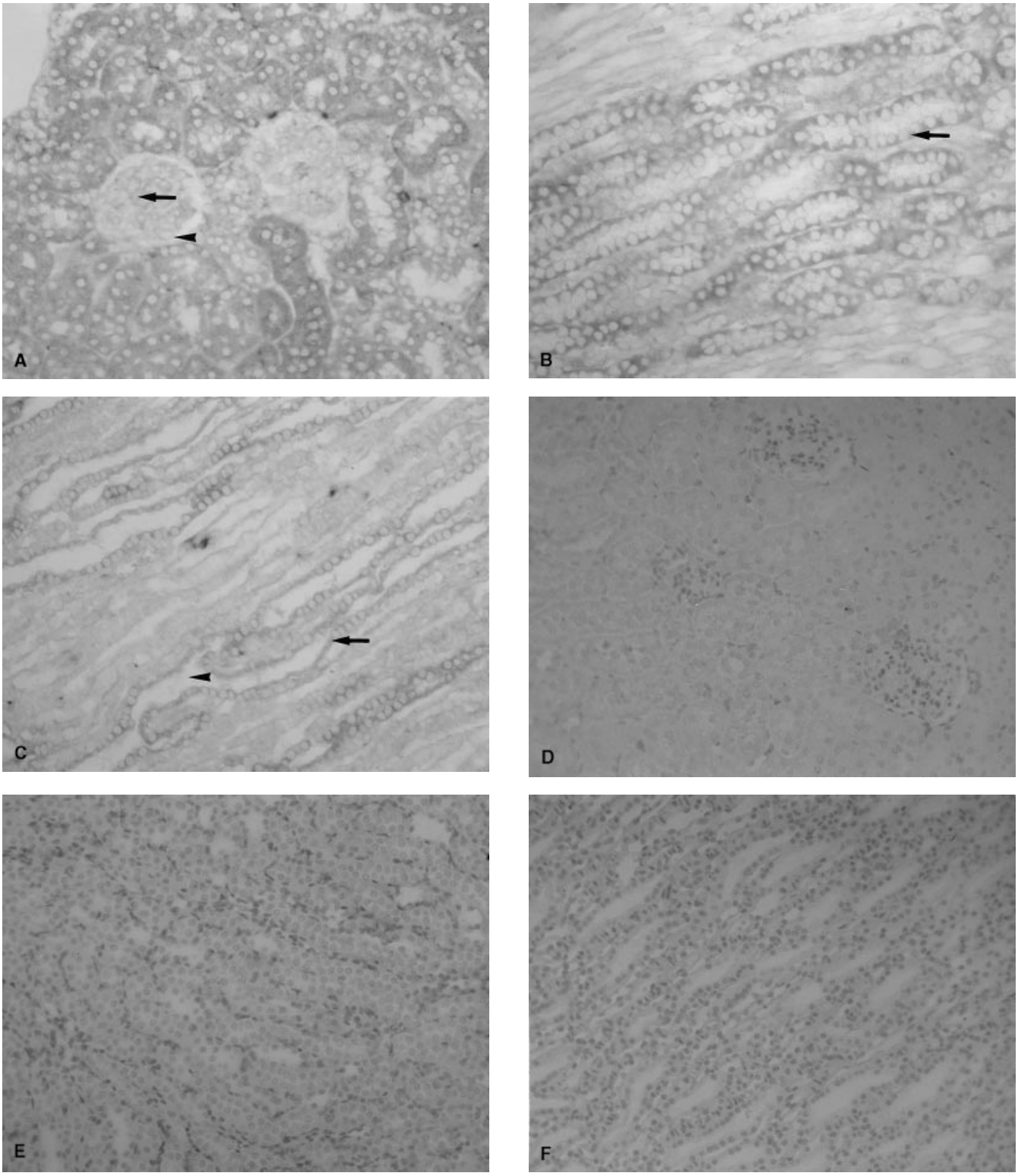


Fig. 5. Immunohistochemical observations of a rat kidney slice. (*A*) Rat glomeruli and proximal tubules (×279). Glomerular mesangial cells, and epithelial cells were immunostained (arrow and arrowhead, respectively). Proximal tubules were also stained by the LTA_4 hydrolase antibody. (*B*) Outer medulla (×279). Proximal straight tubules (arrow) were shown to be immunoreactive against the antibody. The area with weak staining is the vascular bundle. (*C*) Inner medulla (×279). IMCTs (arrow) were strongly stained, while other tubules, mainly TDLs, were not stained. Arrowhead indicates a junction of two IMCTs. Normal rabbit IgG was used instead of specific antibody against LTA_4 hydrolase in cortex (*D*, ×186), outer medulla (*E*, ×186), and inner medulla (*F*, ×186).

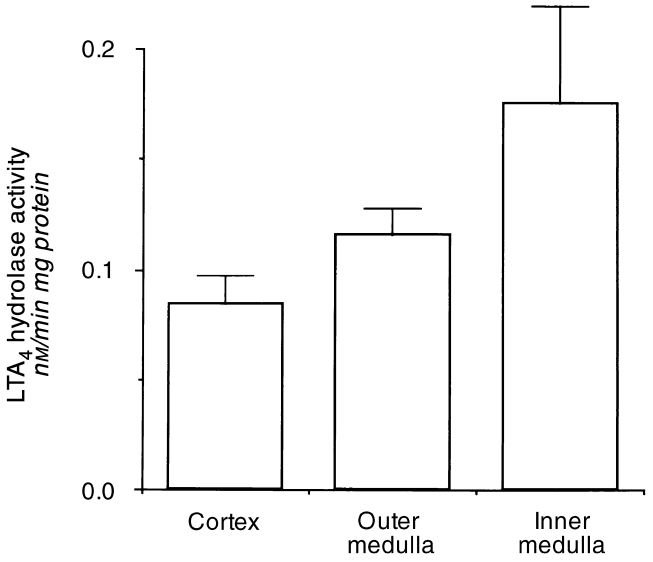


Fig. 6. LTA_4 hydrolase activity in rat kidney cortex, outer medulla, and inner medulla. Cytosolic fractions from each part of kidney tissue were prepared, and LTA_4 hydrolase activities were determined as described in the **Methods** section. Abbreviations are: C, cortex; OM, outer medulla; IM, inner medulla.

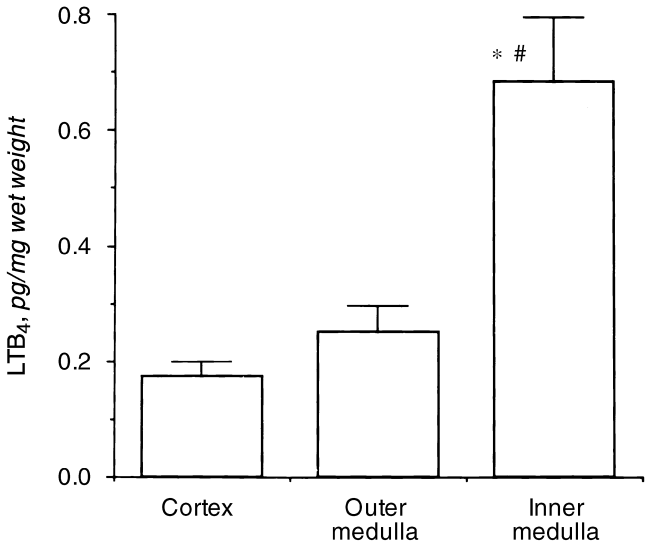


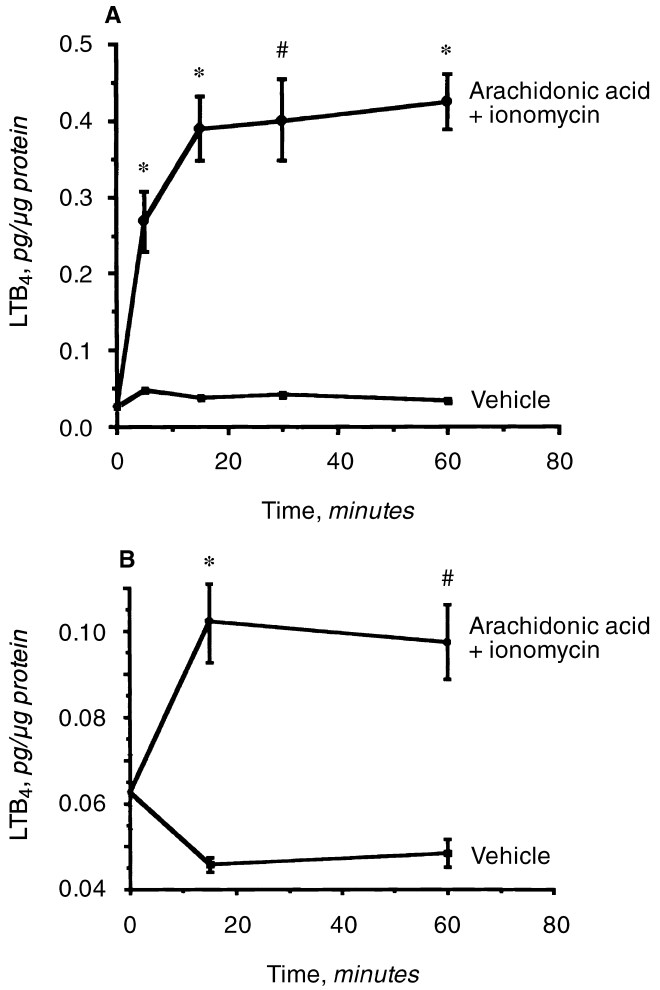
Fig. 7. LTB_4 content in rat kidney cortex, outer medulla, and inner medulla. Each kidney tissue sample was prepared and assayed as described in the **Methods** section. $*P < 0.005$ vs. cortex, and $\#P < 0.05$ vs. outer medulla.

donic acid and ionomycin, LTB_4 was formed time dependently in the isolated glomeruli (Fig. 8A), more was found there than in the tubular and interstitial cell mixture (Fig. 8B), and the LTB_4 synthesis reached a plateau level in 15 minutes. Glomerular LTB_4 synthesis stimulated by arachidonic acid and ionomycin was profoundly reduced by pretreatment with NDGA or AA-861, which are selective 5-LO inhibitors, at 30 minutes (Fig. 8C).

DISCUSSION

In this study, we used the RT–PCR method to determine the presence of LTA_4 hydrolase mRNA, and the results were confirmed by Southern blot analysis. Finally, the direct sequencing of the RT–PCR product showed a 100% match to the LTA_4 hydrolase nucleotide sequences that we attempted to amplify. From these results, we conclude that LTA_4 hydrolase mRNA is ubiquitously distributed along all of the microdissected rat nephron segments of the cortex and outer medulla (Fig. 2). We also tested for the presence of LTA_4 hydrolase protein by Western blot analysis using the affinity-purified specific antibody against human LTA_4 hydrolase, which was prepared per the study of Ohishi et al, utilizing the recombinant human enzyme [17]. Western blotting analyses of various tissues of guinea pig with this antibody revealed a single protein band with a molecular mass of approximately 70 kDa, and immunohistochemical examinations showed immunostaining in various tissues, including epithelial cells of the tracheo-bronchial system [17]. We applied the antibody to the rat kidney as well as to lung tissues. The results showed an approximate 70 kDa protein band in the lung, glomeruli, inner medulla, outer medulla, and cortex. The amount of the protein in the three fractions of rat kidney was greater in the inner medulla, followed by the outer medulla and cortex (Fig. 4). In addition, we examined the specific activity of the enzyme and the basal content of LTB_4 in these three parts of rat kidney. Both were highest in inner medulla, followed by outer medulla and cortex (Figs 4, 6, and 7), indicating that the rank order of the amount of the enzyme protein, the activity of the enzyme, and the product of the enzyme correlated well with one another in these three fractions of rat kidney. Moreover, LTA_4 hydrolase protein could be immunohistochemically detected in all of the nephron segments except thin descending limb cells (Fig. 5 A-C). These results indicate that all of the renal tubular segments in the cortex and outer medulla express LTA_4 hydrolase mRNA and mature enzyme protein. We could demonstrate the distinct presence of the protein in IMCTs, but not in TDLs. We did not perform the RT–PCR of TDLs because of the difficulty of microdissection of this segment and the possible contamination of other tissues such as IMCTs and vascular components. More sophisticated methods are required to determine whether LTA_4 hydrolase mRNA is present in TDLs.

Rat renal tubular segments and isolated glomeruli have not been shown to express 5-LO, nor 5-LO activating protein, the essential enzyme or cofactor protein for LTs biosynthesis. Although LTB_4 formation by the intercellular transfer from cells that produce LTA_4 has been suggested [7, 8], the activity of LTA_4 hydrolase as an amino peptidase has been reported [23, 24]. Therefore, we studied the presence of endogenous LTB_4-forming capacity in rat kidney. LTB_4 production could be



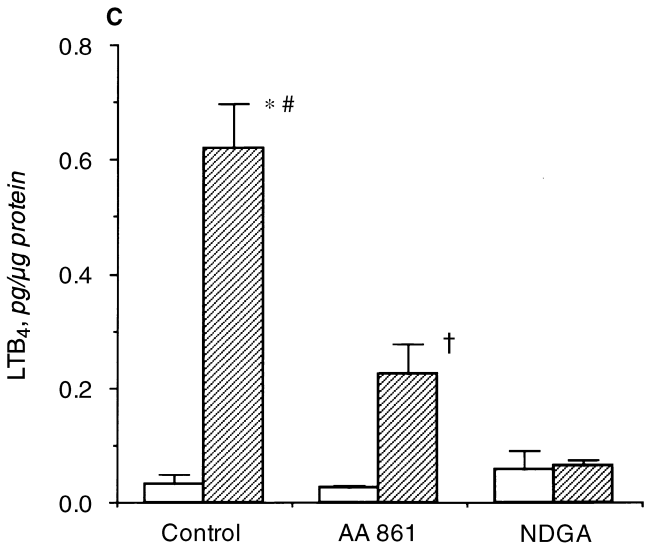


Fig. 8. LTB_4 synthesis by isolated glomeruli and cytosolic fraction of outer medullary homogenates. Arachidonic acid (30 μM) and ionomycin (2.5 μM) were used for stimulation. The reaction was stopped at the indicated time by immersion of sample tubes in boiling water for 5 minutes. (*A*) Glomerular LTB_4 synthesis both under the basal and the stimulated conditions. $*P < 0.002$ vs. values with vehicle at 5 min, 15 min, and 60 min; $\#P < 0.005$ vs. value with vehicle at 30 min. (*B*) LTB_4 synthesis by cytosolic fraction of outer medullary homogenates. (*C*) Reduction of LTB_4 synthesis by 5-LO inhibitors, AA-861 and NDGA. Glomeruli were pretreated with vehicle, 0.2 μM of AA-861, or 4 μM of NDGA for 10 min, and then incubated with or without arachidonic acid and ionomycin for 30 min. Symbols are (□) incubation with vehicle, AA-861 or NDGA alone; (▨) incubation with arachidonic acid and ionomycin after pretreatment with vehicle, AA-861 or NDGA. $*P < 0.005$ vs. vehicle at 15 min, $\#P < 0.01$ vs. vehicle at 60 min. $\dagger P < 0.005$ vs. vehicle at 60 min.

seen in the glomerulus as well as in the outer medullary tissue by stimulation with arachidonic acid and ionomycin (Fig. 8). Furthermore, the presence of LTB_4 could be detected in renal tissue even under the basal condition (Figs. 7 and 8). The selective 5-LO inhibitors, NDGA and AA-861, reduced LTB_4 synthesis stimulated by arachidonic acid and ionomycin, which strongly suggested that we measured authentic LTB_4 and that LTA_4 hydrolase present in glomeruli as well as in outer medullary tissues had activated LTB_4 formation. Therefore, we conclude that all of the nephron segments other than TDLs can form LTB_4 under both steady-state and stimulated conditions, probably by transcellular metabolism of LTA_4 generated by leukocytes or monocytes/macrophages, as has been shown in other tissues [8, 25]. Whether or not LTA_4 hydrolase in these tubular cells acts as an amino peptidase when in physiological conditions needs to be elucidated in the future. The relevance of the absence of the protein in TDL also remains to be clarified.

The pathophysiological significance of glomerular 5-LO products is supported by several studies. Glomeruli subjected to immune injury release a substantial amount of LTB_4 [3, 26]. Selective 5-LO inhibitor also has been shown to attenuate glomerulonephritis in an animal model [26]. However, physiological and/or pathophysiological roles of LTB_4 in the tubular and interstitial cells still remain to be clarified. Because LTB_4, a potent chemotactic agent, is generally involved in inflammatory processes [1, 2], this eicosanoid formed in the tubular and interstitial cells might play a role(s) in the pathogenesis of those renal diseases with interstitial changes, such as interstitial nephritis and nephrosclerosis. Recently, interstitial infiltration of leukocytes in the kidney has been reported to play a pathogenetic role in glomerulonephritis [27, 28]. We believe that tubular LTB_4 may also participate in the pathogenesis of glomerular diseases. Moreover, physiological roles can be speculated when considering the steady-state presence of LTB_4 in tubular cells and in glomeruli. These possible roles of LTB_4 should be tested in the near future. In this context, our study may provide a new insight into the biological roles of LTB_4 in the kidney.

Reprint requests to Akihide Nakao, M.D., First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7–3-1, Hongo, Bunkyo-Ku, Tokyo 113, Japan.
E-mail: akihide-tky@umin.ac.jp

APPENDIX

Abbreviations used in this article are: AMV, avian myeloblastosis virus; DIG, digoxigenin; EI, enzyme immunoassay; IMCTs, inner medullary collecting tubules; LO, lipoxygenase; LT, leukotriene; NDGA, nordihydroguaiaretic acid; RT–PCR, reverse transcription–polymerase chain reaction; TDLs, thin descending limb cells.

REFERENCES

1. Samuelsson B: Leukotriene: Mediators of immediate hypersensitivity reactions and inflammation. *Science* 220:568–575, 1983
2. Henderson WRJ: The role of leukotrienes in inflammation. *Ann Intern Med* 121:684–697, 1994
3. Fauler J, Wierneyer A, Marx KH, Kuhn K, Koch KM, Frolich JC: LTB_4 in nephrotoxic serum nephritis in rats. *Kidney Int* 36:46–50, 1989
4. Rahman MA, Nakazawa M, Emancipator SN, Dunn MJ: Increased leukotriene B_4 synthesis in immune injured rat glomeruli. *J Clin Invest* 81:1945–1952, 1988
5. Samuelsson B, Dahlen S-E, Lindgern JA: Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. *Science* 237:1171–1176, 1987
6. Epstein FH: Leukotrienes and other products of the 5-lipoxygenase pathway. *N Engl J Med* 323:645–655, 1990
7. Fitzpatrick F, Ligget W, McGee J: Metabolism of leukotriene A4 by human erythrocytes. *J Biol Chem* 259:11403–11407, 1984
8. Marcus A, Broekman M, Safier ML: Formation of leukotrienes and other hydroxy acids during platelet–neutrophil interactions in vitro. *Biochem Biophys Res Commun* 109:130–137, 1982
9. Cattell V, Cook HT, Smith J, Moncada S: Leukotriene B_4 production in normal rat glomeruli. *Nephrol Dial Transplant* 2:154–157, 1987
10. Lefkowith JB, Morrison AR, Schreiner GF: Murine glomerular leukotriene B_4 synthesis. *J Clin Invest* 82:1655–1660, 1988
11. Lefkowith JB, Shreiner G: Essential fatty acid deficiency depletes rat glomeruli of resident macrophage and inhibits angiotensin II-induced eicosanoid synthesis. *J Clin Invest* 80:947–956, 1987
12. Taniguchi S, Watanabe T, Nakao A, Seki G, Uwatoko S, Suzuki K, Kurokawa K: Distribution of β2-adrenergic receptor mRNA expression along the hamster nephron segments. *FEBS Lett* 318:65–70, 1993
13. Asano K, Taniguchi S, Nakao A, Watanabe T, Kurokawa K: Distribution of platelet activating factor receptor mRNA along the rat nephron segments. *Biochem Biophys Res Commun* 225:352–357, 1996
14. Makita N, Funk CD, Imai E, Hoover RL, Badr KF: Molecular cloning and functional expression of rat leukotriene A_4 hydrolase using the polymerase chain reaction. *FEBS Lett* 299:273–277, 1992
15. Iida K, Taniguchi S, Kurokawa K: Distribution of 1,25-dihydroxyvitamin D_3 receptor and 25-hydroxyvitamin D_3-24-hydroxylase mRNA expression along rat nephron segments. *Biochem Biophys Res Commun* 194:659–664, 1993
16. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227:680–685, 1970
17. Ohishi N, Minami M, Kobayashi J, Seyama Y, Hata J, Yotsumoto H, Takaku F, Shimizu T: Immunological quantitation and immunohistochemical localization of leukotriene A_4 hydrolase in guinea pig tissues. *J Biol Chem* 265:7520–7525, 1990
18. Ohishi N, Izumi T, Seyama Y, Shimizu T: Purification and characterization of human lung leukotriene A_4 hydrolase. *Methods Enzymol* 187:286–295, 1990
19. Nosaka K, Nishi T, Imaki H, Suzuki K, Kuwata S, Noiri E, Aizawa C, Kurokawa K: Permeable type I collagen membrane promotes glomerular epithelial cell growth in culture. *Kidney Int* 43:470–478, 1993
20. Mancini JA, Evans JF: Cloning and characterization of the human leukotriene A_4 hydrolase gene. *Eur J Biochem* 231:65–71, 1995
21. Evans JF, Dupuis P, Ford-Hutchinson AW: Purification and characterization of leukotriene A_4 hydrolase from rat neutrophils. *Biochim Biophys Acta* 840:43–50, 1985
22. Izumi T, Shimizu T, Seyama Y, Ohishi N, Takaku F: Tissue distribution of leukotriene A_4 hydrolase activity in guinea pig. *Biochem Biophys Res Commun* 135:139–145, 1986
23. Izumi T, Minami M, Ohishi N, Bitoh H, Shimizu T: Site-directed mutagenesis of leukotriene A_4 hydrolase: Distinction of leukotriene A_4 hydrolase and aminopeptidase activities. *J Lipid Med* 6:53–58, 1993
24. Orning L, Gierse JK, Fitzpatrick FA: The bifunctional enzyme leukotriene-A_4 hydrolase is an arginine aminopeptidase of high efficiency and specificity. *J Biol Chem* 269:11269–11273, 1994
25. Maclouf JA, Murphy RC: Transcellular metabolism of neutrophil-derived leukotriene A_4 by human platelet: A potential source of leukotriene C4. *J Biol Chem* 263:174–181, 1988
26. Albrightson CR, Short B, Dytko G, Zabko-Potapovich B, Brickson B, Adams JL, Griswold DE: Selective inhibition of 5-lipoxygenase attenuates glomerulonephritis in the rat. *Kidney Int* 45:1301–1310, 1994
27. Cameron JS: Tubular and interstitial factors in the progression of glomerulonephritis. *Pediatr Nephrol* 6:292–303, 1991
28. Lan HY, Paterson DJ, Atkins RC: Initiation and evolution of interstitial leukocytic infiltration in experimental glomerulonephritis. *Kidney Int* 40:425–433, 1991