Expression of NTPDase1 and NTPDase2 in murine kidney: relevance to regulation of P2 receptor signaling

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Kishore, Bellamkonda K., Jorge Isaac, Michel Fausther, Sheryl R. Tripp, Huihui Shi, Pritmohinder S. Gill, Norbert Braun, Herbert Zimmermann, Jean Sévigny, and Simon C. Robson. Expression of NTPDase1 and NTPDase2 in murine kidney: relevance to regulation of P2 receptor signaling. Am J Physiol Renal Physiol 288: F1032–F1043, 2005. First published January 4, 2005; doi:10.1152/ajprenal.00108.2004.—The regulation of renal function by extracellular nucleotides encompasses alterations in glomerular hemodynamics, microvascular function, tubuloglomerular feedback, tubular transport, cell growth or apoptosis, and transport of water and solutes in the medullary collecting duct. Nearly all cells can release ATP or other nucleotides that are then rapidly hydrolyzed in the extracellular milieu. However, little information is available on the cellular expression of ectoenzymes that hydrolyze extracellular nucleotides within the kidney. Nucleoside triphosphate diphosphohydrolases (NTPDases) are plasma membrane-bound ectonucleotidases. NTPDase1 has identity with CD39, a B lymphocyte activation marker, and hydrolyzes extracellular ATP and ADP to AMP within the vasculature, whereas NTPDase2/CD39L(like)1 preferentially converts ATP to ADP outside of blood vessels. Using immunohistochemical and in situ hybridization approaches, we localized the protein and mRNA of NTPDase1 and 2 in murine renal tissues. In the renal cortex, NTPDase1 is expressed by vascular smooth muscle cells and endothelium in interlobular arteries, afferent glomerular arterioles, and peritubular capillaries. In the inner medulla, NTPDase1 is expressed in ascending thin limbs of Henle’s loop, ducts of Bellini, and in the pelvic wall. In contrast, NTPDase2 is expressed in Bowman’s capsule, glomerular arterioles, adventitia of blood vessels, and pelvic wall. Thus the distribution patterns of NTPDases have parallels to the known distribution of P2 receptors within the kidney. NTPDases may modulate regulatory effects of ATP and degradation products within the vasculature and other sites and thereby potentially influence physiological as well as multiple pathological events in the kidney.

EXTRACELLULAR ATP has the capacity to stimulate extracellular nucleotide purinergic (P2) receptors and elicit various cellular responses. P2 receptor signal modulation is tightly controlled by narrow ranges of the extracellular concentrations of ATP mediated through regulated release from cells via exocytosis or specific transporters (33, 50, 51) and rapid hydrolysis of released nucleotides by ectonucleotidases (66). The latter are a heterologous family of surface-located enzymes that sequentially hydrolyze extracellular nucleotides (46, 64, 66). One such family is that of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase), which hydrolyzes nucleosides 5′-tri- and diphosphates. Structurally, these membrane-bound enzymes have a large extracellular domain containing five apyrase-conserved regions that are potentially implicated in ATP hydrolysis and two transmembrane domains at NH2 and COOH termini (67). Analysis of amino acid sequences of NTPDases has shown that NTPDase1 is identical to CD39, a putative B-cell activation marker (26, 38). Biochemically, the properties of members of the NTPDase family differ from one another (32, 67). Thus NTPDase1 (CD39) hydrolyzes extracellular ATP and ADP to AMP, whereas NTPDase2 (CD39L1; CD39-like protein-1) preferentially converts ATP to ADP (21, 56). By hydrolyzing extracellular nucleotides, the activities of E-NTPDases also prevent receptor desensitization (14). Furthermore, in many tissues, the activities of E-NTPDase are coupled with E-5′-nucleotidase (CD73) activity, which releases adenosine from AMP. Adenosine is a potent agonist of P1 receptors, activation of which often opposes the cellular responses elicited by the stimulation of P2 receptors (15, 16, 30, 34, 45).

In recent years, a large body of pharmacological, electrophysiological and molecular studies has unraveled the autocrine and/or paracrine signaling elicited by extracellular nucleotides in the regulation of glomerular, microvascular, and epithelial functions of the kidney in health and disease (1, 23, 34, 50, 51, 59). Experimental studies demonstrated that under physiological conditions, virtually all renal cells can release ATP (51, 52). However, the various enzymes involved in the extracellular hydrolysis of the released nucleotides in the kidney are ill defined. For example, the cellular expression in the kidney of NTPDases, which are considered as the main class of ectonucleotidases responsible for the sequential hydrolysis of gamma and beta phosphates of tri- and diphosphonucleotides in the vasculature, has been poorly studied (36). The growing interest in the regulation of cellular functions by extracellular nucleotides resulted in focused research on the development of specific agonists and antagonists of P2 recep-

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tors and specific inhibitors for NTPDases (18). In view of these developments, the aim of this study was to localize protein and mRNA expression of NTPDase1 and 2 in murine kidney by immunohistochemistry and in situ hybridization. The term murine is applied here to represent rats and mice, which belong to the subfamily murinae. Knowledge gained by the cellular localization of these important enzymes will aid our understanding of the modulation of P2 receptor signaling in the kidney.

METHODS

Experimental Animals

The animals used in this study were approved by the respective Institutional Animal Care and Use Committees (IACUC) of the University of Utah and/or VA Salt Lake City Health Care System (VA SLC HSC; Salt Lake City, UT), Beth Israel Deaconess Medical Center (Harvard University, Boston, MA), and the University of Laval (Sainte-Foy, Québec, Canada). Specific pathogen-free Sprague-Dawley rats (Harlan, Indianapolis, IN or Charles River, LaSalle, Québec, Canada), CBAxC57bl6J mice (Jackson Laboratory, Bar Harbor, MN), and C57BL/6 mice (Taconic Farms, Germantown, NY) were maintained in a pathogen-free state and were fed ad libitum with commercial rodent diet and had free access to drinking water.

Euthanasia, Collection, and Processing of Tissue Samples

Animals were euthanized by a pentobarbital sodium overdose, and kidneys and spleen were rapidly excised. For immunohistochemistry, the kidneys were cut across in the middle, and one-half of each was fixed in 10% neutral-buffered formalin and processed for embedding in paraffin. For in situ hybridization studies, kidneys were chilled in PBS immediately after excision, and then one-half of each was fixed in 10% neutral-buffered formalin for 1 to 3 h before being embedded in paraffin. In some experiments, animals were euthanized by CO₂ inhalation and tissues were rapidly excised. Liver, heart, and kidney were embedded in O.C.T. freezing medium (Sakura) and snap-frozen in isopentane in liquid nitrogen and stored at −80°C until used.

Immunohistochemistry

Frozen sections were processed as follows. Sections of 6 µm were obtained, fixed in 10% phosphate-buffered formalin mixed with cold acetone, and stained using rabbit polyclonal antibodies to mouse NTPDase1 and 2, as previously described (55). Briefly, sections were incubated overnight at 4°C with primary antibodies, washed in phosphate-buffered saline, and blocked with 5% normal goat serum. Primary antibody binding sites were detected using biotin-labeled goat anti-rabbit secondary antibodies. Endogenous peroxidase activity was previously blocked by incubating with 0.4% hydrogen peroxide in phosphate-buffered saline for 10 min. After several washes with phosphate-buffered saline, the sections were stained with horseradish peroxidase complex (Vector Laboratories, Burlington, Ontario, Canada) as specified by manufacturer’s recommendations. Peroxidase activity was revealed using DAB (Sigma, St. Louis, MO) as a substrate. Sections were counterstained with aqueous hematoxylin (Biomeda, Foster City, CA) for 30 s.

Paraffin sections (3- to 5-µm thickness) were cut on a microtome, and sections were deparaffinized by xylene and graded alcohol and rehydrated. After digesting for 10 min at room temperature with
Protease 2 (Ventana Medical Systems, Tucson, AZ), the sections were incubated with primary antibody at room temperature overnight. Peroxidase-conjugated secondary antibody (rabbit IgG; Sigma) was used at a dilution of 1:300 for 8 min. Detection of antigen-antibody reactions sites was achieved by DAB basic kit (Ventana Medical Systems). Sections were counterstained with hematoxylin for 2 min. Except for the incubation with the primary antibody, sections were processed on a Ventana automated instrument at 40°C. Paraaffin sections were also processed by another procedure, which differed from the above procedure as follows. Deparaffinized sections were processed for antigen retrieval by incubating with DakoCytomation Target Retrieval Solution (Dako Cytomation, Carpinteria, CA) at 95°C for 30 min. After cooling to room temperature, sections were washed three times with PBS and blocked for endogenous peroxidase activity. Sections were blocked for 1 h at room temperature in a blocking buffer consisting of 5% BSA and 0.5% Tween 20 in PBS. Sections were then incubated with primary antibody in blocking buffer overnight at 4°C. After the primary antibody was washed off, sections were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at a dilution of 1:750 in blocking buffer without Tween 20 for 1 h at room temperature. After the secondary antibody was washed off, sections were incubated with ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories) for 30 min at room temperature followed by color development with DAB reagent (Vector Laboratories). Sections were counterstained with hematoxylin, dehydrated, and mounted with Permount under coverslips.

Rabbit polyclonal antibodies to NTPDase1 (C9F, C10F, and C11F) and NTPDase2 (BZ34F) were raised by direct inoculation of the encoding cDNA in pcDNA3 (14). The specificity of these antisera was previously verified by using cytochemistry and Western blot analysis on transfected cells and their protein extracts, respectively (4, 22, 56). For the immunohistochemical studies presented here, the antisera were purified by protein A columns and used after appropriate dilution (1:100 to 1:200). Negative controls for immunohistochemistry were generated by substituting the primary antibody with preimmune or nonimmune rabbit IgG. Positive controls were generated by labeling liver or heart tissue sections. Rabbit polyclonal anti-P2X1 antibody was kindly provided by Dr. I. Oglesby of Roche Palo Alto (Palo Alto, CA). This antibody was previously characterized by immnoblotting (44) and has been used to localize P2X1 receptor in rat kidney by immunohistochemistry (9). Affinity-purified rabbit polyclonal anti-aquaporin-1 (AQP1; L266) antibody was kindly provided by Dr. M. Knepper of the National Heart, Lung, and Blood Institute, National Institutes of Health (Bethesda, MD), and it has been characterized previously (58).

In Situ Hybridization

Generation of digoxigenin-labeled riboprobes. NTPDase1 (CD39) mouse plasmid DNA (pcDNA3-CD39) was generated as described previously (14). Competent cells were transformed with pcDNA3-CD39, grown, and plasmid DNA was harvested and purified by
Probes. cRNA content and DIG labeling as described above for CD39 labeled riboprobes were synthesized. The probes were then assessed for DIG penetration into pieces of ~400 bp using a commercial kit (Novagen, Madison, WI).

Plasmids expressing the open reading frame of rat NTPDase2 (CD39L1) were prepared as described previously (27), pCR2.1-TOPO-CD39L1 Ms (-) plasmid (designated as SR-3) and pCR2.1-TOPO-CD39L1 Ms (+) plasmid (designated as SR-4) were reamplified. After EcoRI restriction digestion, both SR-3 and SR-4 gave a band of ~400 bp as expected. The 400-bp band representing the CD39L1 fragment from SR-4/EcoRI digest was purified on Qiagen columns and ligated into pKS(+)H11001 plasmids. An EcoR1 test digest in parallel with SR-3/EcoR1 plasmid showed that the cloning was successful as both digests gave the same insert. SR-4/EcoRI was digested with BsaH1, resulting in a template for CD39L1 having T3 and T7 transcription initiation sites as flanking regions. Using this template, sense (T3 promoter) and anti-sense (T7 promoter) DIG-labeled riboprobes were synthesized. The probes were then assessed for cRNA content and DIG labeling as described above for CD39 probes.

Hybridization. Kidney sections fixed in formalin for 1 to 3 h and embedded in paraffin were cut to a thickness of 3–4 μm on a microtome. Sections were deparaffinized and postfixed in 4% formalin for 50 min, washed, and quenched in 1.5% hydrogen peroxide for 25 min. Sections were then washed with PBS and treated with proteinase K (10 μg/ml) at 37°C for 30 min, followed by acetylation. After being washed with PBS, sections were prehybridized in hybridization mixture at 42°C for 40 min. Sections were then hybridized with DIG-labeled riboprobes (100 to 400 ng/ml) in hybridization mixture overnight at 42°C. The hybridization mixture contained 250 μg/ml tRNA, 500 μg/ml herring sperm DNA, 2 μM EDTA, 1× Denhardt’s reagent, 10% dextran sulfate, 50% formamide, and 10 mM DTT in 4× SSC. Following hybridization, sections were washed with 1× SSC at 60°C and blocked with maleic acid buffer. Sections were then overlaid with 1:100 or 1:200 dilution of anti-DIG-peroxidase-labeled antibody in blocking buffer at room temperature for 1–2 h. After being washed with wash buffer, color reaction was performed using a DAB substrate kit for 5 min. Sections were counterstained with hematoxylin, dipped in 95% ethanol for 5 min, air dried, and mounted with coverslips using Vecta Mount (Vector Laboratories).

Examination of Sections

Kidney sections processed for immunohistochemistry and in situ hybridization were examined under a Reichert light microscope or a Zeiss Axioskop 2 microscope, and digital pictures were taken with a Nikon 995 Coolpix camera or a Spot Insight Color camera from Diagnostics Instruments.

RESULTS

Western blot analysis of whole tissue homogenates from cortex, outer medulla, and inner medulla of rat kidneys showed that NTPDase1 protein was predominantly expressed in the inner medulla, whereas NTPDase2 protein was abundant in the cortex (data not shown).

Specificity of NTPDase1 and NTPDase2 Antibodies

The NTPDase1 and 2 antibodies used in this study have been extensively characterized previously using brain, liver, and heart tissues and transfected cells (4, 12, 22, 56). In this study, we repeated a few of these control experiments in murine heart and liver. Figure 1A shows labeling of venous sinuses in the mouse liver by NTPDase1 antibody (C9F), which is absent when the liver sections were incubated with preimmune serum (Fig. 1B). Figure 1C shows labeling of blood vessels in mouse heart with NTPDase2 antibody (BZ34F), which is absent when...
the preimmune serum was used instead of the specific antibody (Fig. 1D).

**Cellular Localization of NTPDase1 Protein**

Using specific polyclonal antibodies (C9F, C10F, and C11F), we localized NTPDase1 protein in rat kidney. Immunoperoxidase labeling of NTPDase1 protein in rat kidney showed intense labeling of medium-sized blood vessels (Fig. 2, A and B), peritubular capillaries (Fig. 2, A, C, and E), and mesangial and/or capillary membrane in glomeruli (Fig. 2, C and D) of mouse and/or rat renal cortex. In addition to the smooth muscle layer, labeling of an endothelial layer of blood vessels is also seen (Fig. 2B). Figure 2F is a control panel, where the specific antibody was substituted with preimmune serum. Similar results were obtained with other NTPDase1 antibodies (C10F and C11F).

NTPDase1 antibody also labeled glomerular arterioles in both rat and mouse renal cortex (Fig. 3, A and B). Labeling of serial sections with an antibody specific to P2X1 receptor, known to be expressed only in the afferent glomerular arterioles (9, 25), indicated that the NTPDase1-positive glomerular arterioles were afferent arterioles (Fig. 3, a and b). Figure 3, A and B, also depicts the labeling of mesangial and/or capillary membrane with NTPDase1 antibody.

Longitudinal sections through the papilla or in the inner medulla show that NTPDase1 antibody labeled noncollecting duct structures, which are thin and thus could be considered as either the microvasculature (vasa recta) or the thin limbs of the loop of Henle (Fig. 4A). A cross-sectional profile of inner medulla further confirmed the lack of labeling of collecting ducts (Fig. 4B) and also revealed that the microvasculature (vasa recta), filled with red blood cells, is not labeled with the NTPDase1 antibody (Fig. 4B). Thus the specific labeling is apparently restricted to one subpopulation of thin limbs, either the ascending or descending (one Fig. 4B). Because the AQP1 water channel has been shown to be expressed in the descending, but not the ascending, thin limbs of the Henle’s loop (44), we labeled serial sections of rat kidney with NTPDase1 or AQP1 antibodies. As shown in the serial profiles (Fig. 4, C and D), it is apparent that the NTPDase1 antibody labeled only the ascending thin limbs of Henle’s loop. However, apparently there are also a few noncollecting duct thin structures, which do not label either with the NTPDase1 or AQP1 antibodies. Furthermore, although the collecting duct cells are free of any

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**Fig. 4.** Immunohistochemical labeling of NTPDase1 in the inner medulla of rat kidney. Paraffin-embedded rat kidney sections were labeled with an antibody specific to either NTPDase1 (A, B, and C) or to aquaporin-1 (AQP1; D). A: longitudinal profile through the papilla or inner medulla showing labeling of thin structures (thin and long arrows) but not collecting ducts (thick and short arrows) with NTPDase1 antibody. A, inset: negative control for immunolabeling. B: in cross-sectional profile of inner medulla, it is apparent that the NTPDase1 antibody did not label the collecting ducts (CD) or the microvasculature (vasa recta; small and thin arrows in the top left) but specifically labeled a subpopulation of nonvascular thin structures (*), which may be either ascending or descending thin limbs of the Henle’s loop. The identity of these NTPDase1-positive thin structures is revealed in serial profiles labeled with an antibody specific to NTPDase1 (C) or AQP1 (D). AQP1 has been shown to be expressed in descending thin limbs of Henle’s loop and vasa recta but not in ascending thin limbs (41). As shown in the serial profile presented in C, the NTPDase1 antibody specifically labeled ascending thin limbs of loop of Henle (a) but not the descending thin limbs (d) or vasa recta (v). In contrast, the latter two are clearly labeled with AQP1 antibody in the corresponding serial profile presented in the D.
labeling in the initial part, they nevertheless showed some diffuse labeling in the terminal part (not illustrated). In addition, the ducts of Bellini at the papillary tip are intensely labeled (Fig. 5A). Intense labeling is also seen over the smooth muscle layer of the renal pelvic wall (Fig. 5B).

Specificity of DIG-Labeled Riboprobes for NTPDase1 and NTPDase2

We tested the specificity of our gene-specific DIG-labeled NTPDase1 and NTPDase2 riboprobes on sections of rat spleen, a tissue rich in blood cells. Figure 6, A and C, shows intense labeling of cells in rat spleen with anti-sense riboprobes for NTPDase1 and NTPDase2, respectively. Figure 6, B and D, shows panels from sections labeled with corresponding DIG-labeled sense riboprobes (negative controls).

Cellular Localization of NTPDase1 Messenger RNA

Using specific DIG-labeled riboprobes, we localized NTPDase1 mRNA in rat kidney. NTPDase1 mRNA expression was detected in glomerular arterioles and Bowman’s capsule (Fig. 7, A and B). Several proximal tubular cells also showed diffuse labeling (not shown here). Furthermore, distinct labeling for NTPDase1 mRNA could be detected associated with the walls of medium and larger blood vessels (Fig. 7, C and D), and the epithelial lining, and to a lesser intensity with the smooth muscle layer of the pelvis (Fig. 7E; negative control Fig. 7F).

Cellular Localization of NTPDase2 Protein

Using a specific polyclonal antibody (BZ34F), we localized NTPDase2 protein in rat and mouse kidneys. NTPDase2 immu-
nolabeling was detected on the smooth muscle layer of medium-sized blood vessels (Fig. 8A) as well as adventitia of larger arteries (Fig. 8, B and E). NTPDase2 labeling was also seen in Bowman’s capsule (short arrows in A and B) and glomerular cells and arteriole (long arrow in A) and cells. C and D: distinct labeling of endothelial layer of medium and large blood vessels (arrows), respectively. E and F: sections probed with anti-sense or sense riboprobes, respectively. Note the distinct labeling of the epithelial lining of pelvic wall (arrows in E) and weaker labeling of the underlying smooth muscle layer. G: negative control (sense riboprobe) for A and B.

**Cellular Localization of NTPDase2 Messenger RNA**

Using specific DIG-labeled riboprobes, we localized NTPDase2 mRNA in rat and mouse kidneys. Clear and diffuse labeling of proximal tubular cells with NTPDase2-specific anti-sense riboprobe could be seen both in rat and mouse kidneys (Figs. 9A and 10, A and C). Figures 9C and 10D are negative controls probed with sense probes. Labeling of Bowman’s capsule and some glomerular cells, but not capillary tuft, could also be seen in both rat and mouse kidneys (Figs. 9B and 10B). Furthermore, in rat kidney, distinct labeling of the ducts of Bellini at the papillary tip and epithelial cells of pelvis could be seen (Fig. 9, D and E). Figure 9F is a negative control for labeling of pelvic wall.

**DISCUSSION**

Over the past two decades, several cell membrane P2 receptors, which bind extracellular nucleotides (ATP/ADP/UTP/UDP) with different affinities, have been identified, cloned, and characterized. These receptors, collectively known as extracellular nucleotide receptors, have been classified on the basis of their molecular biology, biological actions, pharmacology, and their tissue and cell distribution. Extracellular nucleotide receptors are classified into ionotropic P2X and metabotropic P2Y receptor families (7, 20). The former are ATP-gated membrane channels that allow a variety of ions and/or small molecules to enter the cells (42, 43), whereas P2Y receptors are traditional G protein-coupled receptors that couple to heterotrimeric G proteins, phospholipases, and the phos-
phosphoinositide signaling pathway (10, 62). Although purinergic regulation of cell function has been an ardent research topic in neurobiology, cardiovascular, and pulmonary fields for a long time, however, only recently the potential regulatory functions of purinergic receptors in renal physiology and pathophysiology are being increasingly recognized. The current status of research indicates that purinergic regulation of renal function encompasses glomerular hemodynamics, microvascular function, tubuloglomerular feedback (TGF), tubular transport, renal cell growth, and apoptosis among others (1, 23, 35, 51).

The release of nucleotides into the extracellular milieu is tightly controlled and regulated. Studies have shown that virtually all nephron and collecting duct cells can release ATP (50–52). However, very little information is available on the expression and cellular localization of enzymes that hydrolyze nucleotides in the kidney. Hence, we carried out studies to localize NTPDase1 (CD39) and NTPDase2 (CD39L1) protein and mRNA expression in murine kidney by immunohistochemistry and in situ hybridization, respectively.

Our studies show that both NTPDase1 and 2 are expressed in several cellular structures, paralleling the known distribution of P2 receptors within the kidney. In the renal cortex, NTPDase1 is expressed in interlobular arteries, glomerular afferent arterioles and endothelial cells, vascular smooth muscle cells, peritubular capillaries, and in tubular cells. In the inner medulla, NTPDase1 is expressed in ascending thin limbs of Henle’s loop, terminal collecting ducts, the ducts of Bellini at the tip of the papilla, and in the pelvic wall. NTPDase2 is expressed in Bowman’s capsule, glomerular arterioles and cells, smooth muscle and adventitial layers of blood vessels, and the pelvic wall. In the following paragraphs, we discuss the relevance of our findings to physiological and pathophysiological roles of purinergic signaling in the kidney.

**NTPDase Expression in Glomeruli and Mesangial Function**

P2Y1, P2Y2, P2Y6, P2X2, and P2X4 receptors are expressed in glomerular mesangial cells, whereas the glomerular endothelial cells express the P2Y2 receptor, and podocytes express P2Y1 and P2Y2 receptors (2, 59). Extracellular nucleotides induce proliferation or apoptotic cell death via P2Y and P2X receptors, respectively, in glomerular mesangial cells of rat (19). Proliferation of mesangial cells, which secrete proin-
flammatory cytokines, leads to synthesis of extracellular matrix components, scarring and glomerulosclerosis (11). Apoptosis of mesangial cells also plays an important role in the pathophysiology of glomerular injury, repair, and scarring. Several in vitro and in vivo studies indicated that extracellular nucleotides may play a role as proinflammatory mediators in glomerulonephritis (49). Interestingly, a recent study demonstrated that the P2X7 receptor, which is not expressed appreciably under normal conditions, is significantly upregulated mainly in the glomerular podocytes of rat models of streptozotocin-induced diabetes mellitus or ren-2-transgenic hypertension (63).

Release of nucleotides into the extracellular spaces usually occurs after the damage to glomerular cells and aggregation of platelets (49). Platelet-dense granules are a rich source of ATP. Our finding of NTPDases expression in glomerular cells and/or capillary endothelium raises the possibility that the extracellular concentrations of nucleotides are kept low during normal conditions by these nucleotidases. Although our light microscopic immunoperoxidase labeling indicates that NTPDases are expressed in glomerular cells and capillary endothelium, the precise cellular localization of these enzymes in different cell populations of glomeruli, such as mesangial or epithelial (podocytes), requires studies at a higher resolution using confocal microscopy, which are beyond the scope of the current work.

**NTPDase Expression in Glomerular Arteriole and Autoregulation of Preglomerular Vasculature**

P2Y1, P2X1, and P2X2 receptors are expressed in glomerular afferent arteriole, whereas the efferent arteriole expresses the P2Y1 receptor (9, 59). P2X and P2Y receptors, expressed in pregglomerular microvascular smooth muscle, have been implicated in mediating autoregulatory behavior (23, 24, 37). A recent study by Inscho et al. (25), using a P2X1 gene knockout mouse, provided compelling evidence that TGF signals are coupled to autoregulatory preglomerular vasoconstriction through ATP-mediated activation of P2X1 receptors. In addition, hydrolysis of ATP to AMP by ectonucleotidases and then to adenosine by ecto-5′-nucleotidase/CD73 appears to be important for the TGF regulation. TGF has been shown to be disrupted in CD73-deficient as well as in adenosine A1 receptor-deficient mice (6, 8). Furthermore, the detection of systemic hypertension in cd39-null mice suggests that NTPDase expression in

Fig. 9. Localization of NTPDase2 messenger RNA in rat kidney by in situ hybridization. Paraffin-embedded rat kidney sections were processed for in situ hybridization using NTPDase2-specific riboprobes. A and B: superficial cortex from kidney sections probed with anti-sense riboprobe, where labeling of glomerular and tubular cells in the cortical region could be seen (arrows). C: negative control probed with sense riboprobe. D: distinct labeling of ducts of Bellini at the papillary tip, when probed with an anti-sense riboprobe. E: labeling of the epithelial cells lining renal pelvic wall (arrows) probed with anti-sense riboprobe. F: corresponding negative control probed with sense probe.
these areas has pathophysiological significance (Enjyoji K and Robson SC, unpublished observations). In view of these reports, our finding of NTPDase expression in afferent glomerular arteriole suggests a role for these enzymes in the regulation of TGF by hydrolyzing extracellular ATP. However, more detailed studies are needed to decipher the exact role of NTPDases in the TGF regulation.

**NTPDase Expression in Blood Vessels and Vascular Function**

Vascular NTPDase2 activates platelet aggregation by preferentially converting ATP to ADP (56), whereas vascular NTPDase1, which hydrolyzes ATP and ADP to AMP, terminates the platelet aggregation response to ADP (26, 39). Our finding of NTPDase1 expression in endothelial and smooth muscle cells in the kidney is consistent with its expression in the blood vessels in all other organs tested so far (4, 36, 53–56, 60, 61). In contrast to the expression of NTPDase1, NTPDase2 is expressed in/by the adventitial cells in the kidney, which is consistent with its expression in other organs (2, 3, 12, 56, 60, 61).

**NTPDase1 Expression in the Inner Medulla and Collecting Duct Transport Function**

Inner medulla consists of collecting ducts, descending and ascending thin limbs of Henle’s loop from juxtamedullary (long loop) nephrons, and descending and ascending vasa recta. The latter are microvascular structures that play a role in maintenance of medullary osmotic gradients by transporting water and urea. In the inner medulla, P2Y2 receptors are expressed in the collecting duct, vasa recta, and thin limbs (29), and there is evidence of expression of the P2X6 receptor in the collecting duct (59). Activation of the basolateral P2Y2 receptor in the inner medullary collecting duct (IMCD) inhibits arginine vasopressin-stimulated osmotic water permeability (28, 51). Experimental evidence also suggests that activation of apical and/or basolateral P2X and P2Y receptors in the IMCD inhibits sodium reabsorption and stimulates chloride secretion (35, 59). Furthermore, recently we reported that activation of the P2Y2 receptor in the IMCD results in enhanced production and release of PGE2 (65), and this purinergic-mediated prostanooid release from the IMCD is markedly accentuated in hydrated rats (57). PGE2 has profound effect on the transport of water, sodium, and urea in the IMCD (40, 47, 48). Thus, in the IMCD, the purinergic and prostanoid systems positively interact, which may potentiate their antagonistic effect on vasopressin-induced water flow. Although no NTPDase immunoreactivity was detected in the proximal part of the IMCD in our study, however, it is possible that released ATP might be hydrolyzed once it reaches the adjacent ascending thin limbs of Henle’s loop, where a prominent expression of NTPDase1 has been documented by us. Identification of NTPDase1-positive thin limbs as the ascending ones was achieved by using AQP1 labeling in serial sections. However, in our study we observed a few thin limbs that did not label either with NTPDase1 or AQP1 antibodies. This may be due to the known axial heterogeneity of AQP1 expression in the descending thin limbs of Henle’s loop (41). The physiological significance of expression of NTPDases in the ducts of Bellini at the papillary tip is not clear at this stage.

![Fig. 10. Localization of NTPDase2 messenger RNA in mouse kidney by in situ hybridization.](http://ajprenal.physiology.org/)

Fig. 10. Localization of NTPDase2 messenger RNA in mouse kidney by in situ hybridization. Paraffin-embedded mouse kidney sections were processed for in situ hybridization using NTPDase2-specific riboprobes. A, B, and C: from sections probed with anti-sense riboprobe. A and C: diffuse but distinct labeling of proximal tubular cells in superficial and deep cortex, respectively. B: labeling of Bowman’s capsule (arrow) and nonendothelial glomerular cells (arrowheads). D: a control, probed with a sense riboprobe showing deep cortical region.
NTPDase Expression in Renal Pelvis

For a long time, it has been considered that the renal pelvis, by virtue of the rhythmic contractions of its muscular wall, facilitates urinary concentration in the inner medulla (13). However, recently it has been proposed that hyaluronan, a glycosaminoglycan, the chief component of a gel-like renal inner medullary interstitial matrix, functions as a mechanosensitive transducer, transferring energy from the contractions of pelvic wall to an axial osmolality gradient in the medulla (31). Our study shows that NTPDases are expressed in the pelvic wall of murine kidneys. The published literature is devoid of information about the expression of any known P2 receptors in the pelvis. However, using a specific antibody and gene-specific DIG-labeled riboprobes, we observed that the epithelial lining of pelvic wall expresses the protein and mRNA of P2Y2 receptor (Kishore BK and Issac J, unpublished observations). These findings raise the possibility that P2 receptor signaling is involved in the peristaltic movements of renal pelvis.

Finally, we also observed varying degrees of NTPDase expression in proximal and distal tubules and in collecting ducts in the terminal part of papilla, the significance of which is yet to be determined. However, considering the fact that almost all renal tubular structures express more than one P2 receptor (59), it is possible that the activity of the NTPDases in tubules may trigger preferential activation of specific subsets of P2 receptors sensitive to ATP or ADP. Furthermore, because ecto-5'-nucleotidase (CD73) is expressed in several structures of the kidney, such as the brush border of proximal tubular cells, apical membranes of intercalated cells in connecting tubules, collecting ducts, and cortical interstitial cells (17), it is possible that NTPDases, in concert with 5'-nucleotidase, play a critical role in terminating P2 receptor signaling and initiating P1 receptor activation. Moreover, given in situ data with respect to specific macula densa labeling, we believe that the levels of NTPDase1 protein in the macula densa are probably very low. The expression of NTPDase protein may be regulated at the translational level under certain conditions. Future studies should address these complex regulatory mechanisms in the kidney in health and disease.

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DISCLOSURES

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