Targeted disruption of the bradykinin B$_2$ receptor gene in mice alters the ontogeny of the renin-angiotensin system

IGOR V. YOSIPIV, SUSANA DIPP, AND SAMIR S. EL-DAHR

Section of Pediatric Nephrology, Department of Pediatrics, Tulane University Health Sciences Center, New Orleans, Louisiana 70112

Received 26 January 2001; accepted in final form 27 June 2001

Yosipiv, Igor V., Susana Dipp, and Samir S. El-Dahr. Targeted disruption of the bradykinin B$_2$ receptor gene in mice alters the ontogeny of the renin-angiotensin system. Am J Physiol Renal Physiol 281: F795–F801, 2001. First published July 12, 2001; 10.1152/ajprenal.0020.2001.—Angiotensin II type 1 (AT$_1$) receptor knockout (KO) mice exhibit an activated kallikrein-kinin system (KKS) that serves to attenuate the severity of the renal vascular phenotype in these mice (Tsudchida S, Miyazaki Y, Matsusaka T, Hunley TE, Inagami T, Fogo A, and Ichikawa I, Kidney Int 56: 509–516, 1999). Conversely, gestational high salt suppresses the fetal renin-angiotensin system (RAS) and provokes aberrant renal development in bradykinin B$_2$-KO mice (El-Dahr SS, Harrison-Bernard LM, Dipp S, Yosipiv IV, and Meleg-Smith S, Physiol Genomics 3: 121–131, 2000). Thus the cross talk between the RAS and KKS may be critical for normal renal maturation. To further define the developmental interactions between the KKS and RAS, we examined the consequences of B$_2$ receptor gene ablation on the expression of RAS components. Renal renin mRNA levels are 50% lower in newborn B$_2$-KO than wild-type (WT) mice. Also, the age-related decline in renin mRNA is greater in B$_2$-KO than WT mice (3.5- vs. 2-fold, P < 0.05). Although renal angiotensinogen (Ao) protein levels are higher in newborn B$_2$-KO than WT mice, Ao mRNA levels are not, suggesting accumulation of Ao as a result of decreased renin-mediated cleavage. Similar age-related increases (8-fold) in angiotensin I-converting enzyme (ACE) activity are observed in B$_2$-KO and WT mice. Renal AT$_1$ protein levels are not different in B$_2$-KO and WT mice. Furthermore, the developmental increases in renal kallikrein mRNA and enzymatic activity are more pronounced in B$_2$-KO compared with WT mice (mRNA: 8- vs. 3-fold; activity: 13- vs. 6-fold, P < 0.05). We conclude that 1) bradykinin stimulates renin gene expression, 2) renal kallikrein is regulated via a negative feedback loop involving the B$_2$ receptor, and 3) Ao, ACE, and AT$_1$ are not bradykinin-target genes.

irkein; kallikrein-kinin; development; angiotensin I-converting enzyme

THE DEVELOPING KIDNEY EXPRESS all the components of the renin-angiotensin system (RAS). Furthermore, RAS activity is greater in the newborn than adult animal (21). Both the pharmacological antagonism and genetic inactivation of the RAS have been shown to impair renal growth and nephrovascular maturation (7, 18). In addition to its role as a renal growth factor, angiotensin II is an important mediator of the elevated renal vascular resistance in the neonatal animal (24). The enhanced activity of the RAS in the developing kidney is counterbalanced by paracrine vasodilators such as bradykinin and its downstream mediators, nitric oxide and prostaglandins (16, 26, 27).

Like the RAS, a complete kallikrein-kinin system (KKS) is expressed within the developing kidney (10). The physiological effects of kinins, including the regulation of growth, vascular tone, and sodium excretion, are mediated by the B$_2$ receptor (3). Previously, we have shown that B$_2$ gene expression and bradykinin levels are higher in the newborn than adult kidney, suggesting an important role for bradykinin in renal maturation (12, 13). Blockade of B$_2$ receptors in newborn rats with a selective antagonist impairs renal growth (31). Moreover, B$_2$ knockout (B$_2$-KO) embryos subjected to salt stress in utero exhibit suppressed renin, an abnormal kidney phenotype, and develop early postnatal hypertension (6, 14). Other investigators have demonstrated that B$_2$ blockade exacerbates the renal vascular thickening in angiotensin II type 1 (AT$_1$)-KO mice (29). Therefore, the functional interactions between the KKS and RAS appear to modulate metanephric structural and functional maturation.

The KKS and RAS also exhibit cross-regulatory influences on gene expression because B$_2$ blockade in newborn rats or AT$_1$ gene disruption in mice blunts the maturational rise in renal angiotensin I-converting enzyme (ACE) (29). To our knowledge, the effects of B$_2$ gene inactivation on the ontology of RAS are not known. The present study was therefore undertaken to examine the potential regulation of the RAS components by bradykinin in the B$_2$-KO mice.

METHODS

Animals. Bradykinin B$_2$ receptor −/− (B$_2$-KO) mice were originally obtained on a mixed genetic background (4) and have since been backcrossed onto the C57BL/6J background for 8–10 generations in our animal facility. B$_2$-KO and C57BL/6J wild-type (WT) mice were maintained on a normal salt diet. Tissues were harvested on days 1, 5, 15, 60, or 90 of postnatal life (n = 4–7/group).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Northern and slot blot analysis. RNA extraction, gel electrophoresis, RNA transfer to membrane, and hybridization procedures were performed as previously described (13). The membranes were hybridized with random-primed 32P-labeled renin, kallikrein, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs. Signals were detected by autoradiography and quantified by scanning densitometry (Ultrorcan, Pharmacia).

Western blot analysis. Kidneys were homogenized in cold lysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.02% Na azide, 0.1% SDS, 1% Nonidet-P-40, and 0.5% deoxycholate) containing a cocktail of enzyme inhibitors added fresh to the lysis buffer (100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aproatin, 1 μg/ml leupeptin, and 10 μg/ml Na3VO4). Insoluble material was removed by centrifugation for 10 min at 14,000 g at 4°C. Proteins were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The adequacy of transfer was assessed by Ponceau S staining of the membranes. Non-specific binding sites were blocked with a blocking solution (PBS containing 0.1% Tween and 3% BSA) overnight at 4°C. Membranes were then incubated with sheep anti-rat angiotensinogen (Ao) antibody at a concentration of 1:2,000 (8), a polyclonal rabbit AT1 receptor antibody directed against the NH2-terminal domain of the human receptor (Santa Cruz Biotechnology, N-10, sc-1173) at a concentration of 1:600 (1), or β-actin (Sigma, 1:4,000) at room temperature for 1 h. After three washes in PBS/Tween, the nitrocellulose membrane was exposed for 1 h at room temperature to the secondary antibody (horse- radish peroxidase-linked goat anti-mouse or rabbit IgG). Immunoreactive bands were visualized using the enhanced chemiluminescence detection system (Amersham). Band signal intensity was determined by scanning densitometry.

ACE and tissue kallikrein activity. Kidneys were dissected free of connective tissue, blotted dry, and immediately homogenized mechanically in ice-cold Tris·HCl buffer (0.1 M, pH 8.2). ACE activity was measured by fluorometric assay of the enzymatic cleavage of hippurate from hippuryl-histidyl-phenylalanine (HHP) at pH 8.2. ACE activity was measured by fluorometric assay of the enzymatic cleavage of hippurate from hippuryl-histidyl-phenylalanine (HHP) at pH 8.2. Kidneys were homogenized in ice-cold Tris·HCl buffer (0.1 M, pH 8.2). The membranes were hybridized with random-primed 32P-labeled renin, kallikrein, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs. Signals were detected by autoradiography and quantified by scanning densitometry (Ultrorcan, Pharmacia).

RESULTS
Downregulation of renin gene expression in B2-deficient mice. To determine whether genetic ablation of B2 receptors alters the developmental expression of renal renin, we compared renin mRNA levels in newborn and adult B2-KO and WT mice. Densitometric analysis of Northern blots hybridized sequentially with renin and GAPDH cDNAs revealed that renin mRNA levels, factored for those of GAPDH, were 50% lower in newborn B2-KO than the WT mice (P < 0.05) (Fig. 1A). There was a significant decrease in renin mRNA levels during postnatal maturation in both groups. However, the age-related decline in renin mRNA was significantly greater in B2-KO than WT mice (3.5- vs. 2-fold, P < 0.05). Furthermore, the differences in renin gene expression between B2-KO and WT mice persisted until adulthood (4-fold, P < 0.02) (Fig. 1, A and B). Thus B2-KO mice exhibited a steeper developmental down-regulation of renal renin gene expression compared with WT mice.

Developmentally restricted effects of B2 ablation on renal Ao. The postnatal expression of renal Ao protein was assessed by Western blotting and is shown in Fig. 2. A and B. Renal Ao/β-actin ratios were threefold higher in the newborn B2-KO than WT mice (0.63 ± 0.10 vs. 0.20 ± 0.03, P = 0.02). On the other hand, RNA slot blot analysis showed no differences in Ao mRNA levels (factored for GAPDH) between newborn B2-KO and WT mice (15.2 ± 2.3 vs. 13.5 ± 4.6 densitometric units, n = 5 and 6, respectively). Therefore, the increase in renal Ao protein in newborn B2-KO mice is likely a result of Ao accumulation from reduced activation by renin. No differences were observed in renal Ao expression between adult B2-KO and WT mice (results not shown).

Ontogeny of renal ACE and AT1 receptors in B2-KO mice. The postnatal changes in kidney ACE activity in B2-KO and WT mice are shown in Fig. 3. ACE activity was relatively low in the newborn kidney and increased progressively during the preweaning period (up to 8-fold). In both B2-KO and WT mice, ACE activity peaked on day 15 of postnatal life and declined slightly thereafter. No significant differences were observed in the age-related changes of renal ACE between the two groups.

Effect of B2 inactivation on AT1 expression. The results of Western blots showed that renal AT1 protein levels, factored for those of β-actin, are not different in the B2-KO and WT mice (Fig. 4, A and B). We and others reported previously that AT1 expression is not different in adult B2-KO and WT mice (6, 20). Collectively, these data indicate that disruption of the B2 receptor gene has no measurable effect on the developmental expression of renal ACE or AT1.

B2 ablation accentuated the postnatal upregulation in kallikrein gene expression. B2 signaling may have a feedback effect on renal kallikrein. The effect of B2 inactivation on renal kallikrein mRNA is shown in Fig. 5. Northern blot analysis demonstrated that kallikrein mRNA levels, factored for GAPDH mRNA, increase during postnatal maturation. This increase was observed in both B2-KO and WT groups. However, the developmental increases in kallikrein mRNA were more pronounced in B2-KO compared with WT mice (8-fold in B2-KO vs. 3-fold in WT, P < 0.05) (Fig. 5A).

Figure 5B depicts the relative changes in renal kallikrein activity in newborn to adult mice. The relative increases in kallikrein enzymatic activity are markedly enhanced in B2-KO compared with WT mice (13-fold in B2-KO vs. 6-fold in WT, P < 0.05) (Fig. 5B). Thus endogenous kinins, acting via B2 receptors, exert a negative feedback effect on the developmental expression of renal kallikrein.

AJP-Renal Physiol • VOL 281 • NOVEMBER 2001 • www.ajprenal.org
DISCUSSION

This study demonstrates that genetic inactivation of the bradykinin B2 receptor in mice alters the ontogeny of the RAS. Of particular interest are the accentuated changes in the developmental expression of renin and kallikrein in the B2-KO mice. Not all the RAS components are influenced by B2 ablation because ACE and AT1 expression remains unchanged. Thus the cross talk between the KKS and RAS is component specific and is mainly exerted at the levels of the rate-limiting enzymatic step in the respective cascades.

The components of the KKS and RAS are expressed in the developing kidney. The developmental expression patterns of RAS and KKS genes have been the subject of intense investigation and are well characterized. The two systems share common developmental features. For example, renal bradykinin and angiotensin II levels are both elevated in the developing compared with the adult kidney (12, 33). Moreover, the developing kidney expresses higher levels of B2 and AT1 receptors than those in the adult (13, 23). On the other hand, ACE (kininase II) gene expression is relatively low at birth and increases during maturation (32). The low ACE activity in the newborn may favor bradykinin accumulation in the developing kidney. The KKS and RAS differ, however, with respect to renin and kallikrein gene expression. Renin synthesis is highly activated in the developing kidney and declines with maturation (20). Kallikrein exhibits the opposite temporal profile, increasing substantially with postnatal maturation (2, 11, 15, 25, 30). Although previous studies have considered the developmental regulation of KKS and RAS separately, the potential cross-regulatory influences between the two systems have not been defined. The availability of genetically altered animal models lacking the RAS or KKS components has opened new avenues to explore the physiological interactions between these two phylogenetically conserved systems.
A new finding of this study is that renin mRNA levels are lower in B2-KO than WT mice at birth. Moreover, the natural postnatal decline in renin was more pronounced in B2-KO than WT mice. A limitation of our study is that renin activity was not measured because changes in mRNA do not always reflect changes in protein levels or enzymatic activity. A role for kinin B2 receptors in the regulation of renin release has been demonstrated recently by Gainer et al. (19), who showed that treatment of salt-depleted human subjects with the B2 receptor antagonist icatibant inhibits the hypotensive effect and the rise in plasma renin activity in response to ACE inhibition. Together, these results and ours suggest that renin gene expression and release are downstream targets of the B2 receptor. Although a previous study localized immunoreactive B2 receptors in the smooth muscle layer of the afferent arterioles in the adult rat kidney (17), fol-

Fig. 2. Angiotensinogen (Ao) protein expression in newborn kidneys of B2-KO and WT mice. A: Western blot of Ao (top) and β-actin (bottom) proteins. B: quantitative analysis of Ao protein factored for β-actin in B2-KO and WT mice. *P < 0.05 vs. B2-WT.

Fig. 3. Postnatal ontogeny of renal angiotensin I-converting enzyme (ACE) activity in B2-KO and WT mice. D, day. *P < 0.05 vs. D1; #P < 0.05 vs. D5.
low-up studies did not confirm this finding (9, 13, 28). It is therefore likely that the effects of bradykinin on renin gene expression are indirect. Because inhibition of nitric oxide synthase activity decreases renin expression at the juxtaglomerular apparatus (5), it is possible that lack of endothelial cell B₂ receptors might have reduced the local nitric oxide concentrations in the vicinity of juxtaglomerular renin-producing cells. Whereas B₂-KO mice on a mixed genetic background have been reported to have a modest elevation of blood pressure (22), the B₂-KO mice used in our study have been backcrossed up to 10 times onto the C57BL6 background and are normotensive (6). Therefore, the decrease in renal renin gene expression in B₂-KO mice is unlikely to be due to changes in blood pressure.

Unlike renin, renal Ao protein levels are higher in newborn B₂-KO than WT mice. This may be due to decreased consumption of Ao by the lower renin levels in B₂-KO mice because Ao mRNA levels were similar in B₂-KO and WT mice. Alternatively, the increased Ao protein levels might be due to enhanced translation of Ao mRNA or possibly inhibition of degradation by alternative pathways. Although kidney ANG peptide levels were not measured here, a previous study showed that kidney ANG II levels were not different in adult B₂-KO and WT mice (6).

ACE is a zinc metalloproteinase that is involved in the posttranslational processing of angiotensin I and bradykinin. We found that ACE activity is low in the newborn mouse kidney and increases substantially during postnatal maturation, peaking during the second to third week of age. Interestingly, this developmental profile of renal ACE expression recapitulates that observed in the rat (32). The conservation of ACE ontogeny attests to the important role of ACE, angiotensin, and bradykinin in renal development. Previously, we reported that blockade of B₂ receptors with Hoe-140 (icatibant) in the newborn rat blunts the maturation of ACE gene expression and activity, suggesting that endogenous kinins function as positive regulators of renal ACE in the developing kidney (34). The results of the present study, however, do not support this conclusion because the developmental changes

![Fig. 4. Renal angiotensin II type 1 (AT₁) receptor protein levels in newborn B₂-KO and WT mice. A: Western blot of AT₁ (top) and β-actin (bottom) proteins. B: quantitative analysis of AT₁ protein levels factored for β-actin.](image)

![Fig. 5. Ontogeny of renal kallikrein in B₂-KO and WT mice. A: kallikrein mRNA levels factored for GAPDH measured by Northern analysis. B: kallikrein activity maturation (relative fold increase from birth until day 60 of postnatal life). *P < 0.05.](image)
in renal ACE activity were almost identical in B2-KO and WT mice. The reasons for the discrepancy in the effects on ACE ontogeny of pharmacological versus genetic B2 inactivation are not clear. Theoretically, B2 inactivation from the time of conception may have allowed enough time for other paracrine systems with overlapping functions to maintain renal ACE.

Intact AT1-mediated signaling is required for normal renal development. Here, we investigated whether genetic B2 ablation alters the ontogeny of renal AT1 protein expression. We found no discernible differences in AT1 protein levels between B2-KO and WT mice. The lack of changes in AT1 expression in B2-KO animals is consistent with previous observations made in adult B2-KO mice (6, 22).

Previous studies have demonstrated that renal tissue kallikrein activity, mRNA expression, and gene transcription rate are remarkably upregulated during postnatal maturation (2, 11, 15, 30). B2 expression, on the other hand, is high in the newborn rat and decreases after weaning (13). Interestingly, B2 receptor blockade enhances kallikrein expression in the newborn kidney (34), suggesting that renal kallikrein is negatively regulated by its product, bradykinin. Here, we tested whether genetic B2 inactivation recapitulates the effects of pharmacological intervention on renal kallikrein. In agreement with our results in the rat (34), we found that renal kallikrein mRNA and activity increase remarkably during maturation in newborn kidneys (34), suggesting that renal kallikrein is negatively regulated by its product, bradykinin. Here, we tested whether genetic B2 inactivation recapitulates the effects of pharmacological intervention on renal kallikrein. In agreement with our results in the rat (34), we found that renal kallikrein mRNA and activity increase remarkably during maturation in mice. More importantly, the developmental upregulation of kallikrein gene expression is more pronounced in B2-KO compared with WT mice. Collectively, these results provide conclusive evidence for the presence of a negative feedback loop between the kinin B2 receptor and kallikrein in the developing kidney.

In summary, the present study demonstrates that mice with targeted disruption of the bradykinin B2 receptor gene exhibit specific alterations in the ontogeny of the RAS. The data suggest that renin is a bradykinin-regulated gene, whereas A0, ACE, and AT1 are not. We propose that intact cross talk between the RAS and KKS is required for normal kidney development because interference with these two systems either worsens the renal phenotype (B2 blockade in AT1-KO mice) or generates a severe developmental renal abnormality (suppression of renin by high salt in B2-KO mice) (14, 29).

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-56264. The angiotensinogen antibody is a gift from Dr. Conrad Sernia (University of Queensland, Australia).

REFERENCES


