Role of Bradykinin B₂ Receptors in Neonatal Kidney Growth

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Abstract. The kallikrein-kinin system is developmentally expressed in newborn kidneys. In addition, bradykinin (BK) is mitogenic in cultured glomerular mesangial cells. However, the role of endogenous BK in postnatal renal development has not been defined. In this study, the role of the BK-B₂ receptor in neonatal kidney growth in the rat was examined. RNA blot analysis and semiquantitative reverse transcription-polymerase chain reaction showed that BK-B₂ mRNA levels were approximately 30- to 40-fold higher in newborn than adult kidneys. Treatment of newborn rats with the selective BK-B₂ antagonist, Hoe 140 (600 μg/kg per day, sc), from days 1 through 14 of life significantly reduced body weight, kidney-to-body weight ratios, and kidney DNA content, compared with saline-treated controls. Hoe 140 treatment had no effect on kidney protein or RNA content or the expression of transforming growth factor-β mRNA. The growth retardation induced by BK-B₂ blockade was observed only in the kidney and, to a lesser extent, in the heart. BK-B₂ blockade had no effect on renal growth in adult rats, suggesting that these effects are developmentally regulated. In contrast to Hoe 140 treatment, neonatal protein undernutrition resulted in a generalized reduction in kidney DNA, RNA, and protein contents; increased renal transforming growth factor-β gene expression; and decreased renal kallikrein expression and enzymatic activity. The results suggest that activation of BK-B₂ receptor expression in the neonatal kidney plays an important role in the regulation of DNA synthesis during the latter stages of nephrogenesis. (J Am Soc Nephrol 8: 920–928, 1997)

The endogenous peptide hormone bradykinin (BK) is formed by the action of plasma kallikrein or tissue kallikreins on high- and low-molecular-weight kininogens. BK is a potent vasoactive peptide that acts via two distinct BK receptor subtypes, B₁ and B₂ (1). Both BK receptors’ cDNA were recently cloned and shown to belong to the seven-transmembrane G protein-coupled family of receptors (2,3). Whereas the BK-B₂ receptor subtype is widely expressed, the B₁ receptor is mainly detected after tissue injury (4,5). The majority of the physiological actions of BK, including the regulation of vascular tone and sodium excretion, are mediated via activation of the B₂ receptor subtype. When activated, the receptor interacts with G proteins to initiate a signal-transduction cascade that stimulates phosphoinositide metabolism and mobilization of intracellular calcium (6,7).

There is increasing awareness that in addition to its vasoactive actions, BK exerts significant effects on cellular growth. For example, BK is mitogenic in murine and human fibroblasts, where it appears to facilitate the stimulation of DNA synthesis produced by epidermal growth factor or platelet-derived growth factor (8–10). BK also stimulates DNA synthesis and proliferation of quiescent rat aortic smooth muscle cells, suggesting that BK may act as a growth modulator in the vessel wall (11,12). In contrast, BK has also been reported to have antiproliferative effects mediated by endothelial-derived nitric oxide (13). We have recently shown that BK, acting via B₂ receptors, stimulates immediate early gene expression, AP-1 binding activity, and DNA synthesis in cultured rat mesangial cells (14). In addition, Bascands et al. (15) reported that BK is mitogenic in quiescent mesangial cells. In vivo studies have corroborated these results. For example, blockade of BK-B₂ receptors in salt-loaded pregnant rats compromises fetal nephrogenesis and is associated with elevated blood pressure in adulthood (16). These findings, combined with our recent observations that renal kallikrein gene transcription and enzymatic activity are upregulated during neonatal development in the rat (17), prompted us to evaluate the potential role of BK-B₂ receptors in postnatal renal growth.

Materials and Methods

Time-dated pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were allowed free access to tap water and regular rat chow. Pregnant rats (day 18 of gestation) were fed either a low- (LP, 6%) or normal- (NP, 24%) protein isocaloric rat chow (Harlan Laboratories Madison, WI). After spontaneous delivery, suckling pups were kept with their mothers, which remained on their respective diets until the end of the study (day 14). Body weights and the amount of food and water consumed were measured daily. To assess whether BK-B₂ receptors modulate somatic and/or organ growth, newborn rats reared by mothers fed LP or NP diets received Hoe 140, a selective and long-acting BK-B₂ antagonist, from days 1 through 14 of postnatal life (18). Hoe 140 was administered subcutaneously in two equally divided doses of 300 μg/kg diluted in 100 μl of saline. We have recently shown that this dose of Hoe 140 does not alter systemic blood pressure or RBF, but abolishes the hypotensive response to 200 ng of intravenous BK in weanling rats (19). Control pups received injections of 0.9% saline. On day 14 of life, the rats were euthanized, and the tissues were harvested and processed as described below. All groups consisted of 8 to 11 animals/group.

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Renal Kallikrein Activity

Kallikrein-like activity in kidney homogenates was measured by the amidolytic assay in the presence of soybean trypsin inhibitor (SBTI) (Sigma Chemical Co., St. Louis, MO) to inhibit related serine proteases, using the fluorogenic substrate D-Val-Leu-Arg-7-AMC (Enzyme Systems, Dublin, CA) (17). Total kallikrein was measured by the same method after activation with 1 µg of bovine pancreas trypsin (Sigma) for 20 min. The activation of prokallikrein was stopped by addition of 20 µg SBTI. The details and validation of this assay for the measurement of tissue kallikrein in the developing kidney have been described elsewhere (17). The intra-assay coefficient of variation was 7.5% for active and 7.9% for total kallikrein, respectively. Kallikrein activity was expressed in milliliters (mU)/mg protein, where one mU is defined as the amount of enzyme capable of hydrolyzing 1 nmol of substrate. Protein concentrations were determined according to Lowry et al., using BSA as standard (20).

Northern Blot Hybridization

Total kidney RNA and oligo dT-selected poly A+ RNA were prepared and used for Northern blot analysis as described previously (21). Slot blots were prepared by dissolving 2.5 to 10 µg of total RNA from individual animals (N = 5 to 6/group) in 0.5 ml of 25 mM sodium phosphate buffer (pH 7.2) and applying this RNA directly to a nylon membrane (GeneScreen Plus; New England Nuclear, Boston, MA) that had been pre-equilibrated with the same buffer by use of a Minifold II Slot-Blotter (Schleicher & Schuell, Keene, NH). All cDNA probes were labeled with 5 µCi [32P]-dCTP (3000 Ci/mmole) using a random priming kit (BRL, Grand Island, NY). The cDNA probes used were: a full-length rat tissue kallikrein cDNA (22), a full-length rat BK-B2 cDNA (2), and a rat transforming growth factor-1 (TGF-β,) cDNA (from American Type Culture Collection. Rockville, MD). The labeled cDNA probes were separated from unincorporated nucleotides by Sephadex G-50 Nick columns (Pharmacia, Piscataway, NJ), and membranes were hybridized with 107 cpm/ml probe for 20 h at 65°C, using hybridization and washing conditions as previously described (21). The membranes were then autoradiographed with intensifying screens (DuPont, Wilmington, DE) at -70°C for 1 to 7 days. Blots were then stripped for 30 min at 95°C with 0.01 × standard saline citrate and 0.01% sodium dodecyl sulfate (SDS) and rehybridized with a probe for human GAPDH (America Type Culture Collection) to account for loading and transfer variations.

Reverse Transcription-Polymerase Chain Reaction of BK-B2 mRNA

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assays were used to estimate the abundance of bradykinin B2 receptor mRNA in neonatal (day 5) and male adult (days 80 through 90) rats. The RT mixture (12 µl) contained 2 µg of total kidney RNA, 1 µg of random hexamers, 1 µl of 10 mM dNTP, 2 µl of 10× RT buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl, 15 mM MgCl2), and 200 U of MLV reverse-transcriptase Superscript II (BRL). RT was performed using the Perkin Elmer Gene Amp PCR System 2400 (Cetus Instruments, Norwalk, CT) at 37°C for 60 min, and the RT enzyme was inactivated by heating at 70°C for 10 min. The template mRNA was degraded by incubating the samples with 2 U of Escherichia coli RNase H at 37°C for 20 min. cDNA was amplified from 25% of RT mixture by PCR using B2 receptor gene-specific primers (25 pmol of each), 1 U of thermostable DNA polymerase from Thermus aquaticus (Taq polymerase), 5 µl of 10× PCR buffer, and 1 µl of 10 mM dNTP (30 cycles; 94°C, 1 min; 58°C, 2 min; 72°C, 3 min). Exponential amplification of B2 receptor cDNA was documented at 15, 25, and 30 cycles of PCR. The upstream primer corresponds to nucleotides 161 through 182 of rat B2 receptor cDNA (5'-AGAACATCTTTTGTCCTCAAGC-3'), and the downstream primer is complementary to nucleotides 714 through 733 of rat B2 receptor cDNA (5'-CGTCTGGACCTCCTCTGA-3') (14). The predicted size of the PCR product is 572 base pairs (bp).

The specificity of the product was confirmed by obtaining two restriction fragments (268 and 304 bp) after digestion of 0.5 µg of the product with 3 U of Nco I restriction enzyme at 37°C for 1 h and by Southern blotting. One half of the PCR mixture was subjected to Southern blot analysis, followed by hybridization with a randomly labeled rat bradykinin B2 receptor cDNA in 6 × saline-sodium phosphate-EDTA, 0.5% SDS, 1% BSA, and 100 µg/ml denatured herring sperm DNA at 65°C for 18 h. The membranes were washed six times in a solution containing 0.1% SDS, 0.1 × SSPE, and 0.5% BSA. Signals were detected by autoradiography at -80°C and quantified by scanning densitometry (Ultrascan; Pharmacia LKB, Uppsala, Sweden).

Analysis of Tissue DNA and Protein Content

DNA was measured in the renal homogenates by a colorimetric reaction with diphenylamine (23). Approximately 100 mg of renal tissue was homogenized in 2 ml of 20 mM phosphate buffer (pH 7.2). The homogenate was then ultra centrifuged at 100,000 × g for 1 h. The pellet was suspended in 3 ml of 1.5 M HClO4, and DNA was hydrolyzed in water bath at 70°C for 15 min. After 24 h at room temperature in a dark room, the absorption was read on a Beckman DU 640 Spectrophotometer (Fullerton, CA) at 595 and 700 nm. In each assay, we checked the linearity of the reaction, adding 1 ml of diphenylamine solution (4% diphenylamine, 0.01% paraformaldehyde in hypochloric acid) to three different dilutions of hydrolyzed DNA (1:100, 1:150, 1:200). Calf thymus DNA (Sigma) was used as standard.

The protein concentration of the homogenate was measured by the Lowry method (20).

Statistical Analyses

After autoradiography, the intensity of each signal on the Northern blots was measured by optical density on an XL densitometer (LKB). In the Slot blots, three measurements of signal intensity at different dilutions of RNA (2.5, 5, and 10 µg) were obtained for each kidney sample. Comparisons among groups were performed by one-way analysis of variance and the Scheffé test or t test. A probability value of less than 0.05 was considered significant. All data are reported as means ± SE.

Results

BK-B2 Receptor Gene Expression Is Activated in the Developing Kidney

To determine whether the BK-B2 receptor gene is expressed in the developing kidney, Northern analysis of total and poly A+ RNA was performed using a radiolabeled full-length rat BK-B2 cDNA (Figure 1A). After 7 days of autoradiographic exposure, kidney BK-B2 receptor mRNA was detectable in the neonatal but not adult kidneys. Densitometrically, the Northern blots indicate that the newborn kidney expressed approximately 40-fold higher levels of BK-B2 mRNA than that of the adult. Figure 1B depicts the expression of BK-B2 mRNA after

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are apparent in the newborn kidney after 25 cycles of PCR within 10 min of autoradiographic exposure. In contrast, a faint signal for the adult kidney was not observed until after 3 h of exposure (Figure 2A). The signal representing the newborn kidney at 15 cycles became visible after 3 h of exposure (Figure 2B), whereas it took 24 h of exposure to visualize the signal corresponding to 15 cycles of amplification in the adult kidney (not shown). Densitometric analysis of the autoradiograms revealed approximate 30-fold-higher levels of B₂ mRNA in newborn than adult kidneys, which is consistent with the findings obtained by Northern analysis (Figure 2C).
Effects of BK-B₂ Blockade on Renal DNA Synthesis
We have recently demonstrated that BK-B₂ mRNA levels are equally elevated on days 5 and 15 of postnatal life and decrease thereafter (21). To evaluate the significance of enhanced BK-B₂ expression in the newborn kidney, neonatal rats were treated with Hoe 140 from days 1 through 14 of life. Hoe 140–treated newborn rats had lower body weights than saline-treated controls (26.0 ± 0.8 versus 31.0 ± 1.6 g, P < 0.05). As shown in Figure 3, kidney weights were lower in Hoe 140– than saline-treated rats (P < 0.05). Importantly, the kidney-to-body weight ratio was lower in Hoe 140–treated rats (11.1 ± 0.1 versus 13.0 ± 0.2 mg/g, P < 0.05) indicating that chronic kinin blockade had a more dramatic effect on kidney growth than somatic growth. To determine whether the decrease in kidney growth induced by chronic Hoe 140 treatment in the newborn rats is the result of inhibition of cell proliferation versus cell hypertrophy, kidney DNA content, protein/DNA ratio, and RNA/DNA ratio were measured (Figure 3). DNA content was lower in Hoe 140– than saline-treated rats, whereas protein/DNA and RNA/DNA ratios were not different, indicating that Hoe 140 resulted in decreased renal cell number but not cell size. In addition, the decrease in kidney weight in Hoe 140–treated rats is tissue-restricted because only the kidneys and (to a lesser extent) the heart manifested a decrease in weight (Figure 4). Plasma protein concentrations were similar in Hoe 140– and saline-treated rats (4.8 ± 0.9 versus 5.1 ± 1.0 g/dl).

Developmentally Restricted Effects of BK-B₂ Blockade
To assess whether the effects of Hoe 140 on renal growth are age-related, adult rats were treated for 14 days with Hoe 140 via Alzet osmotic minipumps (600 µg/kg per day) or an equal volume of 0.9% saline. We have shown that this dose of Hoe 140 blocks the hypotensive effect of intravenous bradykinin (200 ng) (21). The results showed that BK-B₂ blockade did not affect body weight (249 ± 3 versus 242 ± 5 g) or kidney weight (1.1 ± 0.01 versus 1.0 ± 0.02 g). Thus, the effects of Hoe 140 on renal growth are confined to the developmental period.

Neonatal BK-B₂ Blockade Does Not Influence TGF-β₁ Gene Expression
Because BK-B₂ receptors are predominantly expressed in the differentiating renal tubular epithelium rather than nephrogenic mesenchyme (24), we examined the expression of TGF-β₁ (a negative regulator of epithelial cell cycle) in rats treated with Hoe 140 during the neonatal period. Treatment with Hoe 140 did not alter kidney TGF-β₁ mRNA expression on day 14 of postnatal life (Figure 5, top).

Effects of Neonatal Protein Restriction on Kidney Growth, DNA Synthesis, and TGF-β₁ Gene Expression
To evaluate whether the effects of Hoe 140 on renal growth are related to a nonspecific effect of the antagonist on protein intake or synthesis, we also examined the effects of dietary...
protein restriction on normal renal growth (Figure 3). Newborn rats reared by mothers subjected to chronic protein restriction suffered from retardation in somatic (12.5 ± 0.1 versus 31 ± 1.6 g, \( P < 0.001 \)) and kidney growth. However, unlike Hoe 140, protein restriction was associated with a generalized reduction in kidney DNA, RNA, and protein synthesis. These data indicate that kinin receptor blockade during early development suppresses renal cell proliferation, whereas protein restriction inhibits both renal hyperplasia and hypertrophy.

In comparison with Hoe 140, protein restriction during the neonatal period more than doubled renal TGF-\( \beta \), mRNA levels (Figure 5, bottom). GAPDH gene expression was not altered by Hoe 140 (Figure 5) or protein restriction (not shown), indicating that the changes in TGF-\( \beta \), expression are specific and not the result of generalized alterations in gene expression. These results suggest that TGF-\( \beta \) may be involved in the renal growth response to protein restriction but not to kinin blockade in the developing kidney.

Utilizing an identical protocol of Hoe 140 treatment in neonatal rats, we have recently demonstrated that BK-B\(_2\) blockade upregulates kallikrein gene expression and enzymatic activity (21). To further compare the effects of Hoe 140 and protein restriction during the neonatal period, we investigated the effects of protein restriction on kallikrein gene expression. LP resulted in a 50% reduction of renal kallikrein mRNA (13.2 ± 1.3 versus 27.5 ± 3.4 densitometric units, \( P < 0.001 \)) and in active (2.3 ± 0.2 versus 3.5 ± 0.4 mU/mg protein; \( P < 0.05 \)) and total (6.8 ± 0.7 versus 10.8 ± 0.5 mU/mg protein; \( P < 0.05 \)) kallikrein contents (Figure 6). In sharp contrast, LP increased BK-B\(_2\) receptor mRNA levels (13.2 ± 0.7 versus 5.7 ± 0.5 units; \( P < 0.01 \)) (Figure 7). Thus, protein restriction appears to regulate kallikrein and BK-B\(_2\) receptor mRNA reciprocally in the developing kidney.

**Discussion**

Several lines of evidence suggest that BK-B\(_2\) receptors promote cell-cycle progression. First, activation of BK-B\(_2\) receptors stimulates phosphoinositide hydrolysis, intracellular calcium mobilization, and protein kinase C activation (6-8,11). Similar signal transduction events known to occur after stimulation of other G protein–coupled receptors (e.g., AT\(_1\), ET\(_1\)) have been implicated in mediating mitogenesis (25). Second, BK stimulates tyrosine phosphorylation of proteins involved in cell-cell and cell-matrix interactions and proliferation, including p125\(^{Fak}\) and paxillin (26). Third, the mitogen-activated protein kinase pathway, immediate early gene expression, and the DNA binding activity of the transcription factor AP\(_1\) are all activated by BK (14). The latter is believed to transactivate many growth-related genes involved in cell-cycle control. Fourth, BK—acting via B\(_2\) receptors—stimulates DNA synthesis and proliferation of quiescent fibroblasts, mesangial cells, and smooth muscle cells (8-12,14,15).

The study presented here provides new evidence that BK-B\(_2\) receptors are involved in the regulation of DNA synthesis and, presumably, cell number in the neonatal kidney. In the rat kidney, nephrogenesis and DNA synthesis continue postnatally.
Figure 5. (Top) Northern blot analysis of kidney TGF-β mRNA in 14-day-old rats treated since birth with Hoe 140 or 0.9% saline. Each lane contains 20 μg of total RNA. Transforming growth factor (TGF) β mRNA levels factored for GAPDH were not different in Hoe 140–versus saline-treated rats. (Bottom) Slot blot analysis of kidney TGF-β mRNA in 14-day-old rats reared by mothers on low protein (6%) or normal protein (24%) diets. Protein restriction doubles TGF-β mRNA levels in newborn kidneys. (Solid bar represents low-protein diet and shaded bar represents normal-protein diet.)

until days 10 and 40, respectively (27). Kidney-to-body weight ratio and DNA contents per kidney were significantly decreased in Hoe 140–treated rats, compared with saline-treated controls. No effect was observed on total kidney RNA or protein content. Because DNA content per cell is constant, the selective decrease in kidney DNA content by BK antagonism suggests that BK-B₂ receptors stimulate renal hyperplasia, not hypertrophy. Retardation of somatic growth, kidney DNA content, and renal growth also occurs in neonatal rats treated with angiotensin type I (AT₁) receptor blockers (28,29). Thus, it appears that intact function of AT₁ and BK-B₂ receptors may be especially important during the latter part of nephrogenesis and differentiation.

The long-term consequences of kinin blockade during early development on blood pressure and renal function are unknown. Recent studies by Madeddu et al. (30,31) reported higher adult blood pressure in salt-loaded rats treated prenatally and postnatally with Hoe 140. In preliminary studies, we observed that treatment of pregnant rats with Hoe 140, combined with salt-loading, compromises fetal glomerular maturation and tubular differentiation (16). Because similarly treated rats manifest higher blood pressure later in life (30), our observations support Brenner’s hypothesis that congenital or acquired neonatal deficits in nephron number, when combined with an environmental stressor, may be a predisposing factor for the development of hypertension (32,33).

An obvious concern of any pharmacological manipulation during the period of rapid somatic and organ growth is potential interference with normal protein intake. We therefore examined the possibility that the observed effects of BK-B₂ receptor blockade may be mediated by decreased protein intake. However, three lines of evidence suggest that the effects of Hoe 140 on renal growth were not mediated by decreased protein intake during a critical period of development. First, the
effects of Hoe 140 are limited to kidney DNA synthesis, whereas neonatal protein undernutrition decreased kidney DNA, RNA, and protein contents. Therefore, the mechanisms of renal growth retardation induced by Hoe 140 and protein deficiency are fundamentally different; in the former, it is the result of inhibition of cell hyperplasia, whereas in the latter it results from inhibition of both hyperplasia and hypertrophy. Second, the organ-restricted effect of Hoe 140 (kidney and heart) argue against a generalized nonspecific effect of Hoe 140 on organ development. Third, neonatal Hoe 140 treatment increases mRNA and protein expression of kallikrein and BK-B₂ mRNA (21), whereas, as shown in this study, protein restriction decreases renal kallikrein and its mRNA and upregulates BK-B₂ expression. We speculate that the inability of enhanced B₂ receptor expression to counteract the growth-suppressive effects of protein restriction may be a result of the marked downregulation of renal kallikrein synthesis and activity and, presumably, bradykinin levels.

An additional concern is the potential interference of kinin blockade with the maturational rise in RBF and the possible ischemic consequences on nephrovascular development. Our recent finding that neonatal BK-B₂ receptor blockade is not associated with gross alterations in total or outer cortical renal blood flow in weanling rats argues against this possibility (19). TGF-β can act as a negative regulator of nephrogenesis in vitro (34). Thus, we evaluated the effects of Hoe 140 on renal TGF-β₁ gene expression. The lack of significant effects of Hoe 140 on TGF-β₁ mRNA levels suggests that this growth suppressor factor is probably not involved in the effects of BK on restricting renal growth. These findings are consistent with unaltered expression of TGF-β₁ mRNA in BK-stimulated cultured rat mesangial cells (14). On the other hand, the upreg-
Kidney B2 receptor mRNA

6%

Protein diet

24%

Figure 7. Effect of LP intake during neonatal development on renal BK-B2 receptor gene expression. In contrast to kallikrein, renal BK-B2 gene expression is increased by neonatal protein restriction. This finding suggests that the decreased kallikrein-kinin activity resulting from LP intake stimulates expression of the BK receptors.

nary studies indicate induction of proto-oncogene expression and AP1 DNA binding activity in BK-stimulated mesangial cells (14). Thus, transactivation of AP1-inducible growth regulatory genes (e.g., cyclin D1) may play a role in the stimulation of cell-cycle progression by BK.

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