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Ontogeny of Bradykinin B1 Receptors in the Mouse Kidney

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ABSTRACT: Kinins are vasoactive peptides that stimulate two G-protein coupled bradykinin receptors (B1R and B2R). *B2R*-knockout mice are salt sensitive and develop renal dysgenesis and hypertension if salt stressed during embryogenesis. *B1R*-knockout mice, on the other hand, are protected from inflammation and fibrosis. This study examined the spatiotemporal expression of B1R during renal organogenesis. The segmental nephron identity of B1R immunoreactivity was determined by costaining with markers of the collecting duct (Dolichos biflorus), proximal tubule (Dolichos tetraglonus), and nephron progenitors (Pax2). At E14.5, the B1R was confined to few cells in the metanephric mesenchyme. Abundance of B1R increased progressively during development. On E17.5, B1R was enriched in differentiating proximal tubular cells and by postnatal day 1, B1R was clearly expressed on the luminal aspect of the proximal tubule. Quantitative real-time PCR revealed that the levels of B1R mRNA more than double during renal maturation. We conclude that 1) B1R expression correlates closely with nephron maturation; 2) lack of B1R in nephron progenitors suggests that B1R is unlikely to play a role in early nephrogenesis; and 3) enrichment of B1R in maturing proximal tubule suggests a potential role for this receptor in terminal differentiation of the proximal nephron. **(*Pediatr Res* 66: 519–523, 2009)**

Kinins, including bradykinin (BK), are formed from partial hydrolysis of kininogen by a family of serine proteases called kallikreins. Kinins produce their effects by binding and activation of two types of receptors, B1R and B2R (reviewed in Ref. 1). Activation of the B2R by BK stimulates nitric oxide and prostaglandin production and results in vasodilation and natriuresis (2). Hence, mice lacking the B2R gene have the propensity to develop salt-sensitive hypertension (3–5). Accumulating evidence also indicates that B2R plays important developmental functions in the kidney and elsewhere under embryonic stress conditions (6–8).

The B1R is activated by Des-Arg^9-BK, a natural product of BK produced by the carboxypeptidase, kininase I. B1R are induced by tissue injury and are believed to mediate inflammatory responses (9–14). Several transcription factors are known to regulate the B1R and B2R genes including NF-κB and p53 (15–17). In this regard, p53 was reported to repress B1R but to activate B2R gene transcription (18–21). Indeed, kidney p53 levels decrease dramatically during development (22,23), which may account for the maturational decline in B2R gene expression (24). Because p53 represses the B1R promoter (21), it is anticipated that B1R expression is upregulated as p53 levels decline during development. To our knowledge, the developmental expression of B1R is unknown. Accordingly, this study was designed to determine the ontogeny of B1R and map the localization of this receptor in the developing nephron.

METHODS

The study was approved by Tulane University School of Medicine Animal Care and Use Committee.

Immunostaining. Embryos from timed-pregnant CD1 mice (Charles Rivers Laboratories) were harvested on embryonic days E14.5, E17.5, and postnatal days P1, P10, P20, and P90 (n = 4–6 per age group). Immunostaining procedures were performed as described previously (6,25,26). Kidneys were fixed in 10% buffered formalin, dehydrated in graded solutions of alcohol, and embedded in paraffin blocks, and 5-μm sections were cut and mounted on Vectabond-coated slides (Vector Laboratories, Burlingame, CA). Antigen retrieval was performed by immersing slides in 10-mM citric acid and boiling in a microwave for 20 min. Immunostaining was performed using the immunoperoxidase technique with a Vectastain Elite kit (Vector Laboratories). The sections were dewaxed in xylene, rehydrated, and treated with absolute methanol and 1% H_2O_2 for 30 min to block endogenous peroxidase activity. Subsequently, tissue sections were sequentially incubated at room temperature with 1) normal serum for 20 min for blocking; 2) primary antibody, a polyclonal antibody raised in goat directed against an N-terminus epitope of mouse BK B1R (sc-15045, A-16) (Santa Cruz Biotechnology) diluted from 1/50 to 1/500; 3) secondary antibody, biotin-conjugated horse anti-goat IgG for 30 min; and 4) avidin DH, biotinylated horseradish peroxidase H complex for 45 min. Peroxidase activity was visualized with 0.01% 3,3′-diaminobenzidine tetrahydrochloride (polysciences) in the presence of 0.02% H_2O_2 as a source of peroxidase substrate for 4–8 min. Lectins *tetraglobus* (LTA) and *Dolichos biflorus* (DBA) were used to label the proximal tubules and collecting ducts, respectively, and a polyclonal rabbit Pax2 antibody (Zymed) was used to label the nephrogenic zone. Slides were photographed using an Olympus model SC35 camera mounted to an Olympus model BH-2 microscope, and digital images were captured using Adobe Photoshop software.

Specificity of immunostaining. The specificity of immunostaining was determined by 1) preadsorption of the antibody with the amino acid peptide of the B1 receptor protein used to generate the antibody, 2) omission of the primary antibody, and 3) omission of the secondary antibody. For the preadsorption studies, the synthetic peptide (seven dilutions spanning 6 μM–260 mM) was incubated with the antibody overnight at 4°C centrifuged at 10,000 × g for 10 min at 4°C and then added directly to the tissue section (total volume 45 μL).

Section immunofluorescence. After the slides were deparaffinized in xylene followed by an ethanol series rehydration, they were washed in PBS twice before antigen retrieval was performed as described earlier. The sections were again washed twice for 10 min each before blocking with 10% serum in 0.1% BSA in PBS for 30 min in a humidity chamber. The primary antibody was diluted in 3% BSA in PBS and added to the sections overnight in a humidity chamber at 4°C. Primary antibodies included a goat anti-mouse

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Abbreviations: B1R, bradykinin B1 receptor; **B2R,** bradykinin B2 receptor; **BK,** bradykinin

B1R (described earlier) and in some sections, neural cell adhesion molecule (N-CAM; mouse monoclonal, 1:300; Sigma Chemical Co.). The slides were then washed in PBST (PBS with 0.1% Tween) three times for 10 min each before incubation with secondary antibody in PBS in a humidity chamber at room temperature in the dark for 1 h. Secondary antibody was rabbit anti-goat Alexa Fluor 594 (1:3,000). FITC-labeled LTA was used to mark proximal tubules (molecular probes). The sections were again washed three times for 10 min each in PBST followed by a final wash in PBS before being mounted with Dako antifade fluorescent mounting medium under glass coverslips.

Quantitative real-time RT-PCR. Total kidney RNA was extracted using Trizol (Invitrogen) from three to five animals in each group. RNA (20 ng per reaction) was reverse transcribed, and real-time PCR was performed in quadruplicate using the Applied Biosystems 7500 Fast Real-time PCR system and TaqMan GenExpression Assays (Applied Biosystems) for mouse *BdkrB1* (assay ID Mm00432059s1) and gapdh (endogenous control, FAM/MGB probe, nonprimer limited; no. 4352932E). The setup of reaction consisted of 1 μL of cDNA (100 ng), 1 μL of TaqMan primer set, 10 μL *Taq* [TaqMan Fast Universal PCR master mix (2×), No AmpErase UNG; no. 4366072], and 8 μL of H_2O under the following PCR conditions: step 1, 95°C for 20 s (enzyme activation); step 2, 95°C for 3 s (melting); and step 3, 60°C for 30 s (annealing and extension); steps 2 and 3 were repeated 40 times.

RESULTS

Histologic analysis of nephrogenesis. Figure 1*A–C* depicts the stages of nephrogenesis. After induction by the ureteric bud, the metanephric mesenchyme undergoes mesenchyme-to-epithelium conversion leading to successive stages of nephrons in a centrifugal manner. The periphery of the developing kidney contains the most immature nephrons, *i.e.* nephron progenitors, consisting of the vesicle, comma-shaped, S-shaped, and maturing glomeruli/nephrons. The first three stages can be labeled by neural cell adhesion molecule (N-CAM) marking the nephrogenic zone (Fig. 1*A*). The maturing nephrons are situated in the deep cortex and can be labeled by a marker of the proximal tubules (*e.g.* LTA) (Fig. 1*B*). The various stages of nephron maturation are depicted in a histologic section (Fig. 1*C*).

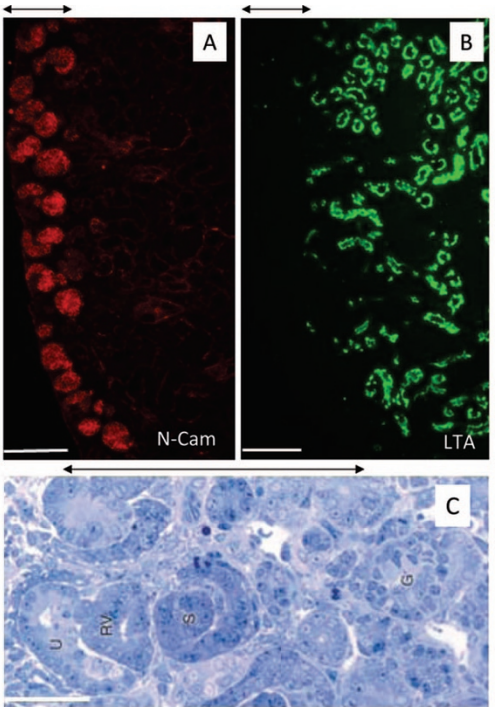

Figure 1. Kidney tissue section from P1 newborn mouse. Immunofluorescence for (*A*) N-Cam and (*B*) LTA to delineate the nephrogenic and differentiation zones, respectively (magnification ×20). *C*, Hematoxylin-stained section showing the various stages of nephrogenesis. *U*, ureteric bud tip; RV, renal vesicle; S, S-shaped body; G, maturing glomerulus. Magnification ×60.

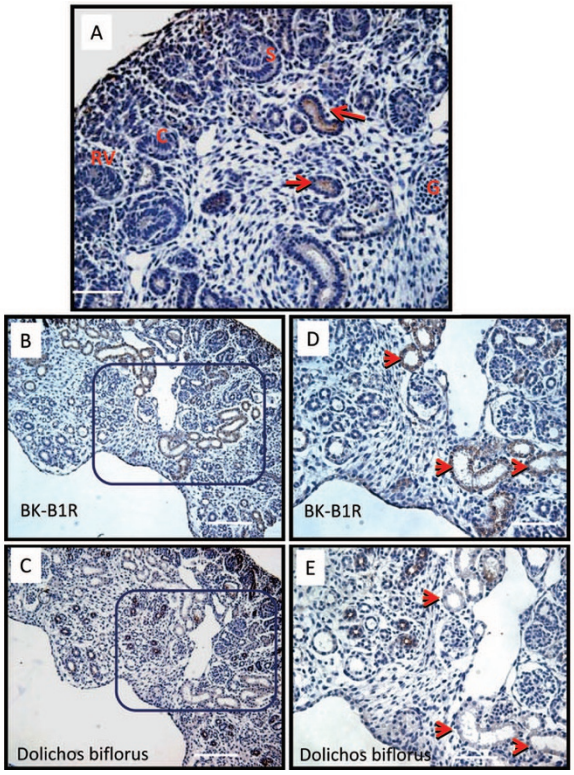

Figure 2. *A*, Tissue section showing B1R immunostaining in E14.5 mouse embryonic kidney (magnification ×40). *Arrows* point to positively stained maturing tubules. Note that nephron precursors, which include renal vesicles (RV), comma- and S-shaped bodies (C, S) are B1R negative. Low (×20) and high (×40) power views of tissue sections stained for B1R (*B*, *D*) or DBA (*C*, *E*) in E17.5 mouse kidney. B1R immunoreactivity is present in the deep cortex in a nonoverlapping manner with DBA, indicating that B1R-nephron segments are derived from the proximal nephron.

Immunolocalization of the B1R. Histologic analysis of the mouse kidney at E14.5 reveals the presence of the four major stages of nephrogenesis: renal vesicle (RV), comma-shaped (C), S-shaped (S), and maturing glomeruli (G) (Fig. 2*A*). Immunolabeling with the B1R antibody was performed using various concentrations ranging from 1/50 to 1/500. B1R immunoreactivity was low at this developmental stage and was only detectable at relatively higher concentrations of B1R antibody (1/50–1/100) (Fig. 2*A*). The nephrogenic zone contained few B1R-positive cells. On the other hand, tubules located in the deep cortex and associated with maturing glomeruli expressed detectable levels of B1R. In these tubules, B1R was observed predominantly on the apical aspect of the tubular cells (Fig. 2*A*, arrows). To determine the origin of these tubules, we stained consecutive sections of E14.5 kidneys with B1R and DBA lectin, a marker of the collecting ducts. DBA-stained epithelia were B1R negative (data not shown). Similarly, glomeruli did not contain B1R immunoreactivity. These findings suggested that B1R-positive tubules are derived from the proximal nephron.

By E17.5, the cortex has demarcated into a well-formed nephrogenic zone and a differentiation zone containing ma-

turing nephrons. The medulla also contains round tubules consisting of the loops of Henle and collecting ducts. Fig. 2*B* and *D* show lower power views of consecutive sections stained for B1R and DBA, respectively. B1R immunoreactivity was observed in elongated and curved tubules in the deep cortex, whereas DBA staining was seen mainly in round tubular sections located in the medulla (medullary-collecting ducts) and straight tubules in the periphery (cortical-collecting ducts). At higher power views, B1R immunoreactivity and DBA are expressed in a nonoverlapping manner (arrows in Fig. 2*D* and *E*). These findings are consistent with the conclusion that B1R are not expressed in the collecting ducts. Of note, B1R immunoreactivity was not detectable in glomeruli or renal microvessels.

To further delineate the regional expression of B1R in the developing kidney, we compared the expression pattern of B1R and Pax2. Pax2 is a transcription factor, which plays a key role in renal development (27) and is expressed in epithelial nephron progenitors and UB branches. This pattern of expression is seen in Fig. 3*A*. In comparison, B1R is expressed in more differentiated tubules in the deep cortex (Fig. 3*B*). Thus, Pax2 and B1R mark different stages of nephron maturation, the nephron progenitors, and differentiated proximal tubules, respectively.

After birth, on postnatal day 1, B1R immunoreactivity increased in abundance and was clearly detected in the proximal tubules (Fig. 4*A* and *B*). Within the proximal tubule, B1R was diffusely expressed in a basolateral distribution and apically. Glomeruli and medullary rays (loop of Henle, vasa recta, and collecting ducts) were B1R negative. To confirm the precise segmental localization of B1R, we performed double immunofluorescence for B1R and LTA (a proximal tubule maker) on tissue sections from P1 kidneys. B1R (red) was expressed in both basolateral and apical aspects of LTA-positive tubules (green) (Fig. 4*C*). LTA-negative tubules were also devoid of B1R immunoreactivity. These results confirm that the major site of B1R expression in the developing kidney is the proximal tubule.

Specificity of immunostaining. To determine the specificity of B1R immunostaining, we performed the following control experiments. First, we preadsorbed the primary antibody with increasing concentrations of the B1R antigenic peptide (×5- to 50-fold). After incubation overnight at 4°C and centrifugation of the antigen-antibody complexes, the sections were incubated with the supernatant. The results revealed that specific B1R immunoreactivity was greatly attenuated by as little as 5-fold excess of antigenic peptide and was completely abolished by incubation with 25-fold or higher concentrations (Fig. 5*A* and *B*). Second, we eliminated the primary or secondary antibodies steps during the immunostaining procedure; this resulted in a low background or no staining (Fig. 5*C*). Collectively, these results demonstrate the specificity of the B1R antibody used in this study.

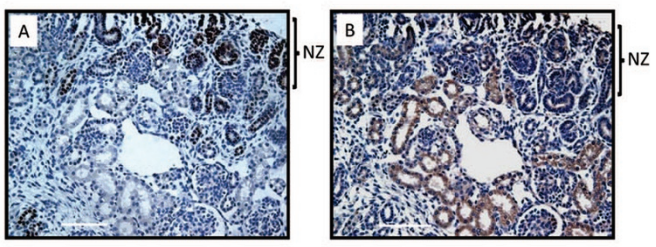

Figure 3. Consecutive sections from a P1 mouse kidney immunostained for Pax2 (*A*) and B1R (*B*). Pax2 marks the nephrogenic zone (*NZ*). B1R-expressing tubules are located in the deep more mature cortical region. Magnification ×40.

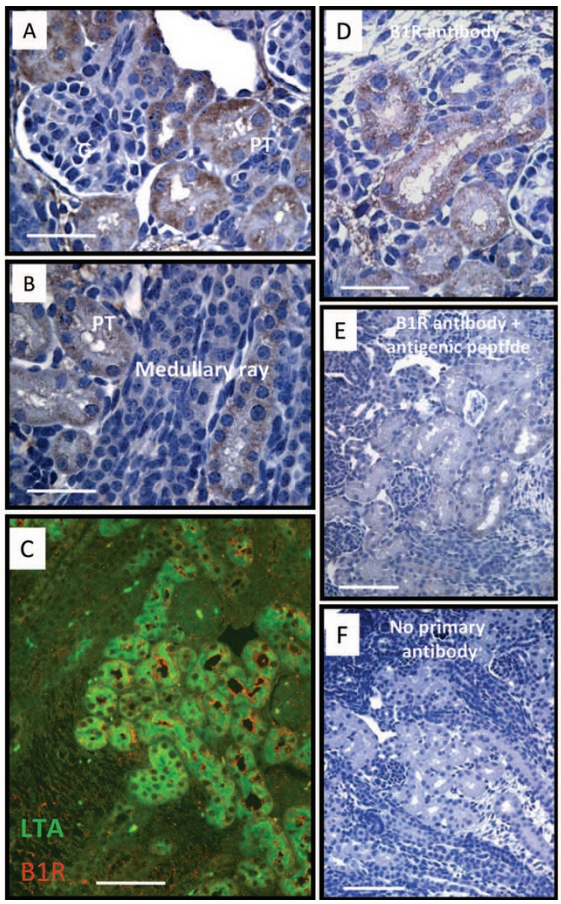

Figure 4. B1R immunostaining in P1 kidney tissue section. *A*, B1R immunostaining is present in proximal tubules but is absent from glomeruli and medullary rays (*B*). Magnification ×60. *C*, Double immunofluorescence for B1R (*red*) and LTA (*green*) in a P1 kidney tissue section (magnification ×40). B1R immunoreactivity is present in proximal tubules on both luminal and basolateral aspects. *D–F*, Immunostaining specificity controls. *D*, B1R immunostaining in P1 kidney tissue section showing the expected localization in the proximal tubules (×60). *E,* Preadsorbption of the primary antibody with ×25 antigen excess eliminates the staining (×40). *F*, Omission of the primary antibody also eliminates specific immunoreactivity (×40).

Developmental changes of B1R gene expression in the kidney. Our immunohistochemical results have shown that the B1R protein is expressed at relatively low levels in E14.5 metanephroi followed by a gradual rise in abundance with maturation. To provide a more quantitative assessment of B1R gene expression, we performed real-time quantitative RT-PCR on RNA isolated from embryonic (E14.5 and E17.5) and postnatal (P1, P10, P20, and P90) kidneys (Fig. 5). *B1R* mRNA levels were factored for the housekeeping gene, β-*actin,* and presented as percent of adult levels. The results demonstrated

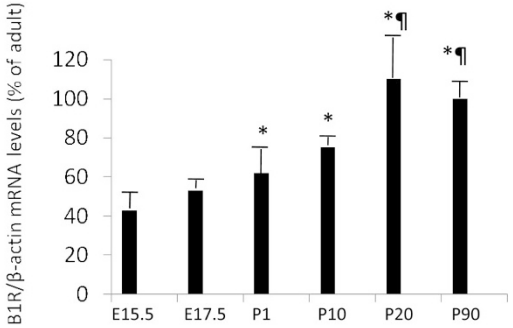

Figure 5. Quantitative real-time RT-PCR of B1R mRNA in mouse kidneys of different developmental stages. *$p < 0.05$ *vs* E15.5; ¶$p < 0.05$ *vs* P1 ($n = 3$ in triplicates).

that metanephric B1R transcript levels double in amounts from E15.5 to P20, when adult expression levels are attained.

DISCUSSION

Previous studies have demonstrated that the developing kidney expresses a local kallikrein-kinin system (KKS), including kallikrein, kininogen, and BK B2 receptors (28–32). Tissue kallikrein is produced by the connecting tubule (33,34). After release, kallikrein cleaves low-molecular weight kininogen, which is synthesized in the collecting duct (34), to release the nonapetide BK. Kininase I, a carboxypetidase, cleaves the terminal arginine of BK to form Des-Arg^9-BK. Although BK preferentially stimulates the B2 receptor, Des-Arg^9-BK stimulates the B1 receptor. B1R and B2R belong to the seven transmembrane G-protein coupled receptor family, and share several signal transduction pathways, such as activation of intracellular calcium and protein kinase C, and downstream transcription factors NF-κB and AP-1 (35,36). However, the two receptors differ in many other respects, *e.g.* B1R is resistant to receptor desensitization and is an inducible receptor. Moreover, although B1R and B2R genes are regulated by common transcription factors such as NF-κB, AP-1, and CREB, the two gene promoters respond in an opposite manner to p53 (18,21,37). Gene disruption strategies in mice have shown that B1R is up-regulated in B2R-null mice (38,39) and plays a role in compensating for B2R in the regulation of cardiovascular protection. Indeed, double B1R/B2R-knockout mice are extremely vulnerable to oxidative stress, DNA damage, and senescence and are at high risk for developing diabetes (40–42).

Although previous studies have carefully characterized the developmental expression of the B2R in the kidney, there is little if any information regarding the ontogeny of B1R. Studies performed in our laboratory and by others have demonstrated that the abundance of B2R mRNA is 10- to 30-fold higher in neonatal than adult tissues (24,43). Receptor autoradiography further indicates that most kinin receptors in the developing kidney belong to the B2 type (24). Immunolocalization studies revealed that B2R are first expressed on the luminal aspect of the upper limb of S-shaped bodies and differentiating cortical collecting ducts. In marked contrast, the metanephric mesenchyme, pretubular aggregates, proximal tubules, and glomeruli display weak or no B2 receptor immunoreactivity (24). After completion of nephrogenesis, B2 receptor expression shifts to both luminal and basolateral aspects of connecting tubules and collecting ducts (43,44).

This study provides new information regarding the developmental expression of the KKS in the mouse kidney. We found that B1R gene expression (mRNA) is low during fetal life and is up-regulated postnatally, and that maturing proximal tubules are the predominant sites expressing B1R protein in the developing nephron. Given these and other results, it is worth considering the differences in the developmental expression of B1R and B2R. First, B2R is constitutively expressed during development and decreases after the end of nephrogenesis (24,31,43). In comparison, B1R is expressed at low levels before birth, after which time B1R increases progressively reaching adult levels by the time of weaning. Second, B2R is enriched in the ureteric bud branches, the precursors of the collecting ducts (43,45); there is also modest amount of B2R in the developing glomerulus (rat) (43). B1R, on the other hand, is not expressed in glomeruli or collecting ducts and is almost exclusively expressed in the proximal tubules. However, this does not rule the possibility that B1R might be induced along new nephron sites in response to stress.

The potential functions of B1R in the developing kidney are unknown. It would be important to know first whether the B1R ligand, Des-Arg^9-BK, is produced by the developing kidney. This will require some knowledge of the ontogeny of kininase I, which generates Des-Arg^9-Bk from BK. B1R-knockout mice are viable but exhibit immune and inflammatory dysfunctions (46,47), but to our knowledge, a formal evaluation of nephrogenesis or differentiation has not been reported. B2R on the other hand, has clear developmental functions that include modulation of renal blood flow through antagonism of angiotensin II (48), protection against early onset of salt-sensitive hypertension (49), and fetal nephroprotection in response to gestational salt stress (6,50). Whether B1R exerts physiologic actions on the structural or functional maturation of the proximal tubule remains to be determined. In this regard, the maturational up-regulation of B1R parallels that of AQP-1, a water channel and a marker of the differentiated proximal tubule (51). A recent study reported that stimulation of B1R on keratinocytes triggers tyrosine kinase signaling and transactivation of the epidermal growth factor receptor (52). Interestingly, B1R-epidermal growth factor receptor cross talk was associated with cellular differentiation rather than proliferation, as assessed by expression of terminal differentiation epidermal markers (52). Future studies aimed at delineating the renal structural, biochemical, and functional phenotypes in B1R-knockout mice are necessary to determine the precise role of B1R in the kidney.

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